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INSIGHTS IN RENAL PHARMACOLOGY: 2021

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Editorial: Insights in renal pharmacology: 2021

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Editorial on the Research Topic
Insights in renal pharmacology: 2021

Kidney disease, either acute (AKI) or chronic (CKD), is an important contributor to morbidity and mortality from non-communicable diseases, and in under-resourced settings, also from communicable and maternal/neonatal diseases (Remuzzi and Horton, 2013; GBD Chronic Kidney Disease Collaboration, 2020). After the 1960s, the availability of renal replacement therapies (RRT) made possible the long-term application of life-saving but costly treatments for patients with end-stage kidney disease (ESKD) (Liyanage et al., 2015). In many countries, however, the lack of access to RRT means an unacceptable premature death for millions of children and adults (Liyanage et al., 2015). The global burden of CKD also extends well beyond the provision of RRT services. Indeed, acute and chronic loss of kidney function has been recognized as a risk factor for cardiovascular events independent of other conventional risk factors for cardiovascular disease (Sarnak et al., 2003; See et al., 2019), and as a risk multiplier for cardiovascular mortality in patients with hypertension and diabetes (Couser et al., 2011). There is evidence that slowing or halting kidney disease progression at early stages prevents the development of ESKD and cardiovascular complications, and can provide substantial economic benefits (Trivedi et al., 2002; KDIGO CKD Work Group, 2013). However, despite the available treatments and interventions, the global all-age burden of CKD has increased in the past three decades, highlighting the need for further research to dissect the in-depth pathophysiologic processes underlying kidney diseases and their progression to ESKD. Such mechanistic research is essential for the continued identification of targets for developing novel treatments for AKI and CKD and their consequences. The importance of this challenge has been further highlighted by the current COVID-19 pandemic, in which kidney involvement, through direct SARS-CoV-2 infection or as a consequence of multi-organ dysfunction, has emerged as a major risk factor for poor outcomes as well as one of the sequelae of long-COVID-19 (Perico et al., 2021a; Perico et al., 2021b).

Against this back-drop, this Research Topic provides a collection of original contributions that describe recent basic, pre-clinical, and clinical progress in defining

the mechanisms and the efficacy of a diverse range of novel drugs, biologic agents and mechanistic targets with the potential to ultimately improve the management of AKI and CKD. Given the heterogeneity of the studies and the wide spectrum of disease-specific pathways and biological processes within the kidney, we briefly describe and discuss, in this Editorial, some of the overarching themes and clinical implications of the sixteen articles that comprise the Research Topic.

Tubular epithelial cells of the proximal segments of the nephron are particularly vulnerable to nephrotoxic and ischemic injuries that contribute to the development of AKI (Lombardi et al., 2016). Accumulating evidence indicates that kidney tubule epithelial cells are also key players in the development of renal fibrosis, the common end-point for all progressive kidney diseases leading to ESKD (Zoja et al., 2006; Boor et al., 2010). Four original articles from this collection have explored the mechanisms of action of novel drugs with respect to tubular cell protection. Tao et al. investigated the protective mechanisms of Dexmedetomidine (Dex), a highly selective α2-adrenoreceptor agonist, in *in vitro* cell damage and *in vitro*, in a mouse model of ischemia/reperfusion (I/R) injury—with a focus on ferroptosis, a non-apoptotic form of cell death that contributes to renal I/R injury (Zhang et al., 2021). It is characterized by mitochondrial shrinkage, increased mitochondrial membrane density, and lipid reactive oxygen species, as well as up-regulation of a unique set of genes including Acyl-CoA synthase long-chain family member 4 (ACSL4) (Li et al., 2019). As previously shown in other models, Dex administration mitigated tissue damage, inhibited ferroptosis, and downregulated the inflammatory response following renal I/R injury. The novelty of the study rests on the documentation, by elegant *in vitro* experiments, that the Dex-mediated protective effects occurred by inhibiting the expression and the activity of ACSL4 via α2-adenergic receptor. A second study (Long et al.) explores the effect and the mechanisms of a complementary therapy with a Chinese medicine formula on hypertension-induced renal injury. Treatment with the traditional medicine, Qingda granule (QDG), significantly attenuated hypertensive renal damage by partially preventing cellular apoptosis induced in renal tissues after angiotensin II (Ang II) infusion in mice, and by suppressing the p53 pathway. TUNEL staining documented that QDG treatment also markedly reduced Ang II-induced apoptosis in a renal tubular epithelial cell line. Besides being an example of a possible new therapeutic strategy for treating hypertensive renal injury, the study highlights the growing literature on the potential value of extracts from traditional Chinese medicines on kidney disease. Evidence suggests that senescence, a distinctive form of permanent cell cycle arrest of kidney tubular cells, influences kidney fibrosis (Schafer et al., 2018). By adjusting the culture conditions of their previously developed, conditionally immortalized proximal tubular epithelial cell line overexpressing the organic anion transporter 1 (ciPTEC-OAT1), Yang et al. documented the validity of this *in vitro* model to study kidney senescence. Indeed, culturing ciPTEC-OAT1 cells at 37°C induced a senescence phenotype characterized by increased expression of cell cycle arrest and anti-apoptosis markers, as well as of senescence-associated secretory phenotype factors. Notably, the model showed responsiveness to treatment with senolytic agents. These findings reveal a new avenue for pharmacological investigations of the sensitivity of senescence to novel drugs that selectively eliminate senescent cells by interfering with anti- and pro-survival signalling (Zhu et al., 2015). If validated *in vivo*, this targeted approach may represent an important new treatment option for preventing the development and progression of kidney fibrosis. Despite decades of calcineurin inhibitor use to modulate the immune system in transplantation and autoimmune diseases (Kamal and Doyle, 2022), the "off target" intracellular pathways underlying cyclosporine (CsA) nephrotoxicity remain ill defined. The study by Karolin et al. reports novel experimental results which suggest that, in kidney epithelial cells, CsA does not act via the calcineurin-NFAT (nuclear factor of activated T cells) axis, as in lymphoid cells, but through inhibition of p38 and PI3K/Akt kinases. It should be noted, however, that these findings do not exclude the possibility that calcineurin inhibitor nephrotoxicity also involves other renal cell molecular pathways.

Three other original articles published within the Research Topic focus on the mechanism(s) of podocyte injury and the potential for innovative agents to modulate this cell damage. Podocyte injury and related proteinuria are the most common features of glomerular disease, which is the leading cause of ESKD (Ruggenenti et al., 2012). The interest of research in this area is highlighted by the bibliometric analysis of Liu et al., who found that, in the last 30 years, global publications on podocyte injury have exponentially increased mainly, but not exclusively, in the field of diabetic kidney disease. Hot topics within this literature include autophagy, oxidative stress, inflammasome and microRNAs (miRNAs). These endogenous, small, single-stranded non-coding RNAs play important roles in many biological processes such as organogenesis, cell proliferation and apoptosis, typically by inhibiting the expression of their target genes at the protein level (Trionfini and Benigni, 2017). Relevant to this, the study by Wang et al. reports that, in a mouse remnant kidney model, overexpression of miRNA-671-5p aggravated podocyte injury, worsened kidney dysfunction and exacerbated renal fibrosis - effects that were limit by treating animals with oligonucleotides targeting miR-671-5p in a murine adriamycin nephropathy model. Thus, inhibiting miR-671-5p could potentially serve as a new approach to prevent podocyte injury and loss of renal function in proteinuric CKD. Other investigators contributing to the Research Topic (Yang et al.) report results that confirm, in a mouse model of diabetes, the beneficial therapeutic effects of SS31, a cell-permeable tetrapeptide that selectively targets the inner mitochondrial

membrane as an antioxidant (Zhao et al., 2004). This study also provides some interesting new mechanistic insights in regard to the protective action of SS31 on podocytes in diabetic kidney disease.

Characterisation of novel pathways for CKD progression that offer insights for therapies is the subject matter of two other articles in the Research Topic. In their review, Curran and Koop expertly summarize the activities of aryl hydrocarbon receptor (AHR), a pleiotropic cell signalling molecule with diverse ligand-specific functions, which are implicated throughout the course of CKD. As the authors describe in detail, various organic endogenous molecules, in addition to exogenous drugs, chemicals, and dietary compounds, bind to and activate AHR to either promote glomerular and tubular damage or protect against kidney injury. These diverse responses are linked to AHR-induced crosstalk with transcription factors associated with kidney fibrosis, metabolism, and the renin-angiotensin system. This incisive review, of particular value to those with deeper interest in the molecular details of CKD pathophysiology, offers an understanding of the key regulatory role of AHR in physiological pathways that may lead to more targeted therapies for CKD. Similarly, in an extensive original study Xuan et al. delineate the pleiotropic effects of SGLT2 inhibitors, beyond the lowering of blood glucose levels and the control of glomerular hyperfiltration (Cortinovis et al., 2022). They show that the SGLT2 inhibitor, dapagliflozin, alleviates renal fibrosis by decreasing necroptosis/inflammation mediated by the receptor-interacting protein kinases 1 and 3 (RIP1 and RIP3) and the mixed-lineage kinase domain-like (MLKL) axis proteins (Choi et al., 2019) in a rat model of unilateral ureteral obstruction (UUO). Suppression of oxidative stress and apoptosis, along with improved mitochondrial function, are suggested to be mechanisms underlying the renoprotective properties of dapagliflozin. These interesting findings provide further rationale for the use of SGLT2 inhibitors to prevent non-diabetic CKD, which is the subject of ongoing clinical trials.

Two review articles address the emerging potential for and hurdles to the promotion of immune regulation as a strategy for modulating autoimmune diseases and kidney transplantation—a topic of growing interest within the biomedicine research community (Juneja et al., 2022). Moving from the observation that defects in interleukin-2 (IL-2) and T regulatory cells (Tregs) are known to contribute to systemic lupus erythematosus and lupus nephritis, Venkatadri et al. summarize the existing evidence for Treg-enhancement strategies involving IL-6, IL-2, IL-33, or a novel hybrid cytokine (termed IL233), that have been shown to induce remission in models of lupus nephritis. A number of alternative strategies for *in vivo* Treg activation and expansion for which encouraging *in vitro* and *in vivo* results have been reported are also described. These include a tumor necrosis factor receptor (TNFR)2-specific agonist, an agonistic OX40:Fc fusion protein, the selective inhibitor of Janus kinase 1 and 2, baricitinib, and mesenchymal stem cells (MSCs). Additionally, regulatory B cells (Bregs) have recently generated interest, having been identified as major drivers of tolerance in kidney transplantation and in autoimmune diseases (Dasgupta et al., 2020). Indeed, in both settings, impaired function and low numbers of Bregs have been reported—leading to a search for strategies to re-establish the lost homeostasis. Here, Garcia et al. discuss the relevance of *in vivo* and *in vitro* induction of Bregs and their potential use as therapeutic agents in kidney transplantation. One approach described by these authors is to boost natural Bregs through specific immunosuppressive agents such as belatacept or alemtuzumab, either alone or in combination with cell therapies (MSCs or Tregs) which have been shown in kidney transplant recipients to induce Bregs. More complex and debateable due difficulties in their identification and culture expansion, is the potential to use Bregs as an adoptive cellular therapy - although improved *in vitro* Breg induction systems are on the horizon.

With the transition from CKD to dialysis-dependent ESKD, comes a further significant increase in the risk for cardiovascular disease (CVD) compared with the general population. This accounts for over 50% of the mortality among dialysis patients (Sharma and Sarnak, 2017). The comprehensive review of Wang and Gao highlights the complex role of inflammatory cells and cytokines in the cross-talk between kidneys and heart in patients on chronic hemodialysis. The authors also propose potential pharmacological interventions that, by addressing key targets in the inflammatory cascade, could improve compliance with hemodialysis therapy and limit the risk of cardiovascular events. Along similar lines, the study by Tsai et al., based on real-world evidence, reports that dialysis patients treated with lipid-lowering agents display a marked reduction in the risk of mortality, hospitalization, and major adverse cardiovascular events. They suggest a clinical benefit of these drugs on major CV outcomes, challenging previous observational and randomized studies showing no advantage of this therapy in dialysis patients (Mach et al., 2020).

Finally, the last part of the collection includes three articles dealing with very disparate topics, but with some intriguing observations. The study by Deng et al. explores the clinical significance of antibody against the M-type phospholipase-A2-receptor (PLA2R), now a routine laboratory test for the characterization of patients with primary membranous nephropathy, as a guide to management. In a large group of patients with seropositive PLA2R-associated membranous nephropathy, they report that PLA2R antibody level above 150 RU/ml (actually a non-validated cut-off value) is associated with higher disease activity and worse prognosis, even under different immunosuppressive regimens. While these findings confirm results of previous studies on the predictive value of PLA2R antibody titre on disease outcome (Ruggenenti et al., 2017), they, unfortunately, include only the

responses to older treatment regimens such as cyclophosphamide and calcineurin inhibitors and not the more recently introduced and better-tolerated B-cell depleting biologics (Ruggenenti et al., 2017). Tabibzadeh et al. focus on a still-debated issue in clinical practice and in the literature, namely the detection and risk of progression of early lithium-related nephrotoxicity. This cross-sectional cohort study of patients prescribed lithium salts for bipolar disorder, confirmed the independent negative effect of lithium exposure on kidney function, especially in patients with microcysts. Since early microcysts—detected only by magnetic resonance imaging (MRI) and not by CT scans or ultrasonography—might also reflect irreversible nephrotoxicity, it has been proposed that systematic MRI in this setting would help clinicians make difficult decisions in patients with a high suicidal risk at treatment discontinuation. The review article by Luo et al. highlights the effect of increased glomerular filtration rate on drug pharmacokinetics—the so-called "augmented renal clearance" (ARC) concept—with an emphasis on antibiotics. Sub-therapeutic exposure in patients with ARC, is an important reason of treatment failure. Thus, the authors examine the debate around the clinical identification of ARC, review the multiple potential mechanisms of this phenomenon, and propose approaches to ameliorate the efficacy of antibiotic treatment in the setting of ARC. Validation of the proposed approaches in future studies will be of high clinical significance.

To conclude, the collection of articles contributed to this Research Topic provides some excellent examples of recent advances which have deepened our knowledge of the complex mechanisms underlying acute and chronic renal tissue injury and explores target cellular pathways of novel potential pharmacologic and non-pharmacologic agents. These articles also serve to highlight that matching fundamental insights into the mechanisms of renal cell apoptosis, ferroptosis, senescence, inflammation and fibrosis to technological advances in the design of target therapies or to the repurposing of medicines with known disease-modulating effects, provides a strong template for the accelerated development of future treatments to slow or halt the progression of kidney diseases and prevent their complications.

Author contributions

NP, MDG, and GR contributed to the concept, design, and writing of the editorial. They approved the manuscript for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
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Augmented Renal Clearance: What Have We Known and What Will We Do?

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Augmented renal clearance (ARC) is a phenomenon of increased renal function in patients with risk factors. Sub-therapeutic drug concentrations and antibacterial exposure in ARC patients are the main reasons for clinical treatment failure. Decades of increased research have focused on these phenomena, but there are still some existing disputes and unresolved issues. This article reviews information on some important aspects of what we have known and provides suggestion on what we will do regarding ARC. In this article, we review the current research progress and its limitations, including clinical identification, special patients, risk factors, metabolism, animal models and clinical treatments, and provide some promising directions for further research in this area.

Keywords: augmented renal clearance, mechanism, clinical treatment, *in vivo* and *in vitro* model, therapeutic drug monitoring

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INTRODUCTION

Since Udy et al. proposed the concept of augmented renal clearance (ARC) in 2010 (Udy et al., 2010), the phenomenon of ARC and the individualization of pharmacotherapy has gradually attracted more attention recently (see **Figure 1A**).

ARC was defined as the enhanced renal elimination of circulating solutes compared to a baseline (Udy et al., 2010, 2011). In clinical practice, creatinine clearance (CL_{cr}) ≥130 ml/min/1.73 m^2 is usually considered a universally standard lower limit (Bilbao-Meseguer et al., 2018; Dhaese et al., 2021; Nicolau et al., 2021; Tang et al., 2021). ARC leads to sub-optimal drug exposure and causes treatment failure (Udy et al., 2010, 2011; Baptista et al., 2011; Baptista, 2018; Van Der Heggen et al., 2019; Avedissian et al., 2020). Although much attention has been given in this area, there still exist some questions, and some new experimental methods and techniques require exploration due to the complexity of the pathophysiological state of ARC.

There are many studies showed that the at-risk groups for ARC include younger patients, especially younger male patients, with lower illness severity scores on the Sequential Organ Failure Assessment (SOFA) or Acute Physiology and Chronic Health Evaluation (APACHE) II (Udy et al., 2013, 2017; Baptista et al., 2020; Nei et al., 2020; Saito et al., 2020; Johnston et al., 2021). Disease-related risk factors include trauma, surgery, sepsis, burn, subarachnoid hemorrhage, and hematological malignancy (Gerlach et al., 2019; Morbitzer et al., 2019; Lannou et al., 2020a; Saito et al., 2020). Patients who are faced with these disease-related factors usually owe their conditions to their underlying systemic inflammation states and receive resuscitation of large volumes of fluid, crystalloid and hypertonic saline solutions, and the administration of vasoactive drugs and vasopressors (Udy et al., 2013; Cook and Hatton-Kolpek, 2019; Beunders et al., 2020).

In this article, we review what we have known on some crucial aspects of ARC and discuss what we will do on ARC in the future. The aim is to provide a comprehensive understanding of ARC and supply some directions for further research in this area.

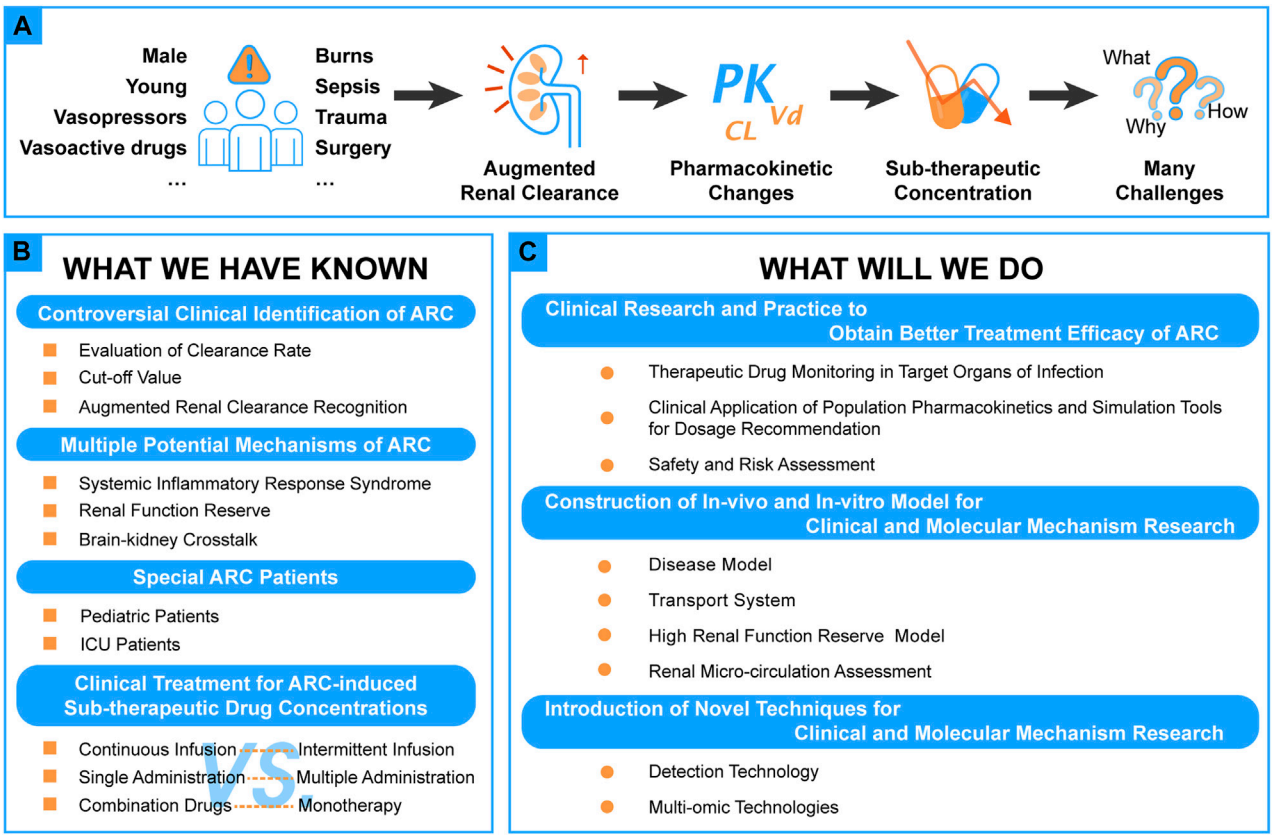

FIGURE 1 | A schematic diagram of main content of this review.

WHAT WE HAVE KNOWN

During the past decades, many studies related to ARC have been conducted and focused on clinical identification, possible mechanisms, ARC-induced sub-optimal concentrations, and corresponding ways of improvement (see **Figure 1B**).

Controversial Clinical Identification of ARC

ARC focuses on two aspects of "augmented" and "renal clearance", that is which methods are to be used to evaluate the kidneys' function and how to judge the status of "augmented". However, there are still controversies about the evaluation methods of renal function and the definition of ARC patients.

Many studies have shown that GFR measured by inulin excretion or radioactive tracer methods, the "gold standard", are the most accurate methods for kidney function assessment (Soveri et al., 2014). There are other methods used for GFR assessment. Creatinine is regarded as the most common endogenous filtration marker and is detected over 24 h to evaluate the excretory function in "normal" conditions (Inker and Titan, 2021). But, a recent study (Collet et al., 2021) showed that compared with the measurement of GFR by iohexol clearance, the 6 h renal creatinine clearance systematically overestimated renal function in adult patients with ARC due to small muscle mass and nutrition, and the mean bias was higher than the calculated using formulas. Additionally, some convenient and simple methods have been developed for the rapid evaluation of kidney function, such as Cl_{cr} calculated by Cockroft-Gault (Cockcroft and Gault, 1976); eGFR calculated by the Chronic Kidney disease Epidemiology Collaboration (Levey et al., 2009) and Modification of Diet in Renal disease Study (MDRD) equations (Levey et al., 1999), which clinicians commonly adopt. But, there are still some issues, including that the calculation results are susceptible to many factors owing to unstable kidney function due to ARC and therefore underestimate renal function when identifying ARC (Baptista et al., 2014; Ruiz et al., 2015; Morbitzer et al., 2019; Gijsen M. et al., 2020). Therefore, more accurate methods and predictive equations for renal function estimation, high-risk screening, and the discovery of optimal surrogate markers are all needed for the rapid and straightforward recognition of ARC.

ARC is defined and recognized as $CL_{cr} \geq 130$ ml/min/1.73 m^2, based on numerous clinical studies finding that $CL_{cr} \geq 130$ ml/min/1.73 m^2 is related to target concentration attainment (Mahmoud and Shen, 2017), while some studies chose other cutoffs such as 120 ml/min/1.73m2, 150 ml/min/1.73m2 or else (Campassi et al., 2014; Carrié et al., 2019; Lannou et al., 2020b; Ong et al., 2021). Some researchers proposed that the ARC duration time should be carefully considered (Udy et al., 2014; Tomasa-Irriguible et al., 2021). In a study of GFR estimation on critically ill patients, conducted by Baptista's team (Baptista et al., 2014), "ARC

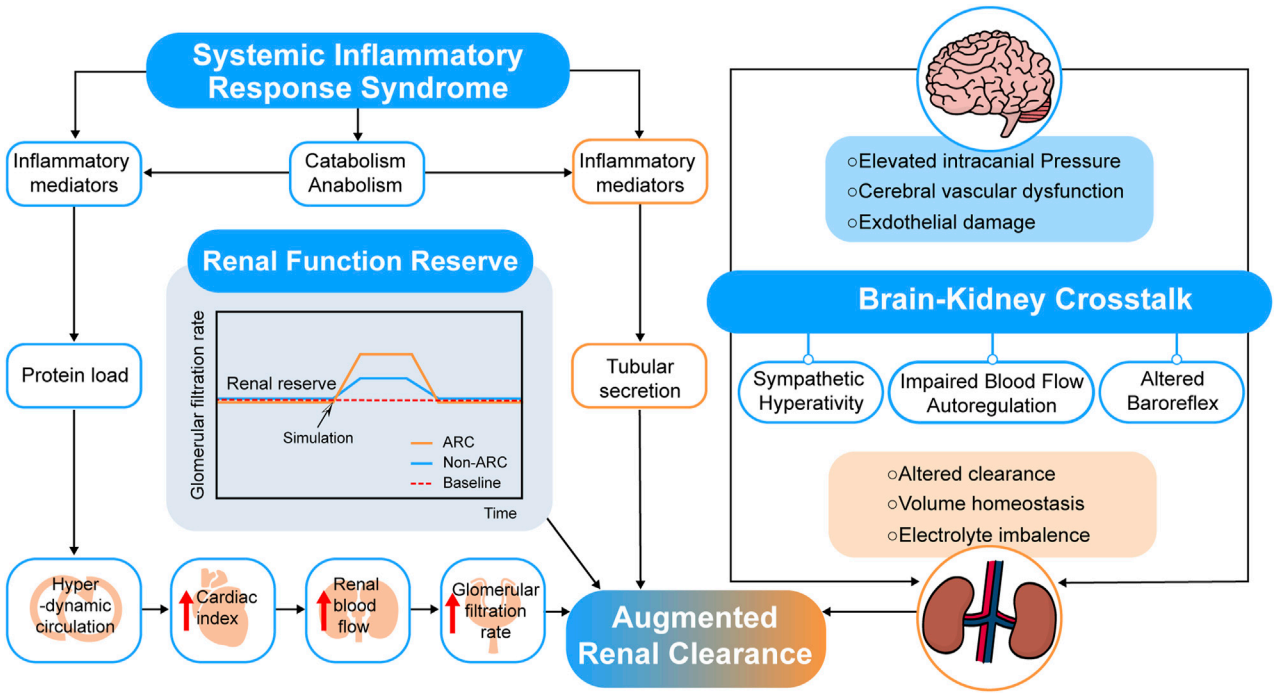

FIGURE 2 | Overview of probable mechanisms of augmented renal clearance.

patient" were defined as ≥50% measurements of CL_{cr} ≥ 130 ml/min/1.73 m^2 during the admission period. And Claus et al. (Claus et al., 2013) found that patients who permanently expressed ARC during antimicrobial treatment had higher treatment failure rates (33.3 vs 17.4%) than patients with transient ARC (1 day). Some exploration has also been done for screening and recognizing ARC patients by using scoring systems (Morbitzer et al., 2019; Gijsen M. P. et al., 2020; Saito et al., 2020). But the risk factors screening and the cutoff value were variant in different studies. Udy's team (Udy et al., 2013) described a model of age, modified SOFA, and diagnostic category, which was used to predict patients manifesting ARC. In 2016, Barletta and others (Barletta et al., 2017) described a predictive model of ARC, which is specific to the intensive care unit (ICU) trauma patients for bedside application and ARCTIC scores ≥6 presented as the cutoff for ARC. Thus, a unified standard of ARC containing cutoff, duration time or scoring criteria should be clearly defined.

Multiple Potential Mechanisms of ARC

The mechanism of ARC is not clear up to now (Bilbao-Meseguer et al., 2018; Baptista et al., 2019) due to the hyperkinetic state, increased cardiac output, and elevated blood flow to major organs of patients at risk of manifesting ARC. Systemic inflammatory response syndrome (SIRS) and renal function reserve (RFR) were proposed to explain the possible mechanisms of ARC (see **Figure 2**).

The theory of SIRS posed that when patients in conditions such as severe trauma, burns, sepsis and major surgery related or irrelevant to infection, cytokines and pro-inflammatory mediators release, which may decrease vascular resistance and increase cardiac output and capillary permeability (Balk, 2014). For critically ill patients, the use of many fluids and positive inotropic drugs for treatment also makes the renal vascular flow further increase, leading to the occurrence of ARC (Sime et al., 2015). A prospective observational study of COVID-19 (Beunders et al., 2021) showed that the detected time point of ARC was strongly related to the day of peak ferritin, C-reactive protein, and D-dimer. The research team of Udy (Udy et al., 2013) has conducted a prospective observational study in 71 patients with sepsis and multi-trauma, 57.7% of whom manifested with ARC, and the results showed that there was a weak correlation between cardiac index (CI) and CL_{cr} ($r = 0.346$; $p = 0.003$). Further, changes in vascular resistance and capillary permeability as well as the influence of inotropic drugs are still unclear and need to be verified by more experiments.

Another theory of RFR suggests that renal reserve plays a role in ARC. RFR refers to the capacity of kidneys under certain physiological conditions or pathological stimuli, such as pregnancies, high-protein diets, high fluid intakes, or uses of high cardiac output drugs (Sharma et al., 2014). The stress tests showed that vasodilation and increased blood flow might be the mechanisms due to the release of endothelium-derived relaxation and prostaglandins locally, displayed as an increase in GFR. Additionally, younger patients tend to have higher renal reserve and function (Ronco et al., 2017). This theory is consistent with the risk factors of ARC, which increases the possibility of the veracity of the RFR theory.

In addition to these two theories, other studies conjectured possible mechanisms of ARC. Dias et al. (Dias et al., 2015) tried linking renal function with traumatic brain injury, and the results showed that PRx (intracranial pressure, cerebral perfusion pressure,

and the cerebrovascular reactivity pressure index) was significantly related to ARC in traumatic brain injury patients. A "brain-kidney crosstalk" theory was proposed by Nongnuch et al. (Nongnuch et al., 2014) in an AKI study, and Khalid et al. (Khalid et al., 2019) also posed that traumatic brain injury-resulted crosstalk between the brain and kidney cause chaotic perturbations and affect their perfusion regulation, enlightened us to a possible link between acute brain injury and kidney function, which still requires further proof in ARC patients (see **Figure 2**).

Special ARC Patients

Pediatric Patients

The modified Schwartz equation is most adopt for evaluating renal function in pediatric patients (Bauters et al., 2019; Gao et al., 2020). There are ten different ARC definitions (CL_{cr} ≥ 130–250 ml/min/1.73 m^2) in pediatrics, although the cutoff commonly defined as the same as adults (CL_{cr} ≥ 130 ml/min/1.73 m^2) (Rhoney et al., 2021). The reported risk factors for pediatric ARC patients include serum creatinine, age, febrile neutropenia, male, septic shock, and antibiotic treatment (Ishii et al., 2018; Van Der Heggen et al., 2019). But increased complexity and difficulty for ARC assessment is in pediatric patients than adults due to rapid growth of the body with age and maturity of kidney and other organs (Rodieux et al., 2015).

ICU Patients

Patients in the ICU always undergo multiple organ failure, illness severity, hemodynamic instability, and exposure to the amount of fluid resuscitation, and more susceptible to nosocomial infections which may lead to comorbidities and complications caused by pneumonia related to mechanical ventilation and infections following trauma or surgery (Eggimann and Pittet, 2001; Vanhorebeek et al., 2020). The evaluation of ARC in ICU patients is more complicated because of these multiple influencing factors.

Clinical Treatment for ARC-Induced Sub-therapeutic Drug Concentrations

Compared to patients with normal kidney function, ARC patients have higher clearances and shorter half-lives of drugs, which promote drug concentrations to fall rapidly. Standard doses make it challenging to meet treatment requirements in these cases (Kaska et al., 2018; Chen and Nicolau, 2020). Some studies have attempted to obtain sufficient drug exposure by changing the way of infusion, altering the frequency of administration, or using drug combinations.

Continuous Infusion vs. Intermittent Infusion

For many drugs such as β-lactams and antiepileptic drugs (e.g., levetiracetam), therapeutic drug monitoring (TDM) is recommended to reach adequate drug exposure. Compared to the intermittent infusion (II), continuous infusion (CI) prolonged T > MIC to achieve pharmacodynamic targets and obtain a higher clinical cure rate (Carrié et al., 2018; Kondo et al., 2020; Goncette et al., 2021). Further, pharmacokinetic/pharmacodynamic (PK/PD) studies have shown that continuous infusion can also increase the probability of target attainment (Roberts et al., 2016; Barrasa et al., 2020).

Single Administration vs. Multiple Administration

Based on the importance of achieving a high bactericidal concentration of aminoglycoside in the initial stage of anti-infective treatment in an ICU (Aréchiga-Alvarado et al., 2020), a once daily dose of amikacin showed a better effect than multiple doses, daily.

Combination Drugs vs. Monotherapy

It was found that under ARC conditions, the intermittent dosing regimen of meropenem and ciprofloxacin as monotherapy is not effective against *Pseudomonas aeruginosa*, even at the maximum approved daily dose for sensitive strains. However, the combination of the intermittent dosing regimen could effectively suppress organisms (Agyeman et al., 2021).

WHAT WE WILL DO

Although many studies have been conducted in the field concerning ARC, there are still some unknown areas in need of further research. In this section, some advanced research methods are provided for further research in this area (see **Figure 1C**).

Clinical Research and Practice to Obtain Better Treatment Efficacy of ARC

The "hyper-dynamic" circulation state of ARC leads to increased renal delivery and elimination of drugs causes sub-therapeutic drug concentrations and sub-optimal antibacterial exposures in ARC patients who are the main reasons for clinical treatment failure (Cook and Hatton-Kolpek, 2019; Tomasa-Irriguible et al., 2020). So there are still some issues should be explored by research or clinical practice, which will provide valuable references to clinical drug therapy to clinicians and clinical pharmacists in the future.

Therapeutic Drug Monitoring in Target Organs of Infection

As a routine clinical test method, TDM can be used to monitor drug concentration in ARC patients. Researches showed that the pharmacokinetic behavior of ARC patients has been changed. The typical values of clearance (CL) and volume of distribution (V) of vancomycin in ARC patients were 8.515 L/h and 2.22 L/kg, which were higher than in the population of normal renal function reported previously (Chu et al., 2020). Some other studies have also found that the CL and V of ceftolozane and tazobactam in ARC patients were both higher than in the healthy subjects (Nicolau et al., 2021). These results pointed out that drugs were distributed widely in patients manifesting ARC, which reminded us that it benefits the treatment of infections in tissues with weak drug penetration or cause unexpected toxicity. But in most centers, serum or plasma drug concentrations are sampled and detected as a surrogate due to practical limitations, which do not reflect the real concentrations at the sites of infection.

So, it is preferred to conduct TDM in target organs of infection, such as cerebrospinal fluid in meningitis and epithelial lining fluid in pneumonia, which will predict and

explain the clinical treatment response better (Felton et al., 2018; Abdul-Aziz et al., 2020; McCreary et al., 2020).

Clinical Application of Population Pharmacokinetics and Simulation Tools for Dosage Recommendation

In view of ARC patients, the dosage regimens need to be optimized in clinical therapy. Tools such as population pharmacokinetics (PPK) and Bayesian estimators combined with other simulation means (e.g., JPKD, Smartdose, Vancomycin Calculator, Monte Carlo simulations) have been used to predict individual pharmacokinetic parameters and to yield clinical dosage recommendations including many antibiotics that are commonly used in clinical practice, such as acyclovir and valacyclovir, linezolid, vancomycin, cefathiamidine, and levetiracetam in patients with ARC (Abdalla et al., 2020; Barrasa et al., 2020; Chu et al., 2020; Gijsen M. P. et al., 2020; Lv et al., 2020; Du et al., 2021; Ong et al., 2021).

The issue is that dosage regimens of ARC patients proposed by pharmacokinetic simulation software are always higher than empirical doses to achieve PK/PD targets (Mahmoud and Shen, 2017; Abdalla et al., 2020; Wang et al., 2021), which might be viewed with caution and have hardly been promoted in clinical practice.

In the follow-up research, more large-scale multi-center PPK studies should be performed to understand the influence factors of ARC in-depth and provide more accurate data for adjusting ARC treatment regimen.

Safety and Risk Assessment

Higher doses for ARC patients are always required to obtain sufficient drug exposure, so the safety and risk assessment should not be ignored.

There are some studies have reported the adverse reactions in ARC patients. In the study of vancomycin administration in patients with different renal function statuses (Yu et al., 2021), the adjusted daily dose of vancomycin in ARC patients was 2.8g/day, higher than the normal renal function group. After treatment with vancomycin, there were 14 cases of ARC changed into normal renal function (NRF) and 3 cases of ARC that changed into impaired renal function (IRF). It has also been reported that acute kidney injury has happened in three ARC patients during vancomycin therapy (1.0–1.5 g, bid), mainly caused by the combined use of nephrotoxic drugs (mannitol and etimicin) and ischemic injury of insufficient renal perfusion (LU et al., 2019).

The main question is that we still lack experience in dosage regimen formulation and adjustments for ARC patients, leading to the safety and risks are still unclear. So in the next step, the evaluation should be performed through multi-center prospective researches, in which factors including combinations of medications, alternatives of tissue toxicity, drug permeability should all be taken into consideration, providing desirable effectiveness and confirmable safety dosing regimens for patients manifesting ARC.

Construction of *In-vivo* and *In-vitro* Models for Clinical and Molecular Mechanism Research

Since the mechanism of ARC is not yet clear, there are only limited animal studies on ARC have been reported. In these studies, iohexol and *p*-aminohippuric acid (PAH) were detected in blood as potential markers to evaluate the GFR, effective renal plasma flow and tubular secretion (Dhondt et al., 2020; Stroobant et al., 2020). Dhondt et al. used lipopolysaccharides (LPS) by continuous infusion to induce a sepsis piglets model, and elevated clearances of GFR marker iohexol and exogenously creatinine and effective renal plasma flow (ERPF) marker PAH were observed (Dhondt et al., 2021b). Decreased systemic exposures of iohexol and amikacin were found after fluid administration, suggesting that fluid therapy is a key factor involved in the development of ARC (Dhondt et al., 2021a).

Considering the mechanism of ARC is unclear till now, animal models based on the risk factors of ARC, including sepsissubarachnoid hemorrhage, burns, and high RFR or modeled by injection of amino acids to stimulate the disease state and augmented GFR (Ge et al., 2019; Lagier et al., 2019; Hu et al., 2020) may be better research method to explore ARC mechanism. Moreover, in the following research, *in vivo* and *in vitro* models such as isolated kidney perfusion, transporter knockout mice for transport system studies on drugs with different excretion mechanisms (Higgins et al., 2012; Ma et al., 2018) and invasive or noninvasive techniques such as synchrotron radiation, fluorescence microangiography methods, and intravital multiphoton microscopy for renal micro-circulation assessment (Li et al., 2021), will help us explore the mechanism and deeply understand the occurrence and development process of ARC.

Introduction of Novel Techniques for Clinical and Molecular Mechanism Research

There are still some uncertainties and unknown areas in the field of ARC research due to a lack of effective research technologies and methods. With the emergence of new detection technology and advanced analytical methods applied in clinical research, the occurrence and development of diseases and their mechanisms can be intensively investigated and deeply understood. Application of ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS), immuno-histochemical staining, multi-omic technologies (metabolomics, proteomics, genomics, lipidomics) research based on biological samples, differential gene expression analysis, biological pathway enrichment analysis, biological function analysis, and other methods will help us obtain and reveal the critical biomarkers, key pathways, and possible pathogenesis of ARC (Guan et al., 2020; Pang et al., 2020; Fukumura et al., 2021).

SUMMARY AND OUTLOOK

The causes of ARC concern a series of endogenous and exogenous factors which lead to elevated levels of GFRs and the hyperperfusion of drugs. Simple and accurate methods and standard cutoff values are still needed to define ARC, and the duration of transient or permanent expression of ARC is still an

unresolved argument. Additionally, the mechanisms of ARC are complex and are not presently clear. Further, doses and medical regimens for treatment choices in ARC patients are facing big challenges. The mechanism of ARC lays the groundwork for the subsequent studies, so optional new technologies such as integrative omics analysis can be performed to explore the differences of metabolites and regulatory genes between ARC and non-ARC patients, which can clarify the mechanism of ARC. The *in vitro* and *in vivo* model of ARC can also be established and employed for a deep investigation into ARC base on the findings of the mechanism. Clinically applicable and practical therapeutic schedules of ARC patients ought to be explored and verified through large-scale multi-center researches.

AUTHOR CONTRIBUTIONS

YC proposed the idea for this article. YL and YW developed the outline, gathered the materials, and wrote the first draft of this manuscript. YM and PW gathered the materials and participated in the discussion of the article. JZ and YC revised the article. All authors approved the final version of this manuscript for submission.

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Knowledge Domain and Emerging Trends in Podocyte Injury Research From 1994 to 2021: A Bibliometric and Visualized Analysis

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Background: Podocyte injury has a direct causal relationship with proteinuria and glomerulosclerosis and, on a chronic level, can lead to irreversible disease progression. Podocyte injury plays a critically decisive role in the development of proteinuric kidney disease. In recent years, the research on podocyte injury has developed rapidly all over the world. However, no report has summarized the field of podocyte injury as a whole to date. Using bibliometric analysis, this study aimed to evaluate the current state of worldwide podocyte injury research in the last 30 years and identify important achievements, primary research fields, and emerging trends.

Methods: Publications related to podocyte injury were retrieved from Web of Science Core Collection. HistCite, VOSviewer, CiteSpace, and the Bibliometrix Package were used for bibliometric analysis and visualization, including the analysis of the overall distribution of annual outputs, leading countries, active institutions and authors, core journals, co-cited references, and keywords. Total global citation score and total local citation score were used to assess the quality and impact of publications.

Results: A total of 2,669 publications related to podocyte injury were identified. Publications related to podocyte injury tended to increase continuously. A total of 10,328 authors from 2,171 institutions in 69 countries published studies related to podocyte injury. China (39.46%) was the most prolific country, and the number of citations of studies in the United States (cited 36,896 times) ranked first. Moin A Saleem, John Cijiang He, and Zhihong Liu were the top three contributing authors, and Journal of the American Society of Nephrology and Kidney International were the most popular journals in the field. "Diabetic nephropathy" is the primary focus area of podocyte injury research, and "autophagy," "microRNA," and "inflammation" were the top keywords of emerging research hotspots, and traditional Chinese medicine monomer may be a neglected research gap.

Conclusion: Our research found that global publications on podocyte injury have increased dramatically. Diabetic nephropathy is the main research field of podocyte injury, whereas autophagy, microRNA, and inflammation are the top topics getting current attention from scholars and which may become the next focus in podocyte injury research.

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INTRODUCTION

Podocytes are highly differentiated epithelial cells attached to the surface of the glomerular basement membrane. They play a prominent role in maintaining the integrity of the glomerular filtration barrier. Podocyte injury and loss are closely related to the development of proteinuria and glomerulosclerosis. In 1994, Kretzler M et al. first attributed glomerulosclerosis to podocyte depletion in a rat model (Kretzler et al., 1994). An important study conducted by Pagtalunan ME et al. found that podocyte loss was related to albuminuria and the deterioration of renal function in Pima Indians with type II diabetes (Pagtalunan et al., 1997). Podocyte depletion is an important morphologic characteristic in the pathogenesis of diabetic kidney disease (DKD) and focal segmental glomerulonephritis (FSGS). Many studies have shown that podocyte injury occur in early-stage DKD (Coimbra et al., 2000) and FSGS (Alachkar et al., 2013), and uncontrollable persistent podocyte injury will lead to glomerulosclerosis and disease progression (Wharram et al., 2005; Makino et al., 2021). Therefore, research focused on preventing podocyte injury or promoting podocyte repair has been the focus and hotspot in this field.

Podocyte injury manifests as hypertrophy, foot process effacement, autophagy, mesenchymal transition, detachment, and apoptosis (Zhou L. et al., 2019). Stress factors that mediate the injury process include mechanical stress (glomerular hypertension or hyperfiltration), oxidative stress, and immune (inflammatory) stress (Nagata, 2016). Once the podocytes are injured, the structure of actin cytoskeleton changes supervenes such that damage to the renal filtration barrier progresses until proteinuria, and glomerular disease occurs (Schell and Huber, 2017). On the other hand, podocyte injury inhibits the synthesis of vascular endothelial growth factor, which in turn affects vascular compartment stabilization and further interrupts the crosstalk between podocytes and vascular compartments, and ultimately leading to the development of glomerulosclerosis. Notably, the proliferation of podocytes is very limited. Although studies have found that podocytes have a certain reserve capacity in infancy, this reserve is rather small (<10% of all podocytes), and becomes depleted with age (Berger et al., 2014; Eng et al., 2015). Mild podocyte injury may be reversible, however, until a certain threshold in podocyte loss is reached (>40% podocyte depletion), and inevitable renal failure occurs (Wharram et al., 2005). Hence, research on podocyte injury is a very promising field, and conducting an analysis of the current status, focus areas, and emerging trends in the field of podocyte injury will yield significant findings.

Bibliometrics is a convenient new approach to the qualitative and quantitative analyses of publications (Chen, 2004; 2017). Using this method, researchers can quickly dig deep into the thematic evolution, main research fields, and new research directions in a certain research field (Chen and Song, 2019). Bibliometrics has become widely used in many disciplines as an auxiliary research method (Wang et al., 2021). However, bibliometric studies on podocyte injury remains lacking.

In this study, we used bibliometric methods to analyze the publications on podocyte injury and systematically evaluate the research status, current research focus, emerging research trends of podocyte injury in the past three decades, highlighting landmark achievements, and pointing out directions for future research.

MATERIALS AND METHODS

Data Source and Search Strategy

We conducted a literature search on the Web of Science Core Collection (WoSCC) on podocyte injury in the past 30 years (from 1990 to 2021). The search formula was as follows: TS = ("podocyte pyroptosis" OR "podocyte apoptosis" OR "podocytopathy" OR "podocytopathies" OR "podocyte injury" OR "podocyte damage", and OR "podocyte dysfunction"). The article language was set to English. In order to avoid deviations from data updates, all the above operations were performed within 1 day, and on July 4, 2021.

Eligibility Criteria and Data Collection

The document types included in the study were only articles and reviews. Meeting abstracts, editorial materials, and proceedings papers, among others, and were excluded. Duplicate studies were also removed artificially. All the information, including the number of papers and citations, titles, authors, affiliations, countries, keywords, journal, publication year, and references, were collected for bibliometric analysis.

Statistical Analysis

In this study, HistCite (version 12.03.17), VOSviewer (version 1.6.16), CiteSpace (version 5.7.R5), and the Bibliometrix 4.1.0 Package (https://www.bibliometrix.org) based on the R language were used to perform the bibliometric analysis.

HistCite (Garfield et al., 2006) was used to calculate the total number of publication records, total global citation score (TGCS), and total local citation score (TLCS) for each publication year, active countries, top institutions, core journals, and authors. More importantly, it was used to identify the representative citation paths of important references.

VOSviewer (van Eck and Waltman, 2010) was used to visualize complex co-citation networks, such as the cooperation and time trends among countries, institutions, and individuals. The size of the nodes represents the number of publications; the thickness of the line represents the strength of the link; and the colors of the nodes represent different clusters or times.

CiteSpace was used to aid visual analysis of the knowledge domain and emerging trends (Chen, 2004), including cluster analysis, dual-map overlay of citations, timeline or time zone

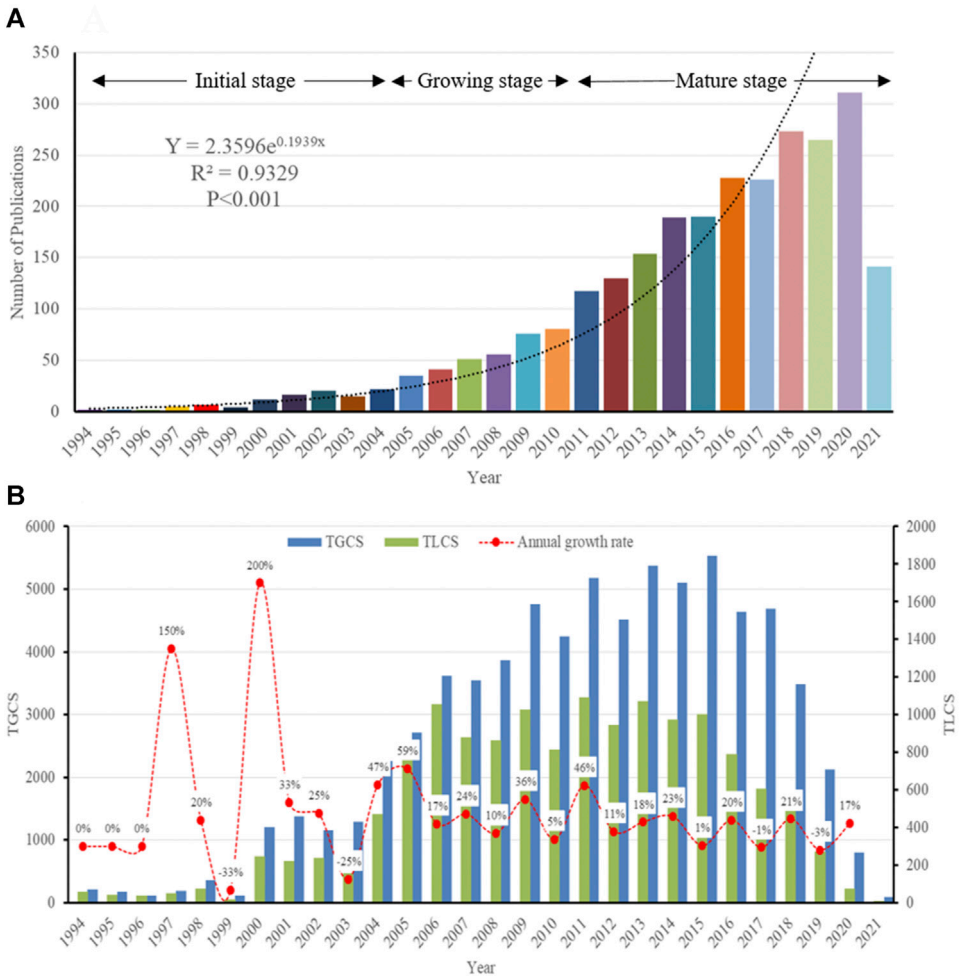


FIGURE 1 | Overall Distribution of publication outputs on podocyte injury **(A)** Global annual output trends; **(B)** TGCS, TLCS and growth rate in annual publications.

views, references, and keywords citation bursts (Chen and Leydesdorff, 2014; Chen and Song, 2019). Cluster analysis can classify references and keywords and identify important research areas on podocyte injury. The modularity Q and mean silhouette are two important evaluation indicators in cluster analysis. Q > 0.3 indicates that the clustering structure is significant enough. Mean silhouette >0.5 indicates that the clustering results are convincing. The bursts of keywords and references are often used to detect new research trends in the field.

The Bibliometrix Package is an established tool based on the R language that is used for bibliometric analysis (Aria and Cuccurullo, 2017). We conducted a thematic evolution analysis using the Bibliometrix Package to categorize the changes in podocyte injury research into different periods.

RESULTS

Overall Distribution

A total of 2,669 publications related to podocyte injury were retrieved from WoSCC, including 2,311 articles, and 358 reviews (**Supplementary Table S1**; **Supplementary Figure S1**). Curve fitting analysis showed that the annual number of publications on podocyte injury has undergone an overall increasing trend since 1994 (R^2 = 0.9329; $p < 0.001$). We artificially divide this period into three stages according to the annual output and growth rate: Initial stage (1994–2004), growing stage (2005–2010), and mature stage (2011–2021). In the initial stage, the total number of publications on podocyte injury was less than 20 per year, and in 1994–1996, only two articles were published in each year, and the only two articles in 1994 were written by Pascua M and Kretzler M, who investigated podocyte injury earlier than researchers in other parts of the world. Pascual et al. (1994) found that complement receptor 1 (CR1) can be used as a marker for podocyte injury (cited 68 times). Over the same period, Kretzler et al. (1994) proposed for the first time that in the uninephrectomized-desoxycorticosterone hypertensive rat model, and glomerulosclerosis may be due to podocyte injury (cited 143 times). In the growing stage, the number of total publications on podocyte injury was less than 100 per year, but increased at an average rate of 9.6 articles per year, and with an average annual growth rate of 25.23%. In the mature stage, the

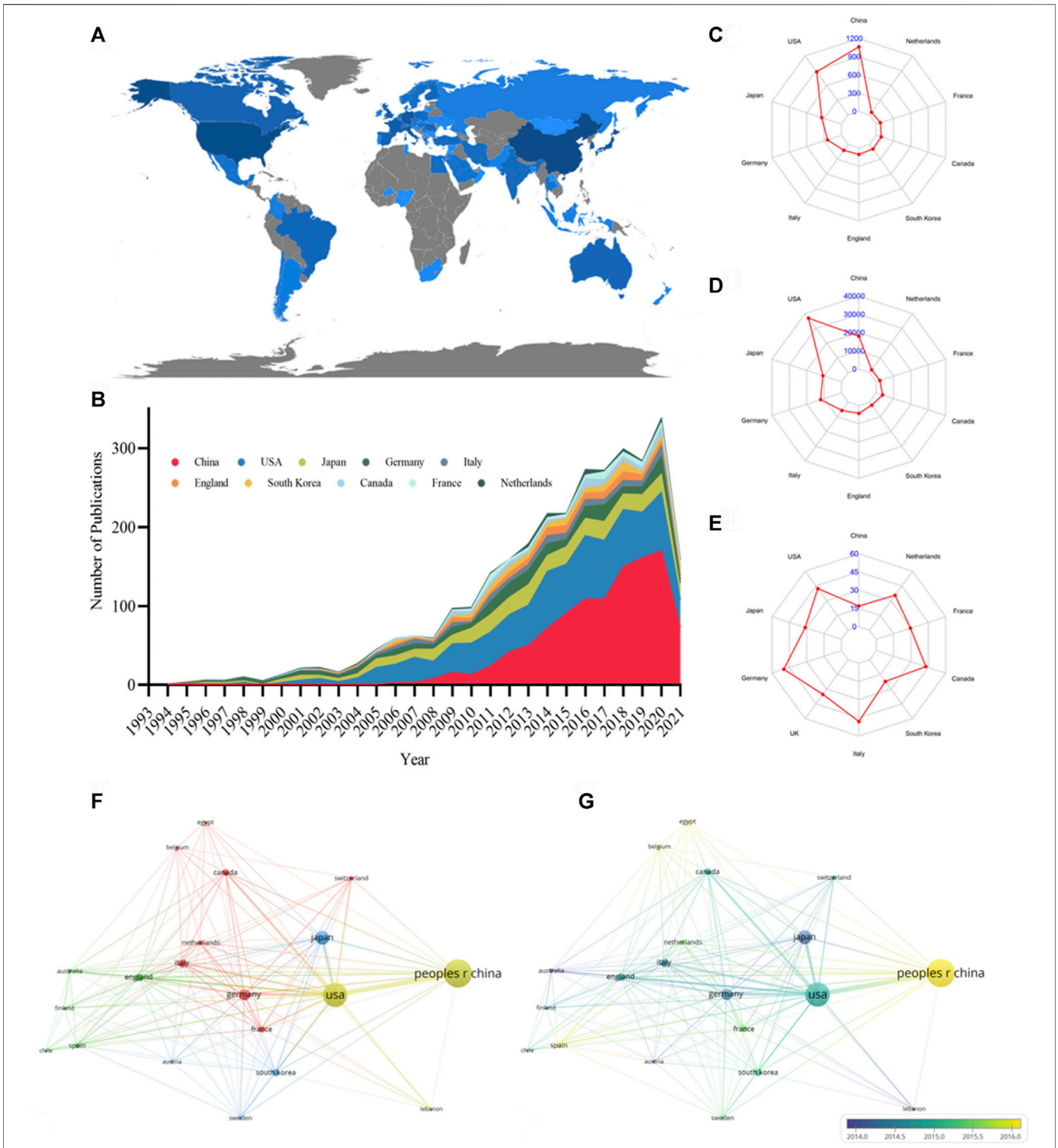


FIGURE 2 | Leading countries in podocyte injury research. **(A)** Geographical distribution of global output; **(B)** Annual output trend of the top 10 productive countries; **(C)** Radar map of the top 10 productive countries; **(D)** Radar map of TGCS of the top 10 productive countries; **(E)** Radar map of average cited of the top 10 productive countries; **(F)** Visual cluster analysis of cooperation among countries; **(G)** Timeline visualization of cooperation among countries.

total number of publications on podocyte injury was more than 100 per year and increased at an average rate of 23.1 articles per year, with an average annual growth rate of 14.76% (**Figure 1A**). The highest number of articles was published in 2020 (n = 311), which was more than the total number of articles published in the previous 15 years (1994–2008, n = 289).

TABLE 1 | The top 10 productive countries concerning podocyte injury research.

Rank	Country	Publications n (%)	LCS	TGCS	Average citation	Centrality
1	China	1,065 (39.90%)	3,739	18,077	16.97	0.03
2	United States	878 (32.67%)	7,651	36,896	42.31	0.54
3	Japan	342 (12.78%)	2,453	10,689	31.35	0.08
4	Germany	242 (9.03%)	2,399	12,008	49.83	0.29
5	Italy	120 (4.57%)	1,030	5,714	35.28	0.16
6	England	107 (4.46%)	648	4,304	48.02	0.09
7	South Korea	93 (3.48%)	393	2056	22.11	0.03
8	Canada	86 (3.22%)	627	3,680	42.79	0.06
9	France	72 (2.66%)	338	2,100	29.58	0.16
10	Netherlands	54 (2.02%)	425	1919	35.54	0.06

TABLE 2 | The top 10 productive institutions concerning podocyte injury research.

Institution	Country	Publication counts	TGCS	TLCS	Average citation
Southern Medical University	China	68	1951	371	28.69
Nanjing Medical University	China	64	1,426	373	22.28
University of Bristol	United Kingdom	62	2067	328	33.34
University of Michigan	United States	62	3,280	739	52.90
Nanjing University	China	60	1,645	352	27.42
University of Washington	United States	57	3,986	1,051	69.93
Shanghai Jiao Tong University	China	56	1,177	193	21.02
Fudan University	China	53	903	191	17.04
Shandong University	China	49	903	197	18.43
Icahn School of Medicine at Mount Sinai	United States	47	973	230	20.70

So far, these articles have been cited 72,836 times, with an average of 27.29 times per article. Since the field of research was then still immature, the TGCS and TLCS of articles published in the initial stage were low. However, the TGCS increased year by year from 2005 to 2008. Since 2009, the TGCS has been relatively stable, indicating that the research on podocyte injury has entered a relatively mature stage (**Figure 1B**).

Leading Countries

From 1994 to 2021, 69 countries published research articles on podocyte injury. The global article productivity is presented in **Figure 2A**. The top 10 countries with the highest number of publications have generated about 84.57% of the articles in the world (**Figure 2B**; **Table 1**). China showed the highest output, publishing a total of 1,065 (39.46%) articles related to podocyte injury, followed by the United States (n = 872; 32.31%), and Japan (n = 341; 12.63%) (**Figure 2C**). The most cited country for published articles is the United States (cited 36,896 times), followed by China (cited 18,077 times), and Germany (cited 12,008 times) (**Figure 2D**). In addition, German publications have the highest average number of citations (average cited 49.83 times), followed by Italy (average cited 48.02 times), and Canada (average cited 42.79 times) (**Figure 2E**). The visualized international collaboration network showed that the cooperation between countries is relatively close. The United States and China showed the closest cooperation, and the United States has been in cooperation with almost all the other countries (**Figure 2F**). Since 1994, research in this field has increased in Germany, Italy, and the United Kingdom, whereas the research on podocyte injury in China, Spain, and Egypt has increased since 2005 (**Figure 2G**).

Active Institutes and Authors

A total of 10,328 authors from 2,172 institutions have published articles on podocyte injury. The top 10 institutions with the highest output in podocyte injury research are shown in **Table 2**. Southern Medical University in China (n = 68) was the leading institution in terms of output, followed by Nanjing Medical University in China (n = 64), the University of Bristol in the United Kingdom (n = 62), and the University of Michigan in the United States (n = 62). The TGCS of the University of Washington in the United States (cited 3,986 times) was the highest, followed by the University of Michigan (cited 3,280 times), and the University of Bristol (cited 2067 times). Cooperation among institutions was relatively close and was divided into four institutional clusters. The cooperation groups led by the University of Michigan showed the closest cooperation with other institutions (**Figure 3A**).

The top three most productive authors were Moin A Saleem of the University of Bristol (published 50 articles), John Cijiang He of the Icahn School of Medicine at Mount Sinai (published 43 articles), and Zhihong Liu of the Medical School of Nanjing University (published 40 articles) (**Table 3**). Kretzler M is an early researcher on podocyte injury. He published his first achievement in this field in 1994. So far, he has been engaged in research in this field for nearly 30 years (1994–2020) (**Figure 3B**). Youhua Liu of

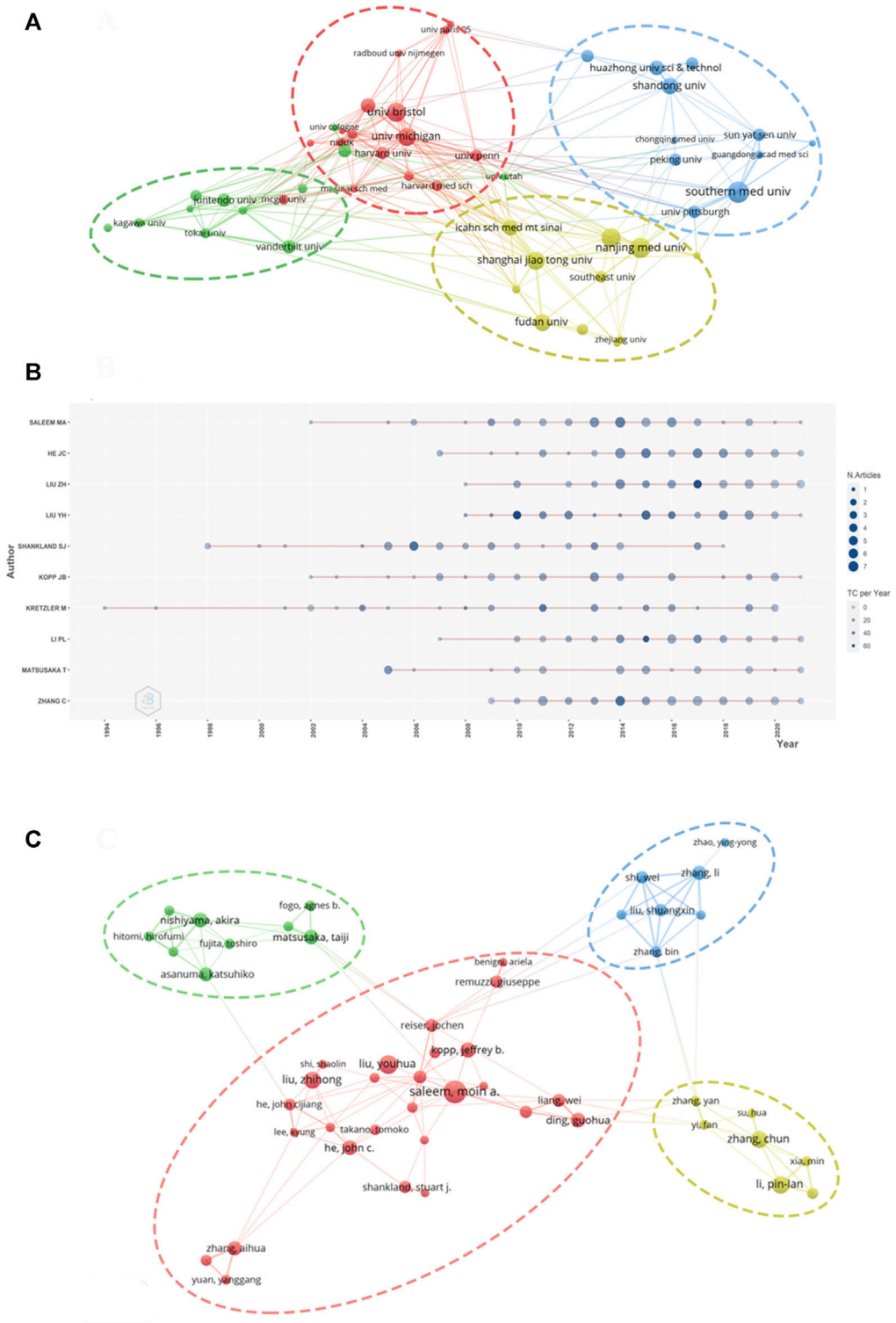


FIGURE 3 | Visualization of active institutes and authors analysis **(A)** Cluster analysis of cooperation among institutes; **(B)** Timeline distribution of the top 10 most productive authors; **(C)** Cluster analysis of cooperation among authors.

TABLE 3 | The top 10 most productive authors in podocyte injury research.

Rank	Name	Country	Institution	Counts	TLCS	TGCS	H-index
1	Moin A Saleem	United Kingdom	Univ Bristol	50	280	1792	24
2	John Cijiang He	United States	Icahn Sch Med Mt Sinai	43	309	1,212	20
3	Zhihong Liu	China	Nanjing Med Univ	40	317	1,365	18
4	Youhua Liu	China	Southern Med Univ	35	688	3,120	25
5	Stuart J Shankland	United States	Univ Washington	37	872	2,730	29
6	Jeffrey B Kopp	United States	NIH	35	270	1,417	22
7	Pin-Lan Li	United States	Virginia Commonwealth Univ	32	279	1,330	19
8	Matthias Kretzler	United States	Univ Michigan	32	563	2,613	24
9	Taiji Matsusaka	Japan	Tokai Univ	29	282	927	15
10	Chun Zhang	China	Huazhong Univ Sci & Technol	29	286	1,004	16

TABLE 4 | The top 10 core journals on podocyte injury research.

Rank	Journal	Counts	TLCS	TGCS	IF (2020)	H-index
1	Journal of the American Society of Nephrology	159	2,110	10,305	10.121	60
2	Kidney International	148	2072	8,895	10.612	55
3	American Journal of Physiology-Renal Physiology	142	1,027	4,476	3.377	38
4	Plos One	94	0	2,182	3.240	26
5	Nephrology Dialysis Transplantation	75	525	2,440	5.992	30
6	Scientific Reports	67	0	944	4.379	18
7	Biochemical and Biophysical Research Communications	49	176	560	3.575	15
8	American Journal of Pathology	36	553	1752	4.307	24
9	Journal of Biological Chemistry	35	462	1857	5.157	24
10	Molecular Medicine Reports	31	62	291	2.952	10

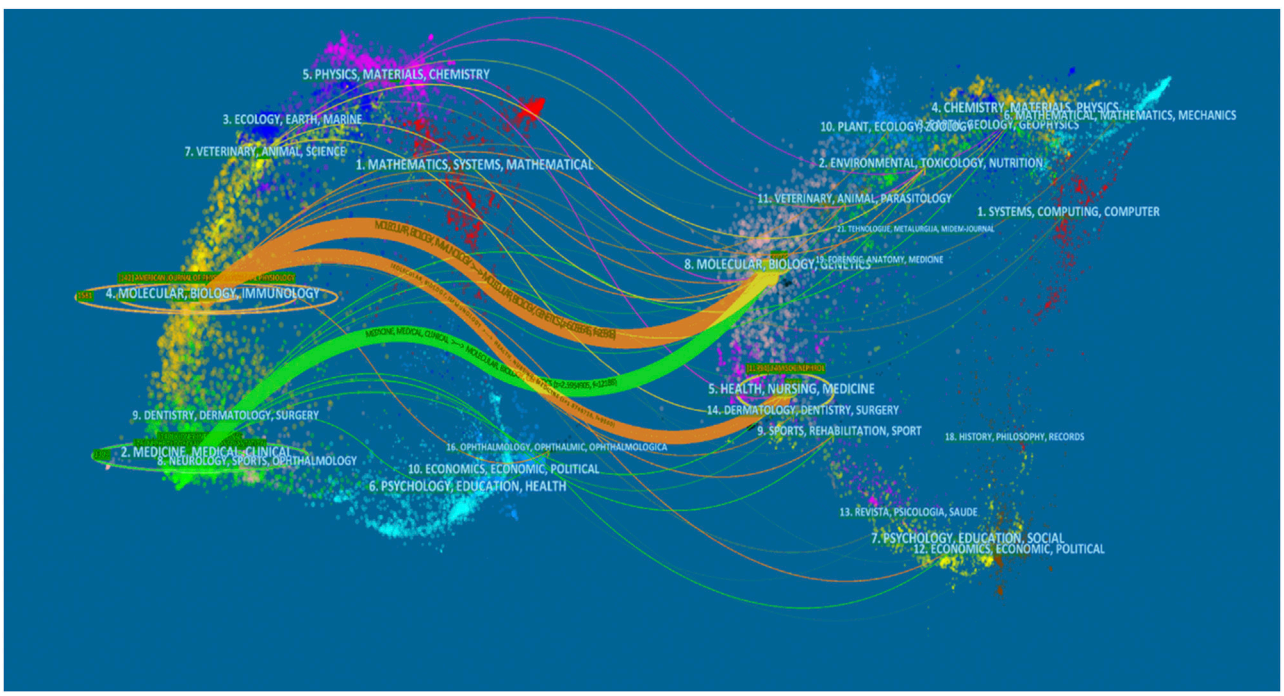


FIGURE 4 | The dual-map overlay of articles citing on podocyte injury research. (The left side were the citing journal, the right side were the cited journal, and the line path represents the citation relationship).

TABLE 5 | The top 10 literatures with the highest number of citations.

Rank	First author	Journal	Year	IF (2020)	Category	Cluster	TGCS	TLCS
1	Susztak K	Diabetes	2006	9.461	Pathophysiology	#2	767	268
2	Shankland SJ	Kidney International	2006	10.612	Pathophysiology	#5	589	249
3	Liu YH	Journal of the American Society of Nephrology	2010	10.121	Pathophysiology	#3	580	53
4	Wiggins RC	Kidney International	2007	10.612	Pathophysiology	#5	494	191
5	Eddy AA	Lancet	2003	79.321	Pathophysiology	#5	460	19
6	Wolf G	Diabetes	2005	9.461	Treatment	#2	454	185
7	Sharma K	Journal of Clinical Investigation	2008	14.808	Treatment	#3	446	44
8	Reiser J	Journal of Clinical Investigation	2004	14.808	Injury mechanism	#5	384	96
9	Wei C	Nature Medicine	2008	53.44	Injury mechanism	#0	375	82
10	Ronconi E	Journal of the American Society of Nephrology	2009	10.121	Treatment	#5	358	55
11	Inoki K	Journal of Clinical Investigation	2011	14.808	Injury mechanism	#1	355	87
12	Abais JM	Antioxidants & Redox Signaling	2015	8.401	Injury mechanism	#5	347	8
13	Coimbra TM	Kidney International	2000	10.612	Injury mechanism	#4	301	50
14	Trimarchi H	Kidney International	2017	10.612	other	#8	289	4
15	Coughlan MT	Journal of The American Society of Nephrology	2009	10.121	Injury mechanism	#4	287	3

TABLE 6 | The top 6 clusters of co-cited references with the highest K value in podocyte injury research.

Cluster ID	Size	Silhouette	Mean year (Range)	Top term	Log (likelihood ratio)
#0	92	0.827	2011 (2004–2018)	Cytoskeleton	20.42
#1	86	0.873	2001 (1996–2007)	Diabetic nephropathy	11.38
#2	79	0.841	2001 (1994–2011)	Stretch	14.03
#3	77	0.847	2012 (2005–2020)	Autophagy	59.85
#4	74	0.866	2016 (2011–2020)	Diabetic kidney disease	20.58
#5	68	0.791	2007 (2001–2016)	Repair	15.70

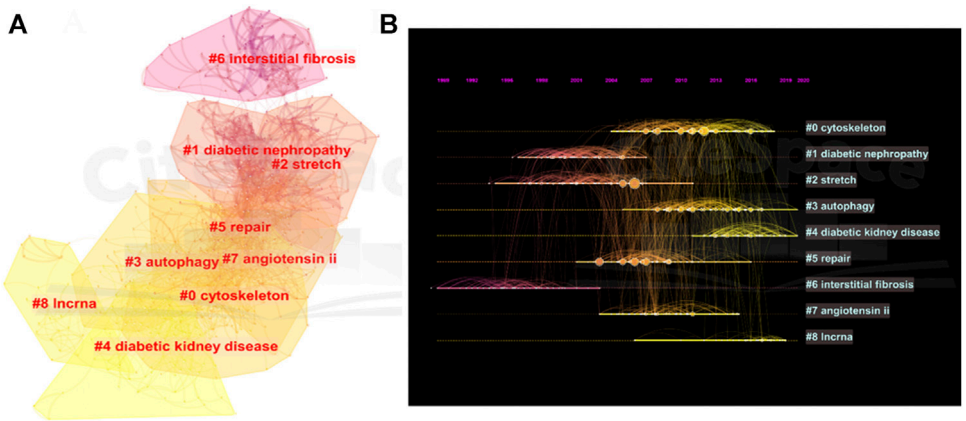
Southern Medical University was the most cited author (cited 3,120 times), followed by Stuart J Shankland of the University of Washington (cited 2,730 times) and Matthias Kretzler of the University of Michigan (cited 2,613 times). The degree of cooperation among authors was relatively low and characterized by cooperation within the institution (**Figure 3C**).

Core Journals

All articles on podocyte injury were published in 413 journals. The top 10 journals with the highest productivity are shown in **Table 4**. About 31.32% of the articles were published in these journals. The Journal of the American Society of Nephrology (JASN) (published 159 articles, cited 10,305 times) was the most prolific journal, followed by Kidney International (KI) (published 148 articles, cited 8,895 times) and American Journal of Physiology–Renal Physiology (published 142 articles, cited 4,476 times). These three journals were also the most cited. The dual-map overlay shows three main citation paths. The published articles were mainly focused on journals in the field of molecular, biology, immunology and medicine, and medical clinical, whereas most of the cited articles were published in journals in the field of molecular, biology, genencs, health, nursing, and medicine (**Figure 4**).

Co-Cited References

The top 15 most cited references includes 7 reviews and 8 research articles (**Table 5**). Most articles focused on the pathophysiology and mechanism of podocyte injury. The most cited article was written by Susztak, who reported that a high-glucose environment can cause podocyte depletion, and leading to early DKD (cited 767 times) (Susztak et al., 2006). Subsequently, Inoki K et al. found that reducing the activity of mammalian target of rapamycin complex 1 in podocytes is a potential strategy for preventing DKD (cited 355 times) (Inoki et al., 2011), which further promoted the development of this field of study. We subsequently constructed a visualization network of cited references and performed a cluster analysis. A total of 14 clusters were found, the modularity Q was 0.6587, and the mean silhouette value was 0.8617 (**Supplementary Table S2**). Six clusters with the highest K values were identified (**Table 6**; **Figure 5A**), which include "cytoskeleton," "diabetic nephropathy," and "stretch," among others. Furthermore, we performed a visualized timeline for clusters (**Figure 5B**). We found that "interstitial fibrosis" is an early field in podocyte injury. However, the current hotspots of podocyte injury are on "autophagy," "diabetic kidney disease," and "lncRNA." Finally, we conducted a reference burst. The top 25 references with the strongest citation bursts are shown in **Supplementary Figure S2**. **Figure 5C** shows the most representative references in terms of burst strength, burst duration, and burst time. We found that the works of Pavenstädt et al. (2003) have the highest bursts strength. In his article, he described the relationship between podocyte



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References	Year	Strength	Begin	End	1994 - 2021
Barisoni L, 1999, J Am Soc Nephrol	1999	24.59	2000	2007	
Kaplan JM, 2000, Nat Genet	2000	19.44	2001	2008	
Boute N, 2000, Nat Genet	2000	18.01	2001	2008	
Schiffer M, 2001, J Clin Invest	2001	18.31	2002	2009	
Mundel P, 2002, J Am Soc Nephrol	2002	25.26	2003	2010	
Pavenstadt H, 2003, Physiol Rev	2003	44.9	2005	2011	
Wolf G, 2005, Diabetes	2005	29.76	2006	2013	
Wharram BL, 2005, J Am Soc Nephrol	2005	23.24	2006	2013	
Winn MP, 2005, Science	2005	17.99	2006	2013	
Susztak K, 2006, Diabetes	2006	34.49	2007	2014	
Shankland SJ, 2006, Kidney Int	2006	38.4	2008	2014	
Wiggins RC, 2007, Kidney Int	2007	27.82	2008	2015	
Nagata M, 2016, Kidney Int	2016	31.16	2017	2021	
Tagawa A, 2016, Diabetes	2016	21.82	2017	2021	

FIGURE 5 | Visualization of co-cited reference analysis. **(A)** Cluster Analysis of Co-cited References; **(B)** Timeline distribution of the top 9 clusters; **(C)** Representative burst references among top 25 references with the strongest citation bursts.

TABLE 7 | Keyword cluster analysis of podocyte injury research.

Cluster ID	Size	Silhouette	Mean year	Top terms	Log (likelihood ratio)
#0	66	0.551	2009	Nephrotic syndrome	128.11
#1	60	0.735	2003	Proteinuria	76.83
#2	59	0.623	2011	Diabetic nephropathy	69.66
#3	51	0.694	2009	Oxidative stress	53.16
#4	34	0.751	2011	Autophagy	50.62
#5	31	0.61	2012	Growth	16.2
#6	31	0.734	2012	Protein	47.27
#7	22	0.779	2011	Disease	56.81

injury and glomerulosclerosis Pavenstädt et al. (2003), indicating that this is the current focus in podocyte injury research. In addition, Nagata M's article has had a strong citation burst in recent years. His article introduced the correlation between autophagy and podocyte injury, indicating that this has garnered increased research interest in recent years (Nagata, 2016).

Analysis of Keywords

We extracted 4,080 keywords at data collection, and a cluster analysis revealed 8 clustering results (**Table 7**). The modularity Q of the cluster was 0.3087, and the mean silhouette value was 0.7322. The timeline of clustering showed that "diabetic nephropathy" and "proteinuria" were the most important areas of podocyte injury research,

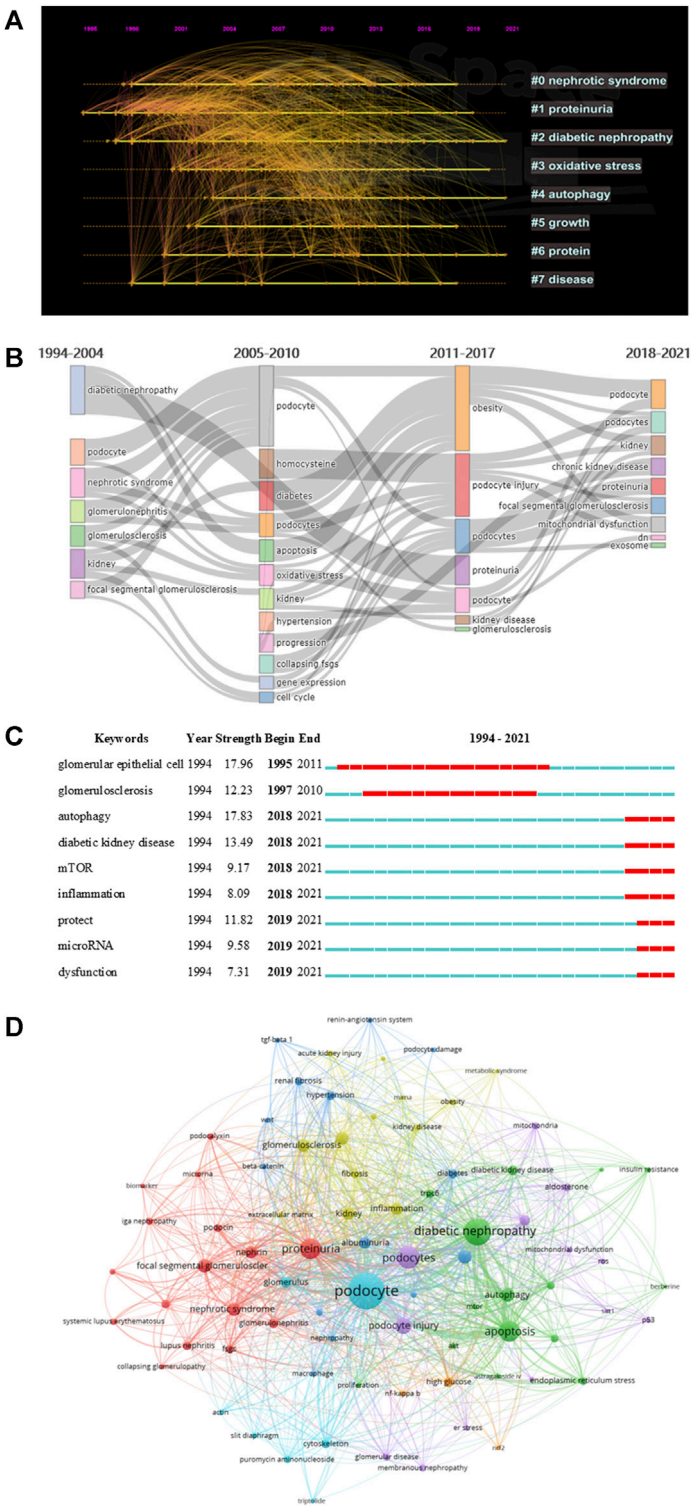

FIGURE 6 | Visualization of keyword analysis. **(A)** Timeline distribution of cluster analysis of keyword; **(B)** Sankey diagram of the keywords evolution of podocyte injury research; **(C)** Representative burst keywords among top 25 references with the strongest citation bursts. **(D)** The network map of keywords.

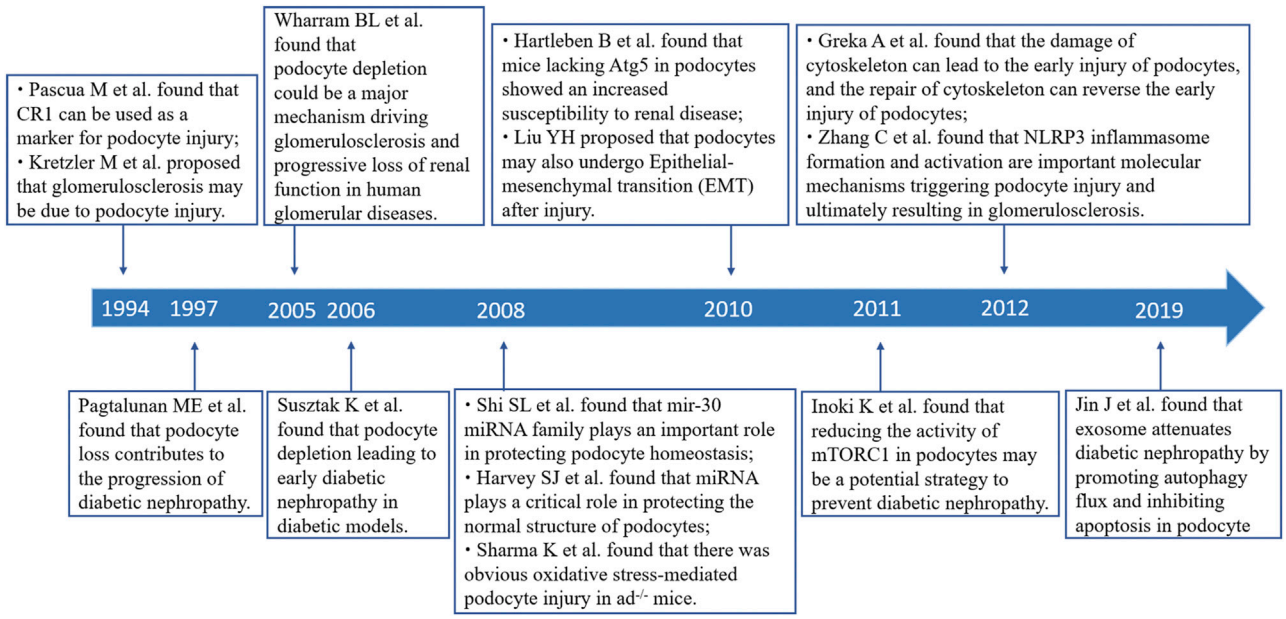

FIGURE 7 | Timeline of part of landmark achievements in podocyte injury research.

whereas "autophagy" was an emerging hotspot in podocyte injury studies (**Figure 6A**). We conducted a thematic evolution analysis on keywords, and found that the initial stages of research on podocyte injury was mainly focused on "diabetic nephropathy." However, with the maturity of the research field, the main research hotspot of podocyte injury has gradually evolved toward "homocysteine," "apoptosis," and "obesity," among others. In the past 3 years, "mitochondrial dysfunctions," "exosome," among others, and has gradually attracted the attention of scholars (**Figure 6B**). A total of 102 keywords were extracted by keyword burst analysis, the top 25 of which are shown in **Supplementary Figure S3**. We found that "autophagy" and "diabetic kidney disease" had the highest burst strength. In addition, we also found that "inflammation," "protect,", and "microRNA" were the latest keywords that emerged in the last 2 years (**Figure 6C**).

In addition, we followed the practice of (Kumar and Goel, 2021; Kumar et al., 2021) to explore the research gaps in the field of podocyte injury. We found that studies on podocyte injury were less exposed in acute kidney injury and IgA nephropathy, which may be related to the pathophysiological characteristics of podocytes. Notably, our study also found that the protective effect of traditional Chinese medicine monomer on podocyte injury is a promising blank field, and such as astragaloside iv (occurrences: 12), triptolide (occurrences: 10), berberine (occurrences: 7), etc. (**Figure 6D**). Xing et al. (2021) found that astragaloside iv can inhibit oxidative stress and alleviate podocyte damage. Coincidentally, Wan et al. (2021) found that triptolide can reverse the epithelial-mesenchymal transition of podocytes and improve podocyte-associated glomerular diseases. Therefore, the protective effect of traditional Chinese medicine monomer on podocyte injury needs more research to fill this gap.

DISCUSSION

In this study, we analyzed the main knowledge domain and emerging trends of podocyte injury using bibliometric analysis. Some landmark articles were also identified using this analysis (**Figure 7**). The results showed that the annual publications on podocyte injury generally show an upward trend and have entered a relatively mature research stage. The study of podocyte injury rose in 1994. Subsequently, Wharram et al. (2005) demonstrated that podocyte depletion may drive glomerulosclerosis and renal function progression in humans. The latest studies found that weight control was effective in preventing the kidney disease progression that occurs because podocytes fail to match the growth of the glomerular tuft (Fukuda et al., 2012).

China was the most productive country, and six of the top 10 productivity institutions are from China. Southern Medical University (published 68 articles, cited 1951 times) is the main representative, and Liu Youhua's team from this institution contributed most of the publications, and with a long-term research focus on Wnt/β-catenin signaling and podocyte injury (Dai et al., 2009; He et al., 2011; Zhou et al., 2013). Their latest study found that the activation of the Wnt/β-catenin signaling pathway mediates oxidative stress-induced podocyte injury (Zhou L. et al., 2019). The United States was the country with eight of the top 10 most cited articles. University of Washington (published 57 articles, cited 3,986 times) is the main

representative. Shankland is a leader in the field of podocyte injury at this institution and has long devoted himself to the study of podocyte injury and glomerulosclerosis. His most cited article describes the relationship between podocyte injury and glomerulosclerosis (cited 589 times) (Shankland, 2006). His latest research found that Krüppel-like factor (KLF) was closely related to podocyte injury in glomerulonephritis. KLF15 expression contributes to the stability of cytoskeleton in podocyte (Mallipattu et al., 2017), whereas the loss of KLF4 activates the STAT3 signaling pathway, which further contributes to podocyte injury (Estrada et al., 2018).

Notably, among the top 10 core journals, JASN and KI had by far, the highest in both the number of articles published and the number of citations, and indicating that these two journals are the most popular journals for scholars who study podocyte injury. Scholars all over the world hope to publish landmark articles in JASN and KI. These two journals have published the most cutting-edge results and major breakthroughs in podocyte injury research. In recent years, Both JASN and KI have mainly focused on research exploring ways of protecting podocytes from damage. For example, a recent article published in KI found that interleukin 9 plays a protective role in the context of podocyte injury (Xiong et al., 2020). In the same period, an article published in JASN found that synaptopodin conferred a protective effect against podocyte injury. Differently, JASN focuses mainly on cutting-edge research, and whereas KI pays concentrates on breakthroughs in basic research.

The timeline view of references and keywords showed that studies related to podocyte injury are mostly basic studies, such as cytoskeleton, apoptosis, autophagy, repair, and interstitial fibrosis. An important review (cited 301 times) discusses the close relationship between cytoskeleton dysfunction and podocyte injury. Cytoskeleton damage can lead to the early injury of podocytes, and cytoskeleton repair can reverse early injury to podocytes (Greka and Mundel, 2012). Similarly, the study conducted by Faul C et al. found that cyclosporine A can prevent synaptopodin dephosphorylation and promote the stabilization of the podocyte cytoskeleton to reduce proteinuria (Faul et al., 2008). Coincidentally, Liu M et al. recently found that sirt6 can maintain cytoskeletal stability and reduce podocyte apoptosis (Liu et al., 2017). Podocyte apoptosis, which is a special form of podocyte injury, exists in the early stages of DKD (Susztak et al., 2006). Schiffer M et al. found that podocyte apoptosis induced by transforming growth factor-beta existed in the early stage in FSGS mice (Schiffer et al., 2004). Oxidative stress has long been considered an important initiating factor of podocyte injury. In a highly cited article, Sharma K et al. found an obvious oxidative stress-mediated podocyte injury in adiponectin knockout (adiponectin$^{-/-}$) mice (cited 464 times). Mo HY et al. found in their latest study that C-X-C chemokine receptor type 4 plays an important role in podocyte injury mediated by oxidative stress and can be used as a new target to improve podocyte injury (Mo et al., 2017).

Among the top 15 most cited articles, studies on DKD accounted for 46.67% (7/15), indicating that DKD is a hot focus of podocyte injury research. Pagtalunan ME et al. first found that podocyte loss was associated with DKD progression in Pima Indians with type II diabetes Pagtalunan et al. (1997). Subsequently, Nakamura T et al. found that the detection of podocytes in the urine was associated with the disease activity of DKD (Nakamura et al., 2000). At the same time, Coimbra TM et al. found that podocyte injury precedes glomerulosclerosis in diabetes mellitus (Coimbra et al., 2000) and that high glucose- and hypertension-related mechanical stress causes podocyte injury and proteinuria, which further progress to early DKD (Wolf et al., 2005). Isermann B et al. found that hyperglycemia caused the interruption of crosstalk between vascular compartment and podocytes, resulting in DKD (Isermann et al., 2007). Identifying new mediators of crosstalk between the vascular compartment and podocytes helps identify more effective treatments for DKD (Siddiqi and Advani, 2013).

With the development of podocyte damage research, some emerging research fields are gradually becoming the topics of interest of researchers. References and key bursts showed some items has had the highest bursts strength in the past 3 years. One of which is autophagy. Substantial evidence indicates that podocyte autophagy is a protective mechanism in kidney disease (Zeng et al., 2014). A landmark article on podocyte autophagy was published in 2010 by Hartleben B et al. who found that mice lacking autophagy-related 5 in podocytes showed an increased susceptibility to renal disease. Thus, they proposed that autophagy is a protective mechanism against glomerular injury (Hartleben et al., 2010). Lenoir O et al. found that hyperglycemia led to enhanced autophagic flux under the diabetic milieu, wherein autophagy in endothelial cells and podocytes inhibit the progression of DKD to glomerulosclerosis (Lenoir et al., 2015). A growing number of protein molecules that protect podocytes from injury by modulating autophagy have been found gradually, include Sirt6 (Liu et al., 2017), heme oxygenase-1 (Dong et al., 2015), and hepatocyte growth factor (Hou B. et al., 2020). Mitophagy has recently emerged as a new focus in podocyte injury research (Li et al., 2017; Zhou D. et al., 2019). Although an increasing number of studies on autophagy have been carried out, more in-depth research on autophagy are expected to be conducted in this field in the future. Another keyword with a significant citation burst is miRNA. Research on miRNA and podocyte injury has exploded because of the critical roles of miRNA. Many miRNA have protective effects on podocytes (Shi et al., 2008; Ishii et al., 2020), such as miRNA-21 (Kölling et al., 2017), miRNA-29 (Lin et al., 2014), and miRNA-30 (Wu et al., 2014). Strikingly, exosomal miRNA seems to be the specific focus in podocyte injury (Jin et al., 2019). Inflammation is the third hotspot in podocyte injury research. Inflammation is associated with podocyte dysfunction (Reidy et al., 2014). Many scholars have found that inflammasome activation leads to podocyte injury and glomerulosclerosis (Zhang et al., 2012; Abais et al., 2013; Shahzad et al., 2015). Notably, inflammation is an important

bridge to podocyte injury studies. It often mediates podocyte injury together with autophagy and oxidative stress (Hou Y. et al., 2020; Dai et al., 2021).

Our research still has some limitations. First, this study only focused on podocyte injury, and we enriched the search strategy as much as possible. Still, some studies about podocytes were not included in the analysis. Second, most of the results of this study are based on machine algorithm, which is slightly insufficient in artificial induction. Finally, some new research fields related to podocyte injury may not have been included, and which may be due to the sensitivity of machine algorithms.

CONCLUSION

Using bibliometric analysis, we found that the research on podocyte injury has a good research prospect. Publications related to podocyte injury are increasing exponentially. DKD is the current focus of research in this field, and autophagy, microRNA, inflammasome, oxidative stress, and exosome are potential hotspots in podocyte injury research, which needs more focus. Notably, the protective effect of traditional Chinese medicine monomer on podocyte injury is less exposed, and more research is needed to fill this research gap.

DATA AVAILABILITY STATEMENT

All datasets presented in this study can be found in the article and **Supplementary Material**.

AUTHOR CONTRIBUTIONS

TL and YZ designed the study. MY and YW collected the data. TL, LY, and HM analyzed the data and drafted the manuscript. All authors contributed to the article and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.772386/full#supplementary-material

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Regulatory B Cell Therapy in Kidney Transplantation

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In the context of kidney injury, the role of Bregs is gaining interest. In a number of autoimmune diseases, the number and/or the function of Bregs has been shown to be impaired or downregulated, therefore restoring their balance might be a potential therapeutic tool. Moreover, in the context of kidney transplantation their upregulation has been linked to tolerance. However, a specific marker or set of markers that define Bregs as a unique cell subset has not been found and otherwise multiple phenotypes of Bregs have been studied. A quest on the proper markers and induction mechanisms is now the goal of many researchers. Here we summarize the most recent evidence on the role of Bregs in kidney disease by describing the relevance of *in vitro* and *in vivo* Bregs induction as well as the potential use of Bregs as cell therapy agents in kidney transplantation.

Keywords: regulatory B cells, kidney transplantation, graft tolerance, cell therapy, immunosuppressive therapy

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INTRODUCTION

B cells are traditionally described to show a primarily effector phenotype: antibody-producing cells with the capacity to present antigen and stimulate T cells through cytokine production (Janeway et al., 1987; Ochsenbein et al., 1999; Harris et al., 2000). However, nowadays it is widely accepted the existence of B cell subsets with regulatory phenotypes (Bregs) involved in suppressing the immune response, inducing tolerance and maintaining homeostasis (Wang et al., 2020). Immunomodulatory functions of Bregs could be mediated by the action of soluble molecules such as IL-10, IL-35, TGF-β or Granzyme B or by cell contact-dependent apoptosis-inducing mechanisms such as PD-L1, FasL or TIGIT (Flores-Borja et al., 2013; Tang et al., 2016; Cai et al., 2019; Chesneau et al., 2020).

Despite essential roles in modulating several diseases, Bregs so far are not known to have a unique or exclusive marker that defines them as a population, but they constitute a heterogeneous cell population that possess a regulatory phenotype and can be found at different stages of B-cell development as reviewed in (Rosser and Mauri, 2015; Oleinika et al., 2019; Long et al., 2021). However, several markers have been proposed as enriched or identifiers of Breg populations. Breg are commonly identified by their expression of IL-10, with transitional phenotypes, $CD19^{+}CD24^{Hi}CD38^{Hi}$, as the most abundant phenotype in peripheral blood. Nevertheless, since the initial description of IL-10 + Breg, other populations such as plasmablast ($CD19^{+}CD27^{Hi}CD38^{+}$), regulatory B1 cells ($CD19^{+}CD25^{+}CD71^{+}CD73^{+}$) and memory IL-$10^{+}$ B cells ($CD19^{+}CD24^{Hi}CD27^{+}$IL-$10^{+}$) have been described as Breg subsets, and different effector molecules have been linked to regulatory B cells identification and function, such as Tim-1, CD5, CD1d, CD25, GMZB, FasL, CD71, etc. as summarized in **Table 1**.

The classic conception of the role of B cells in the field of transplantation was called into question in the last decade, following the publication of two simultaneous studies in 2010, highlighting the

TABLE 1 | Breg Human Cellular markers and Effector molecules.

Human regulatory B cell markers		
Breg subsets and molecules	**Markers**	**Reference**
Transitional or Immature B10 cells	$CD19^{+}CD24^{Hi}CD38^{Hi}IL\text{-}10^{+}$	Blair et al. (2010); Zhang et al. (2012); Flores-Borja et al. (2013); Khoder et al. (2014); Liu et al. (2016)
$CD1d^{Hi}$ B10 B cells	$CD19^{+}CD1d^{Hi}CD5^{+}IL\text{-}10^{+}$	Yanaba et al. (2008); Bankoti et al. (2012); Zhang et al. (2012); van der Vlugt et al. (2014); Khan et al. (2015a)
Memory B10 Cells	$CD19^{+}CD24^{Hi}CD27^{+}IL\text{-}10^{+}$	Iwata et al. (2011); Salomon et al. (2017); Hasan et al. (2019)
Br1 Cells	$CD19^{+}CD25^{+}CD71^{+}CD73^{low}$	Kubo et al. (2012); Kim et al. (2016)
Plasmablasts	$CD19^{+}CD27^{Hi}CD38^{+}$	Matsumoto et al. (2014); Shen et al. (2014)
TIM-1^{+} B cells	$CD19^{+}$ TIM-1^{+}(TIM-1^{+} B cells present in different B cell subsets)	Ding et al. (2011); Xiao et al. (2012); Aravena et al. (2017); Cherukuri et al. (2021)
$GMZB^{+}$ B cells	$CD19^{+}GMZB^{+}$($GMZB^{+}$ B cells present in different B cell subsets)	Hagn and Jahrsdörfer (2012); Hagn et al. (2012); Lindner et al. (2013); Chesneau et al. (2015); Durand et al. (2015); Zhu et al. (2017)
$CD9^{+}$ B cells	$CD19^{+}$ $CD9^{+}$($CD9^{+}$ B cells present in different B cell subsets)	Sun et al. (2015); Brosseau et al. (2018a), Brosseau et al. (2018b); Mohd Jaya et al. (2021)
Circulating B cells	$CD19^{+}CD25^{Hi}$ $CD27^{Hi}CD1d^{Hi}CD86^{Hi}$	Kessel et al. (2012)
$TIGIT^{+}$ memory B cells	$CD24^{Hi}$ $CD27^{+}$ $CD39^{Hi}$ $IgD^{-}IgM^{+}CD1c^{+}$ $TIGIT^{+}$	Hasan et al. (2021)
Human Regulatory B Cell Effector Molecules		
Breg subsets and molecules	**Key Features**	**Reference**
IL-10	Induce Treg, maintain NKT homeostasis, supress effector T cells Modulates plasmacytoid dendritic cells and macrophage and function CD9 and Tim-1 are strongly associated to IL-10 production	Yanaba et al. (2009); Mauri and Bosma (2012); Lykken et al. (2015); Lighaam et al. (2018)
IL-35	Promotes IL-35 and Il-10 production by Treg and Breg Inhibits pathogenic Th1 and Th17 responses	Shen et al. (2014); Wang et al. (2014); Choi and Egwuagu (2021)
PD-L1	Inhibits T cell activation and differentiation by binding PD-1	Khan et al. (2015b); Wang et al. (2019)
FASL	Induces T cell apoptosis by binding FAS	Lundy and Boros (2002); Lundy and Fox (2009); Tang et al. (2016); Wang et al. (2017)
TGF-β	Enhances Treg and Breg induction Inhibits Th1 differentiation by inhibiting STAT4	Natarajan et al. (2012); Lee et al. (2014)
GZMB	Induces T cell apoptosis and strongly suppress T cell proliferation by degradation of the T-cell receptor ζ-chain Present in several B cell subsets, in peripheral, most commonly found in plasmablasts	Hagn and Jahrsdörfer (2012); Hagn et al. (2012); Lindner et al. (2013); Durand et al. (2015); Zhu et al. (2017); Chesneau et al. (2020)
CD73	Supresses effector T cell function by producing adenosine. CD73 catalyses the dephosphorylation of adenine to adenosine	Saze et al. (2013); Kaku et al. (2014)
IDO	Promotes Treg and Breg differentiation	Nouël et al. (2015)

Breg: Regulatory B cell, Br1: Type 1 Regulatory B cell, B10: IL-10-producing regulatory B cells, GZMB: Granzyme B, IDO: indoleamine 2,3-dioxygenase.

relevance of B cells in the development of tolerance in renal transplantation. Both studies involved transplant recipients who had developed spontaneous tolerance and stable patients receiving immunosuppressive therapy. The results obtained in "spontaneously tolerant" patients showed the presence of a higher percentage of B cells in peripheral blood, especially naïve and transitional B cells. At the same time, a higher expression of genes involved in B-cell development was also detected in these tolerant patients compared to stable patients on immunosuppressive therapy (Newell et al., 2010; Sagoo et al., 2010).

Since the emergence of these breakthrough results in 2010, the effect of Breg on the development of tolerance has been described several times as reviewed in (Peng et al., 2018; Cherukuri et al., 2021; Long et al., 2021).

The putative tolerance-inducing power of Bregs makes them an interesting target for the development of therapies to combat transplanted kidney rejection. Among the possible treatment strategies that could be considered, two main groups can be distinguished: those aimed at boosting the natural population of Bregs in the donor and, alternatively, therapy based on the transfer of previously expanded or modified Breg *in vitro*. Here we summarize the most recent evidence on the role of Bregs in kidney transplantation by describing the relevance of *in vitro* and *in vivo* induced Breg (iBreg) as well as the potential use of Bregs as cell therapy agents.

IN VIVO BREGS INDUCTION

Several therapeutic strategies have been proposed or found to induce Breg *in vitro* and *in vivo* in human patients, both in preclinical and clinical trials, despite the aforementioned difficulty of accurately identify Breg. In the following section we discuss the different drugs and therapies involved in Breg induction *in vivo*.

Pharmacological Interventions

The use of immunosuppressive regimes combining different drugs has become a staple of clinical transplantation. For the most part, classical immunosuppressive interventions have little to no effect over B cells, and they have shown not to be active inductors of Breg cells with few exceptions.

Starting from classical immunosuppressive regimes, corticosteroids and calcineurin inhibitors (CNI) mildly reduce the number of total naïve and transitional B cells in renal transplant patients, with the exception of tacrolimus having no effect on B cell subsets (Latorre et al., 2016; Rebollo-Mesa et al., 2016; Tebbe et al., 2016; Bottomley et al., 2017).

In patients with IgA vasculitis that had impaired Breg function, the treatment with glucocorticoid prednisolone, promoted an increase in $CD5^+CD1d^+$, $CD5^+CD1d^+$ IL-10^+, and IL-10^+ B cell subsets, accompanied by an increase in the serum IL-10 concentration (Hu et al., 2016). However, in Lupus Nephritis patients, same treatment with prednisolone correlated with lower percentages of $IL10^+$ B cells (Heinemann et al., 2016).

While low to medium doses of mycophenolate mofetil increase Breg subsets, high doses of mycophenolate reduce both B cell IL-10 and CD80/86 expression on B cells in kidney transplant patients. (Matz et al., 2012; Joly et al., 2014; Rebollo-Mesa et al., 2016; Bottomley et al., 2017).

The effect of mTOR inhibitors over Breg subsets has not been clearly stablished. In kidney transplanted patients sirolimus reduced Transitional B cell populations, while in another report in liver transplant patients, it was described to induce Breg when patients were converted to sirolimus from a tacrolimus based regime (Latorre et al., 2016; Song et al., 2020).

Also the effect of the 6-mercaptopurine analog, Azathioprine, on Bregs has been studied, and it's know to reduce total, naïve and transitional B cells (Rebollo-Mesa et al., 2016; Bottomley et al., 2017).

The B cell depleting agent rituximab induces rapid depletion of $CD20^+$ B cells after administration in a dose-dependent manner, lasting as long as 6 months, followed by a slow recovery (Bergantini et al., 2020). Breg frequencies decrease after administration of the drug, while long term effect of rituximab seems to indirectly stimulate bone marrow to produce transitional B cells when B cells are depleted, coupled with a substantial reduction in $CD27^+$ B (memory) cells at long-term follow-up (Moller et al., 2009; Rehnberg et al., 2009).

The costimulation blocker Belatacept has shown promising results in kidney transplanted patients regarding Breg induction. Belatacept increases IL-10 expression and transitional populations, while reducing plasmablast differentiation (Leibler et al., 2014; Xu et al., 2020).

Finally, common induction therapies, such as basiliximab (chimeric anti-CD25) and Thymoglobulin (anti-thymocyte globulin) show no effect over transitional B cells (Longshan et al., 2014; Alfaro et al., 2021) while CAMPATH-1H (anti-CD52) increased transitional B cells and reduced memory B cells (Thompson et al., 2010; Heidt et al., 2012; Cherukuri et al., 2021).

Other immunomodulatory drugs have been described to induce Breg, such as tocilizumab (anti-IL-6) (Assier et al., 2010; Snir et al., 2011), Fingolimod (sphingosine-1-phosphate receptors modulator) (Grützke et al., 2015) and Laquinimod (quinolone-3-carboxiamide) (Toubi et al., 2012).

Cell and Extracellular Vesicles Therapies

Cell therapies earned a lot of interest as a new approach to induce immunosuppression and tolerance, with an increased presence in clinical trials during the last decade.

Mesenchymal stromal/stem cells (MSC) therapy has been at the forefront of cell therapies in the field of transplantation due to their immunomodulatory and regenerative properties (Pittenger et al., 2019). MSC interact with several cell types, including B cells, inducing regulatory B cells while abrogating plasmablast induction, B cell terminal differentiation and inhibiting antibody production (Comoli et al., 2007; Asari et al., 2009; Guo et al., 2013; Franquesa et al., 2015; Gupte et al., 2017; Perico et al., 2018; Chen et al., 2019, 23). The effect and mechanisms of MSC immunomodulatory action on Bregs has been extensively reviewed (Liu et al., 2020).

A promising alternative to MSC cell therapies are extracellular vesicles (MSC-EVs), reviewed in (Gomzikova et al., 2019; Gowen et al., 2020). EVs emulate parental cell properties, MSC-EVs stimulate tissue regeneration and immune modulation and have been proposed to tackle many diseases including kidney diseases and kidney graft rejection. MSC-EVs have been described *in vitro* to be mediators of Breg induction in a dose dependent manner (Budoni et al., 2013). However, MSC-EV involvement in Breg induction is a complex topic as the EV isolation method used might produce opposite effects as we previously described (Carreras-Planella et al., 2019). Highly purified MSC-EV displayed reduced immunomodulatory capabilities on B cells compared to MSC soluble protein enriched fractions.

In addition to MSC, other cell therapies have been tested and described to induce Breg. Regulatory T cells (Treg) therapy using autologous T cells in kidney transplant patients has been associated with a long-lasting dose-dependent increase of marginal B zone B cells, which are associated with IL-10 production and regulation (Harden et al., 2021). In a different study in a mouse model, CAR-Treg specific to the B cell marker CD19 (Imura et al., 2020) suppressed IgG antibody production and differentiation of B cells in a TGF-β–dependent manner. Regulatory T and B cells work in harmony to stablish homeostasis, and both promote each other induction and expansion as seen in different mouse models (Lee et al., 2014; Wang et al., 2015; Chien and Chiang, 2017) *via* IL-10 and TGF-β.

Tolerogenic Dendritic cells (tolDC) are essential for the induction of Breg in humans and their administration has been described to induce Breg (Boldison et al., 2020). No clinical trials have been performed in kidney transplant patients, but in a phase one safety study in diabetic patients, tolDC increased the frequency of regulatory B cells (Giannoukakis et al., 2011).

Indirect Intervention: The Role of Microbiota

Previous methods described to induce Bregs focus on tackling the specific either cellular or molecular pathways involved in the maintenance or induction of Breg, but a different approach with

growing interest during recent years is to promote balanced stress-free metabolic and immune balance.

In this context, an alternative approach to promote graft tolerance and improve patients' quality of life, most likely in combination with previous drug interventions and/or cell therapies, would be focus on metabolic interventions by modulation of gut microbiota or other metabolic pathways.

Gut microbiota and dysbiosis are linked to adverse events, reduced quality of life, and an increase of graft rejection in kidney transplanted patients (Lee et al., 2019; Swarte et al., 2020; Pacaud et al., 2021). Gut microbiota interacts with the immune system generating a balance of inflammatory and regulatory responses that maintain the homeostasis with metabolic and immune system effects outside the gut, including the generation of Bregs. B cells have the capability to recognize different bacterial and viral elements by the BCR and TLRs (Gallego-Valle et al., 2018; Mu et al., 2020; Pacaud et al., 2021) and also cytokines and metabolites derived from these microbes, such as short chain fatty acids (SCFAs) (Rosser et al., 2020; Daïen et al., 2021; Pacaud et al., 2021; Zou et al., 2021) expanding Breg subsets. Recent studies have elucidated the role of the SCFA pentanoate in the modulation of mTOR activity, leading to a significant boost of IL-10 production by LPS or CpG stimulated Breg, and a substantial reduction of B cell apoptosis, in addition to reducing expression of IL-17A in effector T cells by inhibiting HDAC (histone deacetylase) activity *via* epigenetic modulation (Luu et al., 2019). In a different study, direct inhibition of HDAC by Entinostat, an HDAC inhibitor, increased IL-10 production by LPS-stimulated B cells. Entinostat activity prevented HDAC binding to the proximal region of the IL-10 expression promoter, increasing binding of NF-κB p65, and enhancing IL-10 expression (Min et al., 2021).

IBREG CELL THERAPY: IS IT FEASIBLE

Cell therapy is not a new concept anymore and protocols and clinical trials are being set up to promote tolerance in autoimmune diseases and transplantation in the absence or in a minimized immunosuppressive regime. MSC therapy has taken the lead in this area with several clinical trials already published. In parallel, regulatory immune cell types such as Tregs or tolDCs are the main not-modified immune cell types being studied and used for cell therapy in immune mediated diseases and regenerative approaches. Therefore, the idea of a cell therapy product involving Bregs might sound promising although, to this moment, there are no trials on the use of Breg as a cell therapy. The incomplete knowledge on Breg induction and or expansion, stability, and functional potential and the lack of a consensus Breg signature are just some of the hurdles to be bypassed to generate a safe and efficient cell product. Moreover, we might be dealing with different subsets of Breg depending on the induction cocktail and system used that might present different stability and functionality. We are going in depth on it in the next *In Vitro Breg induction (iBregs)*.

Another matter of concern is the antigen specificity of Breg. Recent studies provide evidence for an essential role of antigen recognition by B cells to generate allograft tolerance in murine models (Kimura et al., 2020; Mohib et al., 2020) and in this line, the critical role of BCR and CD40 expression for Breg development and in transplant models is proven. On the other hand, TGF-β seems to mediate a prominent role in allograft survival (Kimura et al., 2020) while IL-10 essentiality in mediating Breg tolerogenic action is questioned. Insight in the role of Breg antigen specificity may bring the development of chimeric antigen receptor (CAR)-lymphocyte generation to produce cellular therapies with targeted Bregs.

Bregs have also been shown to represent a significant source of serum IgM and IgG during adoptive transfer experiments, and produce antigen-specific, polyreactive and autoreactive antibody specificities (Lo-Man, 2011). However, their role in solid organ transplantation still needs to be defined and new technical advances in nanosciences might bring new opportunities into that area.

The effect that donor or recipient-derived Breg could have in modulating the immune reaction remains unknown if we envision a therapy with autologous or allogeneic Breg in autoimmune diseases or organ transplantation. And as in every cell therapy donor origin (autologous or allogeneic) needs to be carefully considered. The age of the patient appears to be a relevant factor in the capacity of Bregs to produce IL-10 since it is impaired in $CD38^{Hi}CD24^{Hi}$ B cells from old individuals (Duggal et al., 2013). Also, autoimmune diseases have been related to disfunctional Bregs where patient's Breg numbers are normal but they lack the immunomodulatory properties related to this cell "subset" and furthermore, Bregs isolated from patients who had suffered renal graft rejection lost their inhibitory capacity (Nouël et al., 2014). This might be a major problem if we think of autologous cell treatment. There are other mechanistic issues that would have to be addressed such as the time needed to produce enough Breg, infusion timing and dosage, route of administration, and GMP compliance.

In Vitro Breg Induction (iBregs)

The implementation of a reliable and reproducible method of *in vitro* Breg induction and expansion from human B cells is highly required for the development of Breg-based cell therapies. The application of a standardized *in vitro* induction method which generates a well-characterized subset of induced Bregs could compensate for the absence of a human Breg biomarker.

Traditional mechanisms of iBregs induction are mostly based on the stimulation of Toll-like receptors 4 and 9 (TLR4 and TLR9) by bacterial-derived LPS and CpG molecules and the ligation of B-cell antigen receptor (BCR) and CD40 to agonist molecules. In this sense, signaling pathways triggered by TLRs and CD40 accompanied by BCR stimulation might lead to IL-10 production, a conventionally used cytokine for the assessment of *in vitro* Breg induction, *via* activation of transcription factors such as STAT3 (Baba et al., 2015). However, there is also evidence of B cell activation towards effector and/or memory phenotypes when using such strong stimulation methods, and therefore inflammatory cytokines should also be carefully monitored in these induction systems (Lighaam et al., 2018).

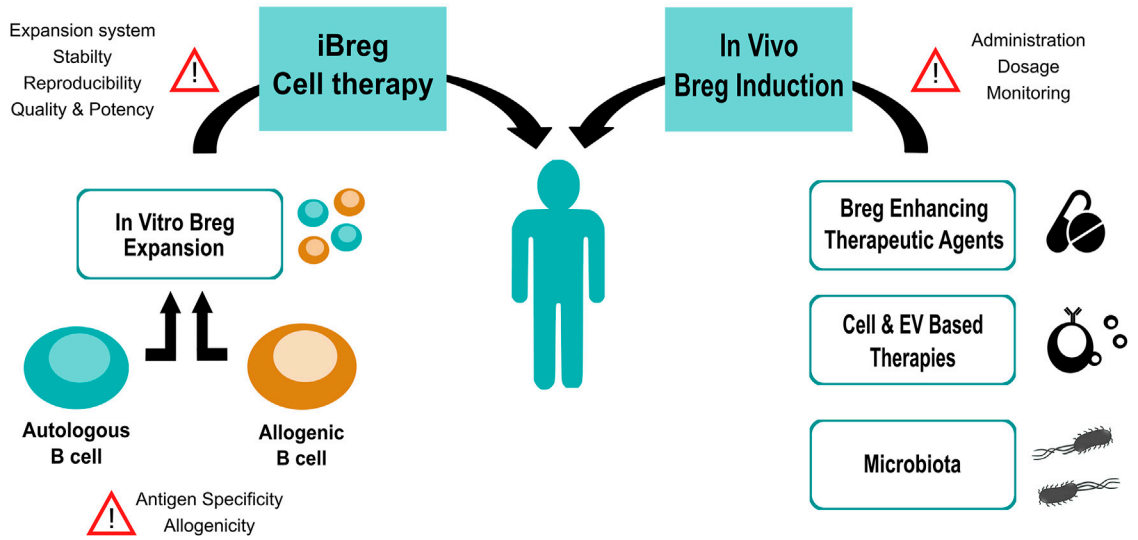

FIGURE 1 | Summarized workflow of Breg-based therapy for kidney transplanted patients, steps and points of concern.

Besides IL-10, several groups have described other molecules as key mediators of iBreg regulatory potential. Several "non-classic" Breg markers have been explored, such as the Granzyme B (GZMB) molecule. Currently, the population of GZMB-expressing B cells is represented as a particular subset of Bregs. In addition, a reproducible protocol for the *in vitro* expansion of this cell subset is already reported in the literature (Chesneau et al., 2020).

As we mentioned in *Indirect Intervention: The Role of Microbiota*, the use of SCFAs is being developed in an attempt to mimic the gut microbiota action. This method would be considered an additional novel form of *in vitro* Breg induction as an alternative to boosting the patient's natural gut microbiota.

Leaving aside the traditional methods and the use of bacterial compounds, our group described the co-culture of tonsil derived-B cells and MSCs from subcutaneous fat as a method of inducing the development of Bregs (Franquesa et al., 2015; Luk et al., 2017).

Common to all the aforementioned methodology, it would be necessary to establish a protocol for the generation of iBregs with a stable phenotype over time. In this regard, the Breg marker CD9 has already been shown to be highly modulated (Mohd Jaya et al., 2021). Therefore, phenotypic and functional stability is a challenge considering the transient nature of some of the Breg phenotypes described (**Table 1**).

DISCUSSION

In this mini-review we aimed to summarize the most relevant and recent evidence on the role of Bregs in kidney disease by discussing the relevance of *in vitro* and *in vivo* Breg induction as well as the potential use of Bregs as cell therapy agents (**Figure 1**). Bregs have been described as major drivers of tolerance in kidney transplantation and in autoimmune diseases. Their upregulated expression has been related to spontaneous tolerant kidney transplant patients, while impaired function and low numbers of Bregs have been associated to several autoimmune diseases.

Breg homeostasis appears to be a cornerstone for immune regulation and tolerance, so several tactics are being approached to reestablish the equilibrium lost in several pathological situations.

One approach is to boost natural Breg populations in patients. Regardless of the limited knowledge of markers, there is already a description of the effect of every immunosuppressant used in kidney transplantation and other anti-inflammatory drugs on Breg populations, allowing for a possible optimization of Breg-induced tolerance. In this line, some immunosuppressants such as Belatacept or Campath, have shown to boost Breg in kidney transplant patients.

Such Breg boosting therapies could be coadjuvant of cellular therapies with MSC or Tregs which have shown capacity to induce Bregs in kidney transplant patients. TolDC have shown similar results in diabetic patients, but have not been tested in clinical trials for kidney transplant. Furthermore, indirect metabolic interventions are slowly gaining track on the field, with increasing publications about the role of gut microbiota and dysbiosis in the maintenance of homeostasis and immune balance, opening new fields of research and clinical research.

On the other hand, Breg application as adoptive cellular therapy is a contentious topic, with no clinical trials on the horizon, due to their difficult identification and expansion. In human, Breg are niche populations in peripheral blood with low expression, with most of them being localized in the spleen, so

in vitro expansion would be necessary to achieve significant therapeutic effects. The lack of consensus on several key points such as; Breg signature, antigen specificity, alloreactivity, expansion system, stability after administration and dosage, would be critical in the efficacy of such therapy.

In recent years, there has been extensive progress in the understanding of Bregs, many researchers are pursuing the definitive human Breg signature, however this seems rather utopic. Many different markers have been associated to Bregs as reviewed in (Rosser and Mauri, 2015; Shang et al., 2020; Catalán et al., 2021), highlighting the fact that they are found across all B cell subsets.

The differences in phenotype and in secreted factors is not just a make-up signature but it affects Bregs' mechanism of action, claiming for proper read-outs for the regulatory activity of Bregs. For long time, IL-10 has been the gold-standard to discern between regulatory and non-regulatory B cells, but evidence has shown that, on one hand IL-10 alone is not enough to truly describe Bregs and iBregs, as IL-10 is also an activation marker, and the ratio between inflammatory markers and IL10 (anti-inflammatory cytokine) might be the real hallmark of Bregs. On the other hand, other secreted factors such as GZMB or the expression of FasL or PD-L1 have also been described as key markers of Bregs and iBregs, opening new doors for their mechanistic paths of regulation.

However, the development of improved *in vitro* Bregs induction systems from human B cells, such as the use of SCFAs or MSCs, opens up the possibility of re-educating the patient's B cells towards a regulatory phenotype and presents a small ray of hope in the context of adoptive Bregs therapy. The ideal *in vitro* induction system should generate, from donor B cells, a sufficient number of iBregs with a stable phenotype and a demonstrable immunodulatory capacity.

AUTHOR CONTRIBUTIONS

SGG, NS-H, and MF summarized the literature and drafted the manuscript. All authors reviewed and approved the final manuscript.

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Targeting Regulatory T Cells for Therapy of Lupus Nephritis

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Lupus glomerulonephritis (LN) is a complex autoimmune disease characterized by circulating autoantibodies, immune-complex deposition, immune dysregulation and defects in regulatory T cell (Tregs). Treatment options rely on general immunosuppressants and steroids that have serious side effects. Approaches to target immune cells, such as B cells in particular, has had limited success and new approaches are being investigated. Defects in Tregs in the setting of autoimmunity is well known and Treg-replacement strategies are currently being explored. The aim of this minireview is to rekindle interest on Treg-targeting strategies. We discuss the existing evidences for Treg-enhancement strategies using key cytokines interleukin (IL)-2, IL-33 and IL-6 that have shown to provide remission in LN. We also discuss strategies for indirect Treg-modulation for protection from LN.

Keywords: IL-2, IL-6, IL-33, Treg cells, SLE, lupus nephritis

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INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a debilitating autoimmune disease characterized by inflammation, increased circulating autoantibodies (autoAb), immune complex (IC) deposition, and multi-organ dysfunction affecting skin, joints, kidneys, brain etc. The cause of SLE is still unclear but genetic, environmental and hormonal factors have been linked to its predisposition. The prevalence of SLE is 366.6/100,000 in USA (2016 estimate) and 97/100,000 in UK (2012 estimate) (Barber et al., 2021). Lupus Foundation of America estimates that at least five million people worldwide may have some form of lupus (https://www.lupus.org/resources/lupus-facts-and-statistics). A majority of the patients affected by lupus progress to lupus nephritis (LN), an end stage renal disease marked by kidney IC deposition and glomerulosclerosis (reviewed in Ward, 2010). There are no approved medications that can cure the disease or provide long term remission with maintenance immunosuppression being the current option. There is thus an urgent need for novel treatment options for LN. Strategies for enhancing regulatory T cells (Treg) have garnered attention recently. While, many studies have employed Tregs for SLE, not enough work has specifically focused on lupus nephritis (LN). Therefore, we also included studies on Treg-based approaches for SLE.

AUTOIMMUNITY AND TREGS

Tregs constitute an important immune cell subset that prevents abnormal activation of the immune system and provide tolerance in allergy and autoimmunity (reviewed in Sharma and Kinsey, 2018). The role of Treg-deficiency in the onset and progression of autoimmunity has long been appreciated. In humans and mice, Tregs are identified as T-cells expressing high levels of IL-2 receptor alpha (CD25) and the transcription factor forkhead box P3 (Foxp3). Alongside IL-2 and CD25, which

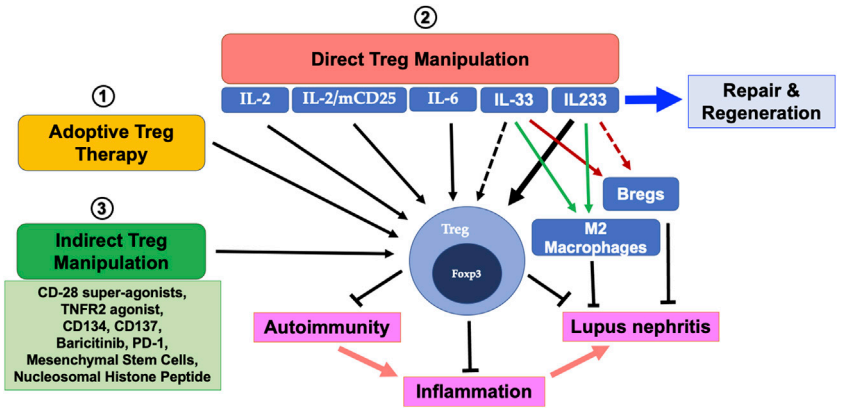

FIGURE 1 | Treg supplementation therapies for autoimmunity and lupus nephritis (LN): **1)** Adoptive Treg transfer therapy has been successfully employed to attenuate inflammation and render protection in acute injury settings and recently, a similar approach was successfully employed in a SLE patient with beneficial outcomes. **2)** Cytokines IL-2, IL-2/mCD25, IL-6 and IL233 have been shown to directly cause robust expansion of Tregs which prevents autoimmunity and LN. Whether IL-33, which is reported to exhibit its protective effects on LN through modulation of M2 macrophages and Bregs have a direct effect on Tregs is yet to be elucidated. Similarly, IL233 has been shown to expand Tregs and induce a M2 phenotype but its effects on Bregs are currently unknown. **3)** Non-cytokine-based approaches using agonists, small molecules, mesenchymal stem cells and nucleosomal histone peptides have been shown to support Treg expansion to target LN.

facilitate the development, function and stability of Tregs, numerous other cell-surface receptors for Tregs have been identified (reviewed in Sharma and Kinsey, 2018). IL-2 supports Treg development in the thymus (Vang et al., 2008) and is also required for their survival and function in the periphery (Fontenot et al., 2005; Barron et al., 2010; Sharma and Kinsey, 2018). While mechanisms of Treg-intrinsic function are still being recognized, factors including excessive production of pro-inflammatory cytokines and resistance to Treg-mediated suppression are being found to contribute to autoimmunity. A strong association of Treg defect in SLE and other human autoimmune diseases is now well-appreciated. Tregs not only control innate and adaptive immunity, but also regulate cellular damage and promote repair (reviewed in Shevach, 2018; Sharma, 2020). Thus, Treg-enhancement strategies remain an attractive strategy for remission from LN (**Figure 1**).

IMMUNE-DYSREGULATION IN LUPUS NEPHRITIS

T cell activation is one of the major pathogenic mechanisms in the immune-dysregulation observed in lupus patients (reviewed in Mountz et al., 2019). Lupus patients have an elevated number of T effector cells, indicating a continued pathogenic response triggered either by pathogens that may mimic self-antigen (Zhao et al., 2019a; Múnera et al., 2020; Munroe et al., 2020), uncleared self-antigen from dying cells (reviewed in Muñoz et al., 2010; Prechl et al., 2016), or altered cytokine milieu contributing to effector cell bias (reviewed in Bengtsson and Rönnblom, 2017; Paquissi and Abensur, 2021). The role of complement proteins in LN is quite complex, where tissue damage is induced by complement deposition, and hypocomplementemia of C3 and C4 is pronounced in active disease, suggesting that complement-mediated clearance of autoantigen and IC play a central role in pathogenesis (reviewed in Li et al., 2021). The effector T-cells on one hand can induce cell-mediated organ dysfunction directly, they also activate innate-immune cells including macrophages and dendritic cells (DC) for fueling the immune dysregulation, as well as differentiate in to T-follicular helper (TfH) cells to help auto-reactive B-cells to produce high-affinity autoAb (reviewed in Mountz et al., 2019; Chen and Tsokos, 2021). The role of cytokines in lupus is supported by reports of elevated Tumor necrosis factor (TNF)-α and Interferon (IFN)-γ in SLE patients (Harigai et al., 2008). Other proinflammatory mediators including IL-6 and IL-17 lead to TH17/Treg dysregulation in lupus patients compared to healthy individuals (Tang et al., 2019).

IL-2: A MASTER REGULATOR OF TREGS IN LUPUS NEPHRITIS

IL-2 was initially discovered as a growth factor that promoted T-cell proliferation *in vitro* (Morgan et al., 1976), but was later realized to be more critical for Treg-maintenance (Fontenot et al., 2005) and T-cell regulation (reviewed in Chen and Tsokos, 2021). IL-2 deficiency may contribute to autoimmunity in SLE patients and lupus-prone mice by inducing paucity of Tregs, defective activation-induced cell death (AICD) and increased IL-17 production (Lippe et al., 2012). Two back-to-back reports first showed the induction of IL-17 producing TH17 cells by TGFβ and IL-6 (Bettelli et al., 2006; Mangan et al., 2006). Subsequently, Laurence et al. showed that IL-2 through STAT5 directly inhibits the STAT3-mediated TH17 differentiation, because blocking of IL-2 or deletion of STAT5 in CD4 T-cells enhanced RORγT, a key transcription factor for TH17 differentiation (Yang et al., 2011). The role of IL-2/STAT5 in TH2 responses as well as inhibition of TH1 was first showed by W. E. Paul and colleagues (reviewed in Zhu et al., 2006). Accordingly, in the setting of multi-organ

inflammatory disease in the Foxp3-mutant scurfy mice, it was shown that deletion of IL-4 or STAT6 (which is downstream to IL-4/IL-13 signaling) enhanced TH1 responses, thus showing the protective effects of TH2 in autoimmune inflammation in several organs (Sharma et al., 2011; Ju et al., 2012). Recently, Hsiung et al. (2020) showed that inflammation leads to lower levels of IL-2R signaling in Tregs, which further lowers their suppressive function. They concluded that low dose IL-2 therapy could possibly overcome the negative effects of inflammation on Tregs. Several investigators have tried to utilize the ability of IL-2 to expand Tregs for protection in autoimmune diseases (Fontenot et al., 2005; Bayer et al., 2007; Grinberg-Bleyer et al., 2010). However, in a study using the NZB/W mice, long-term treatment with a low dose of 1,500 units/day IL-2 or a high dose of 15,000 units/day did not alter the active lupus-like disease or splenocyte populations (Owen et al., 1989). In an interesting approach, a fusion protein of IL-2 with IL-2Ra (CD25) joined by a linker and termed mIL-2/CD25 inhibited LN in female NZB x NZW and male MRL/lpr mice (Xie et al., 2021). The treatment induced Treg expansion without elevation of NK, $CD4^+$/$CD8^+$ T cells and proinflammatory cytokines.

In clinical studies treatment of SLE patients with low dose IL-2 increased Tregs, decreased AICD and restored CTL responses along with a reduction of serum anti-dsDNA antibodies (von Spee-Mayer et al., 2016; He et al., 2016). Another clinical study in refractory SLE patients reported that the SLEDAI scores (a disease activity index for lupus patients) were significantly lowered with low dose IL-2 and rapamycin treatment and normalization of TH17/Treg ratios were significantly reduced (Zhao et al., 2019b). An interesting study focusing on the safety and efficacy of low dose IL-2 in 11 autoimmune disease in 46 patients including SLE found significant Treg-expansion without effector T-cell activation (Rosenzwajg et al., 2019). While low dose IL-2 has shown promise for SLE patients, several study results are still pending. Patients often receive multiple injections due to the short half-life of IL-2 and there is a concern of side effects triggered by low dose IL-2 mediated activation of cytotoxic lymphocytes and NK cells.

Low Dose IL-2 Therapy for Other Autoimmune Diseases

IL-2 therapy has also been successfully tested in other lesser studied autoimmune diseases. A Phase I/II clinical trial on low dose IL-2 therapy in HCV-induced vasculitis increased Treg levels and reduced vasculitis (Saadoun et al., 2011). In a study on Alopecia areata (Castela et al., 2014), low dose IL-2 achieved local recruitment of Treg and also caused regrowth of scalp hair in the majority of participants. This study emphasized the importance of Treg supplementation in conditions involving skin lesions. In a study focusing on autoimmune hepatitis, low dose IL-2 normalized liver enzyme levels (Lim et al., 2018). The TRANSREG clinical trial that comprised of patients with 11 autoimmune diseases also showed promising findings with low dose IL-2 administration (Rosenzwajg et al., 2019). Other reports in primary Sjogren's syndrome (Miao et al., 2018), Polymyositis/Dermatomyositis (Zhang et al., 2021), Psoriatic arthritis (Wang et al., 2020) and Amyotrophic lateral sclerosis (Camu et al., 2020) also showed benefits of low dose IL-2 therapy.

IL-33: AN ALARMIN CYTOKINE THAT REGULATES INNATE IMMUNITY AND INFLAMMATION

Interleukin-33 was identified as a nuclear cytokine belonging to the IL-1 family that is known to induce proinflammatory cytokines and expression of integrins on leukocytes and endothelial cells (Dinarello, 2009; Spooner et al., 2013). The pathway for IL-33 and its receptor ST2 is actively being pursued for immune modulation during autoimmune and inflammatory diseases (Monticelli et al., 2011; Byers et al., 2013). Two forms of ST2: transmembrane (mST2) and soluble isoforms (sST2) have been described (Mueller and Jaffe, 2015). The sST2 has been identified as a biomarker for cardiac, pulmonary and graft-versus-host diseases (Bajwa et al., 2015; Bayes-Genis et al., 2015; Xiao and Zheng, 2016). Recent studies have demonstrated a protective role of IL-33/ST2 in several inflammatory settings (Miller et al., 2008; Mato et al., 2009; Milovanovic et al., 2012). In the hTNF.Tg model of inflammation, IL-33-treatment induced alternatively activated macrophages (AAM) and inhibited TNFα-induced bone-loss (Zaiss et al., 2011). Interestingly, IVIG treatment was shown to suppress autoimmunity through IL-33 induced TH2 responses (Anthony et al., 2011), suggesting that IL-33/ST2 pathway may activate anti-inflammatory mechanisms in other settings. Prior studies on IL-33 have established that recombinant IL-33, both *in vitro* and *in vivo*, expanded $ST2^+$ Tregs. *In vitro*, these $ST2^+$ Tregs were more efficient in suppressing IL-12/IL-33 driven $CD8^+$ T cell IFNγ production than their $ST2^-$ counterpart (Matta et al., 2014). Early IL-33 administration in lupus prone NZB/W F1 mice was shown to induce regulatory B cells and reduce autoantibody levels. Additionally, an M2 macrophage signature was also induced implying a regulatory role of IL-33 in lupus onset (Mohd Jaya et al., 2020). Contrastingly, in a previously published study, antibody-mediated IL-33 neutralization suppressed disease in lupus prone mice along with an increase in Tregs and reduced IL-17 levels (Li et al., 2014). There is thus a disconnect in the available literature on the effects of IL-33 and its role in LN warrants further investigation.

IL-6: A PARADOXICAL TARGET FOR TREG EXPANSION

IL-6 was originally identified as a B-cell differentiation factor that affects the autoantibody and cytokine production. IL-6 plays a key role in defense to infections by regulating the proinflammatory and regulatory T cells as well as contribute to autoimmunity. Elevated serum IL-6 levels correlate with disease severity in SLE patients (Chun et al., 2007). Pathological role of IL-6 has been demonstrated in disease development via targeting IL-6 with anti-IL-6 antibodies or by

gene knockout approaches by several investigators. Indeed, IL-6 suppression was shown to rescue from rheumatoid arthritis (Narazaki et al., 2017), SLE (Tackey et al., 2004), scleroderma (O'Reilly et al., 2013) and many other diseases. Both TH17 and Tregs require TGFβ for their differentiation through induction of both RORγt and Foxp3 expression, where IL-6 negatively regulates Tregs by promoting TH17 (Zhou et al., 2008). The regulation of TH17/Treg differentiation by IL-6 explains the involvement of this pro-inflammatory cytokine in diseases that exhibit a prominent TH17 signature as a causative factor. While underlying mechanism for the observed efficacy of IL-6 blockade therapies are yet to be uncovered, the paradigm that IL-6 is associated with disease initiation and progression stands challenged by recent observations from Steinmetz and colleagues for a protective role of IL-6 in LN (Hagenstein et al., 2019). In 2016, Tregs dually expressing the transcription factors Foxp3 and RORγt were identified and were given the term "biTregs" for their roles in nephrotoxic nephritis (NTN) model of LN (Kluger et al., 2016). It was found that RORγt$^+$Foxp3$^+$ biTregs expanded with LN in the spleens and kidneys. The expansion of this subset of cells were higher than the expansion of conventional Tregs (cTregs: Foxp3$^+$ Tregs) and IL-17$^+$ Tregs. Further characterization also confirmed that the biTregs did not differentiate into cTregs or Th17 cells and that the IL-17 production by these cells was dependent on RORγt. It was concluded that the biTregs are a novel bifunctional Treg lineage with distinct properties and maybe a novel therapeutic target for LN. The observation that IL-6 did not inhibit Treg development or activation in the disease setting, but rather enhanced T effector activation that caused loss of Treg-mediated suppression led Steinmetz group to focus on the biTregs as an active effector Treg lineage (Nish et al., 2014; Ohnmacht et al., 2015; Sefik et al., 2015; Kluger et al., 2016; Yang et al., 2016). Utilizing IL-6 knockout mice, Hagenstein and colleagues (Hagenstein et al., 2019) further showed that IL-6 is required for the anti-inflammatory function of Tregs, as IL-6 treatment resulted in a significant expansion of RORγt$^+$ biTregs and correlating with protection against LN.

IL233: A NOVEL HYBRID CYTOKINE FOR LN REMISSION

IL-2 defect parallels with progression of autoimmunity and LN, and also correlates with reduced levels of Tregs. We found that IL-2 also regulates the expression of IL1RL1 (ST2; IL-33 receptor) on CD4 T-cells (Sharma et al., 2011). In addition, it was found that a major subset of Tregs expresses ST2 and that IL-33 expands Tregs *in vitro* and *in vivo*. Importantly, a novel cooperation between IL-2 and IL-33 was identified, which protected against inflammatory diseases by expanding, activating and mobilizing Tregs more efficiently than either cytokine alone. For more efficient restriction of this cooperativity to Tregs, a fusion cytokine of IL-2 and IL-33 (termed IL233) was generated (Stremska et al., 2017). IL233 exerted a sustained increase in Tregs, specifically in the renal lymph nodes and protected mice from LN more efficiently than IL-2 and IL-33 injected either alone or together. IL233 treatment not only prevented onset of LN, but also induced lifelong remission in moderate to severely proteinuric NZM2328 mice without any detectable side effects. IL233 treatment also inhibited the progression of LN in proteinuric MRL/lpr mice and protected them from early mortality (Stremska et al., 2019). The mechanisms, in addition to increasing Tregs, suggested restoring of IL-2 production by T-cells and tolerance induction via reduced expression of CD80 and CD86 on macrophages and DC. Interestingly, autoAb production and IC deposition were not significantly inhibited, suggesting a disconnect between autoAb and end-organ failure (Clynes et al., 1998; Ge et al., 2013). Thus, IL233 treatment demonstrated therapeutic potential in IFNα-accelerated and spontaneous LN in NZM2328 as well as MRL/lpr mice, indicating its general applicability.

ALTERNATE STRATEGIES FOR TREG EXPANSION

A number of alternate strategies are reported for *in vivo* Treg activation and expansion. These included the use of Treg-related cell surface proteins, signaling and epigenetic modulation by small molecules, as well as approaches using auto-antigen peptides and microbes. CD28 is a co-stimulatory molecule expressed on T-cells and engaged by CD80/CD86 on antigen presenting cells for activation and cell cycle. In a rat model of anti-Thy1 nephritis, it was observed that CD28 super-agonists (CD28SA) at low levels efficiently expanded Tregs, and decreased proteinuria and serum creatinine levels (Miyasato et al., 2011). The roles of TNFRs (Tumor Necrosis Factor Receptors) are being recognized and TNFR2 is highly expressed in Tregs. TNF-α is a pleiotropic cytokine, which exists in both membrane-bound and soluble forms. The membrane bound TNF-α preferentially interacts with TNFR2 and results in suppressive function due to lack of cytoplasmic death domain. In a model of chronic inflammation, use of TNFR2 specific agonist TNCscTNF80 resulted in Treg expansion and reduced inflammation in mouse model of rheumatoid arthritis (Schmid et al., 2017). Similar to TNRF2, Ox40 (CD134) is expressed on Tregs and effector T cells. Ox-40 expression was elevated in several T-cell subsets in proteinuric NZB/W F1 mice. While, treatment with antagonistic Ox40 mAb accelerated autoAb and LN, administration of an agonistic Ox40:Fc fusion protein in an IFNα-accelerated lupus model significantly delayed the onset of severe proteinuria and improved the survival, suggesting a benefit of targeting this pathway (Sitrin et al., 2017). However, the effect of Ox40:Fc on Tregs was not evaluated, despite earlier observations that Ox40-deficient mice had reduced Tregs and that Ox40L overexpression increased Tregs in the spleen without disease suppression, probably due to evasion of Treg-mediated suppression by Ox40 engagement on effector T-cells (Takeda et al., 2004), Analogous to CD134, CD137 is highly expressed on Tregs and a study by Sun et al., showed that treatment with agonistic monoclonal antibody to CD137 blocked spontaneous autoimmunity in MRL/lpr mice (Sun et al., 2002), likely by promoting Treg expansion (Zhang et al., 2007).

Baricitnib, a selective inhibitor of Janus kinase (JAK1) and JAK2 is approved for use in rheumatoid arthritis. In a recent study, Lee et al. observed that treatment with baricitnib could effectively attenuate lupus-like phenotype in MRL/lpr mice. This was accompanied by reduction of total, $CD8^+$ and abnormal double-negative T-cells along-with a greater proportion of follicular ($CXCR5^+$) and extra-follicular ($CXCR5^-$) Tregs, which could have afforded protection (Lee et al., 2021). Checkpoint inhibitors are currently being tested for treatment of several forms of cancer and their effects on Tregs remains controversial. Interestingly, in NZB/W F1 mice, anti-PD1 antibody treatment reduced $CD4^+PD\text{-}1^+$ T cells, promoted suppressive capability of Tregs and ameliorated LN (Wong et al., 2013). Mesenchymal stem cells (MSCs) are multipotent progenitor cells exerting immunosuppressive capacity with respect to both innate and adaptive immune response. MSCs are used in clinical settings to treat various lupus like conditions for over a decade (Zhou et al., 2020). Gazdic et al. (2018) demonstrated that indoleamine 2,3-dioxygenase (IDO) secreted from MSCs stimulates expansion and activation of Tregs to produce IL-10. Whether such effects can have therapeutic efficacy in LN setting is yet to be explored.

Several autoantigen peptide-based approaches are being developed to deliver these molecules to autoreactive T cells to promote clonal deletion or to develop immunoregulation. Kang et al. (2005) showed that a very low-dose of nucleosomal histone peptide efficiently controlled lupus in SNF1 lupus prone mice and induced Treg-subsets. The role of microbiome and probiotics in immune-regulation of SLE is also being actively researched. It was found that certain *Clostridium* strains reduced TH17 cells and induced Tregs. Further, *Bifidobacterium bifidum* supplementation prevented CD4 T-cell overactivation to restore Treg/Th17/Th1 imbalance in SLE via expanding $Foxp3^+IL\text{-}17^+$ populations, however, its effect on LN is still pending (López et al., 2016).

CONCLUSIONS

As of today, there are 173 clinical trials ongoing for therapy of LN. The field of lupus research, having seen some promising therapeutics is now fraught with termination of ongoing trials due to unwarranted effects. There still is no approved drug that can be safely used long term to treat patients with LN. Tregs can be targeted effectively for LN remission, however, there are limited studies focusing on Treg supplementation and its probable adverse effects, if any. With the identification of key cytokines and alternative strategies to regulate Treg function, expansion and activation, it is imperative to continue research efforts on Treg-based strategies for remission from LN.

AUTHOR CONTRIBUTIONS

RS and RV conceived the idea. RS, RV, VS and MD wrote the manuscript.

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Dapagliflozin Alleviates Renal Fibrosis by Inhibiting RIP1-RIP3-MLKL-Mediated Necroinflammation in Unilateral Ureteral Obstruction

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Dapagliflozin, a sodium-glucose cotransporter-2 inhibitor, offers renoprotection in diabetes. However, potential for use in nondiabetic kidney disease remains unknown. Herein, we assessed whether dapagliflozin alleviates renal fibrosis by interfering with necroinflammation in a rat model of unilateral ureteral obstruction (UUO) and *in vitro*. After induction of UUO, rats were administered dapagliflozin daily for seven consecutive days. UUO induced significant renal tubular necrosis and overexpression of RIP1-RIP3-MLKL axis proteins; these coincided with NLRP3 inflammasome activation, and subsequent development of renal fibrosis. Oxidative stress caused by UUO is tightly associated with endoplasmic reticulum stress and mitochondrial dysfunction, leading to apoptotic cell death through Wnt3α/β-catenin/GSK-3β signaling; all of which were abolished by both dapagliflozin and specific RIP inhibitors (necrostatin-1 and GSK872). In H_2O_2-treated HK-2 cells, dapagliflozin and RIP inhibitors suppressed overexpression of RIP1-RIP3-MLKL proteins and pyroptosis-related cytokines, decreased intracellular reactive oxygen species production and apoptotic cell death, whereas cell viability was improved. Moreover, activated Wnt3α/β-catenin/GSK-3β signaling was inhibited by dapagliflozin and Wnt/β-catenin inhibitor ICG-001. Our findings suggest that dapagliflozin ameliorates renal fibrosis by inhibiting RIP1-RIP3-MLKL-mediated necroinflammation via Wnt3α/β-catenin/GSK-3β signaling in UUO.

Keywords: dapagliflozin, unilateral ureteral obstruction, necroinflammation, oxidative stress, endoplasmic reticulum stress, mitochondria

INTRODUCTION

Globally, chronic kidney disease (CKD) causes increased socioeconomic burdens. Despite strict blood glucose control and use of hypotensive or antiproteinuric drugs, growth in the incidence of CKD and progress to end-stage renal disease continues unabated. According to national reports, the prevalence of CKD is proximately 14% in the United State and 10.8% in China (Zhang et al., 2012; Murphy et al., 2016). Renal fibrosis characterized by accumulation of extracellular matrix, infiltration of inflammatory cells,

tubular epithelium cell apoptosis, and activation of fibroblasts, are the common signs of progressive CKD from virtually any etiology. Utilizing a well-known model of unilateral ureteral obstruction (UUO), we and others have defined important players, including oxidative stress, inflammatory mediators, transforming growth factor (TGF)-β1, and programmed cell death (Martínez-Klimova et al., 2019; Jin et al., 2020). Of these, oxidative stress-originated inflammation plays a critical role because it precedes ongoing renal scarring.

Necroptosis is a genetically regulated form of cell death modulated by receptor-interacting protein kinases one and 3 (RIP1 and RIP3, respectively) and downstream substrate pseudokinase mixed-lineage kinase domain–like (MLKL) (Choi et al., 2019). Dying necrotized cells release danger-associated molecular patterns (DAMPs), which subsequently activate innate immunity to evoke sterile inflammatory responses. The sterile inflammation in turn exacerbates necroptosis via two distinct pathways, via tumor necrosis factor-alpha (TNF-α) or interferon-gamma (Mulay et al., 2016). Driven by necrosis and inflammation, this autoamplification loop is referred to as necroinflammation. Emerging evidence demonstrates that RIP1-RIP3-MLKL-mediated necroinflammation is involved in the pathogenesis of obstructive nephropathy (Imamura et al., 2018; Popper et al., 2019), acute kidney injury (AKI) (Mulay et al., 2019), and the progression of AKI to CKD (Chen et al., 2018).

Sodium-glucose cotransporter 2 (SGLT2) inhibitors have been approved for the treatment of all stages of type 2 diabetes mellitus (T2DM). These drugs directly block SGLT2 and thereby inhibit renal glucose reabsorption, promote urinary glucose excretion, and effectively lower hyperglycemia. Because of these activities, SGLT2 inhibitors allow better blood glucose control compared with other antidiabetic agents beyond those with insulin-dependent actions. In addition, SGLT2 inhibitors have several advantages for patients with T2DM, including lower risk of hypoglycemia, reduced body weight (BW), and lower blood pressure (Verma and McMurray, 2018). Furthermore, clinical trials have demonstrated that SGLT2 inhibitors confer renoprotective and cardioprotective effects in patients with T2DM (Kubota et al., 2018; Neuen et al., 2019). Similar SGLT2 inhibitor renoprotective effects have been shown in animal models of renal ischemia/reperfusion injury (I/R) (Chang et al., 2016b; Rezq et al., 2020), gentamicin-induced nephrotoxicity (Mohamed et al., 2019), and chronic tacrolimus nephropathy (Jin et al., 2017). Nevertheless, the benefits of SGLT2 inhibitors in renal fibrosis of nondiabetic CKD have yet to be elucidated.

As such, we sought herein to assess whether dapagliflozin (Dapa) treatment affords renoprotection against renal fibrosis by inhibiting necroinflammation in a rat model of UUO and *in vitro*.

MATERIALS AND METHODS

Experimental Groups and Treatment Protocol

Animal care and experimental procedures were reviewed and approved by the Animal Experimentation Ethics Committee of Yanbian University (SYXK [J]2020–0009) and the Animal Care Committee at the Medical College of Yanbian University (YBU-2019–11–27). Weight-matched male Sprague-Dawley rats weighing 240–260 g were housed in individual cages with a 12 h artificial light-dark cycle and permitted free access to standard chow and water. Following acclimatization for 1 week, rats were randomized into one of five groups and treated daily for 7 days: 1) sham group, sham operated rats without treatment; 2) UUO group, UUO rats without treatment; 3) UUO + Dapa group, UUO rats received Dapa treatment (10 mg/kg oral gavage, T2389/461,432–26–8, TargetMOI®) (Kapoor et al., 2015); 4) UUO + necrostatin-1 (Nec-1) group, UUO rats received Nec-1 treatment (2 mg/kg oral gavage, A4213, APExBIO) (Shen et al., 2019); 5) UUO + GSK872 group, UUO rats received GSK872 treatment (1 mg/kg intraperitoneal, HY-101872, MedChemExpress) (Guo et al., 2019). The UUO model was created as described previously (Jin et al., 2020). Briefly, rats were anesthetized with 1% pentobarbital sodium (40 mg/kg; Sigma-Aldrich) and a flank incision was made. After exposure of the kidney and ureter, the left ureter was ligated with 4–0 silk, and then the incision was sutured. Sham operations were like UUO, without ligation of the left ureter. Administration of Dapa, Nec-1, and GSK872 were started 24 h after UUO and continued for seven consecutive days. Rats were euthanized at the end of the study. Blood, urine, and kidney samples were rapidly collected for further examination.

Biochemical and Functional Measurements

Body weight was monitored daily. At the end of the study, animals were placed individually in metabolic cages (ZH-B6, Anhui, China) and their water intake and urine volume were measured over a 24 h period. Fasting glucose levels (overnight) were measured from a drop of blood obtained from the tail vein, using a rapid glucose meter (ONETOUCH UltraVue, Johnson co, China). Urine protein excretion (UPE) was examined using enzymatic colorimetric methods (Roche Cobas 8,000 Core ISE, Roche Diagnostics, Hoffmann-La Roche Ltd, Basel, Switzerland). Renal function, serum lipid profiles, whole blood hemoglobin A1c (HbA1c), and high-sensitivity C-reactive protein (hs-CRP) were analyzed by an autoanalyzer according to the manufacturer's instructions (Roche Cobas 8,000 Core ISE, Roche Diagnostics, Hoffmann-La Roche Ltd, Basel, Switzerland).

Antibodies

The following antibodies were used: RIP1 (#53286, Cell Signaling Technology; 1:500), RIP3 (#ab62344, Abcam; 1:500), MLKL (#ab243142, Abcam; 1:500), interleukin-1beta (IL-1β, #ab9722, Abcam; 1:500), IL-18 (#ab191860, Abcam; 1:500), NOD-like receptor pyrin domain-containing protein 3 (NLRP3, #ab214185, Abcam; 1:200), ectodermal dysplasia-1 (CD68/ED-1, #ab125212, Abcam; 1:200), transforming growth factor-beta1 (TGF-β1, #ab179695, Abcam; 1:1000), connective-tissue growth factor (CTGF, #ab6992, Abcam; 1:500), 8-hydroxy-2′-deoxyguanosine (8-OHdG, JaICA, Shizuoka, Japan; 1:200), superoxide dismutase-2 (SOD2/MnSOD, #ab13534, Abcam; 1:1000), nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX-4, NB110-58849, Product Datasheet, Novus Biologicals; 1:500), PINK1 (N4/15, #ab186303, Abcam; 1:500), Parkin (#2132, Cell Signaling Technology; 1:1000), p62 (#ab56416, Abcam; 1:500), succinate dehydrogenase complex

subunit A (SDHA, #ab66484, Abcam; 1:1000), C/EBP homologous protein (CHOP, L63F7, #2895, Cell Signaling; 1:500), inositol-requiring protein-1α (IRE-1α, phospho S724, #ab37073, Abcam; 1:500), B-cell lymphoma-2 (Bcl-2, #ab196495, Abcam; 1:1000), Bcl2-associated X (Bax, #ab32503, Abcam; 1:1000), cleaved caspase-3 (#ab2302, Abcam; 1:500), Wingless-type MMTV integration site family member 3a (Wnt3a, #ab219412, Abcam; 1:500), glycogen synthase kinase-3beta (GSK-3β, #12456, Cell Signaling Technology; 1:1000), beta-catenin (β-catenin, #ab32572, Abcam; 1:1000), beta actin (β-actin, #ab8226, Abcam; 1:2000).

Histopathological Examination

The kidney tissues were fixed in periodate-lysine-paraformaldehyde solution and embedded in wax. Following dewaxing, 4 μm sections were conducted and stained with hematoxylin-eosin (HE) and Masson's trichrome. The quantitative analysis of fibrosis was performed using a color image auto-analyzer (VHX-7000, Leica Microsystems, Germany). A minimum of 20 fields per section was evaluated by counting the percentage of injured areas under ×100 magnification. Histopathological analysis was conducted in randomly selected fields of sections by a pathologist blinded to the assignment of the treatment groups.

Immunohistochemistry

Immunohistochemical staining was performed as described previously (Jin et al., 2020). 8-OHdG and ED-1 were detected in 4 μm tissue sections with specific antibodies. Twenty different fields in each section at ×400 magnification were analyzed using a color image analyzer (VHX-7000, Leica Microsystems, Germany).

Transmission Electron Microscopy

Transmission electron microscopy was performed as we previously detailed (Zhao et al., 2021b). Kidney tissues were post-fixed with 1% OSO4 and embedded in Epon 812 following fixation in 2.5% glutaraldehyde in 0.1M phosphate buffer. Ultrathin sections were cut and stained with uranyl acetate/lead citrate, and photographed with a JEM-1400Flash transmission electron microscope (JEM-1400Flash HC, JEOL Ltd, Tokyo, Japan). Using an autoimage analyzer, the number and size of mitochondria were measured in 20 random unoverlapped proximal tubular cells (VHX-7000, Leica Microsystems, Germany).

Immunoblotting Analysis

Immunoblotting was fulfilled as described previously (Zhao et al., 2021b). Images were analyzed with an image analyzer (Odyssey® CL Imaging System, LI-COR Biosciences, NE, United States). Optical densities were obtained using the sham group as 100% reference and normalized with β-actin.

In situ TdT-Mediated dUTP-Biotin Nick End Labeling (TUNEL) Assay

Apoptotic cell death was identified using the ApopTag *in situ* Apoptosis Detection Kit (Sigma-Aldrich,Millipore). The number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells was counted on 20 different fields in each section at ×400 magnification.

Enzyme-Linked Immunosorbent Assay (ELISA)

The urine concentration of the DNA adduct 8-hydroxy-2′-deoxyguanosine (8-OHdG) were measured using a competitive enzyme-linked immunesorbent assay (Japan Institute for the Control of Aging, Shizuoka, Japan) according to the manufacturer's instruction. All samples were performed in triplicate and averaged.

Cell Culture and Treatment

Human kidney proximal tubular epithelial cells (HK-2 cells) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, United States). HK-2 cells were grown in Dulbecco's modified Eagle's medium/Nutrient F12 (DMEM/F12; HyClone; GE Healthcare Life Science, Logan, UT, United States) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc, Waltham, MA, United States), 100 U/mL penicillin, and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc, Waltham, MA, United States). The cells were cultured in a humidified incubator with 5% CO2 and 37°C. Following 24 h incubation, cells were pretreated with or without different concentrations of Dapa (5 and 10 μmol/L) for 1 h and then coincubated with or without H_2O_2 (500 μmol/L), Nec-1 (30 mmol/L), GSK872 (3 μmol/L), and ICG-001 (10 μmol/L) for 24 h.

Cell Viability Assay

The viability of HK-2 cells was evaluated using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. Approximately 1.0×10^4 HK-2 cells/well were seeded in a 96-well plate. All groups of cells were treated as above described, then, 10 μL of CCK-8 solution was added to each well and incubated at 37°C for 3h. The absorbance was measured by determining the optical density at 450 nm (VersaMax Microplate Reader, Molecular Devices, LLC, Sunnyvale, CA, United States).

Measurement of Reactive Oxygen Species (ROS) Production

The levels of intracellular ROS production were measured using 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen) according to the manufacturer's instructions. HK-2 cells were seeded at a density of 2.0×10^5 cells/well in a 6-well plate. All groups of cells were treated as above described, and then the cells were washed three times in PBS and incubated with H2-DCFDA for 30 min. The cells were washed and collected in PBS, and fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, United States).

Apoptosis Assay

Annexin V-positive HK-2 cells were detected using an Annexin V-FITC apoptosis detection kit (Biosharp, Hefei, China)

TABLE 1 | Basic parameters in the experimental groups.

Parameters	Sham	UUO	UUO + Dapa	UUO + Nec-1	UUO + GSK872
ΔBW (g)	57 ± 2.7	38 ± 6.5*	40 ± 5.2*	37 ± 7.8*	40 ± 3.1*
WI (ml)	23 ± 1.8	25 ± 3.2	38 ± 2.7*	27 ± 3.0	21 ± 9.4
UV (ml)	18 ± 2.5	21 ± 1.9	35 ± 1.4*	23 ± 3.4	25 ± 2.6
FG (mg/dl)	107.0 ± 8.2	103.2 ± 11.5	91.2 ± 3.2	100.0 ± 6.1	92.1 ± 5.2
HbA1c (%)	3.6 ± 0.01	3.5 ± 0.03	3.8 ± 0.12	3.6 ± 0.04	3.4 ± 0.02
TG (mg/dl)	82.7 ± 17.5	78.3 ± 24.9	82.4 ± 20.1	76.6 ± 16.1	89.3 ± 15.4
CHO (mg/dl)	66.0 ± 4.6	71.0 ± 7.5	62.9 ± 8.6	64.5 ± 8.0	64.3 ± 3.6
HDL-C (mg/dl)	11.2 ± 0.7	12.3 ± 0.3	10.6 ± 0.8	10.2 ± 1.0	9.8 ± 2.0
LDL-C (mg/dl)	14.2 ± 1.1	16.5 ± 0.9	14.4 ± 1.6	15.5 ± 1.6	13.9 ± 0.8
hsCRP (mg/dl)	0.20 ± 0.03	0.21 ± 0.02	0.20 ± 0.03	0.18 ± 0.06	0.21 ± 0.04
UPE (mg/L)	318.8 ± 35.1	289.1 ± 40.6	319.0 ± 49.6	300.7 ± 38.7	301.8 ± 38.0
Scr (mg/dl)	0.30 ± 0.01	0.35 ± 0.02	0.28 ± 0.03	0.32 ± 0.02	0.34 ± 0.04
BUN (mg/dl)	132.8 ± 5.2	143.6 ± 12.3	139.3 ± 16.4	128.7 ± 9.0	142.5 ± 14.5
Cys-c (mg/L)	3.0 ± 0.20	2.9 ± 0.08	3.1 ± 0.13	2.8 ± 0.13	3.2 ± 0.16

*Values are presented as mean ± SEM. UUO, unilateral ureteral obstruction; Dapa, dapagliflozin; Nec-1: necrostatin-1; ΔBW: body weight gain; WI: water intake; UV: urine volume; FG: fasting glucose; TG: triglyceride; CHO: cholesterol; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; hsCRP: high sensitivity C-reactive protein; UPE, urine protein excretion; Scr, serum creatinine; BUN, blood urea nitrogen; CysC, cystatin C. *p < 0.05 vs sham.*

according to the manufacturer's protocol. All groups of cells were treated as above described, and the cells were harvested, washed three times with PBS, and incubated with 1x binding buffer at a concerntration of 1×10^6 cells/mL. Then, the cells were incubated with 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) at room temperature for 15 min in the dark. The samples were analyzed within 1 h using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, United States). Apoptotic cells were determind as a percentage of the total cell count. The percentage of apoptotic cells was calculated as the number of PI-positive and Annexin-V-positive cells divided by the total number of cells. Three independent experiments were performed.

Statistical Analysis

Data are expressed as mean ± SEM. Multiple comparisons between groups were performed using one-way ANOVA and the Bonferroni post hoc test using SPSS software (version 21.0; IBM, Armonk, NY). Statistical significance was assumed at $p < 0.05$.

RESULTS

Effects of Dapa on Functional Parameters

BW loss was seen in all UUO7 groups, with or without drug treatment. Dapa treatment led to polyuria and increased WI during the experiment period. There were no significant differences in levels of UPE, blood lipid profiles and hsCRP within the experimental groups. Neither UUO nor Dapa and RIP inhibitors influenced renal function, as shown by Scr, BUN, and CysC (**Table 1**).

Dapa Alleviates UUO-Induced Necroptosis

Necroptosis has been strongly linked to the development of renal fibrosis in UUO (Xiao et al., 2017). Gross findings from digital images showed that UUO-induced pyelectasis with destruction of the pelvis and an extremely thin cortex, whereas these exterior changes were prevented by either Dapa or RIP inhibitors (**Figure 1A**). HE staining illustrated that UUO led to a significant renal tubular epithelial cell necrosis, tubular atrophy, and vacuolization (**Figure 1B**). Using electron microscopy, we clearly observed swelling of the tubular epithelium and interstitium, swarms of necrotized bodies, cytolysis, and abscission of microvilli in the tubular epithelial cell lumen of obstructed kidneys (**Figure 1C**). Dapa or RIP inhibitor (Nec-1 and GSK872) treatment alleviated these histopathological alterations compared with their untreated counterparts. Consistently, immunoblotting analysis showed that overexpression of RIP1-RIP3-MLKL axis proteins seen in the UUO group was significantly decreased by treatment of both Dapa and RIP inhibitors (**Figure 1D**).

To establish the rat model of UUO, we performed an *in vitro* study in HK-2 cells subjected to H_2O_2 treatment in the presence or absence of Dapa or RIP inhibitors. Consistent with the results of the *in vivo* study, either Dapa or RIP inhibitors suppressed RIP1-RIP3-MLKL axis proteins induced by H_2O_2 treatment (**Figure 1E**).

Dapa Alleviates UUO-Induced Inflammation

To define the effects of Dapa on interstitial inflammation caused by UUO, we studied the expression of pro-IL-1β, pro-IL-18, and NLRP3 based on previous studies (Gao et al., 2020; Zhao et al., 2021a). As shown in **Figure 2A**, UUO upregulated expression of pyroptosis-related cytokines (IL-1β, IL-18, and NLRP3), resulting in massive ED-1-positive cell infiltration (**Figure 2C**), whereas these were mitigated following Dapa or RIP inhibitors (Nec-1 and GSK872) treatment. In HK-2 cells, both Dapa and RIP inhibitors decreased expression of IL-1β, IL-18, and NLRP3 proteins (**Figure 2B**).

Dapa Alleviates UUO-Induced Renal Fibrosis

Masson trichrome staining displayed that UUO-induced rat kidneys manifested by tubular vacuolization, collagen fibers

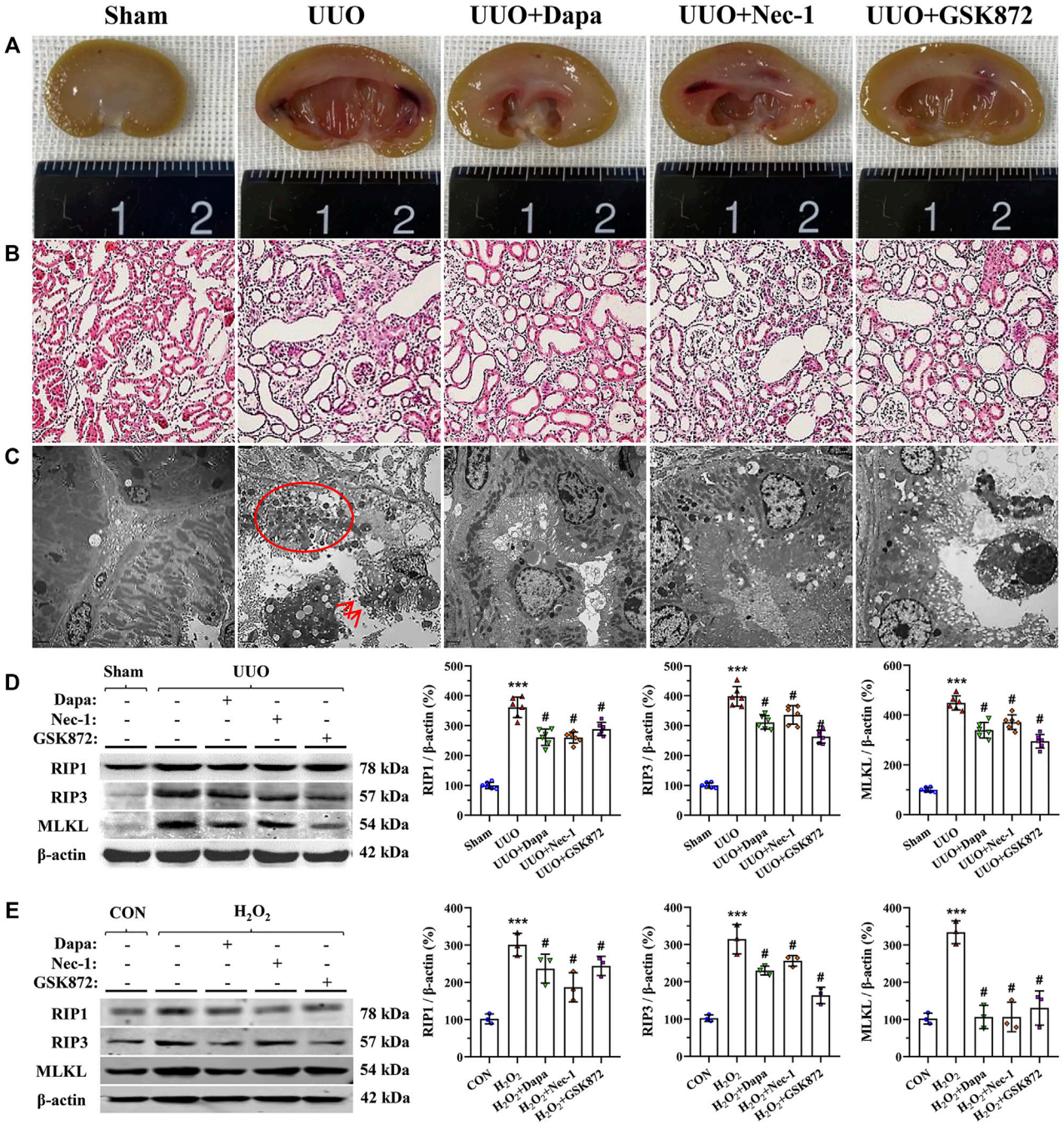

FIGURE 1 | Representative photomicrographs of gross finding **(A)**, HE staining **(B)**, transmission electron microscopy **(C)**, and immunoblotting analysis of RIP1-RIP3-MLKL protein *in vivo* **(D)** and *in vitro* **(E)**. UUO induces a crowd of necrosis within the tubular epithelium (circle) and abscission of microvilli in epithelial cells (arrows), whereas these are improved following administration of Dapa or RIP inhibitor. Data are presented as mean ± SEM (*in vivo*, $n = 6$; *in vitro*, $n = 3$) and analyzed by one-way ANOVA. $^{***}p < 0.01$ vs sham or control; $^{\#}p < 0.05$ vs UUO or H_2O_2.

deposition, and tubulointerstitial fibrosis (**Figure 3A**). Using our quantitative analysis system, increased fibrosis score in the UUO group was significantly decreased with treatment of Dapa or RIP inhibitors (Nec-1 and GSK872). Immunoblotting analysis revealed that either Dapa or RIP inhibitors dramatically inhibited profibrotic TGF-β1 and CTGF expressions compared with UUO alone (**Figure 3B**). Regarding the effect of Dapa and RIP inhibitors on necroptosis, sterile inflammation, and fibrosis, we suggest that Dapa alleviates renal fibrosis in this model of UUO

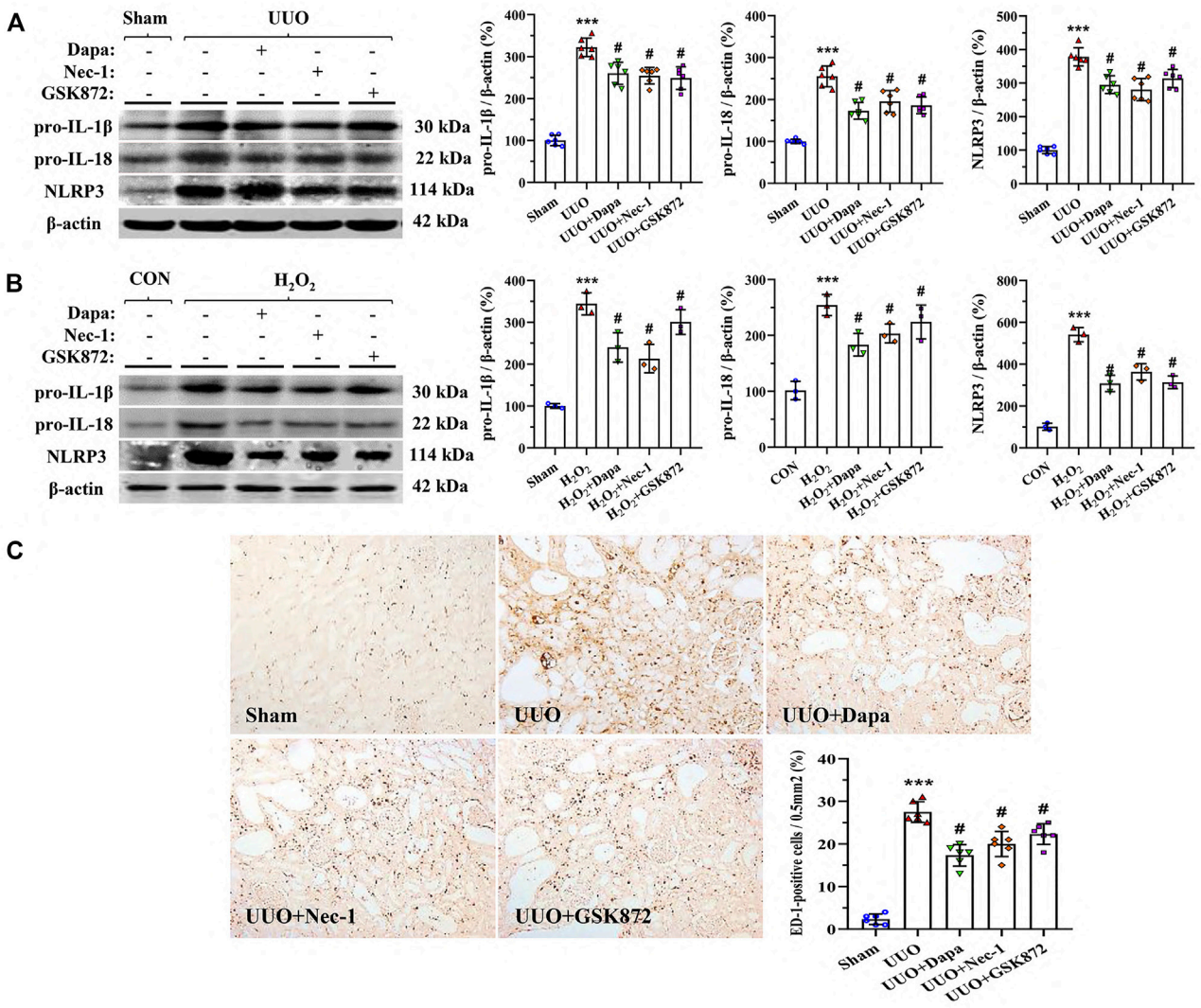

FIGURE 2 | Representative photomicrographs of immunoblotting analysis of a series of pyroptosis-related proteins *in vivo* **(A)** and *in vitro* **(B)** and immunohistochemistry for ED-1 **(C)**. Data are presented as mean ± SEM (*in vivo*, $n = 6$; *in vitro*, $n = 3$) and analyzed by one-way ANOVA. $^{***}p < 0.01$ vs sham or control; $^{\#}p < 0.05$ vs UUO or H_2O_2.

may be associated with suppression of PIR1-PRI3-MLKL-mediated necroinflammation.

Dapa Alleviates Oxidative Stress

Oxidative stress, necrosis, and inflammation are a vicious trio contributing to renal fibrosis during UUO, and an imbalance of oxidant and antioxidant enzymes may play a major role in this process. **Figures 4A,B** revealed that Dapa treatment significantly decreased 8-OHdG expression and urine 8-OHdG concentration in the UUO group. At a molecular level, Dapa counteracted oxidative stress by upregulating MnSOD expression but downregulated NOX-4 expression (**Figure 4C**). In HK-2 cells subjected to H_2O_2, Dapa dose-dependently decreased intracellular ROS production (**Figure 4D**).

Dapa Alleviates UUO-Induced Mitochondrial Dysfunction and Endoplasmic Reticulum (ER) Stress

As delineated in **Figure 5A**, UUO damaged the mitochondrial architecture and fitness, characterized by a significant decrease in the number and size of mitochondria, dilatation of disorganized cristae, vacuolization, mitochondrial fusion, mitophagy formation, and mitochondria divided into two or more daughter organelles (fission). Quantitative analysis revealed that Dapa treatment preserved the number and size of mitochondria (**Figure 5B**). Immunoblotting showed that dysregulated expressions of PINK1, Parkin, p62, and SDHA in the UUO group were balanced by treating Dapa (**Figure 5C**). In addition, UUO was associated with degranulation of ribosomes, disconnected and dilated cisternae and peroxisome vacuolization

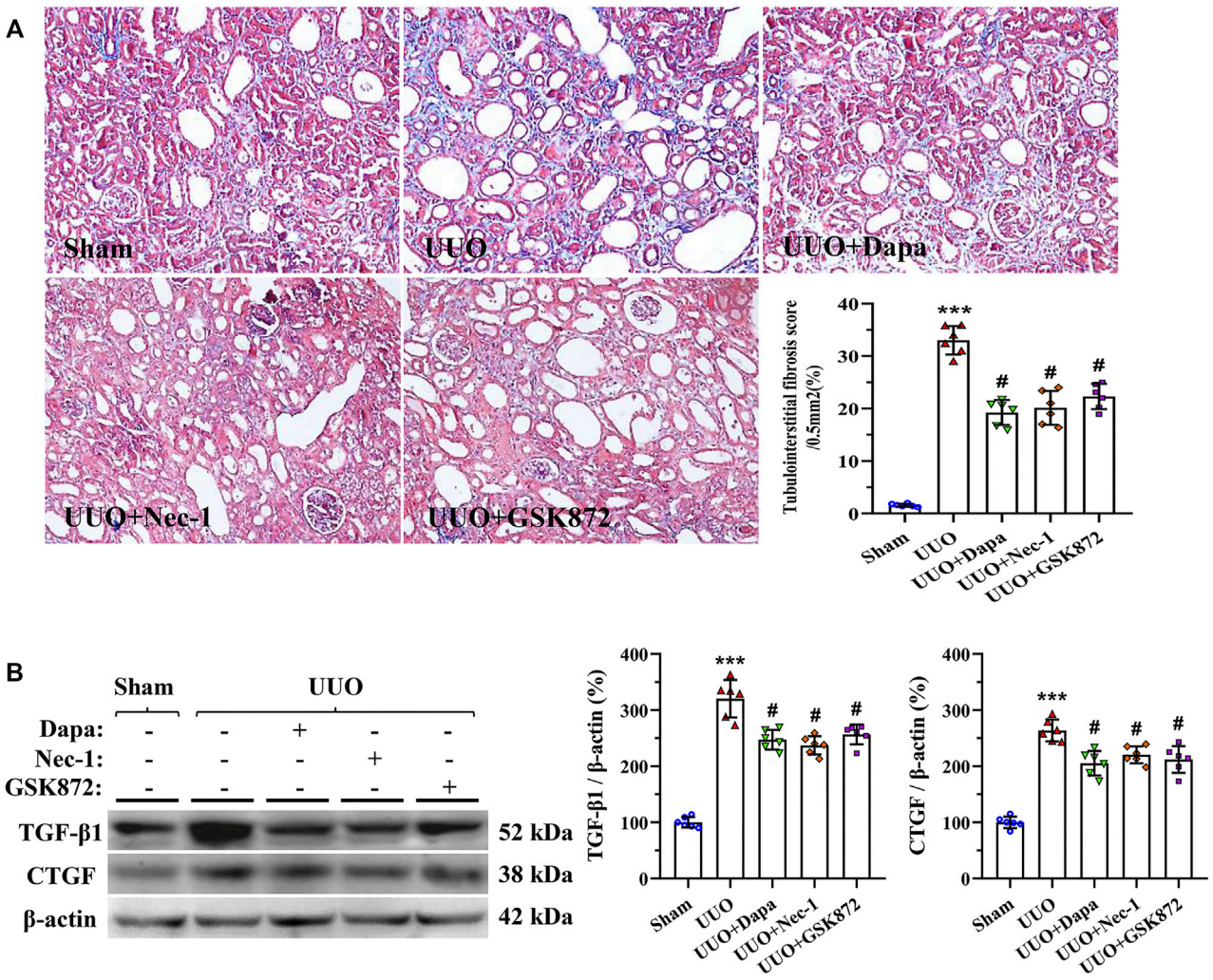

FIGURE 3 | Representative photomicrographs of Masson trichrome staining **(A)** and immunoblotting analysis of TGF-β1 and CTGF **(B)**. Data are presented as mean ± SEM (n = 6) and analyzed by one-way ANOVA. $^{***}p < 0.01$ vs sham; $^{\#}p < 0.05$ vs UUO.

in rough ER, whereas smooth ER remained almost normal in structure. Paralleled with the morphological findings, immunoblotting analysis revealed that UUO increased expression of ER stress-related genes such as CHOP and IRE-1α, but their expressions were decreased by Dapa administration (**Figure 5C**).

Dapa Alleviates Apoptotic Cell Death

Because of its pivotal role in UUO, we evaluated apoptotic cell death and expression of apoptosis-controlled genes in rat kidneys (Yang et al., 2018). Using the TUNEL assay, we found that most TUNEL-positive cells were in the tubular epithelial cells and interstitial cells, where tubular atrophy and fibrosis had developed (**Figure 6A**). Quantifying the number of TUNEL-positive cells revealed that an increase in their number in the UUO groups was significantly decreased in the UUO + Dapa group. Immunoblotting analysis showed that Dapa treatment regulated the Bcl-2/Bax ratio and cleaved caspase-3 expression trended toward cell survival (**Figure 6B**). In vitro study, Dapa dose-dependently improved cell viability and decreased apoptotic cells (**Figure 6C**).

Dapa Inactivates Wnt3α/β-Catenin/GSK-3β Signaling

We evaluated the effects of Dapa on expression of Wnt3α/β-catenin/GSK-3β signaling in a rat model of UUO and in H_2O_2-treated HK-2 cells by immunoblotting analysis. **Figure 7** shows that both UUO and H2O2 activated Wnt3α/β-catenin/GSK-3β protein expression, whereas their expression was significantly inhibited by either Dapa or Wnt/β-catenin inhibitor ICG-001. This finding indicates that Dapa treatment alleviates renal fibrosis through the Wnt3α/β-catenin/GSK-3β-dependent signaling pathway.

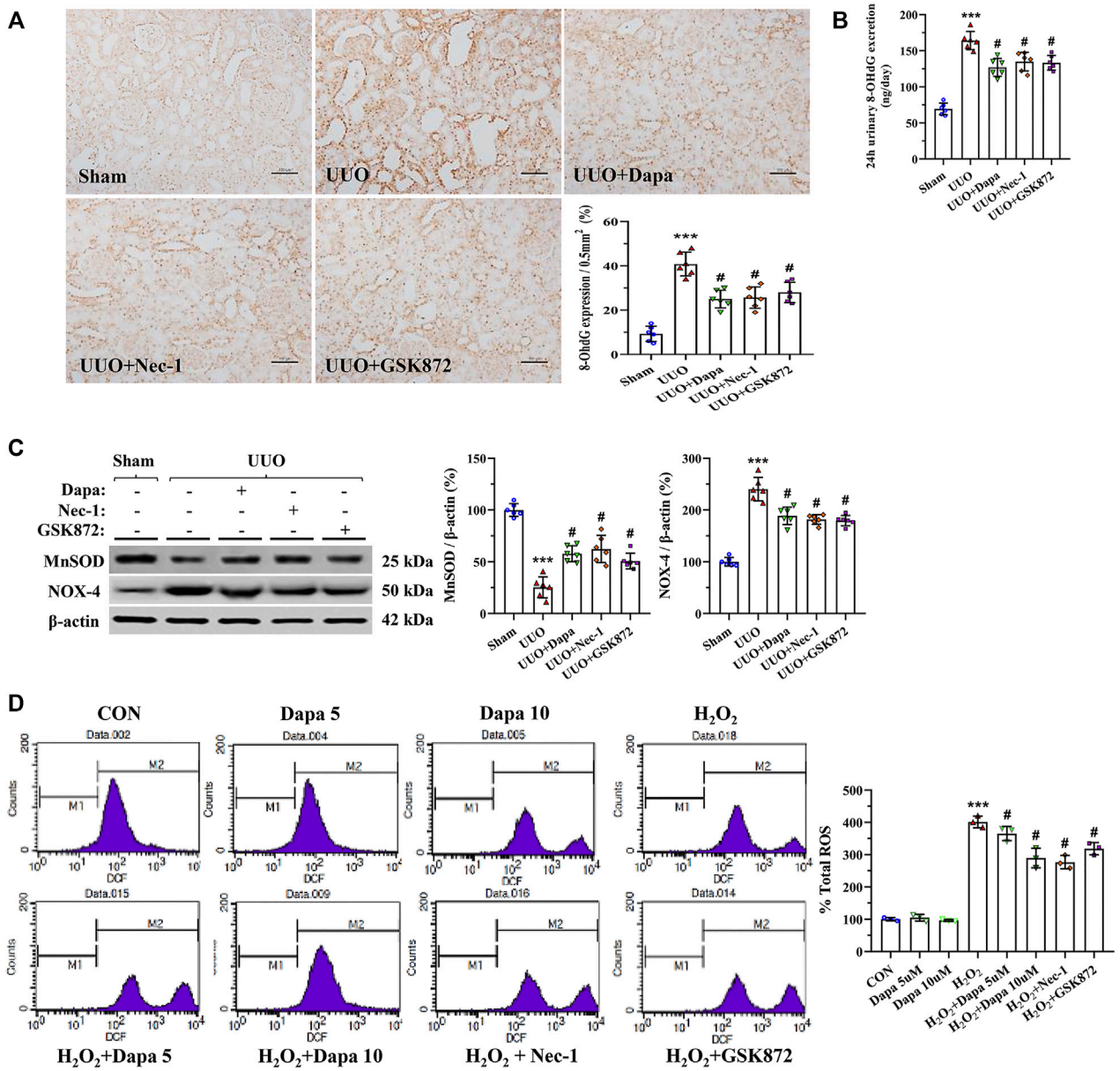

FIGURE 4 | Representative photomicrographs of immunohistochemistry for 8-OHdG **(A)**, urine 8-OHdG concentration **(B)**, immunoblotting analysis of MnSOD and NOX-4 **(C)**, and intracellular ROS production in H_2O_2-treated HK-2 cells with or without Dapa and RIP inhibitors **(D)**. Data are presented as mean ± SEM (*in vivo*, $n = 6$; *in vitro*, $n = 3$) and analyzed by one-way ANOVA. ***$p < 0.01$ vs sham or control; #$p < 0.05$ vs. UUO or H_2O_2.

DISCUSSION

Large clinical outcome trials have validated that SGLT2 inhibitors (Empagliflozin or Canagliflozin) improve renal outcomes, as defined by reducing the risk of deteriorated nephropathy, progression to macroalbuminuria, incidence of renal replacement therapies, and occurrence of doubling of serum creatinine in patients with T2DM (Wanner et al., 2016; Neal et al., 2017). Moreover, a double-blind, placebo-controlled trial enrolling patients from 116 research centers showed that Dapa decreases the urine albumin-to-creatinine ratio and slows kidney disease progression in patients with moderate-to-severe CKD (Pollock et al., 2019). The results herein also reveal that Dapa treatment improves damage to kidney architecture caused by UUO. However, there were no significant differences in FG or HbA1c levels among treatment groups. This implies that Dapa may confer renoprotective effects against nondiabetic CKD beyond hypoglycemia.

A unifying theory of necroinflammation consists of necroptosis and sterile inflammation, which are reciprocally enhanced in an autoamplification loop. Necroptosis regulated by RIP1, RIP3, and MLKL evokes innate immunity by releasing

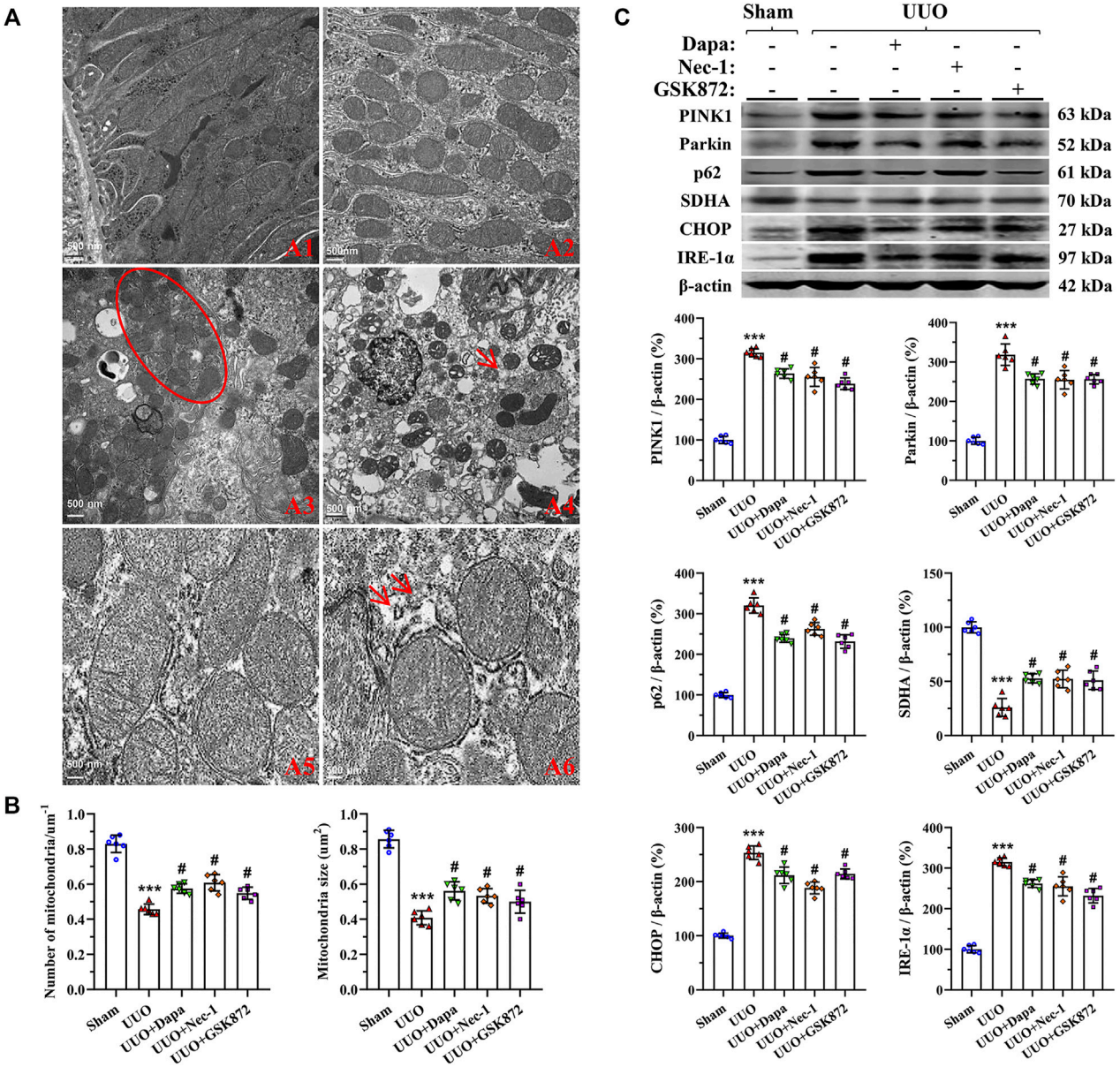

FIGURE 5 | Representative photomicrographs of transmission electron microscopy of mitochondrial and ER morphology **(A)**, quantitative analysis of mitochondrial number and size **(B)**, and immunoblotting analysis of mitochondria- and ER stress-related proteins **(C)**. A1, normal mitochondria; A2, reduced mitochondria number and size and mitochondria fusion; A3, mitochondria divided into two or more organelles (circle); A4, autophagy of mitochondria (mitophagy, arrow); A5, normal rough ER; A6, degranulation of ribosomes and disconnected and dilated cisternae of rough ER (arrows). Data are presented as mean ± SEM (n = 6) and analyzed by one-way ANOVA. $^{***}p < 0.01$ vs sham; $^{\#}p < 0.05$ vs UUO.

DAMPs via the ruptured plasma membrane. Of interest, RIP3-MLKL signaling facilitates inflammation by stimulating NLRP3 inflammasome and thereby regulates the processing and secretion of pro-IL-1β and pro-IL-18 into the mature and active cytokines via caspase-1-activating platforms (Anders and Muruve, 2011; Anders, 2016; Choi et al., 2019). Pharmacological blockade of necroptosis or RIP3 genetic deficiency attenuates UUO-induced inflammation and fibrosis (Xiao et al., 2017; Imamura et al., 2018). Herein, we found that UUO increases expression of RIP1-RIP3-MLKL proteins, which is accompanied by upregulation of pyroptosis-controlling genes and proinflammatory cytokines, and subsequent development of renal fibrosis. However, these effects were all reversed by either Dapa or RIP inhibitors. We propose that Dapa alleviates renal fibrosis by suppressing UUO-induced necroinflammation.

How Dapa treatment abrogates renal necroinflammation and fibrosis in this model is unclear but may be related to oxidative stress. It is well known that UUO is closely associated with

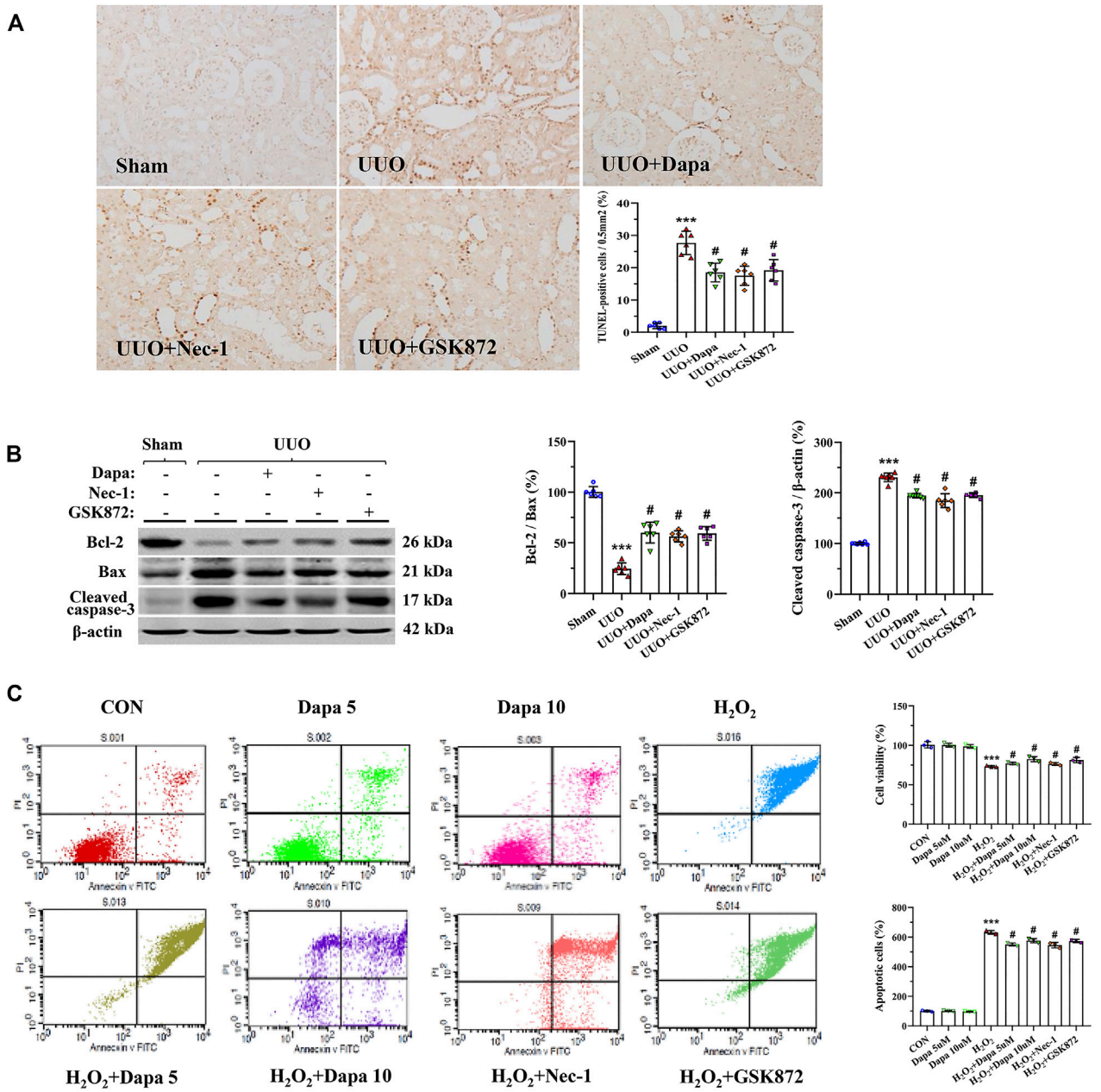

FIGURE 6 | Representative photomicrographs of the TUNLE assay **(A)**, immunoblotting analysis of apoptosis-related genes **(B)**, and apoptotic cell death and cell viability in HK-2 cells **(C)**. Data are presented as mean ± SEM (*in vivo*, $n = 6$; *in vitro*, $n = 3$) and analyzed by one-way ANOVA. ***$p < 0.01$ vs sham or control; #$p < 0.05$ vs UUO or H_2O_2.

hypoxia, which results in oxidative stress. Dapa directly inhibits cytosolic and mitochondrial ROS production in HK-2 cells subjected to H_2O_2 (Zaibi et al., 2021). However, Dapa also indirectly decreases ROS production and expressions of NOX-4 and NOX-2 through its hypoglycemic action in db/db mice and in mProx24 cells (Terami et al., 2014). A similar antioxidative effect of Dapa is also reflected in failing heat stroke (Olgar and Turan, 2019) and hepatic steatosis (Tang et al., 2017). Herein, we observed that Dapa decreased NOX-4 expression and reduced 8-OHdG concentrations, but increased MnSOD expression. These findings suggest that the abrogation of necroinflammation and fibrosis by Dapa seen herein may contribute to its antioxidant property.

Chronic hypoxia may aggravate necrotic effects, causing renal cellular injury by oxidative stress, mitochondrial dysfunction, and ER stress. Inversely, mitochondria are a major source of ROS,

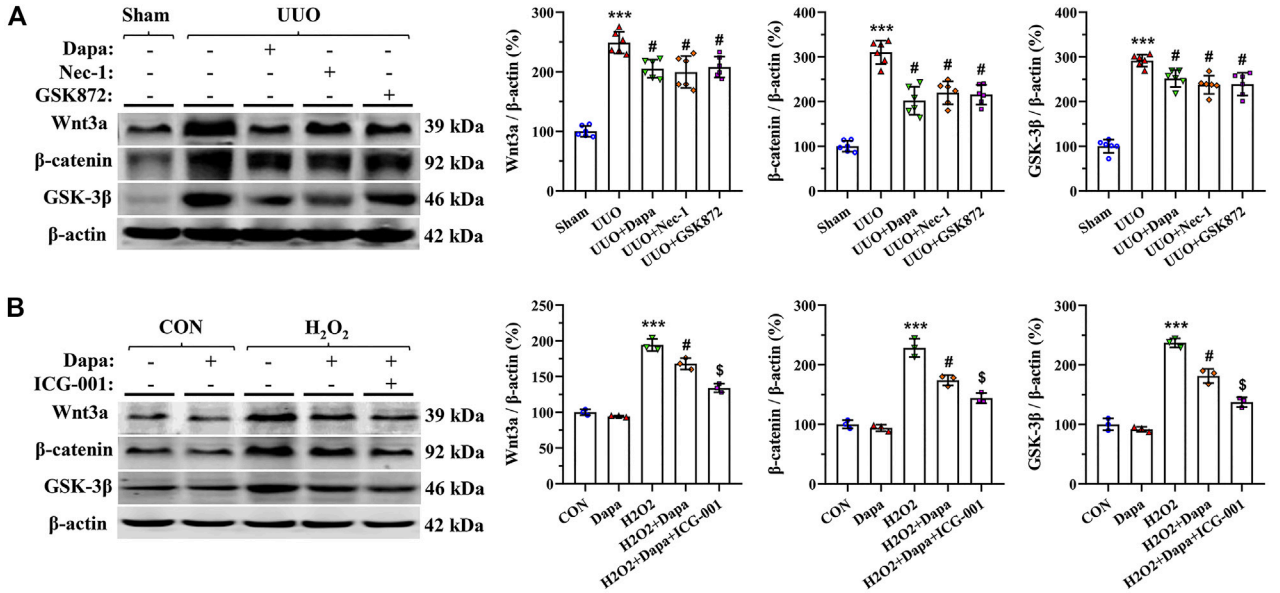

FIGURE 7 | Representative photomicrographs of immunoblotting analysis of Wnt3α/β-catenin/GSK-3 β signaling proteins *in vivo* **(A)** and *in vitro* **(B)**. Data are presented as mean ± SEM (*in vivo*, $n = 6$; *in vitro*, $n = 3$) and analyzed by one-way ANOVA. ***$p < 0.01$ vs sham or control; $^{\#}p < 0.05$ vs. UUO or H_2O_2; $^{\$}p < 0.05$ vs H_2O_2+Dapa.

which is involved in regulation of cell apoptosis (Galvan et al., 2017). We have previously demonstrated that sustained or prolonged ER stress may be cytotoxic, leading to apoptotic cell death (Han et al., 2008). Thus, oxidative stress, mitochondrial dysfunction, and ER stress are interrelated as a vicious cycle in the pathogenesis of UUO-induced renal injury. Using electron microscopy, we clearly observed that Dapa shields mitochondrial and ER structural integrity in UUO-treated rat kidneys. These morphological changes were followed by regulation of an array of mitochondrial- and ER stress-controlling genes, resulting in weakened apoptosis. Our findings are consistent with studies by Chang et al. in renal I/R injury (Chang et al., 2016a) and Shibusawa et al. in diabetic nephropathy (Shibusawa et al., 2019) showing the capacity of Dapa on ER stress and apoptosis.

Our study demonstrates that treatment with Dapa (10 mg/kg/d) alleviates renal fibrosis from UUO by inhibiting necroinflammation, oxidative stress, and apoptosis. This is supported by a variety of nondiabetic CKD studies, such as adenine-induced CKD (Ali et al., 2019) and angiotensin II-dependent kidney damage (Reyes-Pardo et al., 2019). However, an interesting result was observed from subtotal (5/6) nephrectomy. Zhang et al. described that Dapa treatment at 1.0 mg/kg/d did not affect mortality, heavy proteinuria, or declining glomerular filtration rate (Zhang et al., 2016). Nor did Dapa attenuate the degree of glomerulosclerosis, tubulointerstitial fibrosis, or overexpression of TGF-ß1 mRNA in the kidneys of 5/6 nephrectomized rats. Moreover, Ma et al. reported that empagliflozin (10 mg/kg/d) has no effect on either renal dysfunction or profibrotic markers with oxalate-induced nephropathy on days 7 and 14 (Ma et al., 2017). The reasons for this discrepancy in the role of SGLT2 inhibitors in nondiabetic CKD are unknown, but may be related to the optimal dose used, disease model type, or treatment duration. Further studies are needed to resolve these questions.

It is well accepted that activation of Wnt/β-catenin signaling triggers chronic inflammation and overproduction of ROS (MacDonald et al., 2009). In addition, Wnt/β-catenin signaling works in a combination fashion with TGF-β signaling in the process of fibrosis, and TGF-β signaling can evoke expression of Wnt/β-catenin superfamily members, and vice versa (Guo et al., 2012). Thus, Wnt/β-catenin/GSK signaling is involved in the development of renal fibrosis in acute kidney injury (Pan et al., 2021) and diabetic nephropathy (Chang et al., 2018). In our model of UUO, the pathogenesis of renal fibrosis involves a complex network orchestrated by necroptosis, oxidative stress, inflammation, TGF-β1, and activation of Wnt/catenin signaling (Jiang et al., 2015; Dai et al., 2020; Jin et al., 2020). Inhibition of necroptosis or Wnt/catenin signaling alleviates UUO-induced renal fibrosis (Xiao et al., 2017; Gong et al., 2021), implying a relationship between necroptosis and Wnt/β-catenin signaling. Using *in vivo* and *in vitro* studies, we observed that overexpressed Wnt3α/β-catenin/GSK-3β protein in UUO rat kidneys and HK-2 cells was inhibited by either Dapa or ICG-001. Therefore, it is likely that Dapa affords renoprotective effects, probably by interfering with the Wnt3α/β-catenin/GSK-3β signaling pathway.

Clinical trials and animal studies have delineated that SGLT2 inhibitors possess pleiotropic effects beyond hypoglycemia. The

present study demonstrates that Dapa alleviates renal fibrosis via decreased RIP1-RIP3-MLKL-mediated necroinflammation in a rat model of UUO. Suppression of oxidative stress, ER stress, and apoptosis, along with improved mitochondrial fitness, may be a mechanism underlying the renoprotective properties of Dapa. Our findings provide a potential rationale for the clinical use of SGLT2 inhibitors in preventing nondiabetic CKD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal care and experimental procedures were reviewed and approved by the Animal Experimentation Ethics Committee of Yanbian University (SYXK [J]2020-0009) and the Animal Care Committee at the Medical College of Yanbian University (YBU-2019-11-27).

AUTHOR CONTRIBUTIONS

MX and CL designed the research; SP, QN, MP, YJ, HZ, and JJ, performed *in vivo* study; MX and JD conducted *in vitro* study; MX and SP analyzed the data and performed molecular works; MX wrote the manuscript; SP, JJ, and CL revised the manuscript.

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Determinants of Kidney Function and Accuracy of Kidney Microcysts Detection in Patients Treated With Lithium Salts for Bipolar Disorder

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Objectives: Early kidney damage during lithium treatment in bipolar disorder is still hypothetical. We aimed at identifying the determinants of a decreased measured glomerular filtration rate (mGFR) and the accuracy of kidney MRI imaging in its detection.

Methods: In this cross-sectional cohort study, 217 consecutive lithium-treated patients underwent mGFR and kidney MRI with half-Fourier turbo spin-echo and Single-shot with long echo time sequences.

Results: Median age was 51 [27–62] years, and median lithium treatment duration was 5 [2–14] years. 52% of patients had a stage 2 CKD. In multivariable analysis, the determinants of a lower mGFR were a longer lithium treatment duration (β −0.8 [−1; −0.6] ml/min/1.73 m^2 GFR decrease for each year of treatment), a higher age (β −0.4 [−0.6; −0.3] ml/min/1.73 m^2 for each year of age, $p < 0.001$), albuminuria (β −3.97 [−6.6; −1.3], $p = 0.003$), hypertension (β −6.85 [−12.6; −1.1], $p = 0.02$) and hypothyroidism (β −7.1 [−11.7; −2.5], $p = 0.003$). Serum lithium concentration was not associated with mGFR. Renal MRI displayed renal microcyst(s) in 51% of patients, detected as early as 1 year after lithium treatment initiation. mGFR and lithium treatment duration were strongly correlated in patients with microcyst(s) (r = −0.64, $p < 0.001$), but not in patients with no microcysts (r = −0.24, $p = 0.09$). The presence of microcysts was associated with the detection of an mGFR <45 ml/min/1.73 m^2 (AUC 0.893, $p < 0.001$, sensitivity 80%, specificity 81% for a cut-off value of five microcysts).

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Conclusion: Lithium treatment duration and hypothyroidism strongly impacted mGFR independently of age, especially in patients with microcysts. MRI might help detect early lithium-induced kidney damage and inform preventive strategies.

Keywords: nephrotoxicity, lithium, CKD-chronic kidney disease, kidney microcysts, bipolar disorder

INTRODUCTION

Lithium salts are the main prophylactic treatment of bipolar disorder, which is characterized by potentially life-threatening manic and/or depressive episodes. They have proven efficient in the prevention and treatment of acute episodes as well as in the prevention of suicidal risk (Geddes et al., 2004; Investigators et al., 2010; Cipriani et al., 2013; Miura et al., 2014). However, this efficacy is counterbalanced by a narrow therapeutic range that can lead to potentially harmful overdose, and by long-term adverse events (Tabibzadeh et al., 2019). Amongst them, long-term lithium treatment might lead to polyuria and polydipsia, related to impaired urine concentrating ability, and to chronic kidney disease usually with a tubulo-interstitial presentation. The latter is characterized by mild or absent proteinuria (often <1 g/24 h) and cortical and/or medullary small cysts, which supposedly appear at late stages (Grünfeld and Rossier, 2009).

Epidemiological data suggest that chronic use of lithium may lead to impaired glomerular filtration rate (GFR). Indeed, in a retrospective study including 2,500 patients on lithium therapy, Shine et al. showed an increased risk of stage 3 chronic kidney disease (CKD) defined by creatinine-based estimated GFR (eGFR) (Shine et al., 2015). However, in a meta-analysis gathering 385 studies, McKnight et al. showed that eGFR was only 6.2 ml/min/1.73 m^2 lower in patients treated with lithium salts than in control populations, with an overall small risk of renal failure (McKnight et al., 2012). Another study showed that among 145 patients treated with lithium for more than 15 years, only 21% displayed a measured GFR (mGFR) below the -2 SD cut-off for age-adapted GFR (Bendz et al., 1994). It is thus still unclear whether lithium treatment at therapeutic concentrations is harmful in itself or if the GFR decrease in this population is related to age or to other comorbidities such as metabolic syndrome or hypertension as suggested by the cohort study by Clos et al. (2015). If present, the magnitude of the effect of lithium treatment is also unknown.

The aim of our study was to describe clinical, biological and imaging characteristics of a cohort of lithium-treated patients focusing on renal function and radiological kidney features, and to evaluate the correlates of GFR, including the relationship between kidney function and lithium treatment, using a gold standard method for GFR measurement.

METHODS

Study Design and Population

From March 2015 to December 2020, 230 consecutive adult patients were referred by psychiatrists to the Department of Renal Physiology for their first visit with nephrologists. Among these patients, we excluded 13 patients who had discontinued lithium treatment, leaving 217 patients who were referred for a systematic check-up. Eligible patients were ≥18 years of age at inclusion, with various durations of lithium treatment, and had neither started dialysis nor underwent kidney transplantation.

The study was performed according to the Declaration of Helsinki. The study was approved by the local ethics committee from APHP. Nord (Institutional Review Board CER-2021-74). All patients provided written informed consent before inclusion in the study cohort.

Data Collection and Measurements

During a 5 h in-person visit, a large set of clinical and laboratory data were collected, including past medical history, previous use of Non-Steroidal Anti-Inflammatory drugs (NSAIDs) or chemotherapeutic agents, dose and duration of lithium treatment, serum lithium concentration, and current treatment with other psychotropic drugs. Obesity was defined as body mass index (BMI) >30 kg/m^2. Diabetes was defined as fasting glycemia >7 mmol/L or antidiabetic drug treatment. Hypothyroidism was either defined as a thyroid-stimulating hormone (TSH) level >4.1 mUI/L (above the upper normal limit) or a thyroid hormone therapy. Blood pressure (BP) was the average of three measurements in resting conditions. Hypertension was defined as BP ≥140/90 mmHg or the use of antihypertensive drugs. Patients were instructed to fast (not to eat or drink) from 8 p.m. the day before the admission. Patients were asked to collect 24 h urine the day before admission. Indications to discard first morning void on the first day, and then to collect all urine until the first void the next morning were given by a trained nurse and detailed in a written information document. Fasting blood and urine samples were collected. GFR was measured by urinary clearance of ^{51}Cr-EDTA or ^{99}Tc-DTPA (GE Healthcare, Velizy, France and Curium, Saclay, France respectively) depending on the time of the visit due to ^{51}Cr-EDTA shortage (Vidal-Petiot et al., 2021), determined as the average of 7 consecutive 30 min urinary clearance periods, indexed to the standard body surface area of 1.73 m^2 as previously described (Froissart et al., 2005). Previous comparison of the two tracers has shown similar results allowing gathering findings from the two techniques (Vidal-Petiot et al., 2021). Plasma lithium concentration was the last available measurement during the last 6 months. Creatinine was measured using an Isotopic Dilution Mass Spectroscopy-standardized enzymatic method.

Kidney MRI

A renal magnetic resonance imaging (MRI) was proposed to all the patients, and was finally performed in 99 patients due to the remaining patients' refusal (mainly for claustrophobia or

unavailability). MRI was performed with a pre-specified protocol using the following sequences: T2 SSFSE (T2 weighted sequence single-shot fast spin-echo), also called half-Fourier single-shot turbo spin-echo (or HASTE for Siemens), and SSFSE TE long (Single-shot fast spin echo with long echo time). T2 SSFSE is an ultrafast MRI technique with a short acquisition time less susceptible to motion respiratory artifact than other techniques such as echo T2-weighted imaging (Nakayama et al., 2001). SSFSE TE long is a highly T2 weighted sequence allowing an optimal contrast between microcysts and renal parenchyma. This MRI protocol has proven useful in depicting structures containing static fluids including cystic lesions, characterized as T2 hyperintense round areas (Nakayama et al., 2001).

Renal microcysts were defined as small (1–2 mm) round cystic lesions and quantified in both kidneys. As inter and intra-observer reproducibility was poor when counting the total number of microcysts, a semi-quantitative approach was used, and MRI findings were classified as follows: 1/no microcysts, 2/1 to 5 microcysts, 3/6 to 10 microcysts, 4/11 to 20 microcysts, 5/21 to 50 microcysts, 6/51 to 100 microcysts, 7/> 101 microcysts. Images were blindly reviewed and analyzed by 2 senior radiologists and one senior nephrologist. This semi-quantitative scoring was reproducible.

Statistical Analyses

Categorical variables were described as frequencies and percentages, and continuous variables as median [25th-75th percentiles]. Baseline patients' characteristics were compared according to mGFR classes (>90, 60–90, <60 ml/min/1.73 m^2) using Kruskal–Wallis test and Chi-square tests, for quantitative and qualitative variables, respectively. Correlations between mGFR, age and lithium treatment duration (log-transformed) were assessed by Spearman tests. Determinants of mGFR were assessed using multivariable linear regression models. Covariates were selected *a priori* based on potential confounding and included: age, gender, body mass index (<vs. ≥30 kg/m^2), diabetes, hypertension, hypothyroidism, treatment with other psychotropic drugs, lithium treatment duration in years (log-transformed), lithium galenic formulation (extended or immediate-release formulation), serum lithium concentration and 24 h urinary albumin/creatinine ratio (log-transformed). Interactions between treatment duration and age, gender, and BMI were tested. Normality of the distribution of residuals and homoscedasticity were verified. Multiple imputations using the chained equation method (R package *mice*, $n = 50$ imputed datasets, $m = 20$ iterations) were performed for missing data.

In order to specifically analyze the association between mGFR and renal microcysts, a Spearman correlation was performed between mGFR and lithium treatment duration in patients with microcysts and those without microcysts. A linear regression model was built to explain mGFR according to treatment duration, with an interaction term between treatment duration and renal microcysts to assess the difference in mGFR slopes between patients with and without microcysts. The effect of microcysts on slopes was estimated and a Wald test was performed on the interaction term to compare the two groups.

With an mGFR <45 ml/min/1.73 m^2 considered as the state of disease, sensitivity (Se) and specificity (Sp) were calculated for each microcyst cut-off score. A Receiver Operating Characteristic (ROC) Curve was plotted in order to measure the Area Under the Curve (AUC) and the Youden Index allowed identifying the most accurate cut-off value for renal microcysts.

A two-sided p-value < 0.05 was considered statistically significant. Statistical analyses were conducted using in GraphPad Prism 9.0 and R 3.4 software.

RESULTS

Characteristics of the Population

Table 1 summarizes patients' characteristics. Median age was 51 [27–62] years, and 62% were female. Median BMI was 25.9 [22.9–28.8] kg/m^2. Median lithium treatment duration was 5 [2–14] years and median dose was 800 [575–1,000] mg/day. The extended-release form was administered in a majority of patients (63%). Median plasma lithium concentration was 0.72 [0.56–0.87] mmol/L. Median mGFR was 78 [63–91] ml/min/1.73 m^2 with a majority of stage 2 CKD (52%). Microalbuminuria was absent (<3 mg/mmol) in a large majority (83%) of patients and only two patients had a macroalbuminuria (which remained below 0.6 g/24 h). Seven (3%) patients had diabetes mellitus, 26% had hypertension and 36% had hypothyroidism. Among the studied population, three patients were found to occasionally take NSAIDs, with mGFRs of 83, 49.9 and 41.7 ml/min/1.73 m^2. The use of NSAIDs in the latter patient led to a severe lithium overdose (4.1 mmol/L) resulting in acute kidney injury requiring ICU admission the year before referral in the renal physiology unit.

Compared with patients with mGFR higher than 60 ml/min/1.73 m^2, patients in the lowest mGFR group (<60 ml/min/1.73 m^2) were significantly older, with a higher prevalence of hypertension, and were more frequently female. In this population, lithium treatment duration was significantly longer, and daily dose was significantly lower with a lower rate of extended-release lithium preparation. Of note, there was no statistical difference according to GFR groups for serum lithium levels. Even though levels were low in all groups, they also displayed a higher albuminuria and higher proteinuria (**Table 1**).

The prevalence of CKD was higher in patients with a longer treatment duration (**Figure 1**). In patients treated for less than 1 year, only two patients had an mGFR <60 ml/min/1.73 m^2. Among patients treated for more than 25 years, none had a normal mGFR (>90 ml/min/1.73 m^2).

Determinants of mGFR

Lithium treatment duration (log-transformed) and age were inversely correlated with mGFR ($r = -0.51$, $p < 0.001$ and $r = -0.54$, $p < 0.001$, respectively). In multivariable analysis, lithium treatment duration and age were also independently and negatively associated with mGFR, with a stronger association with lithium treatment duration (β-coefficient: −0.8 [−1; −0.6]

TABLE 1 | Characteristics of the patients in the whole cohort and according to measured GFR.

Variables, *n* available data	Total *n* = 217	mGFR >90 ml/min/1.73 m^2 *n* = 57	60≤ mGFR<90 *n* = 113	mGFR <60 ml/min/1.73 m^2 *n* = 47	*p*-value
Age, years *n* = 217	51 [37–62]	38 [31–48.5]	51 [28–61.5]	62 [54.5–69]	<0.001
Male patients, *n* = 217	83 (38.2%)	33 (57.9%)	38 (33.3%)	12 (10.5%)	<0.001
Body mass index, kg/m^2, *n* = 217	25.9 [22.9–28.8]	25.5 [23.1–29.3]	25.6 [22.5–29]	26.8 [23.6–28.8]	0.7
Diabetes mellitus, *n* = 217	7 (3.2)	1 (1.8%)	4 (3.5%)	2 (4.3%)	0.7
Hypertension, *n* = 217	56 (25.8)	6 (10.5%)	27 (23.7%)	23 (48.9%)	<0.001
Hypothyroidism, *n* = 217	78 (35.9)	12 (21.1%)	41 (36%)	23 (53.2)	0.01
Other psychotropic drugs, *n* = 216	152 (70.1%)	35 (61.4%)	83 (73.5%)	34 (72.3%)	0.3
Lithium treatment duration, years, *n* = 217	5 [2–14]	2 (1–5)	5 (2–12)	16 [9.5–30]	<0.001
Extended-release formulation, *n* = 214	138 (63.6%)	41 (71.9%)	74 (64.9%)	23 [48.9%]	0.04
Daily dose, mg/day *n* = 216	800 [575–1,000]	1,000 [788–1,200]	800 [750–1,000]	500 [400–750]	<0.001
Serum lithemia, mmol/L, *n* = 139	0.72 [0.56–0.87]	0.72 [0.53–0.9]	0.75 [0.6–0.9]	0.61 [0.5–0.84]	0.4
mGFR, ml/min/1.73 m^2 *n* = 217	78 [62.8–90.5]	97.3 [94.1–102.9]	77.3 [71.5–83.4]	46.6 [41–53.7]	<0.001
ACR, mg/mmol creatinine, *n* = 214	1.4 [0.9–2.2]	0.9 [0.8–1.9]	1.4 [1.0–2.0]	2.0 [1.4–4.3]	<0.001
PCR, mg/mmol creatinine, *n* = 216	14.1 [10.1–20.2]	11.4 [8.7–15.3]	14.0 [10.1–19.7]	19.6 [15.6–33.2]	<0.001
Renal MRI findings	*n* = 99	*n* = 28	*n* = 48	*n* = 23	
No microcysts	49 (49.4%)	20 (71.4%)	26 (54.2%)	3 (13%)	<0.001
1–10 microcysts	30 (30.3%)	7 (25%)	15 (31.3%)	8 (34.8%)	<0.001
>10 microcysts	20 (20.2%)	1 (3.6%)	7 (14.6%)	12 (52.2%)	<0.001

Categorical and continuous data are expressed in n *(%) and in median [quartile 1- quartile 3], respectively. Baseline patients' characteristics were compared across mGFR, level using the chi-2 test for categorical variables and Kruskal-Wallis Rank Sum Test for quantitative variables. mGFR: measured glomerular filtration rate, ACR: urinary albumin to creatinine ratio, PCR: urinary protein to creatinine ratio, MRI: magnetic resonance imaging.*

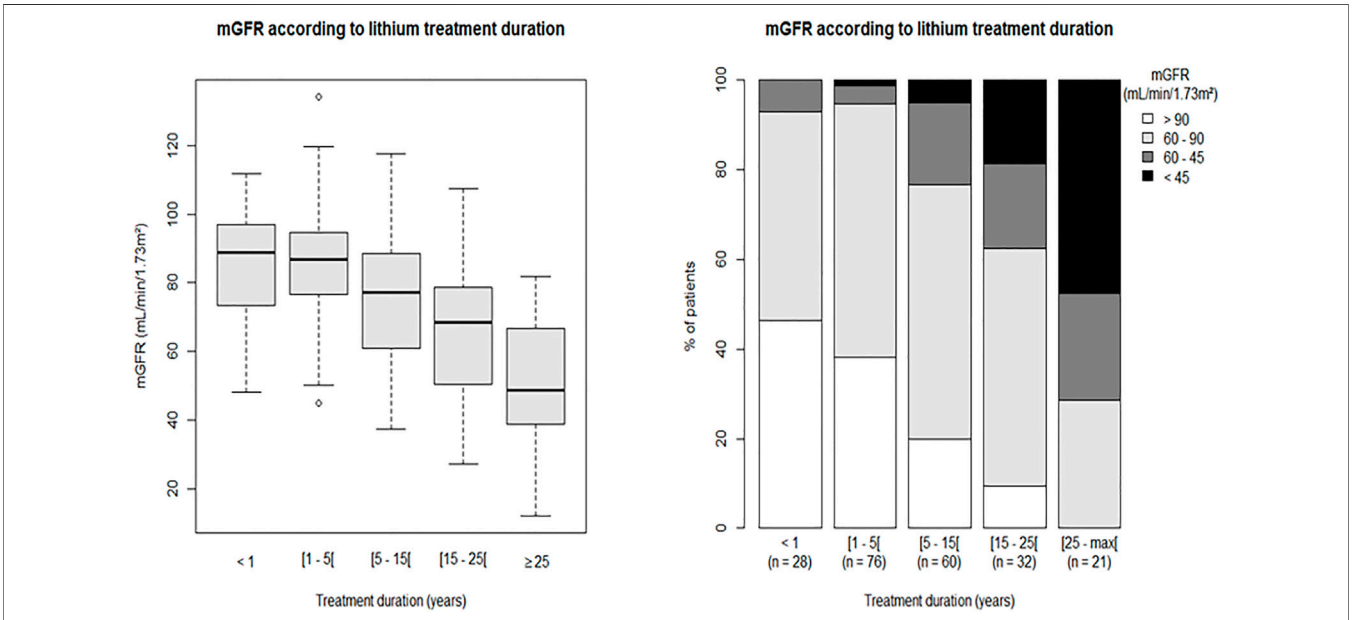

FIGURE 1 | mGFR according to lithium treatment duration. Left panel: median and range of mGFR according to lithium treatment duration. Right panel: stacked bars representing mGFR classes according to lithium treatment duration. mGFR: measured glomerular filtration rate.

ml/min/1.73 m^2 GFR decrease for each additional year of treatment) compared to age (β-coefficient: −0.4 [−0.6; −0.3] ml/min/1.73 m^2 for each additional year of age, $p < 0.001$). Association between treatment duration and mGFR did not depend on age or other covariates (non-significant *p*-values for interaction tests) (**Figure 2**). Albuminuria, hypothyroidism and hypertension were also independently and negatively associated with mGFR (β-coefficients respectively −3.97 [−6.6; −1.3], $p = 0.003$, −7.1 [−11.7; −2.5], $p = 0.003$, −6.85 [−12.6; −1.1], $p = 0.02$). Diabetes was associated with a higher mGFR, but was diagnosed in only seven patients including one with hyperfiltration (defined by an mGFR >120 ml/min/1.73 m^2). The other tested covariables—including serum lithium concentration, the use of extended-release form of lithium carbonate and treatment with other psychotropic agents (including when specifically analyzing the use of neuroleptic agents, SSRI- selective serotonin reuptake

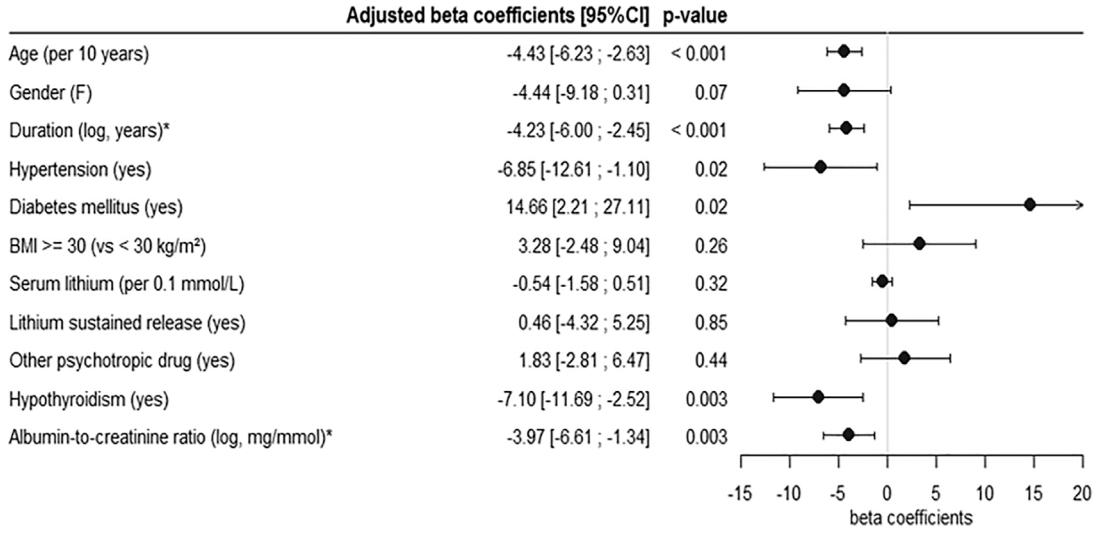

FIGURE 2 | Multivariable analysis of the determinants of mGFR. Determinants were assessed using multivariable linear regression model, with multiple imputations for missing data. All the variables included in the model are represented on the forest plot. F: female, BMI: body mass index, mGFR: measured glomerular filtration rate. * The indicated coefficients correspond to a 2.72-fold [= exp (1)] increase in lithium treatment duration or in albumin-to-creatinin ratio.

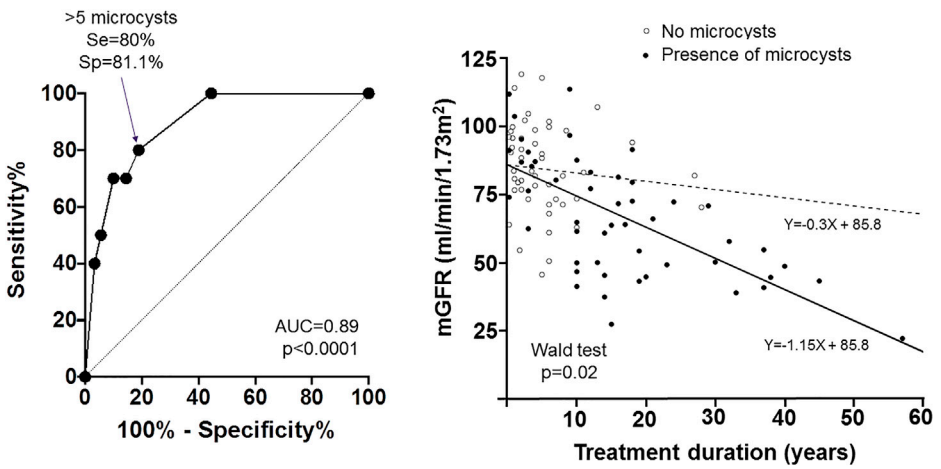

FIGURE 3 | Accuracy of renal microcysts in the diagnosis of lithium-related chronic kidney disease. Left panel: ROC Curve showing the sensitivity and 1—specificity of renal microcysts quantification for the diagnosis of mGFR <45 ml/min/1.73 m^2. The cut-off value of 5 microcysts had the highest Youden Index, with a sensitivity (Se) of 80% and a specificity (Sp) of 81.1%. Right panel: GFR values according to treatment duration and regression lines in patients with (filled circles and black line) and without (open circles and dashed line) microcysts. A Wald test was performed to test the difference in slopes between the two groups.

inhibitors-antidepressants, SNRI-serotonin-norepinephrine reuptake inhibitor-antidepressants, MAO-monoamine oxidase-inhibitors)—were not independently associated with mGFR.

Accuracy of Renal Microcysts for the Diagnosis of Lithium-Related Nephrotoxicity

Among patients who underwent renal MRI 51% displayed renal microcyst(s), appearing as T2 hyperintense round lesions uniformly and symmetrically distributed throughout the medulla as well as the cortex of normal-sized kidneys. Isolated Bosniak type 1 cysts were also unfrequently observed. Microcysts were present in patients as soon as 1 year after lithium treatment initiation. Patients in the lowest mGFR group had a higher prevalence of renal microcysts. When comparing patients with and without microcysts, mGFR and lithium treatment duration were strongly correlated in patients with microcyst(s) (r = −0.64, $p < 0.001$), whereas no such correlation was found in patients with no microcysts (r = −0.24, $p = 0.09$). This was confirmed by the linear regression of mGFR according to lithium treatment duration in patients with microcysts (slope −1.15, 95% CI [−1.44;

−0.86]) and with no microcysts (slope −0.30, 95% CI [−1.03; 0.43]), with a statistically significant difference between the slopes of the two groups (Wald test $p = 0.02$) (**Figure 3**).

Using receiver operating characteristic (ROC) analysis, we determined the renal microcyst quantification threshold associated with the detection of an mGFR below 45 ml/min/1.73 m^2 (**Figure 3**). The AUC was 0.893 ($p < 0.001$). At a cut-off value of 5 microcysts (defined by category 1 = no microcysts, and category 2 = 1 to 5 microcysts), the Youden Index was maximal (0.61), with a Se and a Sp respectively of 80 and 81%.

DISCUSSION

Our study showed a strong relationship between chronic lithium use and GFR decline, independently of other potential confounding factors. The statistical effect of lithium use on GFR decrease was determined as a 0.8 ml/min/1.73 m^2 mean decrease in mGFR per year of exposition. Other determinants of a lower mGFR were higher age, higher albuminuria, hypertension and hypothyroidism.

There are conflicting results regarding renal effects of lithium salts (Tabibzadeh et al., 2019). Several reports suggest that even when CKD is present, it is not clinically significant as no increase in the risk of ESKD has been demonstrated (van Melick et al., 2008; Aprahamian et al., 2014; Kessing et al., 2015). In their meta-analysis, McKnight et al. demonstrated a slight eGFR reduction (mean −6.2 ml/min/1.73 m^2) in patients treated with lithium compared to control groups (McKnight et al., 2012). The main limitation of these studies is the short follow-up duration and the short median treatment duration. In a large retrospective study of laboratory analyses from more than 2,500 patients, Shine et al. observed an increased risk of CKD stage 3 in lithium-treated patients compared to control populations (Shine et al., 2015). In the same line, a French retrospective study showed that 39% of patients aged 20–39 years, and up to 85% in patients aged 70 years or older displayed decreased GFR (defined as an estimated GFR <60 ml/min/1.73 m^2) (Bassilios et al., 2008). Accordingly, a large Swedish cohort study showed that 1.2% of lithium-treated patients had a serum creatinine level >150 μmol/L (Bendz et al., 2010). As controversial as kidney impairment during chronic lithium use may be, the general view is that lithium treatment induces a slowly progressive tubulo-interstitial nephropathy, that cannot be easily detected in longitudinal studies (Davis et al., 2018).

In this perspective, evolution towards end-stage kidney disease (ESKD) is also poorly defined. A retrospective study among patients on dialysis showed that 0.22% of these patients had a diagnosis of lithium-induced nephropathy (Presne et al., 2003). In the Swedish cohort, the prevalence of ESKD in lithium-treated patients was 0.5%—more than 6 times the estimated prevalence in the general population (Bendz et al., 2010)—after a mean treatment duration of 23 years.

Conflicting results have also been reported in experimental studies regarding lithium effect on kidney (Alsady et al., 2016). As few studies have experimented long-term exposure to lithium, a satisfying experimental model of lithium-induced nephropathy with renal insufficiency is still lacking in order to clarify the renal impact of chronic lithium use.

Our study allowed establishing a clear relationship between lithium use and GFR decrease. Indeed, in this population, the strongest determinant of mGFR was lithium treatment duration. The effect of lithium treatment duration was twice that of age, though a cumulative effect of age and treatment duration might potentiate renal lesions secondary to lithium chronic use on an underpinning senescent renal tissue. Of note, a majority of the patients were treated with the extended-release formulation of lithium carbonate, which is supposed to decrease the risk of kidney disease by limiting the risk of acute rises of serum lithium levels (Turan et al., 2002; Carter et al., 2013; Castro et al., 2016). Noteworthy, a higher serum lithium level was not associated with lower mGFR but the high proportion of missing data (36% of missing serum lithium values) might have limited the statistical power of the study. Our results are in line with the study by Bendz et al. showing no difference in terms of plasma lithium levels between patients with CKD and ESKD and those with normal renal function (Bendz et al., 1994). In contrast, Shine et al. suggested a higher risk of adverse renal outcomes with higher serum lithium levels, but the analysis was not adjusted on baseline eGFR (Shine et al., 2015). In any case, a causal relationship between serum lithium level and mGFR should be interpreted with caution in observational studies, as lower mGFR is also responsible of lower excretion rate of lithium and thus higher serum lithium levels (Alsady et al., 2016). Consistently, in our study, the daily dose of lithium was higher in patients with higher mGFR, which is not surprising as serum lithium level depends on lithium glomerular filtration which guides drug dose adaptation. Moreover, a single plasma lithium measurement is probably not representative of the long-term cumulative exposure.

Age was also an independent determinant of mGFR. Age is a well-demonstrated determinant of GFR decline, with a reported annual decline of 0.3–0.8 ml/min/year depending on the studied population[1]. However, GFR decline over time might reflect kidney senescence rather than pathological processes (Delanaye et al., 2019. The combined effect of age and lithium treatment is likely superior to this physiological GFR decrease over time.

Lithium administration is associated with metabolic disorders including weight gain and thyroid disorders, the pathophysiology of which are not fully understood (Gracious and Meyer, 2005). Obesity is a risk factor for CKD and ESKD (Chang et al., 2018). Other factors influencing BMI such as thyroid disorders (Mariani and Berns, 2012) might also promote kidney disease (Brenner and Meyer, 1982). We thus tested whether obesity or hypothyroidism might determine mGFR in these patients. Our analyses ruled out the role of obesity but found a strong association between hypothyroidism and lower mGFR. As previously reported, hypothyroidism was frequent in our study population (36%). The literature suggests a higher prevalence of hypothyroidism in female patients treated with lithium salts, with no reported effect of treatment duration on that risk. However, to the best of our knowledge, the association between kidney impairment and hypothyroidism in patients treated with lithium had not yet been described. Thyroid hormones exert both direct and indirect effects on renal functions, including cardiovascular effects affecting renal

[1]Chapter 2: Definition, identification, and prediction of CKD progression. Kidney Int Suppl. 2013;3(1):63–72. doi:10.1038/kisup.2012.65

hemodynamics (Mariani and Berns, 2012) potentially explaining the link between hypothyroidism and renal risk reported in previous epidemiological data (Rhee et al., 2015). However, our observational study does not establish causality between hypothyroidism and a lower mGFR, and the hypothesis of an association due to a common pathway of susceptibility to kidney and thyroid toxicities induced by chronic lithium treatment cannot be excluded.

The majority of patients (83%) displayed mild CKD with no albuminuria, and only two patients had a macroalbuminuria (<0.6 g/24 h), suggesting that glomerular disease did not participate in the kidney disease and that CKD was mostly not related to other underlying nephropathies. Yet, albuminuria was associated with a lower mGFR in agreement with current knowledge (Levey et al., 2020). The prevalence of diabetes was also very low (3%) in our cohort, and one patient displayed glomerular hyperfiltration, thus inducing a bias in the association between mGFR and diabetes. Conversely, hypertension was observed in 23% of patients and was an independent determinant of mGFR. It has been shown that CKD both contributes to the development of hypertension, and might result from hypertension (Ku et al., 2019). However, the prevalence of hypertension was far lower in our population compared to previously reported CKD populations (Vidal-Petiot et al., 2018), and even less than the reported prevalence in the general population in France (Perrine et al., 2018). Consequently, our results suggest that though hypertension is a relevant comorbidity contributing to the decrease in mGFR in patients treated with lithium salts, it is probably not a feature of lithium-induced nephropathy.

Renal cysts have been reported during long-term lithium use. However, the majority of published data involve small case series or case reports of patients with numerous microcysts and overt chronic kidney disease (Farres et al., 2003; Golshayan et al., 2012; Judge and Winearls, 2015). It is thus not yet clearly established whether renal microcysts are a specific and early feature of kidney impairment of chronic lithium use and if they might be used as a diagnostic tool. Moreover, data regarding the general population is lacking as usual imaging tools detect cysts of greater size (at least >5 mm) (Rahbari-Oskoui et al., 2014) and previous research reported the incidence of the prevalence of renal cysts (>1 cm) (Simms and Ong, 2014). In our cohort we found that even a relatively small number of microcysts was associated with a decrease in mGFR, with acceptable sensitivity and specificity to detect an mGFR <45 ml/min/1.73 m^2. The optimal cut-off value was 5 microcysts. Of major importance, while there was a strong negative correlation between mGFR and lithium treatment duration in patients with at least one microcyst, no such association was seen in patients without microcysts, in favor of a strong relationship between mGFR decrease and the presence of microcysts. The presence of microcysts might thus help inform improved strategies such as decreasing lithium exposure and preventing other comorbidities and nephrotoxic agents. These results must however be interpreted with caution due to the sample size (n = 99). Further investigation is also needed to establish if they reflect the degree of irreversibility, as the potential benefit of treatment discontinuation is still poorly known (Bendz et al., 1994; Markowitz et al., 2000), and shall be weighed against the established suicidal risk in this setting (Baldessarini et al., 1999).

Our study displays some limitations. Regarding lithium treatment, serum lithium level was not measured the same day as renal evaluation. However, our patients were stable, and underwent regular follow-up by psychiatrists. Second, neither information regarding cumulative lithium dose nor episodes of overdose were available. We were thus not able to investigate whether nephrotoxic effect of lithium was related to acute serum lithium level rises, or to a cumulative effect of low dose exposition (Carter et al., 2013). Of note, previous data suggest that renal impairment during chronic lithium use is not related to cumulative lithium dose (Bendz et al., 1994). Finally, the cross-sectional design of the study prevents us from analyzing the predictive value of lithium treatment duration on mGFR decline. Also, we cannot exclude that lithium was initiated in some patients with an already altered GFR, but it can be noted that only 2 out of 42 patients treated with lithium for less than 1 year had an mGFR below 60 ml/min/1.73 m^2.

In conclusion, our study confirmed the independent effect of lithium exposure on kidney function. This effect combined with that of age, hypothyroidism, hypertension and microalbuminuria on mGFR demonstrates that a close monitoring is necessary including blood pressure measurements, GFR estimation, albuminuria and thyroid hormone levels. MRI might also be considered as a useful tool in order to detect microcysts even during early stages of treatment. The association of a lower GFR with microcysts and hypothyroidism questions the issue of a differential individual genetic or acquired susceptibility to lithium treatment. These data might help inform improved strategies to prevent possibly irreversible kidney damage during chronic lithium use.

DATA AVAILABILITY STATEMENT

Data regarding this research is available upon request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board APHP. Nord CER-2021-74. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NT, A-LF, EV-P, FV, and MF designed the study, collected data, analyzed data. NT wrote the first draft of the manuscript. A-LF performed **Table 1**, **Figure 1** and **Figure 2**. NT and CT performed **Figure 3**. BE provided methodological and statistical support. LM, PF, A-LF, and NT blindly reviewed MRIs. A-LF, EV-P, FS, LM, PF, AK, FR, CT, NM, CD, MD, EM, BE, FB, FV, and MF reviewed, drafted and approved the manuscript.

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Role of miRNA-671-5p in Mediating Wnt/β-Catenin-Triggered Podocyte Injury

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Podocyte injury and proteinuria are the most common features of glomerular disease, which is the leading cause of end-stage renal failure. Hyperactivated Wnt/β-catenin signaling is closely associated with podocyte injury, but the underlying mechanisms are incompletely understood. Here we show that miRNA-671-5p (miR-671-5p) plays a crucial role in mediating β-catenin-triggered podocyte injury by targeting Wilms tumor 1 (WT1). Microarray-based expression profiling revealed that miR-671-5p was the most upregulated miRNA in podocytes after β-catenin activation. MiR-671-5p was colocalized with β-catenin in the glomeruli of proteinuric CKD *in vivo*. Bioinformatics analyses and luciferase reporter assays confirmed that miR-671-5p targeted WT1 mRNA. Overexpression of miR-671-5p mimics inhibited WT1 and impaired podocyte integrity, whereas miR-671-5p antagomir preserved the expression of WT1 and other podocyte-specific proteins under basal conditions or after β-catenin activation. In mouse remnant kidney model, overexpression of miR-671-5p aggravated podocyte injury, worsened kidney dysfunction and exacerbated renal fibrosis after 5/6 nephrectomy. In contrast, miR-671-5p antagomir alleviated podocyte injury and attenuated proteinuria and renal fibrotic lesions after glomerular injury *in vivo*. These studies underscore a pivotal role of miR-671-5p in mediating WT1 depletion and podocyte injury induced by β-catenin. Targeting miR-671-5p may serve as a new approach to prevent podocyte injury and proteinuria in proteinuric CKD.

Keywords: podocyte injury, Wnt, β-catenin, miRNA-671-5p, WT1, proteinuria

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INTRODUCTION

Podocyte injury is a major pathological feature of many glomerular diseases such as focal segmental glomerulosclerosis (FSGS), IgA nephropathy (IgAN) and diabetic kidney disease (DKD) (Reiser and Sever, 2013; Fogo, 2015; Assady et al., 2017). As an integral component of the glomerular filtration barrier, podocytes and their foot processes play an essential role in preventing against proteinuria (Pavenstadt et al., 2003; Greka and Mundel, 2012). Increasing evidence demonstrates that podocyte injury not only leads to an impaired glomerular filtration and development of proteinuria, but also is instrumental in causing glomerular sclerosis in proteinuric chronic kidney disease (CKD) (Patrakka and Tryggvason, 2009; Mathieson, 2011). As podocytes are highly specialized, terminally differentiated cells, it is very challenging to restore and repopulate them once they are lost or

dysfunctional (Brinkkoetter et al., 2013; Grahammer et al., 2013; Perico et al., 2016; Djudjaj and Boor, 2019). Therefore, it is of great importance to identify the extracellular culprits and delineate the molecular mechanism underlying podocyte damage.

Wnt/β-catenin is an evolutionarily conserved signaling that plays an imperative role in regulating embryonic development, injury repair and organ fibrosis (Angers and Moon, 2009; MacDonald et al., 2009; Clevers and Nusse, 2012). In many proteinuric CKD such as FSGS, DKD and IgAN, dysregulated activation of β-catenin is evident in the glomerular podocytes (Angers and Moon, 2009; MacDonald et al., 2009; Clevers and Nusse, 2012), suggesting its potential involvement in podocyte injury. Several studies have revealed that activation of β-catenin in podocytes down-regulates Wilms' tumor 1 protein (WT1) and induces the expression of β-catenin downstream target genes such as Snail1, matrix metalloproteinase-7 (MMP-7) and components of the renin-angiotensin system (RAS) (Zhou et al., 2015a; Zhou and Liu, 2015). This leads to podocyte dedifferentiation and mesenchymal transition, which impairs podocyte integrity and disrupts glomerular filtration barrier and causes proteinuria (Matsui et al., 2007; Li et al., 2008; Dai et al., 2009; Heikkila et al., 2010; Kato et al., 2011; Garcia de Herreros and Baulida, 2012; Tan et al., 2019).

As a master transcription factor, WT1 plays a fundamental role in establishing podocyte phenotype and integrity by controlling the expression of a variety of podocyte-specific genes. Our earlier studies have shown that WT1 and β-catenin antagonize each other and competitively bind to the common transcriptional coactivator, the cyclic AMP response element binding protein (CREB) binding protein (CBP) (Zhou et al., 2015b). Activation of β-catenin has no effect on the expression of WT1 mRNA but reduces its protein expression (Zhou et al., 2015b), suggesting that β-catenin inhibition of WT1 occurs at the post-transcriptional level. We further show that β-catenin can induce the ubiquitin-mediated degradation of WT1, but this only partially accounts for the decline of WT1 protein upon β-catenin activation (Zhou et al., 2015b). These findings insinuate that some other unidentified mechanisms may be involved in mediating the loss of WT1 by β-catenin.

MicroRNAs (miRNAs) are endogenous, small single-stranded non-coding RNAs with approximately 22 nucleotides. MiRNA can bind to the 3′-untranslated region (3′-UTR) of its target mRNA and lead to the inhibition of its translation process or directly lead to the degradation of mRNA, thereby inhibiting the expression of the target genes at the protein level (Trionfini and Benigni, 2017; Ishii et al., 2020). MiRNAs play important roles in various biological processes such as organogenesis, cell proliferation and apoptosis, and the pathogenesis of human diseases (Standart, 2007; Mitchell et al., 2008; Bartel, 2009; Inui et al., 2010; Tijsen et al., 2010; Wang et al., 2010). Along this line, we hypothesized that β-catenin may down-regulate WT1 protein by regulating miRNAs in glomerular podocytes.

In this study, we conducted a microarray assay to profile miRNA expression in mouse podocytes after β-catenin activation. We found that miR-671-5p was the top hit among the most differentially expressed miRNAs in β-catenin-overexpressed podocytes. We show that miR-671-5p specifically targets WT1 and inhibits its expression, thereby impairing podocyte integrity. Therefore, targeting miR-671-5p may be a novel strategy in the treatment of proteinuric CKD.

METHODS

Cell Culture and Treatment

Human embryonic kidney 293T cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. The conditionally immortalized mouse podocyte cell line (MPC5) was provided by Peter Mundel (Massachusetts General Hospital, Boston, MA) and maintained as described previously (Dai et al., 2009; Wang et al., 2011). To propagate podocytes, cells were cultured at 33°C with 5% CO_2 in RPMI1640 medium supplemented with 10% FBS and 10 units/ml mouse recombinant IFN-γ (R&D Systems, Minneapolis, MN) to enhance the expression of a thermosensitive T antigen. To induce differentiation, podocytes were grown under nonpermissive conditions at 37°C with 5% CO_2 in the absence of IFN-γ. For some studies, 293T cells or podocytes were transiently transfected with miR-671-5p mimics, inhibitor, and their respective controls (Genepharma, Shanghai, China) and/or N-terminally truncated β-catenin vector (pDel-β-cat) or psiCHECK-2-wide type/mut-WT1 by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Whole-cell lysates were prepared and subjected to real-time PCR and Western blot analyses. Cells were also subjected to immunofluorescence staining and microRNA microarray analysis.

MicroRNA Microarray and Bioinformatics Analysis

MPC5 cells were transiently transfected with expression vector encoding constitutively activated β-catenin (pDel-β-cat) or empty vector (pcDNA3) for 24 h (n = 3) and then total RNA was extracted by using TRIzol (Invitrogen, Carlsbad, CA). MiRCURY LNA microRNA chips (v. 8.0, Exiqon, Vedbaek, Denmark) were used to profile the differences for miRNA expression between two groups. The candidate target genes of miR-671-5p were predicted using TargetScan program (http://www.targe tscan.org).

Luciferase Reporter Assays

The 3′-UTR of WT1 was obtained from mouse genomic DNA by PCR and cloned into the psiCHECK-2 vector (Promega, Madison, WI) and then verified by sequencing. To test the binding specificity, the sequences in the mouse WT1 3′-UTR interacting with the miR-671-5p seed sequence were mutated from GCTTCCA to ATGGTTC. For the luciferase reporter assay, the reporter constructs were co-transfected with miR-671-5p mimic or negative control (NC) into 293T cells using lipofectamine 2000. At 24 h after transfection, luciferase activity was measured using a Dual-Luciferase report assay system (Promega), according to the manufacturer's instructions.

Real-Time Quantitative RT-PCR

Total RNA was isolated from cultured cells or whole kidney lysates using a TRIzol-based RNA isolation protocol (Invitrogen). For miRNA detection, RNA was reverse transcribed using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), and then TaqMan microRNA Assay for mmu-miR-671-5p/U6 was used for PCR according to the manufacturer's instructions. For mRNA, first-strand cDNA synthesis was carried out using 2 μg of RNA in 20 μl of reaction buffer by using a Reverse Transcription System Kit (Promega). Real-time quantitative RT-PCR (qRT-PCR) was performed using a SYBR Select Master Mix (ABI) on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) as described previously (Zhou et al., 2015b). The mRNA levels of various genes were calculated after normalizing with β-actin. The sequences of the primer pairs in qRT-PCR were as follows: mouse WT1, 5′-CATCCAGGCAGGAAAGTGT-3′ and 5′-TGCAGT CAATCAGGTGTGCT-3′; mouse CTGF, 5′-CAAAGCAGC TGCAAATACCA-3′ and 5′-GGCCAA ATGTGTCTTCCAGT-3′; mouse TGF-β1, 5′-GCAACATGTGGAACTCTACCAGAA-3′ and 5′-GACGTCAAAAGACAGCCACTCA-3′; mouse β-actin, 5′-CAGCTGAGAGGGAAATCGTG-3′ and 5′-CGT TGCCAATAGTGATGACC-3′.

Animal Models

All animals were obtained from the Southern Medical University Animal Center (Guangzhou, China) and housed in a standard environment with a regular light/dark cycle and free access to water and chow. Animal studies were approved by the Animal Ethics Committee at the Southern Medical University. For the 5/6 nephrectomy (5/6NX) model, two thirds of the left kidney of the male CD-1 mice (8 weeks) were removed through surgical resection of the upper and lower poles (week -1). One week later (week 0), the entire right kidney was removed via a right back incision. Sham-operated mice had their poles of left kidney (week -1) and right renal artery (week 0) identified, manipulated but not resected (Leelahavanichkul et al., 2010; Yang et al., 2010). Mice were randomly divided into three groups (n = 5 in each group): 1) sham control; 2) 5/6NX mice injected with pcDNA3 vector; and 3) 5/6NX mice injected with pCMV-pri-miR-671-5p plasmid. Plasmids were administered via hydrodynamics-based tail vein injection with a dosage of 1 mg/kg at week 2, 3, 4 and 5, respectively. At week 6, all mice were euthanized, and urine, blood and kidney tissue collected for various analyses.

For assessing the therapeutic effect of antimiR-671-5p, we utilized Adriamycin (ADR) nephropathy model, which developed robust glomerular injury, proteinuria and renal fibrotic lesions in BALB/c mice (He et al., 2011). Briefly, male BALB/c mice (6 weeks) were administered by a single intravenous injection of ADR (doxorubicin hydrochloride; Sigma-Aldrich, St. Louis, MO) at 11.5 mg/kg body weight. Oligonucleotides targeting miR-671-5p (miR-671-5p antagomir) or control antagomir were purchased from Genepharma (Shanghai, China) and injected into mice via the tail vein at 50 μg per mouse per day for 7 days. At week 2, all mice were euthanized, and urine, blood and kidney tissue collected for various analyses.

Urinary Albumin and Creatinine Assay

Serum creatinine levels were measured by an automatic chemistry analyzer (AU480; Beckman Coulter, Pasadena, CA). Urinary albumin was measured by using a mouse Albumin ELISA Quantitation kit, according to the manufacturer's protocol (Bethyl Laboratories, Inc., Montgomery, TX). Urinary creatinine was determined by a routine procedure as described previously (Zhou et al., 2013). Urinary albumin was standardized to creatinine and expressed as mg/mg urinary creatinine.

In Situ Hybridization

Paraffin sections (2 μm) were used to assess miR-671-5p expression in the kidneys of the patients with proteinuric CKD or 5/6NX mice. *In situ* hybridization (ISH) for miR-671-5p transcripts was performed using Enhanced Sensitive ISH Detection kit II (AP) (Boster) and digoxigenin-labeled LNA-miR-671-5p probes (Exiqon, Vedbaek, Denmark), according to the manufacturer's protocol. Human biopsy sections were obtained from diagnostic renal biopsies performed at Nanfang Hospital. All studies involving human kidney sections were approved by the Institutional Ethics Committee at the Nanfang Hospital.

Histology Assessment

Mouse kidney tissues were embedded in paraffin and then routinely proceeded. Quantitation was carried out on the sections stained with Periodic acid-Schiff (PAS) reagents as follows (Raij et al., 1984): grade 0, no mesangial expansion and glomerular hypertrophy; grade 1, 2, 3 and 4, mesangial expansion and glomerular hypertrophy up to 25%, 25–50%, 50–75% and 75–100%, respectively. The glomeruli in each stained section (at least 20 glomeruli) were evaluated under ×40 magnification and results averaged for each kidney. The sclerosis index for each mouse was calculated as follows: (N1 × 1 + N2 × 2 + N3 × 3 + N4 × 4)/n, where N1, N2, N3, and N4 represent the number of glomeruli graded as 1, 2, 3, and 4, respectively, and n represents the number of glomeruli assessed.

The assessment of kidney interstitial fibrosis was performed on the sections stained with Masson's trichrome staining (MTS) under an OLYMPUS BX43 microscope equipped with a digital camera. About 10 nonoverlapping images under high-powered (400×) fields per section were randomly captured, positive areas of MTS staining were quantified by a computer-aided pointing counting technique as described previously (Mo et al., 2017). MTS-positive area (percentage of whole kidney area except tubular lumens) was assessed for collagen deposition in the kidney. Similar methods were used to quantify the fibronectin expression in the kidney sections.

Immunohistochemical Staining

Immunohistochemical staining was performed using the established protocol (He et al., 2012). Antibodies used were as follows: rabbit anti-fibronectin (F3648; Sigma), mouse anti-α-SMA (ab7817; Abcam), mouse anti-β-catenin (610154, BD Biosciences) and rabbit anti-β-catenin (ab15180, Abcam). After incubation with the primary antibodies at 4°C overnight, slides were then stained with Biotin-SP-conjugated secondary

antibody (Jackson ImmunoResearch Laboratories). Images were captured by using OLYMPUS BX43 microscope equipped with a digital camera.

Immunofluorescence Staining and Confocal Microscopy

Kidney cryosections (3 μm thickness) were fixed with 4% paraformaldehyde for 15 min at room temperature. MPC5 cultured on coverslips were fixed with cold methanol: acetone (1:1) for 10 min at room temperature. After blocking with 10% donkey serum for 1 h, slides were immunostained with the following antibodies: ZO-1 (402200; Invitrogen), nephrin (20R-NP002; Fitzgerald Industries International), vimentin (D21H3; Cell Signaling Technology, Danvers, MA) and podocalyxin (AF1556; R&D Systems). The slides were then stained with Cy3-or Cy2-conjugated secondary antibody (Jackson ImmunoResearch Laboratories), and nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) (Sigma-Aldrich). Slides were viewed under a fluorescence microscope (Leica DMi8, Wetzlar, Germany) equipped with a digital camera.

Western Blot Analysis

Protein expression was analyzed by Western blot analysis of whole kidney lysates or whole cell lystaes as described previously (Zhou et al., 2014). The primary antibodies used were as follows: anti-podocalyxin (AF1556; R&D Systems), anti-ZO-1 (402200; Invitrogen), anti-nephrin (ab58968; Abcam), anti-WT1 (sc-393498; Santa Cruz Biotechnology), anti-fibronectin (F3648; Sigma), anti-α-SMA (ab5694; Abcam), anti-collogen Ⅰ (BA0325, Boster Biotechnology), anti-α-tubulin (RM2007; Ray Antibody Biotech) and anti-GAPDH (RM2002; Ray Antibody Biotech). Relative protein levels of Western blots were quantified with densitometries, analyzed by ImageJ software and reported after normalizing to the loading controls. Relative protein levels over the control group (setting as 1.0) were reported.

Statistical Analyses

All data examined are expressed as mean ± SEM. Statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL). Comparison between groups was made using *t* test or one-way ANOVA followed by Student-Newman-Kuels test or Dunnett's T3. $p < 0.05$ was considered significant.

RESULTS

MiR-671-5p Is Induced in Podocytes After β-Catenin Activation and Specifically Targets WT1

To investigate the potential role of miRNAs in mediating Wnt/β-catenin-induced podocyte injury, we used an unbiased approach to profile miRNA expression in podocytes after β-catenin activation. To this end, mouse podocytes were transfected with constitutively activated β-catenin expression vector (pDel-β-cat) or empty vector (pcDNA3) and then subjected to microRNA array analysis. We found substantial changes in the miRNA expression after β-catenin activation, with 119 miRNA up-regulated and 91 down-regulated. The relative expression level of these miRNAs is presented as a heatmap (**Figure 1A**). The miR-671-5p was identified as the most differentially expressed miRNA induced by β-catenin in podocytes. This finding was further confirmed *in vitro* by quantitative, real-time PCR (qPCR). As shown in **Figure 1B**, consistent with the results of miRNA microarray, miR-671-5p was upregulated in podocytes after transient transfection of pDel-β-cat plasmid. *In situ* hybridization (ISH) revealed that miR-671-5p specifically upregulated in the glomerular podocytes of diseased kidney induced by 5/6NX, as compared with sham controls. Co-staining for β-catenin and miR-671-5p on serial sections demonstrated that β-catenin colocalized with miR-671-5p (**Figure 1C**), suggesting a role for β-catenin in upregulating miR-671-5p expression *in vivo* after podocyte injury.

Bioinformatics analyses using miRNA target prediction tools such as TargetScan revealed that miR-671-5p could target WT1, as the 3′-untranslatd region (UTR) of WT1 harbored the conserved site complementary to the seed sequence of miR-671-5p (**Figure 1D**). We found that transfection of mouse podocytes (MPC5) with miR-671-5p mimic did not affect WT1 mRNA expression (**Figure 1E**), suggesting that miR-671-5p may regulate WT1 expression at the post-transcriptional level.

To determine whether WT1 is a direct target of miR-671-5p, the luciferase reporter plasmids containing WT1 3′-UTR (wild type) or mutant sequence corresponding to the miR-671-5p seed sequence were constructed (**Figure 1F**), and transfected into 293T cells, in combination with miR-671-5p mimic or control miRNA (miR-Ctrl). As shown in **Figure 1G**, transfection with miR-671-5p mimic inhibited the luciferase activity of the reporter containing wild-type WT1 3′-UTR but not the mutant WT1 3′-UTR, suggesting that WT1 is a direct target of miR-671-5p.

MiR-671-5p Is Up-Regulated in Glomerular Podocytes in Human CKD

To investigate the clinical relevance of miR-671-5p to the pathogenesis of human proteinuric CKD, we performed ISH for detecting miR-671-5p with a digoxigenin-labeled LNA probes in human kidney biopsies from the patients with various proteinuric CKDs. Kidney biopsies from diabetic kidney disease (DKD), lupus nephritis (LN), focal segmental glomerulosclerosis (FSGS) and IgA nephropathy (IgAN) were subjected to ISH for miR-671-5p, whereas non-tumor kidney tissue sections from renal cell carcinoma patients who underwent cancer resection were used as normal control. As shown in **Figure 1H**, miR-671-5p was barely detectable in normal kidney, but markedly induced in the glomerular podocytes in different proteinuric CKDs. These results indicate a close association between miR-671-5p and the pathogenesis of podocyte injury in human CKD.

MiR-671-5p Targets WT1 and Impairs Podocyte Integrity *In Vitro*

To study the potential role of miR-671-5p in podocyte biology, we maneuvered miR-671-5p expression in mouse podocytes (MPC5)

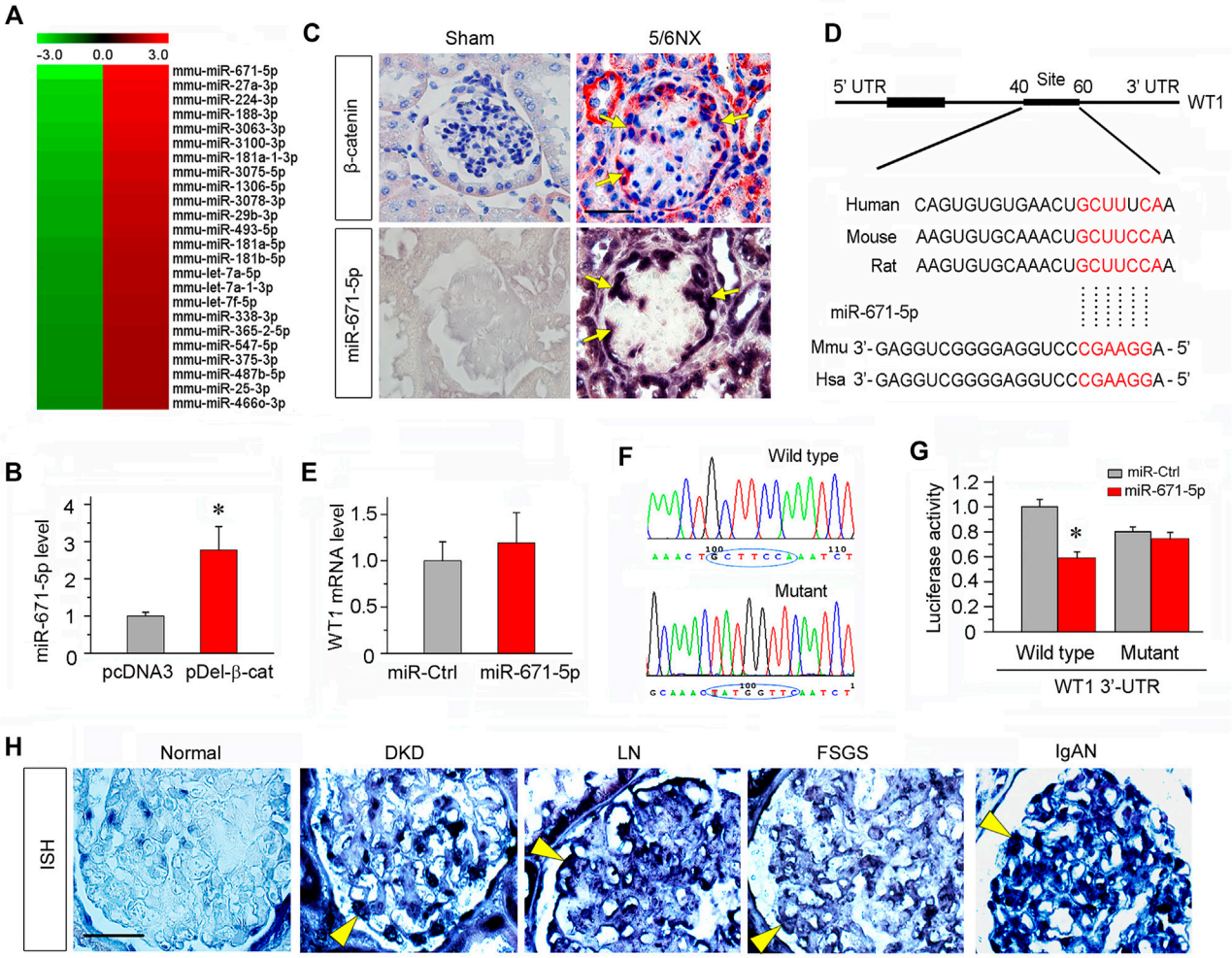

FIGURE 1 | miR-671-5p is induced by β-catenin in podocytes and targets WT1. **(A)** Microarray chip analysis of miRNAs expression in different groups (left: pcDNA3 control group; right: pDel-β-cat group). The red and green colors indicated high or low expression, respectively. **(B)** Mouse podocytes (MPC5) were transfected with empty vector (pcDNA3) or β-catenin expression plasmid (pDel-β-cat) for 24 h. qRT-PCR analysis showed the expression of miR-671-5p in different groups. *$p < 0.05$ ($n = 3$). **(C)** Co-localization of β-catenin and miR-671-5p in glomerular podocytes of diseased kidney. Kidney serial sections (3 μm) of 5/6NX mice were subjected to immunostaining for β-catenin and *in situ* hybridization for miR-671-5p. Representative micrographs from sham group are also shown. Arrows indicate positive staining in podocytes. Scale bar, 20 μm. **(D)** Bioinformatics analysis shows the predicted binding sites of miR-671-5p in the WT1 3′-untranslatd region (UTR) using the TargetScan software. **(E)** qRT-PCR analysis shows that overexpression of miR-671-5p did not affect WT1 mRNA level in mouse podocytes. MPC5 cells were transfected with miRNA negative control (miR-Ctrl) or miR-671-5p mimics (miR-671-5p) for 24 h. **(F)** Sequence validation of the wild type or mutant WT1 3′-UTR for the luciferase reporter construction. The wild-type miR-671-5p binding site in WT1 3′-UTR **(upper)** and the mutated one **(bottom)** in the region corresponding to the miR-671-5p seed sequence are shown. **(G)** Luciferase reporter assay show that miR-671-5p mimics decreased the luciferase activity in 293T cells co-transfected with wild-type WT1 3′ UTR, but not with mutant WT1 3′ UTR. *$p < 0.05$. **(H)** Representative micrographs show miR-671-5p expression in glomerular podocytes of human kidney biopsies from the patients with various CKDs by *in situ* hybridization. Arrowheads indicate the positive staining for miR-671-5p in glomerular podocytes. Kidney tissues adjacent to renal cell carcinoma from patients who underwent carcinoma resection were used as normal control. Scale bar, 20 μm.

by transfecting either miR-671-5p mimic or inhibitor. As shown in **Figure 2A**, transfection with miR-671-5p mimic markedly increased miR-671-5p level, as assessed by qPCR analysis. We found that overexpression of miR-671-5p substantially inhibited WT1 protein expression in MPC5 cells (**Figures 2B,C**), suggesting that miR-671-5p can inhibit its target gene as expected. Interestingly, inhibition of WT1 by miR671-5p down-regulated podocalyxin and ZO-1 expression (**Figures 2B,D,E**). Similar results were obtained by immunostaining for ZO-1 protein (**Figure 2F**). In contrast, inhibition of miR671-5p by anti-miRNA oligonucleotides (antimir-671-5p) in MPC5 cells up-regulated WT1, nephrin, podocalyxin and ZO-1 (**Figures 2G–K**). These findings suggest that miR-671-5p specifically targets WT1 and impairs podocyte integrity.

MiR-671-5p Aggravates β-Catenin-Induced Podocyte Injury *In Vitro*

To validate the role of miR-671-5p in mediating β-catenin-induced podocyte injury, we transfected β-catenin expression

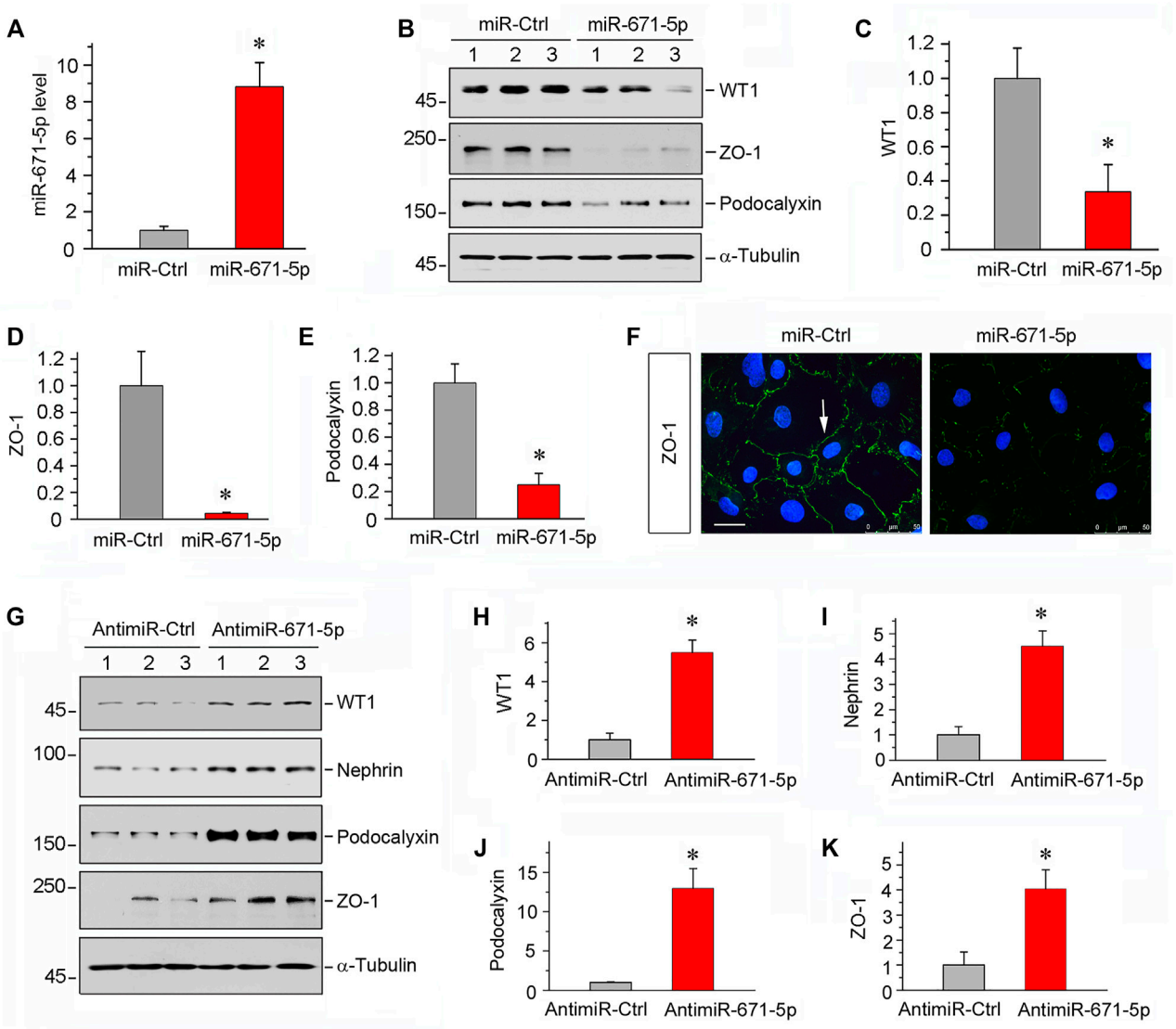

FIGURE 2 | Overexpression of miR-671-5p impairs but knockdown of miR-671-5p protects podocyte integrity *in vitro*. Mouse podocytes (MPC5) were transfected with miR-671-5p mimics (miR-671-5p) or negative control (miR-Ctrl) for 24 h. **(A)** qRT-PCR analysis shows the relative levels of miR-671-5p after transfection. *$p < 0.05$ ($n = 3$). **(B–E)** Representative Western blot **(B)** and graphic presentations of WT1 **(C)**, ZO-1 **(D)** and podocalyxin **(E)** were presented. *$p < 0.05$ ($n = 3$). **(F)** Representative micrographs show the expression and distribution of ZO-1 in podocytes after miR-671-5p overexpression. Scale bar, 50 μm. **(G–K)** Inhibition of miR-671-5p protects podocyte integrity. MPC5 cells were transfected with miR-671-5p inhibitor (AntimiR-671-5p) or control (AntimiR-Ctrl) or for 24 h. Representative Western blot **(G)** and graphic presentations of WT1 **(H)**, nephrin **(I)**, podocalyxin **(J)** and ZO-1 **(K)** were presented. *$p < 0.05$ ($n = 3$).

plasmid (pDel-β-cat), along with miR-671-5p mimic or miR-671-5p inhibitor, into mouse podocytes. Earlier studies show that transfection with pDel-β-cat plasmid induces Snail1 and plasminogen activator inhibitor 1 (PAI-1) expression in podocytes (Zhou et al., 2015b), confirming its ability to stimulate β-catenin downstream genes. As illustrated in **Figures 3A–C**, overexpression of either β-catenin or miR-671-5p inhibited WT1 and podocalyxin. Moreover, combination of β-catenin and miR-671-5p led to further suppression of WT1 and podocalyxin (**Figures 3A–C**), suggesting that miR-671-5p aggravates β-catenin-induced podocyte injury. However, transfection with miR-671-5p inhibitor upregulated podocalyxin, nephrin, ZO-1 and WT1 expression, even in the presence of β-catenin activation (**Figures 3D–H**). Immunofluorescence staining for ZO-1 gave rise to similar results (**Figure 3I**).

MiR-671-5p Accelerates 5/6NX-Induced Podocyte Injury and Glomerulosclerosis *In Vivo*

The finding on the effect of miR-671-5p on podocyte injury *in vitro* prompted us to investigate its potential effect on proteinuric kidney disease *in vivo*. To this end, we used a mouse model of CKD induced by 5/6NX, characterized by

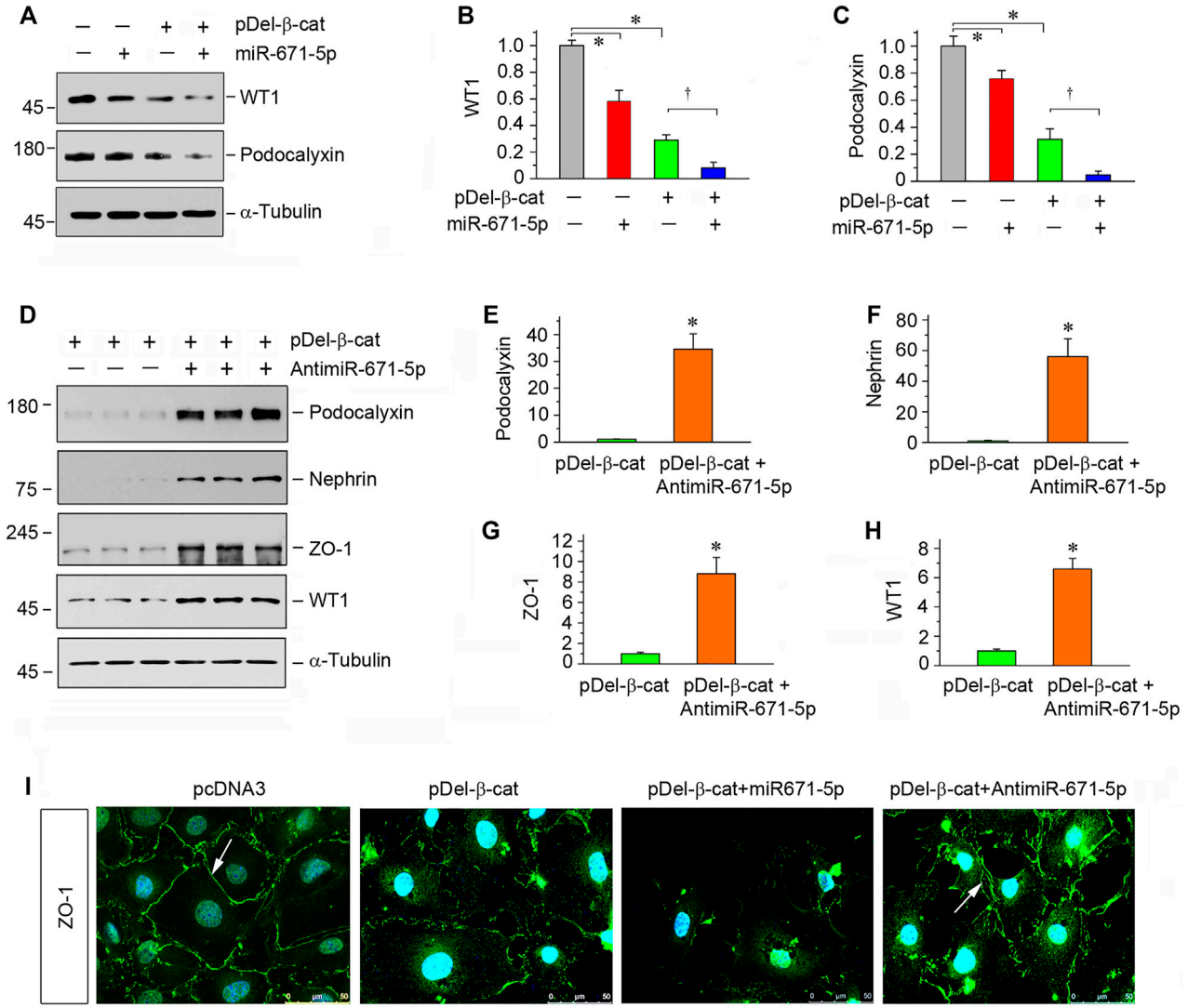

FIGURE 3 | miR-671-5p aggravates β-catenin-induced podocyte injury while miR-671-5p inhibitor ameliorates it *in vitro*. **(A–C)** Representative Western blot **(A)** and graphic presentations of WT1 **(B)** and podocalyxin **(C)** were presented. MPC5 cells were transfected with miR-671-5p mimics (miR-671-5p) or/and β-catenin expression plasmid (pDel-β-cat) for 24 h *$p < 0.05$ versus pcDNA3 controls; †$p < 0.05$ versus pDel-β-cat ($n = 3$). **(D–H)** Representative Western blot **(D)** and graphic presentations of podocalyxin **(E)**, nephrin **(F)**, ZO-1 **(G)** and WT1 **(H)** were presented. MPC5 cells were transfected with β-catenin expression plasmid (pDel-β-cat) or/and miR-671-5p inhibitor (AntimiR-671-5p) for 24 h *$p < 0.05$ ($n = 3$). **(I)** Representative micrographs show the expression of ZO-1 in different groups as indicated. MPC5 cells were transfected with β-catenin expression plasmid (pDel-β-cat) and miR-671-5p mimics (miR-671-5p)/miR-671-5p inhibitor (AntimiR-671-5p) for 24 h, respectively. Scale bar, 50 μm.

progressive podocyte injury, glomerulosclerosis and loss of renal function (Xiao and Liu, 2013). As presented in **Figure 4A**, pCMV-pri-miR-671-5p plasmid or pcDNA3 plasmid were administered via tail vein for 4 times, starting from 2 weeks after 5/6NX surgery (**Figure 4A**). As shown in **Figures 4B,C**, miR-671-5p level was increased in 5/6NX group compared with sham controls, and injections of pCMV-pri-miR-671-5p plasmid further increased miR-671-5p level. ISH revealed that miR-671-5p was mainly expressed in glomerular podocytes (**Figure 4B**).

We next assessed podocyte injury by examining the expression of nephrin and vimentin. As shown in **Figure 4D**, immunofluorescence staining exhibited that overexpression of miR-671-5p accelerated the loss of nephrin and further increased glomerular vimentin expression in 5/6NX mice. Western blot analysis also showed that overexpression of miR-671-5p suppressed the expression of WT1, nephrin and podocalyxin in this model (**Figures 4E–H**). PAS staining showed that 5/6 NX caused mild glomerular hypertrophy and matrix deposition, capillary collapse and tubular dilation with expanded lumen, and overexpression of miR-671-5p markedly worsened these pathological lesions (**Figure 4I**). The extent of glomerular lesions was assessed by semi-quantitative analysis and presented in **Figure 4J**. These data illustrate that miR-671-5p aggravates podocyte injury and glomerular sclerotic lesions.

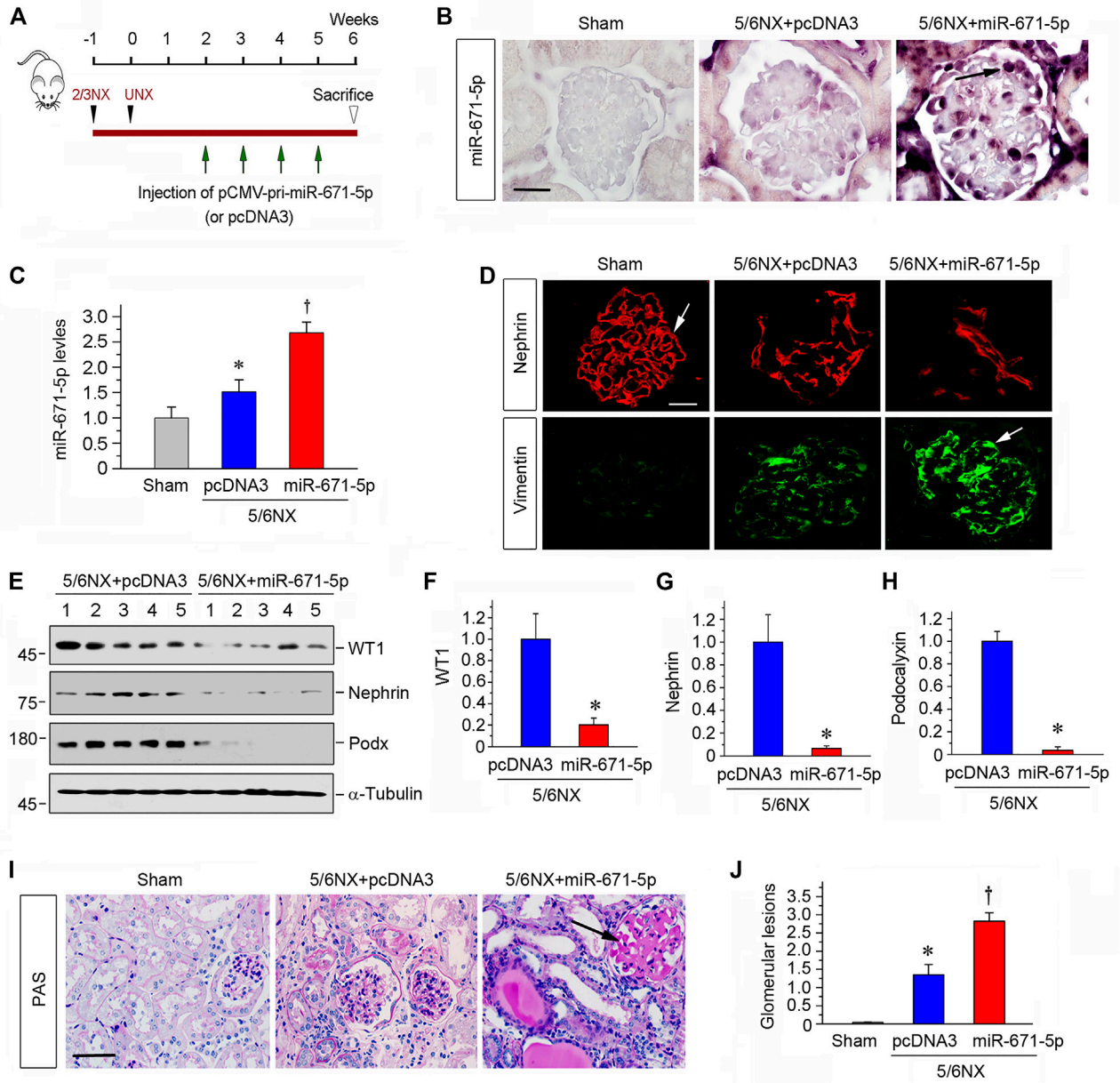

FIGURE 4 | Ectopic expression of miR-671-5p accelerates podocyte injury and glomerulosclerosis in 5/6NX model. **(A)** Experimental design. Black Arrowheads indicate the time of kidney resection. Green arrows indicate the injections of pCMV-pri-miR-671-5p or pcDNA3 plasmid. **(B)** Representative micrographs show miR-671-5p expression in glomerular podocytes in 5/6NX model by *in situ* hybridization. Scale bar, 20 μm. **(C)** qRT-PCR analysis of miR-671-5p levels in different groups as indicated. *$p < 0.05$ versus sham controls; †$p < 0.05$ versus 5/6NX alone ($n = 5–6$). **(D)** Immunofluorescence staining show renal expression of nephrin and vimentin in different groups as indicated. Frozen kidney sections were stained for nephrin and vimentin. Scale bar, 20 μm. **(E–H)** Representative Western blot **(E)** and graphic presentations of WT1 **(F)**, nephrin **(G)** and podocalyxin **(H)** were presented. *$p < 0.05$ ($n = 5–6$). **(I)** Representative micrographs show periodic acid-Schiff (PAS) staining of the kidneys in different groups. Scale bar, 50 μm. **(J)** Quantitative determination of glomerular lesions (based on PAS staining) in different groups. *$p < 0.05$ versus sham controls, †$p < 0.05$ versus 5/6NX ($n = 5–6$).

MiR-671-5p Aggravates Kidney Dysfunction and Exacerbates Renal Fibrosis *In Vivo*

We further assessed kidney function by measuring serum creatinine level. As shown in **Figure 5A**, serum creatinine level was elevated after 5/6NX, and expression of miR-671-5p further increased serum creatinine in this model. We found that overexpression of miR-671-5p also increased the expression of numerous fibrosis-related proteins such as fibronectin, collagen I and α-smooth muscle actin (α-SMA), as demonstrated by Western blot analyses of whole kidney lysates (**Figures 5B–E**). Consistently, qPCR showed that miR-671-5p induced the mRNA

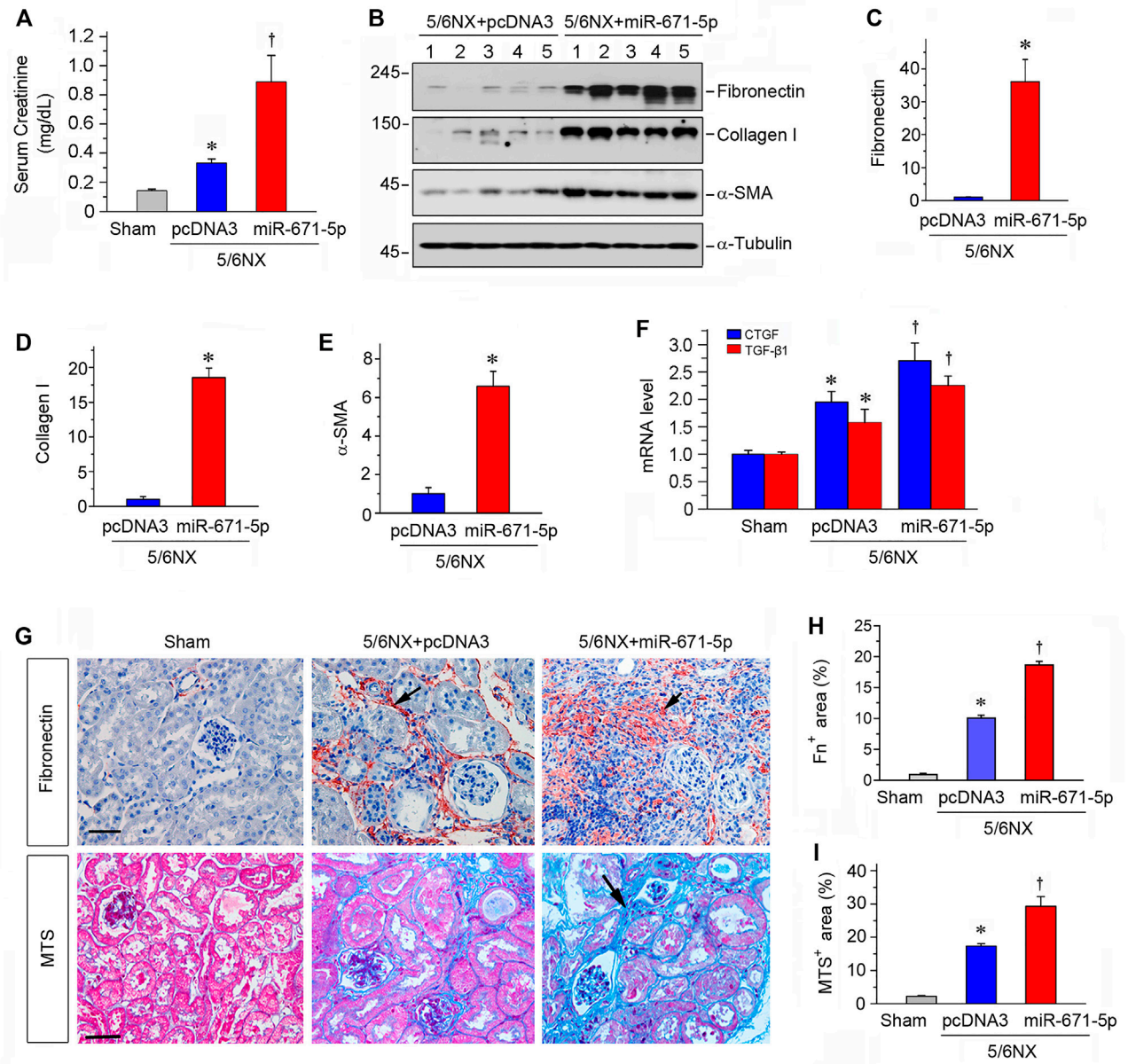

FIGURE 5 | Expression of miR-671-5p *in vivo* aggravates kidney dysfunction and fibrosis in 5/6NX model. **(A)** Expression of miR-671-5p *in vivo* aggravates kidney dysfunction in 5/6NX mice. Serum creatinine was assessed in different groups as indicated. *$p < 0.05$ versus sham controls, †$p < 0.05$ versus 5/6NX ($n = 5–6$). **(B–E)** Representative Western blots **(B)** and graphic presentations of fibronectin **(C)**, collagen I **(D)** and α-SMA **(E)** were presented. *$p < 0.05$ ($n = 5–6$). **(F)** qRT-PCR analysis shows CTGF and TGF-β1 mRNA levels in different groups. *$p < 0.05$ versus sham controls, †$p < 0.05$ versus 5/6NX ($n = 5–6$). **(G)** Representative micrographs show that overexpression of miR-671-5p aggravated fibronectin deposition and fibrotic lesions in the 5/6NX kidneys. Paraffin kidney sections were stained for fibronectin (upper panel) and subjected to Masson's trichrome staining (MTS) (bottom panel). Scale bar, 50 μm. **(H,I)** Quantitative determination of renal fibronectin expression **(H)** and renal collagen deposition (based on MTS) **(I)** in different groups. *$p < 0.05$ versus sham controls, †$p < 0.05$ versus 5/6NX ($n = 4–6$).

expression of TGF-β1 and connective tissue growth factor (CTGF) in 5/6NX mice (**Figure 5F**).

We further assessed the fibrotic lesions in the 5/6NX kidneys after overexpression of miR-671-5p. As shown in **Figures 5G,H**, 5/6NX induced the deposition of fibronectin in kidneys, and overexpression of miR-671-5p aggravated the deposition. Masson's trichrome staining (MTS) also revealed significant collagens deposition in the kidneys after 5/6NX, and overexpression of miR-671-5p further increased their deposition (**Figures 5G,I**). Taken together, these results indicate that miR-671-5p aggravates renal fibrotic lesions in 5/6NX mice *in vivo*.

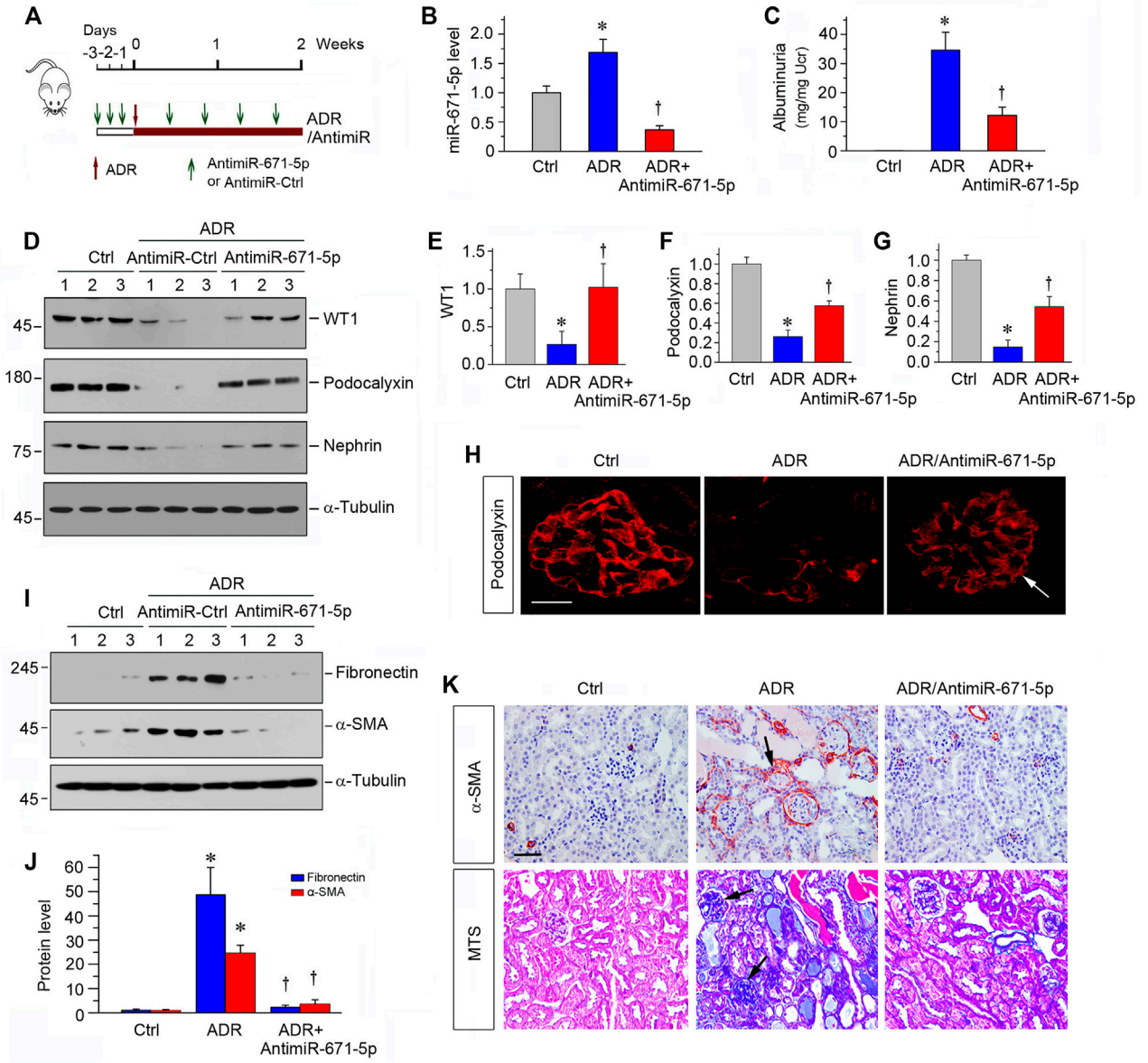

FIGURE 6 | Inhibition of miR-671-5p reduces proteinuria and renal fibrotic lesions in ADR nephropathy. **(A)** Experimental design. Red Arrows indicate the time of ADR injection. Green arrows indicate the different time points of antagomir injections. **(B)** qRT-PCR analysis shows that miR-671-5p level was increased in ADR group compared with control, and injections of antimiR-671-5p decreased miR-671-5p level. *$p < 0.05$ versus normal controls; †$p < 0.05$ versus ADR ($n = 5–6$). **(C)** Inhibition of miR-671-5p reduces proteinuria in ADR nephropathy. Urinary albumin levels were assessed in mice at 2 weeks after ADR injection and expressed as mg/mg creatinine. *$p < 0.05$ versus normal controls; †$p < 0.05$ versus ADR ($n = 5–6$). **(D–G)** Representative Western blots **(D)** and graphic presentations of WT1 **(E)**, podocalyxin **(F)** and nephrin **(G)** were presented. *$p < 0.05$ versus normal controls, †$p < 0.05$ versus ADR alone ($n = 5–6$). **(H)** Immunofluorescence staining shows that antimiR-671-5p preserved renal podocalyxin expression in ADR nephropathy. Arrow indicate positive staining. Scale bar, 20 μm. **(I,J)** Representative Western blots **(I)** and graphic presentations of fibronectin and α-SMA **(J)** were presented. *$p < 0.05$ versus normal controls, †$p < 0.05$ versus ADR alone ($n = 5–6$). **(K)** Representative micrographs show that antimiR-671-5p inhibited α-SMA expression (upper panel) and renal fibrotic lesions (bottom panel) in different groups as indicated. Scale bar, 50 μm.

Inhibition of MiR-671-5p Ameliorates Podocyte Injury and Renal Fibrosis in ADR Nephropathy

To further confirm the role of miR-671-5p in proteinuric CKD, we used another mouse model of podocyte injury and proteinuria induced by ADR, a model of human FSGS (Pippin et al., 2009). As shown in **Figure 6A**, ADR was administered at day 0, and miR-671-5p antagomir was injected intravenously at different time points as indicated. The experiments were terminated at 2 weeks after ADR injection. As illustrated in **Figure 6B**, renal miR-671-5p level was reduced by miR-671-5p antagomir in this model. Urinary albumin levels were elevated at 2 weeks after ADR injection, and antimiR-671-5p largely abolished albuminuria in

this model (**Figure 6C**). We then assessed the level of WT1 protein, the target of miR-671-5p. As shown in **Figures 6D,E**, ADR reduced WT1 expression, whereas antimiR-671-5p largely restored its level. Furthermore, antimiR-671-5p restored the expression of podocalyxin and nephrin, which were down-regulated by ADR (**Figures 6D,F,G**). Immunofluorescence staining also showed that antimiR-671-5p restored podocalyxin level and distribution (**Figure 6H**). Taken together, it appears that miR-671-5p plays a role in podocyte injury by targeting WT1, and antimiR-671-5p restores WT1, thereby preserving podocyte integrity.

We also assessed the renal fibrotic lesions in this ADR nephrology model. As shown in **Figures 6I,J**, renal fibronectin and α-SMA were markedly induced after ADR, and antimiR-671-5p abolished the induction of these proteins (**Figures 6I,J**). Similarly, immunostaining for α-SMA and Masson's trichrome staining demonstrated that antimiR-671-5p ameliorated myofibroblast activation and mitigated renal fibrotic lesions in ADR nephropathy (**Figure 6K**).

DISCUSSION

In this study, using an unbiased microarray expression profiling approach, we have identified miR-671-5p as a key downstream effector of Wnt/β-catenin signaling, which mediates podocyte injury by targeted inhibition of WT1. This conclusion is supported by several lines of evidence. First, miR-671-5p is induced in podocytes after β-catenin activation *in vitro* and colocalizes with β-catenin in glomerular podocytes *in vivo*, and it is specifically upregulated in glomerular podocytes of human kidney biopsies from patients with proteinuric CKD. Second, miR-671-5p targets the 3′-UTR of WT1 and inhibits its expression at the post-transcriptional level. Third, overexpression of miR-671-5p mimic impairs podocyte phenotype and integrity, whereas miR-671-5p antagomir preserves podocyte integrity under basal conditions or after β-catenin activation. Finally, overexpression of miR-671-5p *in vivo* aggravates podocyte injury, glomerulosclerosis and renal fibrotic lesions in 5/6NX mice, while miR-671-5p antagomir ameliorates podocytopathy and renal fibrotic lesions after glomerular injury. These studies underscore a pivotal role of miR-671-5p in mediating podocyte injury and glomerular lesions in proteinuric CKD. Our findings also uncover the intimate interplay among β-catenin, miR-671-5p and WT1, and provide novel insights into the mechanism how Wnt/β-catenin activation triggers podocyte dysfunction, proteinuria and glomerulosclerotic lesions.

Podocytes are highly specialized and terminally differentiated cells, with unique and sophisticated 3-dimensional (3D) structure characterized by foot processes and slit diaphragm (Greka and Mundel, 2012). Such a fine 3D structure of podocytes is largely controlled by WT1, a master transcription factor exclusively expressed in glomerular podocytes in the adult kidney. WT1 controls the expression of a host of podocyte-specific proteins such as nephrin and podocalyxin (Palmer et al., 2001; Guo et al., 2004; Wagner et al., 2004; Lowik et al., 2009; Rachel E.; Wang et al., 2011; Dong et al., 2015). Extensive studies have demonstrated that loss of WT1 is a common feature of podocytopathy that occurs in virtually all forms of proteinuric CKD in animal models and humans (Jian-Kan Guo et al., 2002; Niaudet and Gubler, 2006; Zhou et al., 2015b). Our earlier studies have shown that WT1 and β-catenin functionally antagonize each other in podocytes. Under normal physiological conditions, WT1 is highly expressed and β-catenin is minimal and inactivated (Zhou et al., 2015b). As such, WT1 is dominant over β-catenin in normal glomeruli, which keeps podocyte healthy and fully differentiated. However, in the pathological state, β-catenin is activated whereas WT1 is lost, thereby making β-catenin a predominant regulator in controlling gene transcription (Zhou et al., 2015b). The mutual antagonism between β-catenin and WT1 appears to be mediated through diverse mechanisms. We previously show that β-catenin and WT1 competitively bind to the common transcriptional coactivator CBP in a mutually exclusive manner and thus antagonize each other functionally (Zhou et al., 2015b). In addition, activation of β-catenin also induces ubiquitin-mediated degradation of WT1 protein, providing another means for β-catenin to negatively control WT1 protein in podocytes (Zhou et al., 2015b). Here our present study indicates that by inducing miR-671-5p, β-catenin constrains WT1 protein by the miRNA-mediated inhibition at the post-transcriptional level. Collectively, it is conceivable that β-catenin restrains WT1 activities by three distinctive mechanisms, including competitive binding to CBP, ubiquitin-mediated protein degradation and miRNA-based inhibition.

The findings in the present study could have significant clinical implications, as miR-671-5p is also induced in glomerular podocytes of human kidney biopsies from patients with various CKDs such as FSGS, IgAN, DKD and lupus nephritis (LN) (**Figure 1**). Because these CKDs share several common pathological features characterized by podocyte lesions and proteinuria, it is plausible to speculate that induction of miR-671-5p could represent a convergent response of podocytes, which results in WT1 depletion and podocyte injury. *In situ* hybridization reveals that induction of miR-671-5p in podocytes is common among different proteinuric CKDs, indicating a close association between miR-671-5p and the pathogenesis of human glomerular lesions. This observation is of significance, as miRNAs are not always evolutionally conserved across different species and even conserved miRNAs do not necessarily display the same expression levels or patterns at different stages within a species (Ha et al., 2008; Chen et al., 2020). The comparable induction pattern of miR-671-5p suggests an evolutionally conserved response of glomerular podocytes after injury in mice and humans.

The present study demonstrates that controlling miR-671-5p expression by different maneuvers may be an effective approach to preserve WT1 protein, thereby ameliorating proteinuric CKD. Inhibition of miR-671-5p via antagomir not only preserves WT1 and podocyte integrity under basal conditions, but also ameliorates β-catenin-induced podocyte lesions by restoring WT1 expression *in vitro* (**Figures 2**, **3**). In contrast, overexpression of miR-671-5p decrease WT1 and podocyte-specific proteins such as nephrin and podocalyxin. Furthermore, miR-671-5p appears to work in concert with β-catenin to further aggravate podocyte lesions. The function of miR-671-5p is further confirmed in mouse models of proteinuric CKD induced by 5/6NX and ADR, which are characterized by

podocyte injury, proteinuria and glomerulosclerosis. Consistently, overexpression of miR-671-5p accelerates 5/6NX-induced podocyte injury and renal insufficiency, whereas miR-671-5p antagomir restores WT1 and prevents the progression of ADR nephropathy. Notably, inhibition of miR-671-5p also mitigates kidney interstitial fibrosis. This is probably a consequence secondary to the alleviation of podocyte injury and proteinuria. However, we cannot exclude the possibility that inhibition of miR-671-5p may have direct beneficial effects on tubular epithelial cells, as its expression is also induced in injured tubules (**Figure 1**). Regardless of the mechanisms, the results from the present study provide the proof-of-principle that inhibition of miR-671-5p could be a promising approach for developing therapeutics to treat proteinuric CKDs.

It should be pointed out that, apart from miR-671-5p, there are other miRNAs that may also participate in repressing WT1 protein and cause proteinuria. For example, miR-193a has been reported to target WT1 for inhibition, thereby leading to the pathogenesis of FSGS (Gebeshuber et al., 2013). A recent study has shown that miR-466o-3p also plays a role in mediating Wnt/β-catenin-triggered WT1 repression in podocytes in mice, but not in humans. As such, the clinical relevance of miR-466o-3p to human CKDs remains to be determined. The fact that multiple miRNAs are involved in WT1 regulation in podocytes is not surprising, as many miRNAs targeting the same mRNA is a common feature of miRNA action. On the other hand, bioinformatics analyses show that at least 152 genes are potentially controlled directly by miR-671-5p. Functional and pathway enrichment analyses reveal that miR-671-5p may play an important role in podocyte injury by other mechanisms beyond WT1 inhibition. Therefore, the relative contributions of each miRNA to WT1 suppression in podocytes *in vivo*, as well as the larger landscape of signaling around miR-671-5p remains elusive and deserves further investigation.

In summary, we show herein that miR-671-5p is upregulated in podocytes after β-catenin activation and colocalizes with β-catenin in podocytes of diseased kidneys. We show that miR-671-5p specifically targets the 3′-UTR of WT1 mRNA and inhibits WT1 expression. Overexpression of miR-671-5p reduces WT1 protein and impairs podocyte phenotype and integrity, whereas miR-671-5p antagomir maintains podocyte integrity after β-catenin activation both *in vitro* and *in vivo*. Although more studies are needed, our findings suggest that targeting miR-671-5p may serve as a new approach to prevent against podocyte injury and proteinuric CKDs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Nanfang Hospital Medical Ethics Committee. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Nanfang Hospital Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

LZ and YL conceived the research; CW, and LZ designed the experiments; CW, JL, XZ, QC, XB, and XH performed the experiments; CW, JL, LZ, and YL analyzed the data; CW, JL created the figures, JL and YL wrote the manuscript; and all authors approved the final version of the manuscript.

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Efficacy and Safety of Different Immunosuppressive Therapies in Patients With Membranous Nephropathy and High PLA2R Antibody Titer

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Background: This study aimed to evaluate clinical features and prognosis and therapy option of patients with different risk ranks based on antibody against the M-type phospholipase-A2-receptor (PLA2Rab) level in seropositive M-type phospholipase-A2-receptor (PLA2R)-associated membranous nephropathy (MN) in a large sample size, multi-center study.

Method: Based on the unvalidated cut-off value of PLA2Rab above 150 RU/ml as one of the clinical criteria for high risk of progressive kidney function loss in MN according to 2020 Kidney Disease: Improving Global Outcomes (KDIGO) draft guidelines recommendation, a total of 447 patients who received cyclophosphamide (CTX) or tacrolimus (TAC) combined with corticosteroids treatment for 12 months were divided into high titer (>150 RU/ml) group and non-high titer (20–150 RU/ml) group, which were subdivided into CTX subgroup and TAC subgroup. The overall cohort was classified into CTX group and TAC group as well. Clinical parameters levels and remission rates were recorded at 3, 6, and 12 months follow-up. PLA2Rab was tested by enzyme-linked immunosorbent assay.

Results: Patients with high titer PLA2Rab were associated with more severe proteinuria and hypoalbuminemia compared to those with non-high titer antibody, accompanied by lower complete remission (CR) and total remission (TR) rates at 3, 6, and 12 months, which even took longer to remission. Similar remission rates differences between the two titer groups were observed in the CTX and TAC groups, respectively. PLA2Rab level at baseline was an independent predictive factor for CR and TR. In the high titer group, CR and TR rates in the CTX subgroup were significantly higher than those in the TAC subgroup at 12 months, although serious adverse events were more frequent in the former.

Conclusion: High-risk rank patients with PLA2Rab level above 150 RU/ml have higher disease activity and worse prognosis among patients with seropositive PLA2R-associated MN, even under different immunosuppressive therapeutic models; moreover, CTX combined with corticosteroids was preferred compared to TAC plus corticosteroids, although serious adverse events were more frequent in the former. Additionally, baseline PLA2Rab level was an independent predictive factor for clinical remission.

Keywords: membranous nephropathy, phospholipase A2 receptor, immunosuppressive therapy, remission, prognosis

1 INTRODUCTION

In 2009, Beck et al. (2009) first discovered that M-type phospholipase A2 receptor (PLA2R) was a key target glomerular podocyte antigen, which was abundantly expressed in 70% of patients with primary membranous nephropathy (MN). Subsequent studies showed that antibody against the M-type phospholipase-A2-receptor (PLA2Rab) was found seropositivity in 57%–88.5% in primary MN (Qin et al., 2011; Ramachandran et al., 2016; Huang et al., 2017; Li et al., 2018). A cumulative number of studies have reported a relationship between PLA2Rab levels and clinical parameters, such as 24-h urinary protein and serum albumin (Hofstra et al., 2011; Kaga et al., 2019), and the detection of serum PLA2Rab might help to assess the therapeutic response (Ruggenenti et al., 2015; De Vriese et al., 2017; Guo et al., 2019; van de Logt et al., 2019), time to remission (Qin et al., 2011), prognosis stratification (Liang et al., 2019), and personalized treatment design (Glassock, 2014).

However, because the natural course of MN is long and heterogeneous, the clinical features and prognosis are highly variable. Controversy persists about the association between PLA2Rab levels with clinical characteristics and prognosis (Oh et al., 2013; Bech et al., 2014; Jullien et al., 2017; Pourcine et al., 2017). Moreover, most clinical studies were limited by small sample sizes, single-center studies, and a paucity of studies investigating the relationship according to unified PLA2Rab rank threshold. Different PLA2Rab rank cut-off values have been reported in the respective studies (Hofstra et al., 2012; Hoxha et al., 2014b; Ruggenenti et al., 2015; Ramachandran et al., 2016; Dahan et al., 2017; van de Logt et al., 2018). On the other hand, compared to Kidney Disease: Improving Global Outcomes (KDIGO) recommendations in 2012, there is an important change concerning calcineurin inhibitors (CNIs) in moderate- to high-risk patients, in which CNIs might not seem to be the best therapy for primary MN, and the most effective immunosuppressive therapy is controversial.

It is necessary to abandon the one-therapy-fits-all concept and focus on risk stratification, which included PLA2Rab level in 2020 KDIGO draft guidelines, and to identify the most effective therapeutic options in different MN subsets. The 2020 KDIGO draft guidelines recommend PLA2Rab level above 150 RU/ml as one of clinical criteria for high risk of progressive kidney function loss in MN, while the guidelines also mention that the cutoff value is not verified. To this end, we determined a risk rank threshold corresponding to the draft guidelines recommended PLA2Rab level (150 RU/ml), which help to evaluate clinical features,

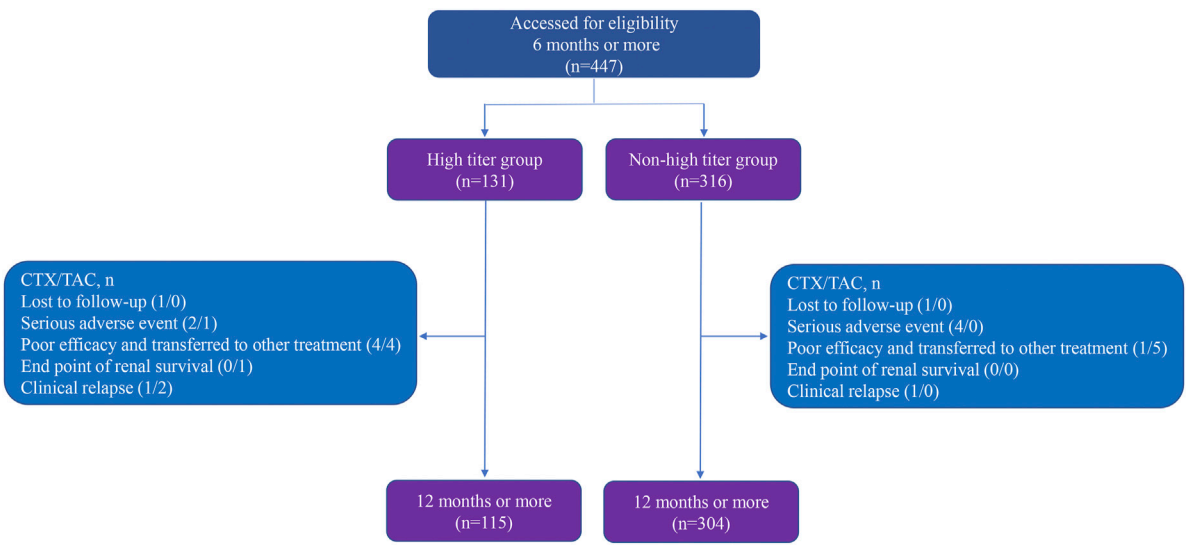

FIGURE 1 | Flow diagram of follow-up for enrollment of participants.

TABLE 1 | Comparison of baseline clinical characteristics between the two titer groups in overall cohort, CTX group, and TAC group.

Parameters	Overall cohort				CTX group				TAC group			
	Total	High titer	Non-high titer	*p*-value	Total	High-titer	Non-high titer	*p*-value	Total	High titer	Non-high titer	*p*-value
	(*n* = 447)	(n = 131)	(*n* = 316)		(*n* = 275)	(*n* = 71)	(*n* = 204)		(*n* = 172)	(*n* = 60)	(*n* = 112)	
Age (years)	54 (44, 64)	55 (47, 63)	53 (42, 64)	0.124	53 (44, 63)	54 (47, 61)	53 (43, 64)	0.394	54 (43, 64)	56 (47, 65)	53 (37, 64)	0.187
Male gender (%)	317 (70.92)	95 (72.52)	222 (70.25)	0.631	198 (72.00)	51 (71.83)	147 (72.06)	0.971	119 (69.19)	44 (73.33)	75 (66.96)	0.389
SBP (mmHg)	129 (120, 139)	130 (122, 139)	129 (120, 140)	0.191	128 (120, 138)	129 (121, 137)	128 (118, 139)	0.379	130 (120, 140)	132 (122, 140)	130 (120, 141)	0.432
DBP (mmHg)	80 (74, 89)	83 (75, 88)	80 (73, 89)	0.079	80 (73, 88)	83 (75, 87)	80 (72, 88)	0.102	82 (74, 90)	83 (72, 92)	81 (74, 90)	0.428
Serum creatinine (μmol/L)	77.89 (63.49, 96.49)	82.20 (64.50, 99.13)	77.44 (63.33, 93.54)	0.186	78.00 (64.10, 97.00)	79.00 (62.63, 98.17)	77.95 (64.34, 94.85)	0.872	77.47 (62.34, 94.95)	85.08 (65.42, 101.63)	75.41 (61.49, 90.95)	0.057
Serum albumin (g/L)	24.87 (20.67, 28.18)	20.62 (18.20, 22.38)	27.10 (23.10, 28.59)	< 0.001	25.61 (20.70, 28.30)	20.57 (18.14, 22.80)	26.87 (23.10, 28.51)	< 0.001	23.37 (20.42, 28.13)	20.74 (18.43, 22.17)	27.25 (22.99, 28.84)	< 0.001
eGFR (ml/min/ 1.73 m^2)	93.02 (74.83, 106.83)<	88.93 (75.22, 103.46)	95.41 (73.72, 107.86)	0.067	92.18 (73.26, 106.92)	89.65 (75.90, 105.05)	94.64 (72.87, 107.18)	0.404	94.27 (75.45, 106.64)	88.90 (69.42, 102.44)	96.32 (76.52, 109.91)	0.070
Urinary protein (g/24 h)	7.02 (4.60, 10.37)	11.18 (9.51, 13.66)	5.62 (4.33, 7.78)	< 0.001	6.80 (4.55, 10.07)	10.53 (9.12, 14.38)	5.78 (4.30, 7.59)	< 0.001	7.90 (4.78, 11.44)	11.69 (10.10, 13.32)	5.51 (4.37, 7.92)	< 0.001
TG (mmol/L)	2.37 (1.67, 3.38)	2.48 (1.75, 3.65)	2.33 (1.64, 3.36)	0.271	2.38 (1.64, 3.39)	2.17 (1.59, 3.58)	2.39 (1.64, 3.38)	0.902	2.36 (1.72, 3.37)	2.53 (1.89, 3.86)	2.23 (1.65, 3.25)	0.064
TCHO (mmol/L)	8.20 (6.81, 9.82)	8.51 (7.22, 10.11)	8.03 (6.65, 9.67)	0.081	8.14 (6.83, 9.82)	8.42 (6.91, 10.11)	8.03 (6.80, 9.67)	0.502	8.21 (6.67, 9.81)	8.78 (7.56, 10.15)	7.97 (6.43, 9.64)	0.065
HDL-chol (mmol/L)	1.50 (1.13, 2.01)	1.45 (1.05, 1.98)	1.51 (1.16, 2.05)	0.161	1.59 (1.12, 2.13)	1.55 (1.02, 2.05)	1.60 (1.14, 2.16)	0.323	1.40 (1.14, 1.88)	1.34 (1.07, 1.87)	1.48 (1.16, 1.88)	0.384
LDL-chol (mmol/L)	4.73 (3.58, 5.95)	4.99 (3.91, 6.14)	4.61 (3.50, 5.90)	0.063	4.71 (3.58, 6.12)	4.84 (3.73, 6.35)	4.61 (3.58, 5.90)	0.378	4.77 (3.58, 5.91)	5.03 (4.25, 6.06)	4.61 (3.14, 5.91)	0.092
PLA2R titer (RU/ml)	87.59 (39.48, 200.00)	280.61 (226.79, 360.18)	59.02 (31.83, 95.10)	< 0.001	80.70 (37.33, 160.50)	280.61 (215.70, 356.63)	61.35 (32.00, 99.92)	< 0.001	94.60 (42.67, 240.58)	281.44 (231.87, 370.74)	52.1 (30.15, 92.18)	< 0.001

Data were shown as median (25–75% interquartile range). p-*value: high titer vs. non-high titer.* p-*value <0.05 was considered statistically significant. CTX, cyclophosphamide; TAC, tacrolimus; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; TG, triglyceride; TCHO, total cholesterol; HDL-chol, high-density lipoprotein cholesterol; LDL-chol, low-density lipoprotein cholesterol; PLA2Rab, antibodies against the M-type phospholipase-A2-receptor*

prognosis, and therapy option in patients with seropositive PLA2R-associated MN in a large sample size, multi-center study.

2 MATERIALS AND METHODS

2.1 Study Design

A total of 447 patients received cyclophosphamide (CTX) or tacrolimus (TAC) combined with corticosteroids treatment were recruited between January 2017 and April 2021 from five Chinese nephrology centers, including the lead center—the Second Affiliated Hospital of Nanchang University, Ganzhou City People's Hospital, Jiujiang Hospital of Traditional Chinese Medicine, the First Affiliated Hospital of Gannan Medical University, and Xinyu City People's Hospital. The present study was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University [No. (2016) No. 120] and conducted according to the ethical principles stated by the Declaration of Helsinki. Informed consent was obtained from all patients.

2.2 Patients

Inclusion criteria were as follows: (1) IMN (stage I–IV) proven by renal biopsy; (2) positive serum PLA2Rab titer at diagnosis; (3) age 18 years or more; (4) urinary protein >3.5 g/24 h, serum albumin <30 g/L, edema, and/or hyperlipidemia after 6 months anti-proteinuria treatment with angiotensin-converting enzyme inhibitor(s)/angiotensin II receptor blocker.

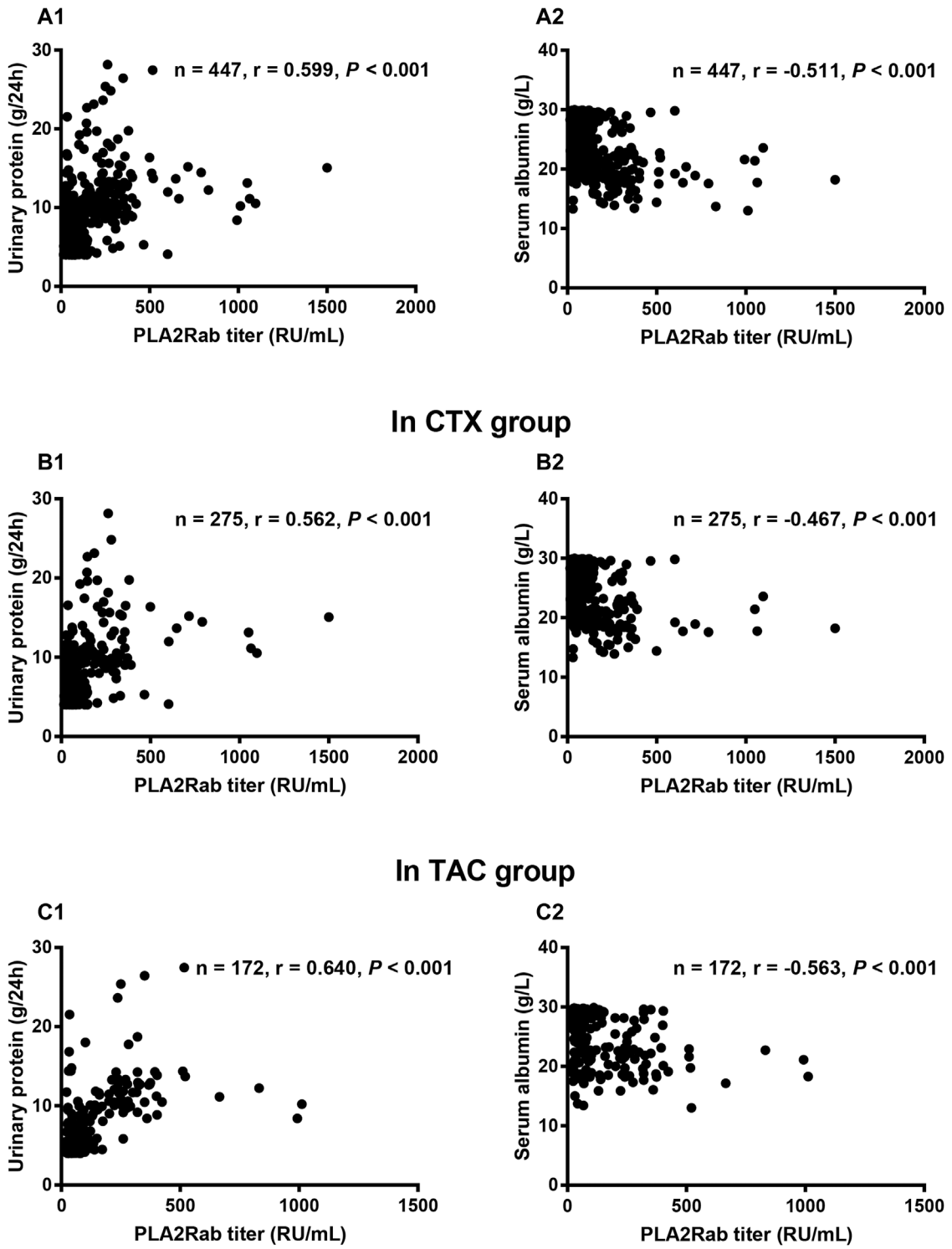

FIGURE 2 | Correlation analyses between serum PLA2Rab titer and urinary protein and serum albumin. The serum PLA2Rab titer positively associated with urinary protein in overall cohort **(A1)**, CTX group **(B1)**, and TAC group **(C1)**; but negatively associated with serum albumin in overall cohort **(A2)**, CTX group **(B2)**, and TAC group **(C2)**. CTX: cyclophosphamide; TAC: tacrolimus; PLA2Rab: antibody against the M-type phospholipase-A2-receptor.

TABLE 2 | Correlation analysis between PLA2Rab and clinical parameters in overall cohort, CTX group, and TAC group.

Parameters	Overall cohort		CTX group		TAC group	
	CO	*p* value	CO	*p*-value	CO	*p*-value
Age (years)	0.096	0.043	0.034	0.578	0.177	0.020
Male gender	0.039	0.413	0.074	0.223	0.005	0.943
SBP (mmHg)	0.077	0.104	0.039	0.518	0.116	0.129
DBP (mmHg)	0.105	0.027	0.112	0.063	0.090	0.242
Serum creatinine (μmol/L)	0.092	0.052	0.053	0.380	0.150	0.049
eGFR (mL/min/1.73 m^2)	–0.110	0.020	–0.047	0.439	–0.194	0.011
TG (mmol/L)	0.122	0.010	0.106	0.080	0.147	0.054
TCHO (mmol/L)	0.110	0.020	0.089	0.142	0.144	0.059
HDL-chol (mmol/L)	–0.051	0.281	–0.041	0.496	–0.078	0.307
LDL-chol (mmol/L)	0.075	0.111	0.106	0.080	0.035	0.653

PLA2Ra, antibodies against the M-type phospholipase-A2-receptor; CTX, cyclophosphamide; TAC, tacrolimus; CO, correlation coefficient; SBP, systolic blood pressure; DBP: diastolic blood pressure; eGFR, estimated glomerular filtration rate; TG, triglyceride; TCHO, total cholesterol; HDL-chol, high-density lipoprotein cholesterol; LDL-chol, low-density lipoprotein cholesterol. $p < 0.05$ *was considered significant difference.*

Exclusion criteria were as follows: (1) patients with secondary forms of MN, including autoimmune diseases, infection-related MN, and MN-related to malignancies or exposure to toxic substances; (2) immunosuppressive therapy in the last 6 months; (3) serum creatinine persistently >309 μmol/L; (4) life-threatening complications; and (5) pregnancy or lactation.

2.3 Interventions and Follow-Up

All eligible patients were divided into high titer group (>150 RU/ml) and non-high titer group (20–150 RU/ml) and subdivided into CTX subgroup and TAC subgroup. Stratified analyses were processed according to different therapeutic models to remove potential confounder in treatment that might cause bias in the association between PLA2Rab levels and outcomes. Therefore, the overall cohort was classified into CTX group and TAC group as well. Patients who received CTX treatment were administered intravenous infusion at 750 mg/m^2 body surface once every month for 6 months and then once every 2 or 3 months (cumulative dosage, 8–10 g). Subjects treated with TAC were initiated oral TAC on a dose of 0.05–0.1 mg/kg/day (no more than 0.15 mg/kg/day), divided into two equal doses at intervals of 12 h. The dose was adjusted according to the target trough blood concentration of 4–8 ng/ml for the first 6 months and tapered gradually until discontinued at the end of 12 months. Both subgroups were combined with oral glucocorticoids therapy.

The follow-up length was at least 6 months, and the mean follow-up period was 11.70 ± 1.22 months. Clinical parameters levels and remission rates were recorded at the given time (3, 6, and 12 months follow-up). The value of estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) 2009 creatinine equation ($CKD\text{-}EPI_{2009scr}$) (Levey et al., 2009). The detection of serum PLA2Rab was performed using enzyme-linked immunosorbent assay (ELISA) kits (EUROIMMUN, Lübeck, and Germany). According to the manufacturer's recommendation, a value ≥20 RU/ml was considered positive.

2.4 Definitions and Outcomes

Based on the KDIGO 2012 guideline (Beck et al., 2013), (1) complete remission (CR) means that urinary protein is <0.3 g/24 h, accompanied by normal serum albumin and serum creatinine; (2) partial remission (PR), urinary protein <3.5 g/24 h and a 50% or greater reduction from peak values, accompanied by an improvement or normalization of the serum albumin and stable serum creatinine; (3) total remission (TR), a composite remission of CR or PR; (4) relapse, new nephrotic syndrome after an achievement of CR or PR, urinary protein >3.5 g/24 h or >50% of the peak values, and with a reduction in serum albumin; (5) end point of renal survival, compared with baseline, double of serum creatine or a 50% decline in eGFR or progression to end stage renal disease (ESRD) with eGFR <15 ml/min/1.73 m^2; (6) serious adverse event, any untoward medical incident, including reaching clinical death, significant or permanent disability or incapacity, and life-threatening illness.

Primary outcomes were CR and TR rates at 3, 6, and 12 months follow-up, time to remission. Secondary outcomes were the evolution of urinary protein, serum albumin, serum creatinine and eGFR over time, end point of renal survival, clinical relapse of nephrotic syndrome, and serious adverse event.

2.5 Statistical Methods

Data were analyzed with Statistical Product and Service Solutions (SPSS) statistical software for Windows, version 24.0 (SPSS Inc., Chicago, IL, United States) and GraphPad Prism (version 7.0; GraphPad Software, La Jolla, CA). One-sample Kolmogorov–Smirnov testing was used to detect whether variables were normally distributed. Continuous variables with skewed distribution were presented as median (25%–75% interquartile range); categorical variables were presented as frequencies or percentages. Categorical variables were compared with Pearson's chi-squared (χ^2) test or Fisher's exact test. Continuous variables were compared with Mann–Whitney U-test. The correlation between two parameters was analyzed by Spearman's rank coefficient of correlation. Cumulative probabilities of remission were assessed according to the Kaplan–Meier survival analysis method and the log-rank (Mantel–Cox) test. Univariable and multivariable Cox regression analyses were used to screen for risk factors affecting prognosis. Based on univariate Cox regression analysis and clinical judgements, variables at baseline that might influence the remission with $p < 0.05$ in the univariable analyses were selected into the multivariable Cox regression analysis. Statistical significance was defined as a two-sided $p < 0.05$.

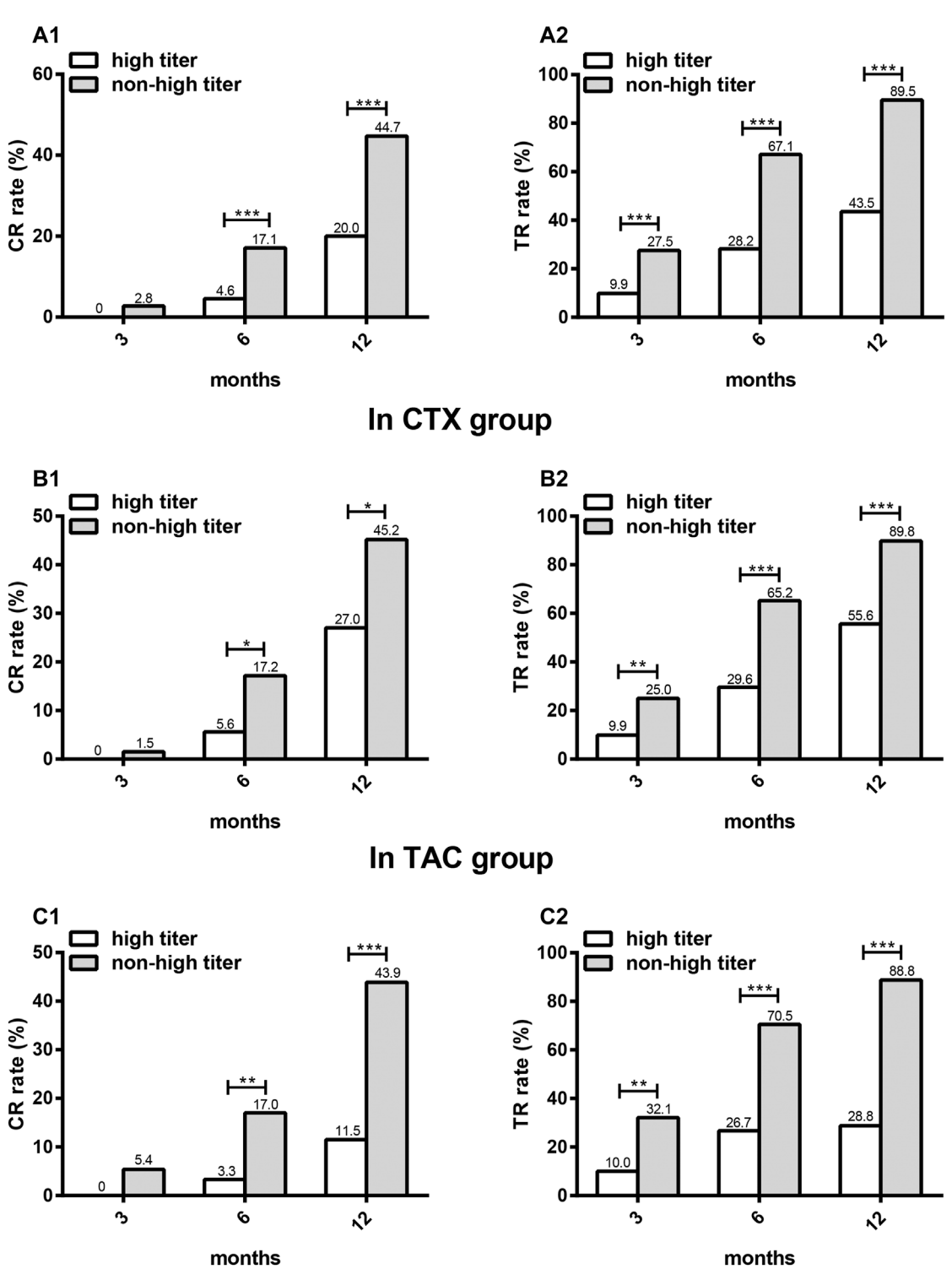

FIGURE 3 | The comparison of CR and TR rates between high titer group and non-high titer group at different time points in different patients. CR **(A1)** and TR **(A2)** in overall cohort, CR **(B1)** and TR **(B2)** in CTX group, CR **(C1)** and TR **(C2)** in TAC group. CR: complete remission; TR: total remission; CTX: cyclophosphamide; TAC: tacrolimus. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

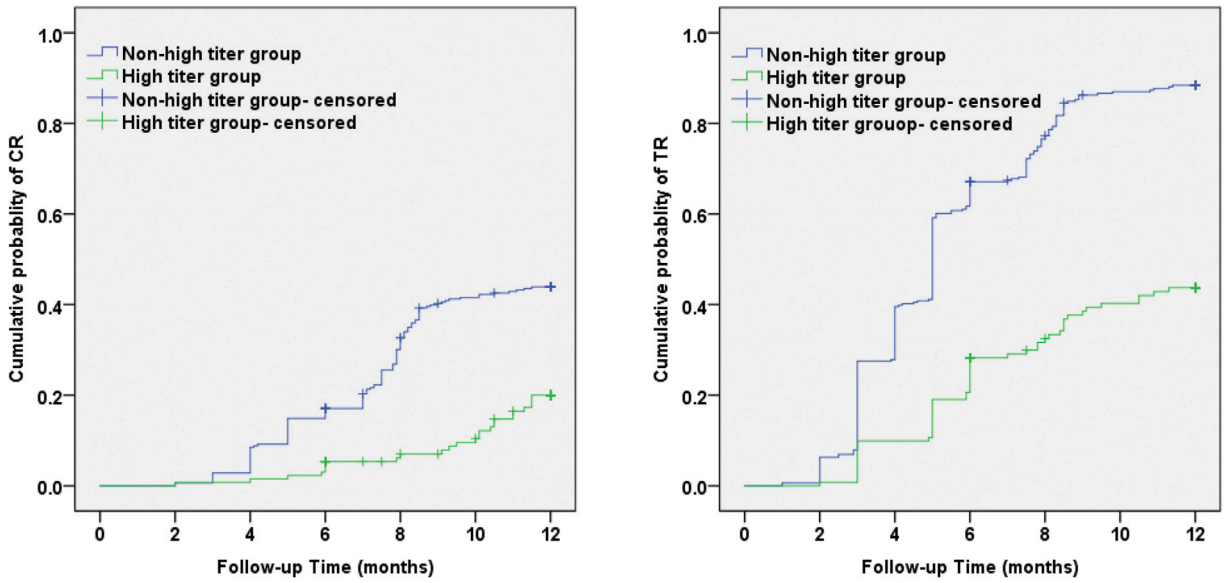

FIGURE 4 | Kaplan–Meier survival analyses of antibody against M-type phospholipase-A2-receptor level and cumulative probability of CR and TR during the 12 months observation period. Incidence of CR and TR over time was significantly higher in the non-high titer group than in the high titer group (Log-Rank, $p < 0.001$). CR: complete remission; TR: total remission.

TABLE 3 | Factors predicting CR and TR for patients with seropositive PLA2R-associated MN.

Variables	CR						TR					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95%CI	*p*-value	HR	95%CI	*p*-value	HR	95%CI	*p*-value	HR	95%CI	*p*-value
Treatment	0.799	0.576, 1.109	0.180	—	—	—	0.822	0.654, 1.032	0.091	—	—	—
PLA2Rab titer (RU/ml)	0.996	0.994, 0.997	**<0.001**	0.997	0.995, 0.999	**0.001**	0.996	0.995, 0.997	**<0.001**	0.997	0.996, 0.998	**<0.001**
Age (years)	0.989	0.978, 1.001	0.066	—	—	—	0.989	0.981, 0.997	**0.009**	0.990	0.976, 1.003	0.142
Gender	0.868	0.621, 1.213	0.407	—	—	—	0.834	0.660, 1.054	0.129	—	—	—
SBP (mmHg)	0.993	0.983, 1.003	0.157	—	—	—	0.995	0.988, 1.001	0.126	—	—	—
DBP (mmHg)	0.984	0.970, 1.000	**0.044**	0.990	0.974, 1.005	0.181	0.991	0.981, 1.001	0.079	—	—	—
Serum creatinine (μmol/L)	0.995	0.989, 1.002	0.166	—	—	—	0.994	0.990, 0.999	**0.020**	0.993	0.981, 1.006	0.291
Serum albumin (g/L)	1.109	1.065, 1.155	**<0.001**	1.083	1.012, 1.159	**0.021**	1.110	1.080, 1.141	**<0.001**	1.094	1.045, 1.146	**<0.001**
eGFR (mL/min/1.73 m^2)	1.006	0.999, 1.014	0.790	—	—	—	1.007	1.002, 1.011	**0.009**	0.994	0.978, 1.009	0.421
Urinary protein (g/24 h)	0.908	0.866, 0.951	**<0.001**	1.033	0.962, 1.109	0.371	0.913	0.886, 0.942	**<0.001**	1.042	0.995, 1.091	0.078
TG (mmol/L)	1.007	0.935, 1.085	0.853	—	—	—	0.987	0.934, 1.044	0.654	—	—	—
TCHO (mmol/L)	1.002	0.938, 1.070	0.959	—	—	—	0.985	0.938, 1.035	0.550	—	—	—
HDL-chol (mmol/L)	0.937	0.720, 1.219	0.629	—	—	—	1.008	0.839, 1.212	0.929	—	—	—
LDL-chol (mmol/L)	0.986	0.913, 1.064	0.719	—	—	—	0.962	0.910, 1.018	0.180	—	—	—

Variables with $p < 0.20$ *in the univariable analyses were included in the multivariable analysis. CR, complete remission; TR, total remission; PLA2R, M type phospholipase A2 receptor; MN, membranous nephropathy; HR: hazard ratio; CI, confidence interval; PLA2Rab: antibodies against the M-type phospholipase-A2-receptor; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; TG, triglyceride; TCHO, total cholesterol; HDL-chol: high-density lipoprotein cholesterol; LDL-chol, low-density lipoprotein cholesterol.The meaning of the bold values is highlight* $p < 0.05$.

3 RESULTS

3.1 PLA2Rab Titers With Clinical Baseline Characteristics at Baseline

Among 447 patients enrolled, 131 (29.31%) patients were in the high titer group and 316 (70.69%) in the non-high titer group, which include 71 (54.20%) CTX subgroup and 60 (45.80%) TAC subgroup in the high titer group, and 204 (64.56%) CTX subgroup and 112 (35.44%) TAC subgroup in the non-high titer group. A total of 275 (61.52%) patients were in the CTX group and 172 (38.48%) in the TAC group. The overview of follow-up for enrolled patients is described in **Figure 1**.

We first evaluated the difference in clinical parameters levels at baseline between high titer group and non-high titer group in the overall cohort, CTX group, and TAC group, respectively. Compared to patients with non-high titer antibody, individuals with high titer antibody had significantly higher urinary protein levels and lower serum albumin levels ($p <$

TABLE 4 | Comparison of baseline clinical characteristics between CTX subgroup and TAC subgroup in the two titer groups.

Parameters	High titer group				Non-high titer group			
	Total	CTX subgroup	TAC subgroup	*p*-value	Total	CTX subgroup	TAC subgroup	*p*-value
	(*n* = 131)	(*n* = 71)	(*n* = 60)		(*n* = 316)	(*n* = 204)	(*n* = 112)	
Age (years)	55 (47, 63)	54 (47, 61)	56 (47, 65)	0.571	42 (53, 64)	53 (43, 64)	53 (37, 64)	0.555
Male gender (%)	95 (72.52)	51 (71.83)	44 (73.33)	0.848	222 (70.25)	147 (72.06)	75 (66.96)	0.344
SBP (mmHg)	130 (122, 139)	129 (121, 137)	132 (122, 140)	0.287	129 (120, 140)	128 (118, 139)	130 (120, 141)	0.117
DBP (mmHg)	83 (75, 88)	83 (75, 87)	83 (72, 92)	0.897	80 (73, 89)	80 (72, 88)	81 (74, 90)	0.244
Serum creatinine (μmol/L)	82.20 (64.50, 99.13)	79.00 (62.63, 98.17)	85.08 (65.42, 101.63)	0.499	77.44 (63.33, 93.54)	77.95 (64.34, 94.85)	75.41 (61.49, 90.95)	0.159
Serum albumin (g/L)	20.62 (18.20, 22.38)	20.57 (18.14, 22.80)	20.74 (18.43, 22.17)	0.895	27.10 (23.10, 28.59)	26.87 (23.10, 28.51)	27.25 (22.99, 28.84)	0.799
eGFR (ml/min/ 1.73 m^2)	88.93 (75.22, 103.46)	89.65 (75.90, 105.05)	89.18 (72.32, 101.82)	0.695	95.41 (73.72, 107.86)	94.64 (72.87, 107.18)	96.32 (76.52, 109.91)	0.289
Urinary protein (g/24 h)	11.18 (9.51, 13.66)	10.53 (9.12, 14.38)	11.70 (10.10, 13.32)	0.149	5.62 (4.33, 7.78)	4.30 (5.78, 7.59)	5.51 (4.37, 7.92)	0.926
TG (mmol/L)	2.48 (1.75, 3.65)	2.17 (1.59, 3.58)	2.53 (1.89, 3.86)	0.231	2.33 (1.64, 3.36)	2.39 (1.64, 3.38)	2.23 (1.65, 3.25)	0.506
TCHO (mmol/L)	8.51 (7.22, 10.11)	8.42 (6.91, 10.11)	8.78 (7.56, 10.15)	0.451	8.03 (6.65, 9.67)	8.03 (6.80, 9.67)	7.97 (6.43, 9.64)	0.455
HDL-chol (mmol/L)	1.45 (1.05, 1.98)	1.55 (1.02, 2.05)	1.34 (1.07, 1.87)	0.371	1.51 (1.16, 2.05)	1.60 (1.14, 2.16)	1.48 (1.16, 1.88)	0.090
LDL-chol (mmol/L)	4.99 (3.91, 6.14)	4.84 (3.73, 6.35)	5.03 (4.25, 6.06)	0.666	4.61 (3.50, 5.90)	4.61 (3.58, 5.90)	4.61 (3.14, 5.91)	0.509
PLA2R titer (RU/ml)	280.61 (226.79, 360.18)	280.61 (215.70, 356.63)	281.44 (231.87, 370.74)	0.705	59.02 (31.83, 95.10)	61.35 (32.00, 99.92)	52.10 (30.15, 92.18)	0.439

Data were showed as median (25%–75% interquartile range). p *value: CTX, subgroup vs. TAC subgroup.* p-*value <0.05 was considered statistically significant. CTX: cyclophosphamide; TAC: tacrolimus; SBP: systolic blood pressure; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; TG: triglyceride; TCHO: total cholesterol; HDL-chol: high-density lipoprotein cholesterol; LDL-chol: low-density lipoprotein cholesterol; PLA2Rab: antibodies against the M-type phospholipase-A2-receptor.*

0.001), while the differences in other clinical parameters levels were not statistically significant (**Table 1**).

We next assessed the correlations between PLA2Rab and clinical parameters at baseline. In the overall cohort, CTX group, and TAC group, correlation analysis indicated that antibody titer positively correlated with urinary protein level (r = 0.599, 0.562, and 0.640, respectively, all $p < 0.001$) and negatively correlated with serum albumin level (r = −0.511, −0.467, and −0.563, respectively, all $p < 0.001$) (**Figure 2**), while the correlations with other clinical parameters were either non-existent or very weak (**Table 2**).

3.2 Primary Outcome

In overall cohort, CTX group, and TAC group, CR occurred in the high titer group significantly less frequently compared with the non-high titer group at 6 and 12 months ($p < 0.001$). There was a nonsignificant trend for lower CR in the high titer group than in the non-high titer group at 3 months (p = 0.064). Then, the same analyses were performed on TR. TR occurred in the high titer group significantly less frequently compared to the non-high titer group at 3, 6, and 12 months ($p < 0.001$) (**Figure 3**).

The cumulative incidences of CR and TR using the Kaplan–Meier method and log-rank test were significantly lower in the high titer group compared with the non-high titer group (χ^2 = 24.02, $p < 0.001$ and 91.97, $p < 0.001$, respectively) (**Figure 4**). The average time to CR and TR in the high titer group was 11.36 ± 0.15 months and 9.42 ± 0.29 months, significantly longer than CR (9.67 ± 0.17 months) and TR (5.82 ± 0.17 months) in the non-high titer group.

Univariate Cox regression analysis of CR showed that baseline levels of PLA2Rab, urinary protein, and serum albumin, and diastolic blood pressure were predictive factors for achieving CR. Then, we performed the same analyses on TR. Univariate Cox regression analysis of TR showed that baseline levels of PLA2Rab titer, serum creatinine, eGFR, serum albumin, urinary protein, and age were predictive factors for achieving TR. Multivariable analysis indicated that PLA2Rab level at baseline—besides baseline level of serum albumin—was an independent predictive factor of CR and TR (**Table 3**).

We next evaluated the differences in remission rates between the CTX subgroup and the TAC subgroup in the two titer groups, respectively. There were no significant differences in the baseline levels of clinical parameters between the two subgroups (**Table 4**). In the high titer group, CR and TR occurred in the CTX subgroup significantly more frequently compared with that in the TAC subgroup at 12 months (p = 0.039 and 0.004, respectively), while in the non-high titer group, and there was a nonsignificant trend for higher CR and TR in the CTX subgroup than in the TAC subgroup at 12 months (p = 0.834 and 0.773, respectively) (**Figure 5**).

3.3 Secondary Outcomes

3.3.1 Evolution of Clinical Parameters

At 3, 6, and 12 months, compared to the non-high titer group, the high titer group had significantly higher urinary protein and lower serum albumin ($p < 0.001$), while it had a nonsignificant trend for higher serum creatinine and lower eGFR ($p > 0.05$). Moreover, at the given time, the percentage improvement in urinary protein and serum albumin was less significant in the high titer group than in the non-high titer group ($p < 0.05$); however, the changes in serum creatinine and eGFR were not significant ($p > 0.05$) (**Table 5**; **Figure 6**).

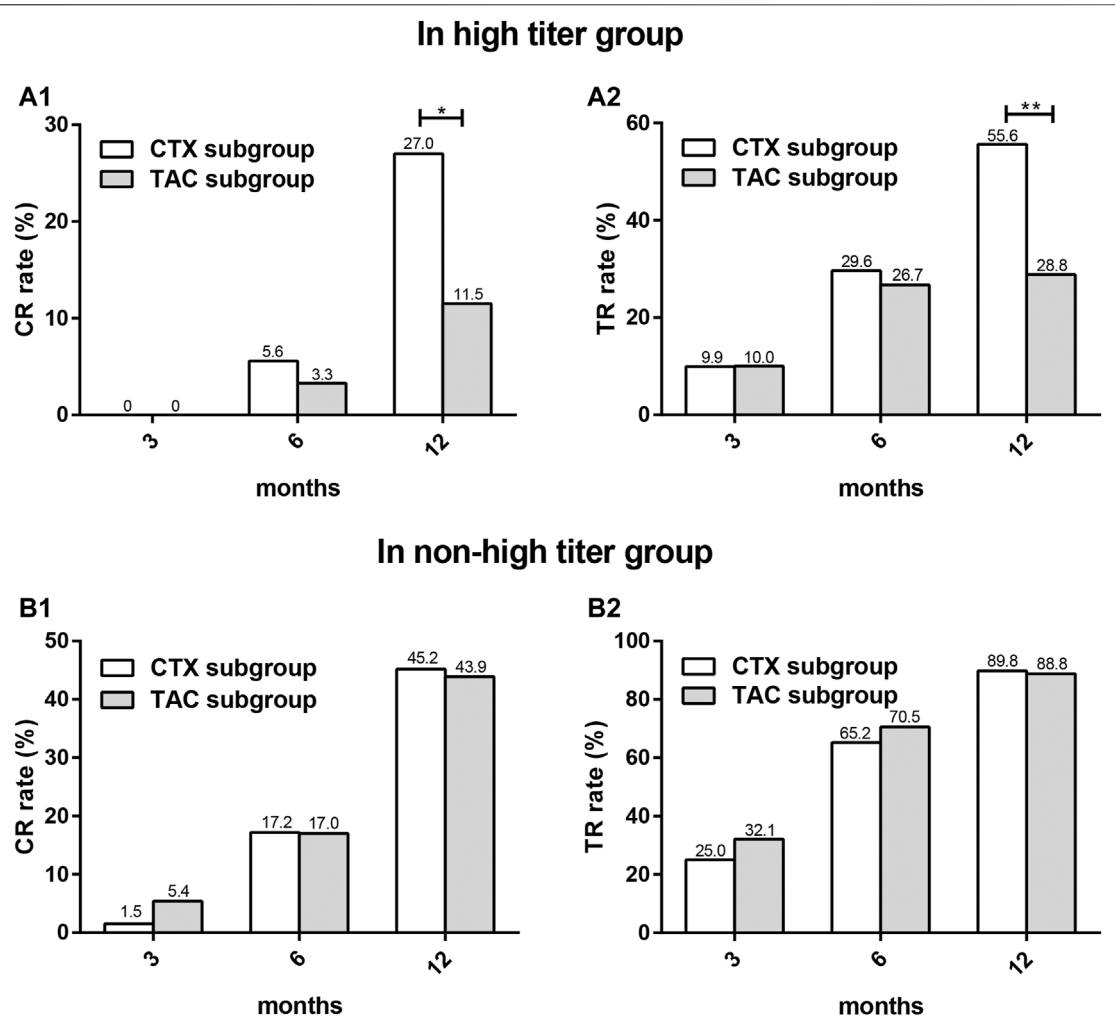

FIGURE 5 | The comparison of CR and TR rates between CTX subgroup and TAC subgroup at different time points in two titer groups. CR **(A1)** and TR **(A2)** in high titer group, CR **(B1)** and TR **(B2)** in non-high titer group.CR: complete remission; TR: total remission; CTX: cyclophosphamide; TAC: tacrolimus. *$p < 0.05$, **$p < 0.01$.

3.3.2 End Point of Renal Survival and Clinical Relapse

By the end of the follow-up period, renal function remained stable in the majority of the subjects. Only one patient who received TAC treatment reached the end point of renal survival in the high titer group, who had no remission of proteinuria. Compared to non-high titer group, a nonsignificant trend for higher incidence of the end point of renal survival in the high titer group was observed during the observation period ($p = 0.293$).

Clinical relapses were recorded 3 (CTX/TAC subgroup: 1/2) out of 131 patients (2.29%) in the high titer group and 1 (CTX/TAC subgroup: 1/0) out of 316 subjects (0.32%) in the non-high titer group, who had achieved PR prior to clinical relapse during the 12 months follow-up period ($p = 0.078$).

3.3.3 Serious Adverse Event

Seven severe infections were observed in 3 (CTX/TAC subgroup: 2/1) out of 131 patients (2.29%) in the high titer group and 4 (CTX/TAC subgroup: 4/0) out of 316 subjects (1.27%) in the non-high titer group ($p = 0.423$). In the high titer group, of the two patients treated with CTX, one developed respiratory failure caused by severe pneumocystis pneumonia, and the other suffered from fatal *Staphylococcus aureus* sepsis, and one patient who received TAC treatment had necrosis of the femoral head. Similarly, in the non-high titer group, four individuals treated with CTX also developed respiratory failure caused by severe pneumonia, including three pneumocystis pneumonia and one pneumocystis combined with bacterial pneumonia. In addition, one patient who received TAC treatment experienced severe cardiovascular event in the high titer group, which was perhaps unrelated to the treatment, as he had a previous history of cardiovascular disease. None of the patients developed thrombosis or thromboembolic events, new tumors, and clinical deaths.

4 DISCUSSION

In this cohort, we first investigated the clinical relevance of PLA2Rab by assessing the association of PLA2Rab with clinical parameters at baseline. Serum albumin and urinary

TABLE 5 | The comparison of clinical parameters over time.

Parameter	Months	Clinical parameters values				Percentage changes of clinical parameters values			
		Overall cohort	High titer	Non-high titer	*p*-value	Overall cohort	High titer	Non-high titer	*p*-value
Up (g/24 h)	Baseline	7.02 (4.60, 10.37)	11.18 (9.51, 13.66)	5.62 (4.33, 7.78)	<0.001	—	—	—	—
	3	3.61 (3.01, 6.25)	8.72 (6.51, 10.57)	3.52 (2.27, 3.69)	<0.001	−40.77 (−62.94, -20.87)	−23.82 (−39.06, −14.00)	−48.44 (−69.58, −25.53)	<0.001
	6	2.22 (0.68, 3.95)	3.84 (2.94, 4.60)	1.41 (0.46, 3.52)	<0.001	−69.65 (−89.54, −53.78)	−65.10 (−77.09, −56.66)	−74.95 (−92.17, −49.73)	0.016
	12	0.51 (0.20, 2.41)	3.54 (0.55, 4.13)	0.34 (0.15, 1.03)	<0.001	−92.13 (−97.19, −71.99)	−71.29 (−95.05, −60.86)	−94.07 (−97.50, −83.20)	<0.001
Salb (g/L)	Baseline	24.87 (20.67, 28.18)	20.62 (18.20, 22.38)	27.10 (23.10, 28.59)	<0.001	—	—	—	—
	3	27.15 (22.56, 31.03)	20.94 (20.01, 24.30)	30.08 (25.61, 31.50)	<0.001	8.54 (3.61, 17.79)	6.88 (0.46, 14.46)	8.96 (4.32, 19.52)	0.002
	6	32.74 (25.15, 36.12)	24.13 (21.30, 30.60)	34.03 (30.32, 37.30)	<0.001	25.88 (11.64, 44.01)	19.71 (8.04, 39.25)	27.21 (13.54, 46.01)	0.016
	12	36.10 (31.75, 40.20)	27.18 (21.04, 34.71)	39.00 (34.81, 41.17)	<0.001	41.76 (23.43, 63.81)	29.94 (6.70, 63.81)	44.22 (30.79, 64.56)	0.001
Scr (μmol/L)	Baseline	77.89 (63.49, 96.49)	82.20 (64.50, 99.13)	77.44 (63.33, 93.54)	0.186	—	—	—	—
	3	67.70 (60.12, 80.57)	69.57 (60.15, 82.50)	66.50 (60.04, 80.45)	0.329	−11.54 (−19.04, −2.81)	−11.11 (−18.97, −2.42)	−11.89 (−19.16, −3.31)	0.825
	6	65.48 (56.90, 75.69)	68.59 (58.19, 78.30)	64.30 (56.58, 75.46)	0.212	−13.95 (−23.84, −3.02)	−14.88 (−23.59, −5.08)	−13.40 (−24.39, −1.48)	0.499
	12	66.13 (58.97, 77.13)	66.32 (60.00, 78.25)	65.97 (58.23, 76.22)	0.351	−14.52 (−23.49, −3.95)	−16.16 (−22.96, −4.54)	−14.09 (−23.62, −3.47)	0.440
eGFR (ml/min/ 1.73 m^2)	Baseline	93.02 (74.83, 106.83)	88.93 (75.22, 103.46)	95.41 (73.72, 107.86)	0.067	—	—	—	—
	3	100.40 (87.40, 111.21)	97.74 (84.73, 109.03)	102.36 (87.69, 111.66)	0.114	7.19 (1.14, 19.25)	6.61 (−0.34, 21.52)	7.25 (1.35, 18.08)	0.971
	6	101.87 (91.10, 111.56)	101.23 (88.26, 108.87)	102.11 (92.08, 113.00)	0.125	9.14 (0.78, 23.54)	10.44 (1.60, 25.09)	7.99 (0.54, 22.79)	0.291
	12	102.36 (91.72, 111.09)	101.03 (88.83, 109.68)	102.65 (92.08, 111.59)	0.153	9.39 (1.86, 23.91)	11.00 (1.78, 25.40)	8.53 (1.81, 23.44)	0.460

Data were showed as median (25%–75% interquartile range). p-*value: high titer vs. non-high titer.* p-*value <0.05 was considered statistically significant. Up, urinary protein; Salb, serum albumin; Scr, serum creatinine; eGFR, estimated glomerular filtration rate.*

protein are generally used to quantitatively evaluate the severity of nephrotic syndrome in MN, which are important indicators for assessing disease activity (Kanigicherla et al., 2013; Lin et al., 2015; Gopalakrishnan et al., 2016; Li et al., 2016). Consistent with data from aforementioned studies (Li et al., 2016; Song et al., 2018; Provatopoulou et al., 2019; Katsumata et al., 2020; Li et al., 2021), we found a significant association of PLA2Rab with urinary protein and serum albumin indicating the correlation between antibody levels and disease activity (Mahmud et al., 2019; Gong et al., 2020; Guo et al., 2020). However, Jullien et al. (2017) claimed that the initial PLA2Rab level did not relate to the degree of proteinuria. It is likely that the distinction may be due, in part, to genetic or racial or environmental background difference.

Additionally, previous studies showed that higher PLA2Rab levels were related to higher levels of serum creatinine and lower eGFR (Hofstra et al., 2011; Provatopoulou et al., 2019), which was inconsistent with the findings of our study. The reason for this discrepancy may be associated with the facts that the kidney function of the subjects in our study was within the normal range or mildly abnormal.

It is known that binding of PLA2Rab in circulation and PLA2R antigen on glomerular podocyte to form an *in situ* immune complex activates a complement to result in podocyte and immune-mediated injury, ultimately causing urinary protein production (Beck et al., 2009; Glassock, 2012; Mcquarrie, 2018). It appears reasonable to speculate that a higher level of PLA2Rab in circulation might result in increased binding to PLA2R on podocyte, more subepithelial deposition of antibody–antigen immune complexes, which lead to more serious damage of podocyte and the destruction of the filtration barrier, and eventually increased levels of proteinuria (Kerjaschki, 2004; Hofstra et al., 2011; Song et al., 2018).

Next, we assessed the differences in remission rates between the two titer groups. CR and TR rates in overall cohort, CTX group, and TAC group were significantly lower in the high titer group than in the non-high titer group, which suggests that the PLA2Rab levels correlate with the rate of clinical remission (Pourcine et al., 2017; Zhang et al., 2017; Han et al., 2019). In the GEMRITUX Study, PLA2Rab titer of <275 RU/ml at baseline was significantly associated with a composite end point of CR or PR (Dahan et al., 2017). Ruggenenti et al. (2015) reported that, compared to those in the highest titer (>204 RU/ml), the

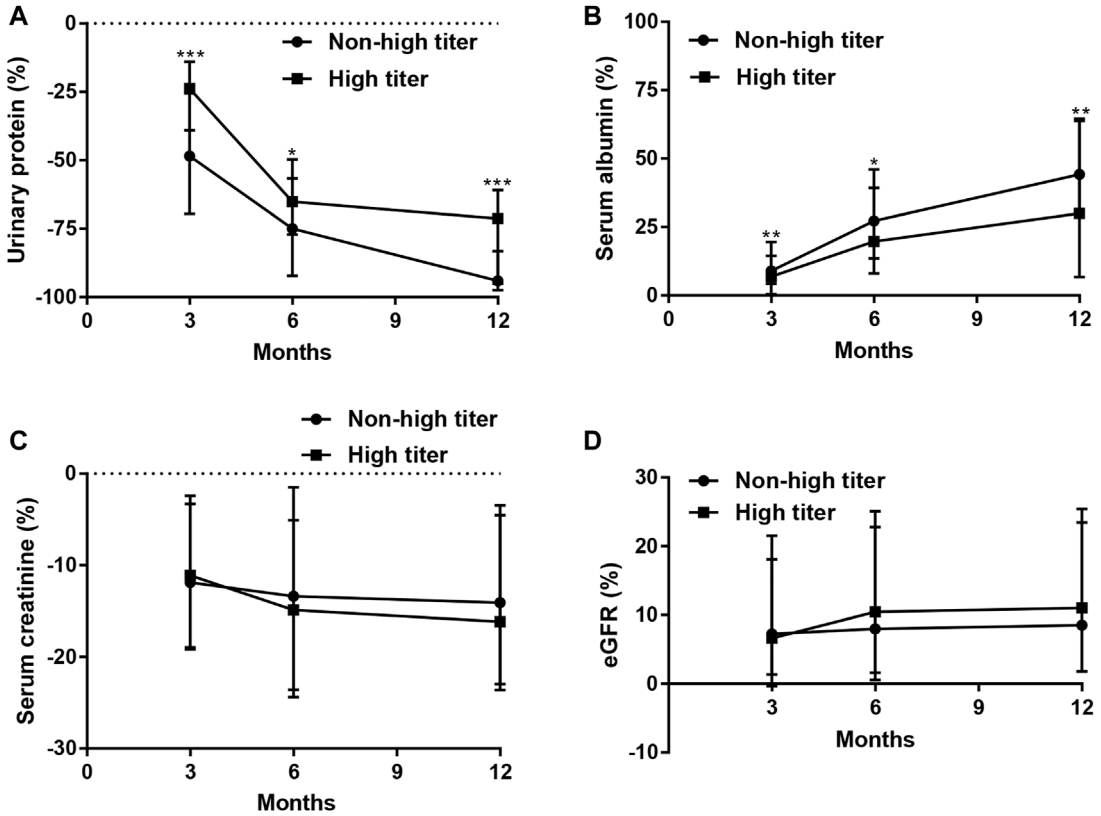

FIGURE 6 | Percentage changes in urinary protein, serum albumin, serum creatinine, and eGFR over time. Compared to baseline, at 3, 6, and 12 months, the decrease of urinary protein **(A)** ($p < 0.001$, $p = 0.016$, $p < 0.001$, respectively) and the increase of serum albumin **(B)** ($p = 0.002$, 0.016, 0.001, respectively) more rapid in non-high titer group than in high titer group, while there were no significant differences in the decrease in serum creatinine **(C)** ($p = 0.825$, 0.499, 0.440, respectively) and in the increase in eGFR **(D)** ($p = 0.971$, 0.291, 0.460, respectively) at 3, 6, and 12 months between the two titer groups. eGFR: estimated glomerular filtration rate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

probability of achieving the composite remission of CR or PR was approximately four- and twofold higher in the lowest (14–86 RU/ml) and middle (87–204 RU/ml) titer, respectively; compared to the highest one, the probability of achieving CR was approximately threefold higher in the lowest and middle titer. The study of Ramachandran et al. (2016) also showed that a greater proportion of patients in the first two tertiles [(20.54–135.90) RU/ml, (149.23–335.18) RU/ml, respectively] had CR compared to individuals in the third tertile (337.16–6,528) RU/ml. Moreover, updated meta-analyses revealed that serum PLA2Rab level at baseline was closely associated with the clinical remission (Rao et al., 2020). Of interest, these conclusion held as well when limiting the analysis to the CTX group and TAC group, which have not been reported so far.

Kaplan–Meier survival analysis and log-rank test also showed that the cumulative probability of CR and TR were significantly lower and remarkably slower in the high titer group than in the non-high titer group at 12 months follow-up, which were basically in agreement with the findings of initial studies (Qin et al., 2011; Hofstra et al., 2012; Hoxha et al., 2014a). In the study by Ruggenenti et al. (2015), over a median follow-up of 30.8 months, time to the composite remission of CR or PR progressively increased from the lowest (14–86 RU/ml) to the middle (87–204 RU/ml) and the highest titer (>204 RU/ml) [5.4 (4.1–8.8), 9.1 (3.5–16.4), and 11.6 (5.3–24.8)]. To exclude influence of other baseline parameters, we performed multivariate Cox regression analysis. Adjusted for age, gender, blood pressure, and other clinical parameters, multivariate Cox regression analysis demonstrated that PLA2Rab level at baseline was an independent predictive factor for achieving CR and TR. Baseline PLA2Rab level can be regarded as an important prognostic factor in primary MN (Kim et al., 2015; Fiorentino et al., 2016; Song et al., 2018).

Unfortunately, in our study, the median time to CR and TR was not obtained due to more than half of patients did not achieve CR and TR in the high titer group, and mean time was described instead of median time. It is possible that "lack of remission" was, in part, because of the nature of chronic course of MN and inadequate follow-up time in 12 months follow-up period. Patients may continue to achieve remission within 12–18 months after completion of the initial treatment regimen (Beck et al., 2013). If the follow-up period is extended, more differences between the two titer groups might be found. Of course, on the other hand, this indicated that the prognosis of patients with high PLA2Rab titer was worse, which was also supported by another result in our study that compared with the non-high titer group, and percentage increase in serum albumin and decrease in urinary protein were significantly lower in the high titer group. It is likely that due the fact that serum PLA2Rab level was

associated with disease activity, the lower remission rates and slower response in the high titer group might be due to the relatively high activity of the disease (Ruggenenti et al., 2015).

Of note, in the high titer group, the proportions of CR and TR at 12 months in the CTX subgroup were significantly higher than those in the TAC subgroup (CR, 27.0 and 11.5%, $p = 0.039$, TR, 55.6 and 28.8%, and $p = 0.004$, respectively), while in the non-high titer group, there was a nonsignificant trend for higher CR and TR in the CTX subgroup (CTX/TAC, CR, 45.2 and 43.9%, $p = 0.834$, TR, 89.8 and 88.8%, and $p = 0.773$, respectively), which appeared to suggest that CTX is preferred for patients with high PLA2Rab levels. The 2020 KDIGO draft guidelines recommend PLA2Rab level above 150 RU/ml as one of the clinical criteria for high risk of progressive kidney function loss and CTX combined with corticosteroids for patients at high risk of progression. A study by Van de Logt et al. showed that no difference was observed in immunological remission rate between rituximab or cyclophosphamide treatments in patients with low (15–84 RU/ml) or moderate (85–151 RU/ml) antibody levels. In contrast, in patients with high (152–776 RU/ml) antibody levels, cyclophosphamide was more effective than rituximab in inducing an immunological remission, and both baseline antibody level and type of therapy were significantly independent predictors, which suggests that the detection of PLA2Rab might be of great value, particularly for the guidance of initial immunosuppressive therapy (van de Logt et al., 2018).

There was a nonsignificant trend for higher incidences of clinical relapse in the high titer group than in the non-high titer group (2.29% and 1.27%, respectively), which was significantly lower than that reported in initial studies. This result might be limited by a relatively short follow-up time. A previous study reported that baseline anti-PL2R levels were not associated with the appearance of clinical relapses (Provatopoulou et al., 2019). However, Mahmud et al. (2019) found that high PLA2Rab level at baseline was a risk factor for clinical relapse. In addition, in our study, there was a nonsignificant trend for higher incidence of the end point of renal survival in the high titer group than in the non-high titer group, whereas Kanigicherla et al. (2013) reported that the risk of doubling of serum creatinine increased remarkably in patients with high PLA2Rab level. These differences may be explained by discrepancies in genetic, environmental condition, ethnic background, study design, state and stage of clinical disease when patient was enrolled, and clinical treatment method.

In the end, we analyzed serious adverse event. There was a nonsignificant trend more likely to occur in the high titer group than in the non-high titer group. It is worth noting that in six patients who received CTX plus glucocorticoids, serious infections occurred. A remarkable and rather unexpected finding of this study was that only one serious adverse event was noted in TAC group. In addition, it is hard to draw definitive conclusions on the risk of cancer within the limited follow-up time.

Even though validation at five different independent institutions without much discrepancy would increase the strength of this study, the foremost limitation of the present study is the retrospective character, which cannot control all potential confounders that might cause bias. Second, a 12-month follow-up time was relatively short. Admittedly, in a relatively short-term follow-up period, the analyses on clinical relapse and the end point of renal survival were limited. Third, all patients were Chinese, which might restrict the applicability to other ethnic groups. Therefore, in future research with a larger dataset and different subset of patients, prospective randomized design with an extended follow-up period should be performed to validate the established relationships and further study the outcomes after 12 months of immunosuppressive treatments.

In conclusion, high-risk rank patients with PLA2Rab level above 150 RU/ml have higher disease activity and worse prognosis among patients with seropositive PLA2R-associated MN, even under different immunosuppressive therapeutic models; moreover, CTX combined with corticosteroids was preferred compared to TAC plus corticosteroids, although serious adverse events were more frequent in the former. In addition, baseline PLA2Rab level was an independent predictive factor for clinical remission.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The present study was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University [No. (2016) No. 120] and conducted according to the ethical principles stated by the Declaration of Helsinki. Informed consent was obtained from all patients. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LD and QH conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and tables, authored or reviewed drafts of the paper, and approved the final draft. JW, KL, and JL performed the experiments, prepared figures and tables, and approved the final draft. WY performed the experiments, authored or reviewed drafts of the paper, and approved the final draft. FJ analyzed the data, authored or reviewed drafts of the paper, and approved the final draft. GX conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and tables, authored or reviewed drafts of the paper, and approved the final draft.

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Nephrotoxicity of Calcineurin Inhibitors in Kidney Epithelial Cells is Independent of NFAT Signaling

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Background: Calcineurin inhibitors (CNIs) such as cyclosporine A and tacrolimus are commonly used after renal transplantation to suppress the immune system. In lymphoid cells, cyclosporine A acts *via* the calcineurin/nuclear factor of activated T-cell (NFAT) axis. In non-lymphoid cells, such as kidney epithelial cells, cyclosporine A induces calcineurin inhibitor toxicity. It is unknown *via* which off-targets cyclosporine A induces calcineurin inhibitor toxicity in kidney epithelial cells.

Methods: To measure a compound's potential to induce nephrotoxicity, the expression of the surrogate marker Fn14 was measured by flow cytometry. Compounds were tested for their potential to induce Fn14 either chemically or plasmid-mediated. Mice were injected with various compounds, and changes in nephrotoxic gene expression levels of the kidney epithelial cells were then analyzed.

Results: Fn14 is specifically upregulated due to calcineurin inhibitor toxicity inducing agents. Inhibition of the NFAT axis showed no increase of the Fn14 expression on the surface of kidney cells. However, inhibition of p38 MAPK, phosphoinositide-3-kinase (PI3K)/Akt, protein kinase C (PKC), and inhibitor of nuclear factor-κB (IκB) kinase (IKK) showed clear induction of Fn14 and increased expressions of nephrotoxic, inflammatory, and fibrotic genes *in vitro* and *in vivo*.

Conclusions: These findings show that cyclosporine A acts independently of NFAT on kidney epithelial cells. Moreover, inhibition of serine/threonine protein kinases mimics cyclosporine A's activity on kidney epithelial cells. This mimicking effect indicates that these protein kinases are off-targets of cyclosporine A and damage structural renal cells when inhibited and therefore contributes likely to the development and progression of calcineurin inhibitor toxicity.

Keywords: calcineurin inhibitor, nephrotoxicity, NFAT-independent, p38 kinase, PI3K - AKT

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INTRODUCTION

Calcineurin inhibitors (CNIs), e.g., cyclosporine A (CsA) and tacrolimus (FK506), are pivotal drugs for the prevention of rejection after solid organ transplantation. CNIs show a highly interindividual pharmacokinetic profile and have a narrow therapeutic range. Even mild overdosing may lead to substantial acute and chronic side effects, including tumor development, infections, metabolic

disturbances, and kidney failure. Calcineurin inhibitors induce nephrotoxicity through (reversible) vasoconstriction in the vas afferent. Vasoconstriction leads to consecutive relative hypoxia and progressive athero- and arteriolohyalinosis, tubular atrophy, and interstitial fibrosis (Feske and Vaeth, 2018). Recent register studies have demonstrated that virtually all kidney transplanted patients develop signs of chronic calcineurin inhibitor toxicity (CNT) within 10 years after kidney transplantation (Stegall et al., 2018; Karolin et al., 2021). In lymphocytes, CsA inhibits the calcineurin/nuclear factor of activated T-cell (NFAT) axis leading to repression of transcriptional programs necessary for activation, proliferation, and cytokine production. This inhibition primarily contributes to the immunosuppressive effect of CsA (Kiani et al., 2000; Casey and Meier-Kriesche, 2011).

Members of the TNF superfamily have a non-redundant role in the pathogenesis of tissue regeneration and wound healing but are also critically involved in chronic inflammatory and fibrosis (Croft, 2009; Burkly, 2015; Croft and Siegel, 2017). We have recently shown that the tumor necrosis factor (TNF)–related weak inducer of apoptosis (TWEAK) is indispensable for developing CNT in mice (Claus et al., 2018). CsA induces the expression of TWEAK's receptor, fibroblast growth factor–inducible 14 (Fn14), in kidney epithelial cells, which facilitates inflammatory and fibrotic signals critical for progressive nephrotoxicity. Deficiency for TWEAK or treatment with TWEAK-neutralizing reagents protected animals from acute CNT lesions (Zhao et al., 2007; Sanz et al., 2008; Xia et al., 2015; Claus et al., 2018). Furthermore, administration of recombinant TWEAK (rTWEAK) induced similar disease as when animals are treated with CsA alone. Interestingly, the combination of rTWEAK and CsA caused severe tubulopathy and interstitial inflammation (Claus et al., 2018). The receptor Fn14 is low ubiquitously expressed on epithelial cells and can be induced upon stress and injury stimuli (Burkly, 2014). Fn14 is the only known receptor to the cytokine TWEAK (Bossen et al., 2006). TWEAK is produced by infiltrating and tissue-resident immune cells, largely monocytes and neutrophils (Campbell et al., 2006; Vince and Silke, 2006; Winkles, 2008).

Currently, it is unclear how the CNI, CsA, interferes with kidney epithelial cells and induces nephrotoxicity. The present work aims to investigate off-targets of CsA in renal epithelial cells. We hypothesize that CsA elicits inflammatory and fibrotic activities in renal epithelial cells independent of NFAT but *via* inhibition of kinase pathways.

MATERIALS AND METHODS

Cell Culture Experiments

The murine kidney epithelial cell line (MCT) has been described previously (Haverty et al., 1988). Cells were grown in 10% fetal calf serum (FBS) supplemented with Dulbecco modified Eagle medium (DMEM; ThermoFisher, 11965092) and sub-cultivated in a 12-well format with 50,000 cells per well. HEK293T cells were cultured in DMEM/10%FBS and 2 mM L-glutamin in a 6-well (800,000 cells/well) and 12-well (300,000 cells/well) format. The next day, cells were stimulated for 24 h with either 10 μg/ml CsA (Sandimmun Neoral® from Novartis) or 10 μg/ml FK506 (Cell Signaling, Daveres, MA). In some experiments, cells were incubated for 24 h with the following reagents: 1 μM 11R-VIVIT (NFAT Inhibitor) (Merck, 480401), 10 μM SB203580 (Sigma, S8307), 10 μM SB202190 (Sigma, S7076), gentamicin, amphotericin B, 10 μg/ml cisplatin (Sigma, C2210000), or rTWEAK (PeproTech, 310-06). HEK293T cells were transfected with 2.5 μg of plasmid DNA using standard lipofectamine 3,000 protocols (ThermoFisher, L3000001). The transfected plasmids were pCMV-VIVIT-GFP (Addgene, 11106), pCMV-p38-CA-EGFP, and pCMV-eGFP-N1 (Addgene, 6,085-1). Following transfection, various treatments were performed the next day for 24 h. For the top-down kinase inhibitor screen, MCTs were cultured in a 12-well format, as described above. The next day, cells were treated with various kinase inhibitors (Enzo, BML-2832) at 10 μM for 48 h and then analyzed by flow cytometry (described below).

Acute Toxicity Mouse Model

As previously described (Claus et al., 2018), acute CNT lesions were induced by injecting (intraperitoneal) animals with 100 mg/kg CsA (Sandimmun Neoral®), 10 mg/kg p38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580, SB202190), and 1 mg/kg 11R-VIVIT on three consecutive days twice daily. The dosage of CsA was chosen based on the publication from Claus et al., 2018. Concentrations for the p38 MAPK inhibitor and 11R-VIVIT were based on previously published *in vivo* studies, where these compounds showed efficacy at indicated concentrations (Seeger et al., 2010; Zhang et al., 2013). Animals were then euthanized, and kidneys and blood were collected for subsequent analysis. Red cell lysis buffer (Roche, 11814389001) was added to the blood cells until all erythrocytes were removed. The white blood cells were used for flow analysis (Described below). Mouse kidneys were digested with 2 mg/ml collagenase I (Sigma, C9891) in 1% BSA in PBS together with DNase for 20min at 37°C. The cell suspension was filtered through a cell strainer, washed with 1% BSA in PBS, and centrifuged at 200xg for 3min in 15 ml total volume. The cell suspension was then stained with antibodies for flow cytometry (described below).

Antibody Staining

Flow cytometry was performed on dissociated MCT or HEK293T cells stained with the Fn14-APC antibody (1:30; Miltenyi, 130-104-281). For negative control, unstained cells or the respective isotype control antibody, mouse IgG2b (1:100, Miltenyi, 130-098-890) was used. Antibody staining was conducted for 30 min in the dark at 4°C. Proliferation assay was conducted as follows: from naïve animal's lymph node/ Spleen, T cells were isolated and stained with 1:1000 e450 proliferation dye (ThermoFisher, 65-0842-85) and treated with various reagents (11R-VIVIT, CsA). Cells were then stimulated with αCD3/αCD28 dynabeads for 5 days. White blood cells were stained for 30 min in the dark at 4°C with CD45-PerCP/Cy5-5 (1:100; Biolegend, 103132) and CD11b-APC/Cy7 (1:100; Biolegend, 101226). The kidney cell suspension was stained with

CD45-PerCP/Cy5-5 and CD326-PE (1:50, Miltenyi, 130-117-667) for 30min in the dark at 4°C. Also, single stains were created to perform proper compensation.

Flow Cytometer and Cell Sort

LSR-II flow cytometer, with the software FACS Diva, was used to determine the Fn14 surface expression, proliferation assay, and blood cell flow analysis. The geometric mean fluorescence intensity (MFI) of antibody staining or total count of cells was calculated by FlowJo Software. Most of the figures showing the Fn14 expression are based on measurements conducted by flow cytometry.

The flow facility at the University of Bern sorted mouse kidney cells. The sort was conducted on the MoFlo Astrios EQ instrument. After DAPI (1:100′000) was added, gating was conducted on DAPI-negative, CD45-negative, and CD326-positive cells. Living CD45-/CD326 + cells were collected in FBS.

RNA Isolation, Reverse Transcription, and RT-qPCR

Total RNA was isolated from samples using TRIzol reagent (Invitrogen, 15596026). A Nanodrop 1000 spectrophotometer was used to measure the RNA concentration and evaluate its quality. A PrimeScript RT Reagent Kit (Takara, RR037A) was used to generate from 1 µg RNA cDNA. cDNA was diluted to 2 ng/µl. An RT^2 Profiler PCR Array for mouse nephrotoxicity (PAMM-094Z) was used for determining the expression of nephrotoxic genes. According to the manufacturer's protocol, the RT^2 Profiler PCR Array analysis was conducted with SYBR green (Qiagen, 330520). The relative gene expression was calculated with the delta–delta Ct method ($2^{-\Delta\Delta Ct}$). Normalization was conducted with provided housekeeping genes on the array.

RNAseq

To the sorted cells from mouse kidneys, TRIzol was added. Total RNA extraction was conducted, as described above. RNA sequencing was performed on Illumina NovaSeq600 with a TruSeq stranded mRNA library. The Bioinformatics facility then normalized the RNAseq data at the University of Bern. First, RNAseq data quality was determined, and reads were mapped to the reference genome. Then, gene expression differences between the various treatment groups were identified. Normalized data were then visualized by using the heatmap tool on Morpheus.

Protein Isolation and Analysis

Cells were lysed in Laemmli buffer and separated by a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Then, transfer on a polyvinylidene fluoride membrane (ThermoFisher, 88518) was conducted. Membranes were blocked with 5% milk in Tris-buffered saline with Tween 20 (TBST) for 1 hour. Incubation with the primary antibody against Fn14 (1:1000; Cell Signaling, 4403S) and GAPDH (1:50′000; Merck, cB1001) was conducted overnight at 4°C. The horseradish peroxidase–conjugated secondary antibody was added to the membrane for 1 hour at room temperature (1:5000, Jackson, 711-035-152 and 1:5000, Jackson, 715-035-151). Visualization of the proteins was conducted using SuperSignal (ThermoFisher, 34076).

Statistical Analysis

Analysis was performed using Prism 5 software (GraphPad, San Diego, CA). Results are the mean ± the standard deviation (SD). Groups were analyzed by Student's t-test (unpaired) or ordinary one-way ANOVA and Tukey's multiple comparison test, with a single pooled variance. p values below 0.05 were considered significant (ns: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, and ****: $p \leq 0.0001$).

RESULTS

Surface Fn14 Is Induced Upon CsA Exposure in Kidney Epithelial Cells but Not in Response to the Cell-Permeable NFAT Inhibitor

We have previously demonstrated that the calcineurin inhibitor CsA induces the Fn14 surface expression on kidney epithelial cells *in vitro* and *in vivo* (Claus et al., 2018). To further support these findings, we challenged MCT cells with various nephrotoxins. Strikingly, FK506, similar to CsA, induced a Fn14 threefold in MCT cells, while gentamicin, amphotericin B, and cisplatin did not do so (**Figure 1A**). As CsA inhibits the NFAT pathway in lymphoid cells, we blunted the NFAT activity in kidney epithelial cells by treatment with direct NFAT inhibitors, namely, either cell-permeable 11R-VIVIT or plasmid-mediated transient expression of the VIVIT plasmid. Chemical 11R-VIVIT was tested on TCR-antibody–stimulated T cells *ex vivo* (**Figure 1B**). T cells treated with 11R-VIVIT or CsA showed no distinct peaks indicating no new cell generation compared to control cells. Meanwhile, treatment of MCT cells with equal doses of 11R-VIVIT or transient transfection of HEK293T cells with the VIVIT plasmid did not increase the Fn14 expression *in vitro* (**Figures 1C,D**). Also, CsA induced further upregulation of Fn14 on cell surfaces, and even the NFAT axis was inhibited (plasmid-mediated) (**Figure 1D**). It has been shown that CsA sensitizes cells to the cytokine TWEAK (Claus et al., 2018). We show in our experiments how cells express significantly higher the adhesion molecule ICAM after TWEAK treatment when pre-sensitized with CsA. This pre-sensitization is no longer observed when NFAT is inhibited in these cells, comparable to control (**Figure 1E**).

Inhibitors of Protein Kinases Mimic Effects of CsA in Kidney Epithelial Cells

To investigate CsA's effect in tubule epithelial cells of the kidneys, we treated MCT cells with the kinase inhibitor library. We investigated the effect of individual compounds on Fn14 induction as a surrogate marker for pro-fibrotic responses. Indeed, only specific protein kinase inhibitors (**Figures 2A,B**)

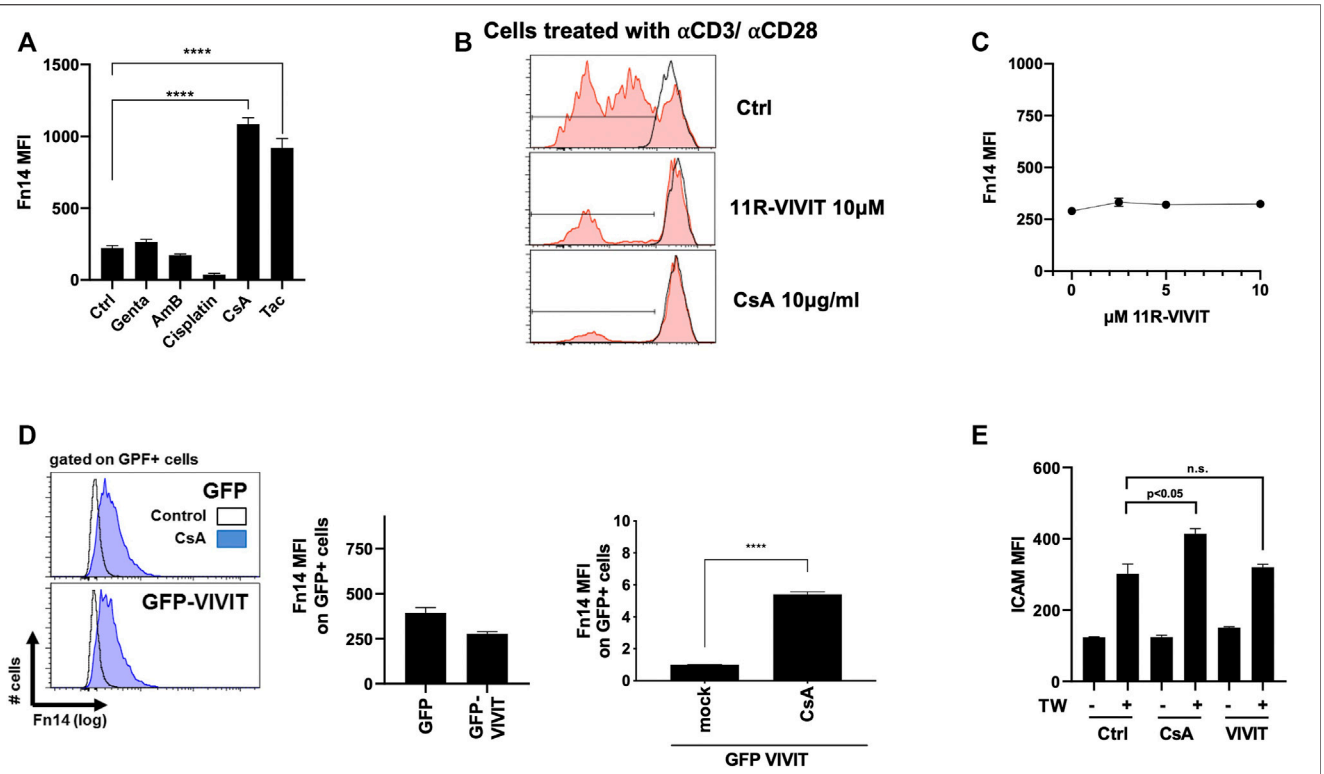

FIGURE 1 | Fn14 induction upon CsA exposure but not upon NFAT inhibition. **(A)** Fn14 induction with CsA and Tac (CNIs) but not with gentamicin, amphotericin B, and cisplatin (MCT). **(B)** NFAT and CsA successfully inhibit proliferation of TCRab-stimulated T cells *ex vivo*. **(C-D)** Inhibition of the NFAT axis, by plasmid chemically (11R-VIVIT) or (GFP VIVIT), shows no upregulation of Fn14 on the cell surface (C: MCT, D: HEK293T). **(E)** Cells sensitized with CsA show upregulation of ICAM upon TWEAK treatment. Inhibition of NFAT combined with TWEAK treatment shows reduced ICAM expression, comparable to control conditions (MCT). For all figures, flow cytometry was used.

significantly upregulated Fn14 on the surface of MCT cells. Interestingly, inhibition of calmodulin kinase II (by KN-62 and KN-93) showed no upregulation of Fn14 (**Figure 2A**). Many inhibitors, such as BAY 11-7082, SB203580, wortmannin, GF 109203X, palmitoyl-DL-carnitine, triciribine, SB202190, and hypericin led to significant induction of surface Fn14. Notably, two p38 MAPK inhibitors (SB203580 and SB202190) showed the potential to upregulate Fn14 on cell surfaces. Titration of both inhibitors revealed a similar potential to induce Fn14 (**Figure 2C**). Protein from SB203580-treated cells showed similar upregulation of Fn14 as cells treated with CsA (**Figure 2D**). Cells treated simultaneously with a p38 MAPK inhibitor and CsA showed an increased expression of Fn14 compared to cells only treated with CsA or one of the p38 MAPK inhibitors (**Figure 2E**). To corroborate these findings, we generated cells expressing a constitutive active p38 MAPK (pCMV-p38-CA-EGFP). The basal Fn14 expression (MFI) was moderate in all untreated mock-transfected (pCMV-eGFP-N1) and p38-CA–expressing cells. Mock-transfected control (GFP) showed a highly significant upregulation of Fn14 upon SB203580 or CsA stimulation. Meanwhile, the p38-CA–expressing cells treated with the p38 MAPK inhibitor or CsA induced a significantly lower Fn14 expression (**Figure 2F**). Furthermore, a gene array revealed that CsA and SB203580 upregulate various genes, whose expression levels did not alter when 11R-VIVIT was used. Nphs2, Cat, Rgn, A2m, Fgb, Ccl3, Cxcl10, Vcam1, and Nox4 and Spp1 are such upregulated genes (**Figure 2G**). Disorders in Nphs2, Cat, and Rgn genes are associated with kidney diseases (Franceschini et al., 2006; Ghaly et al., 2012; Yamaguchi, 2015). Upregulated genes such as Fgb, Ccl3, Cxcl10, Cd44, and Vcam are known to be involved in inflammatory responses (Nguyen and Simpson-Haidaris, 2000; Lim and Luscinskas, 2006; Johnson and Ruffell, 2009; Bhavsar et al., 2015; Lee et al., 2017; Kong et al., 2018). Lastly, also genes indicating the onset of fibrosis (Nox4 and Spp1) are upregulated (Amara et al., 2010; Morse et al., 2019).

CsA, but Not VIVIT, Induces Nephrotoxicity *in vivo*

RNAseq revealed that mice treated with CsA or the p38 MAPK inhibitor show a similar increased gene expression. Gpnmb, Fgb, Vcam1, and Gc are genes indicating ongoing inflammation (Nguyen and Simpson-Haidaris, 2000; Kong et al., 2018; Saade et al., 2021) and are upregulated in these mice. Also, kidney-related genes such as Mgp, Ugt1a1, Abcb1b, and Angptl4 are showing an increased gene expression (Yamakawa et al., 2004; Miyata et al., 2018) (**Figure 3A**). Meanwhile, animals treated with VIVIT showed no such signatures. Moreover, immune cell infiltration ($CD45^+$) into the kidney is upregulated in animals

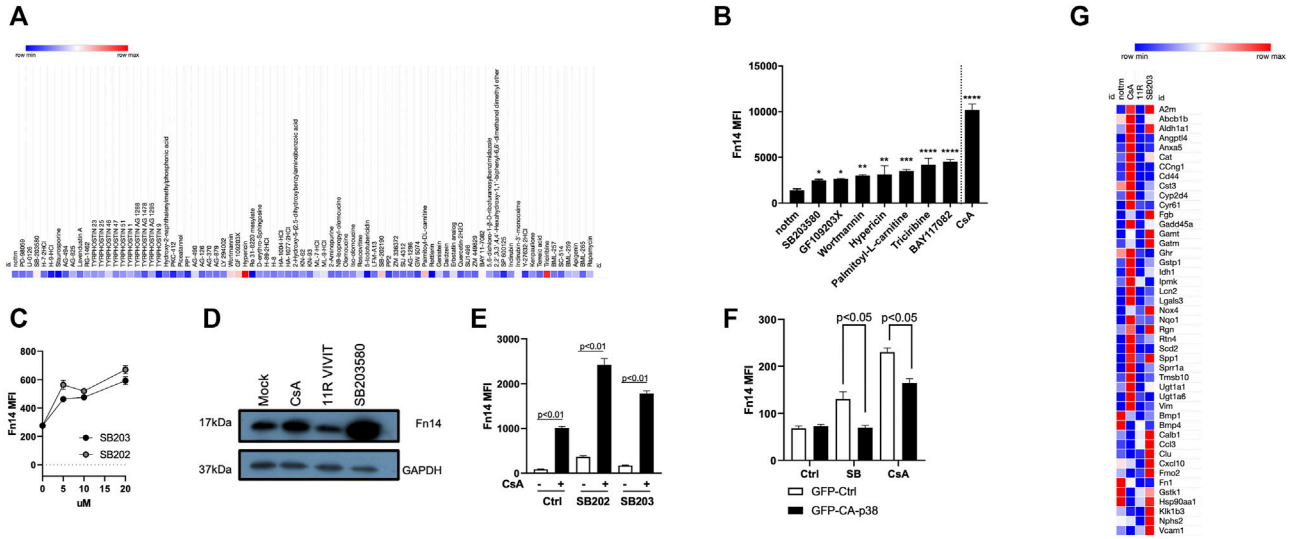

FIGURE 2 | Effects of CsA on kidney epithelial cells are mimicked by protein kinase inhibitors. **(A-B)** Protein kinase library screen revealed that inhibition of PI3K/Akt, IKK, and p38 MAPK induces Fn14 (MCT). **(C)** Titration of two p38 MAPK inhibitors (SB203580 and SB202190) revealed the similar potential to induce Fn14 (MCT). **(D)** Protein levels of Fn14 are increased upon CsA and SB203580 treatment but not when NFAT is inhibited (MCT, Western Blot). **(E)** Increased expression of Fn14 is detected when cells are treated simultaneously with the p38 MAPK inhibitor and CsA compared to single treatment with inhibitors (MCT cells). **(F)** Constitutive active p38 MAPK shows reduced Fn14 expression on cells when treated with either SB203580 or CsA (HEK293T). **(G)** Nephrotoxic genes are upregulated with CsA and with SB203580 (MCT, RT-PCR). If not indicated differently, results were acquired by flow cytometry.

treated with CsA or p38 MAPK inhibitors (**Figure 3B**). Not only could we detect changes in kidney infiltrated cells, but immune cell compartments, notably $CD45^+$ and CD11b + cells, seem to be increased upon CNI and slightly decreased under p38 MAPK inhibition (**Figure 3C**).

DISCUSSION

CNIs play a crucial role in effective immunosuppression after kidney transplantation. However, long-term treatment leads to chronic nephrotoxicity, known as calcineurin inhibitor toxicity. CsA and FK506 interfere with the cytosolic phosphatase calcineurin in lymphoid cells, preventing dephosphorylation NFAT and gene transcription. To date, it is unclear if the biological effects of CNI in non-lymphoid cells result similarly from disrupted calcineurin-NFAT signaling. This work aimed to investigate off-targets of CNI in epithelial cells. We present experimental evidence that CNT lesions are induced in kidney epithelial cells *in vitro* and *in vivo* independent of canonical calcineurin/NFAT signaling yet induced by kinase inhibitors, including inhibitors of the p38 MAPK, phosphoinositide-3-kinase (PI3K)/Akt, protein kinase C (PKC), and the inhibitor of nuclear factor-κB (IκB) kinase (IKK) pathway.

We showed that Fn14 upregulation is achieved by explicitly treating various kidney epithelial cells as well as *in vivo* with CNIs (CsA and FK506). Other compounds, known to be also nephrotoxic, however, failed to upregulate Fn14. These results confirm previous studies (Claus et al., 2018) that Fn14 is specifically upregulated due to calcineurin inhibitor toxicity in kidney epithelial cells. Therefore, the Fn14 is an excellent surrogate marker to measure nephrotoxicity and pro-fibrotic signals induced by CNIs.

Furthermore, we showed that an effective inhibitor (11R-VIVIT) of the NFAT axis fails to induce Fn14 in epithelial cells. When inhibiting the calmodulin kinase II, which is upstream of NFAT, no induction of Fn14 was detected. These results show that the direct NFAT inhibitor cannot mimic CsA's activity in kidney epithelial cells. Therefore, nephrotoxic CNI signaling in kidney epithelial cells is not mediated *via* the canonical NFAT pathway *in vitro* and *in vivo*.

Likely, CNT is the consequence of the off-target effects of CsA in kidney epithelial cells. It has been hypothesized that CsA targets disparate signaling pathways in lymphoid and non-lymphoid cells (Berzal et al., 2012). We showed that CsA's fibrotic effect on kidney cells is stable and easy to measure by the surrogate marker Fn14 and that the Fn14 expression is only upregulated under CNI treatment. As the nephrotoxic effect is NFAT-independent, we show here synthetic compounds and their capacity to induce Fn14. Our screen reveals that protein inhibitors of the serine/threonine p38 MAPK, PI3K/Akt, PKC, and IKK kinases mimic CsA's activity on epithelial cells. These effects are specific for respective pathways since chemically unrelated inhibitors of these routes elicited similar activities (SB203580 and SB202190/wortmannin and triciribine/hypericin, GF 109203X, and palmitoyl-DL-carnitine). Meanwhile, inhibitors of MEK1/2-ERK (PD-98059/U-0126) or tyrosine kinases such as JAK2 and EGFRK (AG-490 and Tyrphostin 23) had no Fn14-inducing activities. Results strongly suggest that the CNT of CsA on kidney epithelial cells acts by inhibiting p38 MAPK and PI3K/Akt kinases. Both kinase pathways p38 MAPK and PI3K/Akt are important in

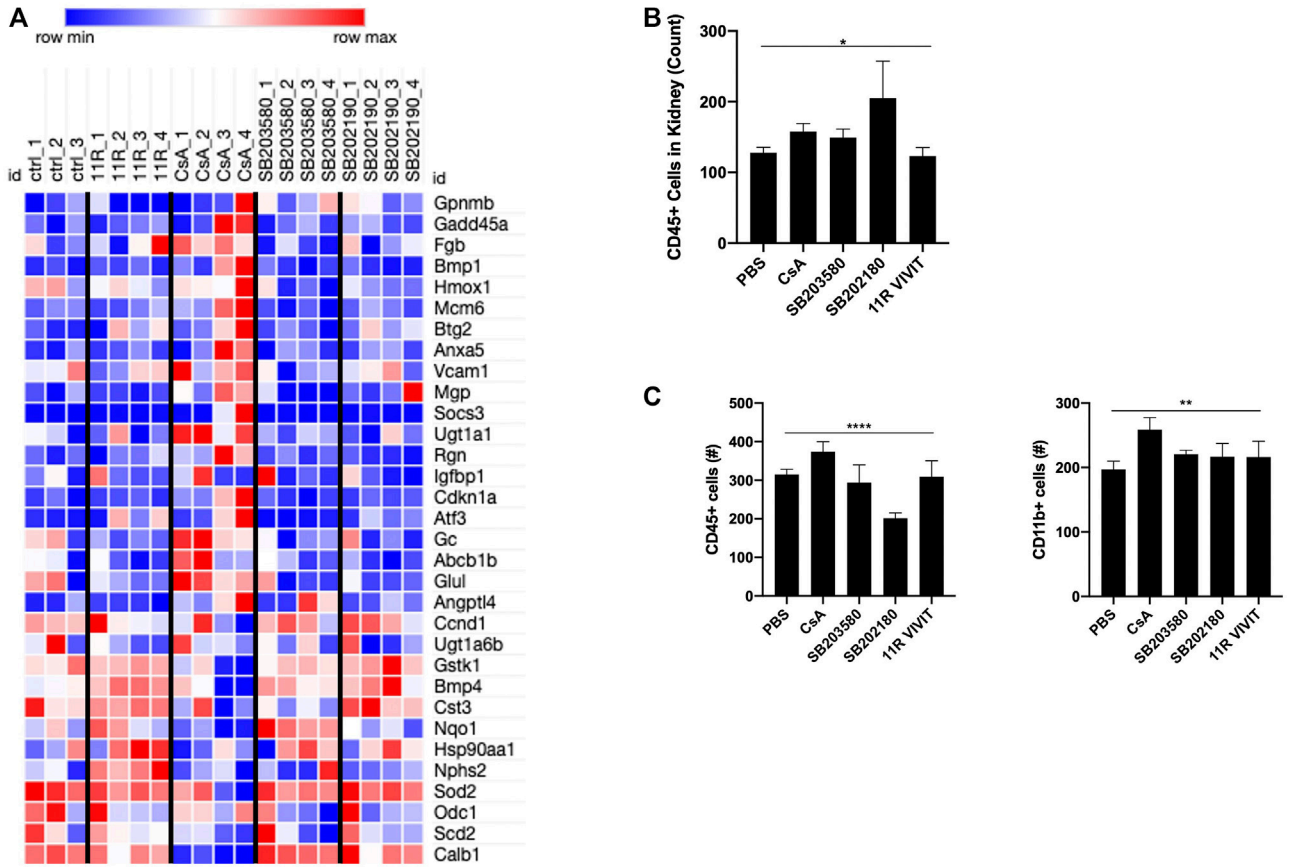

FIGURE 3 | *In vivo* experiments show the development of nephrotoxicity upon CsA and p38-kinase inhibitor. **(A)** Nephrotoxic gene array reveals evident upregulation of genes involved in nephrotoxicity upon CsA treatment. Also, both p38 inhibitors revealed upregulation of nephrotoxic genes (mouse kidney cells, RNAseq). **(B)** Higher CD45 $^{+}$infiltrated cells in the kidneys of CsA, SB203580, and SB202190 were detected. **(C)** Monocytic and lymphocytic cell compartments in the blood show a slight increase in cell number in animals treated with CsA and p38 MAPK inhibitors. B and C were measured by flow cytometry.

epithelial cells for differentiation, proliferation and apoptosis, and extracellular matrix synthesis. In both pathways, the serine/threonine kinase GSK3α is involved (Jacobs et al., 2012). Our top-down approach reveals that the inhibition of GSK3α (by Indirubin-3′-monooxime) shows slight upregulation of Fn14. Lastly, CsA and the p38 MAPK inhibitor induced *in vitro* and *in vivo* nephrotoxic, inflammatory, and fibrotic genes. The p38 MAPK inhibitor upregulates similar genes as CsA, which supports that CsA acts on p38 MAPK as an off-target.

This work has several limitations. First, induction of sustained CNT lesions is cumbersome in inbred mice, and treatments require high supramaximal CsA doses. The reason for this notion is unknown yet likely lies in a greater renal reserve for hemodynamic toxicity, low-renin levels, and other factors (Thomas et al., 1994; Rabe and Schaefer, 2016; Karolin et al., 2021). Possibly, single nucleotide polymorphisms further contribute to this CsA resistance in inbred mice. CNT lesions are not highly specific to CsA or Tac exposure in mice and humans. Similar lesions, including arteriohyalinosis and interstitial fibrosis/tubular atrophy (IFTA), can be identified in patients with chronic active and chronic inactive rejection and kidney transplants from older and hypertensive donors (Myers et al., 1984; Einecke et al., 2017).

This work offers potential for future experimental and clinical projects. First, numerous nephroprotective treatments are available or awaiting clinical approval, including SGLT-2 inhibitors (Vogt, 2021), PLG1-analog (Marso et al., 2016), and finerenone (Bakris et al., 2020). Since CNT is likely an off-target effect of CsA directly on kidney epithelial cells, such nephroprotective drugs could be of great value to prevent/treat developing or existing CNT in solid organ transplant recipients. Second, it remains unknown which subpopulation of kidney cells is affected by CNI treatment. Therefore, the following experiments could be focused on which kidney cell types (i.e., tubules and podocytes) are affected by the nephrotoxic potential of CNIs.

CNT shows a highly variable clinical presentation, and some patients develop early and progressive lesions within months of SOT. In contrast, others tolerate similar CNT doses/through levels over many years or decades without significant graft or kidney function impairment and absence of CNT lesions in biopsy specimens (Baan et al., 2000; Karolin et al., 2021; Xia et al., 2018). The reason for this highly inter-individual predisposition is unclear but likely lies in single nucleotide polymorphisms (from the donor in a setting of kidney

transplantation and the recipient). No GWAS study has yet been performed to decipher risk genes for rapid and progressive CNT after SOT.

In conclusion, these results suggest that CsA acts NFAT-independent on kidney cells yet requires p38 MAP and PI3K/Akt kinase pathways. Furthermore, we could show *in vitro* and *in vivo* that inhibition of proposed pathways (p38 MAPK and PI3K/Akt) mimics the toxicity of CsA on epithelial cells. However, we cannot exclude that CNT is not a highly specific interaction with a single molecular pathway.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE185146 https://www.ncbi.nlm.nih.gov/sra/PRJNA767789, PRJNA767789.

ETHICS STATEMENT

The animal study was reviewed and approved by BE Veterinärdienst des Kantons BE, Sekretariat Tierversuche.

AUTHOR CONTRIBUTIONS

AK and DS performed experiments. AK, DS, and SR planned and analyzed experiments. DS, SR, and GE supervised the findings of this work. AK and DS participated in the writing of the manuscript. Pre-print is available on bioRvix (DOI: 10.1101/2021.07.29.454219).

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The Protective Effects of Lipid-Lowering Agents on Cardiovascular Disease and Mortality in Maintenance Dialysis Patients: Propensity Score Analysis of a Population-Based Cohort Study

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Lipid-lowering agents display limited benefits on cardiovascular diseases and mortality in patients undergoing dialysis. Therefore, they are not routinely recommended for dialysis patients. The aim of this study was to assess the effects of lipid-lowering agents on clinical outcomes in dialysis patients on the basis of real-world evidence. This research used Taiwan's National Health Insurance Research Database to identify dialysis patients from January 2009 to December 2015; patients were then categorized into a case group treated with lipid-lowering agents (n = 3,933) and a control group without lipid-lowering agents (n = 24,267). Patients were matched by age, sex, and comorbidities in a 1:1 ratio. This study used the Cox regression model to estimate the hazard ratios (HRs) for mortality and major adverse cardiovascular events (MACEs) for events recorded until December 2017. During a mean follow-up period of approximately 3.1 years, 1726 [43.9%, incidence 0.123/person-year (PY)] deaths and 598 (15.2%, incidence 0.047/PY) MACEs occurred in the case group and 2031 (51.6%, incidence 0.153/PY) deaths and 649 (16.5% incidence 0.055/PY) MACEs occurred in the control group. In the multivariable analysis of the Cox regression model, lipid-lowering agent users showed a significantly lower risk of death [HR: 0.75; 95% confidence interval (CI): 0.70–0.80] and MACEs (HR: 0.88; 95% CI: 0.78–0.98) than lipid-lowering agent non-users. Moreover, the survival benefit of lipid-lowering agents was significant across most subgroups. Dialysis patients treated with lipid-lowering agents display a 25 and 12% reduction in their risk of mortality and MACEs, respectively. Therefore, lipid-lowering agents might be considered when treating dialysis patients with hyperlipidemia.

Keywords: dialysis, National Health Insurance Research Database, lipid-lowering agents, mortality, major adverse cardiovascular events

INTRODUCTION

Patients with chronic kidney disease (CKD) are at a high risk of morbidity and mortality owing to cardiovascular disease (CVD) (Jankowski et al., 2021); the risk of CVD starts from an early CKD stage and increases along with a decline in the estimated glomerular filtration rate (Yu et al., 2021). While entering into dialysis-dependent end-stage renal disease (ESRD), the risk of CVD significantly increases compared with that in the general population, accounting for >50% of the mortality (Chronic Kidney Disease Prognosis Consortium et al., 2010; Sharma and Sarnak, 2017; Johansen et al., 2021).

Dyslipidemia is a traditional risk factor contributing to CVD, and its correction has been considered the mainstay of treatment in high-risk groups (Lee et al., 2017; Hedayatnia et al., 2020). The first large-scale study investigating the effects of lipid-lowering agents was the Scandinavian Simvastatin Survival Study (4S), which demonstrated that this statin reduced cardiovascular events and mortality in patients with coronary artery disease (Pedersen et al., 1994). Recently, the benefit of statin therapy in CVD is well studied, and it is recommended for the primary prevention of CVD in those at a high risk of atherosclerosis (Ziaeian and Fonarow, 2017; Arnett et al., 2019; Byrne et al., 2019). Furthermore, some studies have suggested that achieving a very low level of low-density lipoprotein cholesterol (LDL-C) (<30 mg/dl) using high-intensity statin was safe and beneficial for the very high–risk population (Nicholls et al., 2016; Giugliano et al., 2017a; Giugliano et al., 2017b). However, the mechanisms and impacts of dyslipidemia remain unclear in patients with ESRD undergoing dialysis. The most widely accepted hypothesis is the carbamylation of LDL-C in the uremia stage (Kraus and Kraus, 2001; Ok et al., 2005). Carbamylated LDL has been shown to be a potent atherogenic factor *in vitro* and *in vivo*. Through endothelial cell damage, the degree of vascular smooth muscle cell proliferation and endothelial cell apoptosis was proportional to the degree of LDL carbamylation and subsequently induced accelerated atherosclerosis (Ok et al., 2005; Apostolov et al., 2010).

Because dyslipidemia is prevalent in patients with ESRD undergoing dialysis (Qunibi, 2015), two large randomized controlled trials have been conducted to investigate its correction by using lipid-lowering agents in merely dialysis patients. The Die Deutsche Diabetes Dialyze Studie (4D study) and A Study to Evaluate the Use of Rosuvastatin in Subjects on Regular Hemodialysis: An Assessment of Survival and Cardiovascular Events (AURORA) showed no benefit of statin use in dialysis patients (Wanner et al., 2005; Fellström et al., 2009). Moreover, the Study of Heart and Renal Protection (SHARP) including both non–dialysis-dependent CKD and dialysis patients showed that statin therapy did not significantly reduce the major adverse cardiovascular events (MACEs) in the subgroup of dialysis patients (hemodialysis and peritoneal dialysis) (Baigent et al., 2011). Therefore, lipid-lowering agents have not been routinely recommended for dialysis patients with hyperlipidemia owing to the aforementioned results in the current guideline (Mach et al., 2020).

Conversely, some previous observational studies have reported that lipid-lowering therapy is associated with reduced mortality in dialysis patients (Seliger et al., 2002; Mason et al., 2005; Jung et al., 2020). A recent study using a population-based nationwide dataset in Korea showed that statins combined with ezetimibe significantly lowered the all-cause mortality in adult patients undergoing maintenance hemodialysis (Jung et al., 2020), and the finding of which suggested that the application of lipid-lowering agents in the dialysis population might be re-evaluated. Moreover, hypertriglyceridemia is commonly observed in dialysis patients, but the therapeutic benefit of using the fenofibrate to lower triglyceride levels is unclear in this group (Hung et al., 2009; Mikolasevic et al., 2017). However, some studies had reported that the use of fenofibrate can provide cardiovascular and mortality benefits in non-dialysis CKD patients (Ting et al., 2012; Yen et al., 2021). Accordingly, we conducted this study to investigate whether the risk of MACEs and all-cause mortality could be reduced by lipid-lowering agents (statin and fenofibrate) in dialysis patients, including hemodialysis and peritoneal dialysis, on the basis of a real-world database.

MATERIALS AND METHODS

Data Sources and Research Samples

In this retrospective study, the source of information was the National Health Insurance Research Database (NHIRD). The NHIRD was established in 1996, including coverage for nearly 99% of beneficiaries who were certified citizens in Taiwan since 1998, and it provides diverse medical information, with inpatient and outpatient demographics, clinical records, diagnosis, procedure codes, and medical expenses (Hsieh et al., 2019). The diagnosis code was based on the International Classification of Diseases (9th and 10th) Clinical Modification (ICD-9-CM; ICD-10-CM) codes. The benefits of using NHIRD include the access to long-term follow-up data.

This study was performed in accordance with the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of Fu Jen Catholic University (IRB approval number: No. C104016). All claim records were anonymized before analysis, and the requirement of written informed consent was waived by the Institutional Review Board of Fu Jen Catholic University.

Study Population and Exclusion Criteria

The data for this study were collected between 1 January 2009 and 31 December 2015. Patients undergoing peritoneal dialysis or hemodialysis for more than 3 months were included (n = 46,261). The codes of dialysis procedures are shown in **Supplementary Table S1**. Exclusion criteria were age less than 18 years (n = 228), missing information (n = 25), cancer diagnosis (n = 726), use of statin or fibrates before entering the dialysis-dependent CKD stage (n = 16,740), and having multiple pancreatic diseases (acute pancreatitis, complications pancreas transplantation, pancreas damage of head, chronic inflammatory pancreatitis, and defect of pancreas, n = 342) before the index date. A total of 28,200

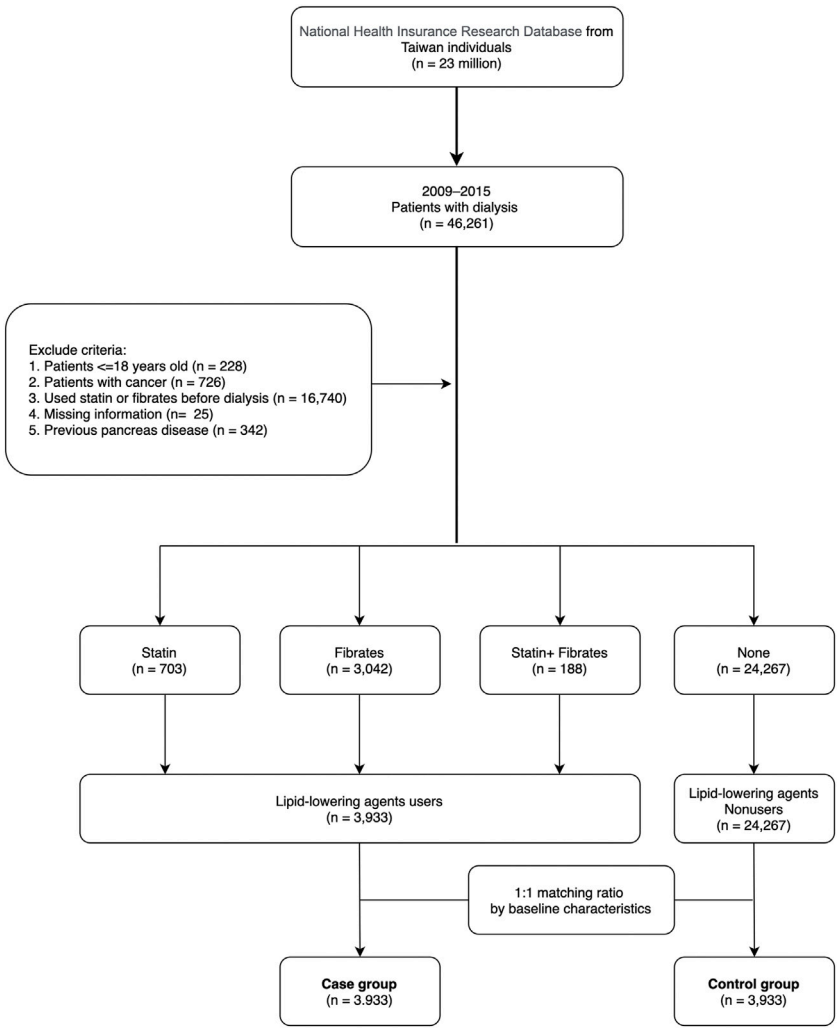

FIGURE 1 | Schematic of patient enrollment in the study.

patients were categorized into case and control groups, and cases using lipid-lowering agents (including statin and fibrate for 2 months) (n = 3,933) were matched with controls (n = 24,267) who did not use lipid-lowering agents within 6 months after entering the dialysis-dependent CKD stage. To reduce bias in our research, we used a 1:1 ratio propensity score matching to the baseline information including sex, age, baseline comorbidities, Charlson comorbidity index score (CCIS) (Charlson et al., 1987), and hospital area (**Figure 1**).

Baseline comorbidities, namely, hypertension, diabetes mellitus, hyperlipidemia, ischemic heart disease, chronic heart failure, stroke, peripheral artery occlusion disease, chronic obstructive pulmonary disease, liver disease, and biliary stone, were defined as diseases when they had at least three outpatient diagnoses or one inpatient diagnosis within 1 year before the index date (**Supplementary Table S1**). The usage of other drugs, namely, angiotensin-converting enzyme inhibitors/angiotensin receptor blockers (ACEI/ARB), calcium channel blockers (CCB), beta-blockers, anticoagulants, digoxin, dipeptidyl peptidase-4 inhibitor (DDP4i), insulin, nonsteroidal anti-inflammatory drugs (NSAIDs), uric acid–lowering agents, and benzodiazepines was considered when the patients used the drug for at least 2 months within 6 months before the index date. The prescription of implanted cardioverter defibrillators (ICDs) was also collected before the index date (**Supplementary Table S2**).

Clinical Outcomes

The index date for users was the date after using lipid-lowering agents for 2 months and that for non-users was the date after entering dialysis for 6 months. Patients were followed up until the occurrence of clinical events including all-cause death, ischemic stroke, and MACEs (the composite cardiovascular events: myocardial infarction, cerebrovascular disease, heart failure, and arrhythmia), or the end of the research (2017-12-31), whichever occurred first. The date of death was collected by linking to the database of the National Register of Deaths. Stroke and MACEs were mainly diagnosed according to the ICD code (**Supplementary Table S1**).

TABLE 1 | Demographic and clinical characteristics of dialysis patients.

Variable	Lipid-lowering agents				
	Before matching			After matching	
	Users (*n* = 3,933)	Non-users (*n* = 24,267)	*p* Value	Non-users (*n* = 3,933)	*p* Value
Gender					
Male	1,765 (44.9)	12,612 (52.0)	<0.001	1,760 (44.8)	0.909
Female	2,168 (55.1)	11,655 (48.0)		2,173 (55.3)	
Age (years), mean (SD)	58.8 (13.8)	63.3 (15.1)	<0.001	59.0 (14.5)	0.512
Comorbidities					
Hypertension	371 (9.4)	2,543 (10.5)	0.045	422 (10.7)	0.056
Diabetes mellitus	1,717 (43.7)	9,801 (40.4)	<0.001	1,686 (42.9)	0.480
Hyperlipidemia	1,662 (42.3)	4,805 (19.8)	<0.001	1,630 (41.4)	0.464
CAD	1,302 (33.1)	7,456 (30.7)	0.002	1,266 (32.2)	0.386
CHF	1,356 (34.5)	8,863 (36.5)	0.013	1,351 (34.4)	0.905
Stroke	756 (19.2)	5,699 (23.5)	<0.001	799 (20.3)	0.223
PVD	526 (13.4)	3,932 (16.2)	<0.001	587 (14.9)	0.048
COPD	729 (18.5)	6,062 (25.0)	<0.001	713 (18.1)	0.641
Liver disease	924 (23.5)	7,886 (32.5)	<0.001	845 (21.5)	0.032
Biliary stone	213 (5.42)	1,887 (7.8)	<0.001	232 (5.9)	0.353
CCIS, mean (SD)	3.5 (2.7)	4.0 (2.9)	<0.001	3.5 (2.9)	0.876
Hospital area					
Central	916 (23.3)	6,208 (25.6)	0.007	979 (24.9)	0.355
Northern	1,622 (41.2)	9,892 (40.8)		1,605 (40.8)	
Southern	1,306 (33.2)	7,576 (31.2)		1,256 (31.9)	
Eastern	89 (2.3)	591 (2.4)		93 (2.4)	
Prescription					
ACEI/ARB	1,107 (28.2)	5,352 (22.1)	<0.001	989 (25.2)	0.002
CCB	2,025 (51.5)	11,083 (45.7)	<0.001	1,904 (48.4)	0.006
Beta-blocker	1,672 (42.5)	8,180 (33.7)	<0.001	1,477 (37.6)	<0.001
Anticoagulants	1,870 (47.6)	10,343 (42.6)	<0.001	1,658 (42.2)	<0.001
Digoxin	62 (1.6)	478 (2.0)	0.095	73 (1.9)	0.339
ICDs	67 (1.7)	386 (1.6)	<0.001	70 (1.8)	0.796
DPP4i	440 (11.2)	1,415 (5.8)	<0.001	280 (7.1)	<0.001
Insulin	977 (24.8)	4,799 (19.8)	<0.001	851 (21.6)	<0.001
NSAID	1,648 (41.9)	10,597 (43.7)	0.038	1,656 (42.1)	0.855
UA-lowering agents	478 (12.2)	1,953 (8.1)	<0.001	367 (9.3)	<0.001
Benzodiazepines	1,817 (46.2)	11,498 (47.4)	0.168	1,776 (45.2)	0.353

Abbreviation: SD, standard deviation; CAD, coronary artery disease; CHF, congestive heart failure; PVD, peripheral vascular disease; COPD, chronic obstructive pulmonary disease; CCIS, Charlson Comorbidity Index Score; ACEI/ARB, angiotensin-converting enzyme inhibitors/angiotensin receptor blockers; CCB, calcium channel blockers; ICDs, implanted cardioverter defibrillators; DPP4i, dipeptidyl peptidase-4, inhibitor; NSAID, non-steroidal anti-inflammatory drug; UA-lowering agents, uric acid–lowering agents.

Statistical Analysis

Continuous data are expressed as mean ± standard deviation and categorical data as proportions (%). Categorical variables were analyzed using chi-squared tests. Continuous variables were analyzed using paired *t*-tests to validate demographic characteristics, including sex and age, and baseline comorbidities between case and control groups. The probability of propensity score was defined by covariates and estimated by using a logistic or probit regression model. There are two important points in propensity matching. First, the researchers have to make sure the matching ratio between the explored and unexplored groups. Second, researchers must determine which baseline variables were the confounding factors and eliminate them (Austin, 2008; Austin and Cafri, 2020). This research used propensity score matching with a 1:1 ratio and used baseline variables to eliminate the discrepancy between lipid-lowering agent users and non-users (Austin, 2011). An intention-to-treatment basis was adopted according to the patients' initial lipid-lowering agent use status without consideration of the subsequent regimen change.

Event-free survival curves were created using the Kaplan–Meier method and tested with the log-rank test. Moreover, the Cox proportional hazard model was adopted to estimate the hazard ratio (HR) with 95% confidence intervals (CI) of clinical outcomes as a function of lipid-lowering agent use. The assumption of proportionality was not violated by testing for an interaction between time and the variables. We also performed a subgroup analysis stratified by comorbidities. All statistical analyses and figures were created using 9.4 version of SAS (SAS Institute, Cary, NC, United States) software. For all tests, two-tailed *p*-values of <0.05 were considered statistically significant.

RESULTS

Patient Characteristics

We enrolled 28,200 dialysis patients in the present study (**Figure 1**). Among them, 3,933 patients (13.9%) used lipid-

TABLE 2 | Risk of clinical outcomes in patients with dialysis comparing lipid-lowering agents users vs. non-users.

Clinical outcome	Before matching					After matching			
	Users (n = 3,933)		Non-users (n = 24,267)		Users vs Non-users	Non-users (n = 3,933)		Users vs non-users	
								Model 1	Model 2
	Events	IR	Events	IR	HR (95%CI)	Events	IR	HR (95%CI)	HR (95%CI)
All-cause mortality	1726	12.30	15,140	20.8	0.60** (0.57–0.63)	2031	15.3	0.80** (0.75–0.85)	0.75** (0.70–0.80)
MACEs	598	4.70	3,914	6.00	0.79* (0.72–0.86)	649	5.50	0.85* (0.76–0.95)	0.88* (0.78–0.98)
Ischemic stroke	137	1.00	817	1.10	0.87 (0.72–1.04)	138	1.10	0.93 (0.73–1.18)	0.87 (0.69–1.11)

Model 1 is crude analysis after matching. Model 2 is further adjusted by medical prescriptions, including angiotensin-converting enzyme inhibitors/angiotensin receptor blockers, calcium channel blockers, beta-blockers, anticoagulants, digoxin, implanted cardioverter defibrillators, dipeptidyl peptidase-4, inhibitor, insulin, non-steroidal anti-inflammatory drugs, uric acid–lowering agents, and benzodiazepines.

Abbreviation: IR, incidence rate (in every 100 person-years); HR, hazard ratio; CI, confidence interval; aHR, adjusted hazard ratio; MACEs, major adverse cardiovascular events.

**p < 0.001. **p < 0.05.*

lowering agents for at least 2 months after entering dialysis. The mean age of lipid-lowering agent users (case group) was 58.8 years, of whom 44.9% were men, 9.4% had hypertension, 43.7% had diabetes mellitus, 33.1% had coronary artery disease, and 19.2% had a stroke history. Moreover, 41.2% of patients were from northern Taiwan and 33.2% were from southern Taiwan (**Table 1**). Before matching, lipid-lowering agent users had lesser comorbidities than lipid-lowering agent non-users. However, the baseline demographic data and comorbidities between lipid-lowering agent users and non-users showed no significant difference after matching. For baseline medical prescriptions after matching, lipid-lowering agent users had a higher prescription rate in ACEI/ARB, CCB, beta-blockers, anticoagulants, DPP4i, insulin, and uric acid–lowering agents than in lipid-lowering agent non-users after matching, as shown in **Table 1**.

In the group of lipid-lowering agents users, the numbers of patients who did not use lipid-lowering agents after the index day were 157 (4%) within 1 year and 297 (7.5%) within 2 years. However, the non-users of lipid-lowering agents were kept free from the lipid-lowering agents for 1 and 2 years after the index day.

Benefit of Using Lipid-Lowering Agents in Dialysis Patients

The total follow-up summation is 86,627 person-year (PY) during the study period (**Table 2**). A total of 16,866 patients (59.8%) died, 4,512 patients (16%) had a new-onset MACE, and 954 patients (3.4%) experienced the ischemic stroke. Before matching, the lipid-lowering agent users had a better incidence rate than non-users in mortality (0.123/PY vs 0.208/PY) and MACEs (0.47/PY vs 0.60/PY) but not in ischemic stroke (0.01 vs 0.01%). Moreover, the Kaplan–Meier event-free curves for all-cause mortality (**Figure 2A**) and MACEs (**Figure 2B**) among lipid-lowering agents users compared with non-users were both significant ($p < 0.05$) after matching. This finding indicated that the use of lipid-lowering agents was associated with a lower risk of mortality and MACEs.

As shown in **Table 2**, we found that treatment with lipid-lowering agents in dialysis patients significantly decreased their risk of mortality (HR, 0.60; 95% CI, 0.57–0.63) and MACEs (HR, 0.79; 95% CI, 0.72–0.86) before matching, and the beneficial effects of lipid-lowering agents were maintained after propensity score matching [HR: 0.80 (0.75–0.85); 0.85 (0.76–0.95), respectively]. After further adjusting the medical prescriptions, such significance was also kept [HR: 0.75 (0.70–0.80) for mortality; 0.88 (0.78–0.98) for MACEs]. The risk of ischemic stroke event had no significant difference between the matching groups [HR: 0.93 (0.73–1.18) in the crude model; 0.87 (0.69–1.11) in the multivariable adjusting model].

In **Table 3**, it shows the events of composites of MACEs between lipid-lowering agents users and non-users during the observation period, of which myocardial infarction had a significant difference between groups (3.5 vs 2.4%, $p = 0.003$).

Subgroup Analysis

We conducted a series of stratified analyses to test the reliability of our results (**Figure 3**). The decreased HRs of mortality among dialysis patients in favor of lipid-lowering agents were consistent across all patient subgroups. Patients of young age, those with diabetes, and those without a history of stroke and congestive heart failure showed a significantly lower risk of MACEs.

DISCUSSION

We have provided real-world evidence on the effects of lipid-lowering agents in reducing cardiovascular events and mortality in dialysis patients using an NHIRD dataset. This finding is crucial to disclose that dyslipidemia correction still plays a role in the improvement of CVD and mortality in dialysis patients, which belong to the high-risk CVD group. Our study extends the current knowledge about the use of lipid-lowering agents in the dialysis population who were eligible to use lipid-lowering agents which may be associated with the reduced mortality and MACEs, and the risk reduction of MACEs may be attributed to the prevention of myocardial infarction. Therefore, we suggested

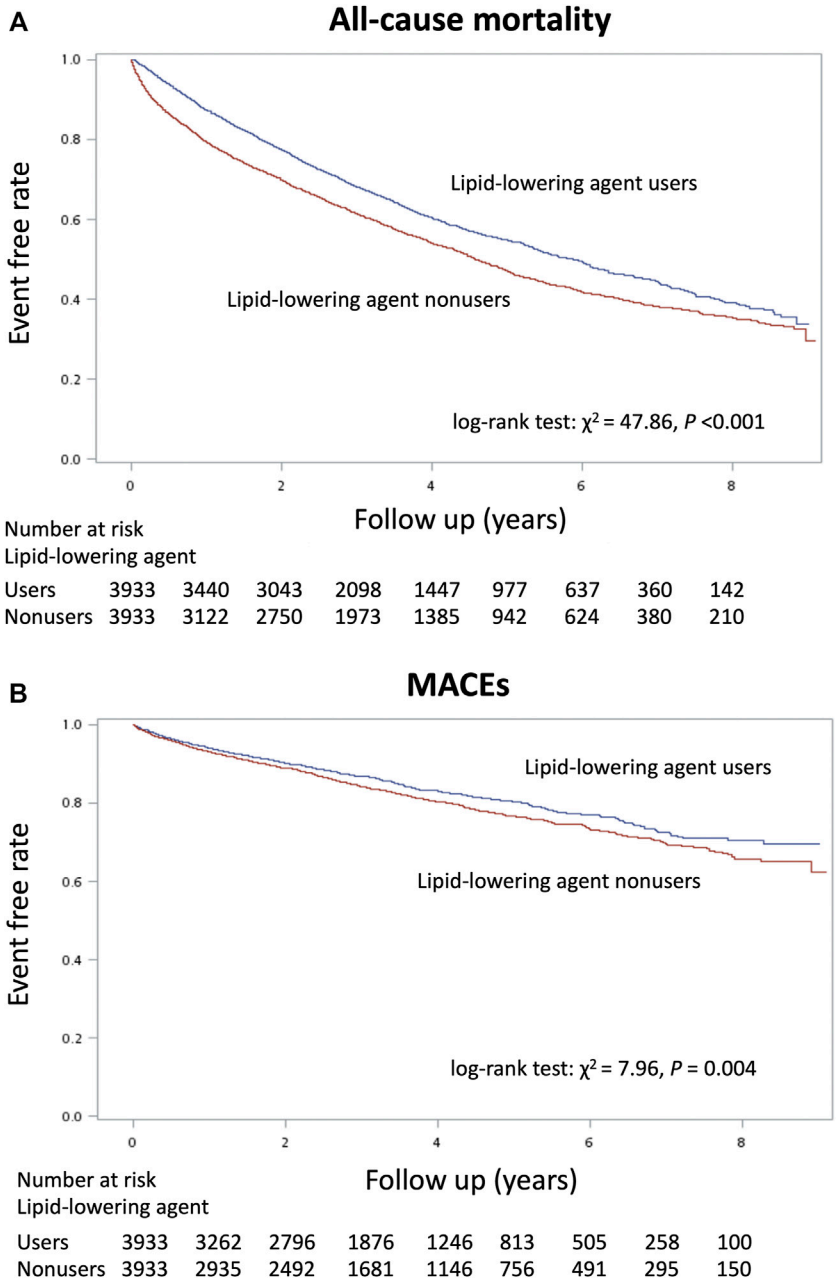

FIGURE 2 | Kaplan–Meier cumulative event-free plots of **(A)** mortality and **(B)** major adverse cardiovascular events (MACEs) in the study population according to whether the lipid-lowering agents were used or not.

TABLE 3 | Components of MACEs between patients with dialysis comparing lipid-lowering agents users vs. non-users.

Components of MACEs	After matching		
	Users (*n* = 3,933)	Non-users (*n* = 3,933)	*p* value
Myocardial infarction	138 (3.5)	94 (2.4)	0.003
Heart failure	319 (8.1)	346 (8.8)	0.273
Cerebrovascular disease	236 (6)	253 (6.4)	0.427
Cardiac arrhythmia	184 (4.7)	211 (5.4)	0.163

Abbreviation: MACEs, major adverse cardiac events.

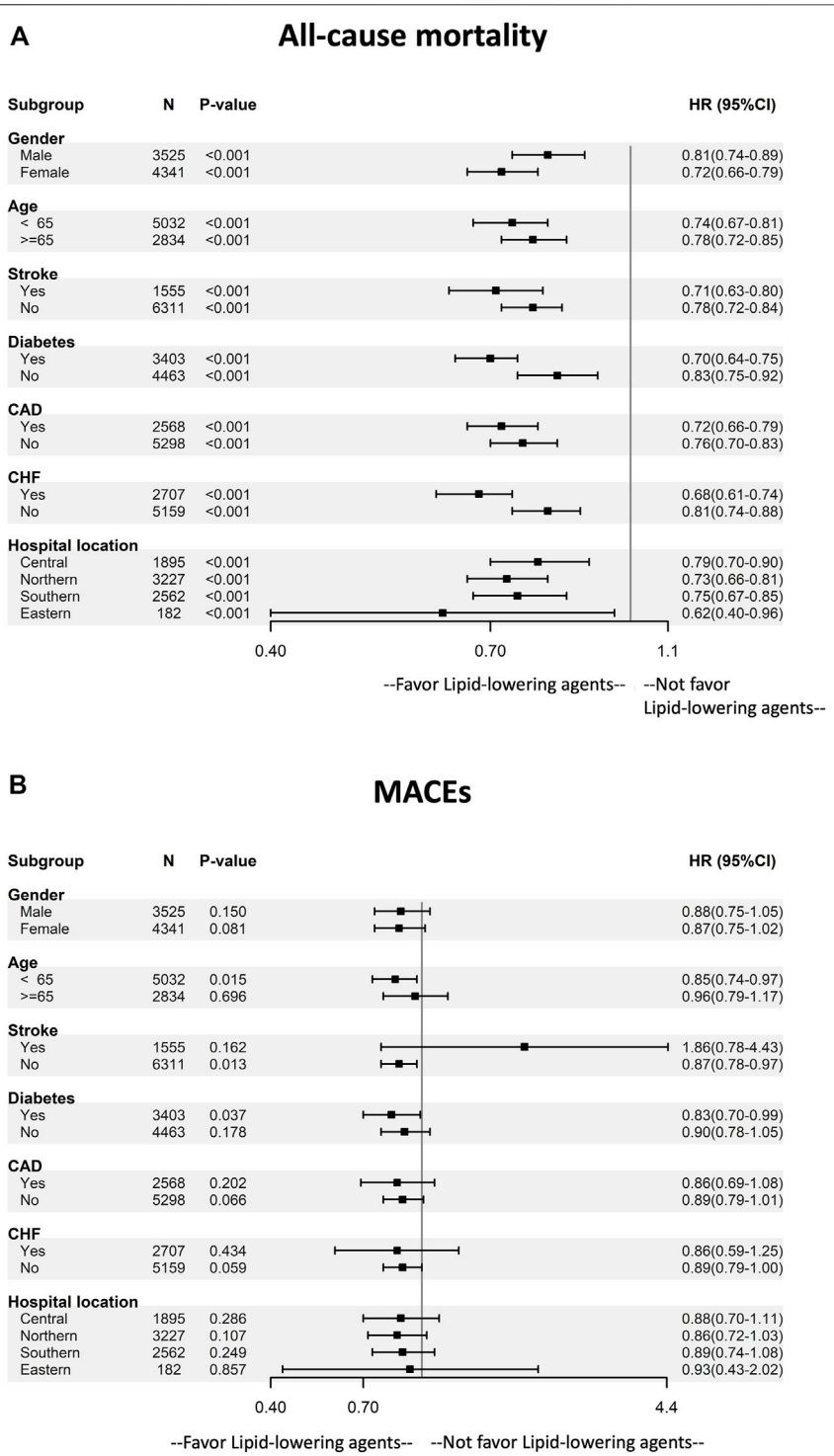

FIGURE 3 | Subgroup analysis of the effect of lipid-lowering agents use on **(A)** mortality and **(B)** MACEs by baseline characteristics. The multivariable adjusting model was the same as model 2 in **Table 2**. Abbreviation: CAD, coronary artery disease; CHF, congestive heart disease; MACEs, major adverse cardiovascular events.

that lipid-lowering agents (stain or fenofibrate) may be applied to dialysis patients with dyslipidemia.

There has been a longstanding debate regarding the use of lipid-lowering agents in chronic dialysis patients. However, this question became unequivocal since the outcomes of the 4D, AURORA, and SHARP trials revealed negative results in the dialysis population without overt CAD (Wanner et al., 2005; Fellström et al., 2009; Baigent et al., 2011). In the 4D study,

diabetes patients undergoing chronic hemodialysis were randomly assigned to receive a statin (atorvastatin) or a placebo. In the statin-treated group, LDL cholesterol levels significantly reduced from baseline since the first 4 weeks and were maintained for 5 years. This reduction in LDL cholesterol did not offer benefit in reducing MACEs compared with placebo, suggesting that it was too late to use statin in the dialysis population (Wanner et al., 2005). Regarding the AURORA study conducted in hemodialysis patients with or without diabetes, similar results were noted between the statin (rosuvastatin) and placebo groups (Fellström et al., 2009). Moreover, the SHARP study showed that after an average follow-up period of 4.9 years, a significant reduction in major atherosclerotic events was observed in the recruited participants. However, this positive finding was not significant in the subgroup based on dialysis at enrollment (Baigent et al., 2011). Moreover, a meta-analysis combined these three studies and showed that statin use had a significant benefit on nonfatal atherosclerotic CV events [odds ratio (OR), 0.89, 95% CI, 0.8–0.99] but not on coronary events (OR, 0.97; 95% CI, 0.88–1.06), ischemic stroke (OR, 1.29, 95% CI, 0.72–2.31), and all-cause mortality (OR, 0.88; 95% CI, 0.74–1.04) (Green et al., 2013).

Because the effects of lipid-lowering agents in dialysis patients were not as expected for the general population in these large randomized controlled trials (RCTs), there are several possible explanations for these negative results. The first one is the complexity of CVD in patients undergoing dialysis. Apart from traditional risk factors, several types of pathogenesis contribute to atherosclerosis and the decline in heart function. The so-called non-traditional factors include mineral and bone metabolism disorder, oxidative stress, and anemia (Yao et al., 2004). Uremic toxin, p-cresol, and indoxyl sulfate have been linked with atherosclerosis and vascular calcification (Barreto et al., 2009; Opdebeeck et al., 2020). In addition, the increased level of fibroblast growth factor 23 as an indicator of renal function decline has shown its deleterious effects on cardiomyocytes (Gutiérrez et al., 2008). These factors render the association of a direct causal relationship between LDL cholesterol and atheroma less relevant (Mihaylova et al., 2012; Fulcher et al., 2015). In addition, there are aspects of CVD in ESRD, such as arrhythmia and heart failure, that affect non-atheromatous pathogenesis (Methven et al., 2017; Saran et al., 2017). Intervention in only one dimension of lipid control will not solve these issues as noted in these large RCT studies.

Despite the negative results from RCT trials, some studies found that ESRD groups undergoing dialysis could benefit from lipid-lowering agents. In the post hoc analysis of the 4D study, statin still lowered the risk of cardiovascular events when the LDL level was >145 mg/dl (März et al., 2011). This suggested that in dialysis patients with a severe degree of dyslipidemia, aggressive lipid-lowering therapy can also reduce cardiovascular mortality in a long-term observational period. Moreover, statin treatment in dialysis patients after acute myocardial infarction improves overall mortality (Chung et al., 2017). Statin treatment significantly decreases overall mortality in ESRD patients with acute myocardial infarction compared with the non-statin group. This is more prominent in the cardiac shock patient subgroup. These results are compatible with those from other studies, supporting a measurable benefit from statins in ESRD patients (Chung et al., 2017).

In our study using Taiwan's NHIRD, chronic dialysis patients using lipid-lowering agents had a better outcome in MACEs, especially the composite of myocardial infarction, and all-cause mortality, suggesting that such agents may be helpful in this population. However, the reasons for the discordance between RCTs and the observational study findings about the use of lipid-lowering agents in dialysis patients are still undetermined. We proposed some possible explanations. In our previous single-center study cohort, we observed an inverted U-curve association between serum indoxyl sulfate levels and cardiovascular events in chronic hemodialysis patients (Tsai et al., 2021). The reported data indicate that although indoxyl sulfate, a recognized uremic toxin, may contribute to atherosclerosis and cardiovascular pathogenesis *in vivo* and *in vitro*, its higher levels led to a better cardiovascular outcome. In our observation, the underlying nutritional status may explain this reverse epidemiology (Zha and Qian, 2017; Hanna et al., 2020). Regarding the lipid-lowering issue, only well-nourished patients in chronic dialysis would be suitable to receive such agents. This may be a reasonable explanation for this finding in our population-based cohort study. Also, the other explanation is that RCTs have a good enteral validity but a relatively low external validity and generalization due to their highly selective population and tightly control setting. Indeed, in real-world practice, patients have higher heterogenicity than those in RCTs. Therefore, a discrepancy exists between RCTs and the studies using real-word data (Kim et al., 2018).

Our study had several strengths. First, we used data from a nationwide database, meaning that the study's results can be generalized. Second, the sample size and observation time were adequate to obtain sufficient inferences. Despite its strengths, our study had several potential limitations. First, NHIRD, an original claim database for reimbursement, does not offer clinical information such as biochemical data, inflammatory burden, blood pressure, and body characteristics (weight, height, waist circumference, and body fat percentage) which might have an impact on the development of MACEs (Sardu et al., 2019; Sardu et al., 2021a; Sardu et al., 2021b). However, our study's large number size with the method of propensity score matching can alleviate this bias. Second, we cannot determine whether the study patients showed regular drug compliance because exposure to lipid-lowering agents was based on prescription information only. Third, this was not a RCT; thus, the unbalanced baseline between the two groups was a major concern, which might induce a bias of confounding by indication (Kyriacou and Lewis, 2016). However, we used the propensity score matching to reduce this bias as the method of propensity score matching is well developed to balance the underlying difference between groups. Also, we further adjusted the medications and ICDs use, the devices could significantly ameliorate clinical cardiovascular outcomes (Sardu et al., 2017), and the significant results in our study were kept.

In conclusion, although lipid-lowering agents are not recommended for routine use in dialysis patients with hyperlipidemia according to the clinical guideline, our results demonstrated significant benefits of the use of lipid-lowering agents on clinical outcomes, including MACEs, and all-cause death, in such a population.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because all claim records were anonymized before analysis. Thus, Fu Jen Catholic University Ethics Institutional Review Board was exempted from a full ethical review, and the requirement to obtain informed consent was waived. Requests to access the datasets should be directed to https://dep.mohw.gov.tw/dos/np-1714-113.html.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the patients/participants or patients/participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Y-WF, MC, and M-HT conceived and designed the experiments. MC, H-HL, and M-HT performed the experiments. Y-CH and MC analyzed the data. Contributed reagents/materials/analysis tools. All authors wrote the manuscript. M-HT and Y-WF approved the manuscript. M-HT and MC contributed equally to this work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.804000/full#supplementary-material

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Corrigendum: The Protective Effects of Lipid-Lowering Agents on Cardiovascular Disease and Mortality in Maintenance Dialysis Patients: Propensity Score Analysis of a Population-Based Cohort Study

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Keywords: dialysis, National Health Insurance Research Database, lipid-lowering agents, mortality, major adverse cardiovascular events

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A corrigendum on

The Protective Effects of Lipid-Lowering Agents on Cardiovascular Disease and Mortality in Maintenance Dialysis Patients: Propensity Score Analysis of a Population-Based Cohort Study *by Tsai, M.-H., Chen, M., Huang, Y.-C., Liou, H.-H., and Fang, Y.-W. (2022). Front. Pharmacol. 12: 804000. doi:10.3389/fphar.2021.804000*

In the published article, Mingchih Chen was incorrectly included as a corresponding author. The only corresponding author should be Yu-Wei Fang.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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SS31 Ameliorates Podocyte Injury via Inhibiting OMA1-Mediated Hydrolysis of OPA1 in Diabetic Kidney Disease

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Diabetic kidney disease (DKD) is currently one of the leading causes of end-stage renal disease (ESRD). Mitochondrial dysfunction in podocyte is involve in DKD development. However, whether early mitochondrial stabilization delays or reverses DKD progression has not been elucidated. SS31 is a novel tetrapeptide compound that targets the inner mitochondrial membrane and protects mitochondria by reducing ROS and inhibiting cardiolipin oxidation. Our study discovered that SS31 might have a long-term podocyte protection in DKD. In this study, we examined the glomerular pathological damage and proteinuria at different stages of diabetes. Results revealed that podocyte mitochondrial injury appeared at the early stage of DKD. Early treatment with SS31 could protect podocyte and alleviate the development of DKD via inhibiting OMA1-mediated hydrolysis of OPA1. Those data indicate that SS31 might be a promising agent in delaying the development of DKD and OMA1-mediated hydrolysis of OPA1 in mitochondria, and SS31 is a novel therapeutic target for the treatment of DKD.

Keywords: SS31, mitochondria, Oma1, OPA1, podocyte, diabetic nephropathy

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INTRODUCTION

Diabetic kidney disease (DKD) is currently one of the leading causes of end-stage renal disease (ESRD) and is the strongest single predictor of mortality in diabetic patients (Reidy et al., 2014). Accumulated evidences suggest that podocyte damage plays a vital role in DKD progression (Bose et al., 2017). Kidney biopsy of Type 1 diabetes mellitus (T1DM) and T2DM shows that podocyte number is highly correlated with proteinuria and acts as an important factor in predicting disease progression (White et al., 2002). As a type of terminally differentiated epithelial cells, podocytes have a limited potential in self-repair and regeneration and are sensitive to various injuries. Thus, effective protection of podocytes is an important strategy for the treatment of DKD.

Podocytes rely on glycolysis and mitochondrial oxidative phosphorylation for ATP synthesis, among which mitochondrial respiration accounts for 77% (Abe et al., 2010). It has been reported that mitochondrial abnormalities are involved in a variety of podocyte injury models (Guan et al., 2015; Szeto et al., 2016; Qi et al., 2017; Fujii et al., 2020), meanwhile mitochondria-targeted drugs significantly alleviate podocyte injury, indicating that mitochondrial homeostasis plays an important role in podocytes (Szeto, 2017). Mitochondrial homeostasis involves mitochondrial biogenesis, mitochondrial dynamics, mitochondrial distribution, mitophagy and mitochondrial DNA content. Among these, mitochondrial dynamics, the balance of mitochondrial fusion and fission, has a vital role in maintaining mitochondrial homeostasis (Cerveny et al., 2007; Chan, 2012; Mishra and Chan, 2016; Wai and Langer, 2016). Enough evidences show that abnormal mitochondrial dynamics is

widely involved in podocyte injury (Wang et al., 2012; Yuan et al., 2018; Ma et al., 2019; Chen et al., 2020), and might be an important target for the treatment of podocyte diseases (Ayanga et al., 2016; Qin et al., 2019). The dynamin-like GTPase OPA1 is a crucial fusion protein in mitochondrial inner membrane. In addition, OPA1 also participates in mitochondrial cristae morphogenesis, apoptosis, and mitochondrial respiration (Olichon et al., 2006). OMA1, a mitochondrial inner membrane zinc metalloprotease, is involved in the proteolysis of OPA1 during stress and apoptosis (Ehses et al., 2009). Under physiological conditions, OMA1 is dormant, but rapidly activated upon mitochondrial dysfunctions (MacVicar and Langer, 2016). Thus, OPA1 and OMA1 play an important role in stabilizing mitochondria. SS31 is a cell-permeable tetrapeptide that selectively targets the inner mitochondrial membrane. Previous studies identified SS31 as a mitochondria-targeted antioxidant (Zhao et al., 2004). Recent research reveals that SS31 selectively interacts with cardiolipin and inhibits cardiolipin peroxidation, thus promoting ATP synthesis and reducing proton leak as well as ROS production (Szeto et al., 2011; Birk et al., 2013). Due to the suppression of cardiolipin peroxidation,SS-31 has shown remarkable efficacy in diverse animal disease models associated with bioenergetic failure, including ischaemia-reperfusion injury, heart failure, skeletal muscle atrophy and neurodegenerative diseases(Yang et al., 2009; Min et al., 2011; Szeto and Schiller, 2011; Kloner et al., 2012; Sloan et al., 2012; Dai et al., 2013; Talbert et al., 2013). The protective role of SS31 was also found in kidney in the progressing of acute kidney injury (AKI), DKD or aging. Mechanically, SS31 could regulate mitochondrial fission and fusion, inhibit mitochondrial ROS-NLRP3 activation, accelerating ATP recovery (Szeto et al., 2011; Yang et al., 2019; Yang et al., 2020). Furthermore, SS31 exerts significant podocyte protective effects in renal injury models such as aging, diabetes and high-fat mice. Nevertheless, the concrete mechanism has not been fully elucidated. In this study, we demonstrate that SS31 restores OPA1 expression by inhibiting OMA1 activation, and preserves mitochondrial function in podocyte during the progression of DKD.

MATERIALS AND METHODS

Reagents and Antibodies

SS31 was obtained from ChinaPeptides (Shanghai, China). Streptozocin (STZ) was purchased from Sigma (Shanghai, China). Antibodies used were as follows: anti-SYNPO (NBP2-39100, Noves), anti-Nephrin (PR52265, Sigma), anti- Caspase-3 (9664s, CST), anti-OPA1 (ab42364, Abcam), anti-OMA1 (sc-515788, Santa) and anti-Tubulin (Sigma, T6074), HRP-conjugated anti-Mouse and anti-rabbit (Sigma) secondary antibody. 1,640 (11879-020) and fetal bovine serum (FBS) (A3160902) were purchased from Gibco.

Animals

All animal care and experiments were performed according to the guidelines for the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University, and the animal ethical approval number is IACUC-1905002 C57BL/6 male mice, weighing 18–22 g, were obtained from Charles River Laboratory Animal Technology (Beijing, China) and randomly divided into normal control group and diabetic model group. Mice from diabetic model group were intraperitoneally injected with streptozotocin (STZ) at 40 mg/kg for 3 days. Two weeks later, mice with random blood glucose higher than 16.7 mmol/L were identified as successful diabetic model mice. The experiment was divided into 2 phases. In the first phase, we observed natural pathological changes in different stages of DN. 15 diabetic mice were successfully modeled and randomly divided into 6 weeks group (n = 5), 12 weeks group (n = 5, one died accidentally halfway), and 20 weeks group (n = 5), while 4 normal mice were used as normal controls. Mice were sacrificed at corresponding time points. Blood, urine and kidney tissues were collected after euthanasia. In the second phase, we explore the podocyte protective role of SS31 in DN. 11 diabetic mice were successfully modeled and randomly divided into STZ group (n = 6) and STZ + SS31 group (n = 5), while 6 mice were used as normal controls. Mice from STZ + SS31 were intraperitoneally injected with SS31(2 mg/kg) every other day for 4 weeks, mice from control and STZ groups were correspondingly intraperitoneally injected with saline for 4 weeks. Mice were sacrificed at weeks 12. Blood, urine, and kidney tissues were collected from mice after euthanasia.

Glomerular Harvest and Primary Podocytes Culture

Glomeruli from 8-week-old C57BL/6 mice were collected by filtering kidney tissues with different pore sizes mesh sieves. In brief, kidneys were cut into small pieces with a scalpel, immersed in 4 ml HBSS and treated with 1 mg/ml collagenase (Sigma, c6885) and 0.5 mg/ml pronase E (Sigma, p6911) at 37°C for 15 min. Then, tissues were filtrated through a 100-um cell strainer (BD Biosciences, San Jose, CA, United States) with a flattened pestle and rinsed with 15 ml HBSS. Afterward, the suspension was flown through a 400-mesh screen. Glomeruli retained on the screen were transferred with a pipette into a new 50-ml tube. Finally, the glomeruli were collected by centrifugation at 3,000 rpm for 10 min.

For primary podocyte culture, glomeruli were suspended with 1,640 containing 10% FBS and seeded on the culture dishes. The culture dishes were kept still for 3 days to allow the glomeruli to adhere. On day 4, culture medium was replaced with fresh one and the unattached glomeruli were washed away. On day 5, cellular outgrowths were detached with trypsin (Gibco) and filtrated through a 40-μm cell strainer to remove the remaining glomerular cores. The filtered cells were collected and seeded in 6-well plates at a density of 1.5×10^5 at 37oC with 5% CO_2.

Adenoviral and siRNA Transfection

6-well plates were seeded with 1.5×10^5 cells per well. After cells adhered and reached 70–80% confluence, the corresponding volume of adenovirus was added according to the MOI and

virus titer. The virus volumes were calculated by the formula "virus volume = (MOI × number of cells)/virus titer". Cells were incubated at 37°C and the medium were replaced after 12–16 h incubation. Specific siRNA was mixed with Lipofectamine® RNAiMAX (Gibco) accordingly. Briefly, cells were transfected with the siRNA- RNAiMAX complex and incubated at 37°C in a CO2 incubator for 24–96 h until gene knockdown could be detected. The mediums were changed after 4–6 h.

Tissue Preparation and Histologic Analyses

A small piece of tissue was immersed in 10% formaldehyde solution for 1 day and then made into paraffin blocks. The blocks were cut into 3-μm sections and fixed on glass slides. Afterwards, the slides were stained with periodic acid-schiff (PAS) agent. The degree of glomerulosclerosis was semi-quantitatively calculated according to the ratio of PAS-positive areas to glomerular area using ImageJ (1.8.0).

Immunofluorescence

A few renal cortices were frozen and cut into 3-μm thickness sections. The sections were fixed with 4% paraformaldehyde, blocked in PBS containing 10%FBS, and then incubated with primary antibodies. 40, 6-diamidino-2-phenylindole (DAPI) was used to visualize the nucleus. Finally, the samples were covered with mounting medium and observed using the fluorescence microscope.

Transmission Electron Microscopy

Transmission electron microscopy was performed as previously described. Briefly, the kidney sections were dissected into 1 mm^3 pieces and fixed in 3.75% glutaraldehyde. After post-fixing in 1% osmium tetroxide, samples were dehydrated in increasing concentration of alcohol, embedded in epoxy resin and then cut into 100 nm ultrathin sections. Then, sections were stained for 10 min in 2% uranyl acetate followed by lead citrate for 5 min at room temperature. Electron micrographs were obtained and analyzed using a FEI Tecnai T20 transmission electron microscope.

Western Blot Analysis

Western blot analysis was performed as previously described (Yang et al., 2017; Wu et al., 2021). Briefly, proteins from cells and isolated glomeruli were lysed by RIPA buffer containing proteinase inhibitors and phosphatase inhibitors. After adding loading buffer, the samples were boiled at 95°C for 5 min 10 and 15% SDS-PAGE were used to separate the samples, followed by transfer of the protein from the gel to the appropriate membrane. Then, the membrane was blocked with 5% no-fat milk for 1 h, and probed with the indicated antibodies overnight at 4°C. After one night incubation, membranes were rinsed 3 times and probed with the second antibody for another 1 h. Finally, membranes were washed again and visualized using a Chemidoc Imaging System.

Measurement of Oxygen Consumption Rate

Cells were seeded in XF24-well microplates (Seahorse Bioscience, North Billerica, MA, United States) at a density of 2.0×10^4 cells per well. After treatment with high glucose (HG) in the presence or absence of SS31, Oxygen consumption rate (OCR) (pmol/min) was evaluated by treating cells with sequential injection of the following compounds: oligomycin (1 μmol/L), carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP, 1 μmol/L), and antimycin A (1 μmol/L) plus rotenone (1 μmol/L). Data were normalized by protein concentration.

Mitochondrial Morphology Staining

Cells were cultured on confocal dishes and mitochondria were labeled with the fluorescent probe Mito-Tracker Red (250 nM, Molecular Probes, Invitrogen) at 37°C for 5 min. Nucleus were labeled with DAPI. The images of mitochondrial morphology were viewed and captured using a confocal inverted laser microscope (LAM 510 Meta, Zeiss).

Urinary Albumin Analysis

Urinary albumin was measured using a mouse albumin ELISA kit (Bethy Laboratories), Urinary creatinine was determined by the QuantiChrom Creatinine Assay kit (DICT-500, Hayward, CA) according to the manufacturer's instructions. Data were normalized by Urinary creatinine.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism Software. Data are presented as the mean ± SD. Differences between multiple comparisons were performed using one-way ANOVA followed by LSD test. Differences between two groups were analyzed with the Student t test. $p < 0.05$ was considered statistically significant.

RESULTS

Podocyte Injury Is Gradually Aggravated During the Progression of Diabetic Kidney Disease

Podocyte injury plays a vital role in the development of DKD. We employed STZ to induce type 1 diabetes in mice (**Supplementary Figure S1A**). As shown in **Supplementary Figure S1B**, mice from the STZ group developed a persistent increasing microalbuminuria at weeks 12 and 20, but no significant change at week 6. PAS and WT1 staining showed that Glomeruli showed significant hypertrophy and mesangial matrix expansion at weeks 12 and 20 (**Supplementary Figure S1C,D**), along with abnormal nephrin distribution and decreased podocyte number (**Supplementary Figure S1E,F**). In addition, Electron microscopic photographs showed a marked basement membrane thickening and foot process widening at week 6 in mice from the STZ group and the slit diaphragm structures almost disappeared at week 20 (**Supplementary Figure S1G–I**). These results revealed that podocyte injury gradually worsened during the DKD progression.

Mitochondrial Dysfunction in Podocyte in Diabetic Kidney Disease

Mitochondrial dysfunction is one of the major mechanisms involved in podocyte injury and death (Hagiwara et al., 2006; Carney, 2015).

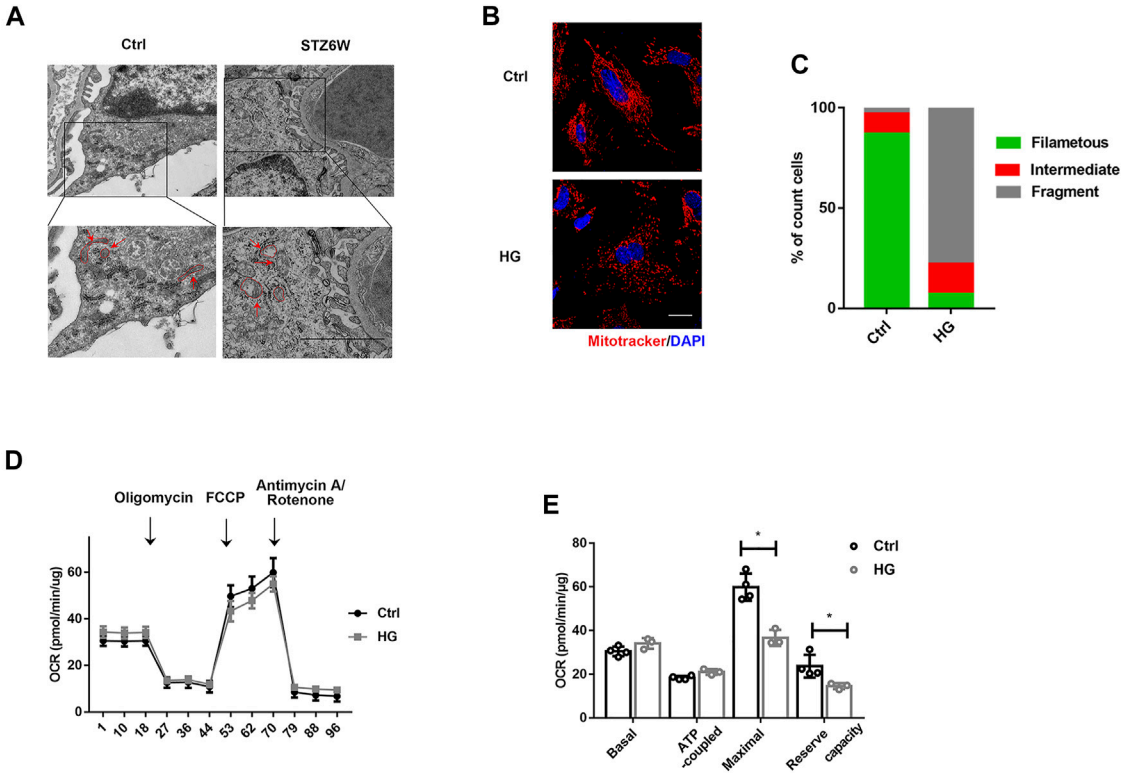

FIGURE 1 | HG induces mitochondrial injury in podocytes *in vivo* and *in vitro*. **(A)** Mitochondrial morphology of podocytes in control and STZ treated mice. The red arrows indicate representative mitochondrial morphology in the groups. Electron microscopic photographs showed that mitochondria of normal podocytes were full and long, and the structure of mitochondrial cristae was clear, while mitochondrial cristae were decreased and vacuolated in podocyte from diabetic mice at weeks 6. (Bar = 2.5 μm). **(B)** Confocal images showing mitochondria. Cells in 6-well plates were challenged with HG (30 mM) for 24H and labeled with MitoTracker Red (red) and DAPI (blue). Mitochondria images were captured using a confocal microscope. As shown in the images, HG treatment shifted the mitochondria from threadiness into fragmentation. (Bar = 2.5 μm). **(C)** The histogram showing the percentage of different mitochondrial morphologies in mice with indicated genotypes. (50 fields were randomly selected for statistics). **(D)** Representative traces showing OCR in control and HG-treated podocytes. Cells in a XF24-well microplate were treated with or without HG (30 mM) for 24 h. **(E)** Statistical analyses of baseline respiratory capacity, ATP-coupled respiratory capacity, maximum respiratory capacity, and reserve respiratory capacity. n = 4,*$p < 0.05$: for maximal, $p = 0.012$(Ctrl vs HG); for reserve capacity, $p = 0.03$(Ctrl vs HG).

Electron microscopic photographs showed that mitochondria of normal podocytes were full and long, and the structure of mitochondrial cristae was clear as indicated by the red arrow in the control group, while mitochondrial cristae were decreased and vacuolated in podocyte from diabetic mice at weeks 6 pointed out by the red arrow in the STZ group (**Figure 1A**). To further determine the changes of mitochondria in high glucose (HG) condition, we observed mitochondrial morphology and function in podocytes treated with HG for 24 h *in vitro*. Compared with control group, HG treatment shifted the mitochondria from threadiness into fragmentation (**Figures 1B,C**). Meanwhile, the mitochondrial maximal respiratory capacity and reserved respiratory capacity were both decreased under HG stimulation (**Figures 1D,E**). Those results further confirmed the mitochondrial dysfunction in the early stage of DKD.

SS31 Halts the Development of Diabetic Kidney Disease

We next administered SS31 to diabetic mice. After successful induction of diabetes at week 2, mice were treated with SS31. As shown in **Figure 2A**, SS31 significantly reduced albuminuria at week 12. Western blot analysis of glomeruli showed that SS31 rescued Nephrin downregulation in diabetic mice (**Figures 2B,C**). Meanwhile, PAS staining revealed that SS31 attenuated mesangial matrix expansion (**Figures 2D,E**). Furthermore, SS31 effectively improved diabetes-induced the loss of podocyte (**Figures 2F,G**) and foot process fusion (**Figures 2H–J**).

We further determine the effect of SS31 on HG-treated podocyte *in vitro*. Western blot analysis showed that SS31 inhibited HG-induced down-regulation of SYNPO and Nephrin (**Figures 2K,L**) as well as up-regulation of cleaved-caspase3 (**Figures 2M,N**), indicating that SS31 also ameliorates HG-induced podocyte injury *in vitro*.

SS31 Improves Mitochondrial Function of Podocyte

Hence, we further examined the effect of SS31 on mitochondrial structure and function *in vivo* and *in vitro*. As shown in **Figure 3A**, SS31 increased mitochondria number and preserved mitochondrial cristae sharp in podocytes from

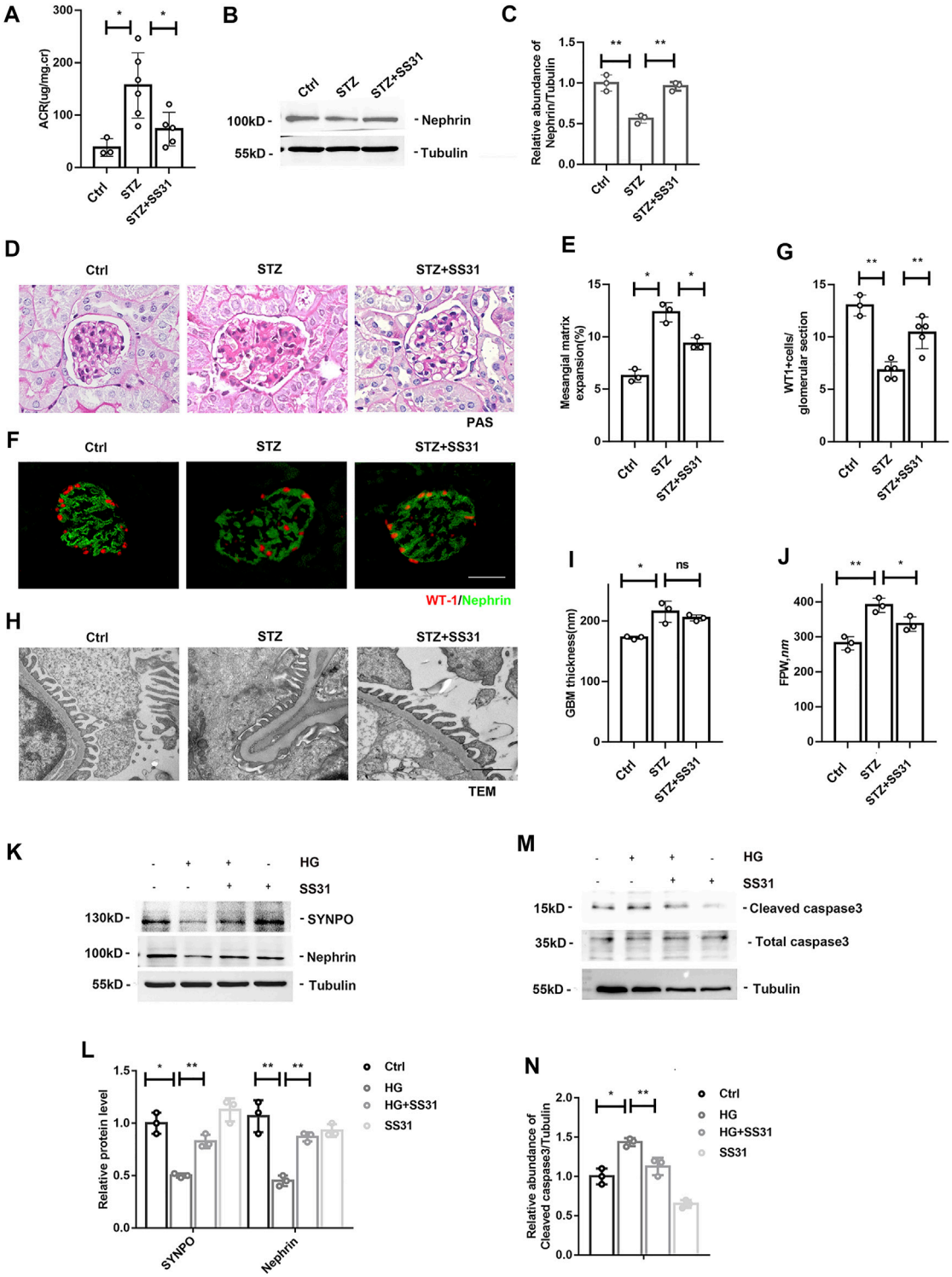

FIGURE 2 | SS31 inhibits HG-induced podocyte injury. **(A)** Urinary albumin-creatine ratio of indicated groups. n = 3–6, *$p < 0.05$: $p = 0.01$(Ctrl vs STZ); $p = 0.02$(STZ vs STZ + SS31). **(B)** Western blot analysis showing protein expression of Nephrin in glomeruli from indicated groups at week 12 after successful induction of diabetes. **(C)** Semi-quantitative densitometry analysis for Nephrin expression. n = 3, **$p < 0.01$: $p = 0.004$(Ctrl vs STZ); $p = 0.002$(STZ vs STZ + SS31). **(D)** Renal histology of glomeruli by PAS staining in indicated groups. (Bar = 25 μm). **(E)** The histogram representing statistical analysis of sclerosed glomeruli in indicated groups. n = 3, *$p < 0.05$:$p = 0.014$(Ctrl vs STZ); $p = 0.018$(STZ vs STZ + SS31). **(F)** Representative immunofluorescent images showing WT1 and Nephrin in indicated groups. (Bar = 25 μm) **(G)** The histogram representing quantification of WT1. n = 3–5, **$p < 0.01$: $p = 0.007$; $p = 0.002$(STZ vs STZ + SS31). **(H)** Electron microscopic pictures of

(*Continued*)

FIGURE 2 | glomerular area showing podocyte foot processes and glomerular basement membrane. As shown in the TEM images, SS31 effectively improved diabetes-induced GBM thickness and foot process fusion. (Bar = 1 μm) **(I,J)** Histograms represent quantification of basement membrane thickness and foot process width. n = 3, *$p < 0.05$, **$p < 0.01$: for GBM thickness, $p = 0.0138$ (Ctrl vs STZ), $p = 0.38$(STZ vs STZ + SS31); for FPW, $p = 0.026$(Ctrl vs STZ), $p = 0.036$(STZ vs STZ + SS31). **(K)** Western blot analysis showing protein expression of SYNPO and Nephrin in podocytes cultured with high glucose in the presence or absence of SS31. Cells were pre-incubated with SS31 (100 nM) for 30 min and then HG (30 mM) for another 24 h. **(L)** Semi-quantitative densitometry analysis for SYNOP and Nephrin. n = 3, *$p < 0.05$, **$p < 0.01$: for SYNPO, $p = 0.012$(Ctrl vs HG), $p = 0.0011$(HG vs HG + SS31), $p = 0.2$(Ctrl vs SS31); for Nephrin, $p = 0.0013$(Ctrl vs HG), $p = 0.0015$(HG vs HG + SS31), $p = 0.2$(Ctrl vs SS31). **(M)** Western blot analysis showing protein expression of Cleaved caspase3 and Total caspase3 in podocyte cultured with high glucose with or without SS31. **(N)** Semi-quantitative densitometry analysis for cleaved caspase3. n = 3, *$p < 0.05$, **$p < 0.01$: $p = 0.016$(Ctrl vs HG), $p = 0.002$(HG vs HG + SS31), $p = 0.06$(Ctrl vs SS31).

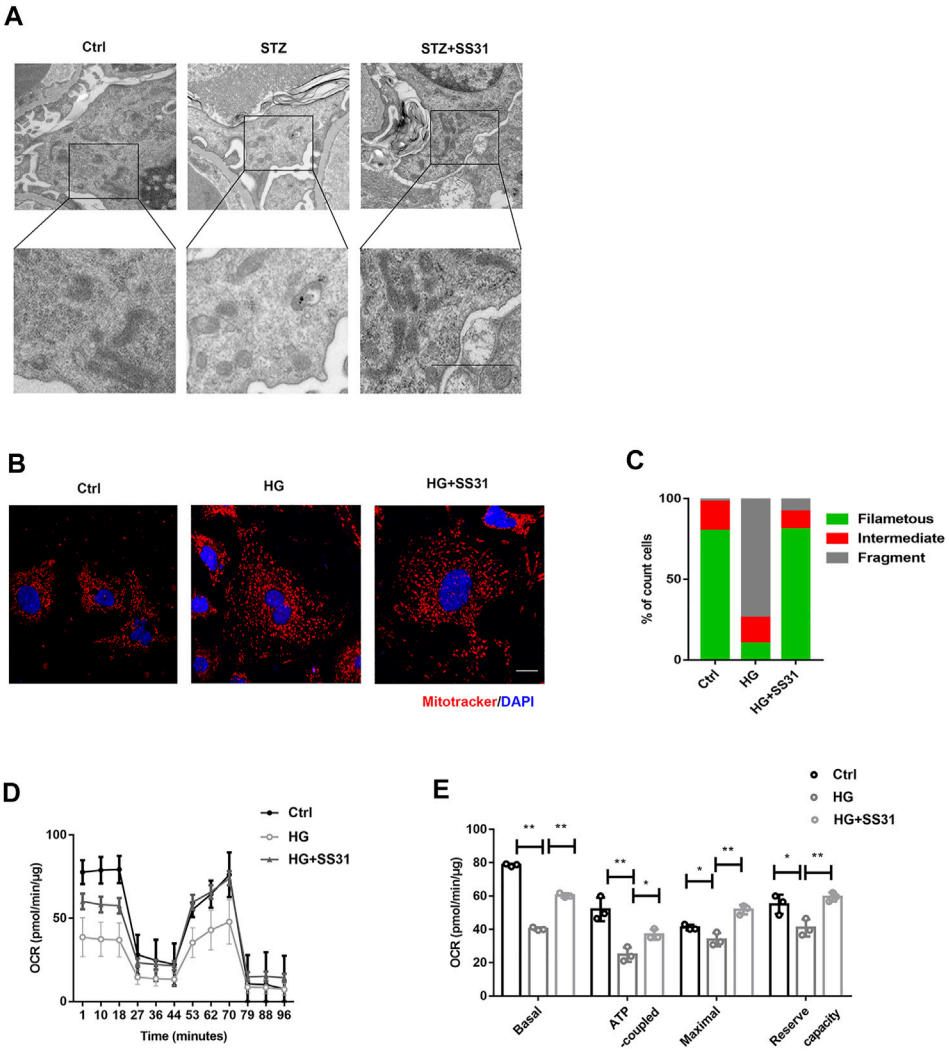

FIGURE 3 | SS31 inhibits HG-induced mitochondrial injury. **(A)** Mitochondrial morphology of podocytes from control and STZ treated mice in the presence or absence of SS31. As shown in the TEM images, SS31 preserved mitochondrial cristae sharp in podocytes from diabetic mice. (Bar = 2.5 μm). **(B)** Confocal images showing mitochondria labeled with MitoTracker Red (red) and DAPI (blue). SS31 markedly ameliorated mitochondrial fragmentation. (Bar = 2.5 μm) **(C)** The histogram showing the percentage of different mitochondrial morphologies in podocytes from the indicated groups. (50 fields were randomly selected for statistics). **(D)** Representative traces showing OCR in indicated podocytes. **(E)** Statistical analyses of baseline respiratory capacity, ATP-coupled respiratory capacity, maximum respiratory capacity, and reserve respiratory capacity. n = 4. *$p < 0.05$, **$p < 0.01$: for basal, $p < 0.001$(Ctrl vs HG), $p = 0.003$(HG vs HG + SS31), for ATP coupled, $p = 0.0048$(Ctrl vs HG), $p = 0.0172$(HG vs HG + SS31), for maximal, $p = 0.044$(Ctrl vs HG), $p = 0.0048$(HG vs HG + SS31), for reserve capacity, $p = 0.03$(Ctrl vs HG), $p = 0.003$(HG vs HG + SS31).

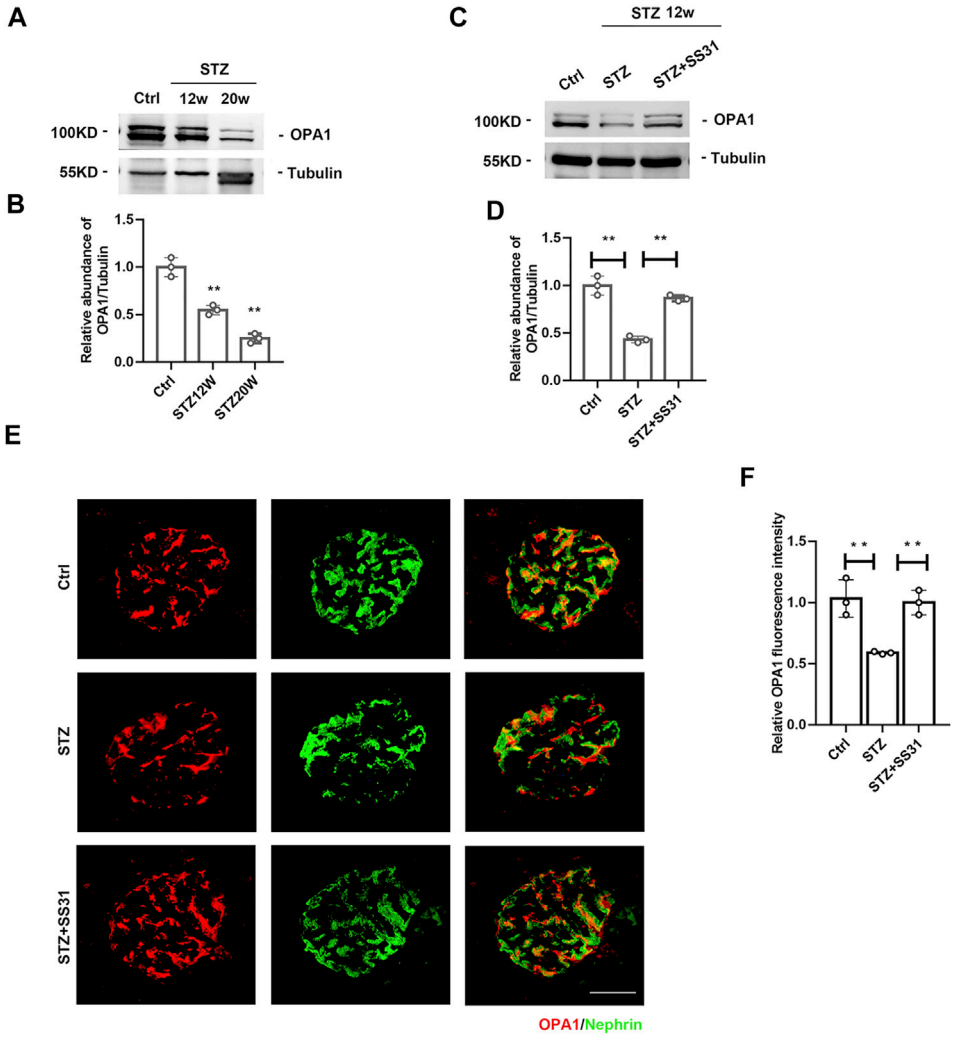

FIGURE 4 | SS31 inhibits STZ-induced downregulation of OPA1 in podocytes. **(A)** Western blot analysis showing glomerular OPA1 expression in mice after STZ injection for 12 and 20 weeks **(B)** Semi-quantitative densitometry analysis for OPA1 expression. n = 3. $^{**}p < 0.01$: $p = 0.004$ (Ctrl vs STZ12w), $p = 0.002$ (Ctrl vs STZ20w). **(C)** Western blot analysis showing glomerular OPA1 expression in diabetic mice with or without SS31 treatment. **(D)** Semi-quantitative densitometry analysis for OPA1 expression. n = 3. $^{**}p < 0.01$: $p = 0.002$ (Ctrl vs STZ), $p = 0.0012$(STZ vs STZ + SS31). **(E)** Representative immunofluorescent images showing OPA1 and Nephrin staining in glomeruli from diabetic mice with or without SS31 treatment (Bar = 10 μm). **(F)** Semi-quantitative densitometry analysis for OPA1 fluorescence intensity. n = 3. $^{**}p < 0.01$: $p = 0.007$ (Ctrl vs STZ), $p = 0.002$(STZ vs STZ + SS31).

diabetic mice. Additionally, SS31 markedly ameliorated mitochondrial fragmentation (**Figure 3B–C**) and OCR decline induced by HG *in vitro* (**Figures 3D,E**). All results indicated that SS31 stabilizes mitochondrial structure and function, thus protecting podocytes against HG-induced injury.

SS31 Inhibits HG-Induced Downregulation of OPA1 in Podocytes

The dynamin-like GTPase OPA1 is a crucial protein that regulates the fusion of the inner mitochondrial membrane and controls mitochondrial cristae morphogenesis. The expression of OPA1 in glomeruli was gradually decreased with the development of DKD (**Figures 4A,B**). SS31 could halt the downregulation of OPA1 in glomeruli from diabetic mice (**Figures 4C,D**). Immunofluorescent staining further confirmed the effect of SS31 on OPA1 in podocyte (**Figure 4E**). In addition, the expression of OPA1 in podocytes was down-regulated under HG condition *in vitro* (**Figures 5A,B**). OPA1 overexpression significantly inhibited HG-induced SYNPO and Nephrin downregulation and podocyte apoptosis (**Figures 5C–H**). SS31 pre-treatment could also restore OPA1 expression in podocyte cultured with HG (**Figures 5I,J**). To further determine the role of OPA1 in podocyte protection of SS31 under HG condition, *Opa1* was silenced by siRNA. SS31 inhibited the HG-induced SYNPO and Nephrin downregulation, but had no effect in *Opa1* knockdown podocyte (**Figures 5K,L**). These data indicate that SS31 protects podocytes by stabilizing OPA1 under HG conditions.

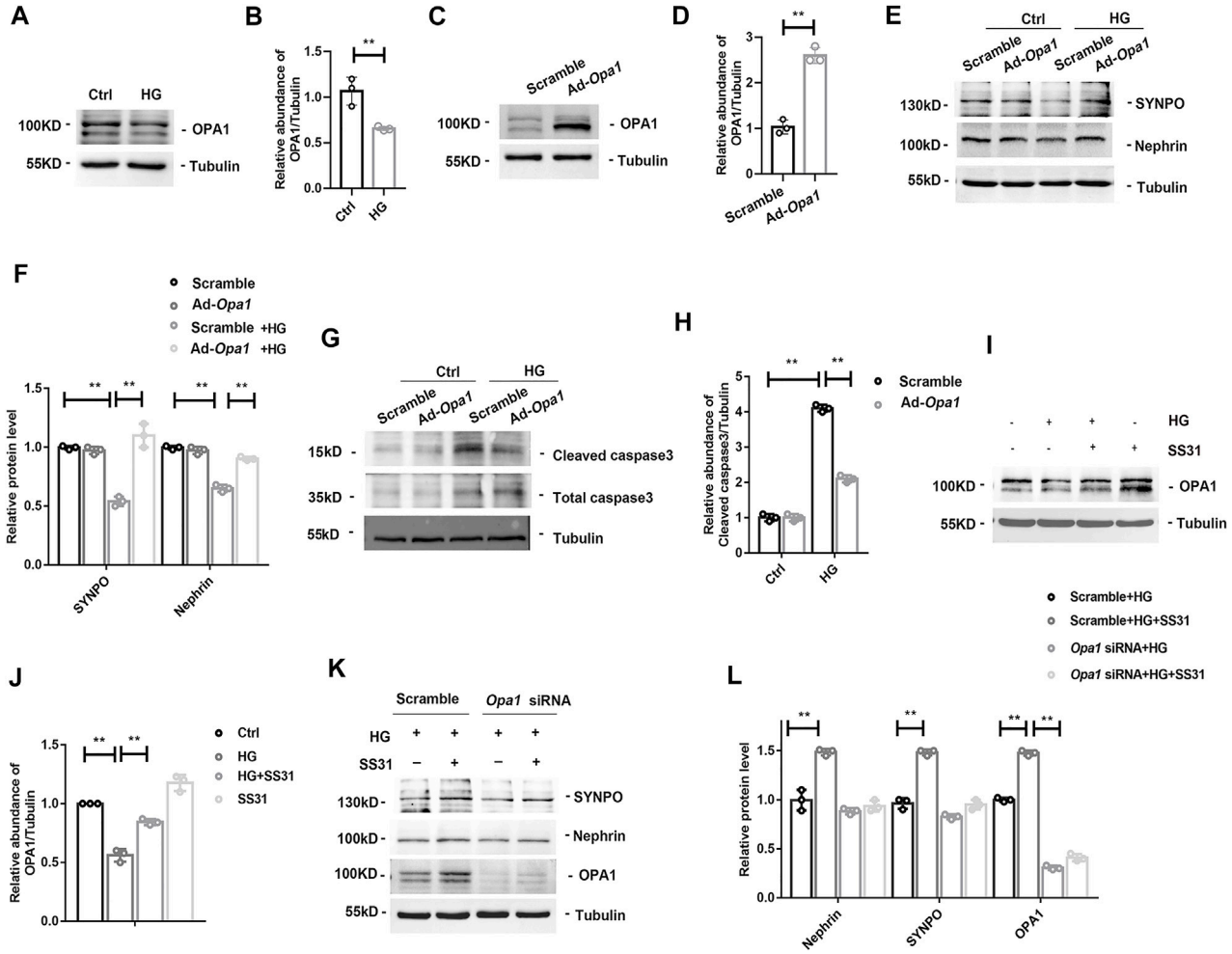

FIGURE 5 | OPA1-dependent protective effect of SS31. **(A,B)** Western blot analysis showing OPA1 expression of podocytes treated with HG (30 mM) for 24H. n = 3. $^{**}p = 0.005$. **(C,D)** Western blot analysis of the proteins of OPA1 in podocyte with Adenovirus-mediated *Opa1* overexpression (Ad-*Opa1*). n = 3. $^{**}p < 0.0018$). **(E,F)** Western blot analysis for SYNPO and Nephrin expression in podocytes with or without overexpression of OPA1. Podocytes were transfected with adenvirus Scramble and Ad-*Opa1* for 24 h, cells were incubated with or without HG (30 mM) for another 24 h n = 3, $^{**}p < 0.01$: for SYNPO: $p = 0.004$(Scramble vs Scramble + HG), $p = 0.003$(Scramble + HG vs Ad-*Opa1*+HG); for Nephrin, $p = 0.004$(Scramble vs Scramble + HG), $p = 0.003$(Scramble + HG vs Ad-*Opa1*+HG). **(G,H)** Western blot analysis showing cleaved caspase3 and total caspase3 expression in HG treated podocyte with or without Ad-*Opa1* transfection. n = 3, $^{**}p < 0.01$: $p < 0.001$(Ctrl vs HG), $p = 0.002$(HG + Scramble vs HG + Ad-*Opa1*). **(I,J)** Western blot analysis showing podocyte OPA1 expression in podocytes cultured with high glucose in the presence or absence of SS31. n = 3, $^{**}p < 0.01$: $p = 0.004$(Ctrl vs HG), $p = 0.006$(HG vs HG + ss31), $p = 0.01$(Ctrl vs SS31). **(K,L)** Western blot analysis for SYNPO, Nephrin and OPA1 expression in SS31 treated-podocytes cultured with HG with or without *Opa1* siRNA transfection. Podocytes were transfected with scramble and *Opa1* siRNA for 24 h. Then, cells were incubated with HG (30 mM) in the presence or absence of SS31 (100 nM) for another 24 h. n = 3. $^{**}p < 0.01$: for Nephrin, $p = 0.004$(Scramble + HG vs Scramble + HG + ss31); for SYNPO, $p = 0.002$(Scramble + HG vs Scramble + HG + ss31); for OPA1, $p = 0.002$(Scramble + HG vs Scramble + HG + ss31), $p < 0.001$(Scramble + HG + ss31 vs *Opa1* siRNA + HG).

SS31 Inhibits the Activation of OMA1 in Podocytes in Diabetic Kidney Disease

OMA1 is a mitochondrial inner membrane zinc metalloprotease and proteolytic cleave OPA1 during stress and apoptosis. We found that glomerular OMA1 was activated in a time-dependent manner in DKD (**Figures 6A,B**), while SS31 significantly inhibited the activation of OMA1 (**Figures 6C,D**). Under high glucose condition, OMA1 was activated in podocyte (**Figures 6E,F**). Furthermore, SS31 pre-treatment could block OMA1 activation (**Figures 6G,H**). We next applied *Oma1* siRNA to knockdown OMA1 expression (**Figures 6I,J**). *Oma1* siRNA transfected podocyte showed an increase in OPA1 expression in HG condition (**Figures 6K,L**). Taken together, these data indicate that SS31 can keep mitochondria fusion by inhibiting OMA1 mediated OPA1 proteolytic cleavage.

DISCUSSION

In this study, we reported that OMA1 activation-mediated hydrolysis of OPA1 participates in HG-induced podocyte

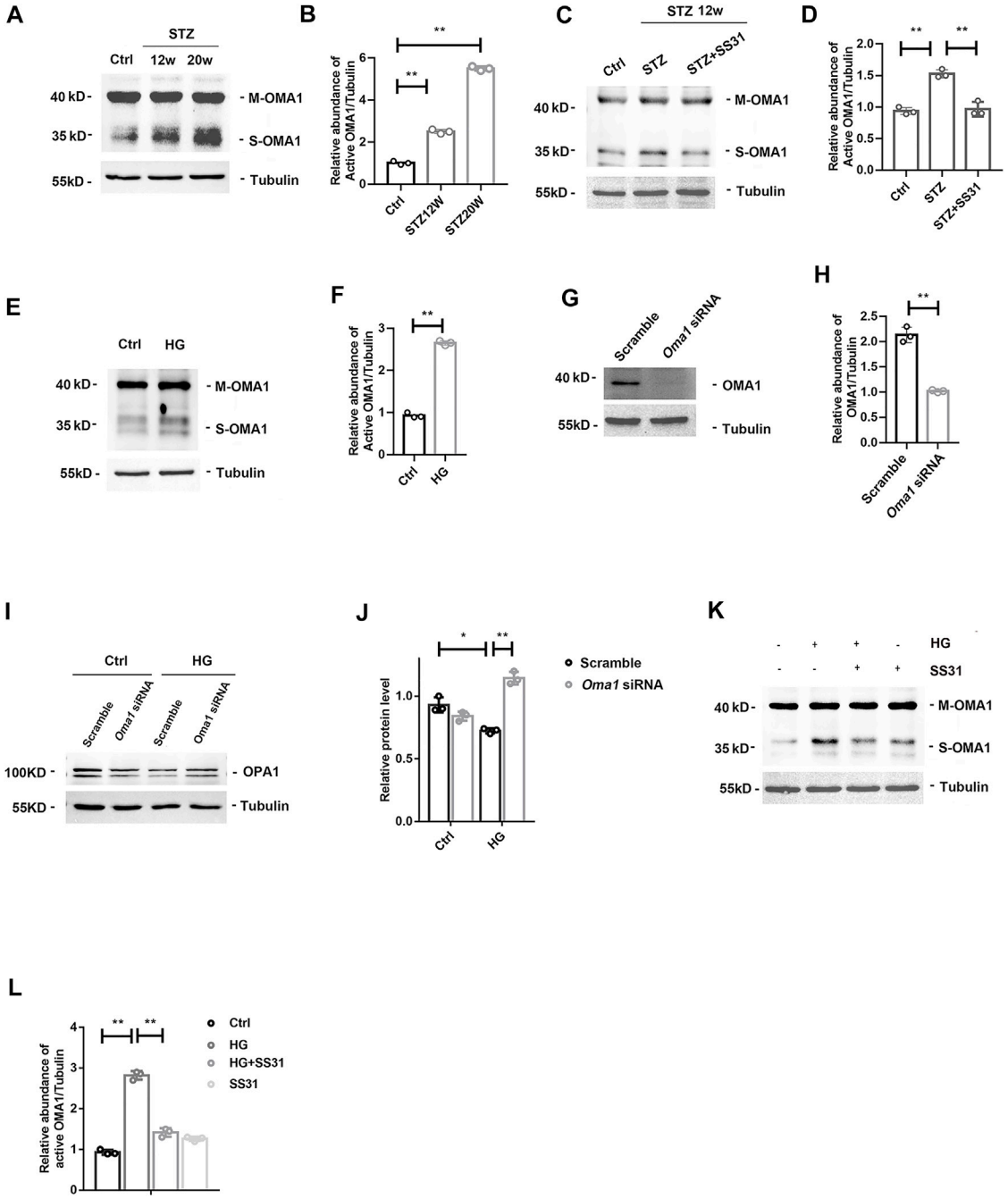

FIGURE 6 | SS31 inhibits HG-induced OMA1 activation to stabilize OPA1 expression. **(A,B)**Western blot analysis showing glomerular mature form of OMA1 (M-OMA1) and short form of OMA1 (S-OMA1) expression in mice after STZ injection for 12 and 20 weeks. n = 3, $^{**}p < 0.01$: $p < 0.001$(Ctrl vs STZ12w), $p < 0.001$(Ctrl vs STZ20w). **(C,D)** Western blot analysis showing glomerular OMA1 expression in diabetic mice with or without SS31 treatment. .n = 3, $^{**}p < 0.01$: $p < 0.001$(Ctrl vs STZ), $p = 0.002$(STZ vs STZ + SS31)**. (E)** Western blot analysis showing mature form of OMA1 (M-OMA1) and short form of OMA1 (S-OMA1) expression of podocytes treated with HG (30 mM) for 24 h. **(F)** Semi-quantitative densitometry analysis for relative S-OMA1 expression. n = 3. $^{**}p = 0.002$. **(G,H)** Immunoblot of OMA1 in podocytes transfected with or without *Oma1*-siRNA. n = 3, $^{**}p = 0.003$). **(I,J)** Immunoblot of OPA1 in HG-treated podocytes transfected with or without *Oma1*-siRNA.n = 3, $^{**}p < 0.01$, $^{*}p < 0.5$: $p = 0.02$ (Scramble + Ctrl vs Scramble + HG), $p = 0.006$ (Scramble + HG vs *Oma1*+HG). **(K)** Western blot analysis showing M-OMA1 and S-OMA1expression of podocytes under HG in the presence or absence of SS31for 24H. **(L)** Semi-quantitative densitometry analysis for relative S-OMA1expression. n = 3. $^{**}p < 0.01$: $p < 0.001$(Ctrl vs HG), $p = 0.003$(HG vs HG + ss31).

mitochondrial injury. SS31 could inhibit the activation of OMA1to stabilize OPA1 expression, and protect podocytes from injury induced by diabetes.

Diabetes is one of the most common diseases affecting more than 350 million people worldwide, and has become an important public health challenge (Thomas et al., 2016). However, the exact molecular pathogenesis of DKD is far from being fully understood. Multiple evidence indicate that podocyte detachment is a vital factor promoting DKD development. In animal renal disease models, more than 20% loss of podocytes results in irreversible glomerular injury, manifesting as albuminuria followed by progression to ESRD (Matsusaka et al., 2005; Wharram et al., 2005). Herein, our study confirms that DKD progression is accompanied by reduced podocyte density, basement membrane thickening and foot processes flattening. As a type of terminally differentiated cells, mature podocytes have a limited capacity to proliferate in adults, susceptible to various injurious factors. Podocyte injury has become one of the major lesions leading to CKD.

Accumulated studies indicated that mitochondria play a critical role in podocyte homeostasis and progression of podocytopathy (Guan et al., 2015; Qi et al., 2017; Szeto et al., 2016; Fujii et al., 2020). Our previous study reported that compared with undifferentiated podocytes, differentiated mature podocytes have increased mitochondrial density and ATP production (Q. Yuan et al., 2020). In the present study, we discovered that mitochondrial abnormalities occur in podocytes in mice with DKD. *In vitro* studies also revealed that HG induces mitochondrial morphology abnormalities and function disorders, suggesting that podocyte mitochondrial injury may be a vital factor in the development and progression of DKD. Podocytes maintain the glomerular filtration barrier by synthesis of GBM components (Pavenstadt et al., 2003), formation of the slit membrane (Saleem et al., 2002), all of which are affected under DKD. As the results showed that diabetes leads to thickening of the GBM and swelling of the foot process. Excitingly, treatment with SS31 for 4 weeks in the initial stage of DKD exhibits significant podocyte protection and inhibits abnormalities in GBM and foot process. The promising long-term renoprotective effect of SS31 encouraged us to further explore the underlying mechanism.

Mitochondria are double-membrane organelles that form a highly dynamic network in the recurrent transformation of fusion and fission. Disruption of mitochondrial dynamics is associated with aging and various human diseases, including neurodegenerative and metabolic diseases and cancers (Bertholet et al., 2016; Chan, 2020). Mitochondrial fusion is regulated by the mitochondrial outer membrane protein MFN and the inner membrane protein OPA1 (Cipolat et al., 2004; Song et al., 2009). In addition to mitochondrial fusion, OPA1 also plays a vital role in regulating mitochondrial functions, including apoptosis and respiratory capacity (Olichon et al., 2006). Our study verified a time-dependent downregulation of OPA1 in podocyte in the progressing of diabetes. Overexpression OPA1 could ameliorate podocyte injury induced by high glucose. Those data indicate OPA1 is a crucial protein for treatment DKD.

The Szeto–Schiller (SS) peptides are cellpermeable tetrapeptides that selectively target mitochondria and concentrate on the IMM instead of penetrate into the mitochondrial matrix. Despite their 3 + net charge, they do not depolarize mitochondria as their mitochondrial uptake is potential independent (Szeto, 2017). Further study provided structural evidence for the interaction of SS31 with mitochondrial cardiolipin (CL) in liposomes, bicelles and mitoplasts. Besides inhibiting cyt c peroxidase activity, researchers have shown that SS-31 can also improve electron transfer through the cyt c/CL complex and promote mitochondrial ATP synthesis (Birk et al., 2014). Following intravenous injection of 1 mg/kg, plasma concentration of SS-31 declined rapidly with an apparent terminal half-life of about 0.8 h. SS-31 is rapidly absorbed after administration, with peak plasma levels detected within 15 min. Bioavailability of SS-31 after subcutaneous administration was higher in the dog (72.7%) and monkey (81.4%) compared to rat (38%) (Szeto and Schiller, 2011). SS31 has a wide range of renal protective effects, including podocytes. It has been reported that SS31 is capable of restoring C3a-induced podocyte motility (Morigi et al., 2020), preserves podocyte number and foot process in mice fed a high-fat diet (Szeto et al., 2016), and inhibits mitochondrial oxidative injury and Cyt c release and elimination of mtROS, thus preventing the activation of podocyte apoptosis pathways in diabetic rats (Wang et al., 2019)and improving podocyte cytoskeletal integrity in mice of advanced age (Sweetwyne et al., 2017). Consisting with previous studies, SS31 has benefit on podocyte in mice with diabetes. The mitochondrial function also is improved by SS31 treatment by restore OPA1 expression.

OMA1 is a distinct metal endopeptidase with little protease activity under physiological conditions but activated upon mitochondrial stress. In response to stress, the C-terminal self-cleavage of OMA1 can promote its activity (Zhang et al., 2014). The substrates of OMA1 include DELE1 and OPA1 (Alavi, 2021). OMA1 controls the dynamic balance of mitochondrial fission and fusion by regulating the hydrolysis of OPA1 (MacVicar and Langer, 2016). OMA1 activity is enhanced by a variety of stresses including accumulation of unfolded polypeptides and dissipation of the membrane potential as well as ROS (Richter et al., 2015). In this study, HG can activate OMA1 which further hydrolyzes OPA1, meanwhile SS31 can protecting podocytes by inhibiting the activation of OMA1 to stabilize OPA1 expression. In the study, we did not exclude the effect of osmotic pressure *in vitro*. However, as shown in **Supplementary Figure S2**, mannitol had no significant effect on Nephrin, OPA1 and OMA1 protein expression in cultured podocytes, while high glucose significantly changed the expression of these molecules, suggesting that the changes *in vitro* were specifically caused by high glucose concentration rather than high osmolality.

In summary, OPA1 plays an important role in podocyte mitochondrial morphology and function, we confirmed that SS31 protects podocytes in DKD via inhibiting OMA1-

mediated hydrolysis of OPA1. Our study provides a new insight into the mechanism of SS31 in protecting podocyte mitochondria under diabetes, indicating that SS31 might be a promising agent in delaying the progression of DKD.

Study Limitation

To better mimic the situation of cells *in vivo*, primary podocytes were performed in our *in vitro* experiments. We utilized filters with different pore sizes to isolate the glomeruli from kidney and acquire primary podocytes. Theoretically, we can completely separate other cells such as macrophages from glomeruli using filters with different pore sizes according to different cell diameters. However, a small number of cells may inevitably aggregate and remain in our extracted cells, resulting in the purity of podocytes that cannot reach 100%, and we could not exclude the effect of the remaining small fraction of cells on the experiment. We tested the purity of our cells above 90% by flow cytometry. Although magnetic beads or flow sorting can further improve the purity of cells, they are costly and the current technology cannot still reach 100% purity. We chosed the affordable approach. The effect of the minimal other cells on experiments was hard to exclude. Maybe more advanced and affordable techniques are needed to improve the purity of primary cells in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University.

AUTHOR CONTRIBUTIONS

JY, LJ, and QS designed the study. QY, WX performed the research. XW, JL analyzed the pathology. QY, JY, LJ, and QS analyzed the data. YZ, HC participated in intellectual discussions. QY and LJ wrote the paper. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.707006/full#supplementary-material

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REVIEW
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Inflammation and Cardiovascular Disease Associated With Hemodialysis for End-Stage Renal Disease

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Chronic kidney disease (CKD) and cardiac insufficiency often co-exist, particularly in uremic patients on hemodialysis (HD). The occurrence of abnormal renal function in patients with cardiac insufficiency is often indicative of a poor prognosis. It has long been established that in patients with cardiac insufficiency, poorer renal function tends to indicate poorer cardiac mechanics, including left atrial reserve strain, left ventricular longitudinal strain, and right ventricular free wall strain (Unger et al., Eur J Heart Fail, 2016, 18(1), 103–12). Similarly, patients with chronic kidney disease, particularly uremic patients on HD, often have cardiovascular complications in addition to abnormal endothelial function with volume overload, persistent inflammatory states, calcium overload, and imbalances in redox responses. Cardiac insufficiency due to uremia is therefore mainly due to multifaceted non-specific pathological changes rather than pure renal insufficiency. Several studies have shown that the risk of adverse cardiovascular events is greatly increased and persistent in all patients treated with HD, especially in those who have just started HD treatment. Inflammation, as an important intersection between CKD and cardiovascular disease, is involved in the development of cardiovascular complications in patients with CKD and is indicative of prognosis (Chan et al., Eur Heart J, 2021, 42(13), 1244–1253). Therefore, only by understanding the mechanisms underlying the sequential development of inflammation in CKD patients and breaking the vicious circle between inflammation-mediated renal and cardiac insufficiency is it possible to improve the prognosis of patients with end-stage renal disease (ESRD). This review highlights the mechanisms of inflammation and the oxidative stress that co-exists with inflammation in uremic patients on dialysis, as well as the mechanisms of cardiovascular complications in the inflammatory state, and provides clinical recommendations for the anti-inflammatory treatment of cardiovascular complications in such patients.

Keywords: inflammation, hemodialysis, cardiovascular disease, chronic kidney disease, oxidative stress, immune response, complement activation pathway

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INTRODUCTION

Chronic kidney disease progressing to the uremic stage requires maintenance dialysis therapy to manage complications, prolong patient survival and improve quality of life. Although dialysis treatment is necessary for such patients, the serious cardiovascular complications resulting from hemodialysis (HD) cannot be ignored. HD superimposes additional cardiovascular risks under the cardiac burden of fluid overload due to pre-existing renal disease. The Kidney Disease: Improving Global Outcomes (KDIGO) conference discussed and made clinical recommendations for volume control (Flythe et al., 2020), blood pressure management (Cheung et al., 2021), and pathophysiological changes occurring in the vasculature of CKD patients in the CKD setting (Johansen et al., 2021), and emphasized the necessity of timely hemodialysis for such patients. The guidelines also state that volume overload in patients with CKD leads to cardiac overload in these patients, making them more susceptible to cardiovascular disease, and therefore blood pressure and volume status are important modifiers of clinical outcomes in patients with CKD (Flythe et al., 2015; Zoccali et al., 2017; Assimon et al., 2018). Meanwhile the CKD environment accelerates the progression of central and peripheral arterial disease, especially the onset and progression of atherosclerosis (Johansen et al., 2021).In HD conditions, the microinflammatory state underlying chronic kidney disease can be exacerbated by abnormalities in the immune system, complement activation triggered by blood contact with the dialysis membrane, accumulation of urotoxins and endotoxin translocation, accumulation of inflammatory factors due to decreased renal filtration capacity and local injury due to arteriovenous fistula formation. Inflammation acts as a catalyst for the development of cardiac insufficiency and contributes significantly to the development of cardiovascular complications in these patients. After ischemic myocardium is reperfused by coronary artery, although there is no myocardial necrosis, but the systolic and diastolic depression persists for more than 1 week, and then gradually improves is myocardium stunned (Braunwald and Kloner, 1982; Kloner, 2020). The release of inflammatory mediators directly leads to hemodynamic overload, increases ventricular wall pressure, induces the onset of myocardium stunned, and long-term involvement leads to irreversible myocardial damage Zuidema and Dellsperger, 2012. Repeated circulatory stress leads to myocardium stunned, and the repeated myocardial injury caused by myocardium stunned leads to irreversible changes in the structure and function of the left heart. Also, vascular calcification progresses rapidly in dialysis patients, and vascular calcification, myocardial fibrosis, and reduced left ventricular compliance may make HD patients more susceptible to hypotension and acute pulmonary edema. Impaired autoregulation and the deposition of abnormal substances such as epicardial adipose tissue (EAT) may cause irreversible damage to the myocardium. This paper provides insight into the inflammatory mechanisms underlying the development of cardiac insufficiency in patients with advanced kidney disease on dialysis and provides more systematic theoretical support for clinical interventions for inflammation in these patients.

ABNORMALITIES OF BIOMARKERS IN DIALYSIS PATIENTS

Troponin

Increased troponin levels in CKD are attributed to a variety of mechanisms, including increased transmural pressure, small vessel coronary artery obstruction, endothelial dysfunction, intracellular edema, and the direct cytotoxicity of uremia (Arcari et al., 2021). Troponin levels are chronically elevated in patients with renal insufficiency in the absence of myocardial infarction (deFilippi et al., 2012). The pathological mechanisms by which this occurs are inconclusive, but the mechanism of occurrence must be multifactorial and include the development of CKD complications such as hypertension, left ventricular hypertrophy, heart failure, and coronary artery disease (Raber et al., 2021a). Dialysis patients are often monitored for elevated troponin during the dialysis process. This is due to the rapid exchange of fluid through the dialysis membrane, which reduces coronary perfusion and thus induces obstruction or microvascular damage. Troponin is metabolized by the kidneys, which may further elevate the peak troponin levels seen in dialysis-induced coronary artery disease (Chan et al., 2021). This elevated troponin level cannot be explained by ischaemic myocardial necrosis, even if the patient has concomitant coronary artery disease.

Dialysis-induced myocardium stunned also results in elevated troponin, but it is unclear whether the elevated troponin originates from cardiomyocytes with reversible or irreversible damage. What we can determine at this time is that myocardium stunned causes troponin elevation that is not entirely consistent with myocardial ischemic necrosis and that it may be associated with the onset of partial apoptosis.

The highly sensitive cardiac troponin (hs-cTn) assay can detect small troponin concentrations and address small changes in biomarker concentrations (Miller-Hodges et al., 2018; Raber et al., 2021b). However, since the specificity of hs-Tn for monitoring myocardial infarction is reduced in patients with CKD (Twerenbold et al., 2018), we propose to confirm the diagnosis of myocardial infarction by continuous monitoring of troponin and comparing the absolute changes in troponin. This can be interpreted as using the degree of change in troponin as a diagnostic indicator, i.e., it is considered more diagnostic when patients with CKD have a >280% increase in high-sensitivity troponin I (hs-cTnI) or a >250% increase in high-sensitivity troponin T (hs-cTnT), but this also reduces the sensitivity of the diagnosis (Kraus et al., 2018). hs-cTnI and N-terminal precursor B-type brain natriuretic peptide (NT-proBNP) were found to be associated with myocardial fibrosis and myocardial edema by Arcari et al. by comparing cardiac MRI and markers of myocardial injury in patients with different renal functions, and with deterioration. As renal function progressively declined, the serological biomarkers hs-cTnT and NT-pro

BNP and imaging markers of structural remodeling correlated more closely with nature T1 (myocardial fibrosis) and T2 (myocardial edema) (Arcari et al., 2021).

BNPs

hs-cTnI and elevated NT-proBNP levels are associated with myocardial remodeling and its prognosis in patients with chronic kidney disease. Increased markers of myocardial injury in chronic kidney disease may be associated with persistent, non-ischaemic, subclinical myocardial injury (Arcari et al., 2021). Patients with CKD with complications of cardiac insufficiency usually present with preserved left ventricular systolic function and reduced ventricular diastolic function (Ponikowski et al., 20162016; Unger et al., 2016; Arcari et al., 2020).

B-type natriuretic peptide (BNP) and NT-proBNP are elevated due to reduced renal clearance, fluid retention, and abnormal left ventricular function. In particular, NT-proBNP is filtered through the kidneys and regulated by dialysis resulting in large fluctuations in patients with advanced kidney disease, making it impossible to define a threshold value to assess the patient's cardiac function (Chan et al., 2021).

sST2

soluble suppression of tumorigenicity (sST2) has received much attention in recent years as a new biomarker for risk stratification in acute and chronic heart failure, for therapeutic assessment, and for predicting patient prognosis (Gaggin and Januzzi, 2013; Januzzi et al., 2015; Dalal et al., 2018; Mirna et al., 2020). A 2018 clinical study assessing the relationship between sST2 and renal function in 842 patients with CKD noted that sST2 was associated with progressive renal function, with higher sST2 suggesting lower estimated glomerular filtration rate (eGFR) (Alam et al., 2019). Subsequently, Mirna et al. [21]studied five new biomarkers in 219 patients with CKD and found that all investigated biomarkers were significantly elevated in patients with CKD, inversely related to eGFR, except for sST2. sST2, as one of the biomarkers with the least impact on changes in renal function, could act independently of renal function. Therefore, sST2 is of great importance for clinical practice in CVD patients with combined CKD.

suPAR

The urokinase plasminogen activator receptor (uPAR) is a binding receptor expressed on the surface of immune cells (mainly neutrophils, activated T cells, and macrophages). Stimulation by inflammation causes cleavage of the uPAR on the cell surface, followed by the production of soluble uPAR, Soluble urokinase plasminogen activator receptor (suPAR) (Huai et al., 2006; Allison, 2016). Thus, elevated suPAR reflects inflammation and immune system activation and is an emerging marker of inflammation (Huttunen et al., 2011).

In 2010, Eugen-Olsen et al. evaluated the correlation between suPAR levels and the risk of cancer and CVD in 2602 general population and suggested that suPAR could be an independent risk factor for predicting the incidence of CVD and all-cause mortality (Eugen-Olsen et al., 2010). In 2014 Borné et al. followed 4,530 general population for a median time of 16.3 years and found that suPAR was associated with elevated NT-proBNP plasma levels and incidence of HF (Borné et al., 2014). Subsequently, Hodges et al. demonstrated that suPAR can outperform traditional inflammatory markers, such as C-reactive protein (CRP), as a biomarker for cardiovascular disease in the prediction of the risk of CVD development (Hodges et al., 2015).

In the kidney, suPAR can induce podocyte dysfunction and impair glomerular filtration function thus leading to the development of CKD (Hayek et al., 2015; Lv et al., 2020), a phenomenon mostly found in focal segmental glomerulosclerosis studies (Wei et al., 2011). 2019 a meta-analysis of the relationship between suPAR and kidney disease pointed out that suPAR was negatively correlated with eGFR, and in patients with CKD, especially ESRD patients suPAR levels were significantly higher than normal (Shuai et al., 2019). Several subsequent studies suggested that close monitoring of suPAR could help in the early diagnosis and treatment of CKD and that suPAR levels were associated with CKD prognosis (Rotbain Curovic et al., 2019; Shuai et al., 2019). 2021 Jhee et al. evaluated the relationship between disease progression and suPAR in 751 CKD patients and demonstrated that suPAR levels were also independently associated with CKD progression (Jhee et al., 2021).

INFLAMMATION

In its normal state, inflammation has a protective effect on infected and damaged tissues by dilating blood vessels and recruiting white blood cells and plasma proteins to abnormal tissues. However, when inflammation persists and is poorly controlled, it can lead to a range of complications. The pathophysiology of chronic inflammation in CKD is not fully understood, but the prognosis of such patients is closely related to their inflammation *in vivo*. The development of inflammation in ESRD patients is multifactorially induced, including 1) exposure of blood to exogenous substances such as dialysis membranes during HD that stimulate inflammation and activate the complement pathway; 2) immune dysfunction, including senescence and apoptosis of immune cells; 3) accumulation of urotoxins *in vivo*, secondary to intestinal dysregulation; and 4) unbalanced oxidative stress (Sun et al., 2016), all of which are discussed later. In addition, increased circulatory preload due to reduced renal function and the presence of metabolic acidosis can lead to increased production of pro-inflammatory factors. Therefore, many inflammatory factors (CRP, Interleukins, etc.) predict the prognosis of ESRD patients by effectively assessing the degree of inflammation in such patients.

Coexistence of Immunity With Inflammation and Ongoing Immune Stimulation

The persistence of immune abnormalities in ESRD patients is a dominant factor in the development and progression of inflammation and mediates inflammation in conjunction with reduced renal clearance due to deteriorating renal function

(Stenvinkel et al., 2005). Among these, neutrophil- and monocyte-mediated innate immune responses and lymphocyte-mediated adaptive immune responses dominate the immune abnormalities in ESRD patients. In addition to this, patients on long-term maintenance dialysis (MHD) are also characterized by normal immunoglobulin levels, disrupted antigen-presenting cells (APCs), and upregulation of phagocyte supramastigote receptors (SRs) (Kato et al., 2008).

Innate Immune Response: Neutrophils and Monocytes

Neutrophils and monocytes, as the most important cells in the immune response to hemodialysis-mediated urotoxicosis and long-term contact with biologically incompatible membranes, coordinate the immune response through the production of cytokines and chemokines by recognizing pathogens or damaged tissues together with dendritic cells and natural killer (NK) cell (Losappio et al., 2020). Therefore, changes in the number of neutrophils and NK cells and changes in phagocytic activity in HD patients may indicate abnormalities in the body's immune system, and such abnormalities are related to the type of biologically incompatible membrane and the dialysis method (Colì et al., 1999; Moore et al., 2001; Pappas et al., 2019). Premature cellular senescence in ESRD patients is caused by persistent DNA damage and epigenetic changes, usually in the form of cellular cessation of proliferation and apoptosis (Kooman et al., 2017; Crépin et al., 2020). This is accompanied by an excessive accumulation of senescent polymorphonuclear neutrophils due to neutrophil dysfunction (Martin et al., 2003). Neutrophils are important cells of the innate immune system and in the early stages of HD treatment, there is a transient decrease in neutrophils due to apoptosis. Anti-myeloperoxidase antibodies (MPO), a type of anti-neutrophil cytoplasmic antibody (ANCA), are a functional marker and activation marker of neutrophils, and changes in their levels and activity are representative of the functional and active status of neutrophilic polymorphonuclear leukocytes (PMN). The excessive apoptosis of neutrophils due to increased MPO release is thought to be the pathological mechanism underlying the development of microinflammation in HD patients (Fukushi et al., 2020), leading to an increased risk of infection and mortality from infection in patients starting HD. This mechanism also confirms the conclusion that plasma MPO is an independent risk factor for all-cause mortality in HD patients, as suggested by two clinical studies in recent years (Nakayama et al., 2018; Kim et al., 2020). Early studies attributed the decrease in neutrophils to their accumulation in the capillaries of the lungs. Recent studies have suggested that the contact of blood with the dialysis membrane leads to the recruitment and activation of neutrophils and monocytes, and that activated neutrophils attach to the endothelial wall of the lung capillaries, the first vascular surface they come into contact with after leaving the dialyzer, resulting in a transient decrease in neutrophils (Hoenich et al., 1986). Transient leukopenia is caused by the activation of the alternative pathway (AP) and the lectin pathway (LP) of complement after the blood comes into contact with the dialysis membrane (Yoon et al., 2007). Further immune dysfunction will result in the release of pro-inflammatory cytokines [e.g., interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor-alpha (TNF-α), monocyte chelator protein-1 (MCP-1), and gamma interferon (Hempel et al., 2017)] from activated neutrophils and monocytes, along with activation of the complement system (dell'Oglio et al., 2017). Activation of the complement system leads to increased expression of adhesion molecules [i.e., CD11b/CD18, also known as complement receptor 3 on leukocytes (CR3)]*in vivo*, which in turn binds to C3b on the dialysis membrane and further leads to neutropenia. The adhesion factors themselves can also induce leukocyte extravasation. CR3 can interact with platelets to cause thrombosis and release factors that stimulate thrombosis (e.g., Von Willebrand factor) (Losappio et al., 2020).

Adaptive Immune Response: Lymphocytes

Patients with ESRD have an abnormal body environment resulting in a chronic inflammatory state characterized by increased production of pro-inflammatory cytokines by T cells, high levels of circulating follicular helper T cells (T_{FH}), and abnormal maturation of plasma cells and T helper (Th) lymphocytes (Losappio et al., 2020). High levels of cytokines are present in ESRD patients due to abnormal renal filtration and a persistent inflammatory state. Notably, IL-18 leads to the development of T cell-mediated adaptive immune changes by inducing the onset of Th1-mediated immune responses and activating Th2 immune responses through the production of IL-4 and IL-13 (Mühl et al., 1996). At the same time in ESKD patients, prolonged inflammatory signaling alters T-cell function and leads to T-cell failure (Wherry and Kurachi, 2015). In a sustained state of inflammation, the abnormal T-cell function changes are irreversible and eventually lead to cell death. A significant reduction in lymphocytes has been identified in several clinical studies of CKD, and the remaining T lymphocytes in such patients may exhibit a more sustained and active pro-inflammatory state (Hartzell et al., 2020). As a subset of $CD4^+$ T cells, T_{FH} helps B cells to produce high-affinity antibodies against pathogens that are potentially pathogenic in chronic inflammatory states (e.g., atherosclerosis, lymphoid tumors, autoimmune diseases) (Crotty, 2019; Hartzell et al., 2020). In addition, the production of IL-4 by T_{FH} may also contribute to the differentiation of macrophages, which play an important pro-inflammatory role in ESRD patients, towards a subset of macrophages that are more capable of producing cytokines and chemokines (Guiteras et al., 2016).

During HD treatment, the body's immune response is weakened due to a significant reduction in levels of B-cell activating factor (BAFF) and IL-17 receptors, followed by a significant reduction in B-lymphocytes. At the same time, the expression of the B-cell lymphoma-2 gene (Bcl-2, an oncogene that significantly inhibits apoptosis) decreases B lymphocytes that are more susceptible to apoptosis. In addition, CD40, a functionally relevant surface antigen for T and B lymphocytes, is essential for B cell growth and primarily promotes the proliferation of immature B cells (Ferraccioli and Gremese, 2017). In patients with CKD, particularly those receiving HD, serum levels of CD40 are elevated (Esposito et al., 2012). Clinical interventions for CD40, such as filtration of CD40 by dialysis

TABLE 1 | Complement is activated by three pathways: the alternative pathway (AP), the lectin pathway (LP), and the classical pathway (CP). Complement activation can be induced by adsorption of complement components to hemodialysis membranes, with AP and LP being the major activation pathways.AP activity is increased by spontaneous C3 hydrolysis that continuously applies minor stimuli to AP.C3b generated by C3 hydrolysis also enhances CP and LP.Under dialysis conditions, covalent binding of C3b to nucleophilic surfaces expressed on dialyzer membranes and adsorption of CHF by dialysis membranes promote activation of the complement substitution pathway, and complement factor B acts as an intermediate mediator involved in the continuous occurrence of complement activation (Flythe et al., 2015). LP is induced by the binding of MBL or Ficolin to carbohydrates and is activated by the adsorption of large amounts of Ficolin-2 and MBL on dialysis membranes during HD, and a significant decrease in Ficolin-2 levels can be found in these patients. CP is induced by the binding of C1q to molecules such as immune complexes or CRP and is activated in HD patients mainly by C1q binding to In HD patients, it is mainly activated by C1q binding to circulating IgG.

Complement activation pathway	Mechanism of occurrence under physiological conditions	Mechanism of complement activation occurring in hemodialysis	Mediating the development of cardiovascular disease
AP	It continuously stimulates complement activation through spontaneous C3 hydrolysis and enhances CP and LP through C3b production	Reduced expression of complement inhibitors leads to AP dysregulation: polysulfone dialyzers can absorb [c]CHF (an important inhibitor of C3 convertase and C3b) and clusterin (blocks activation of the terminal pathway) (Unger et al., 2016)	The Y402H genotype in CFH increases the risk of cardiovascular disease in HD patients (Chan et al., 2021)
LP	[a]MBL or Ficolins recognize carbohydrate-induced	[b]Ficolins-2 initiates complement cascade reactions (including C5a production) and dialysis-induced leukopenia by adsorption to polysulfone dialyzers and leads to substantial depletion of Ficolins2. MBL also activates complement reactions by contact with dialyzers (Unger et al., 2016; Flythe et al., 2020)	MBL is involved in the consumption of atherogenic particles and is beneficial for atherosclerosis in uremic patients. Thus a decrease in MBL (adsorbed to the dialyzer) in HD patients is associated with an increased risk of cardiovascular disease (Cheung et al., 2021) C5a is involved in thrombosis. activation of the LP pathway is significantly associated with increased production of C5a, especially during the first hour of HD onset (Johansen et al., 2021)
CP	Induced by C1q binding to immune complexes or other molecules (e.g., CRP)	C1q binds to immunoglobulin IgG adsorbed by the membrane dialyzer to activate the complement response	C1q, the largest molecular weight gamma globulin among complement components, can promote the release of inflammatory mediators from eosinophils and mast cells under HD conditions, leading to vascular endothelial damage and subsequently atherosclerosis (Zoccali et al., 2017)

[a]*MBL, mannose-binding lectin;*
[b]*Ficolins-2: specific pathogen recognition receptor for LP that acts similarly to MBL;*
[c]*CHF, complement factor H, inhibits C3 convertase and C3b activity while acting negatively on alternative pathways.*

membranes may therefore provide additional benefit to such patients.

Neutrophil-Lymphocyte Ratio

The neutrophil-lymphocyte ratio (NLR), the most sensitive and specific of the inflammatory biomarkers, is an effective indicator of inflammatory status in patients with ESRD (Ahbap et al., 2016). 2 clinical studies published in 2020 showed that NLR is a predictor of all-cause mortality and cardiovascular mortality in patients with chronic kidney disease and that higher NLR indicates higher mortality, so early clinical intervention is indicated in patients with high NLR and CKD (Zhao et al., 2020; Ao et al., 2021). The QRS-T angle is considered to be a response indicator for myocardial inhomogeneity and is the strongest predictor of cardiac death (Yamazaki et al., 2005). Boltuc et al. compared the difference in QRS-T angle between patients with advanced kidney disease on hemodialysis and those with normal eGFR and concluded that the QRS-T angle was significantly increased in HD patients and that NLR was strongly associated with all-cause and cardiac death were strongly associated (Boltuc et al., 2020). Several studies have shown that NLR has a higher sensitivity and a stronger cardiovascular prognostic association than CRP. Thus, NLR may provide additional clinical benefits to HD patients as a new indicator of inflammation.

Weakened immune function in patients with CKD, particularly in MHD, exacerbates pre-existing infections and activates immunity, which in turn leads to inflammation. Alterations in the body's immune cells accelerate atherosclerosis (Fernandez et al., 2019) and weaken the body's ability to clear pathogens and tumor cells, leading to a significantly increased risk of cardiovascular death, infection, and malignancy in patients with renal failure (George et al., 2017). Immune dysfunction is, therefore, an important reason for the reduced survival years of ESRD patients.

Blood Contact With Dialysis Membranes Activates the Complement System

Craddock et al. were the first to find a predisposition to acute cardiopulmonary insufficiency in the early stages of HD treatment. This is since in patients newly receiving HD, blood contact with

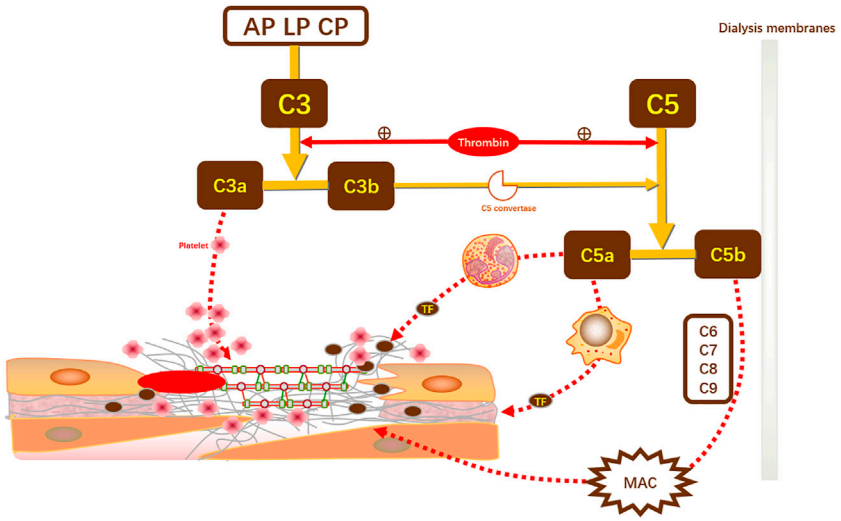

FIGURE 1 | Complement activation promotes coagulation. C3, the initiator of the complement activation pathway, can be cleaved into effector components, namely C3a and C3b. In CKD-induced complement activation, C3a directly promotes coagulation by enhancing platelet aggregation and adhesion. Meanwhile, C3b promotes the synthesis of C5 convertase to induce C5 cleavage to produce C5a and C5b. C5a directly stimulates neutrophils and monocytes to increase the expression of TF and thus induce thrombosis. In renal replacement therapy, C5b comes into contact with the dialysis membrane and, together with multiple complements (C6–C9), mediates the production of MAC and induces coagulation. In turn, coagulation secondary to complement activation can amplify complement and coagulation activation through positive feedback from thrombin on C3 cleavage. TF, tissue factor; MAC, membrane attack complex; AP, alternative pathway; LP, lectin pathway; CP, classical pathway.

dialysis membranes can stimulate innate immune activation and the body recognizes the exposed biological material as a non-self antigen that promotes inflammation through immune cell stimulation, inflammatory cell aggregation, complement activation, and cytokine production (**Table 1**) (Honkanen et al., 1991; Pertosa et al., 1993; Rousseau et al., 1999). Complement is one of the major components of the innate immune system and bridges the adaptive response of the body to abnormal stimuli. c3 acts as a sink for the complement activation pathway and plays a pivotal role in the activation of the complement system and is a key molecule in the activation of the alternative pathway. c5a acts directly on vascular endothelial cells to increase vascular permeability. It is also a chemotactic agent for neutrophils and monocytes at high concentrations, driving the directional movement of these cells, stimulating the oxidative metabolism of neutrophils and monocytes, stimulating neutrophil adhesion, and even increasing oxidative stress (Simone et al., 2014). In addition, C5a enhances the immune response and induces the secretion of cytokines such as IL-1, IL-6, IL-8, and TNF-α by monocytes, which promotes the proliferation of T cells and antibody production by B cells. Meanwhile, increased production of C3a and C5a and lecithin (C3b, iC3b) in HD patients leads to increased cytokine production, exacerbates cytotoxicity, and is mediated by the release of granzyme from neutrophils, promoting inflammation. However, we believe that this complement activation effect is only active in the early stages of HD and gradually decreases during long-term dialysis, and some studies have confirmed a negative correlation between C3 levels and dialysis duration (Girndt et al., 1999; Buraczynska et al., 2009; Mares et al., 2009; Mares et al., 2010; Inoshita et al., 2012; Kishida et al., 2013; Hornum et al., 2014; Poppelaars et al., 2018).

The presence of a procoagulant state during HD due to complement activation increases the risk of inflammatory and cardiovascular events and may result in a poor prognosis (Poppelaars et al., 2018). Complement effectors have a procoagulant effect. First, C3a can activate platelet aggregation and adhesion. Second, C5a can stimulate the expression of tissue factor and tissue-type fibrinogen activator inhibitors *in vivo* by centrophils and monocytes to promote thrombus formation. In turn, the coagulation component thrombin can cleave C3 into C3a and C3b and C5 into C5a and C5b, thereby amplifying the activation of complement. For the treatment of coronary vascular lesions presenting on dialysis, invasive therapy has not been beneficial in most studies due to the risk of contrast nephropathy occurring, and Bangalore et al. even suggested that invasive therapy may increase the risk of stroke in patients with advanced CKD (**Figure 1**). Not only that, with coronary revascularization, but reperfusion to the myocardium may also lead to more severe myocardial injury, i.e., myocardial ischemia-reperfusion injury, due to mechanisms such as oxidative stress, calcium overload, apoptosis, and leukocyte accumulation (Bangalore et al., 2020).

Uremic Toxin Involvement With Endotoxemia

The development of endotoxemia and the accumulation of uremic toxins contribute to the specific pattern of inflammation in ESRD patients (Crépin et al., 2020). Bacterial endotoxin is a lipopolysaccharide of the outer membrane of Gram-negative rods, which accounts for 70% of the total intestinal bacteria in the healthy population. Endotoxin is

broken down by the bacterial cell wall inside and outside the intestinal lumen and is released through host defense and self-integration mechanisms. It has a strong stimulatory effect on the development of inflammation, increasing pro-inflammatory cytokine release by stimulating monocytes/macrophages and circulating lipopolysaccharide receptors to bind to systemic immunoreceptor cells (Yang et al., 1998). Endotoxin translocation by crossing the intestinal barrier into the circulation, i.e., endotoxemia, occurs, which is dependent on abnormal intestinal permeability, and this occurs mainly in cases of intestinal edema and intestinal hypoperfusion. Endotoxemia is associated with systemic inflammation, oxidative stress, malnutrition, cardiac injury, and poor prognosis in cardiovascular disease (Krack et al., 2005; Kotanko et al., 2006).

Uremic toxins are substances that are significantly elevated in patients with renal failure that are toxic, such as urea, creatinine, peptides, potassium ions, and indole sulfate (IS). Gastrointestinal stasis due to excess fluid and reduced clearance of uremic toxins in patients with renal insufficiency results in altered gastrointestinal permeability and loss of intestinal epithelial barrier integrity resulting in exposure to significant endotoxemia, which is characteristic of every patient with CKD (Rysz et al., 2021a; Rysz et al., 2021b). When dialysis is started in CKD patients, the body undergoes significant hemodynamic disturbances, and HD-induced systemic circulatory stress and repeated local ischemia of important organs (here mainly the mesentery) lead to damage of the intestinal mucosa, which subsequently leads to increased translocation of intestinal endotoxins. In normal subjects, endotoxins in the intestine enter the liver through the portal vein and are then removed (Yu et al., 1997; McIntyre et al., 2011). Hemodialysis alters hepatic blood flow and diminishes liver function, leading to endotoxemia and thus increased inflammation (Marants et al., 2021). As a major structural component of the outer membrane of the cell wall of Gram-negative bacteria, the shift of LPS to the inflammatory isoform and the decrease in LPS function is strongly associated with the development of severe inflammation in ESRD patients. Regulation of the gut microbiota may prevent the development of endotoxin-induced inflammation in such patients (Adda-Rezig et al., 2021).

At the same time, uremic toxins mediate abnormal endothelial function in CKD patients. is directly contributes to cardiovascular complications by reducing NO synthesis in endothelial cells and successively impairing endothelial cell proliferation is also indirectly contributes to cardiotoxicity through pro-inflammatory effects and altered immune processes (Düsing et al., 2021; Rapa et al., 2021). Uremic toxin as an intermediate mediator of cardiac and renal damage reflects the complex relationship between heart and kidney, highlighting a potential target for prevention of cardiovascular complications in CKD patients.

Endotoxemia is associated with the occurrence of cardiovascular events in HD patients, highlighting the potential toxicity inherent in HD and providing a clearer therapeutic strategy for improved clinical management.

Hemodialysis Access

Hemodialysis access is usually performed using either an autologous arteriovenous fistula (AVF) or a central venous catheter (CVC). Among them, AVF is the first choice of arteriovenous access for hemodialysis, which can ensure the adequacy of dialysis and maximize the prognosis of patients (Ethier et al., 2008). The risk of sepsis in HD patients is significantly associated with hemodialysis access and is associated with a dramatic increase in mortality (Locham et al., 2021). 2021 Valtuille et al. noted that patients on dialysis with AVF had less oxidative stress or lower severity of oxidative stress relative to patients on long-term central venous catheter dialysis (CVC) (Valtuille et al., 2021). In maintenance hemodialysis (MHD) patients, AVF has a protective effect against unbalanced peroxidation-oxidation. In contrast, patients on dialysis with CVC had a more pronounced inflammatory state (e.g., elevated CRP and IL-6) and higher mortality (Banerjee et al., 2014; Chan et al., 2019). Nevertheless, AVF induces inflammation in HD patients, firstly by stimulating the production of oxidative stress markers during endovascular fistula formation, inducing intimal hyperplasia, and exacerbating local inflammation. Second, AVF for hemodialysis can lead to a hyperdynamic state of circulating blood, stimulating left ventricular hypertrophy and leading to a poor cardiac prognosis (Lee et al., 2021).

High Levels of Inflammatory Mediators

The kidney, as an important organ, receives 1/4 of the total blood flow. Under conditions of impaired renal function, the kidney becomes a target of persistent chronic inflammation due to impaired antioxidant and anti-inflammatory defenses and detoxification. In turn, due to increased inflammatory factor production and impaired proximal tubular clearance, blood concentrations of acute-phase proteins (CRP and fibrinogen) and pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) increase progressively as renal function deteriorates (Gupta et al., 2012; Hartzell et al., 2020). Pro-inflammatory cytokines alter the adhesion molecules on the surface of endothelial cells and leukocytes in the renal vasculature and disrupt the glycocalyx layer (a negatively charged villi-like structure covering the vascular endothelium that is involved in regulating vascular permeability, regulating leukocyte adhesion and flow, responding to vascular mechanical shear, and inhibiting intravascular coagulation), leading to changes in endothelial barrier function, vascular reactivity, and the coagulation system, and even disrupting renal physiology. This alteration also explains the mechanism of cardiovascular complications in CKD patients (Gupta et al., 2012; Hartzell et al., 2020).

Although a small study in 2021 noted that long-term maintenance hemodialysis reduces blood levels of inflammatory factors (including IL-2, IL-6), and high-sensitivity C-reactive protein (hs-CRP)] in patients with renal failure, inflammation remains the dominant factor for cardiac insufficiency that occurs at the start of dialysis (Zhou et al., 2021). In 2016 Sun et al. first compared multiple biomarkers in patients with advanced kidney disease on hemodialysis, and the study suggested that most inflammation-related biomarkers are

TABLE 2 | A recent study of common inflammatory factors associated with cardiovascular complications in hemodialysis patients.

Cytokines	Clinical studies related to cardiovascular complications
IL-1	Several animal studies have shown additional clinical benefits to the kidney with IL-1β inhibitors (Assimon et al., 2018) A 2017 randomized, double-blind, placebo-controlled trial of more than 10,000 patients with CKD by Ridker et al. showed that the use of a human monoclonal antibody targeting IL-1β was associated with a significant reduction in the recurrence of cardiovascular events in such patients (Kloner, 2020)
IL-6	In a 5-year follow-up study of 45 patients on long-term hemodialysis, Thang et al. demonstrated that IL-6) had a more powerful predictive prognostic significance for cardiovascular disease than CRP in HD patients (Braunwald and Kloner, 1982) This was corroborated in a 2015 case study of multiple biomarker levels in 543 ESRD patients, confirming that IL-6 is a strong independent predictor of clinical outcome in patients with CKD (Zuidema and Dellsperger, 2012; Arcari et al., 2021) A case study in 2021 evaluated the genetic phenotypic differences in IL-6 and its predictive value for all-cause mortality in 289 ESRD patients and found that the IL6 (−174G > C) (r1800795) polymorphism regulates the inflammatory response in ESRD patients. The CC genotype, a less common IL6 genotype, causes more severe inflammation and suggests a poorer prognosis in ESRD patients (deFilippi et al., 2012)
IL-18	A 2015 study of the prognosis of patients with CKD who had an acute myocardial infarction 1 year earlier concluded that IL18 was a significant predictor of cardiogenic death at 2-year follow-up (Raber et al., 2021a)
CRP	A 2021 study assessing the correlation between dialysis adequacy and inflammation in 536 HD patients using CRP as an indicator of inflammation noted that inadequate dialysis doses may lead to higher levels of inflammation in chronic hemodialysis patients. And high levels of CRP were directly correlated with neutrophil-lymphocyte ratio and serum albumin (Raber et al., 2021b)
sAlb	The relationship between changes in albumin and sAlb and prognosis in patients transitioning from CKD to ESRD stage was studied for the first time in 2019. Patients just transitioning from CKD to ESRD have a high short-term mortality rate, so improving the nutritional status of pre-ESRD patients, including sAlb levels, is important for the prognosis of such patients (Miller-Hodges et al., 2018). lower pre-ESRD sAlb is significantly associated with higher all-cause, cardiovascular and infection-related mortality and hospitalization rates after ESRD (Twerenbold et al., 2018) In 2020 Amanda et al. compared sAlb and its prognosis across renal function and found that despite the apparent correlation between sAlb and eGFR, a significant correlation between sAlb and mortality in patients with CKD was observed in multiple subgroups classified by renal function (Kraus et al., 2018). It was also noted that sAlb <4.6 g/dl suggested higher mortality and that maintaining sAlb between 4.6 and 4.8 g/dl may be more beneficial in CKD patients with eGFR <60 ml/min/1.73 m^2. Therefore, we suggest that dietary protein intake should be moderate for patients with CKD and that high sAlb should not be the therapeutic goal (Ponikowski et al., 20162016; Arcari et al., 2020)

elevated in such patients, and all of them play a predictive role in the occurrence and prognosis of cardiovascular events in such patients (Mun and Golper, 2000; Sun et al., 2016). There is a correlation between most biomarkers, led by IL-6 and hs-CRP. Multiple biomarkers are collectively involved in the occurrence of cardiovascular events in patients with advanced kidney disease, and the degree of change in biomarkers reflects some extent the possible concurrent diseases in patients with chronic kidney disease, suggesting a general alteration in the signaling pathways of inflammation and apoptosis in uremic and dialysis patients with the uremic disease (Sun et al., 2016). We listed the inflammatory mediators that are closely associated with inflammation and cardiovascular disease development in HD patients and explored the adaptive changes that occur in the organism (**Table 2**).

IL-1

Interleukin 1 (IL-1), also known as a lymphocyte-stimulating factor, is produced by activated monocytes-macrophages. The physiological functions of IL-1 include 1) stimulating the activation of T lymphocytes, 2) stimulating B cell proliferation and antibody secretion, 3) enhancing NK cell activity in concert with IL-2 or interferon, and 4) inducing the release of inflammatory mediators from neutrophils, thus participating in immune regulation. Due to the specific role of IL-1 on the immune system, therapy to inhibit IL-1 function has emerged as a potential therapeutic target to weaken inflammation in CKD patients, especially in HD patients (Barreto et al., 2010; Ridker et al., 2018; Düsing et al., 2021). Inhibition of IL-1β offers a broader prospect for the treatment and prevention of cardiovascular complications in CKD patients (Ridker et al., 2018).

IL-6

Interleukin 6 (IL-6) is an intermediate mediator of the acute response and enhances defense mechanisms against external stimuli by triggering the body's alarm signals through timely expression. IL-6 can stimulate inflammatory and autoimmune processes by stimulating acute phase protein synthesis and neutrophil production (Babaei et al., 2014). IL-6 concentrations are high in CKD patients, especially in maintenance hemodialysis patients, due to the accumulation of uremic toxins, failure of renal filtration capacity, volume overload, and stimulation by oxidative stress (Babaei et al.,

2014; Cao et al., 2014). Likewise, the dialysis process helps stimulate an inflammatory response that further increases IL-6 production.

IL-6 is the only independent predictor of the risk of cardiovascular comorbidity, cardiac mortality, and all-cause mortality in patients with CKD, better reflects the relationship between inflammation and cardiovascular complications than other cytokines (Kisic et al., 2016) and is the most reliable predictor of cardiovascular mortality and total mortality found in patients with CKD (Barreto et al., 2010; Sun et al., 2016).

IL-18

Interleukin 18 (IL-18) acts as a potent pro-inflammatory cytokine, triggering the production of a series of pro-inflammatory cytokines by producing immediate inflammatory factors (i.e., TNF-α and IL-1β), among others.

IL-18 is involved in the development of atherosclerosis and contributes to plaque instability through an immune response that increases the synthesis of endothelial adhesion molecules by inducing the synthesis of interferon-gamma (IFN-γ) and reduces the size of the fibrous plaque cap by promoting the expression of major histocompatibility complex II (MHC II) on macrophages and vascular cells. On this basis, IL-18 regulated matrix metalloproteinase upward expression thereby inhibiting collagen synthesis. Combining several clinical studies, we propose that IL-18 not only accelerates the progression of atherosclerosis in CKD patients but also has a predictive value for cardiovascular prognosis in CKD patients (Sasaki et al., 2021).

In addition, IL-18 is involved in the overall progression of inflammation by mediating the production and activation of various cytokines. in addition to the aforementioned alteration of the Th1/Th2 ratio leading to an altered T-cell adaptive response (Mühl et al., 1996), IL-18 can also synergistically activate Th17 cells with IL-23 to promote the production of the cytokine IL-17 and thus induce the recruitment of neutrophils and monocytes to the site of inflammation (Poulianiti et al., 2016).

C-Reactive Protein

Several previous studies have shown high levels of CRP in patients with intermediate to advanced CKD, with higher levels in ESRD and dialysis patients (Spoto et al., 2015).

It was found that CRP >3 mg/L in dialysis patients may indicate the occurrence of adverse events and a doubling of the risk of cardiovascular disease but has no significant effect on all-cause mortality. Inflammation plays a more important role than oxidative stress in the mechanism of cardiovascular disease in HD patients (Sasaki et al., 2021).

Serum Albumin

Serum albumin (sAlb) is an important biomarker synthesized by the liver and its production is associated with the nutritional status of the body and inflammation (Kim et al., 2013). Patients with CKD often have low sAlb levels, which are associated with lower quality of life and higher rates of hospitalization and mortality due to the imbalance in serum albumin synthesis and consumption caused by the chronic inflammatory state and the susceptibility to other co-morbidities (Friedman and Fadem, 2010; Kato et al., 2010). Serum albumin levels are determined by the rate of hepatic synthesis and catabolism, protein intake, and blood volume distribution, so changes in its concentration can assess the treatment outcome and prognosis of patients receiving renal replacement therapy (Herselman et al., 2010). Not only that, but CKD patients with hypoalbuminemia who develop ESRD are more likely to develop comorbidities such as diabetes mellitus and congestive heart failure (Hsiung et al., 2019). Inflammation interacts with sAlb. Protein-energy wastage (PEW) is often seen in patients with CKD and is an important reason for the poor prognosis of such patients (especially hemodialysis patients). When the body is in a situation of protein and energy depletion, it stimulates inflammation, which in turn leads to PEW, the main indicator of PEW, namely serum albumin (Kovesdy and Kalantar-Zadeh, 2009). On the other hand, lower sAlb is associated with higher validation markers (CRP and leukocytes/neutrophils) (Hsiung et al., 2019). The increased concentration of uremic toxins that occurs due to dietary restrictions in CKD patients leads to altered gut microbiology in such patients stimulating inflammation and a decrease in sAlb (Rothschild et al., 1973). Dietary restrictions in patients with CKD lead to alterations in the gut microbiology of such patients, and the resulting increase in uremic toxin concentrations that occur stimulates inflammation and a decrease in sAlb (Pecoits-Filho et al., 2002; Johansen et al., 2010; Shahzad et al., 2015; Ridker et al., 2017; Thang et al., 2020). In addition, sAlb also decreases due to reduced physical activity in HD patients. In patients on maintenance hemodialysis, the dynamics of serum albumin concentration is an independent predictor of all-cause mortality and cardiac mortality, and in 2010 it was shown that lower serum albumin predicted higher all-cause mortality and cardiovascular mortality (Herselman et al., 2010).

OXIDATIVE STRESS MECHANISMS PARALLEL TO INFLAMMATION

Oxidative stress (OS), which refers to an imbalance between the two antagonistic systems of oxidation and antioxidation in the body, tends to oxidize, causing abnormalities in the biochemical and physiological processes of the body and damage to endothelial tissues (Formanowicz et al., 2015; Kalantar-Zadeh et al., 2016; Kalantar-Zadeh et al., 2017; Ko et al., 2017; Brown-Tortorici et al., 2020; Rocha et al., 2021; Valga et al., 2021). Oxidants of oxidative stress refer to reactive oxygen or nitrogen species (ROS or RNS, respectively) as well as free radicals. Centrophages and monocytes/macrophages are the main sources of ROS, and oxidative stress increases the production of chemokines (MCP-1, CSF-1) and adhesion molecules (ICAM-1), tending the redox balance towards a peroxidized state by promoting the aggregation of these cells (Vaziri et al., 2003). The constant pathological stimulation of macrophages in the inflammatory state can promote oxidative stress and lead to excessive production of ROS, while ROS-induced activation of transcription factors and

pro-inflammatory genes can in turn increase inflammation (Chu et al., 2020; Podkowinska and Formanowicz, 2020).

Oxidative stress in chronic kidney disease is mediated by multiple factors, mainly due to impaired antioxidant mechanisms and increased production of reactive oxygen species (ROS) (Düsing et al., 2021). To achieve redox homeostasis under physiological conditions, highly active antioxidant mediators [including catalase (CAT), glutathione peroxidase (GPX), and the free radical scavenger superoxide dismutase (SOD)] are present in the glomerulus (Siems et al., 2002; Jagiela et al., 2020). The excessive production of ROS negatively affects various components of the renal unit, impairing the glomerular microcirculation and leading to glomerular ischemia in the long term (Luczak et al., 2011). Oxidative stress leads to apoptosis and necrosis of tubular epithelial cells, increased synthesis of collagen and fibronectin by endothelial and thylakoid cells, leading to tubular atrophy and interstitial fibrosis. With the loss of renal function and destruction of a tubular structure, renal regulatory mechanisms, such as the Renin-Angiotensin-Aldosterone system (RAAS), are affected, rendering the kidney unable to compensate for electrolyte and acid-base homeostasis disturbances. OS is more severe in HD patients, and investigators have attributed the oxidative stress state in HD patients to 4 main causes, including urotoxicosis, dialyzer interaction, dialysate contamination, and peripheral blood cell-dialysis membrane interaction (Valtuille et al., 2021).

Reactive oxygen metabolites (d-ROM) are a comprehensive marker of biological oxidative modifications in serum. 2021 A prospective study of 517 hemodialysis patients prospectively studied with d-ROM as a marker of oxidative stress, adjusted for the inflammatory marker CRP, found that CRP and d-ROM had predictive value for cardiovascular event occurrence and all-cause mortality, but in HD inflammation appears to be more important in the occurrence of cardiovascular events in patients. This study demonstrates that there appears to be a common causal pathway between inflammation and oxidative stress and that they can contribute to each other (Sasaki et al., 2021).

Oxidative Stress Under HD Conditions is Associated With Cardiovascular Disease Development

Occurrence of Cardiovascular Disease

Inflammation, an unconventional cardiovascular risk factor, primarily accelerates the onset and progression of atherosclerosis. Long-term maintenance hemodialysis was first proposed to accelerate the progression of atherosclerosis by Lindner et al., in 1974 (Lindner et al., 1974). Subsequently, Gerrity et al. suggested that inflammation mediates the formation and progression of atherosclerosis (Gerrity, 1981). Patients with ESRD have a characteristic accelerated atherosclerotic process in which chronic inflammation is critical to the progression of atherosclerosis, and the coronary arteries are the most severely hit in this pathological process (Papagianni et al., 2003; Pencak et al., 2013). CKD is associated with the development of early atherosclerosis and the degree of atherosclerosis increases with the progression of CKD (Valdivielso et al., 2019). The incidence of atherosclerosis and the rate of disease progression were more significant in CKD patients treated with HD (Ma et al., 1992; Fujisawa et al., 2000).

Lipid Alterations due to Inflammation and Oxidative Stress

Patients receiving HD have abnormalities in certain lipoproteins (including very low density lipoproteins, low density lipoproteins, and intermediate density lipoproteins), which are associated with changes in the arterial wall (Shoji et al., 1998; Nishizawa et al., 2003). 2017 Echida et al. found that higher serum non-HDL cholesterol levels were significantly associated with cardiovascular mortality (Echida et al., 2012). Oxidative modification of low-density lipoprotein cholesterol (LDL-C) affects the onset and progression of atherosclerosis and may lead to cardiovascular disease (Varan et al., 2010; Hopkins, 2013). Oxidatively modified low-density lipoprotein (Ox-LDL) recruits mononuclear macrophages, and scavenger receptors on the cell surface increase macrophage and vascular smooth muscle cell production through uptake of Ox-LDL, ultimately leading to atherogenic plaque formation (increased foam cell formation) and damage to the endothelium. The presence of more significant lipid oxidation due to increased oxidative stress in HD conditions explains the extremely high risk of cardiovascular disease and poor prognosis in HD patients (Kronenberg et al., 2003).

In addition, as the most abundantly expressed protein product in adipose tissue, lipocalin (ADPN) is involved in the regulation of the neuroendocrine system, can regulate lipid disorders, and is positively correlated with the level of inflammation in the body. In hemodialysis patients, abnormal oxidative stress decreases ADPN secretion. In turn, reduced ADPN levels reduce the clearance of ROS, further exacerbating oxidative stress, causing kidney damage, and increasing the incidence of cardiovascular disease (Yu et al., 2015).

Tissue Damage and Endothelial Dysfunction

Atherosclerosis occurs first by macrophages invading the vascular endothelium and transforming into foam cells to form atheromatous material. At the same time, the invading leukocytes release inflammatory mediators that lead to endothelial damage and, in some cases, induce atherosclerotic plaque rupture leading to fatal disease (Liberale et al., 2021). Inflammation and oxidative stress are directly related to the development of cardiovascular disease (CVD) in patients with CKD. Abnormal oxidative stress in ESRD patients can oxidize lipids, proteins and carbohydrates, leading to tissue damage and endothelial dysfunction, exacerbated by the effects of uremic toxins (Dummer et al., 2007; Westerweel et al., 2007).

Serum malondialdehyde (MDA) levels are an important indicator of lipid peroxidation and a strong indicator of cardiovascular disease. Circulating malondialdehyde modified low-density lipoprotein (MDA-LDL) is the main end product of LDL oxidation, negatively correlates with endothelial function, and predicts the onset and progression of atherosclerosis (atherosclerosis and arterial calcification) in the population (Ito et al., 2018). Calcium and phosphorus metabolism is

deranged in uremic patients, leading to activated oxidation of fats and proteins and increased MDA production, exacerbating the risk of cardiovascular disease (Hou et al., 2020). Zhang et al. investigated the factors associated with promoting the development of cardiac insufficiency in uremic patients and evaluated CRP and MDA as markers of inflammatory and oxidative stress mechanisms, respectively, and found that CRP and MDA were negatively correlated with left ventricular ejection fraction (LVEF) (Zhang et al., 2021). Second, elevated serum inorganic phosphorus is often observed in CKD patients. The high phosphate environment leads to increased angiogenesis, endothelial cell senescence, apoptosis, and translocation, thereby disrupting endothelial function and promoting the development of vascular calcification in CKD patients (Di Marco et al., 2008; Rapa et al., 2021).

Zinc (Zn), a biological antioxidant, increases oxidative stress *in vivo* in HD patients due to low plasma zinc levels (mainly due to reduced renal function, diminished intestinal absorption of zinc, and exogenous factors such as diet and medications), causing LDL to differentiate toward electronegative LDL [LDL(-)]. the presence of LDL(-) stimulates the production of many of the inflammatory mediators mentioned previously and promotes cardiovascular disease by recruiting leukocytes to cause pathological changes in the vascular endothelium promoting cardiovascular disease.

Excessive ROS Generation

Patients with chronic kidney disease are constantly exposed to oxidative stress, especially those with ESRD (Witko-Sarsat et al., 1998; Oberg et al., 2004; Tsuchikura et al., 2010). Oxidative stress mechanisms play a more important role in the occurrence of adverse events in HD patients compared to biologic incompatibilities, therefore in uremic patients with HD, increased oxidative stress mechanisms appear to be a more important target for drug and biologic incompatibility therapy in such patients.

Long-term use of AVG and CVC for dialysis in HD patients promotes inflammation and OS due to the metabolic abnormalities associated with uremia and the biologically incompatible system of hemodialysis (Himmelfarb et al., 2002). Biological incompatibility depends on the type of dialysis membrane and the endotoxin dissemination caused by the contaminated dialysate, which activates phagocytes in the blood and leads to the progression of inflammation. At the same time, activated neutrophils and monocytes in the blood produce reactive intermediates that exacerbate oxidative imbalances and further promote inflammation (Tetta et al., 1999; Varan et al., 2010). Hemodialysis activates the complement system, recruiting small molecules such as immunoglobulins (IgG) and complement to attach to the dialysis membrane, thereby promoting the release of ROS (Liakopoulos et al., 2017; Liakopoulos et al., 2019). The above multiple mechanisms explain the induction of oxidative stress and inflammation by biologic incompatibilities, further promoting the development of coronary sclerosis in HD patients (Schettler et al., 1998; Fumeron et al., 2005).

Varan et al. compared the effects of different dialysis membranes on oxidative stress in HD patients and found that all antioxidant enzymes were significantly increased in patients receiving HD treatment. In patients included in the study, even a single dialysis session with a biocompatible membrane (e.g., polysulfone) resulted in a lesser degree of oxidant/antioxidant imbalance *in vivo* than dialysis with a biologically incompatible membrane (e.g, copperane) (Varan et al., 2010).

Angiotensin II, an effector mediating vascular cell hypertrophy, fibrosis, inflammation, and cellular senescence, increases ROS production, and activation of oxidative stress-sensitive transcription factors through activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) activity and promotes inflammation (Griendling et al., 1994). Nox isoforms (especially Nox1, Nox2, Nox4, and Nox5) are involved in intrarenal oxidative stress and stimulate oxidative stress. Nox2, Nox4, and Nox5) are involved in intrarenal oxidative stress and stimulate oxidative stress, which is common in CKD patients (Madero et al., 2009; Bobulescu, 2010; Liesa and Shirihai, 2013; Sedeek et al., 2013; Gondouin et al., 2015; Ziegler et al., 2015; Gamboa et al., 2016; Wan et al., 2016; Ge et al., 2020; Martínez-Klimova et al., 2020; Rayego-Mateos and Valdivielso, 2020; Aranda-Rivera et al., 2021). Physiological doses of Nox4 are involved in cell proliferation, metabolism, and apoptosis (Sedeek et al., 2013), but excessive concentrations of Nox4 can lead to cellular inflammation, fibrosis, and even cellular damage, which subsequently affects renal excretory function. Many studies have suggested that uric acid can be used as a prognostic indicator for cardiovascular disease and CKD, possibly due to increased xanthine oxidase (XO) activity in patients with hyperuricemia, which leads to oxidative stress and endothelial dysfunction. XO is a pro-oxidant enzyme that promotes ROS production and is involved in the uric acid synthesis (Madero et al., 2009). XO activity is increased in HD patients, which is associated with abnormal oxidative stress in HD patients. Because of the pro-oxidant effect of XO, XO activity can be used as a predictor of cardiovascular events in patients with CKD (Gondouin et al., 2015).

Under physiological conditions, mitochondria maintain normal cellular signaling pathways through fatty acid (FA) β-oxidation, tricarboxylic acid cycle (TCA cycle), oxidative phosphorylation (OXPHOS) to produce adenosine triphosphate (ATP), and small doses of ROS, and ATP is also involved in active transport in the renal tubules to maintain normal physiological functions. Abnormalities in many genes related to protein production and mitochondrial activity can be observed in patients on renal replacement therapy (Rayego-Mateos and Valdivielso, 2020; Gamboa et al., 2016). In addition to this, impaired FA β-oxidation and OXPHOS reduce ATP synthesis and increase NOX, hydrogen peroxide (H_2O_2), and CD36 levels in the body, inducing excessive ROS production and stimulating the onset of oxidative stress. The pathological increase of oxidative stress induces the occurrence of mitochondrial autophagy and weakens mitochondrial protein synthesis, further deteriorating mitochondrial function and destroying renal compensatory function (Ziegler et al., 2015). In turn, a pathological increase in ROS further inhibits ATP synthesis, while a decrease in ATP synthesis positively increases

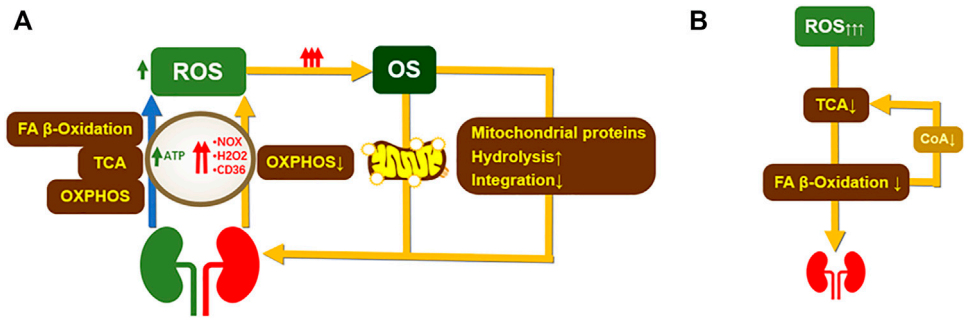

FIGURE 2 | The occurrence of renal oxidative stress affects mitochondrial structure and function. **(A)**. In normal humans, oxidative phosphorylation, tricarboxylic acid cycle, and FA-β-oxidation provide energy to the kidney and induce the production of low doses of ROS. OXPHOS mainly provide ATP to the proximal tubule to maintain the normal physiological function of the kidney. A decrease in OXPHOS capacity during kidney damage leads to an increase in NOX levels. Abnormal NOX levels lead to excessive ROS production exacerbating oxidative stress and inflammation and inducing the development of renal fibrosis. Meanwhile, renal disease impairs mitochondrial function during OXPHOS due to reduced renal degradation of H_2O_2 a FA-βnd disrupts FA-β oxidation. overexpression of CD36 leads to reduced lipid metabolism resulting in involvement and causes impaired FA-β oxidation, leading to excessive ROS production (Ge et al., 2020; Martínez-Klimova et al., 2020). Excess ROS leads to growth mitochondrial damage, 1) decreased mitochondrial protein synthesis, and increased catabolism (Liesa and Shirihai, 2013). 2) Increased mitochondrial autophagy, which occurs mainly through degradation of proteasomes by the PINK1-Parkin pathway and phagocytic receptor interactions. **(B)**. Excess ROS impair FA-β-oxidation by impairing TCA cycle function. reduced FA-β-oxidation leads to lipid accumulation and aggravates renal function. In turn, impaired FA *β*-oxidation leads to a decrease in acetyl coenzyme A, further diminishing TCA capacity (Bobulescu, 2010). ROS, reactive oxygen species; OS, oxidative stress; OXPHOS, oxidative phosphorylation; TCA, tricarboxylic acid cycle; NOX, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; H_2O_2, hydrogen peroxide; CD36, long-chain FA fractionation cluster36.

ROS production, leading to increased oxidative stress and destruction of cellular components resulting in cell necrosis (**Figure 2**) (Aranda-Rivera et al., 2021). Therefore, balancing the redox process can improve mitochondrial function and optimize mitochondrial dynamics by applying antioxidants that target mitochondria.

Anti-Oxidative Stress

Under physiological conditions, free oxygen radicals act as a non-specific defense mechanism against stimuli such as infections. However, when overproduced, they are harmful to the body, affecting lipids, proteins, DNA, and cell membranes, including membrane carbohydrates, leading to membrane damage and cell disintegration. To prevent or mitigate the adverse effects of ROS, antioxidant mechanisms play an important role (Aiello et al., 1999). Uremic patients have a significant disruption of the extracellular redox system, with a tendency toward increased oxidative status and antioxidant depletion, which is exacerbated by HD. ESRD patients treated with HD, therefore, require a more active antioxidant system, yet the available clinical evidence suggests that such patients have an antioxidant dysfunction (Varan et al., 2010). Therefore, the appropriate use of antioxidants in ESRD patients may prevent renal injury and disease progression and reduce the incidence of adverse events by modulating oxidative stress.

Multiple antioxidant stress substances are affected in CKD, especially in HD patients. SOD isomers are important internal enzymes against oxidative stress, and SOD-1, an important isomer of SOD in the kidney, is associated with impaired renal function and reduced renal excretion capacity, so increased SOD-1 synthesis is observed in HD patients (Pawlak et al., 2007; Pawlak et al., 2013). In addition, SOD-1 can be used as a marker of atherosclerosis, vascular abnormalities, and inflammation. However, due to its influence by various factors such as immune status and enzyme activity, the current studies have not reached consistent conclusions about SOD-1. Renal glutathione peroxidase 1 (GPx1) can prevent some damage to the kidney caused by oxidative stress and enhance the kidney's ability to cope with oxidative stress, so proper induction of such enzyme activity and promotion of its gene expression may be clinically beneficial for ESRD patients (Chu et al., 2020). In addition, patients with advanced kidney disease suffer from micronutrient dysregulation due to dietary restrictions, poor appetite, and dialysis depletion, resulting in antioxidant deficiency and insufficient activity. A meta-analysis evaluating the effect of multiple oxidants on all-cause mortality and cardiovascular event rates showed that antioxidants reduced the incidence of cardiovascular events in HD patients without a significant effect on non-dialysis patients (Jun et al., 2012; Baldi et al., 2013; Sepe et al., 2019).

Several studies have confirmed that active components involved in oxidative stress can improve the poor prognosis of HD patients (**Table 3**). In addition, quercetin, amino acids, tea-lipoic acid, and lipocalin are involved in the regulation of oxidative stress.

Medications for Inflammation Control

Drugs commonly used to treat cardiovascular disease may have a higher cardiovascular benefit for ESRD patients due to their potential anti-inflammatory effects (Fiorillo et al., 1998; Helmke and von Vietinghoff, 2016). We have listed the commonly used cardiovascular drugs with anti-inflammatory effects, but there are a variety of drugs with anti-inflammatory effects including colchicine, methotrexate, and interleukin receptor antagonists, and their association with CKD has been little studied and not discussed.

TABLE 3 | Protective effects of common antioxidant substances on cardiac function in dialysis patients.

Antioxidants	Renal protection mechanism
Taurine	Scavenges ROS, reduces inflammatory response, plays a role in phagocytosis and reduces inflammation, and protects against hemodialysis, ischemia, and various renal diseases
I-Carnitine	Reducing the production of acetyl coenzyme a and thus the production of free radicals reduces the production of pro-inflammatory factors in dialysis patients and is beneficial to the kidney
Vitamin C and Vitamin E	In dialysis patients, oxidative stress is associated with reduced vitamin C (Mirna et al., 2020). Infusion of vitamin C and use of vitamin E coated dialyzers in HD patients attenuates oxidative stress by reducing IDO1 activity and NO formation, weakening inflammation and cellular senescence (Januzzi et al., 2015). Vitamin E supplementation may reduce the risk of coronary artery disease by making LDL less susceptible to oxidation (Gaggin and Januzzi, 2013)
Niacinamide	Reduces the production of many cytokines associated with the pathogenesis of cardiac insufficiencies, such as IL-1β, IL-6, IL-8, and tumor necrosis factor

Statins

There is no clear evidence to support the benefit of anti-inflammatory therapy in the dialysis population with cardiac insufficiency, and statins may provide a benefit in the treatment and prevention of cardiovascular disease due to their LDL-lowering and anti-inflammatory effects, but there is no clear evidence for patients on dialysis. The study by Baigent et al. concluded that the use of statins and ezetimibe in combination with lipid-lowering therapy did not result in a better prognosis for dialysis patients compared to non-users. It was also suggested that there was no significant correlation between LDL and cardiovascular prognosis in dialysis patients (Baigent and Landry, 2003). Antibodies to pro-inflammatory cytokines may reduce the risk of adverse cardiovascular events, particularly in patients with CRP <2 mg/L, and anti-inflammatory therapy may be indicated at the start of dialysis, but more clinical findings are needed to support this (Dhorepatil et al., 2019). A 2021 study of a population with coronary heart disease using statins for more than 2 years showed that statins caused a gradual shift to higher density calcification of coronary atherosclerotic plaques while attenuating the progression of coronary atherosclerotic plaque size, but the explanation for the acceleration of coronary atherosclerotic plaque calcification by statins remains controversial (van Rosendael et al., 2021).

PCSK9 Inhibitor

Proprotein convertase subtilisin-kexin type 9 (PCSK9) is the main carrier of LDL-C and causes elevated LDL-C levels, which are associated with inflammation and immunity. Therefore, PCSK9 inhibitors not only reduce LDL-C, but also modulate inflammation and autoimmunity (Zhang et al., 2007). Therefore, PCSK9 inhibitors not only lower LDL-C, but also regulate inflammation and autoimmunity (Frostegård, 2021).

In a study of 9,738 people at high cardiovascular risk receiving statins and PCSK9 inhibitors, Pradhan et al. found that PCSK9 inhibitors significantly lowered LDL-C while reducing hs-CRP by 6.6%. hs-CRP >3 mg/L suggests a high risk of cardiovascular disease and kidney disease (Adejumo et al., 2016). Notably, despite the maximal reduction of LDL in this study, the presence of residual inflammatory risk (hsCRP ≥ 2 mg/L) still put the risk of future cardiovascular disease at a higher risk, whereas the use of PCSK9 inhibitors significantly reduced the risk of cardiovascular events in the high-risk population. This phenomenon cannot be explained by lipid-lowering effects alone, which may be due to the effect of PCSK9 inhibitors on inflammatory regulation in high-risk populations *in vivo* (Pradhan et al., 2018). The FOURIER trial then investigated the efficacy of PCSK9 inhibition in patients with stable coronary artery disease in different hs-CRP strata and found that the positive cardiovascular effects of PCSK9 inhibition were certain regardless of baseline hs-CRP, with patients with higher baseline hs-CRP showing the greatest benefit with PCSK9 inhibition (Bohula et al., 2018).

PSCK9 may serve as a novel cardiovascular risk marker in ESRD patients.2019 Strålberg et al. found that PCSK-9 levels were independently associated with all-cause mortality in 265 ESRD patients receiving long-term HD at a 3-year follow-up (Strålberg et al., 2019). 2021 Vlad et al. found no significant difference in PCSK9 levels in patients with different CKD stages, but PCSK9 > 220 ng/ml was a predictor of cardiovascular events, and PCSK9 > 220 ng/ml and hsCRP >3 mg/L together suggested an increased risk of kidney disease and cardiovascular disease (Vlad et al., 2021). However, PCSK9 inhibitors are less commonly used in patients with ESRD, and their efficacy in such patients is unclear.

Aspirin

The role of aspirin, a drug that provides great benefit for the treatment and prevention of cardiovascular disease, is not promising in patients with CKD. A cross-sectional study of 116 long-term aspirin users in the general population found that CKD patients were more likely to have impaired antiplatelet effects, and that such impaired effects were associated with increased mortality (Mayer et al., 2014; Polzin et al., 2016). A 2016 meta-analysis by Major et al. noted that aspirin did not provide benefit for cardiovascular events as well as prevention in CKD patients, nor did it improve survival in CKD patients, and may also carry a higher risk of major bleeding (Major et al., 2016). A controlled study of 17,762 subjects using aspirin or placebo in 2020 found a significantly increased risk of CVD in 4,768 participants with CKD and that the use of aspirin did not reduce the incidence of CVD in patients with CKD (Wolfe et al., 2021). 2021 et al. studied 91,744 ESRD patients who were not on dialysis and showed that aspirin caused disease progression and increased mortality in these patients (Tsai et al., 2021). Overall, aspirin does more harm than good in patients with ESRD. The ongoing ATTACK trial is the first trial to evaluate

aspirin for the prevention of primary CVD in adults with CKD, and this large study may bring more reliable evidence for the use of aspirin in patients with CKD.

CONCLUSION

Cardiovascular complications in patients with HD are a fatal factor in patients with ESRD. Inflammation is involved in and mediates the development of cardiovascular complications. While hemodialysis benefits the kidney, it exacerbates an already inflammatory state, altering the body's already tolerated microinflammation and volume overload, leading to a "cardiovascular spike". At the same time, the oxidative stress that accompanies inflammation during HD treatment damages renal structures, leading to further deterioration of renal function and increasing the risk of cardiovascular disease. Disturbances in electrolyte and acid-base balance secondary to the deterioration of renal function feedback into the oxidative stress mechanism, leading to a vicious circle between oxidative stress and the kidney. This review details the mechanisms of inflammation in ESRD patients treated with HD, the impact of different processes in the inflammatory cascade on cardiovascular complications, the mechanisms of oxidative stress, and the correlation between oxidative stress and cardiovascular disease. Although the mechanisms of inflammation are vast and complex, as a key factor in the development and prognosis of cardiovascular complications in ESRD patients, interventions that address key targets in the inflammatory cascade (e.g., immune mechanisms, complement activation, etc.) may improve compliance with HD therapy in ESRD patients and provide additional clinical benefits.

AUTHOR CONTRIBUTIONS

YW: reviewed the literature and drafted this review. LG: reviewed the literature, gave critical comments, and revised the manuscript.

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Qingda Granule Attenuates Angiotensin II-Induced Renal Apoptosis and Activation of the p53 Pathway

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Background: Qingda granules (QDG) exhibit antihypertension and multiple-target-organ protection. However, the therapeutic potential of QDG on hypertensive renal injury remains unknown. Therefore, the main objective of the current study is to explore the effects and underlying mechanisms of QDG treatment on renal injury in angiotensin (Ang) II-infused mice.

Methods and results: Mice were infused with Ang II (500 ng/kg/min) or saline for 4 weeks with subcutaneously implanted osmotic pumps. After infusion, mice in the Ang II + QDG group were intragastrically administrated with QDG daily (1.145 g/kg/day), whereas the control group and Ang II group were intragastrically administrated with the same amount of double-distilled water. Blood pressure of the mice monitored using the CODA™ noninvasive blood pressure system revealed that QDG treatment significantly attenuated elevated blood pressure. Moreover, hematoxylin–eosin staining indicated that QDG treatment ameliorated Ang II-induced renal morphological changes, including glomerular sclerosis and atrophy, epithelial cell atrophy, and tubular dilatation. RNA-sequencing (RNA-seq) identified 662 differentially expressed transcripts (DETs) in renal tissues of Ang II-infused mice, which were reversed after QDG treatment. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis based on DETs in both comparisons of Ang II vs. Control and Ang II + QDG vs. Ang II identified multiple enriched pathways, including apoptosis and p53 pathways. Consistently, terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) staining and Annexin V staining revealed that QDG treatment significantly attenuated Ang II-induced cell apoptosis in renal tissues and cultured renal tubular epithelial cell lines (NRK-52E). Furthermore, western blot analysis indicated that Ang II infusion significantly upregulated the protein expression of p53, BCL2-associated X (BAX), cle-caspase-9, and cle-caspase-3, while downregulating the protein expression of BCL-2 in renal tissues, which were attenuated after QDG treatment.

Conclusion: Collectively, QDG treatment significantly attenuated hypertensive renal injury, partially by attenuating renal apoptosis and suppressing p53 pathways, which might be the underlying mechanisms.

Keywords: Qingda granule, hypertension, renal injury, Ang II, p53 pathway

INTRODUCTION

Hypertension is a worldwide health problem with high cardiovascular morbidity and mortality (Aroner et al., 2016). It is reported that 23.2% (estimated 244.5 million) of the Chinese adult population aged ≥18 years had hypertension, and another 41.3% (estimated 435.3 million) had prehypertension according to the Chinese guideline (Wang Z. et al., 2018). Long term hypertension leads to adverse health effects, mainly by accelerating the damage of target organs, including the kidney (Du et al., 2021). The kidney plays an essential role in maintaining renal function and blood pressure and contributes to the development of hypertension, as well as being recognized as one of the primary target organs of hypertension (Ruiz-Hurtado and Ruilope, 2018). Moreover, most current therapeutic strategies still fail to prevent the development and progression of hypertensive renal damage, leading to a large number of hypertensive patients still ultimately suffering from end-stage renal disease (ESRD) (Dong et al., 2021). Therefore, it is urgent to explore novel therapeutic strategies to prevent hypertensive renal damage.

Hypertension is an independent risk factor for chronic kidney disease (CKD). Long term hypertension leads to renal damage, including tubular interstitial fibrosis, vascular sclerosis, and glomerular sclerosis (Johnson and Dipietro, 2013). Multiple contributors, including activation of the renin–angiotensin–aldosterone system (RAAS), inflammation, oxidative stress, endoplasmic reticulum stress, apoptosis, and mitochondrial dysfunction, had been reported to play an essential role in hypertensive renal damage (Linkermann et al., 2014; Padda et al., 2015; Gai et al., 2016; Miloradović et al., 2016). Among them, abnormal activation of the RAAS is an essential cascade mediating a wide variety of physiological and pathophysiological events in kidney diseases (Benigni et al., 2001; Suzuki et al., 2007; Zhou and Liu, 2016).

The "classical" RAAS pathway involves the conversion of angiotensin I (Ang I) to angiotensin II (Ang II) by the angiotensin-converting enzyme (ACE). Then, Ang II interacted with Ang II type 1 (AT1) receptors, resulting in vasoconstriction, aldosterone and vasopressin release, salt and water retention, sympathetic activation, increased oxidative stress, sodium reabsorption, cell proliferation, and vascular hypertrophy (Yanes et al., 2006; Sullivan et al., 2010). Therefore, inhibition of RAAS is an essential strategy for antihypertension. However, currently used antihypertension medicines targeting RAAS failed to prevent Ang II-induced renal damage (Toto, 2006). Therefore, there is an urgent need to further explore novel strategies for antihypertension and prevention of hypertensive renal injury. Moreover, recent studies have revealed that Ang II stimulation significantly induced renal cell apoptosis *in vitro* and *in vivo*, suggesting the importance of preventing or attenuating hypertension-induced renal cell apoptosis (Ning et al., 2011).

Although antihypertensive medicines have made considerable progress, the existing antihypertensive medicines have a ceiling effect (Ferdinand et al., 2020). Therefore, complementary therapies to prevent hypertension-induced renal injury are urgently needed. As a Chinese medicine formula, the Qingxuan Jiang Ya Decoction (QXJYD), prescribed by academician Keji Chen of China, has been used to treat hypertension for decades and exhibits antihypertension and target organ (including vascular and kidney) protection (Xiao et al., 2016; Liu et al., 2017; He et al., 2020). Qingda granule (QDG) was simplified from QXJYD and consisted of *Gastrodia elata* Blume (Tianma), *Uncaria rhynchophylla* (Miz.) Miz. ex Havil. (Gouteng), *Scutellaria baicalensis* Georgi (Huangqin), and *Nelumbo nucifera* Gaertn (Lianzixin) in a ratio of 12:10:6:5.

Our previous studies demonstrated that QDG treatment significantly attenuated the elevation of blood pressure in both SHR- and Ang II-infused mice, promoted vasorelaxation of the thoracic aortic rings, inhibited cell proliferation of vascular smooth muscle cells, and suppressed the activation of multiple signaling pathways (including MAPK, AKT, Ca^{2+}/ERK, etc.) (Huang et al., 2019; Yu et al., 2020; Wu et al., 2021). Moreover, QDG treatment significantly alleviated cardiac inflammation, hypertrophy, apoptosis, and fibrosis and suppressed the activation of NF-κB, ROS/PI3K/AKT, and TGF-β1/Smad2/3 pathways (Cheng et al., 2021; Wu et al., 2020; Chen et al., 2021). In addition, QDG treatment also exhibited neuroprotective effects in both ischemic stroke mice and Ang II-infused mice with brain nerve injury (Zhang et al., 2020; Zhang et al., 2021).

However, the potential role of QDG on hypertensive renal injury remained to be further explored. Therefore, the purpose of the current study was to investigate the renal protection of QDG treatment in Ang II-infused mice or stimulated renal tubular epithelial cells, as well as to further explore its underlying mechanisms by performing multiple biological technologies. These findings may offer a new therapeutic strategy for treating hypertensive renal injury.

MATERIALS AND METHODS

Materials

Ang II was purchased from Abcam (Cambridge, United Kingdom). Isoflurane was obtained from Ruiwode Life Technology Co., Ltd. (Shenzhen, Guangdong, China). Hematoxylin and eosin were purchased from Solarbio Technology Co., Ltd., (Beijing, China). terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) Apoptosis Detection Kit I POD was purchased from Boster Biological Technology Co., Ltd. (Wuhan, Hubei, China). Annexin V-AbFluor™ 647 Apoptosis Detection Kit (cat. no. KTA0004), Cell Counting Kit-8 (CCK8), and antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (cat. no. Abp57259) were obtained from Abbkine (Wuhan, Hubei, China). Radioimmunoprecipitation assay (RIPA) lysis buffer, primary antibody dilution buffer, and secondary antibody dilution buffer were supplied by Beyotime Biotechnology (Shanghai, China). Polyvinylidene fluoride (PVDF) membranes were obtained from Millipore (Billerica, MA, United States). Antibodies against Bcl-2 (cat. no. 12789-1-

AP) and p53 (cat. no. 10442-1-AP) were purchased from ProteinTech (Rosemont, IL, United States). Antibodies against BAX (cat. no. 2772), caspase-3 (cat. no. 9662), and caspase-9 (cat. no. 9508) and secondary antibodies anti-mouse (cat. no. 7076) and anti-rabbit (cat. no. 7074) were purchased from Cell Signaling Technology (Danvers, MA, United States). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, and bicinchoninic acid (BCA) protein assay reagent kit were supplied by Thermo Fisher Scientific (Waltham, MA, United States).

Preparation of QDG

QDG is composed of four herbs: *G. elata* Blume (Tianma), *U. rhynchophylla* (Miz.) Miz. ex Havil. (Gouteng), *S. baicalensis* Georgi (Huang Qin), and *N. nucifera* Gaertn (Lianzixin) in a ratio of 12:10:6:5. QDG was extracted and supplied by Tianjiang Pharmaceutical Co., Ltd. (Jiangsu, China).

For the animal experiment, QDG was prepared as in our previous studies. The concentrations of QDG were selected based on our previous study (Cheng et al., 2021). Briefly, QDG power was dissolved in double-distilled water (dd H_2O) to the indicated concentration immediately before use. For cell culture experiments, the stocking solution of QDG was prepared in serum-free medium to 100 mg/ml and was prepared with completed medium to indicated concentrations of QDG before use.

Animals and Experimental Protocols

Male C57BL/6 mice (28 ± 1 g, 8 weeks) were purchased from SLAC Laboratory Animal Technology Co., Ltd. (Shanghai, China). All mice were raised under specific pathogen-free conditions, with controlled humidity (50%–60%) and temperature (23 ± 1°C), 12 h of light/dark cycle, and free access to food and water. After 1 week of adaptation, the mice were randomly divided into three groups ($n = 5$ each): Control, Ang II, and Ang II + QDG groups. Mice in the Ang II or Ang II + QDG group were infused with Ang II (500 ng/kg/min), while mice in the Control group were infused with saline for 4 weeks by subcutaneously implanting micro osmotic pumps. Mice in the Ang II + QDG group were intragastrically administrated with QDG daily (1.145 g/kg/day; 200 μl for each mouse), whereas mice in both Control and Ang II groups were intragastrically administrated with equal volumes of dd H_2O for 4 weeks. The animal experimental protocols of this study were approved by the Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine and were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Blood Pressure Measurement

The blood pressure, including systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP), was measured prior to the experiment and once a week for 4 weeks by using the tail-cuff plethysmograph method with the CODA™ noninvasive blood pressure system (Kent Scientific, Torrington, CT, United States).

Histological Analysis

Hematoxylin–eosin (HE) staining was performed to evaluate the morphological changes of renal tissues from each group. Briefly, renal tissues were fixed with 4% paraformaldehyde solution for 48 h, then embedded in paraffin, cut into 4 μm sections, and stained with hematoxylin for 1 min and eosin for 2 s. Then, the sections were visualized under an optical microscope (Leica, Wetzlar, Germany) at a magnification of ×400.

RNA Sequencing (RNA-seq)

The renal tissues were stored in RNA later (Takara, Beijing, China) at room temperature for 1 h and moved to −20°C for long-time storage. Total RNA was extracted by a TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, United States) according to the manufacturer's protocol. The concentration and quality of total RNA were measured by both Qubit 3.0 and Agilent 2100 Bioanalyzer. RNA samples with a RIN value of 7 or higher were used for further experiments.

RNA-seq was performed at Capital Bio Technology (Beijing, China). In brief, rRNA was removed from total RNA by a NEB Next rRNA Depletion Kit (NEB, Ipswich, MA, United States) according to the instructions. rRNA-depleted total RNA was fragmented, and the poly(A)-tailed mRNA molecules was generated by a NEB Next Ploy(A) mRNA Magnetic Isolation Module Kit (NEB, Ipswich, MA, United States) according to the manufacturer's instructions. Moreover, the final libraries were quantified on an Agilent 2100 Bioanalyzer using a KAPA Library Quantification Kit (KAPA Biosystem, Wilmington, MA, United States) and subjected to paired-end sequencing on an Illumina HiSeq sequencer (Illumina, San Diego, CA, United States).

The raw data were processed as described previously (Wu et al., 2021). The selected genes were further analyzed in the context of the information obtained from the database of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG).

Cell Culture and QDG Treatment

The renal tubular epithelial cell line NRK-52E was purchased from BeNa (Beijing, China) and cultured in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin. The cell lines were cultured in a humid environment at 37°C and 5% CO_2. The NRK-52E cells were cultured in six-well plates at a density of 0.8×10^5 cells per well. After being cultured for 24 h, NRK-52E cells were incubated in minimum medium (0.5% FBS) for around 8 h and were divided into three groups: Control group, Ang II group, and Ang II + QDG group. Then, cells in Ang II and Ang II + QDG groups were incubated with Ang II (1 μM), followed by treatment with QDG (25 and 50 μg/ml) in the Ang II + QDG group for 48 h.

TUNEL Staining

To assess cell apoptosis in renal tissues from each group, renal tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 4 μm, and stained using a TUNEL kit according to the manufacturer's instructions. The specific experimental procedures for TUNEL assay were performed as previously

described (Cheng et al., 2021). Slides were visualized under an optical microscope (Leica, Wetzlar, Germany) at a magnification of ×400, and five fields of view were randomly selected for each slide. Positively stained cells in each field were determined using ImageJ software.

For NRK-52E cells with or without Ang II stimulation and QDG treatment, the cells were fixed in 4% paraformaldehyde and incubated for 30 min at room temperature. The next step was the same as described above. Cells with a dark-brown nucleus were defined as apoptotic cells, while cells without or with a light-brown nucleus were defined as normal (non-apoptotic cells). The percentage of apoptotic cells was calculated as the ratio of the number of TUNEL-positive cells to the total number of cells examined.

Cell Viability Analysis

The cell viability of NRK-52E cells was determined by CCK8. Briefly, the cells were seeded into 96-well plates (2×10^3 per well) containing complete culture medium and cultured at 37°C in 5% CO_2 overnight. The indicated concentrations of QDG were added to each well for 48 h. At the end of treatment, a CCK8 reagent (10 μl per well) was added to each well and incubated at 37°C for 2 h in the dark. The absorbance was determined by an enzyme-labeling instrument (Multiskan FC, Thermo Fisher Scientific) at 450 nm wavelength. The cell viability of untreated cells was set as 100%.

Annexin V Staining

To provide a comparative assay of apoptosis by Annexin V staining, the NRK-52E cells were seeded into six-well plates at a density of 0.8×10^5 cells per well. After being cultured for 24 h, NRK-52E cells were cultured in serum-free DMEM for 12 h and then were stimulated with Ang II (1 μM) and treated with or without QDG (25 μg/ml or 50 μg/ml) in 0.5% of minimum medium (0.5% FBS) for a total of 24 h. At the end of treatment, cells were harvested and stained with Annexin V and propidium iodide (PI) according to the manufacturer's protocol. Cell apoptosis was detected and quantified by FACS (fluorescence-activated cell sorting) analysis.

Western Blot Analysis

The cell lysis buffer with protease inhibitor and PMSF was added to the renal tissues, which were then ground with a low-temperature grinder, applied on ice for 30 min, and centrifuged at 4°C and 14,000 *g* for 15 min. The protein concentration was quantified by BCA assay. A total of 50 μg protein from each sample was separated on 10% SDS-PAGE gel, transferred to PVDF membranes, and blocked with 0.5% bovine serum albumin for 2 h followed by incubation with the primary antibodies (overnight at 4°C), including rabbit anti-BAX (1:1,000), rabbit anti-BCL2 (1:1,000), rabbit anti-p53 (1:1,000), rabbit anti-cle-caspase-3 (1:1,000), rabbit anti-GAPDH (1:5,000), and mouse anti-cle-caspase-9 (1:1,000). Then, the membrane was washed with TBST and then incubated with secondary antibody at room temperature for 1 h. At the end of incubation, the membranes were washed with TBST, and then detection was performed using an electrochemiluminescence (ECL) kit. The expression of GAPDH protein was used as an internal control. The protein levels were analyzed with ImageJ software.

Statistical Analysis

The data were presented as mean ± SD. All statistical analyses were performed using the SPSS software (Version 22.0) for Windows (SPSS, Inc., Chicago, IL, United States). One-way analysis of variance was used to compare differences among three groups, when conforming to a normal distribution. The nonparametric Kruskal–Wallis test was used to compare differences among the three groups when not consistent with the normal distribution. A p-value <0.05 was considered as statistically significant.

RESULTS

QDG Attenuates Ang II-Induced Elevation of Blood Pressure and Renal Injury

The effect of QDG on hypertension-induced renal injury was assessed using Ang II-infused mice. Consistent with our previous studies (Na et al.; Zhang et al., 2020; Cheng et al., 2021; Wu et al., 2021), Ang II infusion significantly increased the blood pressure, including SBP (**Figure 1A**; $^*p < 0.05$, vs. the Control group), DBP (**Figure 1B**; $^*p < 0.05$, vs. the Control group), and MAP (**Figure 1C**; $^*p < 0.05$, vs. the Control group) of mice, which were attenuated after QDG treatment. Moreover, HE staining revealed significant renal pathological changes, including glomerular sclerosis and atrophy, epithelial cell atrophy, and tubular dilatation, which were alleviated after QDG treatment (**Figure 1D**). These results suggest that QDG treatment might relieve Ang II-induced hypertension and renal injury in Ang II-infused mice.

Identification of QDG Treatment on DETs in Renal Tissues of Ang II-Infused Mice

To explore the underlying mechanisms of QDG on hypertensive renal protection, RNA-seq was performed to identify the differentially expressed transcripts (DETs) in renal tissues among different groups (GSE182517; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE182517).

As shown in **Figures 2A,B** (left panels), Ang II infusion significantly upregulated the expression of 1,427 transcripts and downregulated the expression of 1,173 transcripts in renal tissues of mice, compared to the Control group, while QDG treatment significantly upregulated the expression of 868 transcripts and downregulated the expression of 868 transcripts, compared with the Ang II group (**Figures 2A,B**, right panel). The integrative analysis between the two comparisons (Ang II vs. Control and Ang II + QDG vs. Ang II) identified 261 downregulated transcripts (**Figure 2C**, left panel) and 401 upregulated transcripts (**Figure 2D**, right panel) in the Ang II group, which were reversed after QDG treatment.

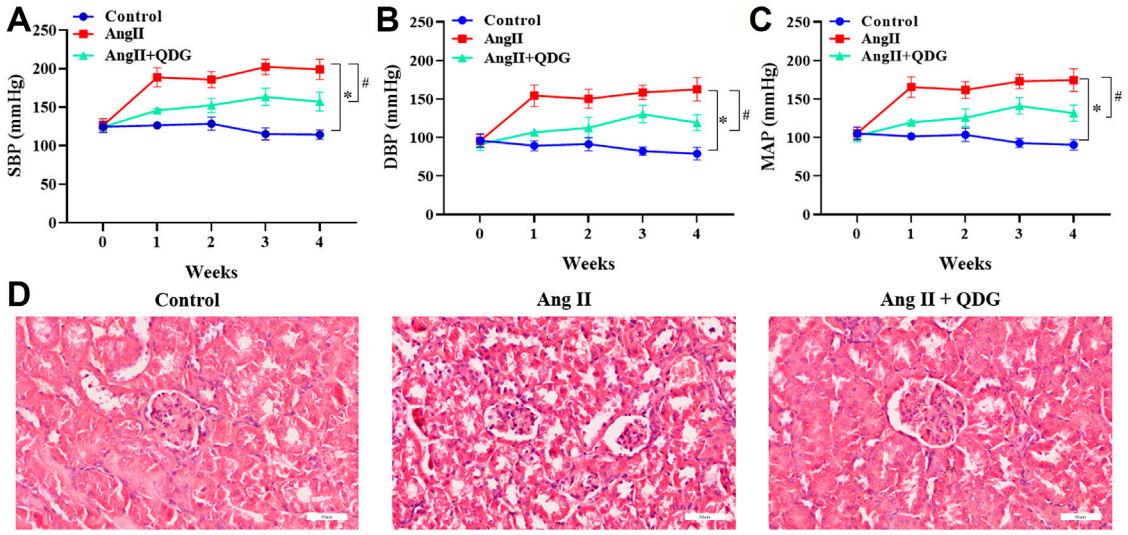

FIGURE 1 | QDG attenuates Ang II-induced elevation of blood pressure and renal injury. **(A)** SBP, **(B)** DBP, and **(C)** MAP of mice from each group were monitored prior to the experiment and once a week for 4 weeks by using the CODA™ noninvasive blood pressure system. **(D)** HE staining was performed to determine the pathological changes of renal tissues from each group. The representative images were taken at a magnification of ×400 (scale bar 60 μm). $n = 5$ for each group. Data were presented as mean ± SD; $^{*}p < 0.05$ vs. the Control group, $\#p < 0.05$ vs. the Ang II group.

Enrichment of Biological Processes and Signaling Pathways After QDG Treatment in Renal Tissues of Ang II-Infused Mice

GO enrichment analysis based on the DETs between Ang II and Control groups revealed multiple predicted potential functions. As shown in **Figure 3A**, cellular component (CC) analysis indicated that DETs were mainly involved in the intracellular part, intracellular membrane bounded organelle, and mitochondrion. Biological process (BP) analysis showed that DETs were mainly associated with metabolism, gene expression, and organelle organization. In the molecular function (MF) category, DETs were significantly enriched in binding, translation factor activity, etc. Moreover, GO enrichment analysis based on the DETs between Ang II + QDG and Ang II groups revealed multiple predicted potential functions. As shown in **Figure 3B**, CC analysis showed that DETs were associated with the cytoplasmic part, intracellular part, and cell part. As to BP, DETs were significantly enriched in the catabolic process, amide biosynthesis, and translation. The MF analysis for these DETs includes binding, catalytic activity, and translation elongation factor activity.

KEGG pathway analysis based on the DETs between Ang II and Control groups revealed multiple enriched signaling pathways, including apoptosis, p53 signaling pathway, and MAPK signaling pathway (**Figure 4A**). Moreover, KEGG pathway analysis based on the DETs between Ang II + QDG and Ang II groups demonstrated multiple enriched signaling pathways, including oxidative phosphorylation, apoptosis, and p53 signaling pathways (**Figure 4A**). The integrative analysis between the two comparisons (Ang II vs. Control and Ang II + QDG vs. Ang II) demonstrated that multiple signaling pathways were enriched in both comparisons (**Figure 4B**). Notably, we find that both cell apoptosis and p53 signaling pathways were significantly enriched in both comparisons (**Figure 4C**), which encouraged us to further explore the regulatory effects of QDG on cell apoptosis and activation of the p53 signaling pathway in renal tissues of Ang II-infused mice.

QDG Attenuates Ang II-Induced Renal Cell Apoptosis *In Vivo* and *In Vitro*

Further verification of QDG treatment on cell apoptosis of renal tissues by performing TUNEL staining revealed an increase of the percentage positively TUNEL-stained cells in renal tissues of Ang II-infused mice (**Figures 5A,B**; $^{*}p < 0.05$ vs. the Control group), while QDG treatment significantly reduced the percentage of positively TUNEL-stained cells (**Figures 5A,B**; $\#p < 0.05$, vs. the Ang II group), confirming the effect of QDG on attenuation cell apoptosis in renal tissues in Ang II-infused mice. We further assessed the effect of QDG treatment on cell apoptosis of a rat renal tubular epithelial cell line (NRK-52E) by performing TUNEL assay and found that 25 and 50 μg/ml of QDG treatment did not affect the cell viability of NRK-52E (**Figure 5C**) but attenuated the Ang II stimulation-induced cell apoptosis of NRK-52E cells (**Figures 5D,E**; $^{*}p < 0.05$, vs. the Control group, $\#p < 0.05$, vs. the Ang II group). Consistently, by performing Annexin V/PI staining, we found that Ang II stimulation significantly increased the percentage of cell apoptosis, which was attenuated after QDG treatment (**Figures 5F,G**; $^{*}p < 0.05$, vs. the Control group, $\#p < 0.05$, vs. the Ang II group).

QDG Activates the p53 Signaling Pathway *In Vivo*

To further verify the regulatory effect of QDG treatment on the activation of the p53 pathway, western blot confirmed that Ang II

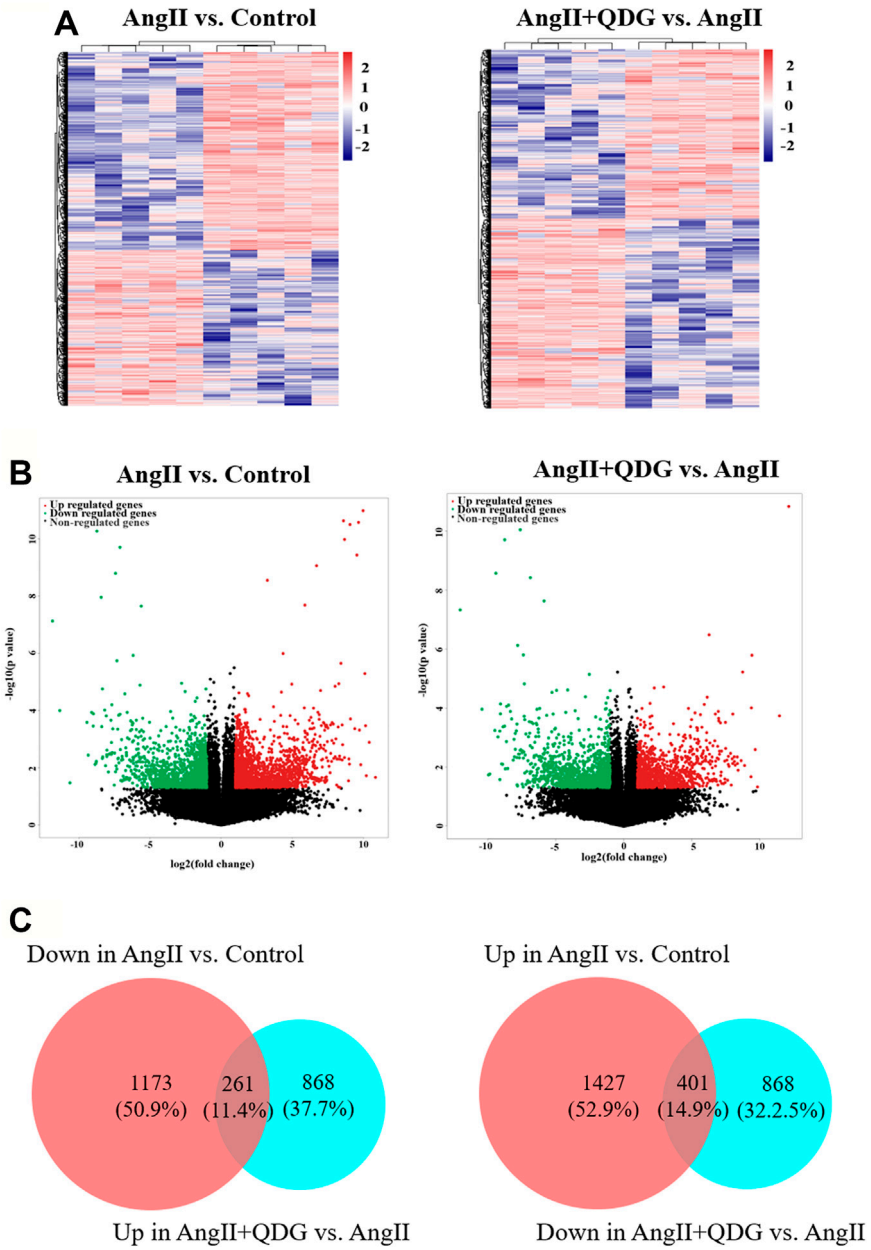

FIGURE 2 | QDG treatment on gene expression profiling of renal tissues in Ang II-infused mice. RNA-seq was performed to determine the DETs in renal tissues from each group. **(A)** Hierarchical clustering plots and **(B)** volcano plots were used to compare gene expression profiles (|fold change| ≥ 2, $p < 0.05$). **(C)** Integrative analysis was performed to identify the integrated transcripts between the two comparisons. The overlapped area in the left panel represents decreased transcript number (261) in the Ang II group but increased in the Ang II + QDG group. The overlapped area in the right panel represents increased transcript number (401) in the Ang II group but decreased in the Ang II + QDG group.

infusion significantly upregulated the protein expression of p53, which was downregulated after QDG treatment. Moreover, by determination of the expression apoptosis-related proteins using western blot analysis, it was revealed that Ang II infusion significantly upregulated the expression of proapoptotic proteins Bax, cle-caspase-9 and cle-caspase-3 but downregulated the expression of antiapoptotic protein Bcl-2 (**Figure 6**; $^{*}p < 0.05$, vs. the Control group), which were all reversed after QDG treatment (**Figure 6**; $\#p < 0.05$, vs. the Ang II group).

DISCUSSION

Long-term hypertension leads to multiple target organ damage, including renal damage. Our previous studies demonstrated that

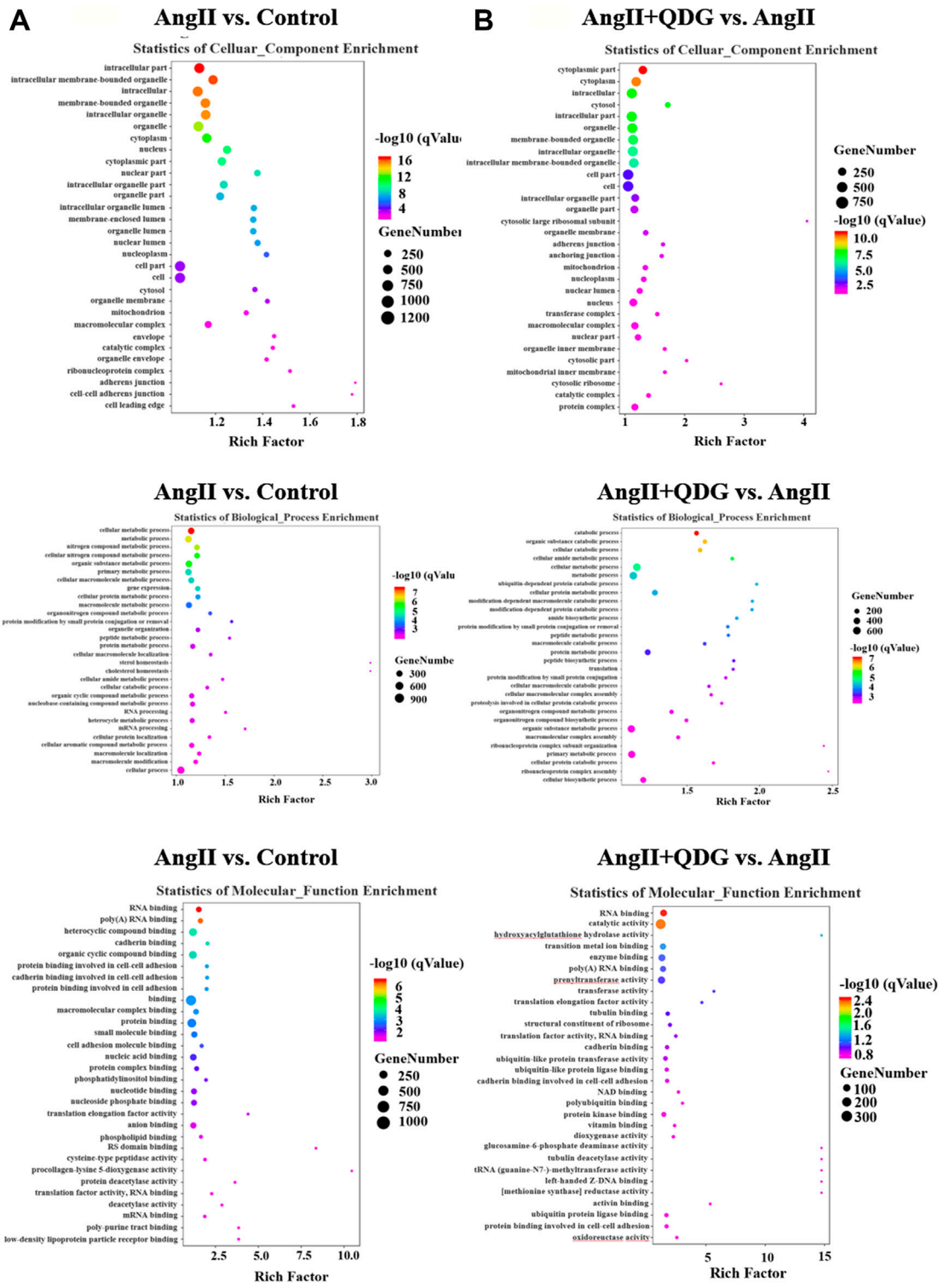

FIGURE 3 | GO enrichment analysis. GO enrichment analysis was performed based on the DETs from both comparisons of **(A)** Ang II vs. Control and **(B)** Ang II + QDG vs. Ang II. The top 30 enriched items of the cellular composition (left panel), biological processes (middle panel), and molecular function (right panel).

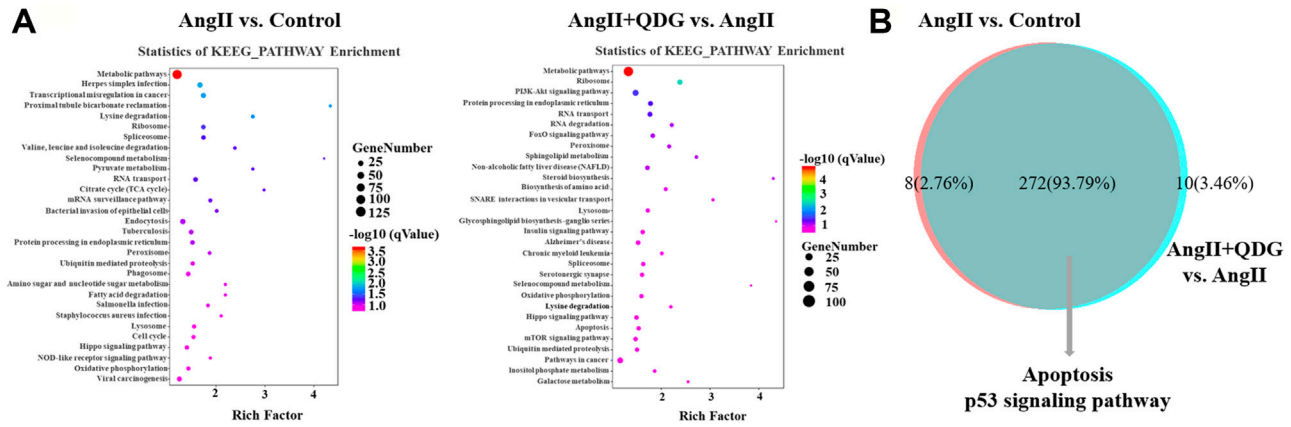

FIGURE 4 | KEGG pathway enrichment analysis. **(A)** KEGG enrichment analysis was performed to identify the enriched signaling pathway in both comparisons of Ang II vs. Control (left panel) and Ang II + QDG vs. Ang II (right panel). The top 30 enriched signaling pathways were presented. **(B)** Integrative analysis was performed to identify the integrated transcripts between the two comparisons. The overlapped area represents the number of enriched signaling pathways in both comparisons of Ang II vs. Control and Ang II + QDG vs. Ang II.

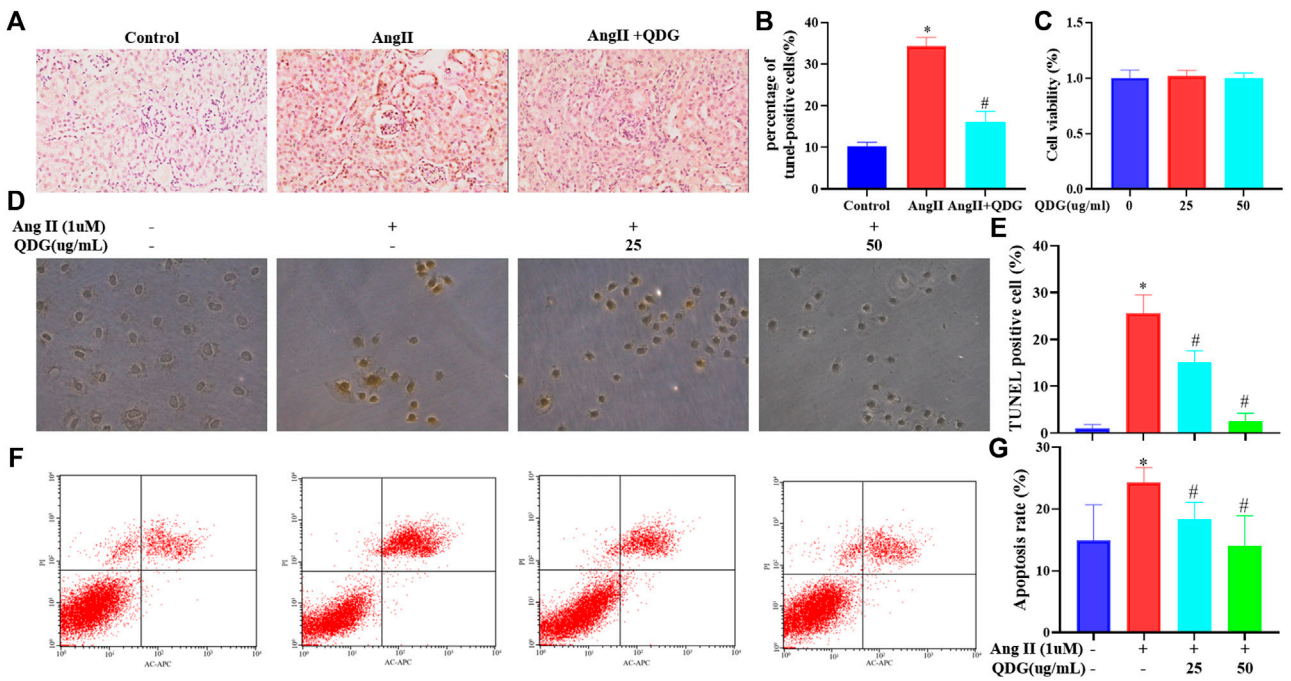

FIGURE 5 | QDG attenuates Ang II-induced renal cell apoptosis *in vivo* and *in vitro*. The TUNEL assay was performed to observe cell apoptosis in renal tissues of mice from each group **(A)** and the percentage of TUNEL-positive cells **(B)**. Viability of NRK-52E cells after treatment with QDG, as analyzed by the CCK-8 assay **(C)**. The TUNEL assay was performed to observe cell apoptosis in NRK-52E cells from each group **(E)** and the percentage of TUNEL-positive cells **(D)**. Representative dot plot of Annexin V- and PI-stained cells **(F)** and a columnar graph that comprised the percentage of apoptosis in NRK-52E cells **(G)**. Data were presented as mean ± SD; **p* < 0.05 vs. the Control group, #*p* < 0.05 vs. the Ang II group.

QDG exhibits antihypertension and multiple-target-organ protection (Huang et al., 2019; Wu et al., 2020; Yu et al., 2020; Zhang et al., 2020; Chen et al., 2021; Cheng et al., 2021; Wu et al., 2021). However, the therapeutic effects of QDG on hypertensive renal injury remained to be further explored. For the first time, the current study revealed that QDG treatment not only attenuated the elevated blood pressure but also alleviated renal cell apoptosis and mediated multiple signaling pathways (including the p53 pathway) in Ang II-infused mice. These results highlighted that QDG exhibited antihypertension and

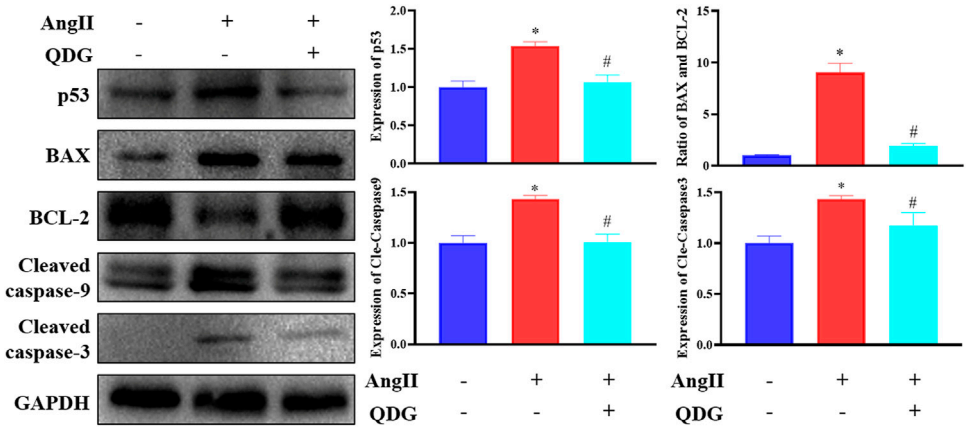

FIGURE 6 | QDG inhibits activation of p53 signaling pathways induced by angiotensin (Ang II). Western blot analysis was performed to determine the protein expression of p53, BAX, BCL2, and cle-caspase-9/caspase-3. GAPDH was used as the internal control. Data were presented as mean ± SD; *$p < 0.05$ vs. the Control group, #$p < 0.05$ vs. the Ang II group.

antiapoptotic effects on hypertensive renal tissues, and mediation by the p53 signaling pathway might be one of the underlying mechanisms.

Despite the development and combination of antihypertension medicines, a large number of hypertensive patients are still ultimately suffering from ESRD (Dong et al., 2021). Therefore, it is urgently required to explore and develop effective strategies for treatment of hypertension and hypertensive renal injury. As a Chinese medicine formula simplified from QXJYD, QDG exhibits antihypertension and vascular and cardiac protection, as well as neuroprotection (Huang et al., 2019; Wu et al., 2020; Yu et al., 2020; Zhang et al., 2020; Chen et al., 2021; Cheng et al., 2021; Wu et al., 2021). In addition, our current study revealed that QDG treatment not only attenuated the elevation of blood pressure but also significantly reduced Ang II-induced renal pathological changes, suggesting that QDG might be used as a complementary therapy to attenuate hypertension and prevent hypertensive target organ injury (including renal injury). However, renal damage should further be confirmed by determination of proteinuria expression or in other hypertensive animal models. Moreover, whether the renal protection of QDG treatment is dependent on blood pressure or not should be further addressed in future studies.

Due to the complicated mechanisms of Ang II-induced renal injury and QDG treatment in attenuating renal injury, we therefore performed RNA-seq, GO, and KEGG enrichment analyses to further explore their complicated mechanisms. Our current study identified 401 upregulated and 261 downregulated transcripts in renal tissues of mice in the Ang II group, which were reversed after QDG treatment. Among these DETs, upregulation of NOX4 (Kumar et al., 2020) and downregulation of ANGPT1 (Loganathan et al., 2018) had been reported to play an essential role in renal injury, which might be the targets of QDG on attenuation of hypertensive renal injury. Therefore, our current study suggests multiple targets of QDG treatment on hypertensive renal injury. However, further validation of QDG targets should be performed in future studies.

Abnormal activation of multiple signaling pathways, including TGF-β1/Smad and NF-κB signaling pathways, had been reported to play an essential role in hypertensive renal injury (Liu et al., 2017; Yan et al., 2018). Based on the DETs in the comparisons of both Ang II vs. Control and Ang II + QDG vs. Ang II, GO analysis enriched multiple biological processes, including translation, translation factor activity, catalytic activity of cell parts, and mitochondria, which might be involved in hypertensive renal injury. Moreover, KEGG enrichment analysis demonstrated that multiple signaling pathways (including MAPK and PI3K-Akt signaling pathways) were significantly enriched in both comparisons. Notably, integrative analysis between two comparisons found that both apoptosis and p53 signaling pathways were enriched. However, the regulatory effects of QDG on renal cell apoptosis and the p53 signaling pathway need to be further investigated.

As one of the characteristics of hypertensive renal injury, renal tubular epithelial cell (RTEC) apoptosis leads to the progression of renal tubulointerstitial fibrosis (Docherty et al., 2006; Ning et al., 2011; Johnson and Dipietro, 2013; Schelling, 2016; Russa et al., 2017; Dan et al., 2018), contributing to the development of end-stage nephropathy. Consistent with a previous study (Gu et al., 2021), the current study revealed that Ang II infusion significantly increased the percentage of cell apoptosis in renal tissues and cultured RTEC, which were attenuated after QDG treatment, suggesting the potential of QDG treatment for both antihypertension and attenuation of renal injury. However, the role of QDG treatment on renal inflammation, oxidative stress, and fibrosis should be further explored in future studies.

As one of the key regulators of apoptosis, p53 can be activated and stabilized through post-translational modification pathways, including ubiquitination, phosphorylation, and acetylation (Kruiswijk et al., 2015). Numerous studies have supported the involvement of p53 in cell apoptosis in multiple renal diseases (Tang et al., 2018; Wang Y. et al., 2018; Liu et al., 2019). Overexpression of p53 increases the expression of apoptotic protein Bax and decreases the expression of antiapoptotic protein Bcl-2, leading to cell apoptosis (Liu and Dan, 2018). Ang II induced

upregulation of BAX and downregulation of Bcl-2 in NRK-52E cells (Ning et al., 2011). Consistently, our current study found that the protein expression of p53, Bax, cle-caspase-9 and cle-caspase-3 was significantly upregulated and that of Bcl-2 was downregulated, while being partly reversed after QDG treatment in renal tissues of mice. These studies suggest that suppression of Ang II-induced p53 signaling pathway activation might be one of the underlying mechanisms of QDG attenuation of hypertensive renal cell apoptosis. In future studies, we intend to perform a series of experiments including using p53 transgenic mice or p53 inhibitors to definitively confirm the antiapoptotic role of QDG through the p53 pathway. In addition, the regulatory effect of QDG on other enriched signaling pathways and DETs should be further addressed in future studies.

CONCLUSION

In summary, the current study proved that QDG treatment significantly attenuated renal cell apoptosis in Ang II-infused mice model or Ang II-stimulated NRK-52E cells, and suppressing the activation of p53 pathway might be one of the underlying mechanisms, which may offer a new therapeutic strategy for treating hypertensive renal injury.

DATA AVAILABILITY STATEMENT

The RNA sequencing data used in present study are deposited at NCBI Gene Expression Omnibus (GEO) under the accession numbers GSE182517; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE182517.

ETHICS STATEMENT

This animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Fujian University of Traditional Chinese Medicine.

AUTHOR CONTRIBUTIONS

AS and JP conceived and designed the experiments. LL, YC, YF, and HL performed the animal experiments. JL, XZ, and YW conducted HE staining. XZ and LW conducted the western blot. XZ, YW, and JC analyzed the data. XZ, LL, and QX drafted the manuscript, which was revised by AS and JP. All authors gave critical contributions to the manuscript and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.770863/full#supplementary-material

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GLOSSARY

Ang II angiotensin II

BAX BCL2-associated X

BCA bicinchoninic acid

BP biological process

CC cellular component

CCK8 Cell Counting Kit-8

CKD chronic kidney disease

DBP diastolic blood pressure

dd H_2O double-distilled water

DETs differentially expressed transcripts

DMEM Dulbecco's modified Eagle's medium

ECL electrochemiluminescence

ESRD end-stage renal disease

FBS fetal bovine serum

GAPDH glyceraldehyde-3-phosphate dehydrogenase

GO Gene Ontology

HE hematoxylin–eosin

KEGG Kyoto Encyclopedia of Genes and Genomes

MAP mean arterial pressure

MF molecular function

PVDF polyvinylidene fluoride

QDG qingda granule

QXJYD Qingxuan Jiang Ya Decoction

TUNEL terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling

RAAS renin–angiotensin–aldosterone system

RIPA radioimmunoprecipitation assay

RNA-seq RNA sequencing

RTEC renal tubular epithelial cells

SBP systolic blood pressure

frontiers in Pharmacology

REVIEW
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Aryl Hydrocarbon Receptor Mechanisms Affecting Chronic Kidney Disease

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The aryl hydrocarbon receptor (AHR) is a basic helix-loop-helix transcription factor that binds diverse endogenous and xenobiotic ligands, which regulate AHR stability, transcriptional activity, and cell signaling. AHR activity is strongly implicated throughout the course of chronic kidney disease (CKD). Many diverse organic molecules bind and activate AHR and these ligands are reported to either promote glomerular and tubular damage or protect against kidney injury. AHR crosstalk with estrogen, peroxisome proliferator-activated receptor-γ, and NF-κB pathways may contribute to the diversity of AHR responses during the various forms and stages of CKD. The roles of AHR in kidney fibrosis, metabolism and the renin angiotensin system are described to offer insight into CKD pathogenesis and therapies.

Keywords: RAAS, aryl hydrocarbon (Ah) receptor, kynurenine, hypoxia, PPAR γ, TGF−β1

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INTRODUCTION

Chronic kidney disease (CKD) affects approximately 37 million (1 in 7) US adults and is the ninth leading cause of death (CDC, 2020). The global incidence of CKD has been estimated at 13.7% (11.7–15.1%) of the nearly eight billion people, or over one billion cases. Current therapies may slow and occasionally halt CKD progression, but most patients continue to progress. Further, these therapies are not fully available to or fully used by many of the globally-affected individuals. CKD is accompanied by premature morbidity and mortality from cardiovascular disease, as hypertension and uremia combine to drive cardiac and vascular damage.

CKD can be caused by systemic diseases and by primary kidney diseases. CKD due to systemic disease is more common; causes include diabetes mellitus (Navaneethan et al., 2021), malignant hypertension (van den Born et al., 2005), systemic lupus erythematosus and systemic vasculitis (Moiseev et al., 2020). Primary kidney diseases, those that chiefly affect the kidney, include polycystic kidney disease, interstitial nephritis, and podocytopathies (minimal change disease, focal segmental glomerulosclerosis and membranous nephropathy) (Curran and Kopp, 2021).

Many CKD patients progress to end-stage kidney disease. Kidney transplant extends life for chronic dialysis patient by a median of 12 years (2014–2017: median 11.7 for deceased donor kidney recipients, median 12.1 years for living donor kidney recipients) (Poggio et al., 2020) and longer in some cases (Zolota et al., 2020). Many patients on chronic dialysis, particularly in the First World, are older and have co-morbidities; their 5-year survival rate has been reported as 56% (Nordio et al., 2012).

Increased rates of cardiovascular disease in CKD patients are the major driver of reduced survival (Jankowski et al., 2021). Thus, better strategies are needed to prevent CKD and to slow or halt progressive loss of kidney function in individuals with CKD.

CKD incidence is linked to several risk factors. Non-modifiable risk factors include genetic variants, low birth weight, and older age. Notable genetic variants include polymorphisms in angiotensin-I converting enzyme (*ACE*), angiotensin II type 1 receptor (*AGTR1*) and apolipoprotein L1 (*APOL1*), the latter accounting for much of the excess CKD risk among populations with sub-Saharan ancestry. Common modifiable risk factors include smoking, obesity, hypertension, diabetes mellitus, excessive alcohol consumption, heavy metal exposure, and excessive use of analgesic medicines (Kazancioglu, 2013). These factors compromise or alter kidney cell function, affecting cells in the glomerulus (podocytes, mesangial cells, and endothelial cells) and in the tubulo-interstitium (tubular cells, endothelial cells, and fibroblasts).

The various factors that induce CKD allow for the development of animal models to mimic the response. These can include 5/6 nephrectomy, unilateral ureteral obstruction nephropathy, angiotensin II (Ang II)-induced hypertension, ischemia/reperfusion-induced acute kidney injury, cisplatin- or cyclosporin A-induced nephropathy or models of systemic disease. An underlying factor in animal or human CKD progression is the aryl hydrocarbon receptor (AHR). This nuclear receptor is activated by endogenous and exogenous ligands and is required for normal kidney development and function. In cultures of murine metanephros, the AHR ligand, benzo(a)pyrene (BaP), disrupted nephrogenesis (Falahatpisheh and Ramos, 2003) and in fetuses from mice fed the AHR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin), hydronephrosis was a common malformation (Peters et al., 1999). AHR renal mRNA levels were also higher in female rats compared to males and at 7 weeks post-5/6 nephrectomy, AHR renal mRNA levels decreased in females, but not males, highlighting a role for AHR in renal gender differences (Lu et al., 2006).

In an animal model of CKD, AHR is not only activated in the kidney but additional organs. Specifically, transgenic mice expressing the AHR responsive-promoter tethered to a β-galactosidase reporter gene and subjected to ischemia/reperfusion-induced acute kidney injury identified AHR activation in the proximal and distal renal tubules, cardiac myocytes, hepatocytes, and microvasculature in the cerebral cortex (Walker et al., 2020). This suggests a presence of AHR ligands in serum, which can activate cells in the kidney, heart, brain, and liver. By culturing serum with an AHR reporter cell line, elevated AHR activity was identified in diabetic nephropathy patients compared to controls (Kim et al., 2013). Serum levels of the AHR ligand, indole-3 acetic acid, measured as a predictor of cardiovascular events and mortality in 120 CKD patients with stage 3–5 CKD and stage 5D CKD (Dou et al., 2015). Indole-3 acetic acid (Lin et al., 2019) and another AHR ligand, indoxyl sulfate (Yeh et al., 2016), are additionally associated with cognitive impairments in CKD patients. The gut is the source of indole, which is processed in the liver to indoxyl sulfate, highlighting AHR ligand crosstalk between organs and within the liver (Lowenstein and Nigam, 2021).

The heterogeneity of CKD presentation and progression may be associated with the diversity of AHR ligands that promote and inhibit disease. As shown in **Table 1**, select AHR compounds that promote kidney injury include non-steroidal anti-inflammatory drugs (NSAIDs), tryptophan metabolites, bacterial pigments, proton pump inhibitors, certain antibiotics, and polycyclic aromatic hydrocarbons (PAHs). Select AHR compounds that inhibit kidney injury include quinoline derivatives, dietary compounds (e.g., resveratrol, indole-3-carbinol), and the tryptophan photo-oxidation product, 6-formylindolo (3,2-b) carbazole (FICZ). The uremic solutes, which include tryptophan metabolites (e.g., indoxyl sulfate, indole-3-acetic acid), contribute to increased vascular permeability, vessel leakage and inflammation (Falconi et al., 2021). The mechanisms may involve indoxyl sulfate-induced endothelial adhesion molecules, which bind to and recruit leukocytes as shown *in vitro* (Ito et al., 2016). In addition, the AHR antagonist, resveratrol, blocked indoxyl sulfate-induced bovine aorta endothelial cell permeability *in vitro* (Assefa et al., 2019), further supporting a function of AHR in CKD pathogenesis.

Independent of the inciting cause of kidney damage, subsequent progression of CKD is associated with metabolic disturbances, oxidative stress, and inflammation, all of which promote fibrogenesis, irreversible nephron loss, and ultimately reduce the glomerular filtration rate (Ruiz-Ortega et al., 2020). Reduced kidney function leads to the retention of various metabolic products, particularly nitrogenous compounds and often excess fluid, sodium, potassium and phosphate, among many small molecules. These metabolic alterations are characteristic of uremia, and some of these in turn, promote cardiovascular disease (Ghoshal and Freedman, 2019).

FIBROSIS IN CKD

Tissue damage from injury or disease induces tissue remodeling and repair. Dysregulation of this process causes an imbalance in extracellular matrix (ECM) homeostasis and the formation of fibrotic tissue. Kidney interstitial ECM consists of diverse molecules, of which the principal families are collagen (types I, II, III, V, VI, VII, and XV), glycoproteins (*e.g.,* fibronectin, laminin), proteoglycans (*e.g.,* biglycan, decorin, versican) and glycosaminoglycans (*e.g.,* chondroitin sulfate, dermatan sulfate, heparin sulfate, hyaluronan). Collectively, these molecules anchor cells within tissue (Bulow and Boor, 2019).

The human genome encodes three isoforms of TGF-β (TGF-β1, TGF-β2, TGF-β3). With regard to extracellular matrix biology; all three isoforms may promote collagen production and tissue fibrosis (Curran and Keely, 2013; Sun T. et al., 2021; Panizo et al., 2021). Activated inflammatory cells secrete inactive TGF-β1 bound non-covalently to a latency associated peptide (LAP), which is disulfide bound to the latent TGF-β binding protein (LTBP). Tissue transglutaminase-2 binds the released complex of LTBP:LAP:TGF-β1 to the ECM by enzymatically cross-linking LTBP to fibronectin and possibly additional ECM proteins associated with elastic fibers (Nunes et al., 1997). Active TGF-β1 is released from the large latent complex by integrin-mediated mechanical deformation of the ECM and/or through degradation of LAP by proteases (*e.g.,* MMPs, thrombospondin-1, and plasmin) (Curran and Keely, 2013).

TABLE 1 | Function of select AHR ligands in chronic kidney disease.

Molecular class	AHR ligand	Experimental results	Setting	Effect on CKD
Non-steroidal anti-inflammatory drug (NSAID)	**Diclofenac** Anti-inflammatory Bass et al. (2009)	Decreased renal perfusion in healthy subjects 1 h after a single oral dose (50 mg) in health subjects Hellms et al. (2019)	Human	Promotes
NSAID	**Sulindac** Anti-inflammatory Ciolino et al. (2006)	Promotes chronic decrements in glomerular filtration rate in patients with renal insufficiency Murray et al. (1995)	Human	Promotes
Quinoline-3-carboxamide derivative that is structurally similar to kynurenine	**Laquinimod** Anti-inflammatory Blocks S100A9 binding to toll-like receptor (TLR)-4 or receptor for advanced glycation end-products (RAGE) Boros and Vecsei (2020)	Oral therapy (1–25 mg, x3/week) delays the development of lupus nephritis in a murine model Lourenco et al. (2014)	Mouse	Inhibits
Quinoline-3-carboxamide derivative that is structurally similar to kynurenine	**Paquinimod** Anti-inflammatory Blocks S100A9 binding to TLR4 or RAGE Boros and Vecsei (2020)	Paquinimod in drinking water inhibits glomeruli complement deposition and hematuria in a murine model (Bengtsson et al., 2012)	Mouse	Inhibits
Quinoline derivative that reversibly inhibits ATP binding to vascular endothelial growth factor receptor-2 (VEGR-2)	**Semaxanib** Anti-inflammatory VEGFR-2 inhibitor Mezrich et al. (2012)	Intravenous (x2/week, 145 mg/m^2) administration induced complete resolution of all metastatic tumors in a renal cell carcinoma patient (Jennens et al., 2004)	Human	Inhibits
4-amino-5-(4-chlorophenyl)-7-(dimethylethyl) pyrazolo [3,4-d] pyrimidine (PP2)]	**PP2** Antagonizes proliferation, inflammation, differentiation, adhesion, migration, apoptosis, autophagy and angiogenesis Src family kinase inhibitor Frauenstein et al. (2015)	Intraperitoneal (2 mg/kg) injection improves kidney function and attenuates kidney tubular injury in a murine LPS-induced acute kidney injury model Pak et al. (2020)	Mouse	Inhibits
Proton pump inhibitor	**Omeprazole** Inhibits parietal cell H+/K + ATP pump Novotna et al. (2014)	Promotes dose-dependent cell death in human and murine proximal tubular cell lines and in human primary proximal tubular cell cultures (Fontecha-Barriuso et al., 2020)	Human cell culture	Promotes
Proton pump inhibitor	**Lansoprazole** Inhibits parietal cell H+/K + ATP pump Novotna et al. (2014)	Intraperitoneal (25 mg/kg) injection increases cell death and inflammation in a murine cisplatin-induced acute kidney model Ye et al. (2021)	Mouse	Promotes
Antibiotic	**Rifampicin** Binds and inhibits bacterial DNA-dependent RNA polymerase Puyskens et al. (2020)	Acute kidney injury in 25 tuberculosis and leprosy patients in response to rifampicin therapy Muthukumar et al. (2002)	Human	Promotes
Tryptophan metabolite	**Kynurenine** Anti-inflammatory Mezrich et al. (2010)	Lower estimated glomerular filtration rate was related to higher plasma kynurenine levels in a meta-analysis Cheng et al. (2020)	Human	Promotes
Tryptophan metabolite	**Indole sulfate** Pro-inflammatory Uremic toxin Schroeder et al. (2010)	Induces glomerular lesions in mice, alters podocyte function and increases inflammation Ichii et al. (2014) Levels are associated with increased mortality in hospital-acquired acute kidney injury Wang et al. (2019)	Mouse Human	Promotes
Tryptophan metabolite	**Indole-3-acetic acid** Pro-inflammatory Pro-thrombotic Uremic toxin Addi et al. (2019)	Blood levels are increased with chronic kidney disease stage 5D and fell substantially after kidney transplantation Liabeuf et al. (2020)	Human	Promotes
Tryptophan metabolite	**Indoxyl glucuronide** Hypoxic transcription factor antagonist Uremic toxin Asai et al. (2018)	Serum levels are elevated in hemodialysis patients Itoh et al. (2013)	Human	Promotes

(Continued on following page)

TABLE 1 | (*Continued*) Function of select AHR ligands in chronic kidney disease.

Molecular class	AHR ligand	Experimental results	Setting	Effect on CKD
Tryptophan photo-oxidation product	**6-formylindolo (3,2-b) carbazole (FICZ)** Promotes IL-22 production Regulates Th17 and T regulatory cell development Concentration-dependent activity Rannug and Rannug (2018)	Intraperitoneally FICZ administered (100 μg/kg/d for 4 days) to mice with rhabdomyolysis and ischemia/reperfusion-induced acute kidney injury attenuated kidney damage Tao et al. (2021)	Mouse	Inhibits
Polycyclic aromatic hydrocarbons (PAHs)	**Benzo (a) pyrene (B[a]P)** Carcinogen Shimizu et al. (2000)	Intraperitoneal injection produces oxidative stress, DNA damage and reduced kidney function Deng et al. (2018)	Mouse	Promotes
Pseudomonas aeruginosa bacterial pigments	**Phenazines** (1-hydroxyphenazine, phenazine-1-carboxylic acid, phenazine-1-carboxamide, pyocyanin) Pro-inflammatory Cytotoxic Moura-Alves et al. (2014)	*Pseudomonas aeruginosa* urinary tract infections are associated with high mortality in hospitalized patients Lamas Ferreiro et al. (2017)	Human	Promotes
Mycobacterium tuberculosis bacterial pigment	**Naphthoquinone phthiocol** Pro-inflammatory Cytotoxic Moura-Alves et al. (2014)	Interstitial nephritis and acute renal failure occur in response to disseminated infection or a localized genitourinary disease Daher Ede et al. (2013)	Human	Promotes
Epstein-Barr virus (EBV) latent protein	**EBV nuclear antigen 3** Function not significantly explored Kashuba et al. (2006)	EBV genome is present in proximal tubule epithelial cells of patients with chronic interstitial nephritis Becker et al. (1999)	Human	N/A
Bioactive compound found in cruciferous vegetables	**Indole-3-carbinol (I3C)** Anti-inflammatory Anti-angiogenic (Popolo et al., 2017)	Oral pre-treatment (20 mg/kg/day) improves cisplatin-induced acute nephrotoxicity indices in rats El-Naga and Mahran (2016)	Rat	Inhibits
Polyphenolic compound present in grapes	**Resveratrol** Antagonizes AHR transcriptional responses in an estrogen receptor-α-dependent manner Chemoprotective Cardioprotective Perdew et al. (2010)	Oral administration (5 mg/day/100 g) at the initiation of a rat anti-glomerular basement membrane nephritis model reduces proteinuria, hypoalbuminemia and hyperlipidemia Nihei et al. (2001)	Rat	Inhibits

Legend. Shown are 12 classes of compounds that bind aryl hydrocarbon receptors (AHR), either activating or suppressing AHR activity. Some compounds affect kidney function. Compounds with negative effects tend to speed chronic kidney disease (CKD) progression, while those with positive effects tend to slow CKD progression in animal models and/or human patients.

Intracellular signals downstream from the TGF-β1 receptor stimulate interstitial myofibroblast proliferation and secretion of collagen and additional ECM proteins (Panizo et al., 2021), promote anaerobic metabolism (Zhao et al., 2020), regulate immune cell differentiation (Sanjabi et al., 2017), and induce the expression of integrins (Curran and Keely, 2013) and the nuclear factor-kappa-B (NF-κB) subunit, p65 (Sun et al., 2015). Peroxisome proliferator-activated receptor-gamma (PPAR)-γ ligands (Guo et al., 2004), estrogen (Ito et al., 2010), and AHR ligands (Woeller et al., 2016; Shi et al., 2020) antagonize TGF-β1 cell signaling. The expression of TGF-β1 is induced by hypoxia (Mingyuan et al., 2018), angiotensin II (Kagami et al., 1994), cytokines (interleukin (IL)-4, IL-13), advanced glycation end products (AGEs) (Li et al., 2004), and autocrine cell signals (Bhogal et al., 2005). These factors are therefore likely to contribute to fibrogenesis in CKD.

With respect to AHR, the quinoline-3-carboxamide derivative and AHR ligand, paquinimod, inhibits fibrosis in murine models of experimental systemic sclerosis (Stenstrom et al., 2016) and liver fibrosis (Fransen Pettersson et al., 2018). The anti-fibrotic functions of paquinimod in CKD have not been significantly explored. Mice fed a diet supplemented with 0.25% adenine generate increased levels of the AHR ligand, indoxyl sulfate, comparable to human CKD patients, leading to periglomerular fibrosis (Walker et al., 2020). These data are supported in rats administered 200 mg/kg indoxyl sulfate drinking water, resulting in the elevated expression of the mesenchymal marker, α-smooth muscle actin, and increased Masson's trichrome-positive fibrosis in the kidney (Bolati et al., 2011). Indoxyl sulfate induces TGF-β1 expression and production in human proximal tubular cells (HK-2 cells) and the antioxidant, indole-3-propionic acid, suppresses this response (Yisireyili et al., 2017). The indole acetic acid derivative, mitochonic acid 5 (MA-5), inhibits mitochondrial reactive oxygen species and improves renal function in an ischemia-reperfusion injury model and a cisplatin–induced nephropathy model (Suzuki et al., 2016). Moreover, mice administered MA-5 through osmotic pump in a model of unilateral ureteral obstruction demonstrated reduced expression of TGF-β1, decreased collagen I staining, and reduced renal fibrosis (Shima et al., 2017). Because dioxin-

induced AHR promotes mitochondrial reactive oxygen species production and in AHR deficient mice, cellular mitochondrial reactive oxygen species are lower compared to controls (Brinkmann et al., 2019), the type of ligand and presence of AHR are likely important to oxidative stress in CKD.

Oxidative stress is associated with the activity of lysyl oxidases (LOX) (Martinez-Revelles et al., 2017) and transglutaminases (Basso et al., 2012), which promote vascular stiffness and neuronal death in murine models, respectively. These pleotropic proteins are involved in cell signaling and post-translational modifications, including the cross-linking of collagen. Calcium-dependent transglutaminases catalyze protein cross-links by introducing glutamyl-lysyl isopeptide bonds between target proteins. LOX catalyzes the formation of aldehydes from lysine residues in collagen and elastin, and this promotes cross-linking of these molecules in tissue (Heck et al., 2013). Transglutaminase-2 and LOX expression are induced by hypoxia and TGF-β (Curran and Keely, 2013), indicating that these molecules facilitate increased cross-linking and fibrosis in tissues with a compromised blood supply.

Excessive ECM accumulation is prevented by the activity of matrix metalloproteinases (MMPs), which cleave fibrillar collagens (Zhao et al., 2020). The AHR ligands FICZ (Shi et al., 2020), kynurenine (Li et al., 2014) and dioxin (Tsai et al., 2014) promote MMP-1 *in vitro*. The AHR ligand indole-3-carbinol inhibits MMP-2 and MMP-9 *in vitro* (Wu et al., 2005) and in a murine model of liver fibrosis, the AHR ligand paquinimod reduced the expression of MMP-2 (Fransen Pettersson et al., 2018), highlighting the diversity of the AHR response in ECM maintenance. Increased production and deposition of type I collagen, primarily by fibroblasts, is associated with increased transglutaminase-2 activity, promoting the formation of a stiff matrix; this can progress to overt kidney fibrosis (Bulow and Boor, 2019; Zhao et al., 2020).

In a study of 202 kidney disease cases of different etiologies, the levels of serum LOX and tissue LOX in renal biopsies were associated with the presence and degree of kidney fibrosis across diseases (Zhang XQ. et al., 2020). In cyclosporin A-induced nephropathy in mice, treatment with LOX inhibitors attenuated inflammation, fibrosis and uremia (Nguyen et al., 2021). Additionally, rats undergoing 5/6-nephrectomy and treated with transglutaminase inhibitors prevented a decline in kidney function and interstitial fibrosis (Johnson et al., 2007), highlighting these cross-linkers as targets in CKD. A role for AHR in regulating these cross-linkers through MMP production or oxidative responses has not been significantly explored.

CKD THERAPIES

Treatment of CKD involves several approaches. Treating the underlying disease, including systemic diseases (diabetes mellitus, systemic lupus, systemic vasculitis, and others), may slow or halt progression (Moiseev et al., 2020; Navaneethan et al., 2021). For example, the sodium-glucose transport protein 2 (SGLT2) inhibitor dapgliflozin was recently approved for progressive non-diabetic kidney disease of many etiologies as a result of studies demonstrating efficacy in reducing the risk for a combined endpoint of kidney function decline, kidney failure, cardiovascular death and hospitalization for heart failure (Wheeler et al., 2021). Pathway-targeted treatments are increasingly available or in development for primary renal diseases or syndromes (*e.g.,* focal segmental glomerulosclerosis, membranous nephropathy, and polycystic kidney disease) (Curran and Kopp, 2021; Finnigan and Leslie, 2021). At the other end of the frequency scale, there are an estimated 10,000 rare diseases (Haendel et al., 2020) and an unknown fraction of these have renal manifestations; many of these lack targeted therapies.

Non-specific therapies for CKD can slow and even halt progressive loss of kidney function. These include diet and medications for blood pressure control (target <130/80) (Faqah and Jafar, 2011), antifibrotic therapies (inhibitors of renin, angiotensin II, and aldosterone, the latter being a potent profibrotic molecule) (Zhang et al., 2019), and dietary sodium restriction and thiazide diuretics (the latter potentiates the antiproteinuric effects of the renin-angiotensin-aldosterone system antagonists) (Park et al., 2014). SGLT2 inhibitors reduce progression of kidney damage in diabetes (Tuttle et al., 2021) and recently this effect has been shown in non-diabetic kidney disease as well, possibly by reducing proximal tubule stress (Almaimani et al., 2021).

There continues to be considerable interest in developing novel anti-fibrotic therapies for kidney disease, although progress has been slow. Pirfenidone is an anti-fibrotic agent (2014 U.S. FDA approval reference ID: 3642437) that showed efficacy in phase two trials for idiopathic pulmonary fibrosis (Raghu et al., 1999; Maher et al., 2020). Preliminary studies of pirfenidone in studies of focal segmental glomerulosclerosis (Cho et al., 2007) and in diabetic nephropathy (Sharma et al., 2011) were encouraging but further development appears to have stalled. The exact mechanisms of action of pirfenidone are not fully characterized. Recent studies suggest the molecule may be a ligand for peroxisome proliferator-activated receptors (e.g., PPAR-α, PPAR-γ) (Hamidi et al., 2021), which appears to inhibit transforming growth factor (TGF)-β1-induced collagen I production (Cui et al., 2020). Pirfenidone may also suppress other profibrotic and pro-inflammatory mediators, including fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) (Chaudhary et al., 2007), interleukin-1β (IL-1β) and tumor necrosis factor (TNF) (Evani et al., 2020).

Similarly, ligands of the aryl hydrocarbon receptor (AHR), specifically tryptophan metabolites [*e.g.,* 2-(10 H-indole30-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) and FICZ], are capable of inhibiting TGF-β1-induced collagen I production in models of systemic sclerosis and thyroid eye disease (Woeller et al., 2016; Shi et al., 2020). An analog (SZR72) of kynurenic acid, a tryptophan metabolite and AHR ligand, inhibits the production of TNF in human blood cultures (Balog et al., 2021). Also, the plant-derived AHR ligand, indole-3-carbinol (I3C), antagonizes IL-1β production in cell lines (Jiang et al., 2013). AHR expression is induced by growth factors (PDGF, FGF) (Vaziri et al., 1996) and in a murine model of

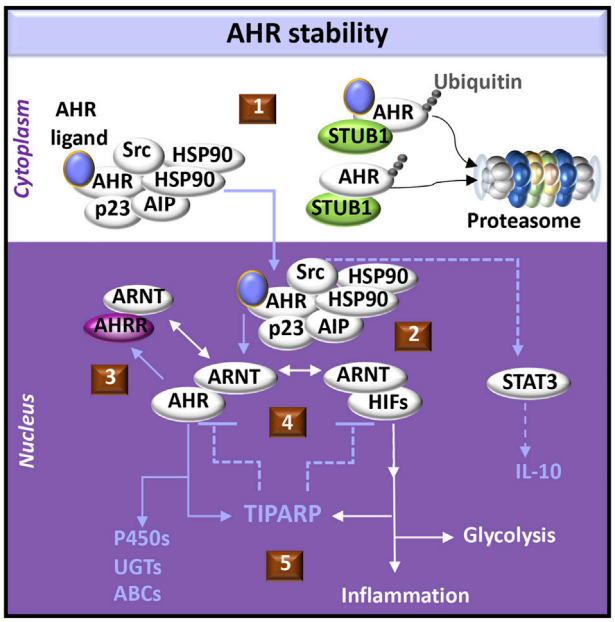

FIGURE 1 | AHR-initiated cell signaling pathways. (1) The aryl hydrocarbon receptor (AHR) forms a complex with chaperone molecules in the cytoplasm. Ligand binding may promote transport of the complex into the nucleus. Alternatively, release of AHR from the complex may promote interactions with an E3 ligase (*e.g.,* STUB1) which acts as a platform for AHR ubiquitination and targeted degradation *via* the proteasome. Both ligand-bound and unliganded AHR can be targeted to the proteasome. (2) AHR dissociates from its cytoplasmic complex to bind the AHR nuclear translocator (ARNT), which alternatively may dimerize with hypoxia inducible transcription factors (HIFs). AHR induces activation of signal-transducer and activator of transcription (STAT3) through Src signaling, acting as a tyrosine protein kinase involved in the production of IL-10. (3) AHR promotes the expression of the AHR repressor (AHRR), which also dimerizes with ARNT and negatively regulates AHR functions by competing with AHR binding sites in DNA regulatory sequences. (4) Activation of AHR or HIFs induces transcription and translation of PARP7 (poly ADP-ribosyl transferase 7), also known as TIPARP (TCDD-inducible poly-ADP-ribose polymerase). TIPARP further promotes polyADP-ribosylation and subsequent degradation of AHR and HIFs (5) Downstream responses of AHR can include activation of the biotransformation enzymes, including cytochrome P450 enzymes (P450s), UDP-gluconosyltransferases (UGTs), and ATP-binding cassette transporters (ABCs). Downstream responses of HIFs can include increased glycolysis and the expression of immunomodulatory genes that provoke inflammation.

cardiac hypertrophy, AHR antagonizes hypoxia-induced VEGF production and the development of fibrosis (Ichihara et al., 2019). These functions of AHR suggest that AHR may be a therapeutic target in CKD. However, the AHR response is defined by a diverse array of toxins, endogenous molecules, drugs, dietary components, and pathogens that may promote or inhibit CKD (**Table 1**). AHR may additionally exhibit crosstalk with estrogen receptors, PPAR-γ, NF-kB, and cell signals in hypoxia and TGF-β1 pathways. In this regard, AHR has shown considerable, and puzzling, diversity of function in the kidney and manifestations associated with CKD. The effects of AHR in cell signaling pathways that influence fibrosis, the renin angiotensin aldosterone system (RAAS), and metabolism are therefore herein described.

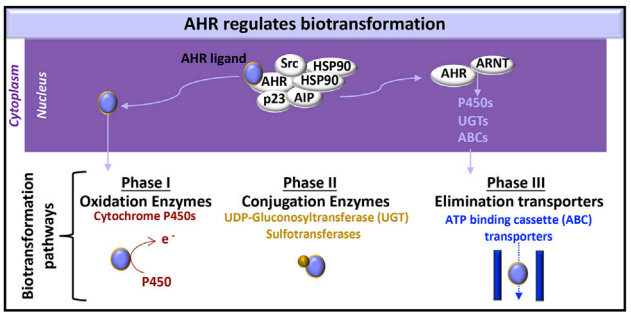

FIGURE 2 | AHR-initiated biotransformation. AHR induces the transcription of certain genes whose products are involved in each of the three phases of drug metabolism.

AHR STABILITY, SIGNALING, AND E3 UBIQUITIN LIGASE ACTIVITY

AHR is a ligand-activated transcription factor and E3 ubiquitin ligase with pleotropic functions in mammalian biology. Sequence homology between circadian [period (*PER*)] and neurodevelopment (single-minded (*SIM*) genes in *Drosophila melanogaster* and a gene in the human dioxin signaling pathway (AHR nuclear translocator (*ARNT*)) established the PER-ARNT-SIM (PAS) domain protein superfamily, of which AHR is a member. PAS domains mediate protein-protein and small-molecule-protein interactions. These include AHR dimerization with ARNT and AHR binding interactions with chaperone proteins and ligands (McIntosh et al., 2010).

Inactive AHR exists in the cytosol in a multi-protein complex, which includes a heat shock protein-90 (Hsp90) dimer, a co-chaperone (p23), an AHR interacting protein (AIP/XAP2/ARA9), and various co-activators, including members of Src family of non-receptor tyrosine kinases (Avilla et al., 2020). AIP (Lees et al., 2003) and p23 (Pappas et al., 2018) block ubiquitination of AHR, possibly by inhibiting AHR binding interactions with the E3 ubiquitin ligase C-terminal hsp70-interacting protein (STUB1/CHIP) (Morales and Perdew, 2007). E3 ubiquitin ligases provide a platform for ubiquitin enzymes to transfer ubiquitin to proteins. Ubiquitin binding can either alter protein signaling or target the protein to the proteasome for degradation (Buetow and Huang, 2016). Both ligand-bound and unliganded AHR can be targeted to the proteasome for degradation (Ma and Baldwin, 2000; Morales and Perdew, 2007).

Ligand binding to AHR in the multi-protein complex induces conformational changes in AHR and reorganization of the chaperones to facilitate nuclear localization of the complex (Avilla et al., 2020). AHR activation induces Src kinase phosphorylation (Xie et al., 2012) and cell signals that promote the production of IL-10 (Zhu et al., 2018). Release of active AHR from the complex allows AHR to partner with ARNT, which can also either dimerize with and activate hypoxia inducible transcription factors (HIFs) or the AHR repressor (AHRR). HIFs compete with AHR for ARNT dimerization and promote transcription of genes expressing enzymes in the glycolytic pathway (Goda and Kanai, 2012). AHR-induced AHRR not only competes with AHR for ARNT but also suppresses AHR

function as a transcription factor by binding AHR responsive elements (AHREs) (Avilla et al., 2020) (**Figure 1**).

AHR IN DRUG METABOLISM

AHR has a pivotal role in regulating the clearance of xenobiotics and particular endogenous compounds (*e.g.,* metabolites of tryptophan, arachidonic acid and hemoglobin), which also bind and activate AHR during various processes, including development (Avilla et al., 2020), hematopoiesis (Angelos and Kaufman, 2018) and disease pathogenesis (Curran et al., 2018; Curran et al., 2019). In the clearance of these molecules, the AHR:ARNT heterodimer induces the expression of proteins involved each of the three metabolic biotransformation pathways. These proteins neutralize the activity of endogenous and xenobiotic molecules and promote the efflux of these molecules from the cell (**Figure 2**).

Phase I metabolism includes oxidation, reduction and hydrolysis of substrates, to generate more water-soluble, but generally still active, xenobiotic molecules (Phang-Lyn and Llerena, 2021). AHR-induced cytochrome P450 enzymes are a common measure of AHR activation in the liver. Their relevance in the kidney (Knights et al., 2013) and the immune system (Effner et al., 2017) are subjects of on-going study. Cytochrome P450 enzymes oxidize substrates and therefore contribute to phase I metabolism (Zanger and Schwab, 2013).

Phase II metabolism is carried out by conjugating enzymes, which add a hydrophilic group to targeted molecules; these protein modifications include glucuronidation, sulfation, acetylation, and methylation (Phang-Lyn and Llerena, 2021). UDP-gluconosyltransferase (UGT)-1A5, UTG1A6, UGT1A7, UGT1A9, UGT2B4, UGT2B4, and UGT2B17 are expressed in the kidney (Knights et al., 2013). AHR can induce UGT1A1 (Yueh et al., 2005) and UGT1A6 (Bock and Bock-Hennig, 2010) in human cell lines.

Phase III metabolism includes ATP-binding cassette (ABC) and solute carrier (SLC) transporters that facilitate xenobiotic efflux (Phang-Lyn and Llerena, 2021). Exposing rat brain capillaries to the AHR ligand, *2,3,7,8-tetrachlorodibenzo-p-dioxin* (TCDD, dioxin), induced production of the ABC transporter, P-glycoprotein, also known as multidrug resistance protein (MRP)-1 (Wang et al., 2011). P-glycoprotein and additional ABC transporters [*e.g.,* MRP2, MRP4, breast cancer resistance protein (BCRP)] are also induced in dioxin exposed killifish renal proximal tubules (Mahringer et al., 2019). Further testing of these responses in human kidney cells would enhance our knowledge of AHR kidney function.

AHR FUNCTION IN ADP-RIBOSYLATION

Both activated AHR (MacPherson et al., 2013) and HIF-1α (Zhang L. et al., 2020) promote transcription of the *TCDD-inducible poly(ADP-ribose) polymerase (TIPARP/PARP7/ARTD14)* gene. Poly-ADP-ribose polymerases (PARPs) post-translationally add a single ADP-ribosyl group (mono-ADP-ribosylation/MARylation) or multiple groups (poly-ADP-ribosylation/PARylation) to substrates (*e.g.*, protein, DNA, and RNA) in regulating DNA repair, transcription, and cell signaling (Sanderson and Cohen, 2020). While the functional attributes of PARPs continue to be characterized, TIPARP uniquely is capable of negatively regulating its transcriptional activators (AHR, HIF-1α) (**Figure 1**). The mechanisms appear to involve TIPARP co-localization with the particular transcription factor and the recruitment of an E3 ligase for ubiquitin-mediated proteasome degradation of the transcription factor (MacPherson et al., 2013; Zhang L. et al., 2020). These integrated networks involving AHR and HIFs implicate AHR in basic metabolic processes and hypoxic responses during the development and progression of diseases such as CKD (Fu et al., 2016).

AHR REGULATORY MECHANISMS IN CKD SIGNALING NETWORKS

CKD manifests hypertension (Panizo et al., 2021), progressive kidney fibrosis (Bulow and Boor, 2019), and tissue hypoxia (Fu et al., 2016). Biochemical features include increased circulating plasma levels of extracellular nicotinamide phosphoribosyltransferase (eNAMPT/visfatin) (Hsu et al., 2016) and elevated blood levels of kynurenine (Cheng et al., 2020) (**Figure 3**). These pathologic processes and biomarkers are regulated by the RAAS, TGF-β1 cell signals, and metabolism. The roles of AHR in each of these systems is yet to be fully characterized.

RAAS AND AHR

The RAAS pathway involves a series of enzymatic reactions that contribute to the homeostatic control of extracellular fluid volume, arterial pressure, tissue perfusion, electrolyte balance, and wound healing. Renin, released from glomerular juxtaglomerular cells, processes liver-produced angiotensinogen into angiotensin I (Ang I), which is further cleaved by a soluble ectoprotein, angiotensin converting enzyme (ACE), into Ang II. The binding of Ang II to the Ang II type 1 receptor (AT1R) promotes vasoconstriction and also induces the production of aldosterone, which promotes renal tubular sodium reabsorption and promotes fibrogenesis. The ACE homolog, ACE2, inactivates Ang II by cleaving and processing Ang I and Ang II into Ang (1–7), which binds the Mas receptor and the AT_2R. By these activities, ACE2 antagonizes the vasoconstrictive, inflammatory, prothrombotic, and fibrotic effects associated with ACE/Ang II/AT_1R activity (Curran et al., 2020).

The functions of AHR in the RAAS signaling pathways appear to depend upon the level of expression of AHR and the AHR ligand. AHR-deficient mice develop cardiac hypertrophy through mechanisms involving HIF-1α cell signals (Thackaberry et al., 2002), Ang II-induced fibrosis (Ichihara et al., 2019), increased plasma levels of endothelin-1, and elevated mean arterial pressures (Lund et al., 2003). In heterozygous AHR (+/−) mice, blood pressure remains normal, and AHR (+/−) mice are more responsive to ACE inhibition and an endothelin-1 receptor antagonist compared to AHR (−/−) null mice (Zhang et al., 2010).

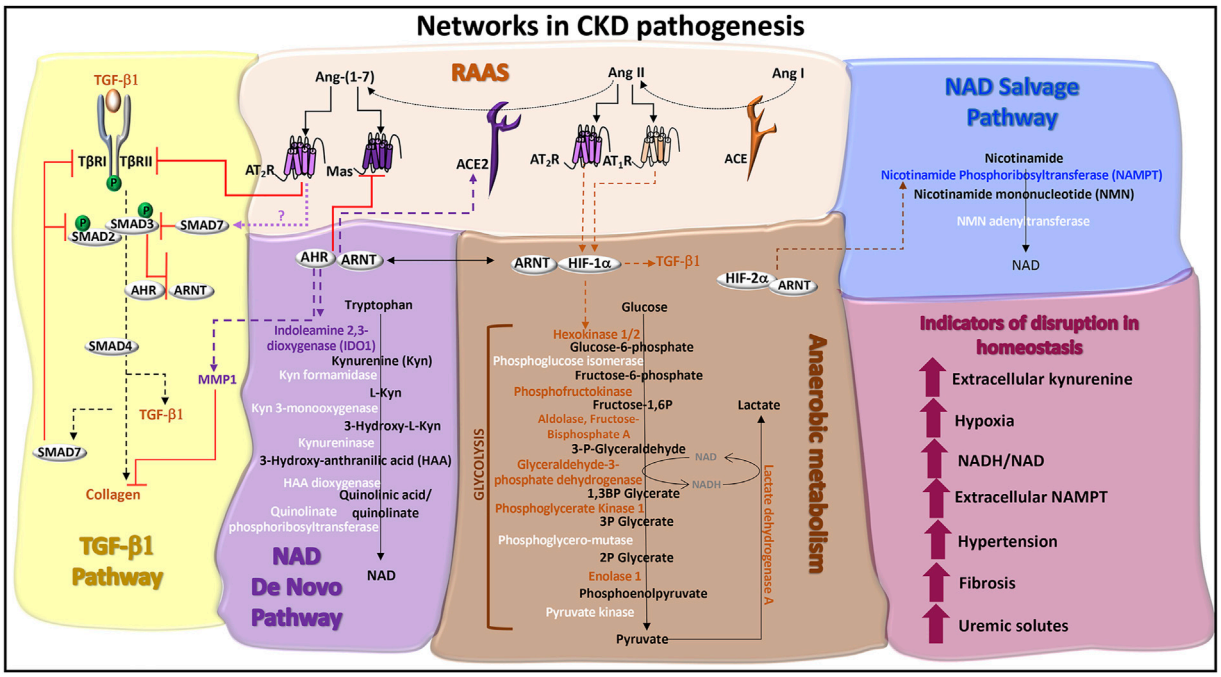

FIGURE 3 | Networks in CKD pathogenesis. **TGF-β1 pathway:** TGF-β1 signaling is initiated through serine/threonine kinase receptors, TGF-β1 receptor (TβR)-I and TβRII. TGF-β binding to TβRII recruits TβRI to form a receptor heterodimer, which is phosphorylated. SMAD2 and SMAD3 are recruited to the receptor heterodimer and are also phosphorylated. SMAD2 and SMAD3 co-localize with SMAD4 and translocate to the nucleus to activate genes, such as collagen and TGF-β1. ACE2 activity promotes the production of SMAD7, potentially *via* Ang-(1–7)-induced Mas or AT_2R receptor signals. SMAD7 is a negative regulator of TGF-β1 by recruiting E3 ligases to TβRI and blocking TβRI-induced SMAD2/3 phosphorylation. Ligand activated AHR antagonizes TGF-β1 and collagen gene expression and protein production, which are associated with fibrosis. Ligand activated AHR also induces the degradation of collagen through the production of matrix metalloproteinase-1 (MMP1). **Renin angiotensin aldosterone system (RAAS):** Angiotensin I (Ang I) is cleaved by angiotensin converting enzyme (ACE) into Ang II. The binding of Ang II to AT_1R or AT_2R promotes the stable expression of HIF-1α and hypoxic responses. The ACE homolog, ACE2, inactivates Ang II by cleaving and processing Ang I and Ang II into Ang-(1–7), which is a ligand for the Mas receptor and AT_2R. AHR regulates the expression of ACE2 and Mas. AT_2R activation promotes TβRII degradation, inhibiting TGF-β1 signals. **NAD de novo biosynthesis pathway (also known as the kynurenine pathway):** Tryptophan catabolism is the defining feature of this pathway. The rate limiting enzymes are indoleamine dioxygenase (IDO) and tryptophan dioxygenase (TDO). IDO1 is induced by AHR:ARNT transcriptional activation of the IDO promoter and promotes the production of kynurenine, which can be released as a cytokine. A series of additional enzymes (highlighted in white) catalyze the production of immunomodulatory and neuroregulatory molecules that are further processed into NAD. **Anaerobic metabolism:** The production of adenosine triphosphate (ATP) in the absence of oxygen occurs through enzymatic reactions in glycolysis and results in the production of lactate and the increased formation of NADH relative to NAD. Enzymes in this pathway are regulated by HIF-1α activated genes (highlighted in orange), which can be stabilized by the RAAS. **NAD salvage pathway:** The primary source of mammalian NAD is from the recycling nicotinamide, which is the amide version of vitamin B3 and a by-product from the enzymatic activity of Poly-ADP-ribose polymerases (PARPs) and sirtuins. The rate limiting enzyme is nicotinamide phosphoribosyltransferase (NAMPT), which is transcriptionally activated by HIF-2α and functions as an extracellular cytokine. **Indicators of disruption in homeostasis:** Factors and conditions along these pathways are induced during CKD pathogenesis.

The effects of overexpression of AHR on RAAS activity have not been extensively examined. AHR overexpression in adipocytes shortens the half-life of PPAR-γ by recruiting PPAR-γ to the cullin 4b (CUL4B)-RING E3 ubiquitin ligase complex (Dou et al., 2019), leading to PPAR-γ degradation in the proteasome. Because reduced PPAR-γ activity induces AT_1 expression and signaling in human fibroblasts (Auclair et al., 2013), overexpression of AHR may also affect the ACE/Ang II/AT_1R pathway. Estrogen receptor (ER)-α can also be recruited by AHR to the CUL4B-RING E3 ubiquitin ligase complex resulting in ubiquitination (Luecke-Johansson et al., 2017) or targeted by TIPARP ADP-ribosylation (Zhang L. et al., 2020). These post-translational modifications that promote ER-α proteasome degradation indicate a potential role of AHR in sex-related differences in RAAS activity (Sabbatini and Kararigas, 2020) (**Figure 4**).

Various AHR ligands increase in abundance during CKD pathogenesis and may affect RAAS activity. Reduced kidney function causes blood retention of a heterogeneous mix of metabolites (uremic solutes), which may be more effectively removed by continuous ambulatory peritoneal dialysis compared to low-flux hemodialysis (Xie et al., 2019). Tryptophan metabolites are uremic solutes and are AHR ligands, which promote CKD progression (**Table 1**). In a small study of CKD patients, an increased ratio of the tryptophan metabolite, kynurenine, to tryptophan was associated with macroalbuminuria and responsiveness to AT_1R blockers (ARBs) (Wu et al., 2020). This study suggests upregulation of the ACE/Ang II/AT_1R pathway in these individuals. However, *in vitro* examination of kynurenine-exposed BEAS-2B lung epithelial cells revealed AHR-induced production of ACE2 (Lv et al., 2021).

Because ACE2 activity antagonizes ACE/Ang II/AT_1R cell signaling (Curran et al., 2020), additional factors in the RAAS

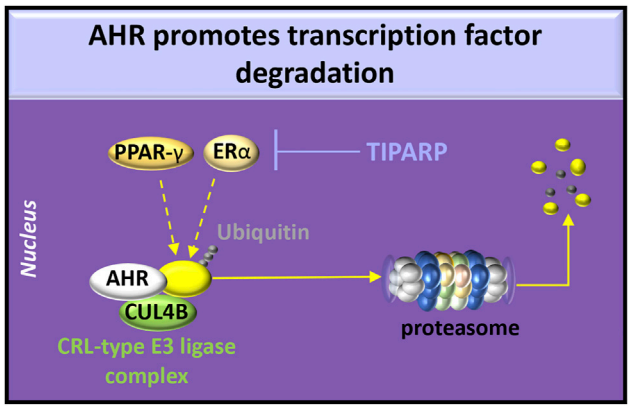

FIGURE 4 | AHR degradative functions. AHR participates in the cullin/RING ubiquitin ligase (CRL-type E3 ligase) complex involving chaperones [*e.g.*, cullin 4b (CUL4B)] to promote ubiquitination of estrogen receptor (ER)-α or peroxisome proliferator-activated receptor (PPAR)-γ. Activation of AHR or HIFs induces transcription and translation of PARP7 (poly ADP-ribosyl transferase 7), also known as TIPARP (TCDD-inducible poly-ADP-ribose polymerase). TIPARP promotes polyADP-ribosylation and subsequent degradation of ER-α.

pathway may be affected by AHR. One such factor is the Mas receptor. In normotensive and hypertensive rats, oral administration of the uremic solute, indoxyl sulfate, reduced kidney Mas receptor expression. This study further demonstrated that indoxyl sulfate-exposed human kidney-2 (HK-2) proximal tubular cells reduced Mas receptor expression and this response was antagonized by Ang-(1–7) pre-treatment or by the absence of AHR (Ng et al., 2014). Thus, AHR expression and activation are integral to the RAAS homeostatic function.

METABOLISM AND AHR

Ischemia and oxidative stress induce metabolic stressors (*e.g.,* hypoxia) and the production of pro-inflammatory mediators (e.g., IL-1β, TNF, IL-6) during the progression of CKD. In a comprehensive pathway map analysis of gene sets from 157 European patients with nine different types of CKD, metabolism and inflammation were identified as the two main pathways in the pathology leading to CKD progression (Stenvinkel et al., 2021). Transcription factors, such as NF-κB and HIFs, regulate these responses and exhibit crosstalk with AHR.

There are three isoforms of HIF-α: HIF-1α, HIF-2α, and HIF-3α, encoded by distinct genes: *HIF1A*, *EPAS1*, *HIF3A*. HIF-1α and HIF-2α are expressed in the kidney, compete with AHR for the dimer partner ARNT, and can be degraded in either the proteasome or the lysosome (Hubbi et al., 2013; Jochmanova et al., 2013). Oxidative stress, hypoxia, Ang II, and certain peptides (*e.g.*, human epidermal growth factor receptor-2 (HER-2), IL-1β, insulin) (Wolf, 2005; Curran and Keely, 2013) reduce HIF post-translational modifications that target the protein for degradation and thereby stabilize HIF expression.

The most well-described HIF-1α transcriptional responses involve the expression of genes whose products are involved in glycolysis and the production of lactate (**Figure 3**). Specifically, the HIF-1α: ARNT heterodimer promotes transcription of *SLC2A1* (Solute Carrier Family two Member 1, GLUT1), *HK1* (hexokinase 1), *HK2* (hexokinase 2), *PFK* (phosphofructokinase), *ALDOA* (aldolase, fructose-bisphosphate A), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *PGK1* (phosphoglycerate kinase 1), and *LDHA* (lactate dehydrogenase A) (Semenza et al., 1996; Jochmanova et al., 2013; Del Rey et al., 2017). Anaerobic metabolism decreases the levels of nicotinamide adenine dinucleotide (NAD) and increases the formation of the reduced form, NADH (**Figure 3**). Despite their differential functions in the RAAS, AT_1R and AT_2R can both induce HIF-1α stabilization in response to Ang II (Wolf, 2005; Liu et al., 2014). Understanding the roles of AT_1R and AT_2R HIF-1α cell signals in CKD requires further study.

The von Hippel-Lindau tumor suppressor (VHL) is an E3 Ubiquitin Ligase Which Ubiquitylates and targets the α-subunit of HIFs for oxygen-dependent proteolysis (Haase, 2009). Deletion of the *VHL* gene or increased production of HIF-2α in mouse podocytes leads to rapidly progressive glomerulonephritis that can be prevented by targeted deletion of the *ARNT* gene (Ding et al., 2013). Understanding whether AHR functions in the absence of ARNT in these cells may not only offer insight to the functions of podocytes, but also mechanisms in NAD metabolism, particularly since ARNT is a factor in both NAD salvage and *de novo* biosynthesis pathways.

The HIF-2α: ARNT heterodimer activates the transcription of nicotinamide phosphoribosyltransferase (NAMPT/visfatin) (Sun et al., 2020), the rate limiting enzyme in the NAD salvage pathway (Garten et al., 2015). Plasma levels of NAMPT are negatively correlated with glomerular filtration rate in nondiabetic hypertensive patients (Hsu et al., 2016), chronic glomerulonephritis patients, and diabetic nephropathy patients (Axelsson et al., 2007). As a cytokine, NAMPT promotes the production of inflammatory mediators, upregulates the expression of adhesion receptors and induces endothelial dysfunction (Romacho et al., 2013). Inside the cell, NAMPT catalyzes the production of nicotinamide mononucleotide (NMN) (Garten et al., 2015) (**Figure 3**). In HK-2 cells exposed to 1 mM hydrogen peroxide plus 1% oxygen, NMN increased cell viability and reduced collagen IV protein production. Similar results were obtained in a murine ischemia-reperfusion injury model, manifesting reduced tubular DNA damage, cellular injury, and fibrosis in response to intraperitoneal NMN therapy (Jia et al., 2021). These data suggest that neutralizing extracellular NAMPT or activating intracellular NAMPT production of NMN may be therapeutic strategies in treating CKD.

The AHR: ARNT heterodimer promotes the transcription of indoleamine 2,3- dioxygenase (IDO1) (Bessede et al., 2014), the rate limiting enzyme in the NAD *de novo* biosynthesis pathway. This pathway catabolizes tryptophan into NAD through a series of enzymatic reactions, which are also part of the kynurenine

pathway (Ralto et al., 2020) (**Figure 3**). In a small study of CKD patients, plasma IDO1 activity level and its downstream metabolites (kynurenine, kynurenic-acid, quinolinic-acid) correlated with disease severity (Schefold et al., 2009). This finding concurs with conclusions from a subsequent study correlating serum IDO1 and kynurenine levels with disease severity (Bao et al., 2013). A meta-analysis indicates that the lower estimated glomerular filtration rate in these patients is associated with higher blood metabolite levels of tryptophan metabolites, including kynurenine, C-glycosyltryptophan (glycosylated amino acid), 3-indoxyl sulfate, and indole-3-lactate (Cheng et al., 2020). Each of these studies demonstrate an increase in the production of AHR ligands and a decrease in the production of NAD from the *de novo* biosynthesis pathway.

Quinolinate phosphoribosyltransferase (QPRT) is the final enzyme in the *de novo* biosynthesis pathway (**Figure 3**). In $QPRT^{+/-}$mice, lower levels of NAD in the kidney and higher urinary levels of quinolinate are identified. In response to renal ischemia-reperfusion injury in wild-type mice, kidney QRPT expression levels are reduced and urinary quinolinate levels are elevated. These models were supplemented with intraperitoneal injections of 400 mg/kg nicotinamide, which ameliorated kidney function (Poyan Mehr et al., 2018), presumably through the production of NAD in the salvage pathway (**Figure 3**). In a small study of patients with COVID-19-related acute kidney injury, 1 g oral nicotinamide/day over 7 days, reduced mortality and renal replacement therapy, highlighting a potential therapeutic function in promoting NAD via the salvage pathway (Raines et al., 2021). Because a primary AHR-induced gene and regulator, TIPARP, also requires NAD for full activation (Zhang L. et al., 2020), further exploration of TIPARP in CKD progression may provide insight into the therapeutic functions of intracellular NAD.

THE TGF-β1 PATHWAY AND AHR

AHR is an integral regulator of extracellular matrix assembly and remodeling. AHR competition with HIF-1α for ARNT may be a mechanism which antagonizes three pro-fibrotic processes: hypoxia induction of TGF-β1, leading to suppressed collagen expression (Mingyuan et al., 2018); hypoxia-induced collagen prolyl hydroxylases (P4HA1 and P4HA2) required for collagen maturation and deposition (Gilkes et al., 2013); and hypoxia-induced enzymes (*e.g.*, LOX, transglutaminases, lysyl hydroxylases) involved in collagen cross-linking (Curran and Keely, 2013; Gilkes et al., 2013). The mechanisms by which ligand activated AHR inhibits alpha-smooth muscle actin and collagen I expression and promotes MMP-1 expression are not fully known but may involve AHR crosstalk with various signaling pathways (Poormasjedi-Meibod et al., 2016; Shi et al., 2020).

Two key signaling pathways in renal fibrosis include the TGF-β1 pathway and the NF-κB pathway, which can also exhibit crosstalk between each other and with AHR. TGF-β1,

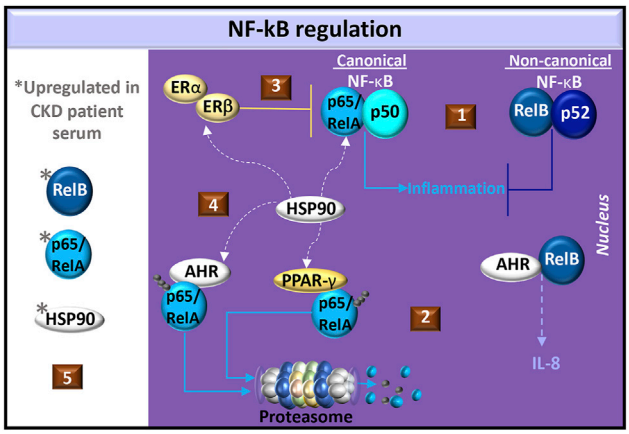

FIGURE 5 | Regulation of NF-κB in CKD. (1) Common NF-κB dimer pairs include p65/RelA and p50 in the canonical pathway and RelB and p52 in the non-canonical pathway. Non-canonical cell signals may antagonize canonical cell signals in fibroblasts. (2) AHR dimerizes with RelB in the production of IL-8. AHR or PPAR-γ activation promotes ubiquitination and degradation of p65/RelA. (3) Estrogen activates estrogen receptors (ER-α/β) to produce the NF-κB inhibitor, IκBα, and promotes the recruitment of ER-β to p65 binding sites, which blocks p65 transcriptional activity. (4) Heat shock protein (HSP)-90 is a chaperone shared by ER-α/β, PPAR-γ, AHR, and canonical NF-κB. (5) Elevated levels of p65/RelA, RelB, and HSP90 are found in CKD patient serum.

β2 and β3 initiate cell signals through activation of TGF-β receptor (TβR)-I and TβRII heterodimers. TβRII phosphorylates TβRI, which recruits and phosphorylates signaling transducer molecules. These receptor-regulated SMADs (*e.g.*, SMAD2, SMAD3) co-localize with the common partner SMAD (SMAD4) prior to localizing to the nucleus and binding to target genes (Chen et al., 2018). SMAD3 also strongly interacts with both AHR and ARNT and promotes dissociation between AHR and ARNT, inhibiting AHR signals (Nakano et al., 2020).

SMAD7 is an inhibitor SMAD that is activated by TGF-β signaling, providing negative-feedback. SMAD7 is recruited to TβRI and prevents receptor-regulated SMAD docking and phosphorylation. SMAD7 also recruits E3 ubiquitin ligases to degrade TβRI, SMAD2 and SMAD3, creating a regulatory feedback loop in TGF-β signaling (Chen et al., 2018).

The RAAS pathway is integrated with the TGF-β cell signal pathway. In an animal model involving Ang II-induced renal fibrosis, Smad7 knockout mice exhibit elevated TGF-β/Smad3 signaling, more severe renal injury and increased progressive fibrosis compared to the wild-type (Liu et al., 2013). However, in Smad3 knockout mice, Ang-II-induced renal fibrosis and NF-κB-driven renal inflammation was significantly lower compared to the wild-type (Liu et al., 2012b), highlighting functions of Ang II in promoting TGF-β cell signals.

Moreover, in mice with unilateral ureteral obstruction nephropathy, deletion of ACE2 results in a fourfold increase in the ratio of intrarenal Ang II/Ang 1-7, which was associated with increased tubulointerstitial fibrosis and inflammation (Liu et al., 2012a). Subsequent mechanistic studies in Ace2 knockout

mice subjected to chronic subcutaneous angiotensin II infusion revealed an increase in Smad-specific E3 ubiquitin protein ligase 2 (SMURF2), a decrease in renal SMAD7, and increased TGF-β and NF-κB (Liu et al., 2017).

Because ACE2 is not known to directly transduce signals, a product of ACE2 activity, Ang (1–7), may function in the regulation of TGF-β cell signaling. Ang (1–7) binds the AT_2R and the Mas receptor (Curran et al., 2020). The AT_2R agonist, CGP42112A, promotes AT_2R co-localization with TβRII for subsequent TβRII degradation (Guo et al., 2016). Possibly, Ang (1–7) activation of AT_2R also induces the production of SMAD7. Because AHR induces ACE2 production (Lv et al., 2021) AHR may play a role in each of these responses (**Figure 3**).

THE NF-KB PATHWAY AND AHR

AHR interacts with both the canonical and non-canonical NF-κB pathways. The NF-κB transcription factor family consists of five proteins: p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), and p100/p52 (NF-κB2). NF-κB1 and NF-κB2 are produced as inactive precursors, p105 and p100, respectively. RelB additionally associates with p100. Cell signals induce proteolytic processing of the inactive precursors, generating functional subunits p50 and p52. The Rel proteins, p65 and c-Rel, are similarly bound to inhibitory protein kinases (*e.g.,* IκBα, IκBβ and IκBε). Cell signal-induced phosphorylation of inhibitory kinases targets the kinases for degradation and release p65 or c-Rel. The various NF-κB proteins form transcriptionally active homo- and heterodimeric complexes. The p65 transcription factor most commonly associates with p50 but can also form homodimers or associate with c-Rel and p52. RelB is only known to form heterodimers with either p50 or p52. The canonical pathway involves the activation of p65/p50 whereas the non-canonical pathway involves RelB (Hayden and Ghosh, 2004; Oeckinghaus and Ghosh, 2009). In mouse embryonic fibroblasts, p65 and RelB exhibit negative crosstalk (Marienfeld et al., 2003; Jacque et al., 2005) and in mouse kidney fibroblasts, RelB suppresses the production of TNF (Xia et al., 1999), which is a common mediator in CKD (Stenvinkel et al., 2021) (**Figure 5**).

In the canonical NF-κB pathway, dioxin-activated AHR promotes p65 ubiquitination for degradation by either the proteasome or lysosome in mouse peritoneal macrophages, (Dominguez-Acosta et al., 2018). AHR also promotes the degradation of ER-α (Luecke-Johansson et al., 2017) and PPAR-γ (Dou et al., 2019), which are both involved in p65 inhibition. Specifically, PPAR-γ promotes p65 ubiquitination for targeted proteasome degradation (Hou et al., 2012) whereas estrogen induces the production of the NF-κB inhibitor, IκBα, and promotes the recruitment of ER-β to p65 binding sites, which blocks p65 transcriptional activity (Xing et al., 2012) (**Figure 5**). Because animal models of glomerulosclerosis treated with 17β-estradiol (Maric et al., 2004) or pioglitazone (Nemeth et al., 2019) exhibit reduced tubulointerstitial fibrosis, estrogen and PPAR-γ cell signals may regulate CKD pathogenesis by inhibiting canonical NF-κB signaling.

A common factor in ER-α/β (Powell et al., 2010), PPAR-γ (Nguyen et al., 2013) and AHR (Soshilov and Denison, 2011) cell signaling is heat shock protein (HSP)-90. This chaperone contributes to the stabilization of IκB kinase (IKK), which is needed for the dissociation of IκB from NF-κB (O'Neill et al., 2015). In chronic glomerulonephritis patients, serum levels of HSP90 and NF-κB are higher than those in healthy individuals. (Chebotareva et al., 2020). The HSP90 inhibitor, geldanamycin, significantly suppresses angiotensin II-induced p65 nuclear translocation in cardiac cells (Lee et al., 2010) and in a murine renal ischemia-reperfusion injury model, pre-treatment with the HSP90 inhibitor, AT13387, improved renal function compared to controls (O'Neill et al., 2015). Understanding the potential competition for HSP90 in the ER-α/β, PPAR-γ, NF-κB, and AHR pathways may help in identifying novel therapeutics for CKD (**Figure 5**).

In the non-canonical NF-κB pathway, dioxin-activated AHR can dimerize with RelB and promote IL-8 production in U937 macrophages. RelB:AHR complexes bind RelB:p52 response elements as well as AHR response elements (Vogel et al., 2007), highlighting the complexity of AHR in the non-canonical pathway. RelB is expressed in renal tubular epithelial cells and levels gradually increase with progressive fibrosis in a mouse renal fibrosis model with unilateral ureteral obstruction. RelB immunohistochemical staining in renal tubular epithelial cells was also positively correlated with the intensity of kidney fibrosis in biopsy specimens in a study of 34 CKD patients. Interestingly, the serum RelB levels from CKD patients also correlated with the intensity of renal fibrosis compared healthy controls (Sun D. et al., 2021). This dysregulated RelB response may also reflect changes in AHR cell signals that regulate fibrosis. Further investigation of RelB:AHR initiated cell signals, particularly in response to uremic AHR ligands, may offer insight into CKD pathogenesis.

CONCLUSION

AHR is a pleotropic cell signaling molecule with diverse ligand-specific functions. The elevated levels of uremic solutes that act as AHR ligands during the progression of CKD highlight the significance of AHR activity in these diseases. AHR contributes to the biotransformation of molecules in the clearance of xenobiotics, and these processes deserve further exploration in kidney pathophysiology. AHR competition with HIF-1α for binding to ARNT in pro-inflammatory and anaerobic responses, together with AHR antagonism of TGF-β1 cell signaling in fibrogenesis, support potential targeting of AHR to slow CKD progression. In the NAD *de novo* biosynthesis pathway, AHR stimulates RNA expression and protein production of the rate-limiting enzyme, IDO1, and binds molecules along this pathway that accumulate in CKD. NAD is required for the activity of TIPARP, which is involved in the recruitment of an E3 ligase for ubiquitin-mediated proteasome degradation of

AHR. It is possible that the extracellular release of kynurenine and NAMPT, rather than intracellular metabolic catabolism of these molecules into NAD, alters the functions of AHR in CKD. Further investigation of AHR regulation of ACE2 and the downstream effects of the ACE2 cleavage product, Ang-(1–7), on Mas and AT_2R may identify novel therapeutic targets in hypertension and fibrosis. Because each of these AHR functions may be regulated by crosstalk with ER-α, PPAR-γ, or NF-κB subunits, a more refined assessment of the binding affinity and availability of HSP90 in these cell signaling pathways during CKD is warranted.

AUTHOR CONTRIBUTIONS

Wrote or contributed to the writing of the manuscript: CC and JK. Illustrations: CC. Developed tables: CC and JK.

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A Human Conditionally Immortalized Proximal Tubule Epithelial Cell Line as a Novel Model for Studying Senescence and Response to Senolytics

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Accumulating evidence suggests that senescence of kidney tubule epithelial cells leads to fibrosis. These cells secrete senescence-associated secretory phenotype (SASP) factors that are involved in diverse signaling pathways, influencing kidney fibrosis. Here, we investigated whether our previously established conditionally immortalized proximal tubule epithelial cell line overexpressing the organic anion transporter 1 (ciPTEC-OAT1) can be used as a valid *in vitro* model to study kidney senescence and senolytics response. CiPTEC-OAT1 proliferates rapidly at 33°C and exhibits a "senescence-like" arrest at 37°C, most likely due to suppression of SV40T expression and subsequent reactivation of the p53 and Rb pathways. To understand how permissive (33°C) and non-permissive (37°C) temperatures of the cell culture affect the senescence phenotype, we cultured ciPTEC-OAT1 for up to 12 days and evaluated the apoptosis and SASP markers. Day 0 in both groups is considered as the non-senescence group (control). Further, the potential of navitoclax, dasatinib, quercetin, and the combination of the latter two to clear senescent cells was evaluated. Maturation of ciPTEC-OAT1 at non-permissive temperature affected mRNA and protein levels of senescence markers. A remarkable upregulation in p21 gene expression was found in the non-permissive temperature group, whereas expression of Lamin B1 decreased significantly. SASP factors, including PAI-1A, IL-1β, CTGF, and IL-6 were upregulated, but no significant difference in Bcl-2 and Bcl-xl were found in the non-permissive temperature group. After culturing ciPTEC-OAT1 up to 12 days, cells in the non-permissive temperature group showed an upregulation in the apoptosis-associated proteins Bcl-2, BID, and Bax, and a downregulation in Mcl-1, Bad, Bak, and Bim at various time points. Further, Bcl-xl, Puma, Caspase 3, Caspase 7, and Caspase 9 showed initial upregulations followed by downregulations at later time points. The loss of Lamin B1, upregulation of SA-β-gal expression and increase in its activity, upregulation of p21 levels and downregulation of p53, along with the upregulation of SASP factors, confirmed that maturation at 37°C promotes senescence features. Finally, the senolytics response was evaluated by testing cell viability following exposure to senolytics, to which cells appeared dose-dependently sensitive. Navitoclax was most effective in eliminating senescent cells.

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In conclusion, culturing ciPTEC-OAT1 at 37°C induces a senescence phenotype characterized by increased expression of cell cycle arrest and anti-apoptosis markers, SASP factors, and responsiveness to senolytics treatment. Therefore, ciPTEC-OAT1 represents a valid model for studying kidney senescence by simply adjusting culture conditions.

Keywords: kidney fibrosis, conditionally immortalized proximal tubule epithelial cell, Bcl-2 family proteins, senescence-associated secretory phenotype (SASP), cell cycle arrest, apoptosis

1 INTRODUCTION

Renal fibrosis is the common end point for all progressive kidney diseases, which leads to kidney failure by an excessive accumulation of extracellular matrix (Boor et al., 2010). Accumulating evidence suggests that senescence of kidney tubule cells influences kidney fibrosis (Schafer et al., 2018). Senescence is a special form of permanent cell cycle arrest, which limits cellular proliferative life span. Some senescent cells can be cleared by immune cells, termed acute (short-term) senescent cells, while chronic (long-term) senescent cells keep accumulating and creating early senescence events, finally aggravating the pathology (Munoz-Espin and Serrano, 2014; Kobbe, 2019). Different hallmarks of senescence have been recognized which are involved in diverse signaling pathways, including apoptosis markers, senescence-associated secretory phenotype (SASP) factors, and cyclin-dependent kinase inhibitors (Hernandez-Segura et al., 2018).

Senescent cells show resistance to apoptosis (Childs et al., 2014) and accumulate dysfunctional mitochondria (Korolchuk et al., 2017). Mitochondrial outer membrane permeabilization (MOMP) is responsible for apoptosis in numerous cell death pathways (Chipuk et al., 2006). In the intrinsic apoptosis pathway, Bcl-2 and the caspase family proteins play important roles (Van Opdenbosch and Lamkanfi, 2019; Ngoi et al., 2020). The Bcl-2 family is divided into three main groups: anti-apoptotic (Bcl-2, Bcl-xl, and Mcl-1), pro-apoptotic (Bax and Bak), and pro-apoptotic BH3-only (Bim, Bid, Bad, and Puma) (Anantram and Degani, 2019). Senescent cells are known to be in a primed apoptotic state, triggered by the abnormal upregulation of anti-apoptotic and pro-apoptotic proteins (Fan et al., 2020). Caspase family proteins are downstream players of MOMP in the intrinsic apoptosis pathway (Shalini et al., 2015), and after the activation of the Bax-Bak-dependent MOMP, cytochrome C is released from the mitochondria stimulating caspase-9 activation and its downstream executioners, caspases-3 and -7, to initiate apoptosis (Van Opdenbosch and Lamkanfi, 2019).

Cell cycle arrest is another typical characteristic of senescent cells, which is largely mediated through activation of either one or both p53/p21$^{CIP1/WAF1}$ (p21) and p16^{Ink4a} (p16)/pRb pathways (Kumari and Jat, 2021). p53/p21 is activated during DNA damage response, resulting in a p21-dependent G0/G1 cell-cycle arrest (Ceccaldi et al., 2012; Ou and Schumacher, 2018). On the other hand, p16 inactivates Retinoblastoma 1 (pRb) thereby inhibiting the action of the cyclin dependent kinases, leading to G1 cell cycle arrest (Rayess et al., 2012). Both p53/p21 and p16/pRb pathways are independent in senescence induction. Acute DNA damage causes a cell cycle arrest via the p53/p21 pathway, while chronic DNA damage followed by the induction of the p16/pRB pathway maintains cell cycle arrest and senescence (Sperka et al., 2012). As a key mediator of cell-cycle arrest, p21 also shows a p53-independent upregulation according to some research (Zhang et al., 2011; Ruan et al., 2020). Furthermore, p53 is also involved in the apoptosis process, as described previously (Rufini et al., 2013; Ou and Schumacher, 2018).

SASP factors are related to a DNA damage response and are generally proinflammatory and/or profibrotic compounds including numerous cytokines (e.g., IL-6 and IL-8), growth factors (e.g., TGF-β and CTGF), chemokines (e.g., CCL2), and matrix-metalloproteinases (e.g., MMP-1 and MMP-3) (Hernandez-Segura et al., 2018; Birch and Gil, 2020). Those proteins induce or maintain senescence through different pathways, contributing to kidney fibrosis (Docherty et al., 2019). Current efforts are focused on clearing senescent cells as a treatment option for prevention of kidney fibrosis development and progression. Senolytics represent a good option as they can selectively eliminate senescent cells participating in senescence associated pathways by interfering with anti- and pro-survival signaling (Zhu et al., 2015). However, suitable cell models are required to evaluate senescence development in kidney tubule epithelial cells and their response to senolytics.

We previously developed a conditionally immortalized proximal tubule epithelial cell line overexpressing the organic anion transporter 1 (ciPTEC-OAT1) and applied it successfully for pharmacological and toxicological investigations, including drug disposition and interaction studies (Wilmer et al., 2010; Nieskens et al., 2016). OAT1 is a first step in the elimination of organic anions in humans and is responsible for the uptake of many anionic (waste) products in kidney proximal tubules (Pou Casellas et al., 2021). Since the expression of OAT1 is rapidly lost when culturing (primary) PTEC *in vitro*, OAT1 was stably expressed in ciPTEC by lentiviral transduction. This cell line now allows prediction of drug-induced nephrotoxicity and drug-drug interactions of organic anions *in vitro* (Nieskens et al., 2016). OAT1 is also involved in the uptake of uremic toxins, known to participate in the uremic syndrome typical of chronic kidney disease (Nigam and Bush, 2019). Since senescence is a key factor contributing to chronic kidney disease, uremic toxins might play a role in this process as well.

CiPTEC was created by means of a temperature sensitive mutant U19tsA58 of SV40 large T antigen (SV40T) and the essential catalytic subunit of human telomerase (hTERT), to keep the characteristics of primary cells (Wilmer et al., 2010).

TABLE 1 | Primers used for real-time polymerase chain reaction.

Gene	Taqman gene expression assay
TBP	Hs00427620_m1
Lamin B1	Hs01059210_m1
p21 (CDKN1A)	Hs00355782_m1
CTGF	Hs00170014_m1
PAI-1(SERPINE1)	Hs00167155_m1
IL-1β	Hs01555410_m1
IL-6	Hs00174131_m1
BCL-2	Hs00608023_m1
Bcl-xl (BCL2L1)	Hs00236329_m1

Temperature-sensitive SV40T allows cells to proliferate at the permissive low temperature of 33°C but induces a proliferation block that resembles senescence at a non-permissive temperature of 37°C (Larsson et al., 2004; Wilmer et al., 2010). The hTERT maintains telomere length, preventing replicative senescence induced by telomere shortening (Bodnar et al., 1998). Some studies already showed a relation between senescence and SV40T conditional models, because both pRb and p53 are activated by SV40T at the non-permissive temperature leading to a senescence-like arrest in the cells (Larsson et al., 2004; Brookes et al., 2015). Therefore, we hypothesized that ciPTEC-OAT1 exhibits a senescence phenotype when cultured at non-permissive temperatures that can be used to study senescence development in kidney tubule epithelial cells and their response to senolytics. In the present study, we evaluated apoptosis markers and other common senescence markers in ciPTEC-OAT1 cultured at permissive and non-permissive temperatures at different time points, to investigate whether these cells can be implemented as a valid *in vitro* model to study kidney senescence. Day 0 in both groups is considered as the non-senescence group (control). Finally, the senolytics response was detected by means of cell viability assessment and senescence-associated β-galactosidase (SA-β-gal) activity.

2 MATERIALS AND METHODS

2.1 Quantitative Real-Time PCR

CiPTEC-OAT1 cells were seeded into 6-well format plates and grown at 33°C; then half of the plates were transferred to 37°C and cultured for up to 7 d. Afterward, cells were lysed in Trizol (Thermo-Fisher, Massachusetts, United States) followed by 5 min centrifugation at 4°C. After RNA isolation, RNA quantity was determined using Nanodrop 2000 (Thermo-Fisher, Massachusetts, United States). For RNA analysis, a cDNA library was synthesized using 3 μg RNA per sample with SuperScript III reverse transcriptase (Thermo-Fisher, Massachusetts, United States). Samples were mixed with TaqMan Gene Expression Assays (**Table 1**) and run on a ViiA 7 real-time PCR system (Applied Biosystems, California, United States). TATA-box binding protein (TBP) was used as an internal reference gene. Samples were run in duplicate and H_2O samples were used to control for potential contamination of reaction. The ΔΔCT method was used to calculate relative expression levels.

2.2 ciPTEC-OAT1 Maturation Process

CiPTEC-OAT1 were grown and expanded at 33°C. Following the seeding, cells were either kept at a permissive temperature of 33°C or incubated for a desired time up to 12 d at a non-permissive temperature of 37°C. The culture medium and cell lysate were collected on Day 0, 3, 6, 9, and 12, and used for the assessment of senescence markers and phenotype.

The ciPTEC-OAT1 cell line was cultured as reported previously (Mihajlovic et al., 2017). Briefly, cells were cultured in phenol-red free DMEM-HAM's F12 medium (Gibco, Life Technologies, Paisley, United Kingdom) supplemented with 10% (v/v) fetal calf serum (FCS) (Greiner Bio-One, Alphenaan den Rijn, the Netherlands), 5 μg/ml insulin, 5 μg/ml transferrin, 5 μg/ml selenium, 35 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor (EGF), and 40 pg/ml tri-iodothyronine to form complete culture medium, up to a maximum of 60 passages. Cells were cultured at 33°C and 5% (v/v) CO_2 to allow proliferation. Cells were grown up to 90% confluence at 33°C, then transferred for 7 d or 9 d at 37°C, 5% (v/v) CO_2 for maturation, refreshing the medium every other day.

2.3 Senescence and Senolytics Response

2.3.1 Cell viability Assay

Cell viability was measured using PrestoBlue® cell viability reagent (Thermo Scientific, Vienna, Austria). CiPTEC-OAT1 cells were seeded into 96-well format plates at a density of 63,000 per well, cultured for 24 h at 33°C and matured for 9 d at 37°C. Matured cells (37°C) were exposed to 100-μl medium with different concentrations of navitoclax, dasatinib, quercetin, or dasatnib and quercetin combinations. Senolytics were obtained from MedchemExpress, the Netherlands. All experiments were performed in a 96-well plate setup in triplicate with a minimum of three independent experiments.

2.3.2 SA-β-Gal Staining Assay

CiPTEC-OAT1 cells were seeded into 12-well format plates, grown at 33°C, then transferred to 37°C for maturation and culturing for 9 d. The cells matured at 37°C for 0 d and 9 d were exposed to 1-ml medium with different concentrations of navitoclax, dasatinib, or dasatnib and quercetin combinations. The SA-β-gal-positive cells were detected using Senescence Detection Kit (ab65351, Abcam, United Kingdom), and evaluated for blue colorization using an optical microscope (200x magnification).

2.4 Western Blot

CiPTEC-OAT1 cells were lysed in ice-cold RIPA Lysis Buffer (Thermo Scientific, Vienna, Austria) for 30 min followed by 20 min centrifugation at 4°C and obtained protein samples were quantified by BCA Protein Assay Kit (Thermo Scientific, Vantaa, Finland). Proteins were loaded and separated on 14–20% acrylamide gradient SDS gels (Bio-Rad Laboratories, Hercules, CA), transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA) in appropriate transferring conditions (25V, 7 min). The membranes were blocked in 5% skim milk-TBST for 2 h and incubated with the primary antibody overnight at 4°C and anti-rabbit (1:3000, Dako, P0448, United States) or anti-mouse (1:3,000, Dako, P0260, United States) secondary antibodies for 1 h at room temperature. The membrane was exposed to Clarity Western ECL Blotting

Substrate following manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA) then imaged using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Hercules, CA) to detect the protein bands, which were quantified using ImageJ software (version 1.53c, National Institutes of Health, United States).

The following proteins were detected by Western blotting: Bcl-2, Bcl-xl, Mcl-1, Bad, Bak, Bim, BID, Bax, Puma, Caspase-3, Caspase-7, Caspase-9, p53, p21, and β-gal, for which primary antibodies were purchased from Cell Signaling Technology (United Kingdom). LaminB1 primary antibody was purchased from Abcam (United Kingdom). The dilution of all primary antibodies was 1:1000.

2.5 ELISA

Cell culture supernatants were centrifuged for 10 min, 240 x g, 4°C, and stored at −20°C. To determine the concentration of SASP factors in the culture supernatants, the ELISA Kits of IL-6 (88-7066-88, Invitrogen, Carlsbad, CA), IL-8 (88-8086-88, Invitrogen, Carlsbad, CA), CTGF (DY9190-05, R&D System, United Kingdom), TNF-α (88-7346-88, Invitrogen, Carlsbad, CA), and TGF-β1(88-8350-88, Invitrogen, Carlsbad, CA) were used according to the manufacturer's instructions.

2.6 Statistics

All data analysis and statistics were performed using the GraphPad Prism (version 8.3.0; GraphPad software, La Jolla, CA), and expressed as mean ± SEM. For comparison of two groups at different temperature and different time points, two-way ANOVA was used followed by Sidak's multiple comparison test. To compare multiple groups in the same condition, one-way ANOVA was used followed by Dunnett's multiple comparison test. $p < 0.05$ was considered significant. Cell viability was expressed as inhibitory constants at 50% of control viability levels (IC_{50} values), which were calculated by plotting log senolytics concentration vs. viability following background subtraction. Nonlinear regression with a variable slope constraining the bottom to 0 was used to fit the normalized data. For cell viability at Day 9 compared to Day 0 evaluations at different concentrations, two-way ANOVA was used followed by Sidak's multiple comparison test. For cell viability of increasing concentrations of dasatinib combined with quercetin compared to dasatinib alone, a two-way ANOVA was used followed by Dunnett's multiple comparisons test.

3 RESULTS

3.1 Growing ciPTEC-OAT1 at Non-Permissive Temperature (37°C) Induces Expression of Senescence-Associated Genes

To understand if non-permissive temperature leads to cellular senescence, we compared expression of several senescence markers on mRNA level after culturing ciPTEC-OAT1 for 7 d at permissive (33°C) and non-permissive temperature (37°C). Compared to the permissive temperature group, no significant difference in the mRNA levels of Bcl-2 and Bcl-xl was observed (**Figures 1A,B**). On the other hand, a significant upregulation of p21 mRNA levels was found in the non-permissive temperature group compared to the permissive temperature group ($p < 0.0001$; **Figure 1C**). Furthermore, Lamin B1 mRNA levels decreased when cells were cultured at non-permissive temperature ($p < 0.01$; **Figure 1D**). SASP factors including PAI-1, IL-1β, CTGF, and IL-6 were all upregulated in the non-permissive temperature group ($p < 0.05$, $p < 0.05$, $p < 0.001$, and $p < 0.05$, respectively; **Figure 1**E-H).

3.2 Maturation at Non-Permissive Temperature of 37°C Affects Protein Levels of Apoptosis-Associated Markers in ciPTEC-OAT1

Senescent cells are characterized by an anti-apoptotic profile (Munoz-Espin and Serrano, 2014). To test whether maturation at the non-permissive temperature of 37°C would affect protein levels of apoptosis-associated markers in ciPTEC-OAT1, both anti- (**Figures 2A–D**) and pro-apoptotic (**Figures 2E–P**) protein markers were evaluated.

Representative Western blots and quantitative relative expression data indicate that there is an increased expression of Bcl-2 (**Figure 2B**) over time regardless of the temperature. However, there is a trend of different expression levels between the two temperature conditions at each day. The protein levels of Mcl-1 on the other hand (**Figure 2C**) decreased with time at all conditions tested, compared to Day 0, the non-senescence group. Bcl-xl (**Figure 2D**) levels showed an upregulation at both 33 and 37°C at almost all time points.

With respect to the pro-apoptotic proteins, we observed that all Bcl-2 family members tested, including Puma (**Figure 2F**), Bax (**Figure 2H**), BID (**Figure 2I**), and Bad (**Figure 2L**) showed an increase at both permissive (33°C) and non-permissive (37°C) groups at different time points. Significant differences in expression levels between 37 and 33°C groups were observed for Bax on Day 3 through Day 12, as well as for Bad on Day 3. Unlike other pro-apoptotic proteins among the Bcl-2 family members, Bim (**Figure 2G**) showed an upregulation at the permissive temperature at various time points, while an initial upregulation on Day 3 through Day 6 followed by a downregulation on Day 9 and Day 12 at the non-permissive temperature. In addition, the protein levels of Bim at 37°C were lower than at 33°C on Day 9 and Day 12. On the other hand, Bak (**Figure 2K**) showed an increased expression at 33°C on Day 3 through Day 12, while a decrease in protein levels was observed at 37°C starting from Day 3.

For the pro-apoptotic caspases, procaspase-3 (**Figure 2N**), procaspase-7 (**Figure 2O**), and procaspase-9 (**Figure 2P**), we found a non-significant trend for overtime upregulation at a permissive temperature, while at a non-permissive temperature, after an initial upregulation, there was a non-significant trend of downregulation at later time points, starting at Day 3 for procaspases-3 and -9, and at Day 6 for procaspase-7.

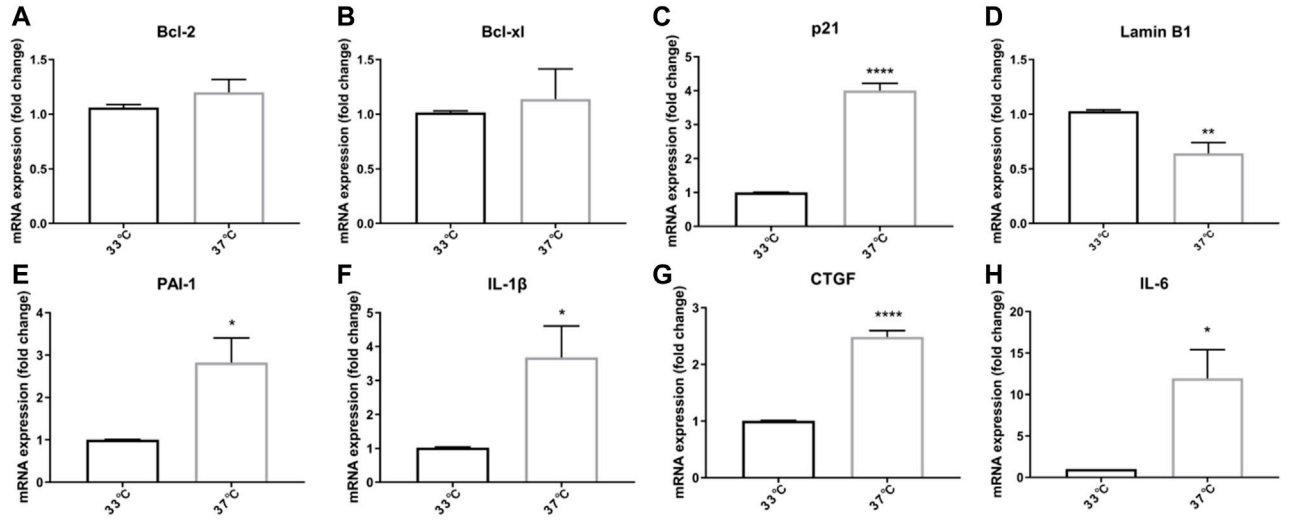

FIGURE 1 | Growing ciPTEC at a non-permissive temperature (37°C) induces expression of senescence-associated genes. **(A)** Bcl-2. **(B)** Bcl-xl. **(C)** p21. **(D)** LaminB1. **(E)** PAI-1. **(F)** IL-1β. **(G)** CTGF. **(H)** IL-6. Four independent experiments in triplicate were performed. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$ (unpaired *t* test). For clarity, only one-sided error bars are shown.

3.3 Maturation of ciPTEC-OAT1 at Non-Permissive Temperature Affects Expression Levels of Common Senescence Makers

To further characterize the development of the senescence phenotype in our ciPTEC-OAT1 model, the expression levels of known senescence hallmarks (p53, LaminB1, p21, and β-gal) were evaluated over time at permissive and non-permissive temperatures of 33 and 37°C, respectively (Hernandez-Segura et al., 2018). The obtained results suggest that the expression of these markers in ciPTEC-OAT1 is influenced by maturation in non-permissive conditions of 37°C (**Figure 3**). The total-p53 levels (**Figures 3A,C**) were markedly upregulated in a time-dependent manner in the proliferation group at 33°C but presented a decreasing trend of expression in the maturation group at 37°C. The expression levels of total-p53 were different on all days tested between the two groups, with higher levels at 33°C. The expression of LaminB1 (**Figures 3B,D**) showed a non-significant trend of decreased expression especially after longer culture (Days 9 and 12) at both temperatures, with the most evident trend of different expression between the two groups being at Day 9. Furthermore, the results show that p21 levels (**Figures 3E,F**) were increased at both 33 and 37°C, and that compared to Day 0, p21 was significantly upregulated on Day 12 at 33°C and on Days 3 through 12 at 37°C. In addition, in non-permissive conditions, there was a significant increase of p21 expression compared to permissive temperature at Days 3 and 6. Regarding β-gal (**Figures 3E,G**), the protein expression levels show an increasing trend at both 33 and 37°C temperatures, there were significant increases of β-gal expression compared to non-senescent cells (Day 0) at Days 3 and 6 at 37°C, with levels being markedly higher at non-permissive compared to permissive conditions at Day 6.

3.4 Maturation of ciPTEC-OAT1 at Non-Permissive Temperature Induces Common SASP Factors Secretion

As shown in **Figure 4**, the secretion profile of some typical SASP factors (IL-6, TGF-β1, TNF-α, IL-8, and CTGF) clearly correlate with prolonged culture at 37°C, which is indicative of a senescence phenotype. IL-6 is increasingly secreted (**Figure 4A**) over time, both at 33 and 37°C culture conditions. The secretion of IL-6 was significantly higher at the non-permissive temperature compared to the permissive temperature at Days 3, 6, and 12. The secreted levels of TGF-β1 did not seem to differ between two culture conditions especially at later time points (**Figure 4B**). The levels of TNF-α (**Figure 4C**) were not different over time and between the culture conditions. The secretion profile of IL-8 (**Figure 4D**) showed a time-dependent increase at both temperature conditions, with a higher trend of expression levels at 37°C compared to 33°C. Finally, the CTGF results (**Figure 4E**) showed an initial increasing trend in secretion, followed by a trend for reduction and return to basal levels starting from Day 3, at both culture conditions. Compared to the permissive culture conditions, the concentration of CTGF at a non-permissive temperature was slightly higher at all time points.

3.5 CiPTEC-OAT1 Exhibiting a Senescence-like Phenotype Are Susceptible to Common Senolytics

Our data suggest that ciPTEC-OAT1 cultured progressively in non-permissive conditions obtains a senescence-like phenotype as indicated by some of the most important markers and SASP factors, especially evident after 9 d of culturing. For that reason, this time point was selected to test the effects of senolytics, a class of small molecules that can selectively eliminate senescent cells participating in associated pathways by interfering with anti- and

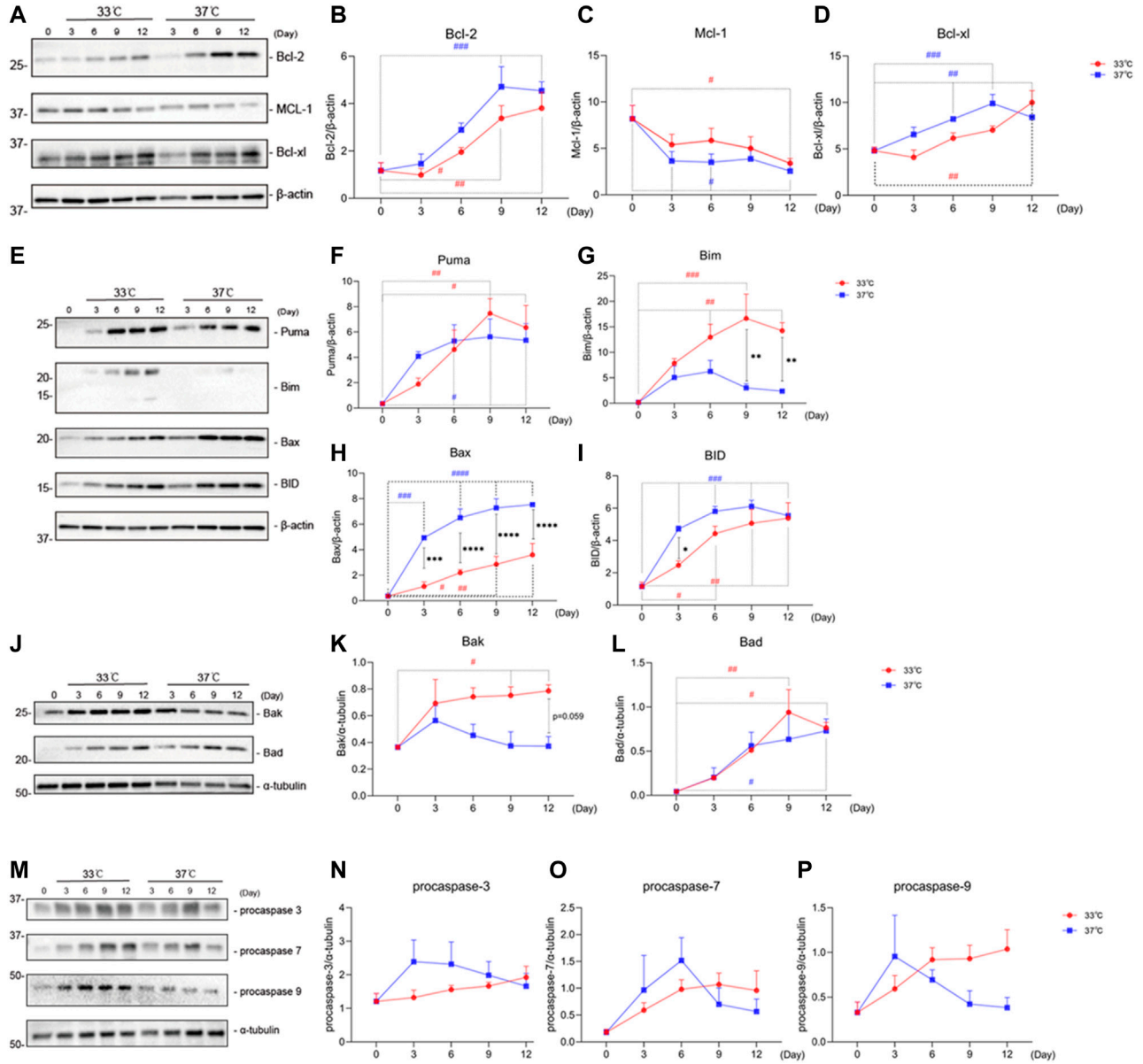

FIGURE 2 | Maturation at a non-permissive temperature of 37°C affects protein levels of apoptosis-associated markers in ciPTEC-OAT1. **(A)** Representative Western blots showing expression of anti-apoptotic proteins Bcl-2, Bcl-xl, and Mcl-1 belonging to the Bcl-2 family. **(B),(C),** and **(D)** Relative expression of Bcl-2, Bcl-xl, and Mcl-1 over time (Day 0 to Day 12) at both permissive and non-permissive temperatures of 33°C and 37°C, respectively. **(E)** and **(J)** Representative Western blots of the pro-apoptotic proteins' expression (Puma, Bim, Bax, and BID shown in **(E)**, Bak and Bad shown in **(J)** of Bcl-2 families. **(F)–(L)** Relative expression levels of Puma, Bim, Bax, BID, Bak, and Bad over time at 33°C and 37°C. **(M)** Representative Western blots showing expression of procaspase-3, procaspase-7, and procaspase-9. **(N),(O),** and **(P)** Relative expression of procaspase-3, procaspase-7, and procaspase-9 at different time points, at 33°C and 37°C. Protein expression levels were normalized against α-tubulin or β-actin and expressed as mean ± SEM. Three independent experiments in triplicate were performed. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (expression levels at 37°C compared to 33°C; two-way ANOVA, Sidak's multiple comparison test). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ (expression levels at Day 3, 6, 9, or 12 compared to Day 0, at 33°C or 37°C; one-way ANOVA, Dunnett's multiple comparison test). Day 0 in both groups is considered as the non-senescence group (control). For clarity, only one-sided error bars are shown.

pro-survival signaling pathways (Zhu et al., 2015) For this, navitoclax, dasatinib, and quercetin were employed to first assess the cell viability after exposures at Day 0 or after 9 d of culture.

As shown in **Figure 5**; **Table 2**, quercetin (**Figure 5A**) did not significantly affect the viability of senescent cells (Day 9 of culture) compared to non-senescent cells (Day 0), while dasatinib (**Figure 5B**) and navitoclax (**Figure 5C**) were more effective in selectively reducing the viability of senescent cells at Day 9 compared to Day 0 and therefore can selectively target ciPTEC-OAT1 at a non-permissive temperature. The combination of dasatinib and quercetin (**Figure 5D**) induced cell death in both culture conditions. The concentrations of

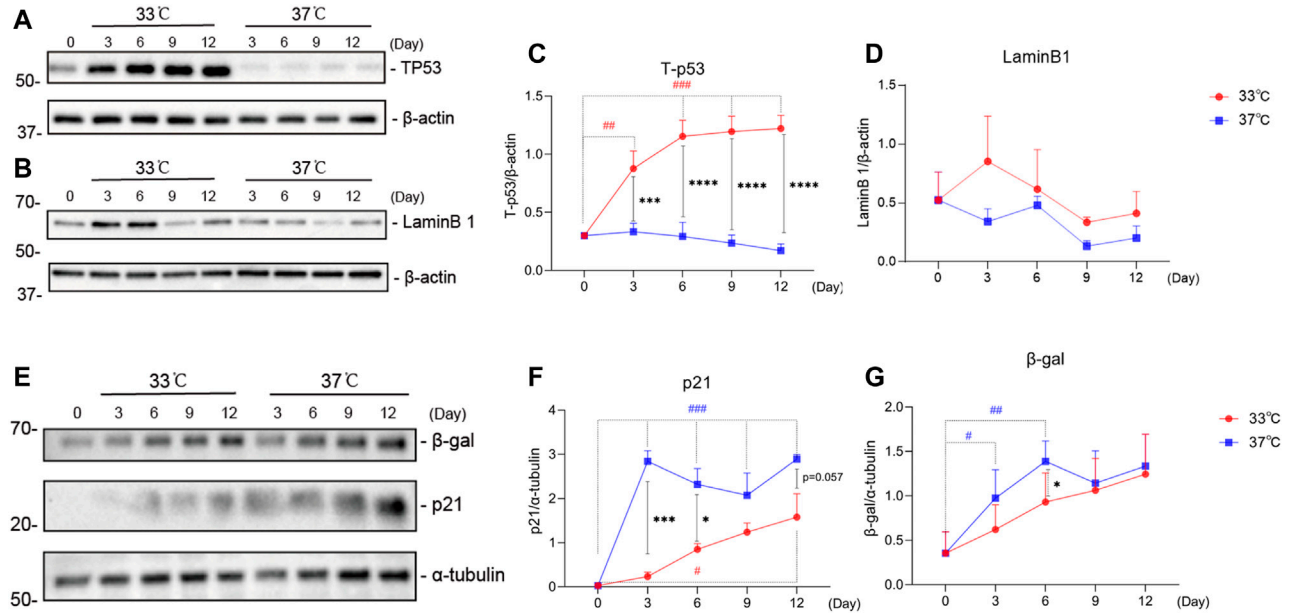

FIGURE 3 | Maturation at a non-permissive temperature of 37°C affects common senescence markers expression in ciPTEC-OAT1. Representative Western blots showing expression of **(A)** total-p53 (Tp53), **(B)** LaminB1, **(E)** p21 and β-gal. **(C),(D),(F),**and **(G)** Relative expression levels of **(C)** total-p53, **(D)** LaminB1, **(F)** p21, and **(G)** β-gal over time (Day 0 to Day 12), at both permissive (33°C) and non-permissive temperatures (37°C). Protein expression levels were normalized against α-tubulin or β-actin and expressed as mean ± SEM. Three independent experiments in triplicate were performed. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (expression levels at 37°C compared to 33°C; two-way ANOVA, Sidak's multiple comparison test). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ (expression levels at Day 3, 6, 9, or 12 compared to Day 0, at 33°C or 37°C; one-way ANOVA, Dunnett's multiple comparison test). Day 0 in both groups is considered as the non-senescence group (control). For clarity, only one-sided error bars are shown.

quercetin used in this combination treatment are non-toxic to the cells regardless of culture conditions. However, after combining quercetin with increasing concentrations of dasatinib, the viability of the cells reduced compared to each single treatment. Notably, dasatinib combined with the highest quercetin dose appeared the most effective in reducing cell viability. This suggests that the co-treatment of dasatinib and quercetin can selectively target ciPTEC-OAT1 presenting senescence-like phenotype (**Figure 5D**). IC50 values of different senolytics are lower at Day 9 of cell maturation at a non-permissive temperature compared to 0 d (**Table 2**). Finally, the cells cultured for 0 or 7 d at 33°C were shown not to be sensitive to navitoclax in contrast to cells cultured at 37°C for 7 d (**Supplementary Figure S2**), which suggests that the cells are senescent when cultured at 37°C for prolonged time (7 d or more).

3.6 Senolytics Clear Senescent ciPTEC-OAT1 as Evaluated by Functional SA-β-Gal Expression

The activity of the lysosomal senescence-associated beta-galactosidase (SA-β-gal) is commonly used as a marker for senescent cells, reflecting increased metabolic activity and enhanced lysosomal content typical of these cells (Hernandez-Segura et al., 2018). We tested the senolytics for their effect on β-gal activity and expression levels. Representative images of SA-β-gal staining upon culturing are shown in **Supplementary Figure S3**, which gives an overview of senescence process during the maturation of ciPTEC-OAT1 cells. The results obtained (**Figures 6A,B**) show that ciPTEC-OAT1 cultured for 9 d at 37°C is positive for SA-β-gal staining and that total β-gal protein was increased. Moreover, following exposure to navitoclax, the number of SA-β-gal positive cells was reduced in a dose-dependent fashion (**Figure 6A**). The expression levels of total β-gal showed a downregulation trend in the presence of navitoclax, with a concentration of 100 nM being the most effective (**Figures 6C,D**). Furthermore, the number of SA-β-gal positive cells was also reduced dose-dependently after the treatment with both dasatinib alone and dasatinib-quercetin combination, which seemed to be slightly more effective than dasatinib alone (**Figure 6B**), accompanied by a downregulation of total β-gal (**Figures 6E,F**). Interestingly, although a lower number of SA-β-gal positive cells was observed after the exposure to higher doses of dasatinib-quercetin combination, there were no differences in protein levels in cells cultured for 9 d at the non-permissive temperature.

4 DISCUSSION

Cellular senescence is an irreversible condition with cell cycle arrest, SASP, and apoptosis resistance, which contribute to chronic kidney disease, leading to fibrosis (Stenvinkel and Larsson, 2013; Hernandez-Segura et al., 2018). We previously developed ciPTEC-OAT1 to be used in drug screening and nephrotoxicity studies (Nieskens et al., 2016). In the present

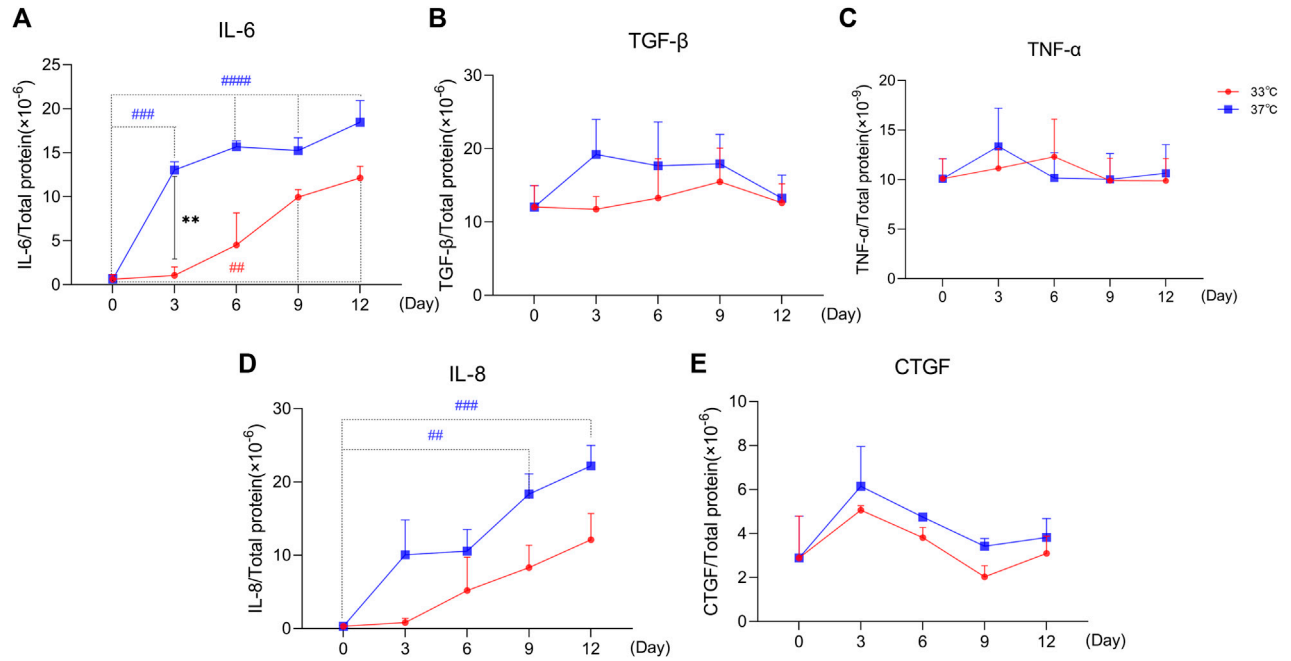

FIGURE 4 | Maturation at a non-permissive temperature of 37°C affects common SASP factors secretion by ciPTEC-OAT1. Release of **(A)** IL-6, **(B)** TGF-β1, **(C)** TNF-α, **(D)** IL-8, and **(E)** CTGF by ciPTEC-OAT1 over time at 33 and 37°C. Concentration is expressed as pg/ml and normalized for total protein (μg/ml). Three independent experiments were performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (secreted levels at 37°C compared to 33°C; two-way ANOVA, Sidak's multiple comparison test). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ (secreted levels at Day 3, 6, 9, or 12 compared to Day 0, at 33°C or 37°C; one-way ANOVA, Dunnett's multiple comparison test). Day 0 in both groups is considered as the non-senescence group (control). For clarity, only one-sided error bars are shown.

study, we demonstrate that the cell model is also suitable for studying tubular senescence in the kidney. We detected differential expression of apoptosis-associated markers, common senescence markers, and some typical SASP factors suggesting that ciPTEC-OAT1 obtains a senescence-like phenotype when cultured at a non-permissive temperature for 9 d. Furthermore, senescent cells appeared sensitive to senolytic drugs.

In the present study, we demonstrated that the ciPTEC-OAT1 cultured at a non-permissive temperature expressed common senescence markers. In particular, the decrease in Lamin B1 (Shimi et al., 2011) and upregulation in p21 (Calcinotto et al., 2019) have been described as characteristic features that are involved in maintaining senescence phenotype by regulating JNK and caspase signaling (Yosef et al., 2017). Further, increased SASP factors have been reported as proinflammatory and matrix-degrading molecules (Childs et al., 2015), including PAI-1 (Sun et al., 2019), IL-1β (Shi et al., 2019), CTGF (Jun and Lau, 2017), and IL-6 (Mosteiro et al., 2018). Another feature entails apoptosis, responsible for cell turnover and maintaining extracellular environment. For instance, the Bcl-2 family members modulate the delicate balance between pro- and anti-apoptosis (Ngoi et al., 2020). The upregulations of Bcl-2 and Bcl-xl suggest that ciPTEC-OAT1 became anti-apoptotic upon maturation in both conditions, whereas culturing at 37°C speeds up the process and induces a more prominent senescence phenotype. Senescent cells show downregulation of Mcl-1 on protein levels (Lee et al., 2018), which is consistent with our findings. Furthermore, the effectors Bax and Bak shuttle between cytosol and mitochondrial outer membrane with different rates (Pena-Blanco and Garcia-Saez, 2018), which might explain why we observed a differential expression in the proteins. BH3-only proteins bind to the BH3 domain of the anti-apoptotic Bcl-2 proteins via hydrophobic interactions, thereby promoting cellular apoptosis (Anantram and Degani, 2019). Of BH3-only proteins, Bid showed higher protein levels at different time points in the maturation group. The increasing trend in all other proteins points toward a priming of cells to undergo apoptosis, but the execution of the death program is restrained. These findings are in line with previous reports showing that following senescence induction by ionizing radiation, senescent cells upregulate pro-apoptosis markers (Chang et al., 2016; Baar et al., 2017). Therefore, cellular senescence in our model with an upregulation of pro-apoptotic markers despite having an anti-apoptotic phenotype argues for cells searching for a new balance to maintain homeostasis.

Caspases are another group of proteins involved in cell death mediated by apoptosis and important senescent markers (Shalini et al., 2015). After MOMP, caspase activation takes place often within minutes, leading to cell death (Pena-Blanco and Garcia-Saez, 2018). Inhibition of caspases therefore blocks apoptosis. Here we detected that the activator (procaspase-9) and executioner (procaspase-3 and procaspase-7) were upregulated in a permissive temperature group but show a differential pattern when cells are cultured at the non-permissive temperature. When mitochondrial-mediated apoptosis is induced and caspase-9 and caspase-3 are activated, the expression of Bax and Bcl-2 has been reported to show different levels to maintain

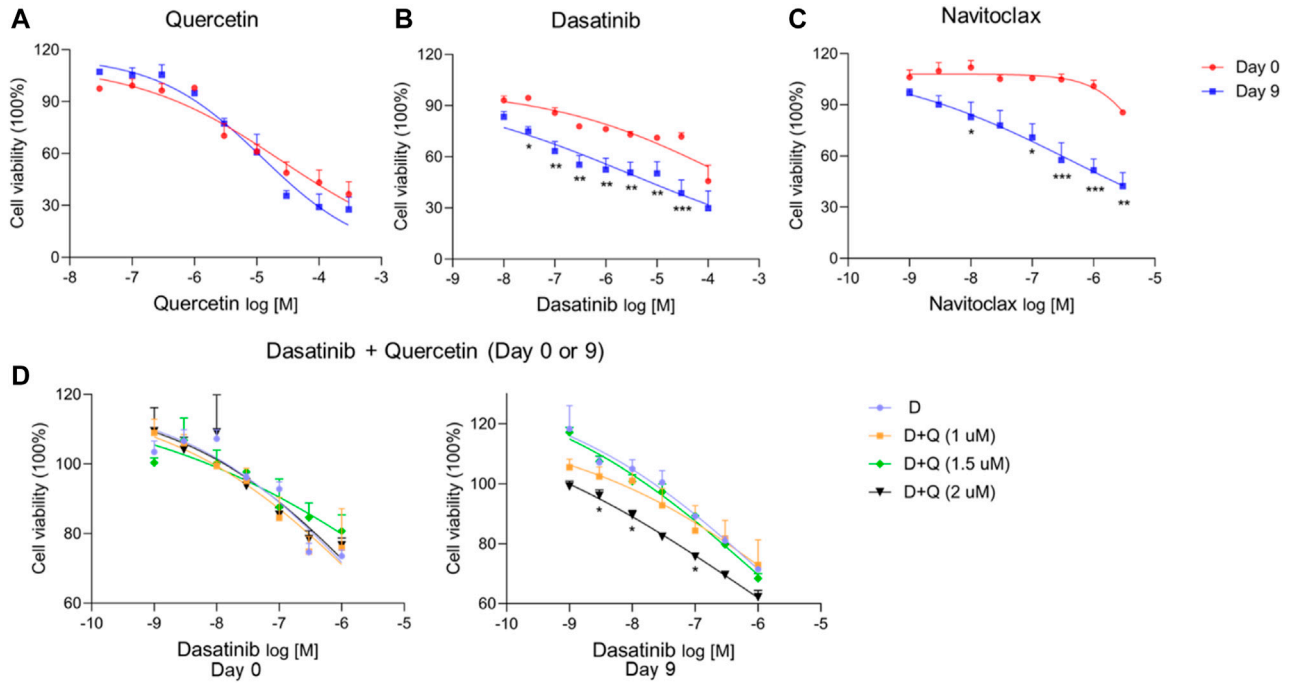

FIGURE 5 | CiPTEC-OAT1 cultured at a non-permissive temperature and exhibiting a senescence-like phenotype is susceptible to common senolytics. Cell viability of ciPTEC-OAT1 cultured for 0 or 9 d at a non-permissive temperature of 37°C and exposed to 100-µl medium with increasing concentrations of **(A)** quercetin (Q), **(B)** dasatinib (D), and **(C)** navitoclax (N) for 24 h. Cell viability of ciPTEC-OAT1 cultured for 0 and 9 d **(D)** at a non-permissive temperature and exposed to 100-µl medium with increasing concentrations of dasatinib combined with quercetin (1 µM, 1.5 µM, or 2 µM) for 24 h. Four independent experiments were performed in triplicate. Data are presented as mean ± SEM, for which results were normalized to unexposed cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (A to C, cell viability at Day 9 compared to Day 0; two-way ANOVA, Sidak's multiple comparison test. D, cell viability of increasing concentrations of dasatinib combined with quercetin (1 µM, 1.5 µM, or 2 µM) compared to dasatinib alone; two-way ANOVA, Dunnett's multiple comparisons test). Day 0 is considered as the non-senescence group (control). For clarity, only one-sided error bars are shown.

TABLE 2 | IC_{50} of (A)-(D) from **Figure 5**.

				D + Q			
IC50	**Q**	**D**	**N**	**D(Q = 0 µM)**	**D(Q = 1 µM)**	**D (Q = 1.5 µM)**	**D(Q = 2 µM)**
Day 0	>10 µM	>3 µM	>3 µM	>3 µM	>4 µM	>3 µM	>4 µM
Day 9	>3 µM	>1 µM	>0.4 µM	>1 µM	>6 µM	>1 µM	>0.7 µM

their balance (Zhang et al., 2021). Irradiation-induced senescence is accompanied by an upregulation of procaspase-3, -7, and -9 (Chang et al., 2016; Baar et al., 2017) but a downregulation of activated caspase-3 (Chang et al., 2016), in line with our results. The final downregulation of procaspases observed in our study might be explained by the cleavage of procaspase-9 finally to active caspase-3 and caspase-7. But because of senescence induction and an adapted balance in Bcl-2 family proteins, procaspase-9 is inhibited, finally leading to apoptosis-resistance (**Figure 7**).

The observed loss of LaminB1 and upregulation of β-gal, well-known indicators of senescence (Hernandez-Segura et al., 2018), in ciPTEC-OAT1 cultured at a non-permissive temperature further confirms the phenotypical changes. Silencing LaminB1 immediately leads to inhibition of proliferation and the induction of senescence (Shimi et al., 2011). Our previous research regarding cell cycle analysis of ciPTEC-OAT1 at permissive and non-permissive temperatures (Mihajlovic et al., 2019) has shown that ciPTEC-OAT1 when cultured at 37°C for 1 or 7 d exhibits significantly reduced proliferation (less cells in the S phase) and an increased number of cells in the G0/G1 phase of the cell cycle, indicating halted proliferation at a non-permissive temperature. This is in line with our current results. The activity of the lysosomal β-gal reflects increased metabolic activity and enhanced lysosomal content typical of senescent cells (Hernandez-Segura et al., 2018). Transfection with the temperature sensitive SV40T gene allows the cells to become conditionally immortalized (Wilmer et al., 2010). Although downregulation of SV40T at 37°C allows activation of p53 and pRb, factors involved in both p53/p21 and p16/pRb pathways, there was no significant difference in their mRNA expression (Supplementary Figure S1) and p16 protein expression appeared undetectable. This suggests that senescence of ciPTEC-OAT1 may not be induced by the p16/pRb pathway. On the other hand, p53 is

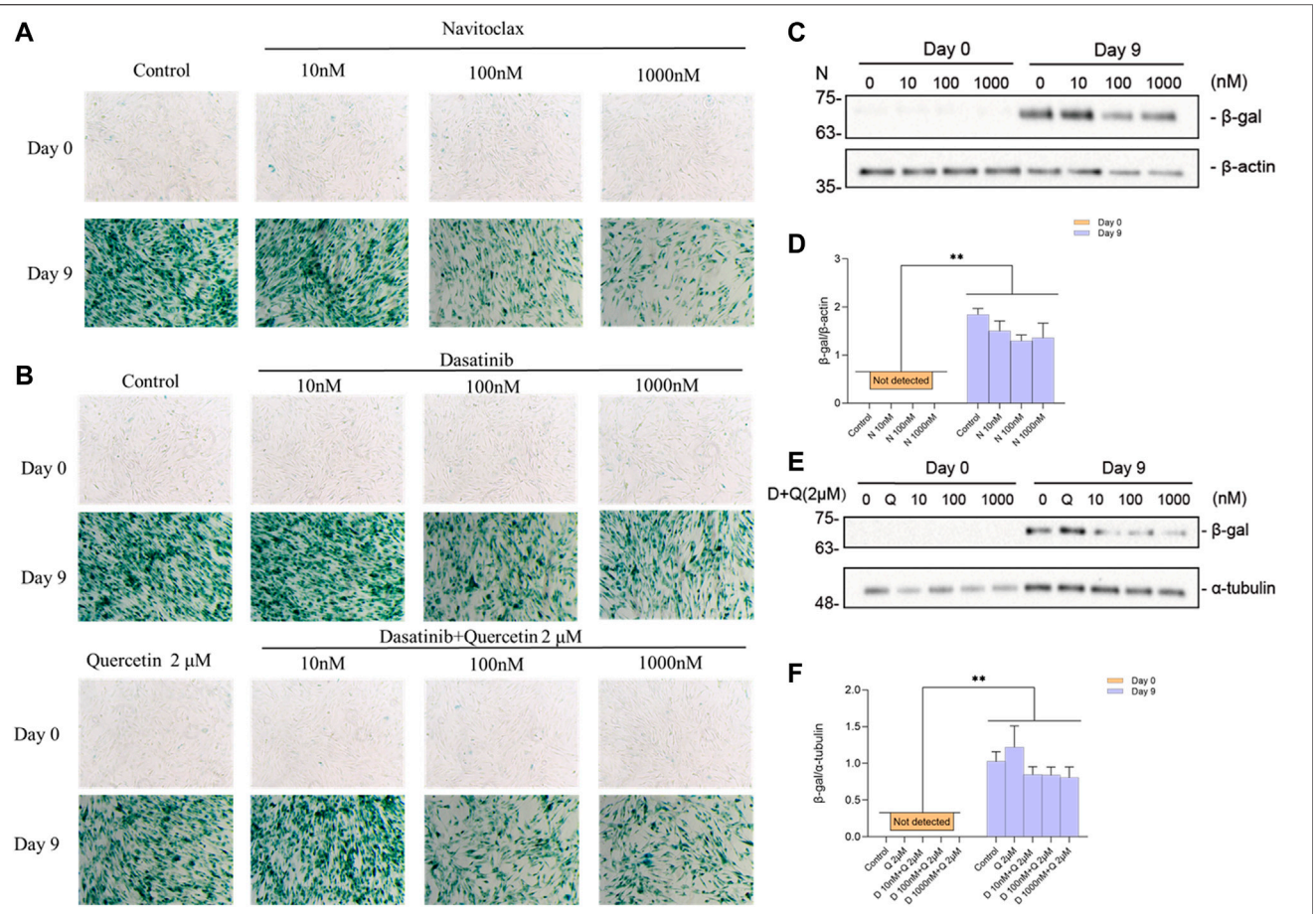

FIGURE 6 | Effects of senolytics on SA-β-gal activity and protein levels in ciPTEC-OAT1 cultured for 9 d at a non-permissive temperature. Representative images of SA-β-gal staining in ciPTEC-OAT1 cultured for 0 and 9 d at 37°C and after 24 h exposure to 1-ml medium with different concentrations of **(A)** navitoclax (N) (10 nM, 100 nM, or 1000 nM) and **(B)** dasatinib (D) alone (10 nM, 100 nM, or 1,000 nM) or combined with quercetin (Q) 2 μM. **(C)** Representative Western blots showing expression of total β-gal after 24 h exposure to different concentrations of navitoclax in ciPTEC-OAT1 cultured for 0 or 9 d at a non-permissive temperature. **(D)** Relative expression of total β-gal after 24 h exposure to different concentrations of navitoclax. **(E)** Representative Western blots showing expression of total β-gal after 24 h exposure to different concentrations of dasatinib combined with quercetin 2 μM in ciPTEC-OAT1 cultured for either 0 or 9 d at a non-permissive temperature. **(F)** Relative expression of total β-gal after 24 h exposure to different concentrations of dasatinib and quercetin 2 μM. Protein expression levels were normalized to α-tubulin or β-actin, and expressed as mean ± SEM. Three independent experiments were performed in triplicate. *$p < 0.05$, **$p < 0.01$ (expression levels at 37°C compared to 33°C at the same time point; multiple *t*-test, Holm-Sidak multiple comparison test). Day 0 is considered as the non-senescence group (control). For clarity, only one-sided error bars are shown.

pivotal in determining the fate of the cells, implying that the p53/p21 pathway is key in the initiation of senescence (Mijit et al., 2020). Previously published works described a decline of total-p53 levels in stress-induced senescence in mice (Feng et al., 2007) and p21's role in maintaining senescence in mice (Yosef et al., 2017), which is also in accordance with our *in vitro* data showing a decline in p53 levels and an increase in p21 levels at 37°C. Therefore, our results suggest that ciPTEC-OAT1 cultured at 37°C promotes senescence through the p53/p21 pathway.

In addition to previously tested markers, SASP factors are also important players in senescence. IL-6 maintains senescence through the p53/p21 pathway (Effenberger et al., 2014; Li Y. et al., 2020), shared by IL-8, which is expressed as a function of IL-6 (Kuilman et al., 2008). TGF-β1 and CTGF are other SASP factors reported to mediate senescence (Jun and Lau, 2017; You et al., 2019). CTGF is a downstream mediator of TGF-β1 and is regulated by TGF-β1 (Ou et al., 2020). TGF-β1 induces kidney fibrosis by accumulation of extracellular matrix and CTGF expression by activation of Smad3 and p53 (Li X. et al., 2020). Meanwhile, both CTGF and TGF-β1 induce senescence and are accompanied with the upregulation of IL-6 and IL-8 (Jun and Lau, 2017; Fan et al., 2019). Although not significant, our results show an increasing trend of both TGF-β1 and CTGF in 37°C group compared to 33°C group. PAI-1 is a major TGF-β1/p53 target gene in kidney fibrosis and is known to be elevated in senescent cells, correlating with increased tissue TGF-β1 levels (Samarakoon et al., 2019; Rana et al., 2020). In our study, PAI-1 and CTGF increased remarkably on the mRNA level, suggesting cellular senescence. TNF-α is another SASP factor and inducer of senescence (Guo et al., 2019), but our results showed no important differences over time and

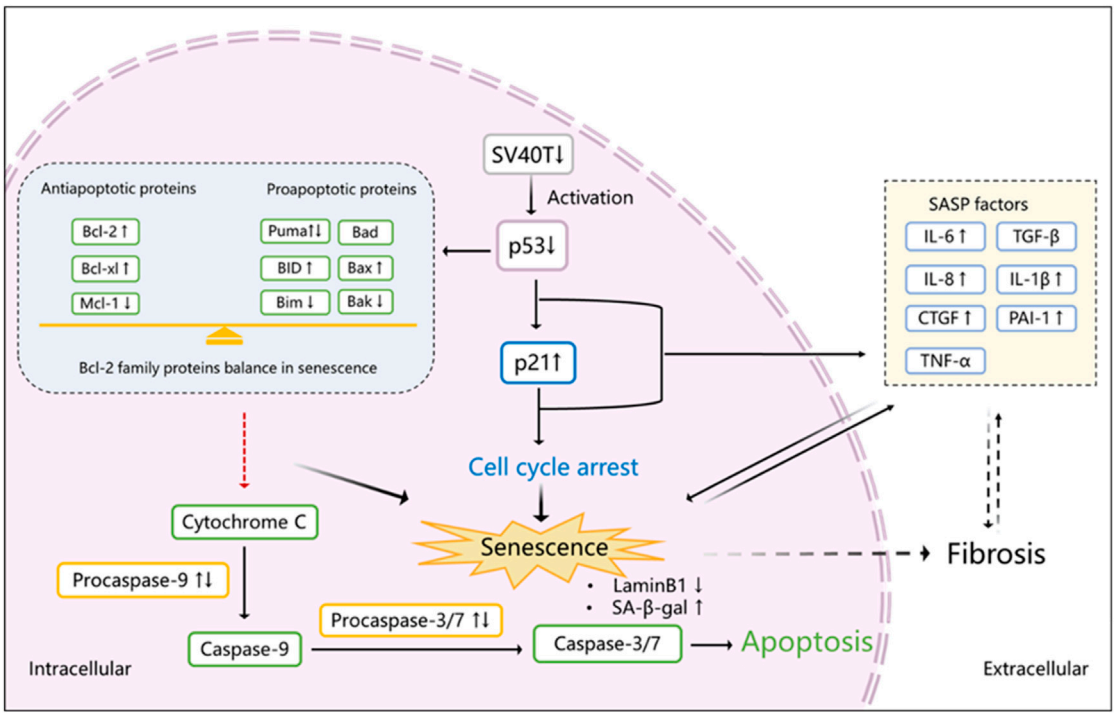

FIGURE 7 | Proposed scheme of senescence induction in ciPTEC-OAT1 after maturation at a non-permissive temperature. After transfer of cells to 37°C and subsequent downregulation of SV40T, p53 is activated which transcriptionally upregulates p21, inducing cell cycle arrest and eventually leading to senescence. SASP factors are released during this process as well. Activated p53 also influences the expression of Bcl-2 family proteins that become abnormally upregulated. Procaspase 9 and its downstream proteins procaspases 3 and 7 are activated at the beginning of this process, and with time going by, the Bcl-2 family reaches a balance between anti-apoptotic and pro-apoptotic proteins expression, halting the activation of procaspases and inhibiting apoptosis. In addition, senescent cells show a downregulation of LaminB1 and an upregulation of SA-β-gal and some SASP factors, including IL-6, IL-8, CTGF, IL-1β, and PAI-1, which may further contribute to kidney fibrosis. ↑, upregulation; ↓, downregulation; ↑↓, initial upregulation followed with downregulation (expression levels at 37°C compared to 33°C).

between the culture conditions, indicating the senescence of ciPTEC-OAT1 is not induced or maintained by TNF-α. There are some discrepancies between mRNA and protein levels of the obtained results. Despite being difficult to explain, these discrepancies might be due to differences in the regulation of transcription and protein translation processes, as well as protein turnover rate. Taken together, the results of the mRNA and protein levels, we believe that the cells exhibit a senescence phenotype at a non-permissive temperature.

Finally, the senolytics navitoclax, dasatinib, and quercetin were evaluated. Our results suggest that ciPTEC-OAT1 cultured at a non-permissive temperature was sensitive to senolytics, and the Bcl-2 family inhibitor navitoclax (Tse et al., 2008; Chang et al., 2016), navitoclax, appeared most effective in selectively reducing viability of cells presenting senescent phenotype. A clinical trial of the dasatinib and quercetin cocktail demonstrated a decrease in p21 and p16 positive human adipose tissue cells and plasma SASP factors of diabetic kidney disease participants (Hickson et al., 2019). Senolytics treatment of ciPTEC-OAT1 led to a dose-dependent reduction of SA-β-gal positive cells, in line with previous results (Zhu et al., 2015). It has been suggested that SA-β-gal activity may be an outcome rather than a cause of senescence (Lee et al., 2006; Piechota et al., 2016), and our findings argue for a clearance of senescent cells leading to a reduction in total cell number. Our follow-up research will focus on investigating the underlying mechanisms of senolytics used in this study.

In conclusion, our results suggest ciPTEC-OAT1 can be used as a valid proximal tubule cell model both for mechanistic studies inherent to kidney senescence and fibrosis and for senolytic effects of newly developed drugs and their combinations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Conceptualization, YY, MM, TN, RG and RM; methodology, YY, MM, FV, TN, RG and RM; investigation, YY and FV; writing original draft preparation, YY; writing review and editing, YY, MM, and RM; supervision, MM, TN, RG and RM; funding acquisition, YY, RG, and RM. All authors have read and agreed to the published version of the manuscript.

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Dexmedetomidine Attenuates Ferroptosis-Mediated Renal Ischemia/Reperfusion Injury and Inflammation by Inhibiting ACSL4 *via* α2-AR

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Ischemia-reperfusion (I/R) injury is a serious clinical pathology associated with acute kidney injury (AKI). Ferroptosis is non-apoptotic cell death that is known to contribute to renal I/R injury. Dexmedetomidine (Dex) has been shown to exert anti-inflammatory and organ protective effects. This study aimed to investigate the detailed molecular mechanism of Dex protects kidneys against I/R injury through inhibiting ferroptosis. We established the I/R-induced renal injury model in mice, and OGD/R induced HEK293T cells damage *in vitro*. RNA-seq analysis was performed for identifying the potential therapeutic targets. RNA-seq analysis for differentially expressed genes (DEGs) reported Acyl-CoA synthetase long-chain family member 4 (ACSL4) related to ferroptosis and inflammation in I/R mice renal, which was validated in rodent renal. Liproxstatin-1, the specific small-molecule inhibitor of ferroptosis, significantly attenuated ferroptosis-mediated renal I/R injury with decreased LPO, MDA, and LDH levels, and increased GSH level. Inhibiting the activity of ACSL4 by the Rosiglitazone (ROSI) resulted in the decreased ferroptosis and inflammation, as well as reduced renal tissue damage, with decreasing LPO, MDA and LDH level, increasing GSH level, reducing COX2 and increasing GPx4 protein expression, and suppressing the TNF-α mRNA and IL-6 mRNA levels. Dex as a α2-adrenergic receptor (α2-AR) agonist performed renal protective effects against I/R-induced injury. Our results also revealed that Dex administration mitigated tissue damage, inhibited ferroptosis, and downregulated inflammation response following renal I/R injury, which were associated with the suppression of ACSL4. In addition, ACSL4 overexpression abolishes Dex-mediated protective effects on OGD/R induced ferroptosis and inflammation in HEK293T cells, and promotion of ACSL4 expression by α2-AR inhibitor significantly reversed the effects on the protective role of Dex. This present study indicated that the Dex attenuates ferroptosis-mediated renal I/R injury and inflammation by inhibiting ACSL4 *via* α2-AR.

Keywords: Dexmedetomidine, Renal ischemia/reperfusion injury, ferroptosis, Inflammation, ACSL4, α2-adrenergic receptor

1 INTRODUCTION

Ischemia-reperfusion (I/R) injury is a common clinical pathology associated with high mortality, occurred in numerous clinical pathologies, such as acute kidney injury (AKI) (Wang et al., 2020b), renal transplantation (Minami et al., 2020), and trauma shock (Hoareau et al., 2019), which leads to systemic inflammation and dysfunction (Hasegawa et al., 2021). Despite several mechanisms of I/R injury have been investigated, the effective treatment remains elusive. Therefore, it is meaningful to develop the potent therapeutic intervention for renal I/R injury.

Recently, increasing studies revealed that a variety of complex pathways were involved in renal I/R injuries, such as apoptosis (Liu et al., 2020), pyroptosis (Xia et al., 2021), and ferroptosis (Ding et al., 2020; Zhang et al., 2021). Attempts to prevent cell death have been strongly biased to the study of apoptosis for it has been considered to be the only form of cell death that can be reversed by pharmacological and genetic intervention. With the acquiring of a better understanding of cell death, ferroptosis has attracted extensive attention due to it is a novel type of cell death caused by iron-dependent phospholipid peroxidation (Doll and Conrad, 2017), which has been confirmed to be related to various diseases such as cancer (Wu et al., 2021b), degenerative diseases (Tang et al., 2021a), antiviral immunity (Xu et al., 2018), stroke (Alim et al., 2019), and I/R injury (Chen et al., 2021). Ferroptosis plays an important role in cisplatin-induced AKI (Hu et al., 2020). In addition, ferroptosis is involved in the AKI model induced by folic acid (Li et al., 2021). The study also found that isoliquiritigenin alleviates septic-induced AKI by suppressing ferroptosis (Tang et al., 2021b). However, the molecular mechanism of AKI is largely unknown. Now, with the direct link between lipid peroxidation and unique cell death pathway, the research on finding new small molecules that can inhibit lipid peroxidation has made progress, and may produce new cell protection strategies.

Ferroptosis is characterized by mitochondrial shrinkage, increased mitochondrial membrane density, and lipid reactive oxygen species (L-ROS), and a unique set of genes, such as Acyl-CoA synthetase long-chain family member 4 (ACSL4), Glutathione peroxidase (GPx4), and Cyclooxygenase2 (COX2) (Li et al., 2019). Lipid peroxidation is essential for ferroptosis and involves the preferential oxidation of arachidonic acid (AA) and its esterifiable production phosphatidylethanolamine (PE) (Lee et al., 2021). One study found that L-ROS inhibitors significantly alleviated myocardial I/R injury through inhibiting glutaminolysis and ferroptosis (Feng et al., 2019). Other studies demonstrated that ACSL4 could facilitate the esterification of AA and adrenoyl into PE, a process closely related to ferroptosis (Doll et al., 2017; Kagan et al., 2017), and suppression of this process by pharmacological ACSL4 inhibition induced anti-ferroptosis and anti-inflammation rescue pathway (Cui et al., 2021). However, the role of ferroptosis and inflammation in renal I/R is still not fully explored and remains elusive.

Dexmedetomidine (Dex) as a highly selective α2-adrenoceptor (AR) agonist alleviates septic heart injury by inhibiting ferroptosis (Wang et al., 2020a), and protects nerve cells from oxidative injury by maintaining iron homeostasis (Qiu et al., 2020). In addition, some studies have found that Dex could improve renal function and reduce I/R injury (Li et al., 2020a; Ma et al., 2020). However, the exact protective mechanism of Dex on renal I/R has not been elucidated. In this study, we hypothesize that Dex could protect the kidney against I/R induced injury through alleviating ferroptosis and inflammation by inhibiting ACSL4 *via* α2-AR.

2 MATERIALS AND METHODS

2.1. Animals

Adult healthy C57BL/6 mice (7–8 weeks, 18–22 g) were obtained from Cavens Biogle Model Animal Research Co., Ltd. (Soochow, China). All mice were kept under controlled temperature of 24–26°C, relative humidity 40–60% and 12 h/12 h light-dark cycle with food and water available ad libitum. Animal care and handling were approved by the Institutional Animal Care and Use Committee of Soochow University (Soochow, China). All experiments were performed in accordance with the Guide for the Care of Use of Laboratory Animals published by the US National Institute of Health.

2.2 RNA Extraction, Library Construction and Sequencing

Total RNA was extracted employing Trizol reagent kit (Invitrogen, United States). Then, mRNA was enriched and fragmented and reverse transcripted into cDNA. After second-strand cDNA were synthesized, purified cDNA with QiaQuick PCR extraction kit (Qiagen, Netherlands), end repaired, poly (A) added, and ligated to Illumina sequencing adapters. PCR amplified, and sequenced using Illumina HiSeq2500 by Gene Denovo Biotechnology Co., (Guangzhou, China). FPKM (fragment per kilobase of transcript per million mapped reads) was calculated using StringTie software. RNAs differential expression analysis was performed by DESeq2 (Love, Huber and Anders 2014) between two different groups and by edgeR (Robinson, McCarthy and Smyth 2010) between two samples. The genes/transcripts with the parameter of false discovery rate (FDR) below 0.05 and absolute fold change ≥2 were considered differentially expressed genes/transcripts.

2.3. Establishment of Renal Ischemia-Reperfusion Model, Cell Culture, OGD/R Procedures and Transient Transfection

The renal I/R model was established as previous described (Liu et al., 2019). Briefly, renal I/R was induced by occluding bilateral renal hilum for 45 min by a microvascular clip and followed by 48 h reperfusion. Human Embryonic Kidney (HEK) 293T cells (ATCC, Rockville, United States) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were incubated at 37°C in an atmosphere of 5% CO_2, and grown to 80% confluence before use. For oxygen glucose deprivation/

reoxygenation (OGD/R) treatment is the same as the description of our previous research (Yang et al., 2021b). Briefly, HEK293T were cultured in an anaerobic chamber containing 95% N_2 and 5% CO_2 for 6 h and then reoxygenated for 6 h to induce OGD/R condition. Cells were pretreated with Dex (1 μM, 24 h incubation before OGD/R) or/and ACSL4 overexpression vector transfection before exposure to OGD/R, as mentioned in this study.

The plasmid was created and synthesized by RiboBio (Guangzhou, China). Before transfection, cells were transplanted to 6-well or 96-well plates and grown to a confluence of 30%. Gp-transfect Mate (GenePharma, China) was used according to the manufacturer's protocol. The ACSL4 plasmid was subcloned into the pcDNA3.1 vector to overexpress ACSL4. All plasmids were incubated with the transfection media for 48 h before OGD/R.

2.4 Lactate Dehydrogenase Assay and Cell Viability Assay

Serum and cellular supernatants were collected for the LDH activity assay (A020, JianCheng, China) according to the manufacturer's instructions. The optical density value for each well at 450 nm absorbance was measured by Spectra Max 190 plate reader. Experiments were repeated at least three times.

To measure cell viability, 6×10^3 cells per well were seeded in 96-well plates and treated as indicated, after which the medium in each well was replaced with 100 μL fresh medium containing 10% Cell Counting Kit-8 reagent (Apex BIO, K1018). After incubation for 1 h at 37°C according to the instructions of the kit, plate was read by a Spectra Max 190 plate reader at a wavelength of 450 nm.

2.5 Iron Measurements and Acyl-CoA Synthetase Long-Chain Family Member 4 Activity

Phosphate-buffered saline (PBS) was used for ischemic renal tissue homogenized. Collecting the supernatant after centrifugation. The content of iron was determined by the Iron Assay Kit (A039, JianCheng, China) according to the manufacturer's instructions. ACSL4 was confirmed facilitate the esterification of arachidonoyl and adrenoyl into phosphatidylethanolamine (PE), a process closely related to ferroptosis (Kagan et al., 2017), so PE was used to evaluate the level of ACSL4 activity (Li et al., 2019). PE kits (ab241005, Abcam) were used according to the manufacturer's instructions.

2.6 Lipid Peroxidation Assay

A lipid peroxidation assay kit (A106, Jiancheng, China) was used to test the lipid peroxidase (LPO) level in lysates of tissue following the manufacturer's instructions. Briefly, lipid peroxide reacts with chromogenic reagents under the condition of 45°C for 60 min and produces a stable chromophore with a maximum absorption peak at 586 nm.

Lipid ROS was measured using the live-cell analysis reagent BODIPY 581/591 C11 (27,086, Cayman). Treated cells were incubated with BODIPY (5 μM) for 1 h at 37 °C in 24-well cultures. After incubation, cells were harvested and washed with PBS, and then resuspended in 500 μL PBS. Images were acquired under an IX83 fluorescence microscope (ECLIPSE Ts2R-FL, Nikon). Cell fluorescence was acquired on a Flow Cytometer (Cytomics^{TM}FC500, Beckman) and analyzed with FlowJo software (FlowJo^{TM}v10, United States).

2.7 Western Blot Analysis

The whole protein of mice renal tissues or HEK293T cells was extracted using the RIPA lysate buffer (Beyotime, China). Protein concentration was measured by a bicinchoninic acid reagent kit (Beyotime, China). The proteins were separated in 8% or 10% gels using a sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, United States). The membranes were blocked with 5% non-fat milk at room temperature for 2 h and then incubated at 4°C overnight with the corresponding primary antibodies: GPx4 antibody (P02794, Abways), ACSL4 antibody (O60488, Abways); COX2 (ab62331, Abcam); β-tubulin antibody (86,298, Cell Signalling Technology). Next, the membranes were washed and incubated at room temperature for 2 h with the secondary antibodies: Goat anti-rabbit IgG antibody (CW0103S, CoWin), Goat anti-mouse IgG antibody (CW0102S, CoWin). The bands were detected using an enhanced chemiluinescence kit (NCM, China) under a luminescent imaging workstation (Tanon5200, China). The protein intensity was analyzed with the ImageJ software and normalized to β-tubulin.

2.8 Real-Time Quantitative Reverse Transcription-PCR (RT-qPCR)

The renal tissue was subjected to RNA extraction, reverse transcription, and then real-time quantitative reverse transcription-PCR as described before (Peng et al., 2020). The following primer sets (Sangon Biotech Inc, China) were used in the process: mouse ACSL4 (forward, 5′-CAA TAG AGC AGA GTA CCC TGA G-3′; reverse, 5′-TAG AAC CAC TGG TGT ACA TGA C-3′), mouse IL-6 (forward, 5′-TGA TGC ACT TGC AGA AAA CAA TCT GA-3′; reverse, 5′-AGC TAT GGT ACT CCA GAA GAC CAG AGG-3′), mouse TNF-α (forward, 5′-TGA TCG GTC CCC AA A GGG ATG; reverse, 5′-TTG GTG GTT TGC TAC GAC GTG G-3′), mouse β-tubulin (forward, 5′-CAG CGA TGA GCA CGG CAT AGA C; reverse, 5′-CCA GGT TCC AAG TCC ACC AGA ATG-3′). human IL-6 (forward, 5′-ATG AAC TCC TTC TCC ACA AGC GC -3′; reverse, 5′-GAA GAG CCC TCA GGC TGG ACT G -3′), human TNF-α (forward, 5′-TGA TCG GTC CCC AA A GGG ATG; reverse, 5′-TTG GTG GTT TGC TAC GAC GTG G-3′), human β-tubulin (forward, 5′-CAG CGA TGA GCA CGG CAT AGA C; reverse, 5′-CCA GGT TCC AAG TCC ACC AGA ATG-3′).

2.9 Transmission Electron Microscope (TEM)

After 48 h of reperfusion, the anesthetized mice were killed, the kidneys were washed with precooled PBS (pH 7.4), cut into 2 mm × 2 mm x 2 mm block tissue and then fixed in 100 mM PBS (pH

7.0–7.5) containing 2.5% glutaraldehyde (Servicebio, China) at room temperature for 2 h, and then transferred to 4°C for storage overnight. Ultrathin sections (70 nm) were cut, stained with uranyl acetate and lead citrate, and viewed under a transmission electron microscope (TEM; HT7700; Japan). Five fields for each sample was randomly chose and counted the mitochondria with ferroptotic features. The number of ferroptotic mitochondria per field in each sample was quantified.

2.10 GSH Assays and Malondialdehyde

Malondialdehyde (MDA) production was determined using a lipid peroxidation assay kit (A003, Jiancheng, China) based on the method of thiobarbituric acid reactive substances (TBARS) to reflect the degree of lipid peroxidation *in vivo*. Mice renal was homogenized and the supernatant was collected for GSH analysis using a GSH assay kit (A06, Jiancheng, China).

2.11 Assessment of Kidney Functions and Injury

Concentrations of serum creatinine and BUN were detected by Automatic biochemical instrument (Siemens ADVIA 1800 Chemistry System, Germany). Hematoxylin and Eosin (H&E)-stained kidney specimens were used to evaluate kidney injury, the renal sections were fixed in 4% paraformaldehyde and embedded in paraffin. Five millimeter slices were cut for H&E staining and then observed under an optical microscope (Olympus, Japan). Renal injury score was evaluated by an established grading by Zhou, W et al. as described before (Zhou et al., 2000). Renal injury was graded as fellow: normal (0 score); <10% necrosis (1score); 10–25% (2 score); 25–75% (3 score); and >75% (4).

2.12 Drug Treatment

Liproxstatin-1(Lip-1, B4987, APExBIO, United States), a ferrptosis inhibitor, was administrated i.p. at a concentration of 10 mg/kg 1 h before ischemia induction, in accordance with previous study protocols (Li et al., 2019). Rosiglitazone (ROSI, S2556,Selleck), a classic peroxisome proliferator-activated receptor-γ agonist that has been used for ACSL4 inhibition, was administered intravenously at a concentration of 0.4 mg/kg 1 h before ischemia induction (Doll et al., 2017; Li et al., 2019). Atipamezole (ATI, abs816081, absin), a α2-AR antagonist, was administered intraperitoneally at a concentration of 250 μg/kg 0.5 h before I/R treatment, and just the renal pedicle clamp was released. Dexmedetomidine (21,090,731, Yangtze River Pharmaceutical group, China) was administered intraperitoneally at a concentration of 50 μg/kg in two time points, including 30 min before I/R and just the renal pedicle clamp was released. Sham mice underwent all the procedures except renal ischemia. Blood and kidney samples were collected 48 h after reperfusion.

2.13 Statistical Analysis

Data were analyzed by GraphPad Prism 8.0 software (GraphPad Inc, La Jolla, United States) and expressed as means ± S.D. Statistical significance was determined by using Student's *t*-test in two groups. ANOVA was used to compare more than two group. $p < 0.05$ was considered statistically significant.

3 RESULTS

3.1 Dex Administration Improved Renal Function and Reversed Acyl-CoA Synthetase Long-Chain Family Member 4's Upregulation During Renal I/R

The renal I/R injury significantly increased serum creatinine (Scr) and blood urea nitrogen (BUN) levels compared with the sham group, Dex administration effectively reduced the increased Scr and BUN levels (**Figures 1A**,**B**), which is consistent with the previous study that Dex has renal protection, but the exact protective mechanism is not clear, so we implemented RNA-seq to find the target gene of Dex. Principal component analysis (PCA) was performed with R package models (http://www.rproject.org/) in this experience. PCA is largely used to reveal the structure/relationship of the samples/datas (**Figure 1C**). The RNA-seq analysis showed 1901 up-regulated after renal I/R compared with the sham group, and 1398 down-regulated genes after Dex administration compared with the I/R group, Among them, 1047 genes have attracted our attention, as shown in the Venn diagram (|log2FCl>1, and FDR<0.05, **Figures 1D–F**). We focused on the ACSL4, a key isozyme for polyunsaturated fatty acids metabolism that dictates ferroptosis sensitivity. RNA-seq result showed higher ACSL4 expression in the I/R group than those in the sham group and then significantly decreased with Dex administration (**Figure 1G**; **Table1**). The qRT-PCR (**Figure 1H**) and western blot (**Figure 1I**) results reveal that Dex significantly reversed the increased expression of ACSL4 following renal I/R injury.

3.2 Ferroptosis and Inflammation Are Involved in Renal Ischemia-Reperfusion

Some core factors, such GPx4, COX2, were confirmed as key and valid proteins in ferroptosis regulation (Li et al., 2019) (Chen et al., 2021). Therefore, to evaluate ferroptosis sensitivity after ischemia in the renal, we determined the expression levels of these proteins under I/R condition. The decreased GPx4 and increased COX2 protein expression in the I/R group were confirmed by western blot analysis (**Figure 2A**). LPO and MDA assay indicated that lipid peroxidation was higher in the I/R group than those in sham group (**Figures 2B,C**). LDH level was also increased after I/R injury (**Figure 2D**). TEM was used to investigate the morphological feature of ferroptosis. The mitochondrial morphological feature of ferroptosis was assessed, and the results showed that shrunk mitochondrial, reputed outer mitochondrial membrane, and the disappearance of mitochondrial cristae in the random field in the I/R group (**Figure 2E**). In addition, tissue GSH level was reduced in the I/R group (**Figure 2F**). And renal I/R led to an accumulation of tissue iron levels (**Figure 2G**). Our results also showed IL-6 mRNA and TNF-α mRNA, the critical indicators that involved in inflammation, were significantly increased during I/R (**Figure 2H**). Taken together, our result indicated that ferroptosis and inflammation may be contributed to renal I/R injury.

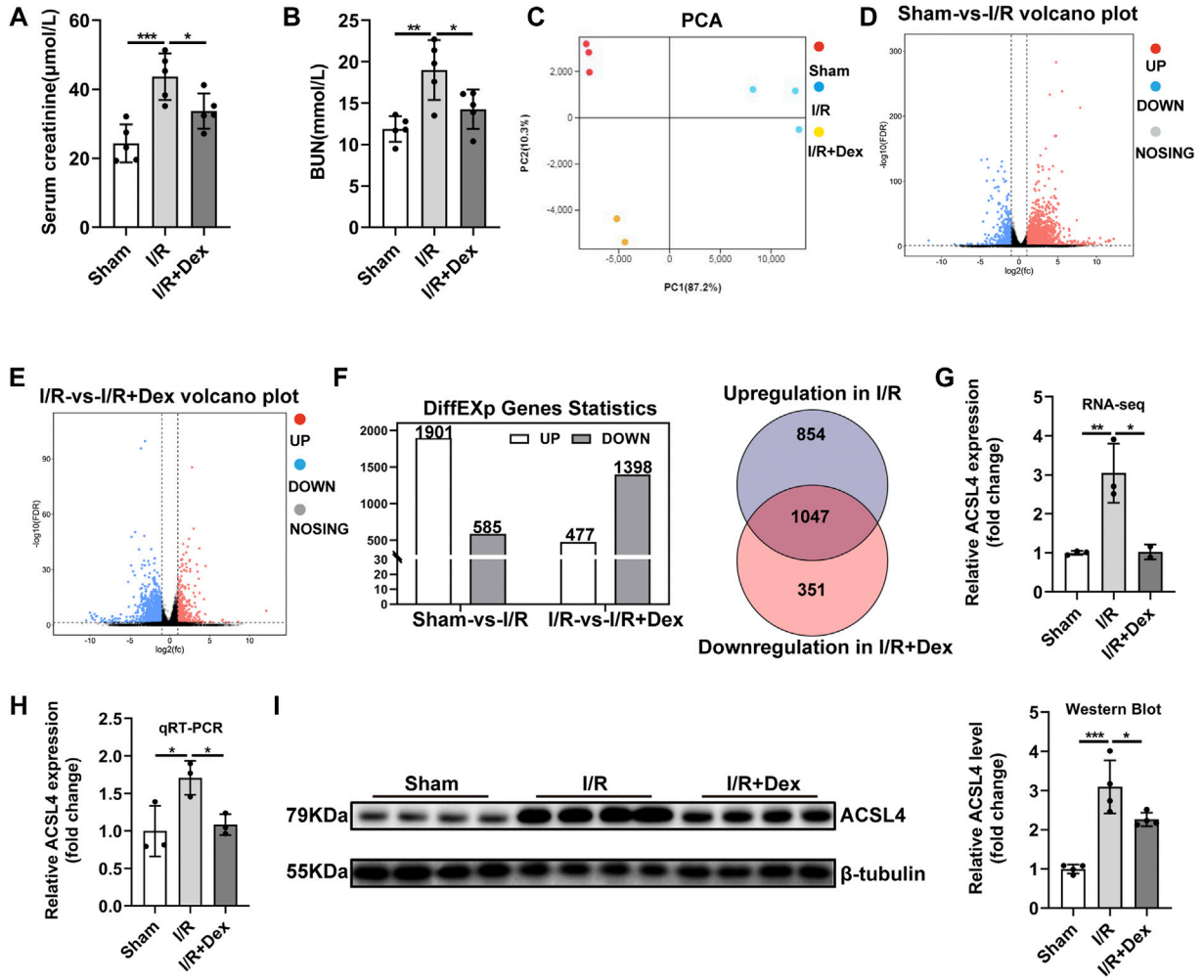

FIGURE 1 | Dex administration improved renal function and reversed ACSL4 upregulation during renal I/R. **(A,B)** The Scr and BUN levels for assessing renal function (n = 5). **(C)** Principal component analysis (PCA) was performed. **(D–F)** Different expression genes statistics ($|\log_2 FC|>1$, and FDR<0.05). **(G)** Expression of ACSL4 gene in renal I/R tissue determined by RNA-sequencing (RNA-seq) (n = 3). **(H)** ACSL4 mRNA expression (n = 3). **(I)** ACSL4 protein expression (n = 4). The data are the Means ± S.D., $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.3 Inhibition of Ferroptosis Alleviates Renal Ischemia-Reperfusion Injury

In order to confirm the role of ferroptosis in renal I/R injury, Liproxstatin-1 (Lip-1), a specific ferroptosis inhibitor, was administrated. Lip-1 treatment significantly reversed the increased COX2 and decreased GPx4 protein expression induced by renal I/R injury (**Figure 3A**). The histopathological scores showed that renal I/R resulted in severe tubular damage, included widespread degeneration of tubular architecture, tubular dilation, tubular cell swelling, pyknotic nuclei, and luminal congestion, which could be significantly improved with Lip-1 treatment (**Figure 3B**). Lip-1 reduced the increased serum LDH level during I/R injury. (**Figure 3C**). Furthermore, Lip-1 also reduced MDA (**Figure 3D**) and LPO (**Figure 3E**), and increased the GSH levels (**Figure 3F**), indicating an improvement in lipid peroxidation and oxidation resistance.

3.4 Inhibition of Acyl-CoA Synthetase Long-Chain Family Member 4 Mitigates Ferroptosis-Mediated Damage and Inflammation

ACSL4, a critical contributor and regulator of ferroptosis, determined the sensitivity of ferroptosis. Rosiglitazone (ROSI), a peroxisome proliferator-activated receptor-γ (PPAR-γ) activator, could inhibit ACSL4 activity. After ROSI treatment, ACSL4 activity was inhibited in the I/R + ROSI group compared with in the I/R group (**Figure 4A**). Meanwhile, the decreased GPx4 and increased COX2 protein expression in the I/R group were also reversed by ROSI treatment (**Figure 4B**). The relative histopathological score was significantly lower in the I/R + ROSI group than that in the I/R group (**Figure 4C**). ROSI treatment significantly decreased LPO, LDH and MDA level, as well as increased GSH level, in the kidney tissue during renal I/R injury

TABLE 1 | Top 15 up-regulated(Sham vs I/R)and down-regulated(I/R vs I/R + Dex)DEGs and ACSL4.

Symbol	Sham mean	I/R mean	log_2(fc)	*p*-Value	FDR
Up-regulated genes(I/R vs Sham)					
Il1f6	0.001	4.753333	12.21472	3.78E-13	4.87E-12
Sprr2f	0.001	3.45	11.75238	3.94E-10	3.69E-09
Ccl20	0.001	3.163333	11.62723	3.85E-11	4.03E-10
Vgf	0.001	3.03	11.5651	3.45E-09	2.88E-08
1700001F09Rik	0.001	3.023333	11.56192	6.44E-11	6.59E-10
Sprr2g	0.001	2.36	11.20457	1.01E-08	7.96E-08
Gm45837	0.001	1.443333	10.49519	1.14E-14	1.69E-13
Crisp1	0.001	1.443333	10.49519	0.000263	0.001007
Il24	0.001	0.85	9.731319	3.42E-07	2.18E-06
Grp	0.001	0.846667	9.72565	2.26E-06	1.25E-05
Gm10375	0.001	0.816667	9.673604	3.75E-06	2.01E-05
Gm3486	0.001	0.68	9.409391	3.70E-05	0.000167
Cxcl17	0.001	0.626667	9.291554	9.61E-05	0.000401
Il6	0.001	0.573333	9.16323	1.63E-05	7.83E-05
Gsta1	0.066667	35.13667	9.041796	1.91E-14	2.77E-13
Acsl4	**10.77**	**34.5633**	**1.68222**	**3.37E-17**	**6.26E-16**
Down-regulated genes(I/R + Dex vs I/R)					
Symbol	**I/R mean**	**I/R + Dex mean**	**log_2(fc)**	***p*-Value**	**FDR**
Crisp1	1.443333	0.001	-10.4952	0.000844629	0.003877536
Gm20683	1.033333	0.001	-10.0131	1.47E-08	0.000000227
Samd1	0.92	0.001	-9.84549	0.000000881	0.00000924
Il24	0.85	0.001	-9.73132	0.0000131	0.000101867
Gm10375	0.816667	0.001	-9.6736	0.0000619	0.00039771
Kank2	0.696667	0.001	-9.44432	7.25E-08	0.000000964
Gm3486	0.68	0.001	-9.40939	0.000384486	0.001957323
Vgf	3.03	0.005	-9.24317	0.00000217	0.0000205
Arhgap36	0.56	0.001	-9.12928	0.00000124	0.0000125
Btbd17	0.386667	0.001	-8.59495	0.0000579	0.000375583
Gml	0.386667	0.001	-8.59495	0.00351278	0.013033653
Tmem59l	0.336667	0.001	-8.39518	0.001163536	0.005081411
Il5ra	0.29	0.001	-8.17991	0.0000738	0.000464371
Gcat	0.283333	0.001	-8.14636	0.00930505	0.029560402
Hmga1b	0.273333	0.001	-8.09452	0.010893458	0.03361304
Acsl4	34.56333	10.385	-1.73474	5.77E-11	1.46E-09

DEG, differentially expressed gene; FDR, false discovery rate.

(**Figure 4D–G**). Besides, the enhanced TNF-α mRNA and IL-6 mRNA levels induced by I/R were effectively reduced by ROSI treatment (**Figures 4H,I**). These results manifested that the suppression of ACSL4 activity alleviated ferroptosis-induced kidney damage and inflammation during renal I/R.

3.5 Acyl-CoA Synthetase Long-Chain Family Member 4 Overexpression Abolishes Dex-Mediated Protective Effects on OGD/R Induced HEK293T Cells Ferroptosis and Inflammation

To assess whether ACSL4 is the mediator of Dex exerting its effects, HEK293T cells were subjected to OGD/R. OGD/R significantly enhanced ACSL4 and COX2 expression and suppressed GPx4 expression (**Figure 5A**). OGD/R fortified the level of LPO (**Figure 5B**), suggesting that OGD/R contributes to lipid peroxidation. OGD/R increased cell death observed in the CCK-8 assay (**Figure 5C**). LDH release was also increased with OGD/R treatment (**Figure 5D**). In addition, OGD/R treatment increased inflammatory cytokines in HEK293T cells (**Figure 5E**). A distinct decrease in LDH release and increase in cell viability was observed in cells after lip-1 treatment (**Supplementary Figure 1A**, **1B**). Then, HEK293T cells were transfected with ACSL4 overexpression plasmid for 48 h. ACSL4 overexpression effectively blocked the decreased ACSL4 in HEK293T cells during OGD/R with Dex treatment. Moreover, ACSL4 overexpression increased COX2 expression and decreased GPx4 expression (**Figure 5F**). As ACSL4 has been confirmed to aggrandize cell sensitivity to ferroptosis, we further evaluated the effects of ACSL4 on HEK293T cell ferroptosis. ACSL4 overexpression could partially reversed the protection of Dex on the inhibition of cell death determined by CCK-8 assay (**Figure 5G**). Dex decreased the density of green fluorescence represented by BODIPY581/591 C11 staining after OGD/R and moderately reversed by ACSL4 overexpression (**Figure 5H**). Lipid peroxidation analysis using BODIPY demonstrating that Dex significantly decreased cell ferroptosis and ACSL4 overexpression could reversed the protection of Dex to OGD/R-induced ferroptosis (**Figure 5I**). ACSL4 overexpression

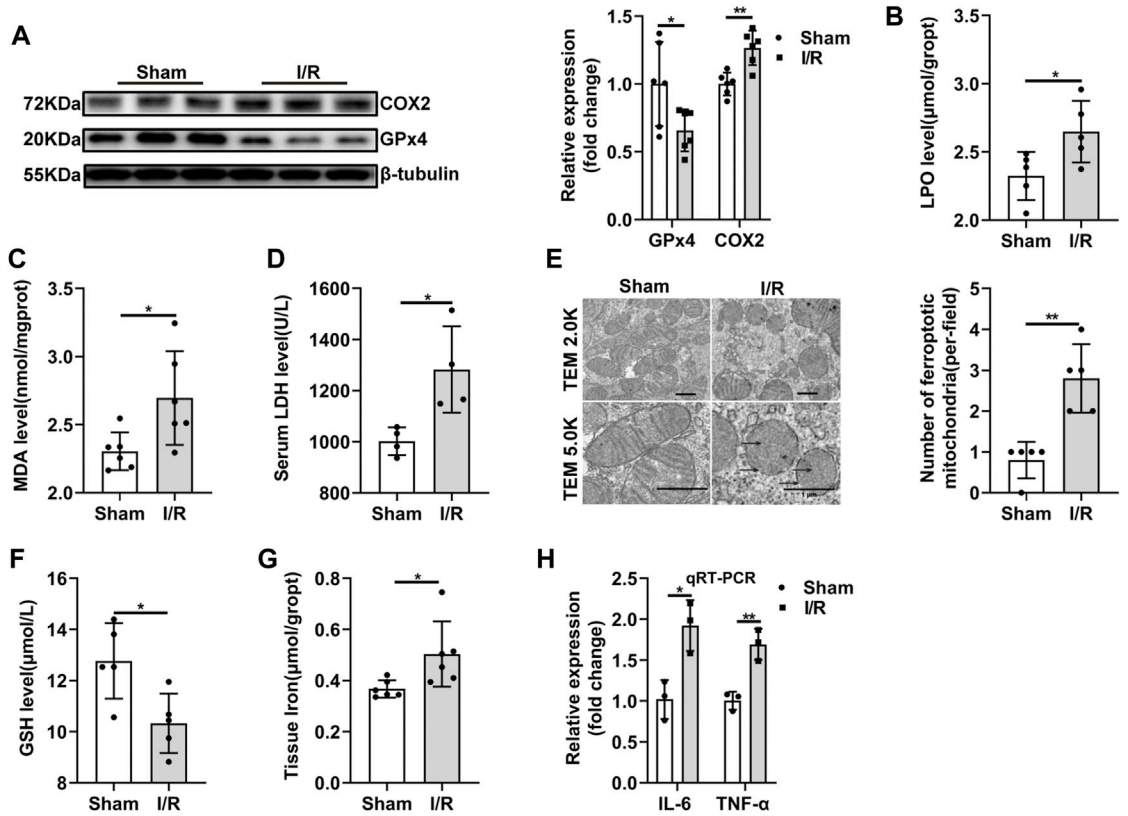

FIGURE 2 | Ferroptosis and inflammation are involved in renal I/R. **(A)** Validation of GPx4 and COX2 protein expression by western blot analysis ($n = 6$). **(B–D)** LPO ($n = 5$), MDA ($n = 6$), LDH ($n = 4$) were measured from each group. **(E)** Representative TEM images (original magnification, × 2.0K or × 5.0K,scarle bar = 1 μm) and quantification were shown ($n = 5$). The black arrows indicate the decline or disappearance of mitochondrial cristae and the rupture of outer mitochondrial membrane **(F,G)** Tissue GSH ($n = 5$) and tissue iron content (n = 6) were measured from each group. **(H)** Validation of IL-6 mRNA, and TNF-α mRNA expression by qRT-PCR analysis ($n = 3$). The data are the Means ± S.D., $^*p < 0.05$, $^{**}p < 0.01$.

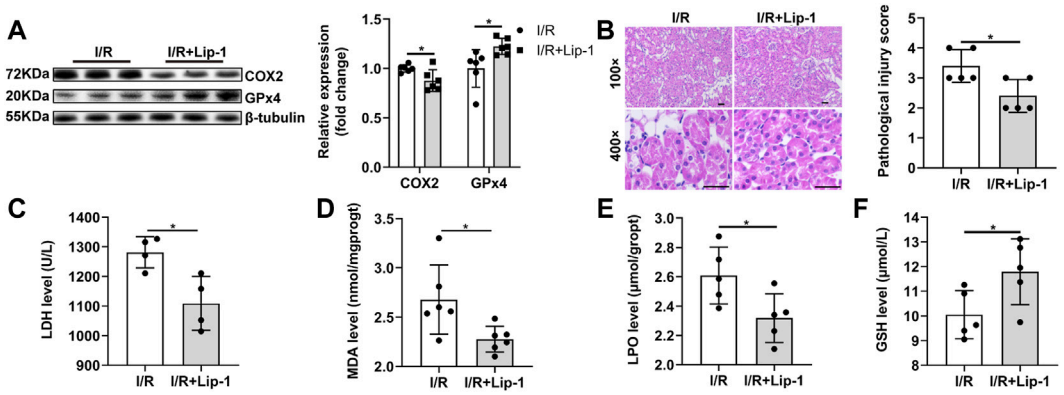

FIGURE 3 | Liproxstatin-1(Lip-1) ameliorates renal I/R injury by inhibiting ferroptosis. **(A)** Western blot analysis of COX2 and GPx4 in the renal tissue ($n = 6$). **(B)** Representative H&E staining (original magnification, × 100 or × 400, scale bar = 20 μm) and corresponding renal injury score (n = 5). **(C)** Serum LDH level ($n = 4$). **(D)** MDA levels in renal homogenates ($n = 6$). **(E)** LPO production in renal homogenates (n = 5). **(F)** GSH levels in renal homogenates ($n = 5$). The data are the Means ± S. **(D)**, $^*p < 0.05$.

blocked the declined LDH (**Figure 5J**), and Dex effectively decreased lipid peroxidation as indicated by the decrease in LPO level (**Figure 5K**). Furthermore, ACSL4 overexpression abolished the decreased inflammatory cytokines (**Figure 5L**, **5M**) in HEK293T cells during OGD/R with Dex treatment. These results demonstrated that Dex inhibited ferroptosis and

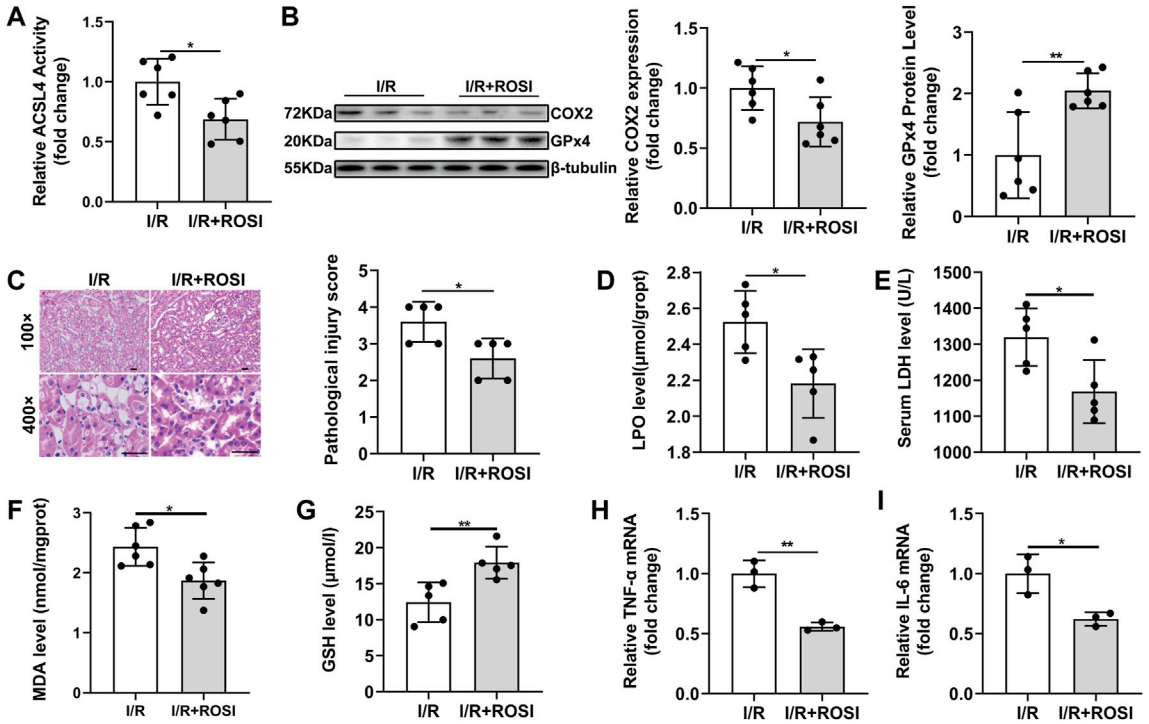

FIGURE 4 | ROSI attenuates ferroptosis-mediated damage and inflammation during renal I/R. **(A)** ACSL4 activity (n = 6). **(B)** Western blot analysis of COX2 and GPx4 in the renal tissue (n = 6). **(C)** Representative H&E staining (original magnification, ×100 or × 400, scale bar = 20 μm) and corresponding relative renal injury score (n = 5). **(D)** LPO production (n = 5). **(E)** Serum LDH levels (n = 5). **(F–G)** MDA and GSH levels in renal homogenates (n = 5). **(H,I)** Expression of TNF-α mRNA and IL-6 mRNA from renal tissue of I/R determined by qRT-PCR analysis (n = 5). The data are the Means ± S.D., $^{*}p < 0.05$, $^{**}p < 0.01$.

inflammation induced by OGD/R, and this effect was partially reversed by ACSL4 overexpression, suggesting that Dex might regulate ferroptosis and inflammation by targeting ACSL4 during OGD/R.

3.6 Dex Attenuates Ferroptosis-Mediated I/R Injury and Inflammation by Downregulating Acyl-CoA Synthetase Long-Chain Family Member 4 Signaling *via* α2-AR

Base on the important role of ACSL4 signaling during I/R injury, we further investigated that whether ACSL4 signaling were involved in Dex-elicited protection against renal I/R injury. ATI administration effectively blocked the decreased ACSL4 and COX2 protein expression and increased GPx4 level suffering from Dex treatment during renal I/R injury (**Figure 6A**). ATI treatment declined the histopathological score (**Figure 6B**), as well as the reversion of increased GSH in kidney tissue induced by Dex treatment during I/R injury (**Figure 6C**). MDA production was higher in the I/R + Dex + ATI group than that in the I/R + Dex group (**Figure 6D**). ATI treatment significantly increased LPO and LDH levels (**Figures 6E,F**). ATI also attenuated the anti-inflammation effect of Dex (**Figures 6G,H**). In brief, ATI treatment significantly reversed the Dex effects on the inhibition role of ACSL4 during renal I/R injury, which suggest that Dex attenuates ferroptosis-mediated I/R injury and inflammation by downregulating ACSL4 signaling via α2-AR.

4 DISCUSSIONS

Ischemia/reperfusion injury is a primary cause of AKI, which often arises from septic shock, major cardiovascular and abdominal surgeries, transplantation, and severe burns (Ergin et al., 2017; Jorge et al., 2019; Nieuwenhuijs-Moeke et al., 2020). Various clinical conditions lead to a decrease in renal blood flow, contributing to renal dysfunction and remote organ damage. Eventually, lead to multiple organ failure and death (Dépret et al., 2017). As the importance of I/R injury is becoming increasingly evident, the prevention of renal I/R injury has been a valid and reliable measure for AKI. Ferroptosis, as a type of regulated cell death, is different from other forms of cell death characterized by the accumulation of iron, lipid peroxidation, and condensed mitochondrial membrane densities. ACSL4 is a key enzyme that regulates lipid composition, has been shown to contribute to the execution of ferroptosis, but its role has not been widely investigated in renal I/R injury induced AKI. As a potent and highly selective α2-AR agonist, Dex has been widely used for long-term sedation and analgesia in an aesthesia because it induces a rapid response and is easily controllable. It is worth noting that some evidence observed in animal experiments

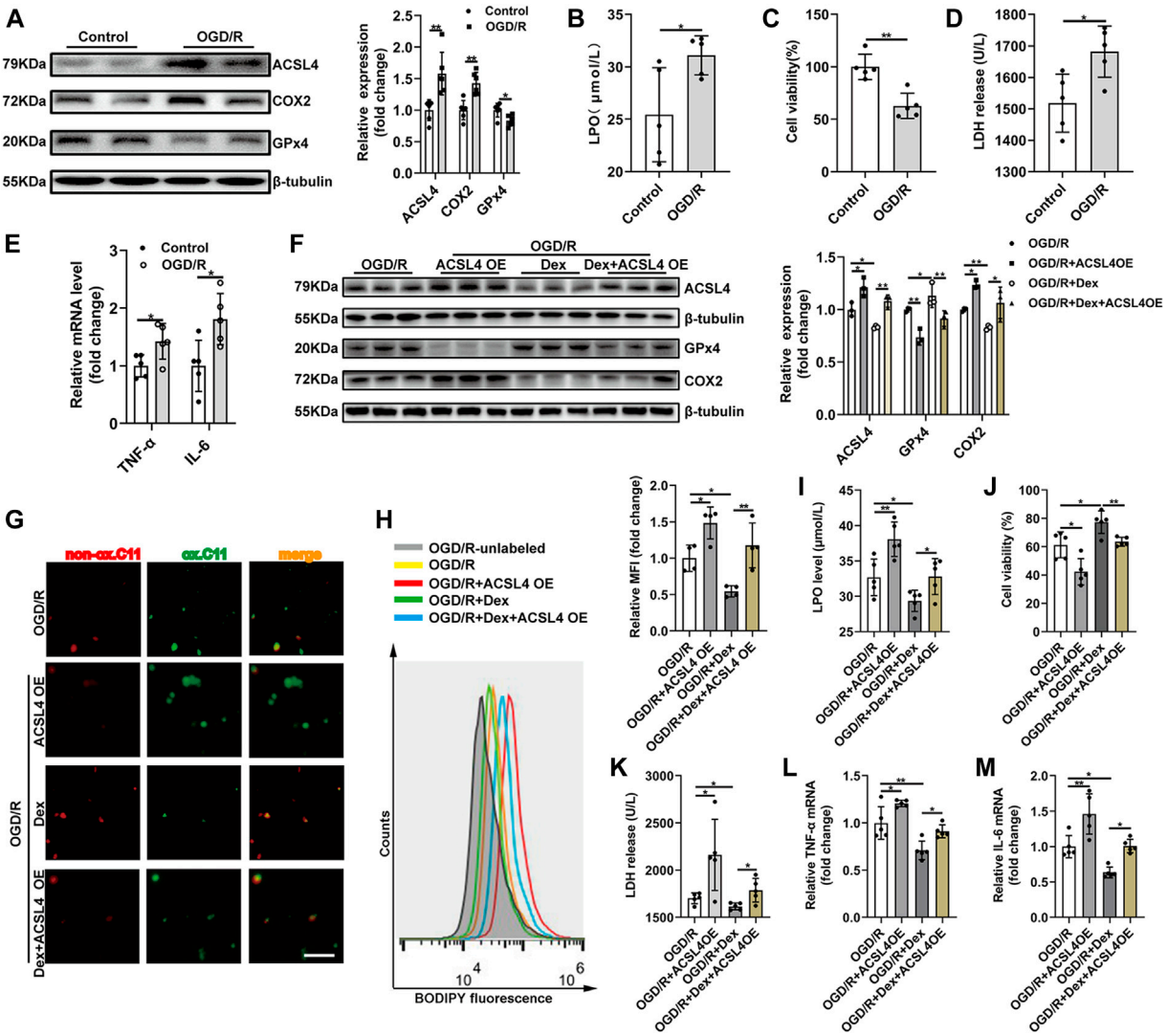

FIGURE 5 | ACSL4 Overexpression Abolishes Dex-mediated Protective Effects on OGD/R Induced HEK293T Cells Ferroptosis and Inflammation. **(A)** Western blot was used to detect the ACSL4, COX2 and GPx4 protein expression in the HEK293T cell. The protein intensity was analyzed by using ImageJ ($n = 6$). **(B)** Cell lipid peroxidation were detected by LPO assay Kits ($n = 5$). **(C)** Cell survival was determined by CCK-8 kit after OGD/R ($n = 5$). **(D)** The level of LDH after OGD/R ($n = 5$). **(E)** The qRT-PCR analysis of IL-6 mRNA ($n = 5$) and TNF-α mRNA ($n = 5$). **(F)** The expression of ACSL4, Gpx4, and COX2 were detected by western blot ($n = 3$). **(G)** Cell survival was determined by CCK-8 kit ($n = 5$). **(H)**Cell lipid peroxidation were detected by BODIPY 581/591 C11 staining use fluorescence microscopy (original magnification, × 100, scale bar = 50 μm). **(I)** Representative histograms and Mean fluorescence intensity (MFI) of BODIPY oxidation in HEK293T cells ($n = 4$). **(J)** The level of released LDH ($n = 5$). **(K)** The level of LPO ($n = 5$). **(L)** The qRT-PCR analysis of TNF-α mRNA ($n = 5$). **(M)** IL-6 mRNA levels ($n = 5$). The data are the Means ± S.D., $^{*}p < 0.05$, $^{**}p < 0.01$.

indicated that Dex reduced kidney tissue damage and improved renal functional recovery during renal I/R injury (Si et al., 2013; Si et al., 2018). To the best of our current knowledge, this is the first study to demonstrate that Dex attenuates ferroptosis-mediated renal I/R injury and inflammation through inhibiting ACSL4 signaling via α2-AR.

A2-adrenoceptors are widely distributed in distal and proximal tubules of the renal and peritubular vascular system (Kang et al., 2018; Bao and Dai, 2020). Studies have confirmed that Dex exerted a protective effects by reducing renal tubular damage and inhibiting apoptosis and inflammation in the tubular epithelial cells (Liang et al., 2017; Wang et al., 2020c). Dex pretreatment was also found to improve I/R renal microcirculation in experimental animals with I/R (Yang et al., 2021a). Renal I/R results in an increase in systemic and local sympathetic activity accompanied by intense vasoconstriction in the renal cortex. Therefore, studies have also confirmed that Dex alleviates renal lesion by resisting inflammatory of the sympathetic nervous system activation (Ma et al., 2020), improving outer renal medullary blood flow through local renal vasodilation(Billings et al., 2008), increasing glomerular filtration, dampening the ability of arginine vasopressin in the

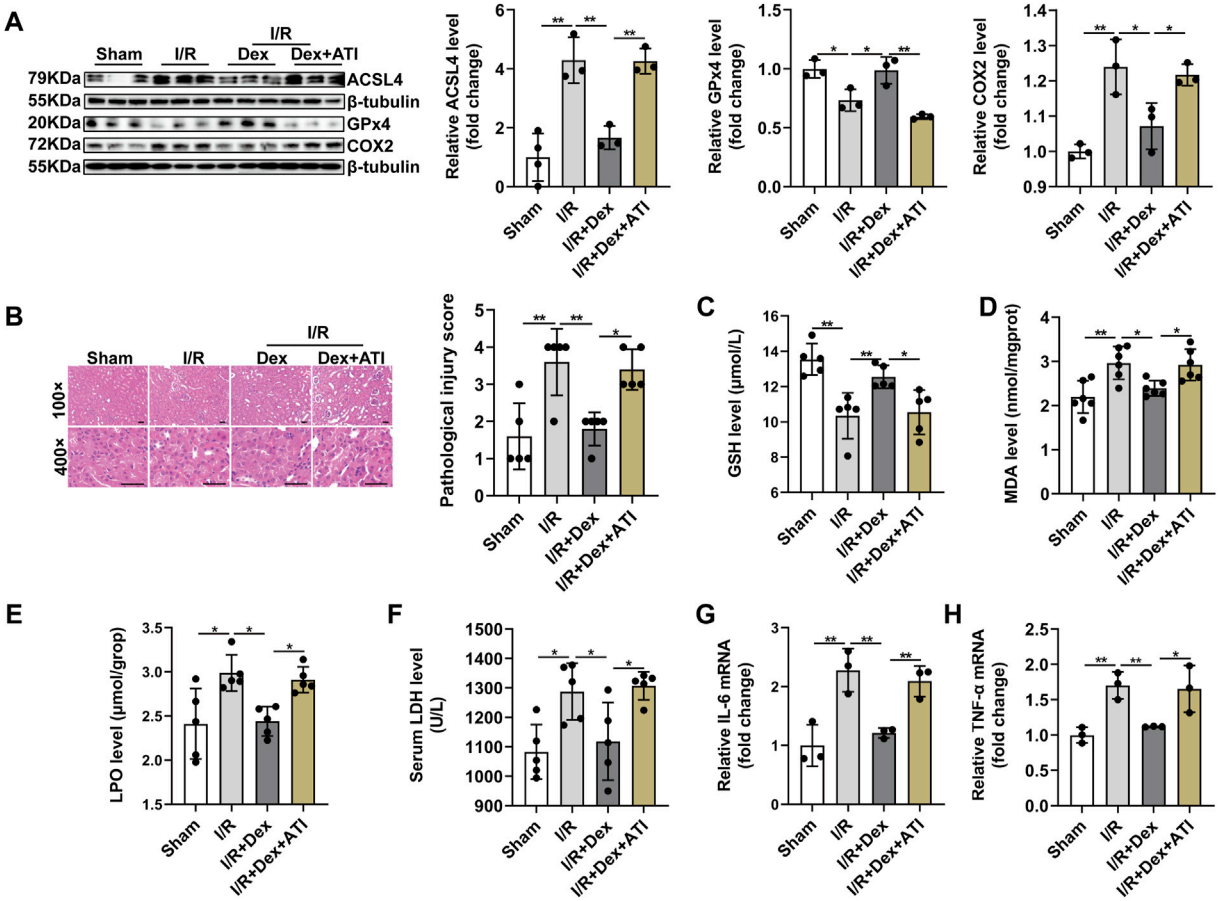

FIGURE 6 | Dexmedetomidine alleviates ferroptosis-induced I/R injury and inflammation through targeting ACSL4 *via* α2-AR. **(A)** Validation of ACSL4, GPx4 and COX2 protein expression by western blot analysis ($n = 6$). **(B)** Representative H&E staining (original magnification, × 100 or × 400, Scale bar = 20 μm) and corresponding relative renal injury score were shown ($n = 5$). **(C–E)** GSH ($n = 5$), MDA ($n = 6$), LPO ($n = 5$) levels in renal homogenates ($n = 5$). **(F)** Serum LDH was measured from each group ($n = 5$). **(G,H)** Validation of IL-6 mRNA and TNF-α mRNA expression by qRT-PCR analysis ($n = 5$). The data are the Means ± S.D., $^{*}p < 0.05$, $^{**}p < 0.01$.

collecting duct, inhibiting the aquaporins along with the transport of $Na^{+}$and water (Rouch et al., 1997), and alpha(2)-adrenoceptor agonist could regulate aquaporin-2 expression to stimulate urination (Junaid et al., 1999). Furthermore, Dex also brings down glomerular congestion, epithelial cell swelling, and stenosis in the luminal (Cakir et al., 2015). At the same time, the renal consists of several intrinsic cells, which injury or dysfunction can cause kidney damage. Therefore, more studies are needed to explore the effect of Dex on renal intrinsic cells and to comprehensively explain the renoprotective effect of Dex.

ACSL4, an important isozyme for polyunsaturated fatty acids (PUFAs) metabolism, has been identified as not only a sensitive regulator of ferroptosis but also an important contributor to the execution of ferroptosis (Doll et al., 2017; Xu et al., 2020). Inactivation of ACSL4 significantly alleviated the tissue damage in a mouse model of ferroptosis, suggesting that ACSL4 may be a target for ferroptosis inhibition (Angeli et al., 2017; Doll et al., 2017). Emerging evidence suggested that ferroptosis occurs in multiple organs under ischemia conditions, such as heart I/R injury (Wu et al., 2021a), renal I/R injury (Martin-Sanchez et al., 2020), intestinal I/R-induced lung injury (Ding et al., 2020) or even liver transplantation in the clinical setting (Yamada et al., 2020). One study found that ischemia-induced ACSL4 activation contributes to ferroptosis-mediated tissue injury in intestinal I/R (Li et al., 2019). And another study demonstrated that inhibiting ACSL4 mediated ferroptosis could prevent myocardial I/R injury (Fan et al., 2021). To date, the functional role of ACSL4 has rarely been reported in renal I/R-related diseases. Our present study found that ferroptosis was involved in the renal I/R injury, which was in line with the previous study (Su et al., 2019). Moreover, we found that ACSL4 expression was up-regulated after renal I/R injury and reversed by Dex administration. Besides, our results showed that the protective effect of Dex against renal I/R-induced injury was blocked by ACSL4 overexpression, revealing the important role of ACSL4 in the I/R injury. In this regard, ACSL4 may be a promising therapeutic target for alleviating ferroptosis-mediated renal I/R injury.

Ferroptosis, a non-apoptotic form of cell death, plays a detrimental role in I/R injury. Morphologically, ferroptosis mainly manifests as

shrinkage of mitochondria, increased mitochondrial bilayer membrane density and reduction or disappearance of mitochondrial cristae, but the cell membrane is still intact. Biochemically, there is intracellular GSH depletion and inactivation of GPx4, lipid peroxides could not be metabolized by the GPx4-catalyzed reduction reaction, resulting in accumulation of ROS production, which triggers ferroptosis (Li et al., 2020b). And the shrunken mitochondria, increased lipid peroxidation and decreased GSH level was confirmed the occurrence of ferroptosis during renal I/R injury in our present study. Furthermore, Liproxstatin-1, the specific small-molecule inhibitor of ferroptosis, significantly attenuated ferroptosis-mediated renal I/R injury with decreased LPO level, reduced MDA level and increased GSH level. Besides, GPx4 serves a pivotal role in ferroptosis, and inhibition of GPx4 activity can lead to the accumulation of lipid peroxides. In GPx4 knockout mice, GPx4 deficiency leads to spontaneous acute renal failure and an increased rate of early mortality, whereas ferroptosis of renal tubular epithelial cells is the main cause of renal failure in GPx4 knockout mice (Friedmann Angeli et al., 2014). Yang and his colleagues demonstrated that COX2 is a suitable marker for the lipid peroxidation that occurs during GPx4-regulated ferroptosis (Yang et al., 2014). The results of the present study demonstrated that the GPX4 activity decreased, COX2 expression increased, and ferroptosis occurred during renal I/R injury in mice. Besides, inhibition of ACSL4 activity significantly reduced the COX2 and enhanced the GPx4 expression. Of note, upregulated COX2 expression has been found in the ferroptosis-induced mice (Yang et al., 2014). These findings indicated that the expression of COX2 might be regulated by ACSL4 activity during renal I/R injury.

Under normal physiological conditions, the resistance of cells and tissues to ferroptosis is higher than that of other forms of cell death (Stockwell et al., 2017). These results showed that the process of I/R changed the lipid metabolism, decreased the resistance of ferroptosis, and finally increased the incidence of ferroptosis in kidney tissue. It could not be ignored is that previous studies pointed out the important role of apoptosis, autophagy, and pyroptosis in renal I/R injury, as well as complex downstream mechanisms and signaling pathways (Fang et al., 2021; Xia et al., 2021; Xu et al., 2021). In addition, the critical role of inflammation in the development of renal I/R injury was confirmed in numerous studies (Qian et al., 2021; Reid and Scholey, 2021). In the precent study, we found that inhibition of ferroptosis or ACSL4 activity significantly decreased the IL-6 and TNF-α levels. And Dex administration attenuated renal I/R injury through suppressing ferroptosis and inflammation. However, we did not further explore the relationship between inflammation and ferroptosis. Clinical and experimental related study indicated that I/R refers to a complex inflammatory process that includes the synthesis of pro-inflammatory cytokines such as IL-6 and TNF-α, and the development of oxidative stress (Leurcharusmee et al., 2018). The oxidative stress caused by inflammatory responses further led to the initiation of lipid peroxidation, DNA damage, and mitochondrial function deterioration. And then further the development of ferroptosis (Gentile et al., 2017; Mao et al., 2020). In addition, Excessive iron is detrimental to the redox balance and can further enhance the production of inflammatory factors, leading to more damage (Gudjoncik et al., 2014).

In conclusion, the present study revealed that Dex exerted its protective effects partially by decreasing ferroptosis and inflammation through regulating ACSL4 via α2-AR. These results address the important role of Dex and ACSL4-mediated ferroptosis during renal I/R injury. DEX may be used as a therapeutic agent for patients with renal I/R-induced AKI.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov, PRJNA766819.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of Soochow University.

AUTHOR CONTRIBUTIONS

W-HT, X-WM, and F-HJ contributed conception and design of the study. W-HT, X-SS, and J-XZ conducted experiments. B-YW, L-GL, and J-KH organized the database. MZ, XW, and KP performed the statistical analysis. W-HT and X-WM wrote the first draft of the manuscript. JD, H-YL, and S-XX wrote sections of the manuscript. All authors contributed to manuscript revision and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.782466/full#supplementary-material

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