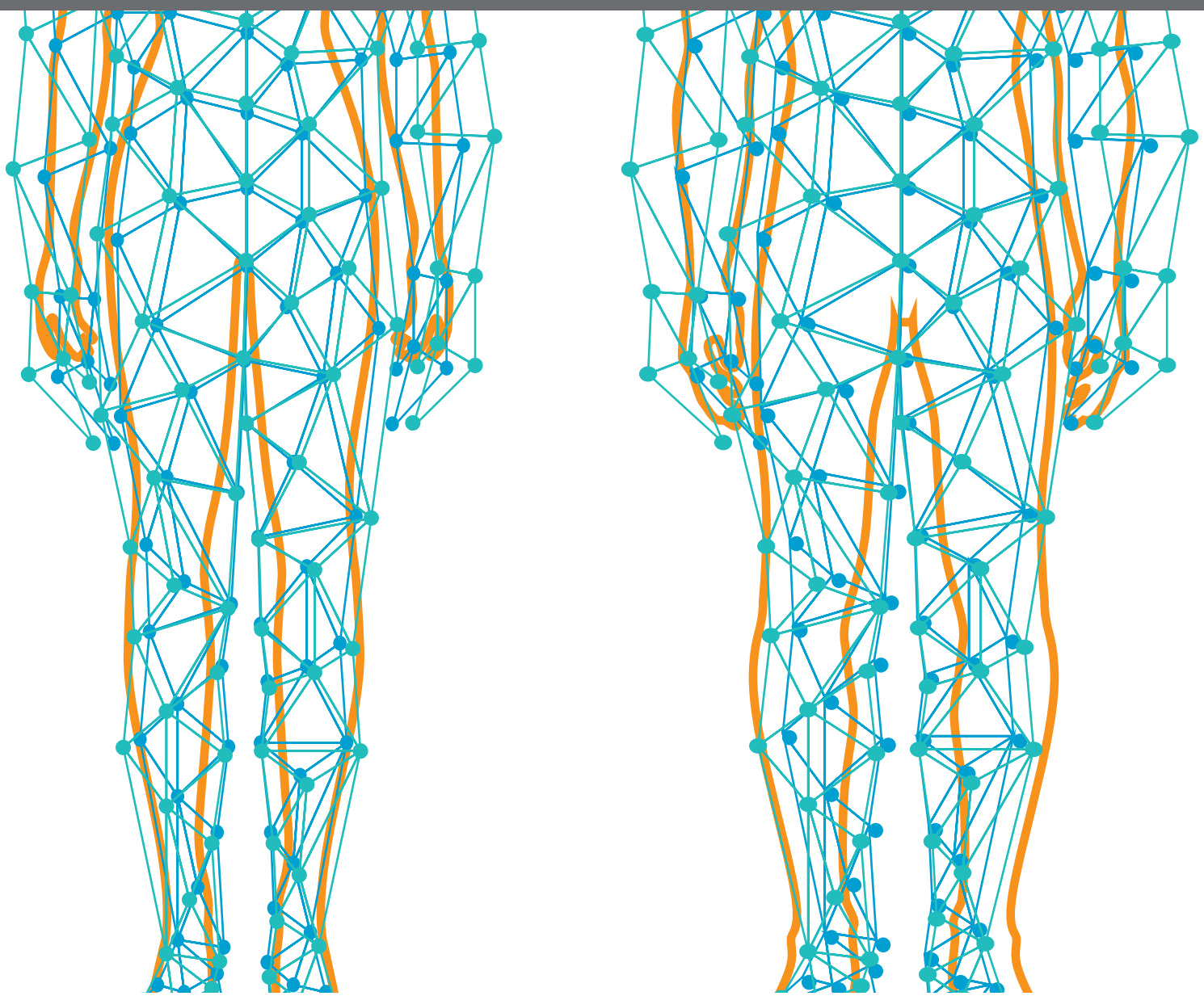
MOLECULAR PATHOPHYSIOLOGY OF DIABETES-RELATED ORGAN INJURY

EDITED BY: Tingting Zhao and Qin Zhou

PUBLISHED IN: Frontiers in Medicine



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ISSN 1664-8714
ISBN 978-2-88974-886-0
DOI 10.3389/978-2-88974-886-0

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MOLECULAR PATHOPHYSIOLOGY OF DIABETES-RELATED ORGAN INJURY

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Citation: Zhao, T., Zhou, Q., eds. (2022). Molecular Pathophysiology of Diabetes-Related Organ Injury. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-886-0

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frontiers in Medicine

EDITORIAL
published: 18 March 2022
doi: 10.3389/fmed.2022.875444

Editorial: Molecular Pathophysiology of Diabetes-Related Organ Injury

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Keywords: diabetes mellitus, diabetic kidney disease, diabetic cardiomyopathy, molecular pathophysiology, diagnostic biomarkers and treatment

Editorial on the Research Topic

Molecular Pathophysiology of Diabetes-Related Organ Injury

INTRODUCTION

The prevalence of diabetes mellitus is increasing rapidly around the world. This disease accompanies by a large variety of multiple organs injuries. For example, diabetic kidney disease (DKD) is affecting one in three patients with diabetes, and cardiovascular (CV) disease is the leading cause of death for these patients. However, the pathophysiology is complex and heterogenic, and the therapeutic strategies are limited. The goal of this Research Topic is to collect the recent advances on pathophysiology, a timely diagnosis and prompt treatment with the final aim to retard the progression of diabetes-related organs injury. Sixty-seven contributions covering the listed Research Topics have submitted to this special issue.

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Pathology, a section of the journal Frontiers in Medicine

Received: *14 February 2022*
Accepted: *17 February 2022*
Published: *18 March 2022*

Citation:
Zhao T and Zhou Q (2022) Editorial: Molecular Pathophysiology of Diabetes-Related Organ Injury. Front. Med. 9:875444. doi: 10.3389/fmed.2022.875444

ADVANCES IN ADIPOSE TISSUES AFFECTED BY INSULIN-DEFICIENT DIABETES MELLITUS

Since adipose tissue is recognized to be an endocrine organ, its role in the pathology of diabetes has aroused great concern. Flenkenthaler et al. provided novel pathophysiologic insights of insulin-deficient diabetes in adipose tissue depots. Multi-omics analysis of mesenteric visceral adipose tissue and subcutaneous adipose tissue of a diabetic, insulin-deficient pig model indicates regionally different metabolic adaptations to overcome energy stress caused by reduced glucose utilization in MIDY adipocytes.

REVIEWS IN MOLECULAR PATHOPHYSIOLOGY OF DIABETIC KIDNEY DISEASE AND CARDIOMYOPATHY

Heart and kidney, both as the higher energy-demanding tissues in the body, shared the common pathogenesis, such as metabolic abnormalities, mitochondrial damage and dysfunction, oxidative stress, inflammation, epigenetic factors, and others. Salvatore et al. summarized the major pathophysiological changes and the underlying mechanisms leading to myocardial remodeling and cardiac functional derangement in diabetic cardiomyopathy. As a conclusion, the main driving force of the pathological processes specific of diabetic cardiomyopathy is hyperglycemia, a factor centrally placed among multiple interwoven pathways involving complex cellular and molecular perturbations, which affect both myocardial structure and function.

Similarly, Chang et al. commented the high glucose transport state and local relative oxygen deficiency (primary and secondary) in proximal tubular might be the initial factors of tubular damage, while excessive mitochondria damages and ROS production are important contributors to the further damage of PTs in DKD. Abnormalities in hemodynamics, glucose and lipid metabolism, mitochondria, oxidative stress, inflammation, and many other factors interact with each other and form a vicious circle, leading to the renal tubular dysfunctions. This review systematically presented the mechanism of the pathogenesis of tubular injury in different stages in the development of DKD, which provide clues to develop specific treatments to prevent and delay the tubular injury in DKD.

Lipid Metabolism in Diabetic Kidney Disease and Cardiomyopathy

Under normal condition, fatty acids are the main energy source of the kidney and heart. In diabetics, the utilization of fatty acid is changed to glycolysis and lipid accumulation. Given the critical role of proximal tubular injury in developing diabetes, Chang et al. and Wang et al., both reviewed that tubular lipotoxicity may occur before mitochondrial dysfunction and is an earlier event in DKD; tubular lipotoxicity may be an indicator for early prediction of DKD.

Ma et al. investigated the susceptibility of 8 polymorphisms in *ApoB* and *PCSK9* genes to DKD in 575 Chinese patients with type 2 diabetes mellitus vs. 653 controls. They indicate that *ApoB* gene is a candidate gene for DKD in Chinese patients with type 2 diabetes mellitus, and its association with hypertension may mediate the association of ApoB gene with DKD.

Advances in Circular RNAs as Diagnostic Biomarkers and Therapeutic Targets in Kidney Disease

Circular RNAs (circRNAs) are a new type of non-coding RNA molecules which regulate gene expression through epigenetic modifications that have attracted more and more attention in recent years. Yu et al. introduce the biogenesis and biological function of circRNAs, and focus on state-of-art regarding circRNAs as novel biomarkers and therapeutic targets in common kidney diseases. They summarized the roles of circRNAs in the diagnosis and prognosis prediction on renal cell carcinoma, acute kidney injury, and glomerular diseases, including DKD. It is worth mentioning that dysregulated circRNAs, especially those from exosomes, are potential biomarkers in the pathogenesis and progression of DKD. Targeting these circRNAs may provide new therapeutic targets for the clinical treatment of DKD.

Advances in Herb Medicine Treating Diabetic Kidney Disease and Cardiomyopathy

Herb medicine is important in current therapies. The ebook included three articles of herb medicine treating DKD and cardiomyopathy. Hu et al. reported Tangdshen Formula, a Chinese herbal medicine for the treatment of DKD may alleviate myocardial fibrosis in KKAy mouse models by inhibiting TGF-β/Smad and Wnt/β-catenin signaling pathways, which proves that there is a common pathogenesis of DKD and cardiomyopathy. Gao et al. sdemonstrated Qing-Re-Xiao-Zheng Formula modulates gut microbiota and inhibits inflammation in mice with DKD, and the underlying mechanism of which was proposed to have an involvement of the inhibition of LPS/TLR4/NF-κB pathway. Liu et al. reviewed the active compounds and therapeutic target of *Tripterygium wilfordii Hook. f.* (TWHF) in attenuating proteinuria in diabetic nephropathy. TWHF widely used to treat DKD in China. Tripterygium wilfordii polyglycosides, triptolide, and Celastrol are the effective medicine against proteinuria and kidney injury in diabetic nephropathy. Their Mechanisms are including anti-inflammation, antioxidation, anti-fibrosis, activating autophagy, and anti-podocyte apoptosis, *via* several mechanisms.

CONCLUSION

In conclusion, these studies improved our understanding on molecular pathophysiology of diabetes-related organ injury. Potential therapeutic methods and targets are also proposed for the future development of effective therapies to the prevention and treatment of the major clinical problem of diabetic kidney disease and cardiomyopathy.

AUTHOR CONTRIBUTIONS

TZ and QZ reviewed the papers included in the Research Topic of *Molecular Pathophysiology of Diabetes-Related Organ Injury* and summarized in the Editorial their main findings, together with a commentary on the current knowledge about diabetic kidney disease and cardiomyopathy. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation of China [No: 81973627] and Beijing Natural Science Foundation [No: 7212195].

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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REVIEW
published: 30 March 2021
doi: 10.3389/fmed.2021.661076

Update on the Mechanisms of Tubular Cell Injury in Diabetic Kidney Disease

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Increasing evidence supports a role of proximal tubular (PT) injury in the progression of diabetic kidney disease (DKD), in patients with or without proteinuria. Research on the mechanisms of the PT injury in DKD could help us to identify potential new biomarkers and drug targets for DKD. A high glucose transport state and mismatched local hypoxia in the PT of diabetes patients may be the initiating factors causing PT injury. Other mechanism such as mitochondrial dysfunction, reactive oxygen species (ROS) overproduction, ER stress, and deficiency of autophagy interact with each other leading to more PT injury by forming a vicious circle. PT injury eventually leads to the development of tubulointerstitial inflammation and fibrosis in DKD. Many downstream signaling pathways have been demonstrated to mediate these diseased processes. This review focuses mostly on the novel mechanisms of proximal renal tubular injury in DKD and we believe such review could help us to better understand the pathogenesis of DKD and identify potential new therapies for this disease.

Keywords: tubulointerstitial fibrosis, diabetic kidney disease, proximal tubular cell, proximal tubular, pathogenesis

OPEN ACCESS

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Specialty section:
This article was submitted to Pathology, a section of the journal Frontiers in Medicine

Received: *30 January 2021*
Accepted: *08 March 2021*
Published: *30 March 2021*

Citation:
Chang J, Yan J, Li X, Liu N, Zheng R and Zhong Y (2021) Update on the Mechanisms of Tubular Cell Injury in Diabetic Kidney Disease. Front. Med. 8:661076. doi: 10.3389/fmed.2021.661076

INTRODUCTION

Diabetic kidney disease (DKD) is a progressive microvascular complication of diabetes mellitus. Within the kidney, the glomeruli, tubules, vessels, and interstitium are disrupted, resulting in impaired renal functions and eventually end-stage renal disease (ESRD). Epidemiological studies have shown that the current global burden of diabetes affects more than 425 million people. Without intervention, the number of individuals with diabetes worldwide will rise to an estimated 629 million in 2045 (1). Given the high prevalence of diabetes, accordingly, the incidence of DKD is rising rapidly with ∼30–40% of diabetic patients develop DKD and a third of these patients progress to ESRD, which brings tremendous impacts on the socio-economics (2, 3).

Based on the distinct pathological glomerular changes (4), DKD has previously been regarded as a glomerular disease, and the injury to the renal tubules considered as secondary to glomerular lesions. In the natural history of DKD, the development of persistent microalbuminuria (presence of albumin in the urine) progresses to overt proteinuria, followed by a gradual decline in the glomerular filtration rate (GFR) and eventually renal failure (5). Although albuminuria represents an independent risk factor for DKD, about 20% of patients with non-albuminuric DKD progress to advanced ESRD within 10 years (6). The in-depth understanding of the disease has enabled the identification of some patients with decreased renal function before the presence of microalbuminuria according to creatinine-based estimated glomerular filtration rate (eGFR) (7–10), and these are the patients that tend to progress more rapidly. When compared with patients with proteinuria, these patients tend to have more severe

tubulointerstitial fibrosis and tubular atrophy, suggesting that renal tubular injury plays a key role in the progression of DKD in the absence of proteinuria. In general, renal tubular injury is closely correlated with the decline of eGFR in chronic kidney disease (CKD) patients. Recent evidence has suggested that the proximal tubular (PT) injury develops in the early stage of DKD and promotes DKD progression (11). Therefore, this review focuses on the mechanisms of PT injury in DKD.

HYPOXIA

The kidney is an oxygen-intensive organ that receives 20% of the cardiac ejection fraction. The activity of renal tubular transport is accountable for major oxygen consumption in kidney metabolism. The process of renal oxygenation consists of a fine and balanced physiological process, which includes oxygen supply determined by renal blood flow as well as arterial oxygen content and oxygen consumption governed by renal tubular reabsorption. The oxygen in the renal cortex is mainly utilized for glomerular filtration and solute reabsorption. Majority of oxygen supply goes to the renal cortex with a low supply to the renal medulla. This ensures an effective countercurrent multiplication system while the oxygen supply of the medulla is extremely limited, albeit slightly higher than its oxygen utilization (12). Therefore, an imbalance between the oxygen supply and oxygen demand in the medulla will result in hypoxic damages to the medulla tubules (13), whereby the renal tubules in the medulla are the most vulnerable to renal ischemic injury (14).

The development of hypoxia depends on three factors: increased oxygen consumption, oxygen utilization disorder, and reduced oxygen supply, which often co-exists simultaneously and interacts with each other to form a vicious circle (15). Studies have shown that 60% of the overall energy consumption of kidneys is devoted to sodium reabsorption with the PT responsible for almost two-thirds through basal Na^+/K^+ ATPase activity primarily and quantified as ouabain-sensitive O_2 consumption (16). The glucose in the tubule fluid is delivered into the cell by secondary active transport mostly via the sodium-dependent glucose transporters 2 (SGLT-2) in the apical membrane of the proximal tubular epithelial cell (PTEC). Although this is not an energy-dependent process, the sustainability of this activity demands a persistent electrochemical gradient of Na+ produced by Na^+/K^+ ATPase activity. Therefore, excessive glucose reabsorption in PT will invariably lead to increased oxygen consumption in type 2 diabetes (17). Moreover, the diabetic kidney is constantly in a state of high oxygen consumption due to hyperfiltration and increase tubular reabsorption, which increases further the severity of renal tubular hypoxia. This situation is then exacerbated by subsequent mitochondria dysfunction (15). Besides, the most classic complication of diabetes is systemic microangiopathy which is characterized by basement membrane thickening with hyaline deposition. This vascular injury will lead to decreased blood supply and oxygen supply in the kidney (18, 19). Furthermore, gluconeogenesis is a major source of oxygen and energy consumption in the kidney, accountable for 25% of the energy required for sodium reabsorption (20). For diabetic kidneys, the degree of gluconeogenesis in the kidney is increased significantly (14, 21).

In studies using diabetic animal models, outer medullary hypoxia has been demonstrated using blood oxygen level-dependent (BOLD) MRI. Also, both cortical and medullary hypoxia has been reported in the diabetic animal models as well as humans with DKD (22–25). With increased oxygen utilization, hypoxia inducible factor (HIF)-1 α has been implicated in the correlation of hypoxic and tubulointerstitial fibrosis (26–30). On the other hand, SGLT-2 inhibitors have been shown to poses a renal protective effect on diabetes patients by inhibiting glucose reabsorption and its associated high oxygen consumption (21, 31), in addition to targeting HIF-1 α protein to inhibit mitochondria oxygen consumption (32, 33). Furthermore, these factors inhibit and internalize megalin O-GlcNAcylation to reduce the reabsorption of plasma proteins (e.g., albumin and neutrophil gelatinase-associated lipoprotein) in PT, which is renal protective (34).

MITOCHONDRIAL DYSFUNCTION

PTEC demands substantial energy to maintain a normal function (35), whereby 65% of the electrolytes and 100% of the glucose and amino acids filtered by the glomeruli are reabsorbed by the PT. PTEC is rich in mitochondria which is an important organelle performing oxidative metabolism in eukaryotic cells mainly through the β-oxidation of fatty acids to produce adenosine triphosphate (ATP) (35). Mitochondria is also a place for aerobic respiration and energy supply of cells, which produces 95% of the energy needed in cellular activities through oxidative phosphorylation and therefore is regarded as the power plant of a cell. Mitochondria is the center of ATP production and its dysfunction leads to apoptosis.

Mitochondrial homeostasis is strictly essential for an optimally functioning kidney, given that the kidney is an organ that demands high energy consumption (36). In diabetes, the epithelial cells of the S1 segment of the PT require a large amount of ATP as an energy source to reabsorb excess glucose. However, ATP production brings superoxide (O_2^-) production concurrently, which can be converted into excessive reactive oxygen species (ROS), leading to mitochondrial damage and disorders in ATP production (36). Indeed, a reduction in the ATP pool represents the initial event of PTEC damage, with the degree of ATP reduction correlates with the severity of the damage (37). Studies have demonstrated that the production level of ROS may exceed the capacity of the local antioxidants, which is the biomarker of renal mitochondrial dysfunction in diabetes (38–41). This is further supported by the changes of bioenergetics and kinetics of mitochondria that may precede the development of DKD (38).

In addition to the driving force of cells, mitochondria have also been regarded as the judge and executor of programmed cell death (42, 43). In mitochondrial homeostasis, a balance in the mitochondrial biogenesis, including fusion and mitophagy, is required (35). Both Fission and fusion complement each

other to maintain the mitochondrial morphology under different metabolic conditions, while mitophagy removes damaged mitochondria from the network (35). Mitochondrial swelling is considered an indicator of mitochondria dysfunction (44), which can be confirmed by electron microscopy (45, 46). Uncontrolled mitochondria dysfunction eventually leads to the activation of the intrinsic cell death pathway and cell death (47, 48). Cell death may present in various forms, including apoptosis, autophagic cell death, pyroptosis (49).

In recent years, increasing research studies have been performed on the role of oxidative stress in cell death, given its integral role in tubule injury in DKD. Studies have shown that AOPPs (50, 51) induces oxidative stress and DKD mitochondria dysfunction through CD36/β-Catenin and PKC pathways, leading to tubulointerstitial fibrosis. On the contrary, in animal studies using DKD mice, PGC-1 α (52) ameliorates renal fibrosis via an antioxidant mechanism. Antioxidants (tempol and ramipril) inhibit NADPH upregulation by negatively regulating the endoplasmic reticulum stress (ERS) and inflammation to improve renal damage in DKD (53). Oxidative stress and endoplasmic reticulum stress positively regulate by each other, forming a vicious cycle (54). Sirt3-CD38 has also been shown to play a role in diabetic renal tubule damage by regulation of mitochondrial oxidative stress (55, 56).

Given that the mitochondria may be a target for therapeutic intervention, the mechanisms of some potential drugs have been explored. SS31, a novel antioxidative peptide that targets mitochondria, has been specially designed to concentrate in the inner mitochondrial membrane (57), which reduces renal tubulointerstitial damage in diabetic mice by decreasing mitochondrial fragments and restoring mitochondrial morphology through the inhibition of Drp1 expression and upregulation of Mfn1 expression in renal tubular epithelial cells. Also, the role of SS31 has been associated with CD36 (58). Besides, Na2S4, a polysulfide donor that directly sulfhydrates SIRT1, reduces high glucose-induced oxidative stress, cell apoptosis, inflammatory response in renal tubular epithelial cells, and the progression of epithelial-to-mesenchymal transition (EMT) (59). Also, Carnosine has been shown to significantly decrease the production of ROS, alleviate oxidative stress, and inhibit apoptosis through mitochondrial pathway *in vitro* (60) and *in vivo* (61). This may be a promising drug for the treatment of DKD. All these studies shed light on the new potential therapeutic agents in the prevention of renal tubulointerstitial damage through regulation of mitochondrial function and ROS production.

INNATE IMMUNITY

A persistently high glucose can cause abnormal activation of mitochondrial endoplasmic reticulum stress and intracellular signal transduction pathways, leading to cell stress and cellular dysfunction. The abnormal activation following each stress response promotes further activation of downstream inflammatory factors, the release of damps, and induction of innate immune response. The innate immune response induces a continuous process of chronic inflammatory reaction in the kidney, leading to substantial mesangial hyperplasia and renal interstitial fibrosis, which lays the foundation for the occurrence and development of DKD (62). Compared with adaptive immunity, the mechanism of an innate immune response plays an integral role in the occurrence of diabetic kidney injury (63, 64), which is composed of pattern recognition receptors that recognize pathogenic and endogenous ligands. The bindings of ligands trigger several complex inflammatory cascade reactions, including Toll-like receptor (TLR) signaling, nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs), the kallikrein–kinin system (KKS), protease-activated receptor (PAR) signaling, and the complement cascade, resulting in further renal fibrosis and other renal damages (65). In particular, the complement cascade plays a key role in innate immunity that is responsible for the pathogenesis of several immune-mediated inflammatory diseases (66). A study has shown that the novel aptamer (NOX- D21) improves renal function and reduces tubulointerstitial fibrosis by inhibiting the expression of C5a in db/db mice (67). TAM receptors (Tyro3, Axl, and Mer) have been implicated in the innate immunity (68). Studies have demonstrated an obvious TAM shedding in DKD patients, though the mechanism of this observation remains unclear. Further research is warranted to establish the role of TAM in the development of renal injury and DKD.

ANGIOTENSIN II

Angiotensin II (AngII) is also recognized as a mediator of hyperglycemia-induced renal damage. The concentration of renal Ang II is ∼1,000-folds higher than that of circulating AngII (69). An increased AngII level is implicated in the development of renal fibrosis by directly upregulating the pro-fibrosis genes (70). Early studies have revealed that AngII induces cellular hypertrophy of tubular cells that is mediated by the activation of endogenous TGF-β (71, 72). Also, the study of primary PT has demonstrated that glucose significantly increases the concentration of AngII in cell lysates, while angiotensin receptor blocker (ARB) significantly reduces this effect of AgnII (73, 74). Furthermore, AngII induces ROS (75) and EMT (76, 77), leading to tubular cell damage. Importantly, recent studies have revealed a high affinity of angiotensin II type 2 receptor (AT2R) in the mitochondria of renal tubules. In the early stage of diabetes, AT2R inhibits the production of mitochondrial reactive oxygen species and cell proliferation. Overexpression of AT2R in tubular epithelial cells contributes to the decreased mitochondrial bioenergy efficiency and increased mitochondrial superoxide production (78).

In the current clinical practice, angiotensin-converting enzyme inhibitors (ACEI), and ARB are the first-line drugs being used in the prevention of DKD. Several recent studies have shown that the combination therapy of renin-angiotensin system (RAS) inhibitor together with neprilysin inhibitor was more effective in preventing renal fibrosis than using RAS inhibitor alone in the development of DKD [LCZ696 and angiotensin receptor blocker (79); combination of sacubitril [NEPi] and valsartan

(80); combination of thiorphan [NEPi]/telmisartan [ARB]; and thiorphan/Dize [ACE2 activator] therapies (81)]. Moreover, the combination of PGE1 with ACE inhibitor protects renal function more as compared with PGE1 or ACEI monotherapy (82). These studies provide evidence on the alternative options of effective clinical treatment with RAS blockers.

FATTY ACIDS

In healthy kidneys, ATP is primarily generated via oxidative phosphorylation (OXPHOS) of fatty acid (FA). However, in diabetics, the utilization of fatty acid is changed to glycolysis and lipid accumulation, which also represents an important pathway of DKD due to lipid accumulation in the renal tubular epithelia (83) via increased absorption and synthesis of fatty acids, in addition to decreased utilization. The toxic effect of FA on the renal tubular epithelial cells is associated with hypoxia and mitochondrial dysfunction (33, 84). A recent study has shown that FATP2, a member of the fatty acid transporter family, regulates DKD pathogenesis through a combined lipotoxicity and glucotoxicity (glucolipotoxicity) mechanism (85). Nevertheless, PBI-4050, which is a fatty acid receptor modulator, attenuates the development of DKD in type 2 diabetes (86). Saturated fatty acid (SFA)-related lipotoxicity is also the pathogenesis of diabetes-related PT cell damage. Therefore, increasing the enzymes that metabolize free fatty acid (FFA) can theoretically protect the PT cells from SFA-related lipotoxicity. The study by Iwai et al. has found a significantly lower expression of Stearoyl-CoA Desaturase-1 (SCD1) in the kidney of diabetic mice induced by a high-fat diet (HFD) than that of non-diabetic mice. Thus, enhancing SCD1-mediated desaturation of SFA and subsequent formation of neutral lipid droplets may provide a promising therapeutic target to reduce SFA-induced lipotoxicity (87). Besides, through restoring functional lymphatic vessels, SAR13175 was able to eliminate inflammatory cells and toxic lipid metabolites in the kidney that can also improve lipotoxicity-related fibrosis in diabetes (88).

AUTOPHAGY

Autophagy is a highly conserved pathway through which cells degrade and recycle macromolecules and organelles. Growing evidence shows dysregulated autophagy in DKD (89). The well-known autophagy regulation pathways include mammalian target of rapamycin (mTOR), Adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) and sirtuins (SIRT). In addition, a variety of stress conditions, including hypoxia, oxidative stress, ERS, and metabolism, have been shown to regulate autophagy (90, 91). In general, mild to moderate ERS and activation of autophagy play a protective role in kidney cells. When the harmful stimulus cannot be effectively alleviated, this leads to the sustained ERS creating an imbalance between ERS and autophagy. This will lead to kidney cell injury and progression of DKD (92).

mTOR can interact with several proteins to form two different complexes, namely mTORC1 and mTORC2, to regulate autophagy. There is ample evidence that mTORC1 is a key regulator of autophagy, which regulates different steps of autophagy such as nucleation, elongation, maturation, and termination (93). mTORC2 indirectly regulates autophagy by activating mTORC1. In general, mTORC1 is a negative regulator of autophagy by inhibiting the activity of Ulk1 complex through direct phosphorylation. On the contrary, AMPK and SIRT1 are effective positive regulator of autophagy (89). In recent years, some new findings have been made in this field. Huang et al. identified KCa3.1 (calcium-activated K+ channel) involved in renal tubular autophagy dysfunction through PI3K/Akt/mTOR signaling pathway in DKD (94). Theodomir et al. confirmed that P2Y2R deficiency increased the expression of sirtuin-1 and FOXO3a, which enhanced autophagy and improved renal insufficiency in DKD (95). In addition, Yang et al. found that Smad3, the downstream transcription factor activated by TGFβ (transforming growth factor β), suppressed lysosome biogenesis in a TFEB-dependent manner (96). Furthermore, ATF4 (activating transcription factor 4) (97), TRAIL (TNF related apoptosis inducing ligand) (98), Soluble epoxide hydrolase (sEH) and lys63 UB proteins were also confirmed to be involved in the regulation of autophagy in the kidney cells.

Targeting various components of autophagy pathway may become a new strategy for clinical treatment of DKD. As a potential target for regulating autophagy, Mikhail V blagosklony proposed rapamycin (sirolimus) for the treatment of diabetic kidney injury (99). However, clinical studies have found that rapamycin and its analogs can cause immunosuppression, glucose intolerance, increased risk of type 2 diabetes, and other side effects (100). In particular, it has been reported that long-term use of rapamycin can aggravate glomerular damage and increase albuminuria (101). Recently, Dudley W. Lamming group discovered the highly selective compound DL001, which inhibits mTORC1, could be developed for the treatment of DKD (100). In addition, several other drugs have been shown to improve DKD *in vivo* and *in vitro* models by regulating autophagy (102–104). SGLT2 inhibitors are also thought to increase autophagy in diabetic kidneys (105). The role of autophagy in the development of diabetes is still insufficient, and more experiments are needed to further elaborate in this field.

INFLAMMATION AND EMT

In the development of tubulointerstitial fibrosis, the complicated process of inflammation not only is the initiating factor but also the result of the development of several other factors. Local inflammation in renal tubules is a marker of progressive renal disease (106). Additionally, systemic inflammation exists in patients with type 2 diabetes, which involves the production of a large variety of chemokines that promotes inflammation in the microenvironment, thus increasing renal damage. Inflammation promotes renal infiltration of monocytes and lymphocytes, which augments further the inflammatory response and the development of cell damage and fibrosis (71). Additionally,

a large number of macrophages, lymphocytes, and mast cells infiltrate and secrete copious pro-inflammatory cytokines and oxygen-free radicals, which could provoke renal tissue damage and accelerate the process of renal fibrosis (107). Renal tubular inflammation is associated with several triggers, including local hyperglycemia, advanced glycation product, mitochondria oxidative stress, angiotensin II, PKC, and other factors (108). Recent evidence on the effects of histamines in renal function suggests that histamines may also contribute to glomerular hyperfiltration, inflammation, fibrosis, and tubule hypertrophy (109).

OTHER PATHWAYS DISCOVERED IN RECENT YEARS

Numerous cell signaling pathways have been confirmed to play a role in the progress of DKD. Here, we discussed some of the new pathways discovered in recent years.

HIPPO SIGNAL PATHWAY

The Hippo signal transduction pathway has been heavily researched in recent years. Experimental studies have shown the important roles of the Hippo signal transduction pathway in regulating organ size, carcinogenesis, tissue regeneration, and functions of stem cells. YAP (Yes-associated protein) and its homologous protein, TAZ (transcriptional coactivator with PDZ-binding motif), are the main effector molecules of the Hippo pathway. The study by Yang et al. has demonstrated that the activated YAP induced by the inhibition of MST1 up-regulates the activation of TEAD directly by binding to TEAD to form YAP-TEAD heterodimer, which promotes the expression of pro-fibrosis genes in the renal tubular epithelial cells (110). A high expression of YAP, TEAD, and CTGF was found in renal tissue of patients with type 2 DKD suggesting a key role of YAP in renal damage, while YAP expression is also correlated with Systolic BP, BUN, Cr, DKD stage, DKD pathological grade, serum albumin, and eGFR (111). The expression of YAP protein and its phosphorylation were also upregulated in the renal PTs of diabetic mice. Further studies have revealed that the activated EGFR-PI3K-Akt-CREB signaling pathway mediates the YAP gene expression, nuclear translocation, and interaction with the TEAD transcription factor complex (112). Besides, TAZ has been shown as a novel non-SMAD downstream effector of renal TGF-β1 signaling, which is activated in fibrotic kidney via TGF-β1-dependent mechanisms, while a sustained TAZ signaling promotes epithelial maladaptive repair (113).

NOD-LIKE RECEPTORS (NLRS)

NLRs are a family of cytoplasmic pattern-recognition receptors, which play several key roles in both innate and adaptive immunity (114–116) by inducing inflammation and cell death while facilitating rapid removal of invasive pathogens. Different NLRs poses distinct roles in regulating immunity and inflammation (117). NLRC3 inflammasome aggravates tubular injury through promoting pro-inflammatory and pro-fibrotic response of renal tubular cells (118). A study demonstrated that the reduction of NLRP3 inflammasome suppressed by the TNF-α inhibition alleviated tubular injury in DKD rats (119). Moreover, NLRP3 exerts inflammasome-independent effects on TGFβ signaling, which contributes to renal fibrosis in DKD (120). The role of NLRC5 has been shown to be multifaceted in the progression of DKD. Under high-glucose conditions, NLRC5 enhances IκB phosphorylation and reprograms macrophages toward the M1 phenotype in addition to activating the TGFβ signaling (121). Macrophages are closely related to interstitial fibrosis (122). Among the variety of phenotypes of macrophages, macrophages of M1 phenotype infiltrated the diabetic kidneys at the early stage play mainly the pro-inflammatory role, while the activation of macrophages M2 occurs in the late stage to promote renal fibrosis in DKD (123–125).

PTEN

PTEN decreases in diabetic renal tubular epithelial cells when cultured with high glucose, contributing to impaired autophagy and renal fibrosis (126, 127). Animal studies have demonstrated that although the level of unmodified Pten decreases, the level of $Pten^{K27-polyUb}$ increases significantly with the damaged renal tubules. Sufficient serine/threonine phosphatase activity can be obtained after the modification of $Pten^{K27-polyUb}$ to remove the phosphate groups of TWIST, SNAI1, and YAP. Consequently, these pro-fibrosis transcriptional factors activate the pro-fibrosis genes (128). Li et al. have proposed that the unmodified PTEN (EMT prophylaxis) and $Pten^{K27-polyUb}$ (EMT promotion) are dynamically regulated in kidney disease, in which the identification of $Pten^{K27-polyUb}$ may help in the early diagnosis of DKD and represent a potential therapeutic target.

ZINC TRANSPORTER

Zinc transporters are categorized into Zrt/Irt-related protein (ZIP) and zinc transporters (ZnT), which function together to maintain intracellular zinc homeostasis. In the cytoplasm, both ZIP and ZnT are zinc transfer proteins (129). Studies have demonstrated the subcellular localization of ZnT8 on the insulin secretory vesicle membrane of the islet β cells, which promotes the synthesis, storage, and secretion of insulin and regulates the homeostasis of intracellular free zinc ions. The study by Zhang et al. has found that ZnT8 is highly expressed in the tubular epithelial cells but only weakly expressed in the glomeruli or podocytes, and confirmed the protective effect of ZnT8 against tubulointerstitial fibrosis by inhibiting the TGF-β1/Smads signal pathway. However, in normal circumstances, overexpression, or knock-down of ZnT7 does not alter the phosphorylation level of Smad2/3 (130). On the other hand, Zhang et al. (52) suggest the important anti-fibrotic role of Zn via the PI3K/Akt/GSK-3β signaling pathway. Nevertheless, the role of Zn in the pathogenesis of DKD requires further research and clarification.

OTHERS

In addition to the above, several other signaling pathways have been studied in PT in DKD. These include FoxO1-STAT1 signaling (131), TSC1-mTORC1 signaling (132), HSP70-TLR4 axis (133), and PDGFRβ/Akt/mTORC1 nexus (134).

MICRORNA

MicroRNA (miRNA) is a small molecule that attracts great interest in the field of DKD research, given that it has been implicated in the occurrence and development of DKD. In particular, miRNAs participate in the progress of tubulointerstitial fibrosis, leading to structural changes and dysfunction of renal tubules. Also, miRNA and RNA-induced silencing complex (RISC) form a complex (135), which inhibits the expression of target genes by promoting mRNA degradation or inhibiting mRNA translation. Thus, whether miRNA promotes or inhibits fibrosis will depend on their specific target genes related to fibrosis as summarized in the table below (**Table 1**).

TABLE 1 | miRNAs related to tubulointerstitial fibrosis in diabetic kidney disease.

Oxidative stress	miR-25	PTEN (127) NOX4 (136)
	miR-146a	NOX4 (137)
	miR-4756	Sestrin2 (87)
Autophagy	miR-22	PTEN (127)
	miR-155-5p	Sirt1 (138)
EMT	miR-23a	SnoN (139)
	miR-27a	PARγ (140)
	miR-30b-5p	SNAI1 (141)
	miR-30c	SNAI1 (142)
	miR-30c-5p	JAK1 (143, 144)
	miR-34a-5p	SIRT1 (145)
	miR-98	Nedd4L (146)
	miR-130b	SNAI1 (147)
	miR-133b	SIRT1 (148)
	miR-145	ZEB2 (149)
	miR-181a-5p	Egr1 (150)
	miR-184	LPP3 (151)
	miR-192	Egr1 (152) ZEB1/ZEB2 (153)
	miR-199a-3p	IKKβ (154)
	miR-199b	SIRT1 (148)
	miR302a-3p	ZEB1 (155)
	miR let-7c	HMGA2 (156, 157)

BIOMARKERS OF TUBULAR CELL INJURY

Several biomarkers of tubular cell injury have been identified in patients with DKD. TNFR1 and TNFR2 have been shown as reliable biomarkers for predicting the progression of DKD (158) and their levels also correlate with tubular cell injury and inflammation (159). Kim1 is known as an early biomarker for DKD and its level increases even prior to the onset of microalbuminuria (160). Urinary N-acetyl-beta-d-glucosaminidase (NAG) is also considered as a potential early biomarker for DKD (161).A cross-sectional study shows that u-NGAL and RBP-4 are potential markers of tubular damage which can be used as complementary measurements to albuminuria and GFR in the early diagnosis of DKD (162).

ACUTE KIDNEY INJURY (AKI) AND DKD

Patients with DKD were susceptible to severe AKI and usually had a worse prognosis following AKI (163). Advani recently summarized clearly that diabetes may increase the risk of AKI while AKI may increase the risk of CKD in diabetes (164). PT suffers from more severe renal tubular hypoxia and mitochondria dysfunction in diabetic kidney. Inflammatory cytokines have been also reported to be upregulated in diabetic kidney leading to serial cascades of inflammation (165). Hyperglycemia, advanced glycation end products (AGEs) and albuminuria itself can induce the expression of adhesion molecules and chemokines in proximal tubular cells to aggravate injury. In a separate study (166), STZ-induced and Akita diabetic mouse models exhibited heightened susceptibility to increased tubule cell damage and programmed cell death caused by ischemia reperfusion injury (IRI). Proximal tubule cells exposed to high glucose exhibited higher apoptosis following depletion of ATP or exposure to severe hypoxia. The authors (166) identified activation of the intrinsic pathway of apoptosis characterized by mitochondrial Bax accumulation and cytochrome c release, and the activation of the intrinsic pathway of apoptosis which was induced by the upregulation of p53 in tubule cells exposed to high glucose and ischemic insult. The studies by Kelly et al. also showed that DKD patients are more susceptible to renal ischemia leading to more severe tubular cell apoptosis (167, 168).

In addition, miRNAs have been shown to be highly promising diagnostic markers for early DKD and it may have a potential role in the treatment of DKD. However, one miRNA often has multiple target genes, while one target gene may also be regulated by multiple miRNAs. Given this complexity, further studies are warranted to ascertain the specific roles of miRNA in renal fibrosis before considering their potentials application in clinical setting.

CONCLUSIONS

PT injury appears in the early stage of DKD and continues throughout the progression of DKD (169). Based on its structural and functional characteristics, PTs are vulnerable to injury in hyperglycemic states and difficult to recover. In diabetic patients, a high glucose transport state and local relative oxygen deficiency (primary and secondary) in PT may be the initial factors of

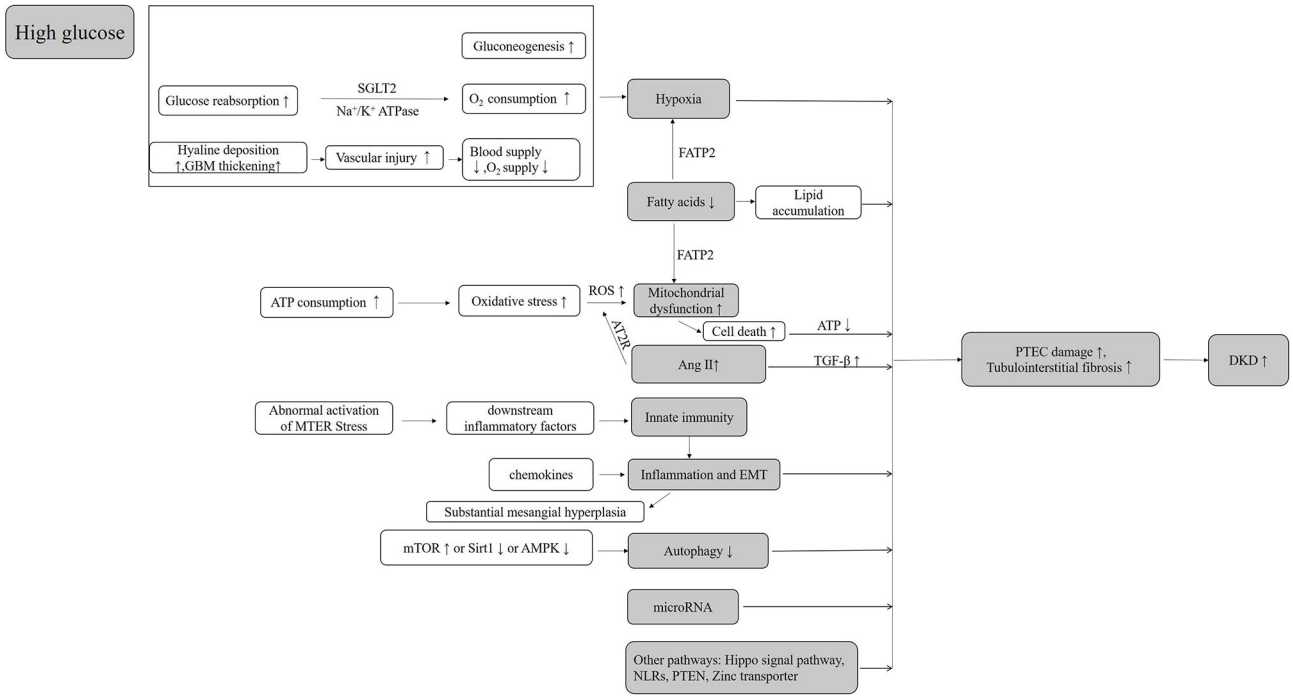

FIGURE 1 | The mechanisms of tubular cell injury in DKD.

tubular damage, while excessive mitochondria damages and ROS production are important contributors to the further damage of PTs in DKD. Abnormalities in hemodynamics, glucose and lipid metabolism, mitochondria, oxidative stress, inflammation, and many other factors interact with each other and form a vicious circle, leading to the renal tubular dysfunctions (**Figure 1**).

In this review, we discussed the potential mechanisms of renal tubular damage in DKD and potential therapeutic targets to prevent or treat the tubular cell injury. Renal tubular damage is a complex and dynamic process involving a "tubulocentric view" or "glomerulocentric view," which represents a manifestation of different stages in the development of DKD. New studies are required to further understand the pathogenesis of tubular injury in DKD and to develop specific treatments to prevent and delay the tubular injury in DKD.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

YZ was supported by The National Natural Science Foundation of China [81973772]; The 2018–2020 Three-year Action Plan for Traditional Chinese Medicine Further Development in Shanghai [ZY(2018-2020)-CCCX-2002-02]; the Shanghai leadership program for Chinese Medicine [ZY(2018-2020)-RCPY-1007]; Shanghai Shuguang Scholar [16SG37]; Shanghai Municipal Key Clinical Specialty [shslczdzk04201]; RZ was supported by Senior Talents Program of Integrated Traditional Chinese and Western Medicine in Shanghai [ZY(2018-2020)-RCPY-2002].

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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.

frontiers in Medicine

ORIGINAL RESEARCH
published: 06 April 2021
doi: 10.3389/fmed.2021.659188

Susceptibility of *ApoB* and *PCSK9* Genetic Polymorphisms to Diabetic Kidney Disease Among Chinese Diabetic Patients

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This study aimed to investigate the susceptibility of 8 polymorphisms in *ApoB* and *PCSK9* genes to diabetic kidney disease (DKD) in Chinese patients with type 2 diabetes mellitus. This is a case-control association study, including 575 DKD cases and 653 controls. Genotypes were determined using ligase detection reaction method, and data are analyzed using STATA software. The genotype distributions of rs1042034 and rs12720838 differed significantly between the two groups ($P < 0.001$ and $P = 0.008$, respectively). After adjusting for confounding factors, the mutations of rs1042034 and rs12720838 were associated with the significantly increased risk of DKD. For instance, carriers of rs1042034 T allele (CT and TT genotypes) were 1.07 times more likely to have DKD than carriers of rs1042034 CC genotype [odds ratio (OR) = 1.07, 95% confidence interval (CI): 1.03–1.10, $P < 0.001$]. Further, haplotype T-A-G-T in *ApoB* gene was overrepresented in cases (18.10%) compared with controls (12.76%) ($P_{Simulated} = 0.045$), and haplotype T-A-G-T was associated with a 33% increased risk of DKD (OR = 1.33, 95% CI: 1.04, 1.70). In further haplotype-phenotype analysis, significant association was only noted for hypertension and omnibus haplotypes in *ApoB* gene ($P_{Simulated} = 0.001$). Our findings indicate that *ApoB* gene is a candidate gene for DKD in Chinese patients with type 2 diabetes mellitus.

Keywords: diabetic kidney disease, single nucleotide polymorphism, association, *ApoB*/*PCSK9* genes, risk

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Edited by:
Qin Zhou,
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Reviewed by:
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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *27 January 2021*
Accepted: *12 March 2021*
Published: *06 April 2021*

Citation:
Ma L, Wang S, Zhao H, Yu M, Deng X, Jiang Y, Cao Y, Li P and Niu W (2021) Susceptibility of ApoB and PCSK9 Genetic Polymorphisms to Diabetic Kidney Disease Among Chinese Diabetic Patients. Front. Med. 8:659188. doi: 10.3389/fmed.2021.659188

INTRODUCTION

As a major microvascular complication of diabetes mellitus, diabetic kidney disease (DKD) has skyrocketed to epidemic proportions. Latest statistics indicates that the age-standardized prevalence of DKD worldwide was 15.48/1,000 and 16.50/1,000 in men and women, respectively (1). Although global DKD prevalence has remained stabilized during the last three decades, the mortality rate of DKD is growing in comparison to that of other types of chronic kidney disease (2). Effective strategies should be developed to curb this global burden (3). A practical strategy is the identification of possible risk factors to potentially predict subjects who are more likely to pre-dispose to DKD, thereby helping doctors and healthcare professionals to make immediate prevention and control measures against this disease.

It is widely accepted that DKD is a complex multifactorial disease, partly under genetic control (4). Previous evidence revealed that DKD occurs in familial clusters, indicating a strong heritable component in the pathogenesis (5, 6). The results of recently completed genome-wide association studies have advanced knowledge on the genetic underpinnings of DKD (7–10). Despite much endeavors, the causal genetic determinants of DKD are not yet fully understood. As such, candidate gene approach represents an alternative strategy by focusing on the genes with strong biological or clinical implications (11). The genes encoding apolipoprotein B (ApoB) and proprotein convertase subtilisin/kexin type 9 (PCSK9) are such candidate genes for DKD. Clinical studies have shown that circulating ApoB served as an independent predictor of renal replacement therapy in DKD patients (12, 13), and PCSK9 can promote hypercholesterolemia, and PCSK9 concentrations were associated with chronic kidney disease stages (14) and lipid-lowering regimens (15). However, in the medical literature, the genetic pre-disposition of *ApoB* and *PCSK9* genes to DKD is rarely reported.

To fill this gap in knowledge and yield more information for future research, we genotyped 5 single nucleotide polymorphisms (SNPs) in *ApoB* gene and 3 SNPs in *PCSK9* gene in 575 DKD patients and 653 controls, and investigated their susceptibility to DKD risk, both individually and jointly.

METHODS

Study Design and Ethical Approval

This is a case-control association study officially approved by the institutional review boards of the China-Japan Friendship Hospital. All study subjects were enrolled from this hospital during the period between August 2016 and December 2018, and they gave written informed consent prior to drawing blood samples for genetic analysis and filling out questionnaires.

Study Subjects

A total of 1,228 patients, who were diagnosed with type 2 diabetes mellitus, were enrolled in this study. Of these patients, 575 had newly-diagnosed and histologically-confirmed DKD, as the case group. The remaining 653 patients who had experienced type 2 diabetes mellitus for 7 or more years, were clinically confirmed to be free of DKD, and had no history of severe kidney diseases formed the control group.

Eligibility Criteria

DKD was diagnosed according to the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF-K/DOQI) guidelines (16).

In detail, subjects in the case group were included if they had a clinical diagnosis of type 2 diabetes mellitus and 24h urinary albumin >500 mg/24 h or an albumin creatinine ratio (ACR) >30 mg/g, and subjects were excluded if they had no previous history of kidney diseases, or if they had primary or secondary kidney diseases that caused proteinuria, such as IgA nephropathy, membranous nephropathy, lupus nephritis, obstructive renal disease, and acute urinary tract infection.

Subjects in the control group were included if they had a clinical diagnosis of type 2 diabetes mellitus and ACR <30 mg/g. Exclusion criteria were identical as the case group.

Demographic Data Collection

A structured questionnaire was designed to collect information on age, gender, body weight/height, cigarette smoking, hypertension status, and duration of type 2 diabetes mellitus. Body mass index (BMI) was calculated as body weight (in kilometers) divided by body height (in meters) squared.

Laboratory Biomarker Measurement

Laboratory markers included 24 h urinary albumin excretion, ACR, high density lipoprotein cholesterol (HDLC), low density lipoprotein cholesterol (LDLC), total cholesterol (TC), triglyceride (TG), hemoglobin A1c (HbA1c), and homocysteine (HCY). Serum concentrations of fasting TG, TC, HDLC, LDLC, and HCY were measured using an automated biochemical analyzer (AU5800 Clinical Chemistry System, Beckman Coulter, Brea, CA, USA). HBA1c was measured using the D-10 Hemoglobin Testing System (Bio-Rad, Hercules, CA, USA).

Genomic DNA Extraction and Genotyping

Genomic DNA was extracted from whole blood cells according to the manufacturer instructions, and quantified using the NanoDrop 1000 spectrophotometer (ThermoScientific). DNA samples were frozen at −20°C until mass genotyping.

Five SNPs in *ApoB* gene, including rs1042034, rs679899, rs676210, rs1367117, rs12720838, and three SNPs in *PCSK9* gene, including rs662145, rs45448095, rs11583680, were genotyped by use of the ligase detection reaction (LDR) method.

In detail, 50 ng DNA was amplified in 15 μl reaction mixture containing 7.5 μL of Premix Ex Taq, and 10 pmol of each primer for the amplification of genomic sequences. Pre-heating of the mixture at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and elongation at 75°C for 90 s. LDR ligation reaction was in 10 μl reaction mixture containing 3 μl of polymerase chain reaction (PCR) product, 1 μl of 10* Taq DNA ligase buffer, 0.125 μl of Taq DNA ligase, and 0.02 μl of mix probes.

The primer sequences and probe sequences of each SNP are summarized in **Supplementary Tables 1, 2**, respectively.

Statistical Analysis

Continuous data are expressed as median (interquartile range), and categorical data as percentage. Two-group comparisons were completed using the Wilcoxon rank-sum test or χ^2-test when appropriate. The genotypes and alleles of each SNP under study, as well as the tests for Hardy-Weinberg equilibrium, were compared using χ^2-test between controls and cases. Risk prediction of each SNP for DKD, summarized as odds ratio (OR) and 95% confidence interval (CI), was calculated separately under additive and dominant models before and after adjusting for confounding factors. Linkage patterns of SNPs under study in each gene were examined using the HaploView software (version 4.2, Cambridge, MA, USA).

The frequencies of derived haplotypes were estimated using the Haplo.Stats program in the R software (version 3.6.1). Comparison of derived haplotypes between the two groups, prediction estimates of each haplotype for DKD risk, and association of omnibus haplotypes with baseline characteristics were completed using the Haplo.Stats program as well.

Unless otherwise stated, statistical analyses were performed using the STATA software (version 14.1, Stata Corp., College Station, TX). The power to detect statistical significance was derived using the PS Power and Sample Size Calculations (version 3.0) (17).

RESULTS

Baseline Characteristics

Table 1 shows the baseline characteristics of study subjects. Cases were significantly older than controls (median age, 62 vs. 60 years, $P = 0.001$). There was a slightly high proportion of males in cases relative to controls (66.4% vs. 60.8, $P = 0.041$), as well as a high proportion of smokers ($P = 0.041$) and hypertension ($P < 0.001$).

Linkage Disequilibrium

In *ApoB* gene, two SNPs, rs1042034 and rs676210, were in complete linkage, and in *PCSK9* gene, rs45448095 and rs11583680 were in complete linkage. As such, rs676210 and rs11583680 were removed from the following analyses.

Genotype and Allele Distributions

The genotype and allele distributions of the remaining 6 SNPs between controls and cases are presented in **Table 2**. The genotype distributions of rs1042034 and rs12720838 differed significantly between the two groups ($P < 0.001$ and $P = 0.008$, respectively). No significance was noted for the comparisons of the other SNPs. The genotype distributions of all study SNPs satisfied the Hardy-Weinberg equilibrium at a significance level of 10%.

Single-Locus Analysis

The risk prediction of the remaining 6 SNPs for DKD risk under both additive and dominant models is provided in **Table 3**. After adjusting for confounding factors, including age, gender, smoking, BMI, hypertension, and duration of diabetes, the mutations of rs1042034 and rs12720838 were associated with the significantly increased risk of DKD under both models of inheritance. For instance, carriers of rs1042034 T allele (CT and TT genotypes) were 1.07 times more likely to have DKD than carriers of rs1042034 CC genotype, independent of these confounders (OR = 1.07, 95% CI: 1.03–1.10, $P < 0.001$).

Haplotype Analysis

Table 4 shows the estimated frequencies of haplotypes separately in *ApoB* and *PCSK9* genes, as well as their prediction for DKD

TABLE 1 | The baseline characteristics of the study subjects.

Characteristics	Controls ($n = 653$)	Cases ($n = 575$)	*P*
Age, years	60 (53, 67)	62 (54, 70)	0.001
Males, %	60.8	66.4	0.041
Smokers, %	32.0	37.6	0.041
Hypertension, %	51.6	78.1	<0.001
BMI, kg/m^2	25.31 (23.40, 27.70)	25.78 (24.00, 28.39)	0.003
Duration of diabetes, years	14 (10, 18)	15 (9, 21)	0.208
TC, mmol/L	4.15 (3.50, 4.88)	4.19 (3.43, 5.03)	0.715
HbA1C, mmol/L	7.85 (6.80, 9.20)	7.80 (6.70, 9.30)	0.984
HCY, μmol/L	11.10 (9.41, 13.26)	13.37 (10.74, 16.81)	<0.001
TG, mmol/L	1.45 (1.03, 2.18)	1.65 (1.13, 2.44)	<0.001
HDLC, mmol/L	0.99 (0.83, 1.20)	0.97 (0.79, 1.19)	1.578
LDLC, mmol/L	2.44 (1.93, 3.06)	2.39 (1.83, 3.08)	1.768

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HbA1C, hemoglobin A1c; HCY, homocysteine; TG, triglyceride; HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol. Data are expressed as median (interquartile range) or percentage, when appropriate. P was calculated using the Wilcoxon rank-sum test for continuous data and the χ^2-test for categorical data.

TABLE 2 | The genotype and allele distributions of studied polymorphisms between controls and cases.

SNPs	Genotype/ allele	Controls	Cases	χ^2	*P**
***ApoB* gene**					
rs1042034	CC	373 (57.12%)	319 (55.48%)	18.11	<0.001
	CT	270 (41.35%)	221 (38.43%)		
	TT	10 (1.53%)	35 (6.09%)		
	T	290 (22.21%)	291 (25.30%)	3.25	0.071
rs679899	AA	453 (69.37%)	409 (71.13%)	3.27	0.195
	AG	188 (28.79%)	148 (25.74%)		
	GG	12 (1.84%)	18 (3.13%)		
	G	212 (16.23%)	172 (15.11%)	0.57	0.448
rs1367117	GG	496 (75.96%)	434 (75.48%)	0.04	0.981
	AG	147 (22.51%)	132 (22.96%)		
	AA	10 (1.53%)	9 (1.57%)		
	A	167 (12.79%)	150 (13.04%)	0.04	0.850
rs12720838	CC	455 (69.68%)	393 (68.35%)	9.70	0.008
	CT	192 (29.4%)	162 (28.17%)		
	TT	6 (0.92%)	20 (3.48%)		
	T	204 (15.62%)	202 (17.57%)	1.68	0.195
***PCSK9* gene**					
rs662145	TT	505 (77.34%)	434 (75.48%)	1.34	0.511
	CT	137 (20.98%)	134 (23.30%)		
	CC	11 (1.68%)	7 (1.22%)		
	C	159 (12.17%)	148 (12.87%)	0.27	0.603
rs45448095	CC	544 (83.31%)	454 (78.96%)	3.84	0.147
	CT	103 (15.77%)	115 (20.00%)		
	TT	6 (0.92%)	6 (1.04%)		
	T	115 (8.81%)	127 (11.04%)	3.45	0.063

*SNPs, single nucleotide polymorphisms. *P was calculated using the χ^2-test.*

risk. In *ApoB* gene, the most common haplotype was C-A-G-C (alleles in order of rs1042034, rs679899, rs1367117, and rs12720838), and its frequency was 66.54% in controls and 60.90% in cases. In *PCSK9* gene, the most common haplotype was T-C (alleles in order of rs662145 and rs45448095), and its frequency was 80.19% in controls and 82.28% in cases. Comparison of haplotypes between the two groups revealed that haplotype T-A-G-T in *ApoB* gene was overrepresented in cases (18.10%) compared with controls (12.76%) (Simulated $P = 0.045$), and haplotype T-A-G-T was associated with a 33% increased risk of DKD (OR = 1.33, 95% CI: 1.04, 1.70). The power to detect the significant association of haplotype T-A-G-T with DKD was estimated to be 79.9%.

No significance was noted for the comparisons of other haplotypes and risk predictions.

TABLE 3 | Risk prediction of 8 studied polymorphisms for diabetic kidney disease under both additive and dominant models.

SNPs	Additive model		Dominant model	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Before adjustment				
rs1042034	1.06 (1.03, 1.10)	<0.001	1.06 (1.03, 1.10)	<0.001
rs679899	1.02 (0.99, 1.05)	0.257	1.02 (0.99, 1.05)	0.257
rs1367117	1.00 (0.99, 1.01)	0.845	1.00 (0.99, 1.01)	0.845
rs12720838	1.06 (1.02, 1.10)	0.005	1.06 (1.02, 1.1)	0.005
rs662145	1.00 (0.98, 1.01)	0.459	1.00 (0.98, 1.01)	0.459
rs45448095	1.02 (0.97, 1.08)	0.391	1.02 (0.97, 1.08)	0.391
After adjustment*				
rs1042034	1.07 (1.03, 1.10)	<0.001	1.07 (1.03, 1.10)	<0.001
rs679899	1.02 (0.99, 1.05)	0.287	1.02 (0.99, 1.05)	0.287
rs1367117	1.00 (0.99, 1.01)	0.883	1.00 (0.99, 1.01)	0.883
rs12720838	1.06 (1.02, 1.11)	0.002	1.06 (1.02, 1.11)	0.002
rs662145	0.99 (0.98, 1.01)	0.334	0.99 (0.98, 1.01)	0.334
rs45448095	1.02 (0.97, 1.07)	0.506	1.02 (0.97, 1.07)	0.506

*SNPs, single nucleotide polymorphisms; OR, odds ratio; 95% CI, 95% confidence interval. *Adjusting for age, gender, smoking, body mass index, hypertension, and duration of diabetes.*

Haplotype-Phenotype Analysis

Further, an analysis on the association of haplotypes as a whole with baseline characteristics was done in both genes (**Table 5**). Of all characteristics, significant association was only noted for hypertension and omnibus haplotypes in *ApoB* gene (Simulated $P = 0.001$).

DISCUSSION

To the best of our knowledge, this is the first study that has explored the susceptibility of *ApoB* and *PCSK9* genetic alternations to DKD risk in a large Chinese diabetic population. The key finding of this study is that *ApoB* gene is a candidate gene for DKD. Particularly, two SNPs, rs1042034 and rs12720838, in *ApoB* gene were individually associated with the significant risk of DKD under both additive and dominant models of inheritance, and in the presence of other two SNPs in this gene, the risk was clearly enhanced, indicating the possible synergistic contribution.

DKD is a polygenic disease (18). A long list of genes have been identified to play a contributory role in the pathogenesis of

TABLE 4 | The frequency of derived haplotypes between controls and cases, and their risk prediction for diabetic kidney disease.

Haplotype*	Controls (%)	Cases (%)	Hap. score	*P*	Simulated *P*	OR (95% CI)
***ApoB* gene**						
C-A-G-C	66.54	60.90	−1.39	0.163	0.175	Reference
T-A-G-T	12.76	18.10	1.81	0.042	0.045	1.33 (1.04, 1.70)
C-G-A-C	6.28	4.64	−0.83	0.408	0.410	0.94 (0.66, 1.35)
T-G-A-C	4.35	4.92	0.68	0.497	0.530	1.22 (0.82, 1.81)
T-G-G-C	2.99	3.23	0.32	0.749	0.700	1.14 (0.70, 1.87)
C-G-G-C	1.84	2.21	−0.10	0.924	0.925	1.22 (0.64, 2.35)
C-A-A-C	1.34	2.49	1.24	0.213	0.210	1.94 (0.97, 3.89)
C-A-G-T	1.64	1.46	−0.62	0.537	0.555	0.94 (0.47, 1.89)
T-A-G-C	0.98	1.05	0.13	0.900	0.920	1.15 (0.49, 2.69)
***PCSK9* gene**						
T-C	80.19	82.28	−1.34712	0.178	0.185	Reference
C-C	8.77	8.91	−0.13257	0.895	0.885	1.01 (0.75, 1.35)
T-T	6.94	5.54	1.47107	0.141	0.105	1.28 (0.91, 1.8)
C-T	4.10	3.26	1.20819	0.227	0.210	1.3 (0.82, 2.06)

OR, odds ratio; 95% CI, 95% confidence interval.

**In ApoB gene, alleles in a haplotype are assigned in the order of rs1042034, rs679899, rs1367117, and rs12720838; in PCSK9 gene, alleles in a haplotype are assigned in the order of rs662145 and rs45448095.*

TABLE 5 | Association of all derived haplotypes as a whole in each gene with baseline characteristics of study subjects.

Characteristics	*ApoB* gene			*PCSK9* gene		
	Global statistics	*P*	Simulated *P*	Global statistics	*P*	Simulated *P*
Age	13.08	0.442	0.269	2.57	0.462	0.451
Gender	24.27	0.029	0.069	6.00	0.112	0.104
BMI	8.93	0.778	0.497	1.46	0.692	0.698
Duration of diabetes	11.72	0.468	0.307	1.28	0.734	0.759
Hypertension	104.82	<0.001	0.001	1.12	0.773	0.794
Smoking	13.81	0.387	0.283	0.78	0.854	0.862
TC	12.14	0.516	0.257	1.37	0.712	0.709
HbA1C	17.48	0.178	0.101	0.29	0.963	0.962
HCY	18.18	0.151	0.057	1.61	0.656	0.651
TG	9.51	0.733	0.371	0.44	0.933	0.950
HDLC	6.61	0.921	0.656	2.43	0.488	0.507
LDLC	13.25	0.429	0.210	2.69	0.443	0.407

BMI, body mass index; TC, total cholesterol; HbA1C, hemoglobin A1c; HCY, homocysteine; TG, triglyceride; HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol.

DKD, such as *ADIPOQ* gene (19) and *IL-6* gene (20). However, the results are not often reproducible at a population level. The reasons behind this poor reproducibility are manifold. The most possible reason is the divergence in the genetic underpinnings of different origins of populations. For instance, a polymorphism may be in close linkage with another nearby causal locus in one ethnic group but not in another (21). As such, it is necessary to establish a candidate list of culprit genes and mutations in each ethnic group. Another reason lies in the fact that the net impact of a single gene or single mutation on complex diseases such as DKD may be small or moderate, or its impact may be offset or antagonized by other cellular regulators (22). To shed some light on this issue, besides single-locus analysis, we also interrogated the contribution of unlinked SNPs as a haplotype, and importantly, the risk magnitude in *ApoB* gene was reinforced in haplotype analysis. In particular, the significant haplotype T-A-G-T in *ApoB* gene, harboring the mutant alleles of both rs1042034 and rs12720838, was associated with over 30% increased risk of DKD, in comparison with the increased risk of both SNPs at 6 and 6%, respectively.

The third possible reason is the involvement of environmental or intermediate phenotypes in the development of DKD, such as hypertension, which has been established as a promising risk factor for DKD (23, 24). As expected, we have observed a significant association between omnibus haplotypes in *ApoB* gene and hypertension. Dozens of studies have evaluated the genetic susceptibility of *ApoB* gene to hypertension (25–27). In view of our haplotype-disease and haplotype-phenotype analyses, it is reasonable to speculate that the association of *ApoB* gene with DKD may be mediated by its association with hypertension, which further precipitates the development of DKD. We agree that further studies exploring the concurrent association of *ApoB* genetic defects with hypertension and DKD are needed to confirm or refute this speculation.

Several limitations should be acknowledged for this association study. First, this study is cross-sectional and hospital-based in design, and all study subjects were diagnosed to have type 2 diabetes mellitus. It is of added interest to enroll healthy subjects free of type 2 diabetes mellitus as the controls. Second, only 8 candidate SNPs were selected in *ApoB* and *PCSK9* genes. Third, the sample size is insufficient for further subsidiary investigations, such as upon stratification by gender and hypertension. Fourth, all study subjects are exclusively Chinese, and it leaves an open question for the generalizability of our findings to other ethnic groups.

Conclusions

Despite these limitations, our findings indicate that *ApoB* gene is a candidate gene for DKD in Chinese patients with type 2 diabetes mellitus. Although no hint of association was detected for *PCSK9* gene, its candidacy in the development of DKD cannot be excluded, and is subject to a matter of debate. For practical reasons, further studies are needed to investigate the underlying mechanisms of ApoB, *in vitro* or *in vivo*, in the pathogenesis of DKD.

DATA AVAILABILITY STATEMENT

The original contributions generated 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 Institutional Review Boards of the China-Japan

Friendship Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LM, YC, PL, and WN: conceptualization. LM, SW, WN, HZ, XD, YJ, and MY: data collection. LM: funding acquisition. LM, SW, and YJ: investigation. LM, HZ, and XD: data detection. WN: statistics. WN, LM, YC, and PL: writing. All authors read and approved the final manuscript prior to submission.

FUNDING

This study was supported by the National Natural Science Foundation of China (Grant Nos: 82074221 and 81703892).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.659188/full#supplementary-material

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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.

frontiers in Medicine

REVIEW
published: 30 June 2021
doi: 10.3389/fmed.2021.695792

The Diabetic Cardiomyopathy: The Contributing Pathophysiological Mechanisms

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Individuals with diabetes mellitus (DM) disclose a higher incidence and a poorer prognosis of heart failure (HF) than non-diabetic people, even in the absence of other HF risk factors. The adverse impact of diabetes on HF likely reflects an underlying "diabetic cardiomyopathy" (DM–CMP), which may by exacerbated by left ventricular hypertrophy and coronary artery disease (CAD). The pathogenesis of DM-CMP has been a hot topic of research since its first description and is still under active investigation, as a complex interplay among multiple mechanisms may play a role at systemic, myocardial, and cellular/molecular levels. Among these, metabolic abnormalities such as lipotoxicity and glucotoxicity, mitochondrial damage and dysfunction, oxidative stress, abnormal calcium signaling, inflammation, epigenetic factors, and others. These disturbances predispose the diabetic heart to extracellular remodeling and hypertrophy, thus leading to left ventricular diastolic and systolic dysfunction. This Review aims to outline the major pathophysiological changes and the underlying mechanisms leading to myocardial remodeling and cardiac functional derangement in DM-CMP.

Keywords: diabetes mellitus, cardiomyopathy, heart failure, pathophysiology, insulin resistance

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Pathology, a section of the journal Frontiers in Medicine

Received: *15 April 2021*
Accepted: *07 June 2021*
Published: *30 June 2021*

Citation:
Salvatore T, Pafundi PC, Galiero R, Albanese G, Di Martino A, Caturano A, Vetrano E, Rinaldi L and Sasso FC (2021) The Diabetic Cardiomyopathy: The Contributing Pathophysiological Mechanisms. Front. Med. 8:695792. doi: 10.3389/fmed.2021.695792

INTRODUCTION

Diabetic Cardiomyopathy (DM-CMP) is a form of heart disease associated with diabetes mellitus (DM), which causes significant structural and functional changes in the myocardium. The pathogenesis has been a hot topic of research since its first description (1), and it is still under active investigation, as a complex interaction among multiple factors play a role at systemic, myocardial, and cellular/molecular levels. The current pathogenic hypotheses mostly derive from translational models, with human evidence far less developed due to limited access to human tissue samples.

This review aims to outline the state of the art about the major pathophysiological changes and underlying mechanisms leading to myocardial remodeling and cardiac functional derangement in DM-CPM.

DIABETES MELLITUS AND HEART FAILURE: A BIDIRECTIONAL EPIDEMIOLOGIC ASSOCIATION

The risk for heart failure (HF), as well as that for all components of cardiovascular disease (CVD), is higher in individuals with diabetes as compared to non-diabetic people.

The Framingham Heart Study, published in 1974, is among the first studies to demonstrate this association, reporting an incidence 2.4- and 5-fold higher, respectively, in men and women, after adjustment for common CVD risk factors (2).

A robust epidemiological evidence has confirmed that HF is among the most common complications of DM. A prevalence of ∼20% (4–28%) has been found in clinical trials of glucose-lowering drugs in DM, consistent with a recent position paper of the Heart Failure Association of the European Society of Cardiology (ESC) (3).

DM patients without HF at baseline are more likely to develop this complication over time as compared to non-diabetic people (4), whereas subjects without diabetes at 45 years are more than 60% less likely to manifest HF (5). In the Kaiser Permanente system, out of more than 8,000 patients followed for up to 6 years, the risk of new-onset HF resulted 2.5-fold higher in patients with type 2 DM (T2DM) rather than their non-diabetic counterparts (6). In a large population-based study of 34,198 T2DM patients initially free from overt CVD, HF was even more common than myocardial infarction (MI) as first presentation of CVD (7). In T2DM subjects with newly-recognized HF, the incidence was almost 5-fold higher for HF with preserved ejection fraction (HFpEF) (about 23%) vs. HF with reduced EF (HFrEF) (about 5%) (8).

A low annual incidence of HF (0.2%) and myocardial dysfunction (−0.1%) is reported in type 1 DM (T1DM), likely dependent on the younger age of the studied population (9). Nevertheless, there is a well-documented prevalence of early subclinical cardiomyopathy in children and adolescents with T1DM (10). A meta-analysis of subjects included in clinical trials demonstrated that the presence vs. absence of DM in hypertensive individuals increased the risk of HF by more than 4-folds (11).

Subjects with impaired glucose tolerance (IGT) or insulin resistance (IR) have a 1.7-fold increased risk of HF (12). A community-based cohort study that followed patients for almost 30 years revealed that several biomarkers reflecting IR and dyslipidemia, predicted HF independently of ischemic CVD and other established CV risk factors (13).

HF is a frequent as serious complication of diabetes. Its prognosis is worse than in non-diabetic subjects, with a 75% higher risk of CV death or HF hospitalization (14), and a frequent progression to end-stage HF, which may require heart transplantation despite optimal medical therapy (15). In a prospective study from the mid-1990s, HF 1-year mortality was 30% in people with DM, about 1.5-fold higher than in those without (16). The HF mortality risk was 10-fold higher in a diabetic population older than 65 years (17). Currently, the clinical impact of DM–CMP and other chronic diseases in hospitalized elderly subjects is affected by both gender, and frailty (18–20).

In the CHARM (Candesartan in Heart Failure Assessment of MoRtality and Morbidity) study, DM was associated with a higher relative risk of HF hospitalizations or CV death in patients with HFpEF than HFrEF (21).

On the other hand, as HF is common in DM, so DM is highly prevalent in people with HF, hence one condition increases the incidence and worsens the prognosis of the respective other. Patients with HF have a 4-fold higher prevalence of T2DM (20%) than patients without (4–6%) (22). In a CHARM study group analysis, more than 25% of patients with HF has diabetes (23). When admitted with HF, one-third of patients without a previous diagnosis of diabetes results affected by DM or impaired glucose tolerance (IGT) (24). This prevalence rises to 40% in a large multicenter European study (25), as confirmed in the EVEREST analysis (26).

The mechanism responsible of the increased risk of T2DM in HF is the impaired insulin signaling induced by loss of skeletal muscle mass, sedentary lifestyle, and increased circulating cytokines, which trigger a vicious cycle in which IR and HF deteriorate each other (27). In patients with advanced HF, hemodynamic recovery after ventricular assist device placement is associated with improvements in both systemic and cardiac insulin sensitivity, glucose homeostasis, and toxic lipid products (28). Likewise, IR significantly affects HF prognosis (29).

THE DIABETIC CARDIOMYOPATHY

Based on the observation that two-thirds of elderly patients with diabetes presented with a myocardial dysfunction, Lundbæk has firstly suggested in 1954 the concept of a specific DM-related cardiomyopathy (30). This term refers to the current definition proposed by the European Society of Cardiology (ESC), that is a "cardiomyopathy is defined as a heart muscle disease in which the myocardium is structurally and functionally abnormal in the absence of coronary artery disease (CAD) as well as hypertensive, valvular, or congenital heart disorders" (31). Almost 20 years

Abbreviations: AGE, advanced glycation and product; AMPK, 5′-AMP-activated protein kinase; CaSR, calcium sensing receptor; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CAN, cardiovascular autonomic neuropathy; CCR2, C-C chemokine receptor type 2; cGMP/PKG, cyclic guanosine 3′, 5′-monophosphate/ protein kinase G; CTGF, connective tissue growth factor; CV, cardiovascular; CVD, cardiovascular disease; DAG, diacylglycerol; DM, diabetes mellitus; DM-CMP, diabetic cardiomyopathy; ECM, extracellular matrix; ED, endothelial dysfunction; eNOS, endothelial NO synthase; ER, endoplasmic reticulum; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; HIF1α, hypoxia-inducible factor 1α; IGT, impaired glucose tolerance; FA, fatty acid; LV, left ventricle; FDA, Food and drug administration; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; lncRNAs, long non-coding RNAs; MI, myocardial infarction; MAPK, mitogen-activated protein kinase; miRNAs, MicroRNAs; MMPs, matrix metalloproteinases; NCX, Na^{+}/Ca^{2+} exchanger; NFkB, nuclear factor kappa B; NO, nitric oxide; NOX, NADPH oxidase; NRLP3, NLR family pyrin domain-containing 3; O-GlcNAc, O-linked β-N-acetylglucosamine; PARP, poly ADP ribose polymerase; PGC-1α, PPAR-γ coactivator-1α; PI3K/Akt, phosphatidylinositol 3-kinase/protein kinase B; PKC, protein kinase C; PKG, protein kinase G; PPAR, peroxisome proliferator-activated receptor; RAAS, renin angiotensin aldosterone system; RAGEs, receptor for advanced glycation end products; ROS, reactive oxygen species; SERCA, sarcoplasmic/endoplasmic reticulum calcium-ATPase; SGLT2, sodium glucose co-transporter 2; sRAGE, soluble RAGE; S1PR1, sphingosine 1-phosphate receptor 1; SR, sarcoplasmic reticulum; SREBP-1c, sterol regulatory-element-binding protein-1c; T1DM,Type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; TGF- β, transforming growth factor-β; TIMPs, tissue inhibitors MMPs; TZDs, thiazolidinediones; UCP, uncoupling protein; VEGF, vascular endothelial growth factor.

later, Rubler et al. reported the post-mortem findings of four diabetic patients with glomerulosclerosis and advanced symptoms of HF unrelated to valvular, congenital or hypertensive heart disease, alcoholism or significant epicardial coronary artery atherosclerosis. These data thus provided evidence that a cardiomyopathy could directly result from DM, likely in dependence of myocardial microangiopathy or metabolic derangements (1).

Currently, DM-CMP is widely recognized as a specific form of cardiomyopathy which occurs independently of other cardiac risk factors and is promoted by the long-standing metabolic perturbations of diabetes, thus exerting a direct toxic effect on the myocardium (30). Although Rubler originally reported a dilated cardiomyopathy manifesting with the characteristic symptoms of HF, the restrictive LV remodeling with diastolic LV dysfunction is the more frequent picture in DM (32).

In the current clinical practice, DM-CMP diagnosis is still challenging, as it requires the identification of distinct functional and structural changes in the LV and the concomitant exclusion of other cardiac diseases and risk factors for CVD. Due to the very frequent confounding of other HF risk factors such as hypertension, CAD, and renal disease, the burden of a "pure" diabetic cardiomyopathy is conceivable not as high as the cardiomyopathy of heterogeneous etiology, with a calculated prevalence of 16.9% of diabetic patients in a small study (33).

PATHOPHYSIOLOGY OF DIABETIC CARDIOMYOPATHY

Several mechanisms determining molecular, cellular and interstitial changes, as well as activation of renin-angiotensin aldosterone axis and adrenergic systems, are involved in the development of DM-CPM. These include imbalance of myocardial energy substrates, gluco- and lipotoxicity, altered insulin signaling, mitochondrial defects, endoplasmic reticulum (ER) stress, deranged intracellular calcium handling, oxidative stress, endothelial dysfunction, deposition of advanced glycation end products (AGEs), maladaptive immune responses, and so on. Each of them contributes to the structural remodeling and functional defects in diabetic myocardium, including impairments in cardiac relaxation, compliance, and contractility (**Figure 1**).

Metabolic Abnormalities in the Diabetic Heart

Changes to the metabolic milieu associated with DM, such as lipotoxicity, glucotoxicity and impaired insulin signaling, emerge as crucial pathogenic factors for DM-CMP. Together, they exert, both directly and indirectly, a detrimental increase in oxidative stress, endothelial dysfunction and inflammation, thus making a strong contribution to the myocardium structural and functional derangement.

Myocardial Energy Substrate Changes and Lipotoxicity

Energy Substrates in Healthy Myocardium

Due to the constant cardiac activity, the myocardium is the higher energy-demanding tissue in the body. To absolve this function, it is equipped with an efficient metabolic machinery, mainly represented by the mitochondrial oxidative phosphorylation (34). Under normal conditions, most of energy for myocardium (∼60–90%) is supplied by fatty acid (FA) oxidation, whereas the remaining ∼10–40% of ATP derives from the oxidation of pyruvate produced in equal amounts by glycolysis and lactate oxidation. Of note, the heart is a net consumer of lactate, both at baseline and upon increase in workload. Ketone bodies are not immediately available from food but produced in the liver by incomplete oxidation of FAs released from the adipose tissue in response either to fasting or energy depletion. They provide, mainly the D-beta-hydroxybutyrate, an alternative substrate for oxidative phosphorylation. Under physiological conditions, aminoacids represent a minor source of energy (21, 35).

The healthy heart is commonly defined a "metabolic omnivore," due to its crucial capacity to shift between different substrates, according to their availability, in order to ensure a continuous energy supply (36). This metabolic flexibility is mainly determined by the "Randle cycle," by which high circulating levels of glucose decrease the FA oxidation rates and vice-versa (37). Another metabolic regulator is 5′-AMP-activated protein kinase (AMPK), which acts as a cellular "fuel gauge" (38). In the long term, the nuclear peroxisome proliferator-activated receptor (PPAR)-α, abundantly expressed in the myocardium, play a pivotal role upregulating the transcription of genes related to FA uptake and oxidation (39). Overall, the relative substrate contribution to ATP production can vary mostly depending on energy demand, substrate availability and hormonal milieu. For instance, exercise induces a switch from FAs to glucose oxidation, whereas during prolonged fasting or poorly controlled diabetes, ketone bodies can represent the main energy supplier (34).

Changes of Energy Substrates in Failing Heart

In HF the mitochondrial oxidation of FAs is decreased, more likely due to the PPARα signaling suppression and the activation of hypoxia-inducible factor 1α (HIF1α)–PPARγ signaling axis, which impair FA transport into mitochondria and downregulate FA oxidative enzymes (34). Due to the stimulation of lipolysis by sympathetic activation in HF, an increased FA delivery to cardiac myocytes is responsible of this shift away from FA oxidation (40). This imbalance between FA uptake and oxidation leads to cytosolic overload of triglycerides and accumulation of metabolic intermediates generated by non-oxidative pathways such as ceramide and diacylglycerol (DAG), exerting toxic effects and maladaptive signaling, including IR (34, 41). These deposits promote inflammation, cell damage and, eventually, cell death, hence a condition of "lipotoxicity" which contributes to the HF progression (42, 43).

The glucose metabolism of the failing heart is characterized by an enhanced glucose uptake, not accompanied by a concomitant

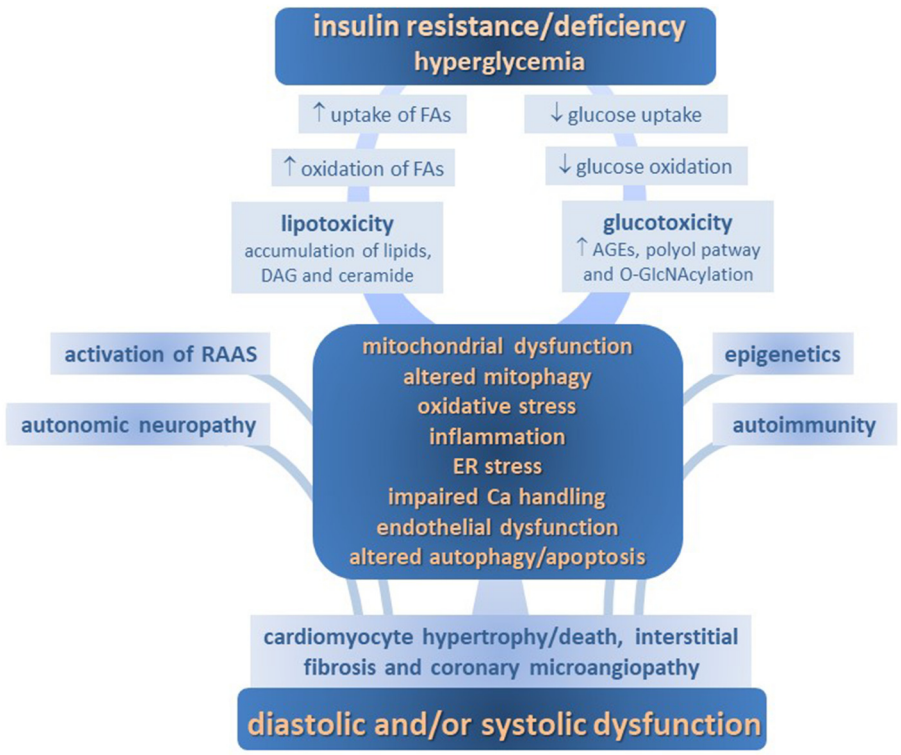

FIGURE 1 | Pathophysiology of diabetic cardiomyopathy.

increase in glucose oxidation. Therefore, despite an increase in the relative contribution of glucose oxidation to ATP production, the absolute substrate flux through glucose oxidative pathways actually reduces (34). During the late stages of HF, the glucose availability for ATP production is further impaired by the association of a marked IR (44), even though some authors suggest that the cardiac IR may represent a beneficial mechanism protecting the heart from fuel overload (45).

Ketone bodies may represent a relevant energy source in the HF setting when metabolism of other energy substrates falls (46). Indeed, an upregulation of the enzymes involved in ketone body metabolism is reported in both murine models (47) and patients with advanced HF (48). Since the ATP production/oxygen consumption ratio of the β-hydroxybutyrate is higher (2.50) than that of FA palmitate (2.33), this ketone body has been proposed as a "super fuel" enhancing cardiac metabolic efficiency (34).

Metabolic Disturbances in Diabetic Heart

Exposure to hyperglycemia by itself decreases insulin signaling and glucose uptake in cardiomyocytes (49). Essentially, due to the impaired capacity to transport and metabolize glucose determined by insulin deficiency in T1DM and IR in T2DM, the diabetic heart shifts away from glucose as an energy source and gets in a "metabolically inflexible" and less efficient FA-dependent state. This is a crucial pathophysiological condition if considering that glucose is the unique cardiac substrate able to provide ATP during hypoxia or ischemia (50).

Consistent with a prevalent FA utilization, the diabetic heart shows an increased expression of the FA transporter CD36 on both sarcolemmal and endosomal membranes, with an enhanced subcellular vesicular recycling from endosomes to plasma membrane (51). Excess FAs activate PPAR-α, which increases expressions of genes involved in FA oxidation, but also suppresses glucose utilization (52). These typical derangements in the myocardial energy metabolism of diabetic heart are mimicked in mice with cardiac-restricted overexpression of PPAR-α (39). However, studies in diabetic patients either with or without HF argue against an activation of the PPAR-α signaling axis which drives the increase in FA uptake and oxidation (53, 54). Another proposed mechanism for enhanced FA oxidation may be the increased acetylation of mitochondrial β-oxidation enzymes observed in an obese animal model (55).

Excessive FA oxidation increases ATP expenditure for futile cycling of metabolic intermediates, inhibits ATP shuttling from mitochondria to the cytosol, and increases the expression of mitochondrial uncoupling protein (UCP) 3 through PPAR-α, thereby dissipating the mitochondrial proton gradient and deteriorating the ATP production efficiency (56, 57). Finally, these changes produce oxidative stress and mitochondrial dysfunction (58). Moreover, the dissipation of the mitochondrial membrane potential might interfere with excitation–contraction coupling and mitochondrial Ca^{2+} uptake, thus potentially underlying arrhythmias (59).

A deposition of lipids and their metabolites in the cytosol of cardiomyocytes has been documented in DM animal models (60). Human studies using Oil Red-O staining of explanted hearts at the time of heart transplantation have demonstrated cardiac steatosis (28, 41). This excess accumulation of lipids leads to myocardial IR and reduced bioavailability of nitric oxide (NO) (61). Increased levels of DAG in cardiomyocytes activates protein kinase C (PKC) isoforms, thus reducing insulin metabolic signaling and NO production. Similarly, ceramide directly activates atypical PKCs to phosphorylate and inhibit the insulin metabolic Akt signaling and disrupts endothelial NO synthase (NOS) signaling impairing NO bioavailability (62, 63). As well, ceramide may activate caspase 3 and stimulate cytochrome C release, thus inducing cellular apoptosis, and inhibit key pathways involved in defense against DNA damage, such as Poly ADP ribose polymerase (PARP) (64).

A relevant question is whether the altered substrate metabolism is cause or consequence of the failing heart in diabetes. A ventricular biopsy study has showed that even in the absence of contractile failure the diabetic heart exhibits a decreased mitochondrial capacity for β-oxidation, increased accumulation of intracellular lipids, ER stress, and a higher degree of apoptosis (65). Another very recent human bioptic study suggests the crucial role of the toxic metabolic milieu of DM in the early progression of DM-CMP (66). A lipid accumulation in cardiomyocyte was found after only 3 months in non-DM hearts transplanted to diabetic patients. Moreover, triacylglycerol and ceramide contents were both related with early dysfunctions in DM recipients after 12 months. Levels of myocardial insulin receptor were lower in healthy hearts transplanted in DM than non-DM recipients, and SREBP1c (sterol regulatory-element-binding protein-1c) and PPAR systems were highly expressed in cardiomyocytes of DM recipients.

Hyperglycemia and Glucotoxicity

Sustained exposure to high glucose levels is a major driver of cardiac pathology in DM (67–69). In an observational study on individuals with T1DM, the incidence of HF increased monotonically with the HbA1c, with a range of 1.42–5.20 per 1,000 patient-years between patients in the lowest (<6.5%) and highest (>10.5%) HbA1c categories (70). In a similar study on T2DM patients, each 1% increase in the HbA1c corresponded to an 8% increase in the HF risk (71). Conversely, in T2DM patients of UK Prospective Diabetes Study, each 1% reduction in HbA1c level corresponded to a 16% reduction in the risk of HF (72).

The detrimental effect of chronic hyperglycemia, referred to as "glucotoxicity," is mainly mediated by oxidative stress, increased formation of AGEs and enhanced substrate flux through alternative metabolic pathways (50).

Oxidative Stress

Hyperglycemia contributes to oxidative stress in diabetic heart by excessive oxygen radical formation from the auto-oxidation of glucose, formation of glycated proteins, and impaired buffering capacity due to glycation of metfodant enzymes (73, 74).

The mitochondrial electron transport chain is among the first targets of high glucose levels, with a direct increase in superoxide anion formation. Moreover, high glucose activates protein kinase C (PKC), thus leading to up-regulation of NADPH oxidases (NOX), xanthine oxidase, uncoupling of NO synthase (NOS), microsomal P-450 enzymes, and arachidonic acid metabolism pathways (75). The consequent increased reactive oxygen species (ROS) impair cardiac structure and function by directly damage DNA, proteins and phospholipids, and promote myocytes apoptosis. Kuster et al. found that a short-period of exposure to H_2O_2 of *in vitro* rat ventricular myocytes determined a progressive decrease in cell shortening, followed by diastolic arrest. The possible mechanisms were the direct oxidative modification of sarcoplasmic/endoplasmic reticulum calcium-ATPase (SERCA) and Na^+/Ca^{2+} exchanger (NCX) (76). One harm of the superoxide generation stands in its interaction with NO to form peroxynitrite, a potent oxidant involved in enhanced apoptosis of both animal and human cardiomyocytes (77, 78).

Antioxidant response may be a determinant of the heart health in diabetes. Other findings reveal that the mitochondrial isoform of aldehyde dehydrogenase (ALDH2) may play a role in the development of DM-CMP, possibly through protection against oxidative stress and preservation of mitochondrial integrity (79). Evidence from literature indicates that diabetes upregulates the Ras-related small G protein RhoA, a factor that may impair cardiac function determining uncoupled eNOS, reduced NO bioavailability, and enhanced O_2^-. IGF-I is a crucial cardiac survival factor that downregulating RhoA produces beneficial effects also mimicked by the Rho kinase inhibitor Y27632 and BH4, a finding indicating that the selective IGF-I overexpression may represent a therapeutic potential for DM-CMP (80).

Enhancing cardiac endogenous antioxidant capacity is an attractive way to prevent DM-CMP. A pivotal target may be represented by Nrf2, an important regulator of cellular detoxification responses and redox status that can lead to antioxidant response elements (ARE)-mediated basal and inducible expression of more than 200 genes (81). Sulforaphane, a molecule within the isothiocyanate group of organosulfur compounds from cruciferous vegetables, such as broccoli, Brussel sprouts or cabbage, is a potent Nrf2 activator (82). A study on *db*/*db* mice fed with broccoli sprout extract or sulforaphane for 3 months showed significant prevention of diabetes-induced cardiac oxidative damage and inflammation by up-regulating Nrf2 transcriptional activity (83). A recent study on mice provided the direct evidence that the preventive effect of sulforaphane against DM-CMP depends on AMPK resulting from both improvement of AMPK-mediated lipid metabolism and potentiation of antioxidative pathway mediated by AMPK/AKT/GSK3β signaling (84).

Accumulation of Advanced Glycation End Products

Persistent hyperglycemia causes the non-enzymatic glycosylation of proteins and enzymes with production of toxic AGE adducts, irreversibly altering their structure and functions (85). As an example, AGEs formed on SERCA2a in diabetes impair the sarcoplasmic reticulum (SR) Ca^{2+} reuptake in cardiomyocytes and slow cardiac relaxation (86), whereas long-term treatment

with an AGE crosslink breaker partially normalized SR Ca^{2+} signaling (87).

A significant increase in AGE compounds and their binding to cell surface specific receptors (RAGEs) trigger a cascade of pathophysiological responses responsible of severe cardiac damage. Among these, the activation of PKC and NOX lead to the fabrication of peroxide and, ultimately, of ROS, and to the maladaptive activation of mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NFκB) signaling, followed by the production of several inflammatory and/or profibrotic factors, as well as upregulation of apoptosis (*via* p53 and calcineurin signaling) and autophagy (88–92). All these mechanisms may cause functional and structural damage, till cardiomyocyte death and eccentric LV remodeling with systolic dysfunction.

Interestingly, metformin induces activation and phosphorylation of MAPK, which could mediate its several extraglicemic effects (93, 94).

As shown by light microscopic immune-histochemical visualization, AGEs also accumulate in the myocardial interstitium between cardiomyocytes (95). The non-structural compartment of extracellular matrix (ECM) is represented by a variety of proteins (including collagen IV, laminin, fibronectin, myelin, tubulin, plasminogen activator 1, and fibrinogen), vital for ECM plasticity and with glycosylation as a common denominator (96). Besides ECM disturbation by oxidative stress and inflammation, accumulation of AGEs in the interstitium stimulates the differentiation of fibroblasts into myofibroblasts (*via* Janus kinase-signal transducer and activator of transcription, JAK-STAT signaling), which produce excess matrix proteins, and the crosslink matrix metalloproteinases (MMPs), which indeed impair ECM degeneration. The increased resistance of connective tissue to enzymatic proteolysis and the enhanced collagen cross-linking lead to myocardial fibrosis and stiffness, thus resulting in impaired compliance and diastolic LV relaxation (97–99). This process is potentially mediated by the up-regulation of pro-fibrotic cytokines such as transforming growth factor-β (TGF-β) and connective tissue growth factor (CTGF) (100).

In DM-CMP, an abundant AGEs deposition even involves both endothelial and smooth muscle cells of myocardial microvasculature by triggering vascular inflammation and dampening endothelial NO production (101, 102).

As evidence for the role of AGEs in DM-CMP pathogenesis, the cleavage of preformed AGE crosslinks with ALT-711 attenuates the diabetes-associated cardiac abnormalities in rats (103), and the administration of a RAGE antagonist in a rat model of T1DM prevents AGEs/RAGE signaling-mediated increases in myocardial collagen, fibrosis, stiffness and diastolic dysfunction (104).

The soluble RAGE (sRAGE) is the circulant isoform of RAGE which, by competing with cellular RAGE, may inhibit the pro-inflammatory and pro-fibrotic activity of AGE (105). Unsurprisingly, lower levels of circulating soluble receptors for AGEs predict incident HF in patients with DM (106).

A recent study on experimental diabetes has demonstrated that the inhibition of AGE formation by aminoguanidine exerts a beneficial effect against cardiac remodeling and contractile dysfunction, likely through the regulation of autophagy and ER stress (107).

Activation of Polyol Pathway

In In a high-glucose state as diabetes, aldose reductase converts a part of glucose overload to sorbitol, which is oxidized to fructose by sorbitol dehydrogenase. The first reaction produces a depletion of NADPH, a molecule essential for the functioning of various endothelial enzymes, including cytochrome P450 and NO synthase, and a cofactor in the generation of the reduced glutathione. The second reaction increases the cytosolic NADH: NAD^+ ratio, which can inhibit the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and increase the concentrations of triose phosphate, with consequent formation of AGE and DAG (108).

The chronic elevation of DAG in diabetes (and in part the increased circulating levels of FAs) activates PKC, a central player in signal transduction and intracellular crosstalk, by phosphorylating a huge array of substrates on serine/threonine residues. PKCβ2 isoform is over-expressed in the myocardium of diabetic animal models and patients with HF (109, 110), and the activation of the PKC/DAG signaling pathway is associated with biochemical and structural changes typical of DM-CMP (e.g., reduced blood flow, increased vascular permeability, basal membrane thickening, ECM deposition, and cardiac hypertrophy) (111–113). On the contrary, PKC inhibition may reverse structural and functional derangements in the diabetic heart (114).

Maladaptive Hexosamine Biosynthesis

During chronic hyperglycemia, a small percentage of glucose is shuttled through the hexosamine biosynthesis pathway, thus generating the O-linked β-N-acetylglucosamine (O-GlcNAc). This metabolite may rapidly bind to a multitude of proteins altering their function *via* the O-GlcNAc transferase (115). The ones specifically involved in the progression of DM-CMP include Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), phospholamban and myofilaments, with a negative impact on cardiac contractility and relaxation (116).

Several studies have suggested that O-Glc-N-Acylation of cardiomyocyte proteins might be associated with the development of cardiac hypertrophy (117, 118). This pathogenic mechanism of myocardial hypertrophy has been recently confirmed both in cultured cells and *in vivo*, as triggered by high carbohydrate diets (119). The reduction of the excess cellular O-Glc-N-Acylation, indeed, obtains beneficial effects on calcium handling and diabetic cardiac function (120).

Many mitochondrial proteins are highly susceptible to O-Glc-N-Acylation, which suggests another way for hexosamine pathway to induce cardiac dysfunction in diabetes (121).

Insulin Resistance

Increasing evidence points to IR as a primary etiologic factor in DM-CMP development.

IR impairs the myocardial glucose utilization and increases the expression of myocardial UCPs. The resulting decline in

the efficiency of high-energy phosphate production prevents the myocardial adaptive response to injury, as observed in patients with HFpEF (122, 123).

IR impairs the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signal transduction pathway to elicit normal metabolic responses. The resultant reduction of glucose oxidation decreases the Ca^{2+} ATPase activity and moves Ca^{2+} back into the SR, thus increasing the intracellular content of ion (30, 124). Since PI3K/AKT can also activate endothelial NOS (125), the reduced NO production in IR states further increases the intracellular Ca^{2+} levels and Ca^{2+} sensitization in cardiomyocytes *via* the cGMP/PKG signaling pathway (30, 51, 126). On the other hand, through the PI3K/Akt pathway, the higher insulin levels associated to IR may induce the titin switching toward the stiff N2B isoform, thus impairing cardiomyocyte distensibility (127).

All these abnormalities may promote cardiac stiffness and diastolic dysfunction, being mainly relevant to restrictive/HFpEF phenotype of DM-CMP, especially in obese T2DM patients. Other contributing mechanisms of IR to myocardial injury are lipotoxicity, sympathetic up-regulation, inflammation, oxidative stress, and fibrosis (128).

The impact of IR on cardiac morphology and function has been extensively documented in clinical studies. In the Framingham Heart Study, LV mass and wall thickness increased with worsening glucose intolerance, and the relation between IR and LV mass observed only in women, was largely dependent on obesity (129). A recent longitudinal study with a 25-yrs follow-up period revealed that cumulative exposure to DM or higher IR adversely affects LV remodeling and function (130). A link between IR and concentric LV remodeling and hypertrophy is confirmed in studies using cardiac magnetic resonance imaging (131, 132).

Intriguingly, opioid system, which seems related to IR (133), play a role in HF (134).

PATHOPHYSIOLOGICAL MECHANISMS PROMOTING DM-CMP

A plethora of mechanisms mostly connected to the above-described metabolic alterations, act in unison to promote cardiomyocyte injury and cardiac dysfunction in DM.

Altered Calcium Homeostasis and Calcium/Calmodulin Dependent Protein Kinase II

Perturbations in the cytosolic calcium trafficking and ventricular excitation-contraction coupling at cardiomyocyte level are the mechanistic hallmark of cardiac dysfunction in diabetes (124). Physiologically, the excitation of the cardiomyocyte determines the actin-myosin interaction and contractile activity by inducing Ca^{2+} influx *via* L-type Ca^{2+} channels in the plasma lemma and subsequent Ca^{2+} transient, i.e., Ca^{2+} release from sarcoplasmic reticulum (SR) through ryanodine receptors. During cardiomyocyte relaxation, Ca^{2+} actively moves from cytoplasm into SR by SERCA, with the contribution of sarcolemma Ca^{2+} extrusion by NCX and Ca^{2+} ATPase (135).

In diabetic cardiomyocytes, the activity of SERCA and NCX is impaired, likely by either reduction in protein levels or its post-translational modification because of non-enzymatic glycosylation (136). The slower Ca^{2+} transients and leaky Ca^{2+} release channel, result in an impaired calcium load of SR, which is the primary organelle for handling intracellular calcium. To support the correlation between Ca^{2+} handling and cardiac dysfunction, cardiac overexpression of SERCA2a significantly improves myocardial contractility in streptozotocin-induced diabetic rats (137). Being the calcium efflux from cytosol depressed, the cardiomyocyte relaxation impairs, and the action potential duration prolongs (138). These changes are likely associated with the clinical finding of diastolic dysfunction.

CaMKII is a multifunctional serine/threonine kinase physiologically activated in response to β-adrenergic receptor signaling, which targets a number of Ca^{2+} homeostatic proteins in the heart (139). During acute cardiomyocytes activation, CaMKII stimulates glucose uptake, energy production, sarcolemmal ion fluxes, SR Ca^{2+} release/reuptake and myocyte contraction/relaxation coupling, all mechanisms empowering the physiological cardiac adaptation. In diabetic myocardium, as a result of impaired Ca^{2+} handling and oxidative, nitrosative and hyperglycemic stresses, CaMKII is in a state of chronic maladaptive upregulation leading to inefficient substrate utilization, mitochondrial dysfunction, inflammation, fibrosis, ion channel remodeling, impaired intracellular Ca^{2+} handling, contractile dysfunction, and increased risk of arrhythmias (140, 141). In a recent study, the cardiac tissue from both T2DM patients and rats presents an elevated CaMKII activation as compared to non-diabetic controls. Moreover, the trabeculae from diabetic rats have reduced contraction and relaxation performance, which may be restored by the inhibition of this kinase (142).

Mitochondrial Dysfunction, ER Stress, and Altered Mitophagy

The increased β-oxidation exceeding the respiratory capacity of mitochondria in diabetic hearts induces accumulation of toxic lipid metabolites and generation of oxidative stress and inflammation, which further deteriorate mitochondrial function, possibly culminating in cardiomyocyte death (143). In addition, the signaling pathways by which AMPK activates the PPAR-γ coactivator-1α (PGC-1α), the master metabolic regulator of mitochondrial biogenesis and respiratory function, is impaired in advanced DM-CMP (51).

The hyperglycemia-stimulated ER stress may be the initiator, concomitantly with the FA overload of cardiomyocytes, of an adverse mitochondrial remodeling in human diabetic myocardium (144). ER stress is a condition of over-accumulation of misfolded proteins triggered by intracellular buildup of saturated FA and oxidative stress (145). If the activation of the "unfolded protein response" aiming to restore a normal ER function fails, the cardiomyocyte may go toward a profound mitochondrial dysfunction, including decreased ability to process

FA up to self-destruction by apoptosis (146). Upregulation of GRP78 and induction of CHOP, two markers of ER stress response, has been recently described in LV myocardium from diabetic patients (65), consistent with previous findings in animal models of T2DM (147).

Mitophagy, a type of selective autophagy where the damaged or unnecessary mitochondria are sequestered by auto-phagosomes and degraded by lysosomes, is an essential step in maintaining mitochondrial homeostasis in the heart, together with mitochondrial fission, fusion, and biogenesis (148). Increasing lines of evidence suggest that mitophagy is significantly changed in diabetic cardiomyocytes, and some vital proteins involved in this process have been found altered in many diabetic tissues, including heart (149, 150). Even in the context of metabolic syndrome, cardiac mitophagy is altered (151).

Autophagy, Apoptosis, and Senescence of Myocytes

Adult cardiomyocytes rarely proliferate, thus their death may represent the *primum movens* for the cascade of hypertrophic and fibrotic LV remodeling leading to progressive heart dysfunction, till congestive HF. Higher rates of myocyte death, as determined by autophagy, apoptosis, and senescence, characterize DM-CMP (152).

Constitutive autophagy, a highly conserved process for bulk degradation and recycling of cytoplasmic components in lysosomes, is a homeostatic mechanism crucial to counter oxidative stress and AGE formation and to protect cardiomyocytes from aging-related and ischemia-induced cardiac hypertrophy (153, 154). Bellot et al. reported that ROS and autophagy mutually regulate and that elimination of ROS-damaged cells *via* autophagy is a protective mechanism (155). Indeed, if autophagy is suppressed and excessive ROS persists, the cardiomyocytes would eventually go toward apoptotic death (156). On the other hand, excessive induction of autophagy may indiscriminately destroy cytosol and organelles and determine hypertrophy and fibrosis, with an accelerated progression to ventricular dilatation and decline in systolic performance (157).

The concomitant release of autophagy-related factors, as observed under high-glucose conditions, may contribute to cell death and cardiac dysfunction (158).The activation of PI3K/Akt/mTOR signaling pathway, instead, an essential regulator of cardiac autophagy (159), ameliorates hyperglycemia–induced cardiac hypertrophy (160). A study convincingly supports insulin signaling as a significant regulator of myocardial autophagy, mediating in early life its physiological postnatal suppression, thereby linking nutrient sensing to postnatal cardiac development (161).

Whether the autophagic responses are adaptive or maladaptive remains controversial. Likewise, the role of autophagy in diabetic heart has been not fully understood yet. Several reports show an increased/decreased/unchanged autophagy in the hearts of either humans or animals with T2DM (162). In a study on animal models, autophagic adaptations in DM-CMP seem remarkably different between T1DM and T2DM, being overactivated in the first, but suppressed in the second (163), but even on this topic data are controversial (164). Likely, autophagy regulates both cell survival and cell death in diabetic heart through a strict cross-talk with apoptotic pathways (152), and apoptosis is involved in DM-CMP mainly as a consequence of autophagy dysregulation (165, 166).

A significant increase of apoptosis and cell necrosis characterizes both animal models and patients with DM. Endomyocardial biopsies in diabetic patients with dilated cardiomyopathy show a 4-fold increase of necrosis in cardiomyocytes, 9-fold in endothelial cells, and 6-fold in fibroblasts as compared to their non-diabetic counterparts (167). Hyperglycemia-induced ROS production speeds up apoptosis, some of which is elicited by angiotensin II and glycosylation (168). Many other factors (e.g., mitochondrion damage, oxidative stress, ER stress, inflammation, and even fibrotic signaling) can activate either pro-apoptotic or necrosis signaling pathways in the diabetic heart (169).

The phenomenon of senescence is typically attributed to telomere shortening after repeated cell division. Currently, we know that senescence is also inducible by a series of pathogenic stimuli involved in apoptosis, such genotoxic, mitochondrial and oxidative stresses, as well as inflammation. Moreover, the accumulation of senescent cells can itself cause persistent inflammation and oxidative stress *via* a so called "senescence-associated secretory phenotype" leading to organic dysfunction (169). It is also well-known that senescent cells contribute to the outcome of a variety of cardiac diseases, including age-related and -unrelated cardiac diseases like DM-CMP (170). In this context, DM may impair the *in vitro* proliferation and differentiation potential of adult cardiac stem/progenitor cells, further worsening their senescence phenotype, even when compared to non-diabetic ischemic patients (171).

Inflammation

Likewise to the known contribution of inflammation to other HF etiologies, both systemic and local maladaptive inflammation responses are strongly concerned with the progression of DM-CMP (172, 173).

Exposure of heart to glucose or FA excess activates NFκB, a protein complex which controls DNA transcription and induces the expression of proinflammatory cytokines (IL6, pro-IL18, pro-IL1β, and TNF-α) and the assembly of NLR family pyrin domain-containing 3 (NLRP3) inflammasome (30, 51). Similarly, AGE/RAGE signaling promotes NF-κB activation and mediates an inflammatory reaction by heterodimerizing with toll-like receptor-4, thus leading to the production of NLRP3, pro-IL1β, and pro-IL18 (104). Activated NLRP3 inflammasome plays a crucial role in the pathogenesis of HF in diabetes, resulting in amplification and infiltration of inflammatory cell, whereas a decrease in NLRP3 attenuates cardiomyopathy in a T2DM rat model (174–176).

Monocytes/macrophages are leading players in DM-CMP pathogenesis. Particularly, macrophage proinflammatory M1 polarization is increased and macrophage M2 anti-inflammatory response inhibited in diabetic heart (177). The recruitment of these cells to sites of inflammation is induced by the C-C chemokine receptor type 2 (CCR2) (178), and macrophages

derived from CCR^{2+} monocytes are required for adverse left ventricle remodeling (179). A recent study on mice demonstrated that the heart expression of CCR2 associated to persistent hyperglycemia leads to DM-CMP development, whereas the inhibition of this chemokine could inhibit oxidative stress and M1 macrophage infiltration in diabetic hearts (180).

Apart from macrophages, an involvement of neutrophil and lymphocyte regulation in DM-CMP has emerged. Chronic systemic inflammation in diabetes leads to leukocyte activation and recruitment to various organs with further inflammatory tissue remodeling over time ultimately evolving in fibrosis. At heart level, this may result in reduced cardiac output that ultimately stimulates further cardiac inflammation and fibrosis leading to dilation and established heart failure (181). These pathways may be critical to the discovery of new targeted therapies for controlling DM-CMP progression. As an example, the T cell-specific deletion of sphingosine 1-phosphate receptor 1 (S1PR1), as well as the administration of the S1PR1 antagonist FTY720, are able to exert protection against cardiac fibrosis in a streptozotocin-induced diabetic model (182, 183).

Recently, the role of adipokines (e.g., adiponectin) on the cardiovascular outcome has been well-described (184). Moreover, several less investigated mechanisms might be involved in cardiovascular inflammation (185–187).

Endothelial Dysfunction

Regardless of the relevance for both accelerated atherogenesis and microvascular diabetic complications, the impaired endothelial function of coronary microvessels is a key feature of DM-CMP (188), especially contributing to diastolic dysfunction and HFpEF (189).

The hallmark of ED is the impaired endothelium-mediated arterial vasodilation as a consequence of depressed bioavailability of nitric oxide (NO), a short-living mediator generated from L-arginine by endothelial NOS (eNOS) (190). During the early stages of IR and DM-CPM, the impaired NO-induced vasodilatation may be balanced by the either preserved or even enhanced endothelium-derived hyperpolarizing factor (EDHF)-mediated vasodilatation. Later, even this mechanism degenerates, thereby promoting microvascular dysfunction (30, 191).

Exposure of endothelial cells to excessive and/or fluctuating blood glucose levels can stimulate the generation of ROS and AGEs, with the consequent downregulation of eNOS and production of NO and cGMP (192, 193). In addition, superoxide anion inactivates NO by forming the more powerful oxidant peroxynitrite, thus triggering nitrosative stress and premature endothelial senescence (188).

The low NO bioavailability to adjacent cardiomyocytes decreases cGMP production and protein kinase G (PKG) activity, with consequent increased ratio of titin isoform N2B:N2BA expression and of intracellular Ca^{2+} content and sensitization. These changes result in a slow relaxation, high diastolic stiffness, and impaired cardiomyocyte elastance (194). As support to the relevance of this mechanism, PKG administration to cardiomyocytes isolated from DM-CMP patients with this phenotype corrects their high resting tension (99). Similar alterations have been observed in cardiomyocytes isolated from patients suffering from both aortic stenosis and DM (195). In addition, ED is associated with microvascular inflammation due to an increased expression of adhesion molecules and local infiltration and accumulation of macrophages expressing TGF-β. As a consequence, myocardial fibroblasts transform into myofibroblasts responsible of interstitial fibrosis (188). The role of TNF-alpha on ED has also been observed (196).

Notably, an increased albuminuria, marker of renal ED, is strictly related to a poor CV outcome in diabetic patients (197–200).

Microvascular Rarefaction

Similar defects in endothelium-dependent/independent vasodilation involve coronary microcirculation in both T1DM and T2DM patients (201). In addition, structural microvascular alterations impairing the capacity of coronary vascular bed independently of coronary atherosclerosis, may also contribute to DM-CMP (202).

In the myocardium of a well-recognized murine model of diabetes, a significant decline in microvessel density, reduced expression of selected VEGF isoforms, and increase in oxidative stress have been described, all significantly associated with measures of LV performance (203). In a study on patients with end-stage HF, capillary rarefaction and pericyte loss, accompanied by decreased contractility and increased stiffness, characterize diabetic human myocardial explants as compared to non-diabetic samples (204). In the same study, *in vitro* experiments on murine endothelial cells have shown that hyperglycemia attenuates tube formation, migration, and pericyte attraction upon proangiogenic stimulation (204). Moreover, the relative microvascular rarefaction resulting from cardiomyocyte hypertrophy is itself sufficient to induce cardiac fibrosis and diastolic dysfunction (205).

Autoimmunity

Immune inflammation is involved in the pathogenesis of myocarditis and cardiomyopathy (206). An immune biopathology has also been suggested in the pathogenesis of DM-CMP, especially in autoimmune-prone T1DM patients.

MI has been reported to induce sustained proinflammatory $CD4^{+}$ T-cell and auto-antibody responses against α-cardiac myosin heavy chain, a major autoantigen in myocarditis, both in mice models and in patients with T1DM, but not in control mice and T2DM subjects. Shared cardiac myosin autoantibody signatures between post-MI in T1DM patients and non-diabetic patients with myocarditis also suggests a post-infarction autoimmune syndrome in T1DM patients (207).

Some authors suggested that the cardiac insults of severe diabetic ketoacidosis might initiate the synthesis of antibodies directed to cardiac self-antigens involved in the early immunopathogenesis of cardiomyopathy in young patients with T1DM (208). By measuring prevalence and profiles of cardiac autoantibodies in longitudinal samples of T1DM patients from the Diabetes Control and Complications Trial, poor glycemic control has been demonstrated as associated with cardiac autoimmunity, as shown by the presence of multiple cardiac autoantibody types (209).

Epigenetics

Epigenetics, the inheritable changes in gene expression without change of DNA sequences, represents a significant link between environmental exposure, as hyperglycemia, inflammation, and oxidative stress, and alterations in gene activity (210).

MicroRNAs (miRNAs) are a group of small, single-strand RNA molecules belonging to the non-coding RNA family, which affect their target genes at a post-transcriptional level by either inhibiting mRNA or degrading protein production (211), whose dysregulated expression is highly implicated in the pathophysiology of DM-CMP.

Some miRNAs abundantly expressed in cardiomyocytes, such as miR-1 and miR-133a, are reduced in T2DM patients (212). In streptozotocin-induced diabetic rats, miR-133a overexpression is able to improve myocardial contractility through the upregulation of tyrosine aminotransferase, a known regulator of norepinephrine production and β-adrenergic receptors (213). Jeyabal et al. found a considerably decreased miR-9 expression in high glucose-cultivated cardiomyocytes and human DM myocardium (214). Downregulation of miR-30c mediates the pro-hypertrophic effects of hyperglycemia in diabetic cardiomyopathy by upregulating Cdc42 and Pak1 genes (215). Li et al. established that miR-30d leads to cardiomyocyte pyroptosis in DM-CMP by direct repression of Foxo3a expression (216). Cardiac-enriched miR-1 and miR-206 are responsive to hyperglycemia and favor the apoptosis of cardiomyocytes through the negative regulation of the heath shock protein 60 (217). Recent evidence demonstrates that miR-208 and miR-499, together with miR-1 and miR-133, might play a role in the differentiation of stem cells into cardiomyocytes (218). A proposed role for miR-208 in diabetic heart disease is the regulation of myosin heavy chain gene expression (219).

Some literature suggests an involvement of exosomes in DM-CMP, the extracellular vesicles containing a variety of biological components, including miRNAs, proteins and lipids, which mediate the intercellular communication (220). The stress induced by hypoxia, inflammation, and hyperglycemia has been reported to increase protein and mRNA content in endothelial cell-derived exosomes, and the exosomes released from diabetic cardiomyocytes could deliver detrimental components able to initiate endothelial cell dysfunction and impair angiogenesis (30). Of note, heat shock protein 20-engineered exosomes exert beneficial effects *via* the modulation of cardiomyocyte exosome secretion with restoration of normal cardiac function under hyperglycemic conditions (221).

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts longer than 200 nucleotides with both nuclear and cytoplasmic location which regulate gene expression through a variety of molecular mechanisms, including the interaction or competition with other RNAs, DNA binding proteins, and specific regulatory DNA sequences (222). Recently, the lncRNA H19 has been found remarkably reduced in a murine model of DM-CMP as a consequence of hyperglycemia, and to regulate cardiomyocyte apoptosis by targeting VDAC1, a mitochondrial porin involved in ATP transport (223).

Histone acetylation is a rapid and dynamic process mainly regulated by histone acetyltransferases (promoting gene transcription) and histone deacetylases (preventing gene transcription), which represent a major epigenetic mechanism whose deregulation may induce the development of several diabetic complications (224). BRD4, a histone acetylated reader protein which regulates either the activation or repression of gene transcription, has been recently identified as a critical mediator of hyperglycemia-induced cardiomyocyte hypertrophy and cardiac fibrosis through the AKT pathway (225).

A study in streptozotocin-induced diabetic rats has recently found that DNA methyltransferase-1 enhances cardiac fibroblast autophagy in diabetic cardiac fibrosis through inhibiting androgen receptor axis (226).

Activation of the Renin-Angiotensin-Aldosterone System

In a context of IR and hyperglycemia, the inappropriate activation of RAAS despite a state of salt and volume excess, plays an important role in the development of DM-CMP (30), whereas the RAAS block protects against cardiac damage (227).

Beyond receptors AT1 and AT2, Ang-II interacts with NOX, resulting in an overload of oxidants and free radicals in the body, with the subsequent exacerbation of oxidative stress and inflammation (169). This effect is supported by studies showing the effectiveness of ramipril in preventing upregulation of p47phox, p22phox, and reducing NADPH driven oxide production (228). Blocking of Ang-II also reduces the expression of p22phox, NOX and hyperglycemia-induced p47phox (229).

Activation of RAAS may induce systemic and cardiac IR through the mTOR–S6K1 signal transduction pathway (230). Meanwhile, enhanced angiotensin II type 1 receptor and mineralocorticoid receptor signaling in the myocardium enhance the adaptive proinflammatory immune response and inflammation, including increases in leukocyte adhesion, cytokine expression and macrophage infiltration (231).

Cardiovascular Autonomic Neuropathy

Diabetes is often associated to both neurosensorial damage and neuropathy (232). In particular, diabetic Cardiac Autonomic Neuropathy (CAN), in the absence of cardiac disease, seems associated with LV systolic and mainly diastolic dysfunction, even though it is difficult to assess its independent role among the multitude of factors involved in DM-CMP (233).

Due to an initial predominant parasympathetic denervation, excessive sympathetic activation in the early stages of diabetic CAN may promote LV hypertrophy, thus affecting both sympathovagal balance and baroreflexes (234). Moreover, an abnormal norepinephrine signaling may induce myocardial injury and LV remodeling *via* the cytotoxic effects of the increased catecholamine heart content observed in diabetic rat ventricles (235), eventually mediated by oxidative stress, inflammation, and apoptosis (236–238).

On the other hand, the sympathetic denervation associated to long-lasting diabetic CAN may impair β-adrenergic signaling and reduce myocardial contractile strength, relaxation kinetics, and diastolic distensibility (63, 239).

By changes in myocardial neurotransmitters, CAN may also alter myocardial blood flow and directly deteriorate LV

function. A diastolic dysfunction associated to abnormal cardiac sympathetic function appears early in the course of T1DM, as assessed by cardiac sympathetic imaging (240). Among subjects with T2DM or IGT referred for elective coronary angiography, those suffering from CAN have a higher prevalence and a more severe form of LV diastolic dysfunction (241). In a study based on cardiac magnetic resonance imaging in a large cohort of patients with T1DM, the presence of CAN is associated with an increased LV mass and concentric remodeling (242).

STRUCTURAL CHANGES IN DIABETIC CARDIOMYOPATHY

The above-described detrimental pathways elicited by diabetes at a systemic level and in the myocardium itself collectively promote myocardial hypertrophy and interstitial fibrosis, the two structural hallmarks identified in animal models and patients with both T1DM and T2DM (32, 243). Depending on the combination patterns of these two structural changes, the clinical phenotype of DM-CMP varies from a subclinical diastolic dysfunction to diastolic HFpEF and, eventually, to systolic dysfunction and HFrEF (30, 244). In the HFpEF phenotype, the LV is usually hypertrophied and stiff with normal LV volume. At the cellular level, cardiomyocytes appear hypertrophied with a normal structure of the sarcomere accompanied by increased collagen deposition in the interstitial space. HFrEF phenotype is usually associated with increased LV volume due to dilation, and cardiomyocytes appear damaged, with loss of sarcomeres, and at times replaced by fibrosis (63).

Cardiac Hypertrophy

Epidemiological data report diabetes and high-sugar diets as risk factors for cardiac hypertrophy and other complications (63, 243–245), a condition highly prevalent (up to 56%) in asymptomatic T2DM patients (246–248). Cardiac hypertrophy is strongly associated with the progression to HF, particularly if hypertension coexists (249), and with a higher incidence of other clinical events, including stroke and sudden death (250).

Cardiac myocytes are differentiated cells which have lost the propensity of proliferation after birth. When exposed to high glucose stress, they increase in size by enhanced protein synthesis and addition of sarcomeres, but not in number, with a resulting greater length (eccentric hypertrophy) or width (concentric hypertrophy) (251). A re-expression of fetal genes has been observed, such as myosin heavy chain (β-MHC) and GATA-1, and activation of early response genes (252).

The microvascular endothelial dysfunction may contribute to the cardiomyocyte enlargement through the parallel addition of sarcomeres due to the removal of a NO-dependent brake on pro-hypertrophic stimuli (188). The increased thickness of ventricular walls in hypertrophied diabetic hearts may partly depend on ECM enlargement. Accordingly, abnormally increased myocardial echodensity, more likely related to collagen deposition, has been detected in asymptomatic diabetic patients with normal ventricular mass (253).

Hypertrophy and fibrosis are two coexisting structural aspects of DM-CMP, likely generated by common pathophysiological mechanisms. As an example, the loss of cardiomyocytes typical of the diabetic heart stimulates the resident cardiomyocytes to compensatively work and become hypertrophic, but at the same time it evocates inflammation pathways generating fibrosis.

Extracellular Remodeling and Interstitial Fibrosis

Myocardial fibrosis is a main pathological feature of the diabetic heart which involves both left and right ventricular walls, and can lead to cardiac remodeling, dilation and dysfunction, as well as to arrhythmias and, eventually, congestive HF (101) (**Figure 2**).

Cardiac fibroblasts, the primary matrix-producing cells in the myocardium, help maintaining ECM homeostasis in healthy hearts (254). The majority of resident cardiac fibroblasts responsible for fibrotic response arise from the embryonic epicardium. During development, these cells undergo epithelial-mesenchymal transition under the influence of several growth factors; subsequently, a portion of these mesenchymal cells invade the myocardium to become the resident cardiac fibroblasts. Studies have also revealed that cells of the endocardium, a specialized cardiac endothelial lining, and endothelial cells of the coronary vessel may migrate into the *interstitium* where they undergo endothelial-mesenchymal transition and respond to pro-fibrotic stimuli in a manner similar to resident fibroblasts. Other cells such as pericytes of cardiac vessels can differentiate into collagen-producing cells and may contribute to the fibroblast population following cardiac injury. Finally, the circulating fibrocytes are bone marrow-derived cells considered a potential source of fibroblasts in the fibrotic heart. They represent a unique fibroblast progenitor population that co-express fibroblast markers, along with typical hematopoietic markers (181).

Mechanical or bioactive pathological insults may induce the phenotypic transition of fibroblasts from a resting to an active state characterized by heightened proliferation, migration, contractility, and ECM production (255). A high activation of fibroblasts has been observed in hearts of db/db mice and atrial tissue derived from T2DM patients, resulting in a dynamic balance disorder of cardiac ECM synthesis and accumulation, along with an excessive collagen deposition (256–258).

A dysregulation of specific collagen degrading metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), two crucial determinants of interstitial accumulation of secreted matrix proteins, also contributes to increased extracellular collagen content in the diabetic heart (259, 260). Recently, the enhanced expression of two isoforms of MMP-2 has been induced by high glucose *in vitro* and in a T1DM murine heart model (261).

The pathological processes referring to diabetes which mainly remodel ECM include hyperglycemia, AGE accumulation, inflammation, oxidative stress, and increased levels of neuro-hormones (258).

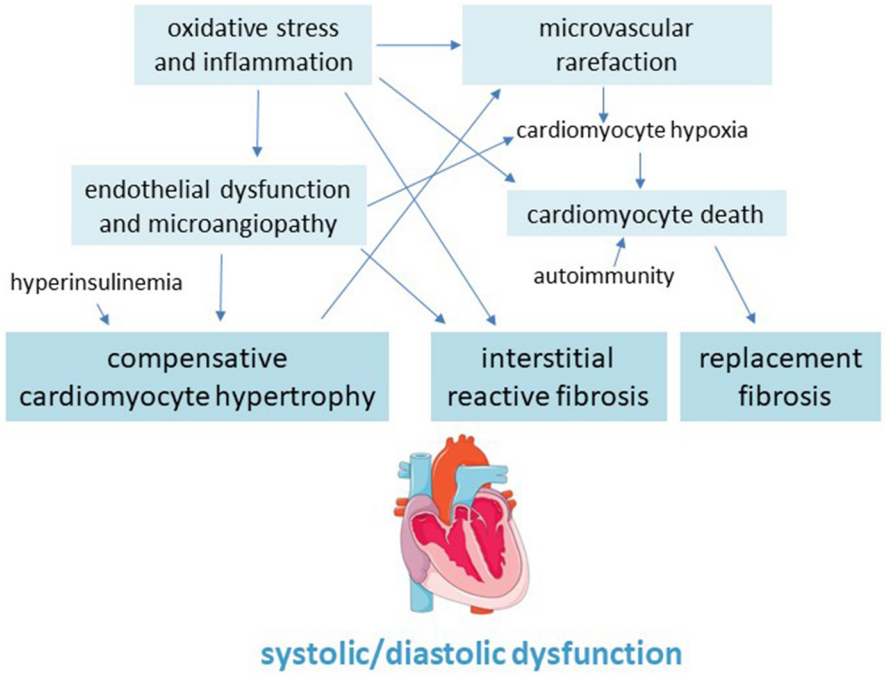

FIGURE 2 | Pathogenic scheme of hypertrophy and fibrosis in the diabetic cardiomyopathy.

Excessive collagen deposition may derive from either hyper-expression of TGF-β or CTGF, two key modulators of collagen production. The former is either mediated by angiotensin II activation or induced by high glucose and leptin *via* increasing transcription, secretion, and activation (169, 262, 263). On the other hand, in a study on a murine model of obesity and IR given a diabetogenic diet for 11 weeks, cardiac fibroblasts acquired enhanced myofibroblastic/fibrotic gene expression but reduced responsiveness to TGF-β1 (264). Similarly, cardiac fibroblasts isolated from db/db mice exhibited elevated collagen synthesis but weakened TGF-β1 response (256).

Myocardium tissues of diabetic rats and cardiac fibroblasts treated with high glucose show a significant increased expression of the calcium sensing receptor (CaSR), a member of the C family of the G protein coupling receptor superfamily widely expressed in both prokaryotic and eukaryotic cells (265). A CaSR inhibitor may alleviate the myocardial fibrosis induced by high glucose (266).

The integrins, a family of transmembrane proteins able to integrate and transduce mechanical and biochemical signals, may have a key role in myocardial fibrosis by inducing myofibroblast differentiation (267). Collagen treated with methylglyoxal, a major cell-permeant precursor of AGEs, appears to initiate a forward-feedback loop where glycated ECM increases the expression of integrins. The stiffed myocardial matrix further activates integrins and up-regulates TGF-β, with worsened cardiac fibrosis. Indeed, the deletion of the α11 integrin in streptozotocin-treated diabetic animal models attenuates the cardiac fibrosis (64, 268).

In addition to fibroblasts, even fibrogenic actions by monocytes and macrophages, lymphocytes, endothelial cells and pericytes, mast cells, and cardiomyocytes may contribute to the diabetes-associated heart fibrosis (169).

DIABETES-INDUCED LEFT VENTRICULAR DYSFUNCTION

Even though some authors have postulated that HFpEF and HFrEF represent distinct phenotypes of DM-CMP (63), these clinical patterns are traditionally described as two stages occurring during diabetes progression. An early stage characterized by increased myocardial stiffness, enhanced atrial filling pressure and impaired diastolic function, may be followed, even though not so commonly, by a late stage of further impairment in diastolic function and appearance of a systolic dysfunction (269).

LV diastolic and systolic dysfunctions can be efficiently detected by echocardiography thanks to its large availability and low cost (243). Unfortunately, screening approaches including B-type natriuretic peptide, exercise stress testing, and more sensitive echocardiographic measurements, have not been fully validated yet to identify subclinical dysfunction in diabetic patients (270).

Diastolic Dysfunction

LV diastolic dysfunction displays from heart stiffening due to both myocardial fibrosis and hypertrophy (100) and represents the initial and most common functional deficit of diabetic heart, generally previous the appearance of systolic dysfunction (271, 272).

An impaired diastolic functioning of LV is detected in 40–75% of asymptomatic T1DM/T2DM patients by conventional echocardiography and tissue Doppler imaging, being characterized by a delayed and extended diastolic phase, with impaired early diastolic filling, prolongation of isovolumetric relaxation, increased atrial filling and increased myocardial stiffness, predominantly in late diastole (249).

Changes in diastolic function have also been widely reported in diabetic animals without evidence of heart disease by other factors (273). In a study of the 90s, diastolic dysfunction has been associated with aging, long duration of diabetes, increased blood pressure, interventricular septal thickness, dyslipidemia, and high HbA1c (274).

Systolic Dysfunction

As DM-CMP insidiously proceeds and eccentric cardiac remodeling develops, systolic dysfunction may appear, a condition associated with a poor prognosis with an annual mortality of 15–20% and a higher incidence of congestive HF and sudden death (249).

Defects in excitation-contraction coupling at the cardiomyocyte level, including impairment in cardiomyocyte contraction, relaxation, and cytosolic calcium trafficking, as well as epigenetic mechanisms and enhanced mitochondrial ROS generation, may all contribute to this progressive worsening (32, 124).

Even though systolic dysfunction usually follows diastolic dysfunction at a later stage of the DM-CMP course, some studies have detected systolic dysfunction in diabetic patients with normal diastolic function, suggesting that diastolic dysfunction may not necessarily be the first functional alteration (275). In a T2DM population with no documented cardiovascular disease and no signs of ischemia at stress test, asymptomatic LV dysfunction was detected in 262 patients. Among these, 27% had isolated systolic dysfunction and 16% isolated diastolic dysfunction (276).

EFFECTS OF ANTI-HYPERGLYCEMIC DRUG THERAPY ON HEART FAILURE IN DIABETES

Along with the classic outcome of major adverse CV events, recently published CV outcomes trials of anti-hyperglycemic drugs include analysis of HF data, especially the rate of hospitalization for this event.

The ancient drug metformin was absolutely contra-indicated in patients with HF until 2007 when FDA removed this limitation. The controversy regarding its safety and effectiveness in the setting of HF was resolved by the results of a later systematic review of observational studies including 34,000 patients favoring the metformin as the treatment of choice in patients with diabetes and HF (277).

In addition to raised concerns about increased MI, the use of the thiazolidinediones (TZDs) rosiglitazone and pioglitazone, was associated with fluid retention and increased risk of HF, as indicated by three randomized controlled trials, DREAM, ProACTIVE, and GSK211, reporting a respective relative risk of HF of 2.17 (95% CI 0.96–0.91), 1.49 (1.23–0.80), and 7.09 (1.60–0.96) (278). The main mechanisms accounting for TZD-related fluid retention is the PPAR-γ stimulation of EnaC-mediated renal salt absorption in the collecting duct, with the likely contribution of stimulation of sodium transporters in the proximal tubule. Concurrently, the reduction of systemic vascular resistance by TZD might expose the capillary networks to higher perfusion pressures thereby precipitating fluid extravasation. Additionally, TZDs increase the plasma concentration of the VEGF, a potent inducer of vascular permeability, further predisposing patients to oedema (279).

Three new classes of anti-hyperglycemic agents have been introduced in recent years.

The dipeptidyl peptidase-4 (DPP-4) inhibitors exhibited increased HF hospitalization in the SAVOR-TIMI 53 trial evaluating saxagliptin and in the secondary analysis of the EXAMINE trial for alogliptin. A recent pooled analysis illustrates that DPP-4 inhibitors do not increase the HF risk among T2DM patients with a previous history of HF, but they increased this risk among patients without history of HF (HR 1.21, 95% CI 1.04–1.41, $p = 0.01$), possibly because nearly all studied subjects had established CVD (280). Basic research suggests that the inhibition of DPP-4 may exert beneficial actions on heart, mainly by inhibiting the degradation of stromal cell-derive factor-1, a chemokine produced by stromal and endothelial cells that promotes regeneration and repair during organ damage, and that of GLP-1, thus restoring cardiac remodeling and apoptosis caused by the pathological decline in circulating GLP-1 in response to pressure overload (281, 282). On the other hand, since DPP-4 involves in the degradation of vasodilator factors and the NO-dependent mechanism, its inhibition can exert important systemic vasodilator effects that reduce heart load (283). Unfortunately, these beneficial results on animal studies were not replicated in humans.

The antagonists of the GLP-1 receptors (GLP-1RAs) represent the other incretin-based therapy potentiating endogenous GLP-1. Based on the evidence from RCTs, none of the six available GLP-1RAs has displayed benefits against HF, despite demonstration in animal models and humans of ameliorated endothelial dysfunction, improved myocardial function, and cardiomyocyte protection against glucolipotoxicity and ROS (280). A novel GLP-1RA, the oral hypoglycemic peptide 2 (OHP2), has demonstrated to protect against DM-CMP in high-fat diets and continuous streptozocin injection induced rat models. Both hyperlipidemia and myocardium lipid accumulation were decreased by OHP2 treatment. In addition, OHP2 reversed oxidative stress and mitochondrial dysfunction in diabetic hearts (284).

The inhibitors of the sodium glucose co-transporter 2 (SGLT2) are the first class of glucose-lowering agents that have demonstrated in large-scale studies an impressive reduction in

the risk of serious new-onset HF events by ≈30% in T2DM patients with or without established CVD (285). Of note, in none of the trials this benefit is explained by the glycemic control. Several mechanisms have been postulated for such a striking cardioprotective effect. The primary action of SGLT2 inhibitors reducing sodium and glucose uptake in the nephron, leads to a decrease in preload and afterload through osmotic diuresis. Additional beneficial effects are improvement of the composition of proinflammatory and anti-inflammatory cytokines in the body, as well as reduction in cardiac fibrosis (286). Other potential cardioprotective mechanism includes the increase in hematocrit, determined by erythropoietin hyperproduction by renal fibroblasts when the stimuli of hyperglycemia and excess glucose reabsorption are removed, the increase in fasting levels of ketone bodies with enhanced utilization of this efficient metabolic fuel in the failing heart, and the inhibition of the sodium hydrogen exchanger-1 in the myocardium, whose overactivity may lead to increase in intracellular sodium and calcium (287).

DIFFERENT ASPECTS OF CARDIOMYOPATHY IN T1DM AND T2DM

Various small and large animal models of T1DM and T2DM have been generated to investigate the impact of diabetes on the heart and a lot of clinical studies have been published in the last decades on DM-CMP. Nonetheless, the complex pathophysiology of this condition remains still less than fully clear. The topic is further complicated by the different etiology of T1DM and T2DM that make partially distinct the mechanisms involved in their cardiac dysfunction (288).

Although etiologically different, the two types of diabetes share common metabolic disturbances, including hyperglycemia, dyslipidemia and associated glucotoxicity, lipotoxicity, and oxidative stress that are the predominant pathological mechanisms driving the development of DM-CMP as determined by insulin deficiency in T1DM and insulin resistance in T2DM.

On the other side, the development of HF in T1DM appears more closely related to glycemic control than in T2DM as indicated by the reported different increase in HF risk, 30% in T1DM and 8% in T2DM patients, for each additional percentage point of HbA1c (270). Likely, a good metabolic control obtained by insulin therapy in patients with T1DM may normalizes the metabolic derangements induced by insulin deficiency and attenuate the detrimental effects of diabetes on the heart (288). Instead, the insulin resistance typical of T2DM leads to increase in circulating triacylglycerol levels and FA delivery to cardiomyocytes that result in impaired mitochondrial β-oxidation, with greater mitochondrial dysfunction and accumulation of toxic lipid metabolites in the heart of patients with T2DM than in patients with T1DM (30).

Differences in pathophysiology of heart damage between the two types of diabetes also result in different clinical pictures. In T2DM-associated DM-CMP, there is a prevalence of mechanisms mediating concentric LV remodeling and hypertrophy with increase in ventricular stiffness leading to diastolic dysfunction. The corresponding clinical features include reduced ventricular compliance with increased systemic and pulmonary venous pressures and congestion despite preserved systolic function. By contrast, T1DM-associated diabetic cardiomyopathy is characterized by cardiomyocyte loss, LV remodeling and increased myocardial collagen deposition, which increase LV end-diastolic volume and impair systolic function. As a consequence, symptoms of systolic dysfunction are more typical in patients with T1DM with earlier clinical manifestations of HFrEF (289).

A similar progression of DM-CMP has also emerged in preclinical studies in diabetic animal models. A study comparing cardiac performance in rat models of T1DM (streptozotocin induced) and T2DM (Zucker diabetic fatty rats) by a pressure-volume conductance catheter system, suggested that a decreased systolic performance and a delayed relaxation mainly characterize T1DM, whereas an increase in diastolic stiffness of the heart is more remarkably in T2DM (290). A recent study using speckle-tracking echocardiography with invasive hemodynamics for the detection of cardiac dysfunction in rat models of T1DM and T2DM confirmed these results (291). It was found that contractility and active relaxation were deteriorated to a greater extent in T1DM compared to T2DM. In contrast, diastolic stiffness was more pronounced in T2DM. Correspondingly, systolic function was markedly altered in T1DM but preserved in T2DM, a disease profile resembling that observed in T2DM patients with HFpEF.

CONCLUSION

Diabetic cardiomyopathy is a common complication of diabetes which deserves a special clinical attention due to its insidious subclinical progression that, in some cases, may culminate in a manifest and rapidly evolving HF burdened by a very poor outcome.

The main driving force of the pathological processes specific of DM-CMP is hyperglycemia, a factor centrally placed among multiple interwoven pathways involving complex cellular and molecular perturbations which affect both myocardial structure and function.

Despite the current large knowledge, the pathophysiology of DM-CMP development and progression is still far from being fully elucidated. Consequently, effective therapies targeting this diabetic complication are lacking.

In-depth knowledge of etiologic and pathogenic mechanisms is crucial for the development of target-specific treatments to reduce the risk of HF in diabetic patients. Since subclinical cardiac abnormalities could be reversible when early detected, prevention-oriented therapies can even hopefully be identified.

AUTHOR CONTRIBUTIONS

TS and FS: conceptualization. TS, PP, RG, GA, AD, AC, EV, LR, and FS: investigation. TS: writing—original draft preparation. TS, FS, and PP: writing—review and editing. TS, FS, and RG: supervision. All authors have read and agreed to the published version of the manuscript.

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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.

frontiers in Medicine

ORIGINAL RESEARCH
published: 16 September 2021
doi: 10.3389/fmed.2021.719950

Qing-Re-Xiao-Zheng Formula Modulates Gut Microbiota and Inhibits Inflammation in Mice With Diabetic Kidney Disease

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *03 June 2021*
Accepted: *17 August 2021*
Published: *16 September 2021*

Citation:
Gao Y, Yang R, Guo L, Wang Y, Liu WJ, Ai S, Woon TH, Wang Z, Zhai Y, Wang Z and Peng L (2021) Qing-Re-Xiao-Zheng Formula Modulates Gut Microbiota and Inhibits Inflammation in Mice With Diabetic Kidney Disease. Front. Med. 8:719950. doi: 10.3389/fmed.2021.719950

Evidence indicates that the metabolic inflammation induced by gut microbiota dysbiosis contributes to diabetic kidney disease. Prebiotic supplementations to prevent gut microbiota dysbiosis, inhibit inflammatory responses, and protect the renal function in DKD. Qing-Re-Xiao-Zheng formula (QRXZF) is a Traditional Chinese Medicine (TCM) formula that has been used for DKD treatment in China. Recently, there are growing studies show that regulation of gut microbiota is a potential therapeutic strategy for DKD as it is able to reduce metabolic inflammation associated with DKD. However, it is unknown whether QRXZF is effective for DKD by regulating of gut microbiota. In this study, we investigated the reno-protective effect of QRXZF by exploring its potential mechanism between gut microbiota and downstream inflammatory pathways mediated by gut-derived lipopolysaccharide (LPS) in the kidney. High-fat diet (HFD) and streptozotocin injection-induced DKD mice model was established to assess the QRXZF effect *in vivo*. Mice treated with QRXZF for 8 weeks had significantly lower levels of urinary albumin, serum cholesterol and triglycerides. The renal injuries observed through histological analysis were attenuated as well. Also, mice in the QRXZF group had higher levels of Zonula occludens protein-1 (ZO-1) expression, lower levels of serum fluorescein-isothiocyanate (FITC)-dextran and less-damaged colonic mucosa as compared to the DKD group, implying the benefit role for the gut barrier integrity. QRXZF treatment also reversed gut dysbiosis and reduced levels of gut-derived LPS. Notably, the expression of toll-like receptor 4 (TLR4) and nuclear factor-κB (NF-κB), which are important inflammation pathways in DKD, were suppressed in the QRXZF groups. In conclusion, our results indicated that the reno-protective effects of QRXZF was probably associated with modulating gut microbiota and inhibiting inflammatory responses in the kidney.

Keywords: Qing-Re-Xiao-Zheng formula, gut microbiota, inflammation, diabetic kidney disease, TLR4/NF-κB pathway

INTRODUCTION

According to the International Diabetes Federation (IDF) Diabetes Atlas 2019, nearly 10% of adults suffer from diabetes mellitus. This translates to 463 million people worldwide, and the number is expected to increase to 700.2 million (1). Diabetic kidney disease (DKD) is a common microvascular complication of diabetes, and remains as the main cause of end-stage renal disease (ESRD). However, there is no cure available and it has caused a large financial burden (2, 3).

Evidence suggests that gut microbiota has been implicated in the pathogenesis of several risk factors of DKD involving obesity, insulin resistance and diabetes (4–7). The gut microbiota helps to supply nutrients and vitamins, fights off invasive pathogens and protects intestinal barrier function (8–10). Accumulating studies have proposed gut-kidney axis plays great role in DKD by several gut-derived factors (11). Studies show that regulation of gut microbiota is a potential therapeutic strategy for DKD as it is able to reduce metabolic inflammation associated with DKD (12, 13). In particular, gut-derived endotoxins such as LPS, an inflammatory marker involved in the pathogenesis of DKD (14), Gut dysbiosis suppresses the expression of tight junction proteins, leading to increased intestinal permeability and the translocation of Gram-negative bacteria-derived LPS into the blood (15), that might be involved in metabolic inflammation and DKD progression (16, 17).

Prebiotic supplementations are non-digestible food ingredients, which play renal protective effect mainly by enhancing the growth of specific beneficial bacteria in the gut. Prebiotics not only alter the intestinal microbiota but also improve intestinal tight junction integrity and decrease blood endotoxemia caused by LPS. Traditional Chinese Medicine (TCM) is an alternative treatment for patients with DKD in China (18, 19). Qing-Re-Xiao-Zheng formula (QRXZF) which was formulated based on the "ZhengJia" theory in TCM has been commonly used for DKD treatment (20). It comprises of *Astragali radix IV* (Huang Qi), *Radix angelicae sinensis* (Dang Gui), *Concha Ostreae* (Mu Li), *Rheum officinale Baill* (Da Huang), and four other herbs. Since gut microbiota and inflammatory responses might lead to the progression of DKD (13, 14), our study aims to investigate the anti-inflammatory and reno-protective effects of QRXZF in DKD mice by observing alterations in gut microbiota and levels of gut-derived LPS and identifying the relationship between gut microbiota and DKD.

MATERIALS AND METHODS

Herbal Formation and Component

QRXZF consists of *Astragali radix IV* (Huang Qi), *Radix angelicae sinensis* (Dang Gui), *Concha Ostreae* (Mu Li), *Rheum officinale Baill* (Da Huang), and four other herbs. Herbs were weighed and boiled at 10°C for 1 h and the final concentration was extracted into 2 g/ml. Herbs were purchased from Beijing Tong Ren Tang, which has high quality control standards validated according to the Chinese Pharmacopeia (China Pharmacopoeia Committee, 2015).

Animals and Ethics Statement

Seven-week-old male C57BL/6J mice were purchased from Jiangsu-Jicui Yaokang Lab Animal Ltd. Mice in the control group were fed with common feed while mice in the high fat diet (HFD) group were fed with high fat food (60 kcal% fat, D12492, Research Diets, New Brunswick, NJ, United States). Mice were kept 3 per cage in specific pathogen-free (SPF) conditions, under controlled environmental conditions (a 12-12 h light-dark cycle, 22 ± 2°C room temperature, and 60-65% relative humidity while free access to water as well as food). All experimental procedures were approved by the Ethics Committee of Beijing University of TCM and performed following the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

Experimental Design

After fasting for 16 h, mice in the HFD group fed for 7 weeks were treated daily with streptozotocin (STZ) (40 mg/kg/d, i.p; Sigma, USA) freshly dissolved in citrate buffer (0.1 mol/L, pH 4.3) for 5 days consecutively, while the control group were treated with citrate buffer.

Seven days after the last injection, blood glucose levels were tested by obtaining blood from the tail vein after an overnight fast. Mice with glucose levels over 16.7 mmol/L were randomly assigned to either the DKD ($n = 6$) or the QRXZF ($n = 6$) group. Mice in the DKD group were gavaged with saline water 0.25 ml/d, while mice in the QRXZF group were treated with QRXZF at a dose of 15.6 g/kg/d. Treatment was done via intragastric gavage daily for 8 weeks.

Fresh fecal samples were frozen in liquid nitrogen and stored at −80°C before further processing. Urine samples were collected with metabolic cages and stored at −20°C. 4 h before sacrifice, fluorescein-isothiocyanate (FITC)-dextran (44 mg/100 g, 4 kDa; Sigma), a high molecular weight glucose polymer, which cannot be digested, were fed to the mice to assess changes in intestinal permeability. Blood samples were collected without anticoagulants and centrifuged at 3,000 rpm for 15 min. Organs including kidney and colon issues were stored at −80°C before further analysis.

Serum and Urine Biochemical Assays

Blood glucose levels were tested by One Touch Ultra 2 glucometer (Johnson, USA). Serum cholesterol, triglyceride, creatinine, and urine creatinine were measured using ELISA kits purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). Urine albumin and serum LPS were detected using commercial assay kits (Bethyl Laboratories, USA) and (LONZA, USA), respectively. Serum was diluted in phosphate-buffered saline (PBS) (1:1) and analyzed for FITC-dextran concentration by a fluorescence spectrophotometer (485 nm excitation, 535 nm emission).

Histological Examination

Kidney and colon tissues were fixed in 10% formalin, embedded in paraffin while cut into 2 μm-thick sections for staining. Kidney tissues were investigated after hematoxylin-eosin (HE)

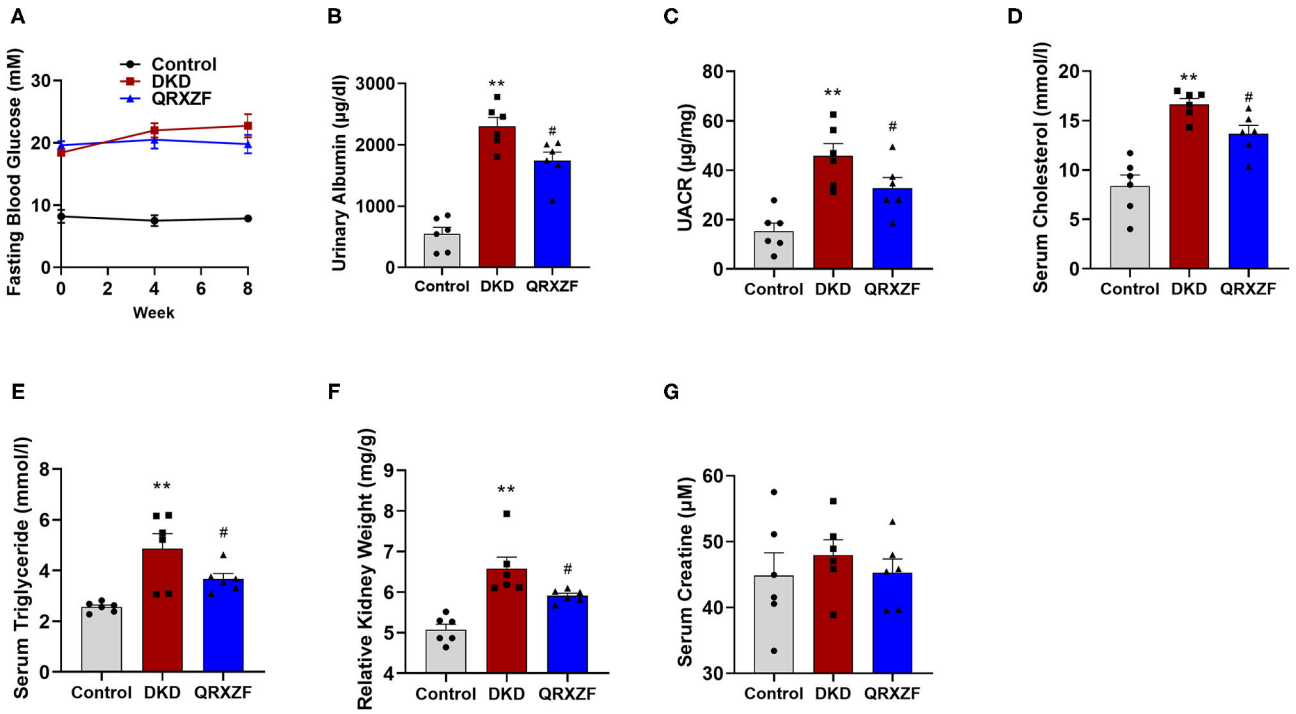

FIGURE 1 | QRXZF decreased urinary albumin and ameliorated disorder of lipid metabolism in DKD mice after 8 weeks ($n = 6$, means ± SEM). **(A)** Serum glucose; **(B)** Urinary albumin content; **(C)** Urine albumin to creatinine ratio (UACR); **(D)** Serum cholesterol; **(E)** serum triglyceride; **(F)** Kidney weight/body weight; **(G)** Serum creatinine. $^{*}P < 0.05$, $^{**}P < 0.01$ DKD group vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ QRXZF group vs. the DKD group.

staining, Masson trichome staining as well as periodic acid-Schiff (PAS) staining. Colon tissues were investigated after HE staining. Histological analysis was conducted on 50 full-sized glomeruli obtained from each specimen after PAS-staining. The level of glomerulosclerosis was scored as follows: 0, no sclerosis; 1, sclerosis observed in <10% of glomeruli; 2, sclerosis observed in 10-25% of glomeruli; 3, sclerosis observed in 25–50% of glomeruli; 4, sclerosis observed in >50% of glomeruli.

Western Blotting Analysis

Kidney and colon tissues from each mouse were homogenized in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors. The amount of protein in the samples were quantified using the Bradford assay and equal quantities of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After protein transfer, membranes were blocked in 5% BSA for 1 h and incubated at 4°C overnight with specific primary antibodies, and then incubated with horseradish peroxidase (HRP) linked secondary antibody. Antibodies specific to Zonula occludens protein-1 (ZO-1) (ab96587), Toll-like receptor 4 (TLR4) (ab13867), nuclear factor-κB (NF-κB) p65 (ab16502) and phospho-NF-κBp65 (Ser536, ab86299) were purchased from Abcam.

Microbiota Analysis

Total genome DNA was collected from fecal samples using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). Assessment of DNA quality was conducted with 1% agarose gel electrophoresis. 16S rRNA gene sequencing was performed on gut microbiota composition in the mice. The V3-V4 hypervariable regions of the 16S rRNA gene were amplified by universal primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) as well as 806R (5′-GGACTACHVGGG TWTCTAAT-3′) incorporating sample barcode sequences. After the quality assessment, the library was sequenced on the MiSeq platform (Illumina) to generate 300-bp paired-end reads. In order to obtain effective reads, the Trimmomatic software was used to filter the poor-quality reads. Chimera sequences were removed by using the UCHIME algorithm. The sequencing data were submitted to the National Center of Biotechnology Information (NCBI) Sequence Read Archive Database with the accession no.PRJNA729207.

Operational taxonomic units (OTUs) were identified as 1 cluster with the similarity cutoff of 97%. We used the Mothur software to plot the rarefaction curve. Chao1 index and observed_species indices were performed to quantify and compare the alpha diversity. The principal component analysis (PCA) analysis and the Non-metric multidimensional scaling (NMDS) were performed using QIIME (http:qiime.org/) to compare beta diversity. Bacterial taxa of the groups were analyzed according to their relative abundance (false discovery rate < 0.05). Inner to outer rings were organized following the order of phylum, class, order, family, and genus.

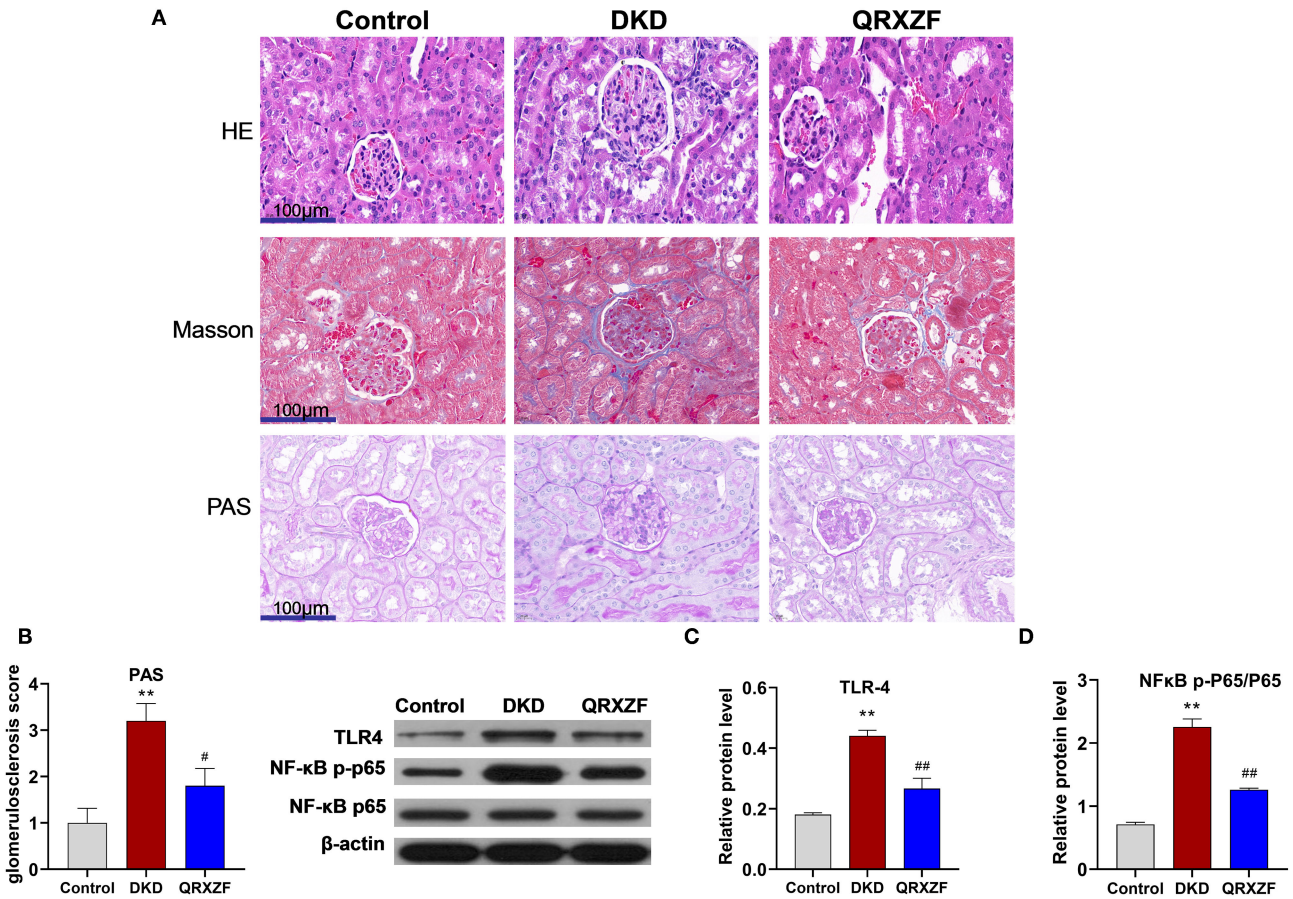

FIGURE 2 | QRXZF attenuated renal injury and inhibited inflammatory response in the kidney. **(A)** HE, Masson, and PAS stained at 400×magnification, respectively; **(B)** PAS score; **(C,D)** western blot, expression of TLR4 and the ratio of NF-κB p-p65 to NF-κB p65 ($n = 3$, means ± SEM). $^{*}P < 0.05$, $^{**}P < 0.01$ DKD group vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ QRXZF group vs. the DKD group.

Statistical Analysis

Statistical analysis was performed using SPSS 22.0. All experimental data are presented as means ± SEM. Comparisons within multiple groups were measured by ANOVA. $p < 0.05$ indicated statistical significance.

RESULTS

QRXZF Decreased Urinary Albumin and Regulated Lipid Metabolism in DKD Mice

Diabetes was induced in HFD-fed mice after the STZ injection. These mice developed hyperglycemia at the first week (termed week 0) and high levels of blood glucose were maintained throughout the experiment. However, there were no significant differences in serum glucose between the QRXZF group and the DKD group at the end of both week 4 and week 8 (**Figure 1A**). Mice in the DKD group had remarkably increased urinary albumin content and urine albumin to creatinine ratio (UACR), which were reversed after treatment with QRXZF (**Figures 1B,C**). Similarly, mice in the DKD group had significantly higher levels of serum cholesterol and triglyceride, which were significantly reduced after treatment with QRXZF (**Figures 1D,E**). In addition, kidney weight/ body weight ratio of mice which received QRXZF were significantly lower than mice in the DKD group (**Figure 1F**). However, there were no significant differences of serum creatinine among the three groups, which may be because we only established an early stage DKD animal model (**Figure 1G**).

QRXZF Attenuated Renal Injury and Inhibited Inflammatory Responses in the Kidney

Histological features of kidneys from mice in the DKD group include glomerular hypertrophy, glomerular basement membrane (GBM) thickening, mesangial matrix expansion and vacuolar degeneration of tubular epithelial cells. After treatment with QRXZF, glomerular hypertrophy, mesangial matrix expansion and tubulointerstitial injury were partially ameliorated (**Figure 2A**). Sections stained using the Masson's trichrome stain showed that renal fibrosis was improved after QRXZF treatment (**Figure 2A**). In addition, and the PAS score

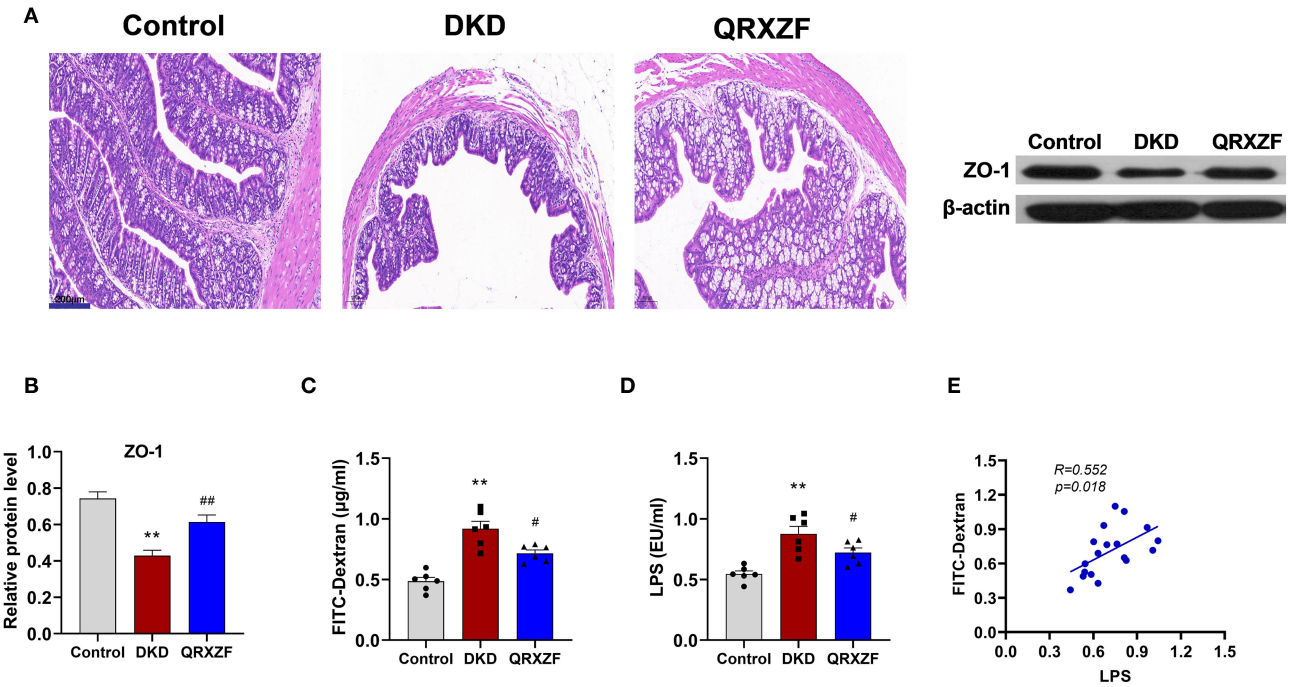

FIGURE 3 | QRXZF improved gut barrier. **(A)** HE stained at 100×magnification; **(B)** Expression of ZO-1 evaluated by western blot analysis ($n = 3$, means ± SEM); **(C)** serum FITC-dextran ($n = 6$, means ± SEM.); **(D)** serum LPS; **(E)** Correlation analyses between serum LPS and FITC-dextran. $^{*}P < 0.05$, $^{**}P < 0.01$ DKD group vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ QRXZF group vs. the DKD group.

to examine extracellular matrix (ECM) accumulation was also decreased after QRXZF treatment (**Figures 2A,B**).

The TLR4/NF-κB signaling pathway is crucial in the regulation of inflammation, while dysregulation might lead to higher levels of inflammation and subsequent DKD. As shown in the Western blot analysis, both the levels of TLR4 expression and the ratio of NF-κB p-p65 to NF-κB p65 were higher in the DKD group as compared to the control group (**Figures 2C,D**). In contrast, QRXZF inhibited the expression of TLR4 and reduced of the ratio of NF-κB p-p65 to NF-κBp65 in the kidney. These results show that QRXZF could suppressed the TLR4/NF-κB inflammation signaling pathway in the kidney.

QRXZF Enhanced Intestinal Barrier Integrity

HE staining of colon tissue obtained from the DKD group showed greater damage to the intestinal mucosa as compared to the control group, which was ameliorated after QRXZF treatment (**Figure 3A**). Western blotting results showed that expression of ZO-1 protein in the colon was significantly upregulated after administration of QRXZF as compared with the DKD group (**Figure 3B**). Compared to the DKD group, levels of serum FITC-dextran, a marker of intestinal permeability was significantly lower in the QRXZF group (**Figure 3C**). Levels of serum LPS, an important indicator of inflammation, also decreased significantly after treatment with QRXZF (**Figure 3D**) and showed a positive correlation with levels of FITC-dextran (**Figure 3E**). These results show that QRXZF is effective in maintaining intestinal barrier integrity, which could be the reason for the reduced levels of circulating LPS.

QRXZF Modulated the Gut Microbiota in DKD Mice

When analyzing the composition of gut microbiota, sequences were divided into operational taxonomic units (OTUs) with a similarity cutoff of 97%. Rank abundance curves and rarefaction curves of each of the 18 samples being investigated plateaued with the depth of sequencing, indicating that the entire microbial community was captured (**Figures 4A,B**). The Chao 1 index and the observed species index selected to assess alpha diversity were significantly lower in the DKD group as compared to the control group. After treatment with QRXZF, both indices were further reduced in QRXZF group, though no significant differences were observed for the Chao 1 index ($P = 0.055$), suggesting that QRXZF treatment did not enrich the microbiota diversity (**Figures 4C,D**). Non-metric multidimensional scaling (NMDS) as well as principal component analysis (PCA) were conducted to assess beta diversity. Results indicated that the main components of the three groups could be well-distinguished and differences were identified in each group (**Figures 4E,F**).

To investigate the regulatory effect of QRXZF, a LEfSe analysis and cladogram were performed to reveal the dominant genera of the gut microbiota (**Figures 5A,B**). As shown in **Figure 5C**, the composition of gut microbiota of each group at the

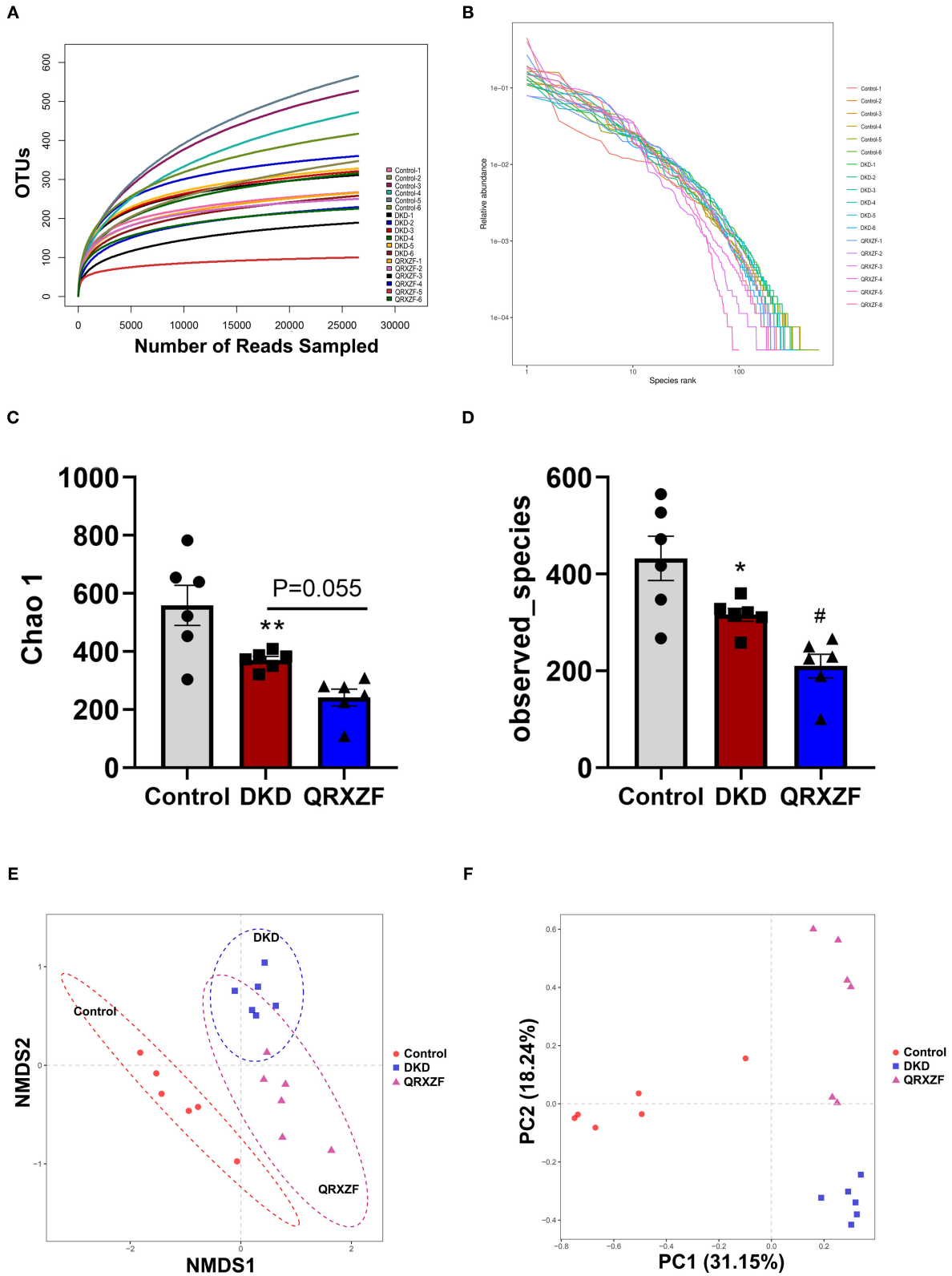

FIGURE 4 | (A) Rarefaction curve; **(B)** Rank abundance curve; **(C,D)** Chao1 index and the observed species index ($n = 6$, means ± SEM); **(E)** Non-metric multidimensional scaling (NMDS) analysis between three groups; **(F)** Principal component analysis (PCA). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ DKD group vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ QRXZF group vs. the DKD group.

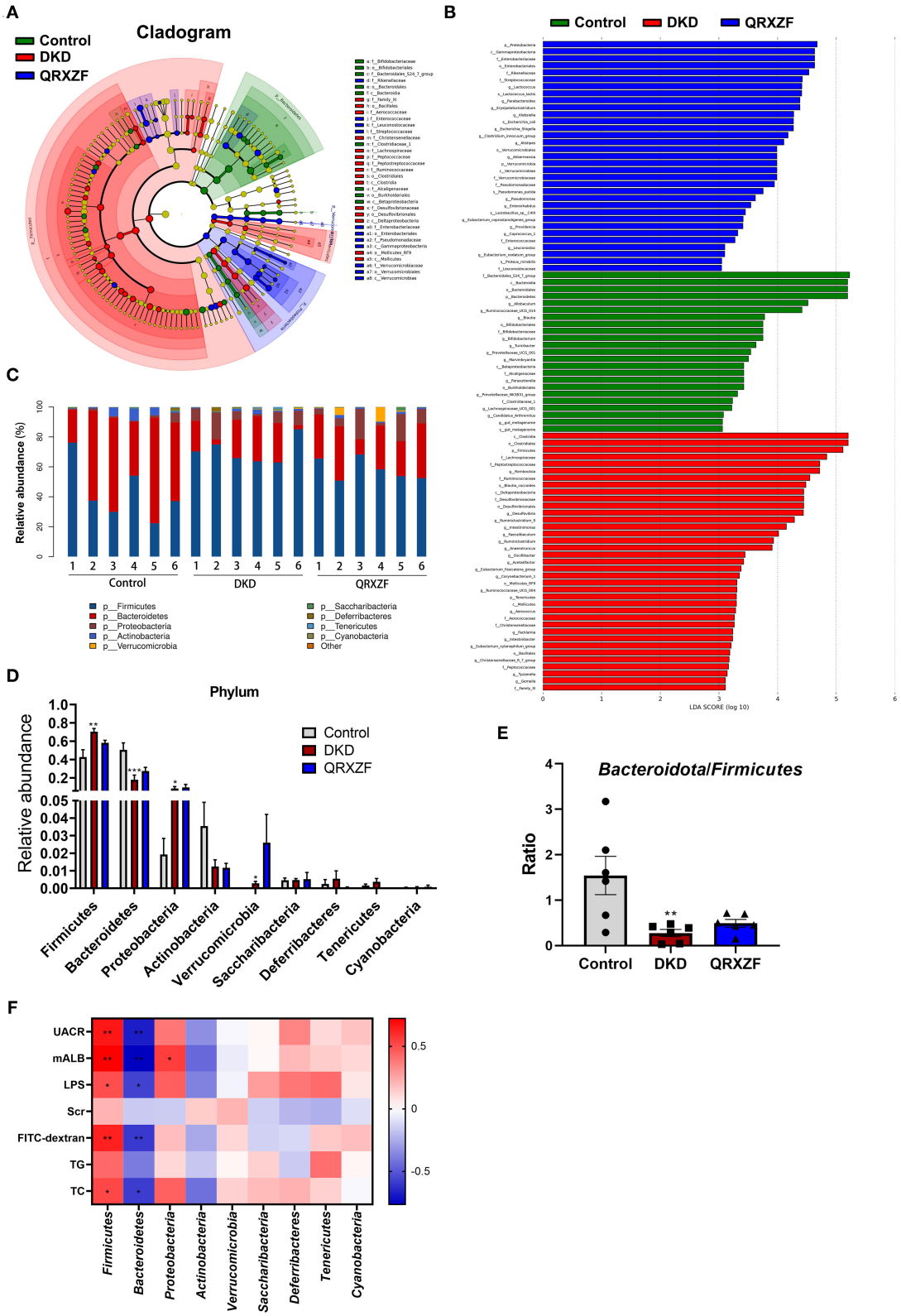

FIGURE 5 | Key biomarkers of gut microbiota between each group. **(A)** Taxonomy analysis; **(B)** LEfSe analysis; **(C)** Relative abundance at phylum level; **(D)** Relative abundance at the phylum level between three groups; **(E)** *Bacteroidetes*-to-*Firmicutes* ratio ($n = 6$, means ± SEM); **(F)** Correlation analyses between metabolic

(Continued)

FIGURE 5 | parameters and relative abundance of gut microbiota at phylum level. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ DKD group vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ QRXZF group vs. the DKD group; Colors ranged from blue to red, where blue indicates negative correlation and red indicates positive correlation. Significant correlations were marked by $^{*}P < 0.05$, $^{**}P < 0.01$.

phylum level including *Bacteroidetes, Actinobacteria, Firmicutes, Proteobacteria, Saccharibacteria, Deferribacteres, Tenericutes,* and *Cyanobacteria. Firmicutes* and *Bacteroidetes* were the most dominant, with a total of >90%. As compared to the control group, the DKD group had a significantly lower abundance of *Bacteroidete* but higher abundance of *Firmicutes, Proteobacteria,* and *Verrucomicrobia* (**Figure 5D**). Ratio of *Bacteroidetes-to-Firmicutes* in the DKD group was also significantly decreased (**Figure 5E**).

We conducted Spearman's correlation analysis to establish correlation between parameters tested and relative abundances of gut microbiota at the phylum level. As shown in **Figure 5F**, results suggest that *Firmicutes* exhibited a positive correlation with UACR, microalbumin (mALB), TC, LPS and FITC-dextran, while *Bacteroidetes* had a negative correlation. *Proteobacteria* showed a positive correlation with mALB.

The abundance of *Desulfovibrionaceae* and *Desulfovibrio* were higher in the DKD group, which were closely related to increased leakage of LPS. Meanwhile, the DKD group had lower abundance of *Parasutterella*, a bacterium capable of producing short-chain fatty acids (SCFAs). SCFAs have anti-inflammatory and protective effects on the intestinal barrier. Mice treated with QRXZF had a higher abundance of *Rikenellaceae*, which might have enhanced the levels of SCFAs in the intestine.

Collectively, based on the spectrum of gut microbiota in the three groups, we speculated that injury to intestinal and renal tissues in the DKD group was related to the increased LPS-releasing bacteria but decreased the levels of bacteria with gut protective effects. QRXZF could protect the intestinal barrier, reduce LPS and kidney injury which may be related to changes of gut microbiota.

The results showed that *Peptostreptococcaceae*, *Rikenellaceae, Desulfovibrionaceae, Desulfovibrio*, *Corynebacterium_1, Anaerotruncus*, *Gemella*, *Tyzzerella, Oscillibacter*, *Ruminiclostridium, Parasutterella*, *Alistipes* and *Akkermansia* at the family and genus levels were selected to be meaningful gut microbiota (**Figure 6A**). Spearman's correlation analysis suggested that *Peptostreptococcaceae, Gemella* and *Corynebacterium_1* exhibited a positive correlation with levels of UACR, mALB, LPS, FITC-dextran, TG and TC. *Desulfovibrionaceae, Desulfovibrio* and *Oscillibacter* exhibited a positive correlation with levels of UACR, mALB, LPS, FITC-dextran and TC. *Anaerotruncus* exhibited a positive correlation with levels of mALB, LPS, FITC-dextran and TC. *Ruminiclostridium* exhibited a positive correlation with levels of mALB, LPS and TG. *Tyzzerella* exhibited a positive correlation with levels of UACR, mALB, LPS and TC. However, *Parasutterella* have negative correlations with levels of UACR and mALB, respectively (**Figure 6B**).

DISCUSSION

DKD is the main cause of ESRD. As DKD progresses, it results in glomerular hyperfiltration, increasing albuminuria, and declining estimated glomerular filtration rate (eGFR), which ultimately leads to ESRD (21). In this study, we successfully induced an appropriate mouse model with early-stage DKD through HFD feeding followed by low-dose STZ injection. The DKD mice exhibited a rise in blood glucose levels, weight/body weight ratio, UACR and more severe pathological damage of kidney tissue. Excepting of blood glucose, these indices were improved after treatment with QRXZF. In addition, QRXZF was also effective in improving lipid metabolism.

Evidence show that dysbiosis of gut microbiota could lead to metabolic diseases and kidney disease (22, 23). Our results showed that there were significant differences in alpha and beta diversity for three groups. These results indicate that QRXZF had significant effects on the diversity of gut microbiota. *Firmicutes* and *Bacteroidetes* were the dominant gut microbiota at phyla level (24). In our study, the DKD group had a significantly higher relative abundance of *Firmicutes*, but lower abundance of *Bacteroidetes* as well as the ratio of *Bacteroidetes* to *Firmicutes*. These changes were closely related to HFD (25), as well as obesity and lipid deposition (26). Composition of gut microbiota was altered after treatment with QRXZF, which could have resulted in improved regulation of lipid metabolism.

At the family and genus level, differences in gut microbiota exist amongst three groups. The abundance of *Desulfovibrionaceae, Desulfovibrio*, *Peptostreptococcaceae*, *Corynebacterium_1* was higher in the DKD group. Desulfovbrionaceae and *Desulfovibrio* are LPS-producing bacteria (27, 28), where LPS produced by Desulfovbrionaceae have potent inflammation-inducing capacities, usually 100- to 1,000-fold higher than LPS from *Bacteroides spp* (29), which are involved in gut permeability and chronic inflammation (30). As a potential human pathogen, *Corynebacterium_1* could enhance an individual's susceptibility of LPS (31) and increase of the levels of inflammation (32). *Peptostreptococcaceae*, a bacterium that promotes inflammation, was more abundant in the DKD group (33).

Additionally, *Anaerotruncus, Gemella*, *Tyzzerella, Oscillibacter,* and *Ruminiclostridium* were more abundant in the DKD group as compared to the control group, while *Parasutterella* and *Alistipes* were less abundant. *Alistipes* and *Parasutterella* (23, 34) synthesize SCFAs. while *Anaerotruncus, Tyzzerella* and *Gemella* were negatively correlated with levels of plasma SCFAs (35). SCFAs are an essential source of energy and contribute to gut barrier integrity (36, 37), down-regulation of inflammatory factors and inhibition of kidney inflammation (38, 39). *Oscillibacter* and *Ruminiclostridium* were negatively correlated with the expression of ZO-1 protein, a protein

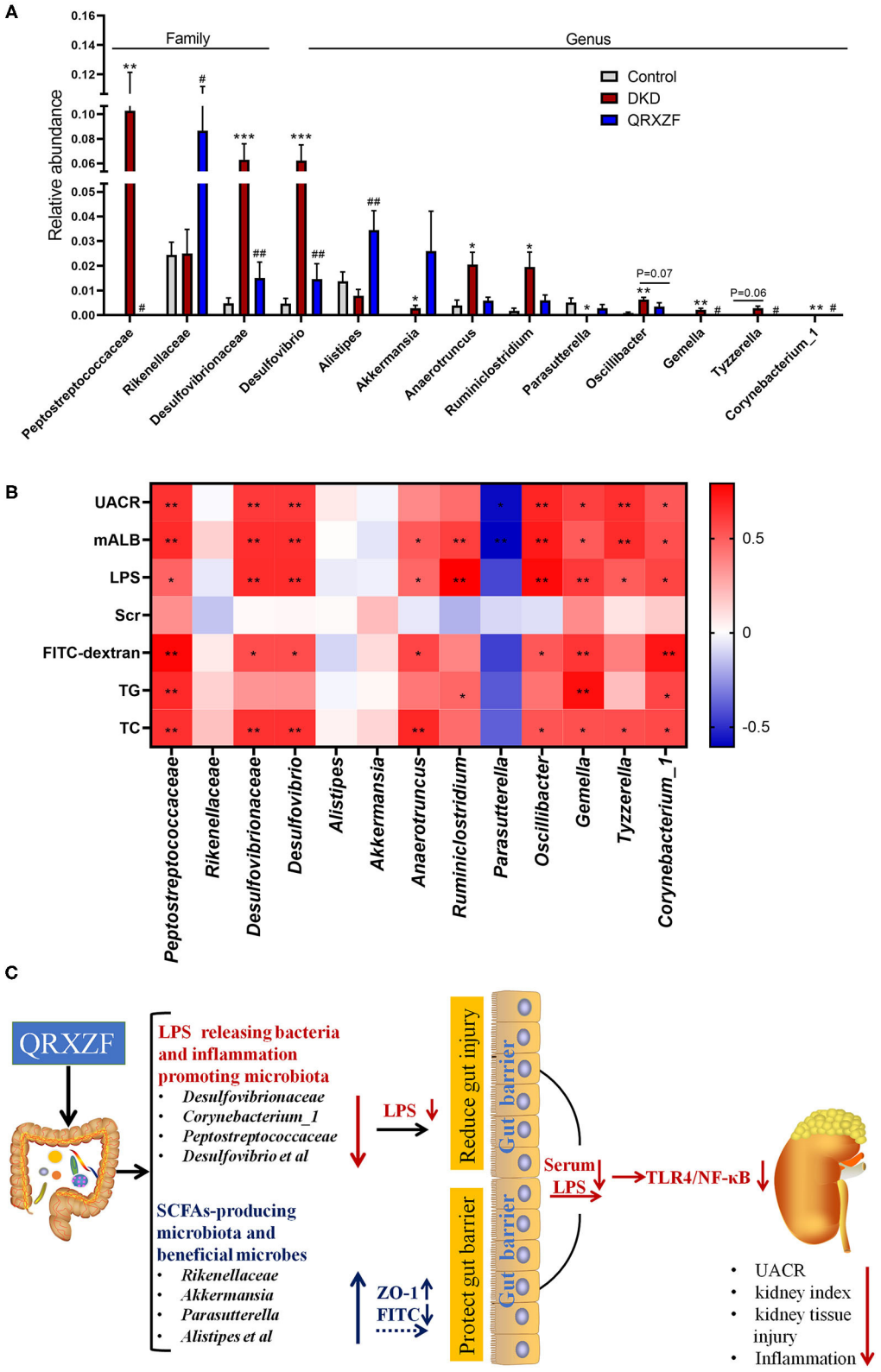

FIGURE 6 | (A) Relative abundance at the family and genus level ($n = 6$, means ± SEM); **(B)** Correlation analyses between metabolic parameters and relative abundance of gut microbiota at family and genus level; **(C)** The mechanisms schematic of action of QRXZF in High-fat diet (HFD) and streptozotocin injection-induced

(Continued)

FIGURE 6 | DKD mice model. QRXZF treatment could prevent the gut dysbiosis, reduce the intestinal permeability and gut-derived LPS into blood. The reno-protective effect of QRXZF might be associated with inhibition of the LPS/TLR4/NF-κB inflammation signaling pathway in the kidney. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ DKD group vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ QRXZF group vs. the DKD group; Colors ranged from blue to red, where blue indicates negative correlation and red indicates positive correlation. Significant correlations were marked by $^{*}P < 0.05$, $^{**}P < 0.01$.

important for the maintenance of gut barrier (40–42). However, the reason for this is unclear. We postulate that *Oscillibacter* and *Ruminiclostridium* could possibly regulate mechanisms associated with gut barrier integrity or it could alter the composition of gut microbiota, leading to changes in the levels of ZO-1 protein. After treatment with QRXZF, almost all of the above results were reversed.

After administration of QRXZF, the abundance of *Rikenellaceae* and *Akkermansia* were enriched. *Rikenellaceae* are also positively correlated with the production of butyric and valeric acids, which are important component of SCFAs (43). *Akkermansia* are beneficial microbes (44), which could reduce levels of serum LPS, relieve intestinal mucosal damage and contribute to better metabolism (45, 46). Taken together, our findings suggest that QRXZF could decrease LPS-producing microbiota and increase SCFAs-producing microbiota as well as gut barrier protective microbiota, which suggests that QRXZF has positive effects on gut dysbiosis and the gut barrier function.

DKD is a chronic inflammatory disease accompanied by lipid disorders (47, 48). Evidence shows that gut-derived LPS may be crucial in chronic inflammation and progression of DKD (14, 17). In our study, we have shown that damage to the gut barrier led to a lower expression of tight junction proteins ZO-1, higher levels of the serum FITC-dextran and subsequently increased intestinal permeability, which may facilitate the passage of gut-derived LPS into the blood. Gut-derived LPS could initiate inflammatory responses through TLRs, in particular through the TLR4-related pathway, where LPS mediates the activation of NF-κB (13). This would cause chronic inflammation and accelerate DKD. Evidence also shows that the TLR4/NF-κB pathways in the kidney are closely related to the development of DKD (49, 50). Our study shows that the LPS/TLR4/NF-κB pathway was up-regulated in DKD group, as we found increased levels of serum LPS and overexpression of TLR4 and NF-κB in the kidney. However, the LPS/TLR4/NF-κB pathway was downregulated after QRXZF treatment.

Besides, to further test our hypothesis that the gut microbiota and the inflammatory responses were inevitable correlation with the reno-protective effect of QRXZF against DKD, the fecal microbiota transplantation (FMT) (51, 52) experiment or germ-free mice would be performed in further studies to provide more evidences about QRXZF.

CONCLUSION

Our study demonstrates that QRXZF could prevent the gut dysbiosis, reduce the intestinal permeability and gut-derived LPS into blood. The reno-protective effect of QRXZF might be associated with inhibition of the LPS/TLR4/NF-κB inflammation signaling pathway in the kidney (**Figure 6C**).

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/, PRJNA729207.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Beijing University of TCM.

AUTHOR CONTRIBUTIONS

ZW and LP: conceptualization, funding acquisition, and writing—review and editing. YW and WL: supervision. YG, RY, ZW, and YZ: formal analysis, investigation, writing—original draft, and visualization. SA and TW: writing—review editing. All authors approved of the final submission.

FUNDING

This study was supported by the National Natural Science Foundation of China (81804032, 81904105, and 81970713), Research Start up Fund for Doctor of Medicine of the First Affiliated Hospital of Henan University of Traditional Chinese Medicine (2021BSJJ021), and the top talent training program of Henan Province of traditional Chinese Medicine.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.719950/full#supplementary-material

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frontiers in Medicine

REVIEW
published: 16 September 2021
doi: 10.3389/fmed.2021.714958

Circular RNAs as Novel Diagnostic Biomarkers and Therapeutic Targets in Kidney Disease

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Circular RNAs (circRNAs) are a novel type of non-coding RNAs that have aroused growing attention in this decade. They are widely expressed in eukaryotes and generally have high stability owing to their special closed-loop structure. Many circRNAs are abundant, evolutionarily conserved, and exhibit cell-type-specific and tissue-specific expression patterns. Mounting evidence suggests that circRNAs have regulatory potency for gene expression by acting as microRNA sponges, interacting with proteins, regulating transcription, or directly undergoing translation. Dysregulated expression of circRNAs were found in many pathological conditions and contribute to the pathogenesis and progression of various disorders, including renal diseases. Recent studies have revealed that circRNAs may serve as novel reliable biomarkers for the diagnosis and prognosis prediction of multiple kidney diseases, such as renal cell carcinoma (RCC), acute kidney injury (AKI), diabetic kidney disease (DKD), and other glomerular diseases. Furthermore, circRNAs expressed by intrinsic kidney cells are shown to play a substantial role in kidney injury, mostly reported in DKD and RCC. Herein, we review the biogenesis and biological functions of circRNAs, and summarize their roles as promising biomarkers and therapeutic targets in common kidney diseases.

Keywords: circular RNAs, biogenesis, biological functions, biomarkers, therapeutic targets, kidney diseases

OPEN ACCESS

Edited by:
Weiren Luo,
The Second Affiliated Hospital of Southern University of Science and Technology, China

Reviewed by:
Weiqiang Lin,
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Specialty section:
This article was submitted to Pathology, a section of the journal Frontiers in Medicine

Received: *26 May 2021*
Accepted: *16 August 2021*
Published: *16 September 2021*

Citation:
Yu J, Xie D, Huang N and Zhou Q (2021) Circular RNAs as Novel Diagnostic Biomarkers and Therapeutic Targets in Kidney Disease. Front. Med. 8:714958. doi: 10.3389/fmed.2021.714958

INTRODUCTION

Circular RNAs (circRNAs) are a new type of non-coding RNA molecules that have attracted more and more attention in recent years (1). Although initially they are overlooked as redundant products from mis-splicing events yielding at low expression levels (2), a growing number of studies indicate that circRNAs are widely expressed in eukaryotic cells, from fungi, plants, to metazoans, such as fruit fly, mouse as well as human (3–6). Unlike linear RNAs, circRNAs are single stranded and covalently closed RNA transcripts. The structure characteristic renders circRNAs naturally resistant to exonuclease-mediated degradation, thus producing high stability (7). Many circRNAs are abundantly expressed, evolutionarily conserved, and exhibit cell-type-specific, tissue-specific and developmental-stage-specific expression patterns (3, 8–10). CircRNAs are formed by a non-canonical splicing event called back-splicing during which a downstream 5′ splice donor site is covalently joined to an upstream 3′ splice acceptor site (11, 12). They mainly arise from protein-coding exons, but can also from introns, untranslated, or intergenic regions of

the genome (12). According to formation modes and sequences, circular RNAs can be classified into three types: exonic circRNA (ecircRNA), which is generated from back-spliced exons (8); intronic circRNA (ciRNA), which arises from intron lariats (13); exon-intron circRNA (EIciRNA), which consists of both exons and introns (14). The biological functions of circRNAs have been extensively investigated in this decade. The most frequently proposed mechanism of action is to act as microRNA (miRNA) sponges (12, 15). Moreover, circRNAs have been shown to function through interacting with proteins, regulating transcription, or directly undergoing translation (1). Nevertheless, the functions of most circRNAs identified to date remain largely elusive.

Since the recent findings that circRNAs are ubiquitous in human tissues and differentially expressed under pathological states, their functional relevance in diseases have been increasingly explored. The majority of researches have been focused on their roles in cancer (16), cardiovascular diseases (17), diabetes mellitus (18), and neurological disorders (19). For example, as a well-characterized circRNA in human diseases, ciRS-7 is initially found to be abundantly expressed in neuronal tissues and participated in neuronal development by acting as a sponge for miR-7 (12, 15). Subsequent studies reveal it could also exert oncogenic functions during tumorigenesis and modulate insulin secretion (20, 21), indicating that circRNAs may play important roles under diverse pathophysiological states. In recent years, there has been an increasing focus on characterizing the roles of circRNAs in kidney diseases, including renal cell carcinoma (RCC), acute kidney injury (AKI), diabetic kidney disease (DKD), and other glomerular diseases (22–24). These studies explored the feasibility of circRNAs as non-invasive biomarkers for the diagnosis and outcome prediction of specific kidney diseases. Furthermore, the functions of circRNAs expressed by kidney resident cells in the pathogenesis and progression of renal disorders have also been actively investigated. This review will introduce the biogenesis and biological function of circRNAs, and focus on state-of-art regarding circRNAs as novel biomarkers and therapeutic targets in common kidney diseases.

BIOGENESIS AND PROPERTIES OF CircRNAs

Biogenesis and Regulation of CircRNAs

CircRNAs are transcribed from pre-mRNAs through a non-canonical splicing event called back-splicing (**Figure 1**), which is regarded as a type of alternative splicing by a broad definition. In general, during a back-splicing event, a downstream 5′ splice donor site is linked to an upstream 3′ splice acceptor site via covalent bonds to form a closed-loop circRNA. Though circRNA circularization and linear splicing compete against each other, mutagenesis analyses in circRNA expression vectors as well as blocking experiments using the inhibitor for spliceosome assembly have shown that both canonical splice sites and spliceosomal machinery are required for circRNA biogenesis (25, 26). The processing of back-splicing circularization has not been fully elucidated, and several working models have been proposed (**Figure 1**). In the first model, the direct base paring between inverted repeat elements in the flanking intron sequences (such as Alu elements) brings a downstream splice donor site into close proximity with an upstream acceptor site, which may facilitate back-splicing looping by the canonical splicing machinery (27). RNA binding proteins (RBPs) including Quaking (QKI) and fused in sarcoma (FUS) could also drive exon circularization in a similar manner by dimerization of RBPs that bind to specific motifs in the flanking introns (28). These two proposed models lead to the formation of ecircRNAs or EIciRNAs (circRNAs with retained introns). Other hypothesis includes the exon-skipping model, in which alternative exons are spliced out to form exon lariat that end up as ecircRNAs by internal back-splicing (29). Last but not the least, intronic lariat precursors that escape from the debranching step of canonical linear splicing can result in the production of ciRNAs (13). Following biogenesis, ecircRNAs are normally translocated into the cytoplasm, while ciRNAs and EIciRNAs are predominately retained in the nucleus.

The regulation of circRNAs biogenesis is crucial for characterizing their functional roles under pathophysiological conditions, but is still largely unclear. Emerging lines of evidence have identified that several regulatory mediators are involved in circRNAs formation (**Figure 1**). As mentioned above, QKI was found to promote circRNAs production during human epithelial–mesenchymal transition (EMT) (28). QKI could bind to specific motifs in introns flanking circularized exons and bring these exons closer together via dimerization, which facilitates circularization and results in enhanced circRNAs formation. Overexpression of the splicing factor Muscleblind (Mbl), or the insertion of synthetic Mbl-binding sites into the introns flanking circRNA-forming exons in an expression vector, promoted exon circularization, suggesting that Mbl can enhance circRNAs formation in a similar way (25). Adenosine deaminase 1 acting on RNA (ADAR1) has been found to suppress circRNAs biogenesis by disrupting the looping of flanking introns relying on base pairing between inverted repeats. Knockdown of ADAR1 promoted the up-regulation of certain circRNAs (10). ATP-dependent RNA helicase A (also known as DHX9) has also been shown to negatively regulate circRNAs expression via a similar mechanism (30), while immune factors NF90/NF110 can promote circRNAs production by stabilizing intronic RNA pairs in viral infection (31). The elimination and degradation of circRNAs undoubtedly play a role in their expression levels, yet remains poorly characterized to date. Future studies are warranted to uncover the turnover process of circRNAs.

The Properties of CircRNAs

One prominent characteristic of circRNAs is that they are strikingly more stable than other types of RNAs, including linear mRNAs and other non-coding RNAs. One study compared the half-lives of four ecircRNAs and their associated linear transcripts and found the ecircRNAs consistently exhibited long half-lives exceeding 48 h in contrast to linear counterparts with half-lives <20 h (8). Another study calculated the half-lives of 60 circRNAs and their linear isoforms in mammary cells and concluded that the median half-life of circRNAs was at least

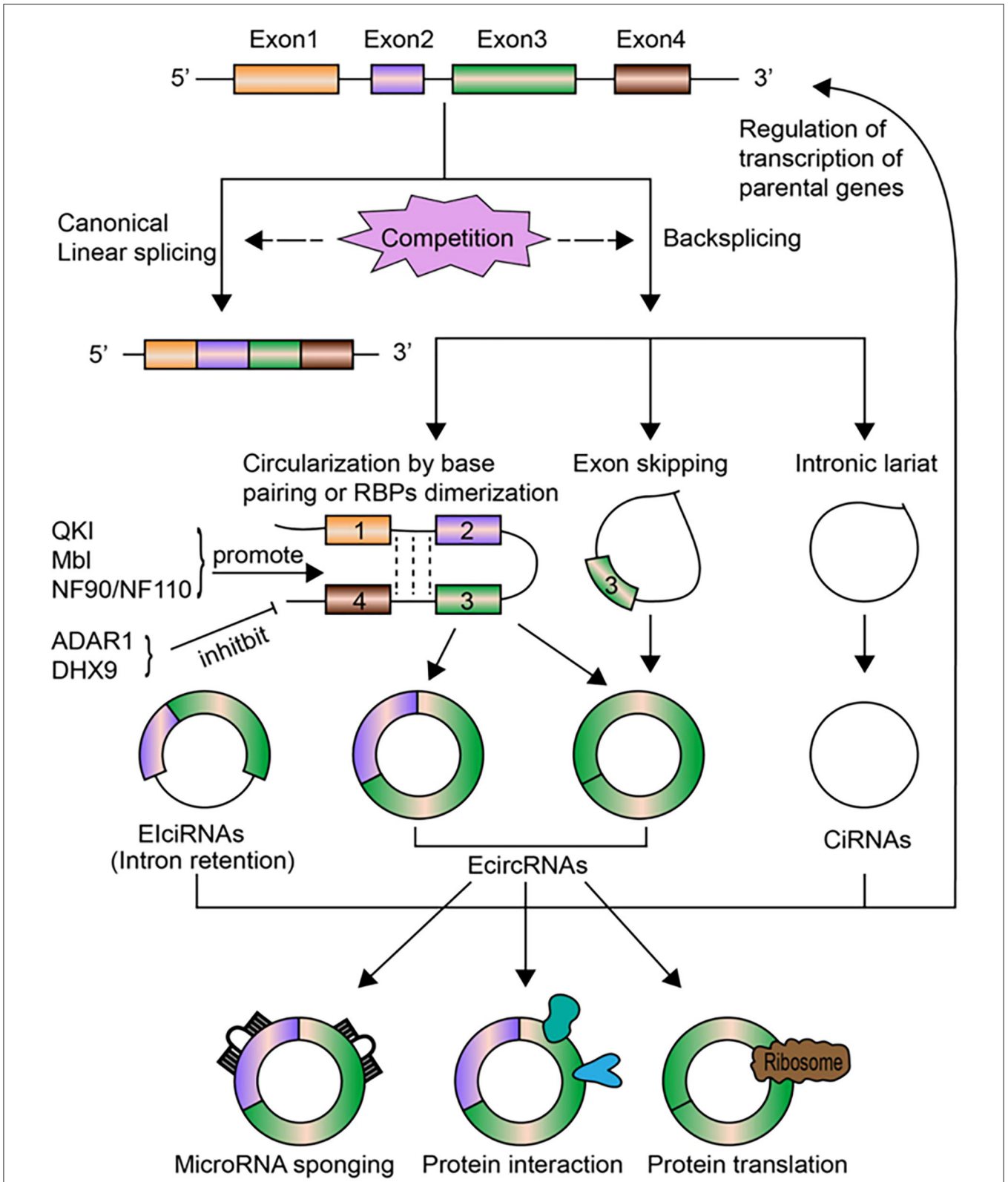

FIGURE 1 | The biogenesis, regulation, and functions of circRNAs. CircRNAs are transcribed from pre-mRNAs through the non-canonical back-splicing event. The back-splicing process involves different mechanisms, including circularization by base paring of intronic sequences or RBPs dimerization, exon skipping, and intronic

(Continued)

FIGURE 1 | lariat, which generates three types of circRNAs: ecircRNAs, ciRNAs, and EIciRNAs. Some molecules, including QKI, Mbl, NF90/NF110, positively regulate the biogenesis of circRNAs, while ADAR1 and DHX9 inhibit circRNAs formation. CircRNA biogenesis *per se* competes with canonical linear splicing. The most well-studied functions of circRNAs is to act as miRNA sponges to modulate the expression of miRNA target genes. CircRNAs can bind to RNA pol II or other RBPs to affect transcription levels of their parental genes, especially for nuclear-retained ciRNAs and EIciRNAs. CircRNAs can act as sponges, decoys, scaffolds for proteins or regulate protein trafficking through interacting with specific proteins. Certain circRNAs can be translated into proteins under stress circumstances.

2.5 times longer than that of linear mRNAs (32). The high stability of circRNAs is mainly attributed to their special closed-loop structures which are resistant to exonuclease-mediated degradation, yet the precise structural mediators involved remain to be investigated. Though circRNAs were initially overlooked as "junk" byproducts of transcription with low expression levels, emerging evidence suggests that circRNAs are widely expressed in a variety of eukaryotic cells and most human tissues (4, 11). As for the abundance of circRNAs, some studies showed they were weakly expressed in certain cell types (32), while others demonstrated the abundance of certain circRNAs was comparable to or considerably higher than that of associated linear counterparts (8, 10, 11). The discrepancy among studies regarding the expression abundance of circRNAs might be due to their cell-type-specific or tissue-specific expression patterns. A striking example is the mouse circRNA circRims2, which is expressed substantially higher than its linear mRNA in mouse adult brain, but is weakly expressed in other mouse tissues (10). Furthermore, many circRNAs are well-conserved in expression and sequence among species (8, 10). Highly expressed circRNAs are more likely to be conserved, indicating that they are functionally important. These characteristics make circRNAs as promising biomarker candidates for human diseases.

BIOLOGICAL FUNCTIONS OF CircRNAs

The biological functions of circRNAs have been extensively investigated in recent decade. Similar to miRNAs and long non-coding RNAs (lncRNAs), circRNAs have also been found to play an important role in the regulation of gene expression. They may modulate gene expression by distinct modes of action at transcriptional or post-transcriptional levels. The most well-characterized mechanism is to act as sponges for miRNAs [(12, 15); **Figure 1**]. Meanwhile, circRNAs could affect splicing and regulate transcription levels (14, 25), interact with specific proteins and modulate their functions (33, 34), or directly undergo translation under stress conditions [(35, 36); **Figure 1**].

CircRNAs Act as MiRNA Sponges

MiRNAs are an important type of small non-coding RNAs that negatively regulate gene expression by binding to the 3′ untranslated regions (3′ UTR) of messenger RNAs (mRNAs). Many studies have found that circRNAs harboring miRNA binding sites could compete with mRNAs for miRNAs binding and function as miRNA sponges, thus indirectly affect gene expression. The first-characterized and most well-known circRNA to support this model is ciRS-7 (circRNA sponge for miR-7), which contains more than 70 conserved binding sites for miR-7 (12, 15). CiRS-7 functions to bind miR-7 and the interaction is conserved among species and several disease models. Introduction of ciRS-7 expression vector *in vitro* produced a substantial reduction of knockdown effect by miR-7 on its targets, while repressing ciRS-7 levels by miR-671 regained the inhibition on targets genes by miR-7, supporting the role of ciRS-7 as a highly functional sponge for miR-7 (15). However, genetic ablation of the ciRS-7 locus in mice led to the down-regulation of miR-7 and promoted the expression of its targets (37), arguing for the functional significance of the ciRS-7/miR-7 interaction *in vivo*. These discrepant data suggest that whether ciRS-7 exerts as a sponge for miR-7 may depend on specific biological circumstances. In addition to ciRS-7, many other circRNAs have been shown to function as miRNA sponges. The testis-specific circRNA, sex-determining region Y (Sry), which harbors 16 putative target sites for miR-138 in mouse, was demonstrated to serve as a sponge for miR-138 *in vitro* (15). Transcriptomic analysis revealed that circHIPK3 could sequester a group of miRNAs, including miR-124-3p and miR-338-3p, to regulation β-cell functions (21). Another study showed that circHIPK3 was capable to sponge 9 miRNAs with 18 potential binding sites to regulate cell growth (38). In addition, circCCDC66 was found to exert a novel oncogenic function in colorectal cancer via acting as sponges for a set of miRNAs which target oncogenes (39). Although the mechanism as miRNA sponges has been widely studied, partly due to relatively easy operability, only a minority of circRNAs contain multiple miRNA target sites (13, 40), implying most circRNAs might not function by sponging miRNAs.

CircRNAs Affect Splicing and Regulate Transcription

CircRNAs are generally produced cotranscriptionally via exon circularization from protein-coding genes (27). It has been shown that circRNAs biogenesis, which is dependent on both canonical splice sites and spliceosomal machinery, competes with pre-mRNA splicing on a global cell level, and then affects the expression of transcribed genes (25). In principle, the more an exon is circularized, the less it will appear in the processed mRNA (29). Therefore, the processing of circRNAs *per se* could regulate gene expression at splicing levels. Furthermore, certain circRNAs could also modulate gene transcription. Unlike ecircRNAs which are mainly localized in the cytoplasm, intron-containing circRNAs including EIciRNAs and ciRNAs are more likely to be retained in the nucleus in human cells (13, 14). These two types of circRNAs have been found to directly participate in transcription regulation. Several abundantly expressed EIciRNAs (including EIciEIF3J and EIciPAIP2) and ciRNAs (including ci-ankrd52) were demonstrated to physically interact with polymerase II complex and promote transcription of their parental genes in *cis* (13, 14). Depleting or knockdown of these circRNAs led to the

reduced transcription of their parental genes. Whether additional nuclear-retained circRNAs could regulate transcription in a similar manner or in *trans* remains to be explored.

CircRNAs Function Through Interaction With Proteins

Increasing studies have found that circRNAs may exert their biological functions by interacting with various proteins. In addition to those nuclear-retained circRNAs binding to polymerase II complex to enhance transcription as mentioned above (13, 14), circRNAs can also act as protein sponges or decoys (25), or as scaffolds to facilitate protein complex formation and reaction (33, 34), or participate in protein trafficking (41). One example of circRNAs as protein sponges was from the study of the splicing factor Muscleblind (MBL) and its circular isoform circMbl (25). MBL could directly promote the biosynthesis of circMbl which was found to contain a binding site for MBL, and a strong interaction between them was confirmed. The cooperative association raises the possibility of an autoregulatory loop in which MBL will decrease the level of its own mRNA by enhancing circMbl production, and then circMbl could sponge out excessive MBL protein by binding to it. Circ-Foxo3 is another circRNA functioning as protein sponges as well as scaffolds for protein complex which could bind to the cell cycle proteins cyclin-dependent kinase 2 (CDK2) and cyclin-dependent kinase inhibitor 1 (CDKA1 or p21), resulting in the formation of a ternary complex and cell cycle arrest (33). Another study found that circ-Foxo3 could bind to both p53 and the mouse double-minute 2 (MDM2). As MDM2 mediates both the ubiquitylation of p53 and FOXO3 protein, the interaction thus might sponge the ubiquitylated effect on FOXO3 and facilitate MDM2-dependent ubiquitylation of p53, leading to cell apoptosis (34). Furthermore, circRNAs may participate in protein trafficking. The ecircRNA FECR1 binding to the promoter of its host gene FLI1 could recruit TET1 demethylase to induce DNA hypomethylation and activate transcription (41). The cooperative interactions between circRNAs and specific proteins may be one underappreciated mechanism of their modes of action.

CircRNAs Undergo Translation

Though devoid of 5′ cap and 3′ polyadenylated tails structure, recent studies indicate that a subset of endogenous circRNAs can be translated in a cap-independent manner (35, 36, 42). Circ-ZNF609 was found to be associated with heavy polysomes and could be translated into a protein during muscle differentiation, albeit with a lower translation efficiency compared to its linear counterpart (42). In drosophila heads, a group of circRNAs was shown to be associated with translating ribosomes and one circRNA generated from the *muscleblind* (*mbl*) locus could synthesize a protein product as detected by mass spectrometry (35). The internal ribosome entry sites (IRESs) embedded within sequences or modification of N6-methyladenosine (m^6A) was demonstrated to be capable to drive translation of circRNAs (36). However, the biological significance of circRNA translation remains largely unknown. It was shown that the efficiency of circRNA translation was altered under cellular stress (35, 36), indicating that cap-independent translation of circRNAs might be an adaptive mechanism under stress conditions.

CircRNAs AS NOVEL BIOMARKERS FOR KIDNEY DISEASES

Mounting evidence suggests that circRNAs are abundant in a variety of body fluids, such as saliva, blood, urine, and exosomes secreted by most cell types (43–46). Furthermore, since circRNAs are exceptionally stable molecules, along with their cell-type-specific and tissue-specific expression patterns, their potentials as novel biomarkers in liquid biopsy have attracted an increasing interest of research. To date, results from many studies indicate that circRNAs could be represented as novel diagnostic and prognostic biomarkers in multiple human diseases, including cancer (47) and cardiovascular diseases (48). Preliminary attempts have been made to clarify the roles of circRNAs as biomarkers in kidney diseases in recent years. At present, this field of research are mainly focused on renal cell carcinoma (RCC), acute kidney injury (AKI), and glomerular diseases, including diabetic kidney disease (DKD), which are to be discussed in detailed (**Table 1**).

CircRNAs and RCC

RCC is the most common type of kidney neoplasm, accounting for 85–90% of adult renal malignancies (63). Early diagnosis of RCC and timely identification of post-operative recurrence and metastasis are crucial for improving the outcome of patients with RCC. Reliable biomarkers to fulfill these clinical expectations are urgently needed. Recently, dysregulated circRNA expression profiles have been reported in RCC. Five hundred forty-two circRNAs were identified as differentially expressed by using RNA microarray data from online RCC database (64). Among these, 324 circRNAs were down-regulated, whereas 218 were up-regulated in the ccRCC group. Another study performed a genome-wide screening of dysregulated circRNAs using 7 matched clear cell RCC (ccRCC) tissue samples by microarray analysis and 78 circRNAs were up-regulated while 91 were down-regulated in malignant tissues compared to adjacent normal samples (49). The expressions of three selected circRNAs (circEGLN3, circNOX4, and circRHOBTB3) were validated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) assays. By performing receiver-operating characteristics curve (ROC) analysis, these circRNAs demonstrated excellent diagnostic values to diagnose ccRCC, with the AUC values of circNOX4, circRHOBTB3, and circEGLN3 as 0.81, 0.82, and 0.98, respectively. Moreover, the predictive accuracy of a clinical model based on clinicopathological variables of ccRCC was significantly improved by including the expression signature of these three circRNAs. Another study found that the expression of hsa_circ_0001451 was shown to be significantly decreased in ccRCC tissues and correlated with tumor staging and metastasis (50). The AUC-ROC value of this circRNA for diagnosis of ccRCC was 0.704 and regression analysis disclosed that the level of hsa_circ_0001451 was an independent predictor for overall

TABLE 1 | Summary of candidate circRNAs as biomarkers in kidney diseases.

CircRNA	Disease	Specimen	Expression change	Significance as biomarker	References
circEGLN3	RCC	Kidney tissues	Up	As diagnostic biomarker of RCC; up-regulation predicts better prognosis	(49)
circRHOBTB3	RCC	Kidney tissues	Down	As diagnostic biomarker of RCC; down-regulation predicts poor prognosis	(49)
hsa_circ_0001451	RCC	Kidney tissues	Down	As diagnostic biomarker of RCC; down-regulation predicts poor prognosis	(50)
circPCNXL2	RCC	Kidney tissues	Up	Up-regulation predicts poor prognosis	(51)
circ-ABCB10	RCC	Kidney tissues	Up	Up-regulation predicts poor prognosis	(52)
hsa_circ_001895	RCC	Kidney tissues	Up	Up-regulation predicts poor prognosis	(53)
circ_001842	RCC	Kidney tissues	Up	Up-regulation predicts poor prognosis	(54)
circPRRC2A	RCC	Kidney tissues	Up	Up-regulation predicts poor prognosis	(55)
circ-EGLN3	RCC	Kidney tissues	Up	Up-regulation predicts poor prognosis	(56)
circ_0001368	RCC	Kidney tissues	Down	Down-regulation predicts poor prognosis	(57)
cRAPGEF5	RCC	Kidney tissues	Down	Down-regulation predicts poor prognosis	(58)
ciRs-126	AKI	Blood	Up	Up-regulation predicts poor 28-day survival	(59)
hsa_circ_0001334	acute kidney rejection	Urine	Up	As diagnostic biomarker of acute rejection; up-regulation predicts poor 1-year graft function	(45)
circ_101319	IMN	Blood	Up	As diagnostic biomarker of IMN	(60)
circ_002453	LN	Blood	Up	As diagnostic biomarker of LN	(61)
hsa_circ_0123190	LN	Blood	Down	As diagnostic biomarker of LN	(62)

RCC, renal cell carcinoma; AKI, acute kidney injury; IMN, idiopathic membranous nephropathy; LN, lupus nephritis.

survival of ccRCC patients, supporting this circRNA as a reliable diagnostic and predictive indicator of ccRCC. Other circRNAs that have been reported to predict prognosis for RCC included circPCNXL2, circ-ABCB10, hsa_circ_001895, circ_001842, circPRRC2A, circ-EGLN3, circ_0001368, and cRAPGEF5 (51–58). The former six circRNAs were all found to be up-regulated in tumor tissues and associated with poor clinical outcomes in ccRCC patients. The latter two was shown to be significantly reduced in RCC samples. The decreased level of cRAPGEF5 was negatively correlated with tumor growth and metastasis and shown to be an independent factor for poor prognosis in RCC patients. These findings supported the roles of circRNAs as novel potential biomarkers for the diagnosis and outcome prediction of RCC.

CircRNAs and AKI

AKI is an increasingly common clinical syndrome characterized by the rapid decline in kidney function and has a relatively high mortality rate with no specific treatment beyond supportive care. Exploration of novel biomarkers besides serum creatinine and urine output for early diagnosis of AKI remains an area of utmost interest. In recent years, a large number of circRNAs have been found to be differentially expressed in AKI animal models induced by ischemia and reperfusion (I/R) or cisplatin treatment (65, 66). Interestingly, the dysregulated circRNA profiles following I/R treatment could be restored by pre-treatment with the drug losartan accompanied by improvements in the functional and histological indicators of kidney injury (65), implying that circRNAs might play a role in AKI development and mediate the renoprotective effect of losartan. As AKI is a severe complication in critically ill patients in intensive care unit (ICU), a genome-wide circRNA expression analysis was performed using RNA isolated from whole blood of ICU patients and revealed that ciRs-126 (circRNA sponge of miR-126) was significantly increased in AKI patients compared to healthy and disease controls (59). Further Cox regression and Kaplan Meier curve analysis identified ciRs-126 as a strong independent risk factor for 4-week-survival. By ROC curve analysis, ciRs-126 levels yielded an AUC value of 0.92 with 91% sensitivity and 74% specificity. Though this is a single-center experience

with relatively small sample size, these data suggest ciRs-126 might act as a useful biomarker for predicting the outcome of AKI patients in ICU settings. In another study conducted by the same study group, the global expression profile of urinary circRNAs was identified in patients with acute renal allograft rejection and hsa_circ_0001334 in urine was shown to be up-regulated in patients with acute rejection compared to controls and normalized following successful anti-rejection therapy (45). Importantly, the elevated levels of this circRNA could be measured at subclinical time points of rejection while there are no elevations in serum creatinine.Hsa_circ_0001334 yielded an AUC value of 0.85 with a sensitivity of 70.11% and specificity of 92.31% in diagnosing acute rejection. In addition, the increased expression of hsa_circ_0001334 was positively associated with decline of kidney function 1 year after transplantation. Thus, urinary hsa_circ_0001334 might serve as a novel non-invasive marker of acute kidney rejection and predictor of graft function.

CircRNAs and Glomerular Diseases

Glomerular diseases refer to a large group of diseases that injuries mainly involve bilateral glomeruli, which can be classified into three categories: primary, secondary, and inherited glomerular diseases. Glomerular diseases have a very high morbidity worldwide and remain the primary cause of chronic kidney disease (CKD) and end-stage renal disease (ESRD), which pose huge burden on society and economy. At present, the diagnosis of glomerular diseases mainly depends on invasive kidney biopsy and reliable indicators for predicting clinical outcomes of specific glomerular diseases are lacking. Recently, increasing studies have focused on the expression and roles of circRNAs in the diagnosis and prognosis prediction of various glomerular diseases.

Diabetic kidney disease (DKD) is one of the most frequent complications of diabetes mellitus and the leading cause of CKD worldwide, which is pathologically characterized by mesangial cells (MCs) proliferation, extracellular matrix (ECM) accumulation, and basement membrane thickening. Nevertheless, there are few reports evaluating the roles of circRNAs as biomarkers in DKD up to date. One study investigated the differentially expressed (DE) circRNA profiles in the *db*/*db* mouse model by microarray analysis (67). Another study performed high-throughput circRNA sequencing using the same model and identified 40 DE circRNAs, among which 18 were up-regulated and 22 were down-regulated in diabetic mouse kidneys (68). Circ_0080425 was found to be significantly increased in kidneys of streptozotocin-treated diabetic model and positively correlated with the severity of pathological abnormalities (69). Another study constructed an *in vitro* high glucose (HG)-treated glomerular endothelial cells (GECs) model and obtained exosomal circRNA profiles secreted by GECs using high-throughput sequencing. Compared to normal glucose (NG)-treated GEC exosomes, 217 circRNAs were significantly down-regulated while 484 were up-regulated in HG-treated GEC exosomes (70). The level of circ_DLGAP4 was also shown to be increased in exosomes isolated from HG-treated MCs (71). Moreover, its expression was consistently elevated in DKD rat models, DKD patients, and further increased in DKD patients with macroalbuminuria, suggesting it might correlate with DKD progression. These preliminary studies suggest that dysregulated circRNAs, especially those from exosomes, are potential biomarkers for DKD. However, further studies are needed to verify these DE circRNAs as reliable biomarkers of DKD. Furthermore, there is a lack of research to conduct global screening for biomarkers using samples from DKD patients. It would also be interesting to search for circRNAs that could distinguish true DKD patients from those with non-DKD (NDKD).

IgA nephropathy (IgAN) is the most common type of primary glomerulonephritis worldwide. To date, only two studies have analyzed the dysregulated circRNAs profiles in IgAN patients (72, 73). The first study conducted circRNA sequencing using RNA isolated from peripheral blood mononuclear cells (PBMCs) of three pairs of IgAN patients and healthy controls (72). A total of 145 circRNAs were identified as differentially expressed, among which 112 circRNAs were up-regulated while 33 were down-regulated in IgAN group compared to controls. A recent study investigated the circRNAs profiles in urinary exosomes from five pairs of IgAN patients and healthy controls by high-throughput RNA sequencing (73). In total, 1,322 circRNAs were detected in the urinary exosomes and 476 were aberrantly expressed, including 450 up-regulated and 26 down-regulated circRNAs. These two studies are relatively preliminary with small sample sizes. The roles of these dysregulated circRNAs as biomarkers in IgAN warrant further exploration.

Membranous nephropathy (MN) is another common type of glomerulopathy with increasing frequency over the past decades (74). A preliminary study profiled the expression of circRNAs in exosomes from both serum and urine in patients with idiopathic membranous nephropathy (IMN) and identified 89 DE circRNAs in serum exosomes and 60 DE circRNAs in urinary exosomes (75). Another study conducted microarray analysis to identify circRNA profiles in the peripheral blood of IMN patients and showed that 955 circRNAs were differentially expressed, of which 645 were up-regulated and 310 were down-regulated (60). The increased expression of circ_101319 in the IMN group was validated by qRT-PCR. ROC curve analysis revealed that the AUC value for circ_101319 to diagnose IMN was 0.89, with a sensitivity of 93.33% and specificity of 70.00%, suggesting circ_101319 might act as a reliable biomarker for the diagnosis of IMN. As there is increasing focus on the role of anti-PLA2R antibody as a key biomarker in the diagnosis and monitoring of IMN (76), it would be intriguing to search for candidate circRNAs that could enhance the diagnostic and predictive power of the anti-PLA2R antibody.

Lupus nephritis (LN) is the most common complication of systemic lupus erythematosus (SLE) and 10–30% of LN patients will progress to ESRD (77). Initially, several studies used microarray or high-throughput RNA sequencing to screen the circRNAs profiles in peripheral blood of SLE patients (78, 79). One study identified hsa_circ_0000479 was significantly increased in SLE patients compared to controls and its high expression was associated with low albumin levels and positive urine protein (79). Another study profiled the circRNAs expression by RNA sequencing using renal biopsy tissues from

LN patients (80). Among dysregulated circRNAs, circHLA-C positively correlated with proteinuria, serum creatinine, percentage of crescentic glomeruli, and renal activity index. Subsequent studies made further efforts to explore the diagnostic values of circRNAs in LN. One study showed that plasma circRNA_002453 was significantly up-regulated in patients with LN compared to SLE patients without LN, rheumatoid arthritis (RA) patients, and healthy controls (61). Interestingly, the level of circRNA_002453 was positively associated with proteinuria and renal SLEDAI score but not with systemic activity makers. ROC analysis revealed that circRNA_002453 yielded an AUC value of 0.906 to diagnose LN. Another study identified hsa_circ_0123190 was down-regulated in both renal tissues and peripheral blood of LN patients (62). There was no significant association between the tissue levels of hsa_circ_0123190 and clinical parameters, while its expression in blood was negatively correlated with serum creatinine, and the AUC-ROC value diagnosing LN was 0.900. Results of these studies suggested that circRNA_002453 and hsa_circ_0123190 could be reliable biomarkers for the diagnosis of LN.

CircRNAs AS THERAPEUTIC TARGETS FOR KIDNEY DISEASES

Although lots of efforts have been made in kidney diseases field, the pathogenesis of most renal disorders remains largely unclear, which hinders the development of specific therapeutic strategies to treat these diseases. Since circRNAs could regulate gene expression and are differentially expressed under pathological conditions, they are probably biologically functional in diseases. In fact, current data indicate that circRNAs are involved in the pathogenesis and progression of cancer (16), cardiovascular diseases (17), and neurological disorders (19). Recently, studies have found that a great number of dysregulated circRNAs expressed by kidney resident cells contribute to the initiation and development of multiple kidney diseases, mostly reported in DKD, RCC, as well as other types of renal disorders (22, 23). Mechanistically, the majority of these circRNAs were shown to exert their biological functions by acting as miRNA sponges. Although most of these findings are from *in vitro* cell culture experiments, these studies provide novel avenues to elucidate the mechanisms underlying kidney diseases and make circRNAs novel promising therapeutic targets in this realm.

CircRNAs and RCC

In recent years, a growing number of studies have shown that circRNAs play critical roles in the tumorigenesis and progression of RCC (**Table 2**). These circRNAs are involved in various processes of RCC development, including cell proliferation, migration, invasion, apoptosis, and EMT. The majority of circRNAs identified to date are shown to exert oncogenic effects, and the most common studied mechanism is to act as miRNA sponges. For instance, circPCNXL2 was highly expressed in ccRCC tissues compared to adjacent non-tumor tissues (51). Functionally, circPCNXL2 inhibition markedly repressed RCC cells proliferation and invasion, suggesting it serve as an oncogenic circRNA in RCC progression. Mechanistically, circPCNXL2 functioned as a sponge for miR-153 to modulate the expression of its target gene ZEB2, which is a known positive driver in tumor progression. Another study reported that the high expression of circ_000926 facilitated the development and progression of RCC by sponging miR-411 to up-regulate CDH2, which is a marker of EMT and contributor to RCC aggressiveness (81). A recent study found a novel circRNA (circPRRC2A) promoted angiogenesis and metastasis of RCC (55). Further mechanistic experiments revealed that circPRRC2A could directly bind to miR-514a-5p and miR-6776-5p to manipulate the control of TRPM3-induced EMT. On the other hand, several circRNAs were shown to function as tumor suppressors in RCC. Circ-AKT3 was stably decreased in both ccRCC cell lines and tumor tissues (82). Restoration of circ-AKT3 inhibited cell migration and invasion by acting as a sponge of miR-296-3p and up-regulating E-cadherin expression, supporting a protective role of circ-AKT3 in ccRCC metastasis. Like circ-AKT3, cRAPGEF5 was significantly down-regulated in RCC (58). Functional assays demonstrated that cRAPGEF5 suppressed tumor growth and metastasis of RCC by sponging oncogenic miR-27a-3p, which targets the suppressor gene TXNIP. Circ_0001368 was also identified as a novel anti-tumor RNA via negatively regulating the miR-492/LATS2 axis (57). Several other circRNA-miRNA-mRNA interaction cascades have been reported in RCC, most of which were found with tumor promotive effects, including circ_001895/miR-296-5p/SOX12 (53), circ_001842/miR-502-5p/SLC39A14 (54), circ-EGLN3/miR-1299/IRF7 (56), circ_0039569/miR-34a-5p/CCL22 (83), circ-ZNF609/miR-138-5p/FOXP4 (84), and circ_0054537/miR-130a-3p/cMet (85). Intriguingly, several circRNAs have been reported to work as miRNA reservoirs to regulate RCC development (86, 87). CircHIAT1 was involved in the androgen receptor (AR)-driven RCC progression by serving as a miRNA reservoir to increase the stability and availability of miR-195-5p/29a-3p/29c-3p, leading to the suppression of AR-enhanced ccRCC migration and invasion (86). CircATP2B1 was found to be repressed by estrogen receptor beta (ERβ), which functions as an oncogene in RCC metastasis (87). Overexpression of circATP2B1 could increase miR-204-3p stability by acting as a miRNA reservoir to partially reverse ERβ-promoted ccRCC invasion. In addition to the manipulation of hub genes, recent evidence has revealed that the circRNA-miRNA network regulate RCC progression through tumor-associated signaling pathways. For instance, circ-0072309 played anti-tumor roles by sponging miR-100 to block the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway signaling cascades, which are pivotal pathways in RCC development (88). Recently, an interesting study attempted to explore the roles of circRNAs in chemotherapy resistance of RCC patients (89). This study revealed that hsa_circ_0035483 was highly expressed in RCC and facilitated gemcitabine resistance and tumor growth by modulating the hsa-miR-335/CCNB1 axis. Importantly, silencing hsa_circ_0035483 enhanced gemcitabine sensitivity and repressed tumor growth *in vivo*, suggesting hsa_circ_0035483 could be a promising therapeutic target of

gemcitabine resistance in RCC treatment. *In vivo* manipulation of circRNAs expression is vital to clarify their functional relevance. In fact, many studies have already explored the effect of interfering with circRNAs expression on RCC growth and metastasis *in vivo* (51, 53–55, 57, 58, 81, 82, 86, 87). These studies mainly adopted the strategy of injecting RCC cell lines stably overexpressing or knocking down specific circRNAs, most of which were achieved by transfection with lentivirus plasmids, into nude mice. One study performed subcutaneous inoculations of RCC cell lines stably depleting circ_000926 by transfection with siRNA against circ_000926 and observed inhibitory effects on tumor growth (81). Findings from *in vivo* experiments further confirmed the potential of dysregulated circRNAs as therapeutic targets for RCC. Nevertheless, more efforts are needed to fully evaluated the safety and specificity of these ectopic circRNA intervention strategies.

CircRNAs and DKD

Hyperglycemia is an essential contributor for the pathogenesis of DKD and additional mechanisms, such as oxidative stress, inflammation, participate in the development of DKD as well (90). Almost all types of kidney resident cells are affected and altered under the diabetic milieu. The studies evaluating the functional relevance of circRNAs in DKD have just begun to be increasing in recent 2 years (**Table 3**). Most adopted *in vitro* cell culture models induced by HG stimulation, among which mesangial cell lines were the most frequently used. MCs proliferation, ECM production, and fibrosis were the most frequently studied biological phenomena, and most circRNAs identified to date were found to be increasingly expressed in DKD. For instance, the first study regarding circRNA in DKD showed that circRNA_15698 was significantly up-regulated in both HG-treated MCs and DKD mice (67). CircRNA_15698 knockdown suppressed the synthesis of fibrosis-related proteins in HG-treated MCs, suggesting this circRNA positively regulate the fibrotic process. Further experiments revealed that circRNA_15698 functioned as a sponge for miR-185 and subsequently increased the expression of its target gene TGF-β1, one of the master regulators in fibrosis, leading to enhanced ECM accumulation in DKD. The second relevant study found circLRP6 was highly expressed in HG-treated MCs and could promote cell proliferation, oxidative stress, ECM accumulation, and inflammation (91). This circRNA exerted its functions by sponging miR-205 to activate the classical pro-inflammatory TLR4/NF-κB pathway. The elevated expression of circ_0080425 exerted positive effect on cell proliferation and fibrosis in MCs via sponging miR-24-3p to up-regulate FGF11 (69). Circ_0000491 aggravated ECM and fibrosis-associated protein synthesis through suppressing miR-101b which targets TGFβRI (68). Circ_0123996 was able to promote MCs proliferation and fibrosis through functioning as the sponge for miR-149-5p and inducing Bach1 expression (92). Circ_00037128/miR-17-3p/AKT3 axis also facilitated DKD progression via modulating MCs proliferation and fibrosis (93). On the contrary, another two circRNAs, circ-AKT3 and circ_LARP4, were found to be down-regulated in HG-stimulated MCs model (94, 95). Circ-AKT3 inhibited ECM accumulation via modulating miR-296-3p/E-cadherin signals, while circ_LARP4 overexpression could repress MCs proliferation and fibrosis but increase cell apoptosis by sponging miR-424. As discussed above, the circ-AKT3/miR-296-3p/E-cadherin axis also plays a role in suppressing renal cancer metastasis (82), implying that circRNA-mediated regulatory networks might function under diverse disease conditions. Though current data was mainly derived from MCs, circRNAs may also play a role in other kidney cell types, such as tubular epithelial cells (TECs) and podocytes. The expression of several circRNAs, including hsa_circ_0003928, circ_WBSCR17, circACTR2, and circEIF4G2, were shown to be increased by HG stimulation in TECs model (96–99). Interference of hsa_circ_0003928 alleviated HG-induced secretion of inflammatory cytokines and repressed apoptosis in TECs by sponging miR-151-3p partly through regulating Anxa2, suggesting this circRNAs could positively modulate inflammation and apoptosis (96). Circ_WBSCR17 aggravated HG-induced inflammatory responses and fibrosis in HK-2 cells by activating SOX6 via targeting miR-185-5p (97). CircACTR2 was shown to promote HG-induced pyroptosis, inflammation, and fibrosis in TECs (98), yet the underlying mechanisms remain to be defined. CircEIF4G2 positively regulated the synthesis of fibrosis-related proteins in HG-induced TECs via the miR-218/SERBP1 pathway (99). One circRNA, circ_0000285, was found to be significantly increased in podocytes treated with HG as well as in DKD mouse kidneys (100). Up-regulation of circ_0000285 contributed to the development of DKD by triggering podocyte injuries through sponging miR-654-3p and activating MAPK6. Interestingly, results of several studies in DKD indicated that different circRNAs could act as the same miRNA's sponge. For example, miR-143 was confirmed to be targeted by both circ_DLGAP4 and circ_0000064 in MCs, whose increased expression consistently promoted cell proliferation and fibrosis (71, 101). As mentioned above, circ_WBSCR17 functioned through sponging miR-185-5p in TECs. Another study revealed that circHIPK3 could target miR-185-5p in MCs (102). These two circRNAs exhibited promotive functions on fibrosis both through negatively regulating miR-185-5p, in tubulointerstitial and glomerular compartments, respectively. Nevertheless, circHIPK3 was demonstrated to protect TECs from HG-induced toxicity via sponging miR-326/miR-487a-3p in another report (103), suggesting the same circRNA could regulate different miRNAs in different cell types and exert diverse effects. At present, there is a lack of research on *in vivo* manipulation of circRNA expression in DKD animal models. A recent study attempted to interfere with the expression of circRNA_010383 *in vivo* (104). They used a well-established ultrasound-microbubble-mediated gene transfer technique to specifically deliver the circRNA_010383 expression plasmid into the kidneys. By intermittent ultrasound-mediated circRNA_010383 transfer, its expression level was markedly restored in diabetic mouse kidneys, leading to amelioration of renal fibrosis. These studies suggest that circRNAs may play vital roles in the pathogenesis of DKD and can act as potential therapeutic targets for DKD, which undoubtedly requires further explorations.

TABLE 2 | Summary of candidate circRNAs as therapeutic targets in renal cell carcinoma (RCC).

CircRNA	Expression change	Function	Target miRNA	MiRNAs target genes/pathways	References
hsa_circ_0001451	Down	Inhibiting RCC cell proliferation and promoting apoptosis	ND	ND	(50)
circPCNXL2	Up	Promoting RCC cell proliferation, invasion, and tumor growth	miR-153	ZEB2	(51)
circ-ABCB10	Up	Promoting RCC cell proliferation and inhibiting apoptosis	ND	ND	(52)
hsa_circ_001895	Up	Promoting RCC cell proliferation, migration, invasion, and inhibiting apoptosis	miR-296-5p	SOX12	(53)
circ_001842	Up	Promoting RCC cell proliferation, migration, invasion, EMT, and tumor growth	miR-502-5p	SLC39A14	(54)
circPRRC2A	Up	Promoting RCC cell proliferation, migration, invasion, angiogenesis, EMT, tumor growth, and metastasis	miR-514a-5p, miR-6776-5p	TRPM3	(55)
circ-EGLN3	Up	Promoting RCC cell proliferation, migration, invasion, and inhibiting apoptosis	miR-1299	IRF7	(56)
circ_0001368	Down	Inhibiting RCC cell proliferation and invasion	miR-492	LATS2	(57)
cRAPGEF5	Down	Inhibiting RCC cell proliferation, migration, invasion, and tumor growth and metastasis	miR-27a-3p	TXNIP	(58)
circ_000926	Up	Promoting RCC cell proliferation, migration, invasion, EMT, and tumor growth	miR-411	CDH2	(81)
circ-AKT3	Down	Inhibiting RCC cell migration, invasion, and tumor metastasis	miR-296-3p	E-cadherin	(82)
circ-0039569	Up	Promoting RCC cell proliferation, migration, and invasion	miR-34a-5p	CCL22	(83)
circ-ZNF609	Up	Promotes RCC cell proliferation and invasion	miR-138-5p	FOXP4	(84)
hsa_circ_0054537	Up	Promoting RCC cell proliferation, migration, and inhibiting apoptosis	miR-130a-3p	c-Met	(85)
circHIAT1	Down	miRNA reservoir; inhibiting RCC cell migration and invasion	miR-195-5p/29a-3p/29c-3p	CDC42	(86)
circATP2B1	Down	miRNA reservoir; inhibiting RCC cell invasion	miR-204-3p	FN1	(87)
hsa-circ-0072309	Down	Inhibiting RCC cell proliferation, migration, invasion, and promoting apoptosis	miR-100	PI3K/AKT, mTOR	(88)
hsa_circ_0035483	Up	Promoting autophagy and the resistance of RCC to gemcitabine	hsa-miR-335	CCNB1	(89)

RCC, renal cell carcinoma; ND, not determined; EMT, epithelial–mesenchymal transition.

CircRNAs and AKI

Although accumulating evidence suggests that circRNAs are aberrantly expressed in AKI animal models and clinical samples (59, 66), there are few studies to characterize their roles in the pathogenesis of AKI. One study found a novel circRNA, circular antisense non-coding RNA in the INK4 locus (cANRIL), was induced by lipopolysaccharides (LPS) treatment in HK-2 cells (105). Silencing cANRIL alleviated LPS-induced inflammatory injuries and oxidative stress in HK-2 cells by blocking NF-κB and c-Jun N-terminal kinase (JNK)/p38 pathways via increasing miR-9 expression. Another study performed high-throughput RNA sequencing using renal tubular tissues from cisplatin-induced AKI mice models and identified a novel circRNA (circ-0114427) in human by comparing homologous genes between mouse and human (106). Circ-0114427 was remarkably increased in several AKI cell models and could exert anti-inflammatory

TABLE 3 | Summary of candidate circRNAs as therapeutic targets in diabetic kidney disease (DKD).

CircRNA	Expression change	Function	Target miRNA	MiRNAs target genes/pathways	References
circRNA_15698	Up	Promoting ECM-related proteins synthesis	miR-185	TGF-β1	(67)
circ_0000491	Up	Promoting ECM-related proteins synthesis	miR-101b	TGFβRI	(68)
circ_0080425	Up	Promoting MCs proliferation and fibrosis	miR-24-3p	FGF11	(69)
circ_DLGAP4	Up	Promoting MCs proliferation and fibrosis	miR-143	ERBB3/NF-κB/MMP-2	(71)
circLRP6	Up	Promoting MCs proliferation, oxidative stress, ECM accumulation, and inflammation	miR-205	HMGB1/TLR4/NF-κB pathway	(91)
circ_0123996	Up	Promoting MCs proliferation and fibrosis	miR-149-5p	Bach1	(92)
circ_0037128	Up	Promoting MCs proliferation and fibrosis	miR-17-3p	AKT3	(93)
circ-AKT3	Down	Inhibiting ECM-related proteins synthesis	miR-296-3p	E-cadherin	(94)
circ_LARP4	Down	Inhibiting MCs proliferation and fibrosis and promoting MCs apoptosis	miR-424	ND	(95)
hsa_circ_0003928	Up	Promoting TECc apoptosis and inflammation	miR-151-3p	Anxa2	(96)
circ_WBSCR17	Up	Promoting TECs apoptosis, inflammation, and fibrosis	miR-185-5p	SOX6	(97)
circACTR2	Up	Promoting TECs pyroptosis, inflammation, and fibrosis	ND	ND	(98)
circEIF4G2	Up	Promoting fibrotic markers synthesis	miR-218	SERBP1	(99)
circ_0000285	Up	Promoting podocyte injury	miR-654-3p	MAPK6	(100)
circ_0000064	Up	Promoting MCs proliferation and fibrosis	miR-143	ND	(101)
circHIPK3	Up	Promoting MCs proliferation and fibrosis	miR-185	ND	(102)
circHIPK3	Down	Promoting TECs proliferation and inhibiting apoptosis	miR-326/miR-487a-3p	SIRT1	(103)
circRNA_010383	Down	Inhibiting ECM-related proteins synthesis	miR-135a	TRPC1	(104)

ECM, extracellular matrix; MCs, mesangial cells; ND, not determined; TECs, tubular epithelial cells.

effects in early stages of AKI development. Mechanistically, circ-0114427 directly sponged miR-494 to up-regulate the expression of activating transcription factor 3 (ATF3) and then inhibited the secretion of downstream cytokine IL-6. These findings identified a novel circ-0114427/miR-494/ATF3/IL-6 regulatory axis in AKI progression. Different from the roles in DKD and RCC, circ-AKT3 was shown to promote cell apoptosis and enhance oxidative stress in AKI induced by I/R treatment (107). Results of this study revealed that circ-AKT3 could activate the Wnt/β-catenin signal via functioning as a sponge for a different miRNA (miR-144-5p), implying that the same circRNA could regulate different miRNAs to exert multifaceted functions under diverse pathological circumstances. Of note, the research regarding the roles of circRNAs in AKI is still in the preliminary stage, and more in-depth research is warranted in the future.

CircRNAs and LN

As discussed above, many circRNAs were found to be differentially expressed in patients with SLE or LN. These dysregulated circRNAs may play roles in the pathogenesis and progression of SLE and LN. However, only two studies have made initial attempts to explore the functions of circRNAs in LN at present. One study identified that circHLA-C was the most significantly increased circRNA in LN and displayed a tendency of negative correlation with miR-150 (80). Further bioinformatic analysis revealed circHLA-C harbored

a perfect match binding sequence for miR-150. Since miR-150 was previously reported to promote renal fibrosis in LN, these results suggest that circHLA-C might participate in the development of LN by sponging miR-150. The other study found hsa_circ_0123190 was down-regulated in LN and could serve as a sponge for hsa-miR-483-3p, which targets apelin receptor (APLNR) (62). APLNR has been shown to be involved in renal fibrosis by acting on TGF-β1 and its expression was associated with chronicity index (CI) of LN. Therefore, hsa_circ_0123190 might contribute to renal fibrosis in LN by modulating the hsa-miR-483-3p/APLNR/TGF-β1 axis. Nevertheless, results of these two studies are relatively preliminary. Future investigations by gain-of-function and loss-of-function *in vitro* and *in vivo* experiments are needed to clarify the functional roles of candidate circRNAs as potential therapeutic targets in LN.

CircRNAs and Other Kidney Diseases

Focal and segmental glomerulosclerosis (FSGS), a common histopathological lesion which is characterized by segmental glomerular scarring, is one of the leading causes of adult nephrotic syndrome and ESRD worldwide. One study found that circZNF609 was up-regulated in both adriamycin-induced FSGS mouse kidneys and bovine serum albumin (BSA)-treated HK-2 cells, while miR-615-5p showed the opposite trend (108). The intra-renal expression of circZNF609 was positively correlated while miR-615-5p was negatively correlated with podocyte injury and renal fibrosis. Moreover, perfect match sequences between circZNF609 and miR-615-5p were predicted by bioinformatics tools. These results suggest circZNF609 might be involved in the pathogenesis of FSGS by targeting miR-615-5p. However, these preliminary findings are simply correlation data of expression changes, which undoubtedly need to be validated in future functional experiments.

Hypertension is a common chronic disease with a high prevalence in the general population which frequently produces adverse effects on certain target organs, including heart, blood vessels, and kidney. Recent studies indicate many circRNAs were aberrantly expressed in blood samples of hypertensive patients or kidneys of hypertensive models (109–111). Among them, circNr1h4 derived from the Nr1h4 gene was significantly decreased in hypertensive mouse kidneys (111). Mechanistic investigations revealed that circNr1h4 modulated renal injury by sponging miR-155-5p to regulate its target gene fatty acid reductase 1 (Far1). These results provide novel insights into underlying mechanisms of hypertension and hypertensive nephropathy, which require further investigations.

Vascular calcification (VC) is one of the common complications in CKD patients. One study using RNA sequencing identified that a large number of circRNAs changed significantly in a cellular model of VC (112). Among them, circSamd4a played an anti-calcification role in VC as overexpressing it could reduce VC. Mechanistically, circSamd4a acted as a sponge for miR-125a-3p and miR-483-5p to regulate downstream genes related to calcium modulation. This study provides novel mechanisms for the development of VC and circSamd4a may serve as a promising therapeutic target for VC in CKD patients since it is conserved in humans.

CONCLUSIONS AND FUTURE PERSPECTIVES

Recent advances in the circRNA research field have uncovered their diversified functions in health and disease. In the past 5 years, increasing endeavors have identified numerous circRNAs involved in the pathogenesis and progression of various kidney diseases. These putative circRNAs function mainly through circRNA-miRNA-mRNA networks, expanding our understanding of regulatory mechanisms underlying kidney disorders. In addition, owing to their impressive stability, circRNAs have been validated as reliable biomarkers for diagnosis and prognosis prediction of multiple kidney diseases. Results of these investigations support that circRNAs possess the promising potential as both biomarkers and therapeutic targets in the kidney realm.

However, we have to admit that there are shortcomings regarding current research and many challenges remain to be overcome in this field. Firstly, most studies investigating circRNAs as biomarkers are from single-center, with small sample sizes, and lack of independent external cohort validation. Larger independent cohorts from multi-center settings are highly desirable to validate candidate circRNAs as promising biomarkers. In addition, there are few studies searching for biomarkers to monitor treatment efficacy or predict relapse of glomerular diseases, including nephritic syndrome, lupus nephritis, and vasculitis-associated renal lesions. Exosomal circRNAs in blood and urine are promising biomarkers for non-invasive liquid biopsy and more standardized techniques are desired to reliably detect these exosomal circRNAs. Secondly, the vast majority of aberrantly expressed circRNAs have not been studied functionally and those possibly as hub genes in signaling pathways underlying kidney diseases remain to be identified. To date, most studies on circRNAs are focused on their function as miRNA sponges. It is likely that those dysregulated circRNAs may function through other regulatory mechanisms, such as transcription regulation or acting as RBP sponges, which warrants further research. Last but not the least, the biological functions and therapeutic potential of candidate circRNAs need to be verified in animal models, including non-human primates. There is still lack of ways to specifically and efficiently deliver circRNAs into recipient cells *in vivo* and whether interfering specific circRNAs expression would produce off-target effects remains uncharacterized. Nevertheless, it is becoming clear that increasing exploration into the potential roles of circRNAs will extend our understanding of kidney diseases and hopefully will become an intense area of research in the near future.

AUTHOR CONTRIBUTIONS

JY and DX wrote the manuscript. NH drew the figure and tables. QZ supervised the manuscript. All authors contributed to article design, revision, read, and approved the submitted version.

FUNDING

This study was supported by program from Guangdong Basic and Applied Basic Research Foundation (2020A1515010247); Guangzhou Science and Technology Innovation Commission (201806010123); Kelin Young Talents Program of the First Affiliated Hospital of Sun Yat-sen University (Y50179); National Key R&D Program of China (2016YFC0906101); Operational Grant of Guangdong Provincial Key Laboratory (2017B030314019); Guangdong Provincial Programme of Science and Technology (2017A050503003 and 2017B020227006); Guangzhou Municipal Programme of Science and Technology (201704020167); Natural Science Foundation of Guangdong Province, China (Grant No. 2017A030310199); and National Natural Science Foundation Grant (82170732).

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frontiers in Medicine

REVIEW
published: 21 September 2021
doi: 10.3389/fmed.2021.747922

The Active Compounds and Therapeutic Target of *Tripterygium wilfordii Hook. f.* in Attenuating Proteinuria in Diabetic Nephropathy: A Review

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: 27 July 2021
Accepted: 25 August 2021
Published: 21 September 2021

Citation:
Liu P, Zhang J, Wang Y, Shen Z, Wang C, Chen D-Q and Qiu X (2021) The Active Compounds and Therapeutic Target of Tripterygium wilfordii Hook. f. in Attenuating Proteinuria in Diabetic Nephropathy: A Review. Front. Med. 8:747922. doi: 10.3389/fmed.2021.747922

Tripterygium wilfordii Hook. f. (TWHF) is a traditional Chinese herbal medicine and widely used to treat diabetic kidney disease in China. Emerging evidences have revealed its ability to attenuate diabetic nephropathy (DN). Tripterygium wilfordii polyglycosides (TWPs), triptolide (TP), and celastrol are predominantly active compounds isolated from TWHF. The effects and molecular mechanisms of TWHF and its active compounds have been investigated in recent years. Currently, it is becoming clearer that the effects of TWHF and its active compounds involve in anti-inflammation, anti-oxidative stress, anti-fibrosis, regulating autophagy, apoptosis, and protecting podocytes effect. This review presents an overview of the current findings related to the effects and mechanisms of TWHF and its active compounds in therapies of DN, thus providing a systematic understanding of the mechanisms and therapeutic targets by which TWHF and its active compounds affect cells and tissues *in vitro* and *in vivo*.

Keywords: diabetic nephropathy, *Tripterygium wilfordii Hook f.*, tripterygium wilfordii polyglycosides, triptolide, celastrol

INTRODUCTION

Diabetic nephropathy (DN) is defined as decreased renal function with persistent clinically detectable proteinuria (1). As a serious microvascular complication of types 1 or 2 diabetes mellitus (DM), DN occurs in ∼25–40% of patients with DM, and has become the leading cause of end-stage renal disease (ESRD) in China (2, 3). Approximately 463 million people suffers from DM worldwide in 2019, and are expected to raise up to 700 million untill 2045 (4).

Proteinuria, an independent risk factor of disease progression, is the most important clinical characteristic of DN. The presence of microalbuminuria can increase all-cause mortality in patients with diabetes mellitus (DM) (5). Without early intervention, ∼50% of DM patients with microalbuminuria will progress to macroalbuminuria (6, 7). Although several recent studies have confirmed that angiotensin-converting enzyme inhibitors (ACEIs)/angiotensin receptor blockers (ARBs) can reduce DN proteinuria and delay disease progression (8, 9), these have been shown to be ineffective in DN patients with normal blood pressure (10).

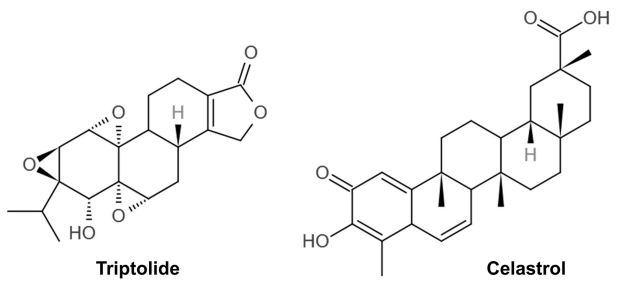

FIGURE 1 | The chemical structure of triptolide and celastrol.

Various traditional Chinese herbal medicine (CHM) has been shown to be effective in the treatment of proteinuria (11, 12). *Tripterygium wilfordii Hook. f.* (TWHF), also known as Lei Gong Teng, is a traditional CHM which is widely used in the treatment of the inflammation and autoimmune disorders (13–15). Based on its diverse pharmacological activities, TWHF has been used to treat different diseases, such as cancer, rheumatoid arthritis, and Crohn's disease (16–18). Recent experimental and clinical studies have demonstrated that TWHF could significantly reduce proteinuria, protect renal function, and attenuate kidney injury (19–21).

Several randomized controlled clinical trials have found that TWHF possibly imparts nephroprotective effects by decreasing proteinuria, serum creatinine (Scr) levels, and blood urea nitrogen (BUN) levels (22–24). A network pharmacology research showed that TWHF may play a role in treating DN through AGE-RAGE signaling pathway, TNF signaling pathway, IL-17 signaling pathway, insulin resistance, and calcium signaling pathway (25). However, the underlying mechanisms by which TWHF and its active compounds attenuate proteinuria in DN remain unclear. This review discusses the molecular mechanisms of TWHF therapies in proteinuria in DN.

MAIN ACTIVE COMPOUNDS OF TWHF

TWHF belongs to genus Tripterygium of family celastraceous, and its main bioactive ingredients include terpenoids, tripterygium wilfordii polyglycosides (TWPs), lignans, glycosides, and alkaloids. The terpenoids of TWHF are constituted by sesquiterpenes, diterpenes (triptonide, tripdiolide, and triptolide), triterpenes (wilforlide A, pristimerin, and celastrol) (26, 27).

TWPs, triptolide (TP) and celastrol, predominantly active natural products isolated from TWHF, are mainly used to treat DN (**Figure 1**). As the fat-soluble mixture extracted from the root of TWHF, TWPs are the first CHM studied and used in anti-inflammatory and immune regulation (28). In 1972, Kupchan et al. first isolated and characterized TP from TWHF (26). Celastrol was first isolated from TWHF for the activator of the mammalian heat shock transcription factor 1 (29). The pharmacological activities and mechanisms of TWHF and its active compounds have been extensively investigated in many kidney disease models (**Table 1**, **Figures 2**, **3**).

EFFECTS, MECHANISMS, AND THERAPEUTIC TARGETS OF TWPS AGAINST PROTEINURIA AND KIDNEY INJURY IN DN

Anti-inflammatory Effects

Chronic systemic inflammation is associated with kidney injury, and animal and human studies have established that inflammation is a cornerstone in the development and progression of DN (68, 69). Inflammation can alter or interfere with the regulation and perfusion distribution can induce kidney injury, thereby enhancing the DN progression. Overproduction of Advanced glycation end products (AGEs)or damage from degradation may activate inflammation, which, in turn, promotes DN (70). Thus, the regulation of inflammation is key to the development of treatment schemes for kidney disease.

TWPs exhibit anti-inflammation activity in DN rats. TWPs improve renal inflammatory injury in DN rats by reducing the levels of inflammatory cytokines, such as IL-1, IL-17 and interferon- γ (IFN-γ) (30). TWPs downregulate TNF-α, whereas it upregulated IL-4 (anti-inflammatory T-helper cell type 2 cytokine) in renal tissues (31). The JAK2/STAT3 signaling pathway regulates a broad range of biological effects such as cell proliferation, differentiation, inflammation, and apoptosis (71). Inhibiting JAK2/STAT3 activation, which contributes to the pathogenesis of DN, has been shown to be a novel therapeutic scheme for the treatment of this disease (72). In DN rats, TWPs reduce the levels of BUN, Scr and improve kidney function, and also effectively blank the inflammatory response by inhibiting the activity of JAK/STAT pathway (32). Treatment with TWPs also inhibit inflammation via regulating the signal pathway of MAPK/NF-κB in renal tissues (33).

In bovine serum albumin induced chronic glomerulonephritis rat model, TWPs inhibit the inflammatory factor (TNF-α, IL-1β) expressions, and improve the renal pathological damage via regulating MAPK signaling pathway (34). In immunoglobulin A nephropathy (IgAN) rats, TWPs decrease the levels of serum IL-1β, IL-6, and reduce the pathological damage of renal tissue (35) (**Table 1**, **Figures 2**, **3**).

Antioxidative Stress Effects of TWPs

Oxidative stress is associated with inflammation in DN progression. The presence and severity of systemic inflammation contribute to kidney injury-related oxidative stress (73). Oxidative stress caused by the overaccumulation of reactive oxygen species (ROS) induces protein and nucleic acid damage, thereby leading to impaired cellular damage and tissue pathology (74). The mitochondria are the major sources of ROS as well as the main targets of ROS (75). The damaged mitochondria with impaired respiration block the transfer of electrons along the respiratory chain, which then react with O_2 in upstream respiratory chain components to form superoxide free radicals and ROS (76). In response to the excessive production of ROS, mammalian cells have evolved various peroxidases that catalyze the conversion of intracellular hydrogen peroxide to water. These include catalase,

TABLE 1 | Pharmacological activities of *Tripterygium wilfordii Hook. f.* and active compounds against proteinuria and kidney injury in DN.

Natural product	Underlying mechanisms	Model	Experimental detail	Underlying targets	References
TWPs	Anti-inflammatory	STZ-induced DN male SD rats	9 and 18 mg/kg by gavage for 8 weeks	Reducing serum IL-1, IL-17, IFN-γ levels	(30)
		High-sugar and high-fat diet and STZ-induced DN male SD rats	6, 12, and 24 mg/kg by gavage for 4 weeks	Reducing renal TNF-α expressions, increasing renal IL-4 expressions	(31)
		High-sugar and high-fat diet and STZ-induced DN male SD rats	8 mg/kg by gavage for 8 weeks	Inhibiting the activity of JAK/STAT pathway	(32)
		STZ-induced DN male SD rats	8 mg/kg by gavage for 4 weeks	Inhibiting the activity of MAPK/NF-κB pathway	(33)
		Fetal Bovine serum albumin induced chronic glomerulonephritis Wistar rats	15 mg/kg by gavage for 4 weeks	Inhibiting the activity of p38MAPK pathway	(34)
		Fetal bovine serum albumin to stimulate activated macrophages induced IgAN Wistar rats	20 mg/kg by gavage for 4 weeks	Reducing serum IL-1β, IL-6 levels	(35)
	Antioxidative stress	STZ-induced DN male SD rats	4.5, 9, and 18 mg/kg by gavage for 8 weeks	Reducing renal MDA expressions, increasing renal GPxs expressions	(36)
	Anti- fibrosis	High-sugar and high-fat diet and STZ-induced DN male SD rats	50 mg/kg by gavage for 16 weeks	Reducing renal TGF-β1 and gremlin expressions, increasing renal BMP-7 expressions	(37)
		Male db/db mice	25, 50, and 100 mg/kg by gavage for 8 weeks	Promoting AKT/mTOR pathway	(38)
		STZ-induced DN male SD rats	50 mg/kg by gavage for 8 weeks	Inhibiting renal RhoA and Rock1 expressions	(39)
		Unilateral ureteral obstruction SD rats	10 mg/kg by gavage for 14 days	Inhibiting renal miR-192 and collagen I expressions	(40)
	Anti- podocyte apoptosis	High-sugar and high-fat diet and STZ-induced DN male SD rats	1, 3, and 6 mg/kg by gavage for 8 weeks	Reducing renal VEGF expressions, increasing renal nephrin and podocin expressions	(41)
		Adriamycin- induced nephropathy male SD rats	50 mg/kg by gavage for 8 weeks	Increasing renal nephrin and CD2AP expressions	(42)
		Sunitinib-induced podocytes	40 ng/ml for 48 h	Increasing celluer nephrin and CD2AP expressions	(43)
TP	Anti-inflammatory	High-sugar and high-fat diet and STZ-induced DN male Wistar rats	100 μg/kg by gavage for 8 weeks	Inhibiting of inflammation and macrophage infiltration	(44)
		Cationic bovine serum albumin induced MN male SD rats	200 μg/kg by gavage for 4 weeks	Inhibiting NF-κB Signaling Pathway	(20)
		Fetal bovine serum albumin to stimulate activated macrophages induced IgAN male Wistar rats	200 μg/kg by gavage for 16 weeks	Reducing serum TNF-α, IL-17A, IFN-γ, and IL-4 levels, inhibiting renal NLRP3, and TLR4 expressions	(45)
		Bovine gamma globulin induced IgAN male SD rats	100 and 200 μg/kg by gavage for 8 weeks	Reducing serum IL-1β and IL-18 levels, inhibiting renal IL-1β, Case-1, IL-18, and NLRP3 expressions	(46)
		Female MRL/lpr lupus mice	125 μg/kg by gavage for 9 weeks	Inhibiting renal JAK1/STAT1 Pathway	(47)
LLDT-8 (a TP derivative)		Female MRL/lpr lupus mice	125 μg/kg/2 d by gavage for 9 weeks	Reducing renal IFN-γ, IL-17, IL-6, and TNF-α expressions	(48)
		Murine anti-glomerular basement membrane (GBM) glomerulonephritis male NZW parental mice	125 μg/kg/2 d by gavage for 14 days	Promoting renal Fcγ receptor signaling	(49)

(Continued)

TABLE 1 | Continued

Natural product	Underlying mechanisms	Model	Experimental detail	Underlying targets	References
TP	Antioxidative stress	High-sugar and high-fat diet and STZ-induced DN male SD rats	200 μg/kg by gavage for 8 weeks	Reducing renal COX-2 and iNOS expressions	(50)
		STZ-induced DN male SD rats	200 μg/kg by gavage for 4 weeks and 8 weeks	Reducing renal NF-κB, iNOS, eNOS, and VEGF expressions	(51)
		Puromycin aminonucleoside-mediated PAN male SD rats	200 μg/kg by gavage for 21 days	Promoting renal RhoA signaling	(52)
	Anti- fibrosis	High-sugar and high-fat diet and STZ-induced DN male SD rats	100 μg/kg by gavage for 12 weeks	Inhibiting renal miR-137/Notch1 pathway	(19)
		High-fat diet and STZ-induced DN male SD rats	200 μg/kg by gavage for 12 weeks	Inhibiting renal miR-141-3p/PTEN/AKT/mTOR pathway	(53)
	Activating autophagy	STZ-induced DN male C57BL/6 mice	200 μg/kg by gavage for 12 weeks	Increasing renal Podocin, Bax, and Caspase-3 expressions	(54)
		Puromycin amino nucleotide-cultured mouse podocytes	100 ng/ml for 4 h	Inhibiting renal mTOR pathway	(55)
		algA1 from IgAN patients -cultured mouse podocytes	10 ng/ml for 24 h	Inhibiting cellular mTOR pathway	(56)
	Anti- podocyte apoptosis	Glucose and TGFβ1 -cultured mouse podocytes	0.5, 1, and 3 ng/ml for 36 h	Inhibiting phosphorylation of GSK3β	(57)
		Glucose cultured mouse podocytes	8, 16, and 32 ng/ml for 24 h	Increasing cellular nephrin expressions	(58)
		Glucose cultured mouse podocytes	10 ng/ml for 48 h	Increasing cellular synaptopodin and desmin expressions	(59)
		Bovine serum albumin, carbon tetrachloride, and lipopolysaccharide induced IgAN male SD rats	100, 200, and 400 μg/kg by gavage for 4 weeks	Increasing renal nephrin and podocin expressions	(60)
Celastrol	Anti-inflammatory	STZ-induced DN male SD rats	50, 100 μg/kg by gavage for 4 weeks	Inhibiting the activity of MAPK/NF-κB pathway	(33)
		Male db/db mice	1 mg/kg by gavage for 8 weeks	Inhibiting the activity of NF-κB pathway	(61)
	Activating autophagy	High-sugar and high-fat diet and STZ-induced DN male SD rats	1.5 mg/kg by gavage for 4 weeks	Promoting renal PI3K/AKT pathway	(62)
		Glucose cultured mouse podocytes	0.1, 0.2, 0.6, 1.0, 1.5, and 2 μM for 48 h	Promoting cellular HO-1-mediated autophagy	(63)
TWPs	Improving renal hypoxia	STZ-induced DN male SD rats	8, 16 mg/kg, by gavage for 8 weeks	Reducing renal HIF-1α and endothelin-1 expressions	(64)
	Improving renal glucose transport	STZ-induced DN male SD rats	1.8 g/kg by gavage for 8 weeks	Reducing renal GLUT-1 expressions	(65)
TP	Improving renal glucose transport	STZ-induced DN male SD rats	1.8 g/kg by gavage for 8 weeks	Reducing renal GLUT-1 expressions, increasing renal GLUT-4 expressions	(66)
TWHF	Anti- fibrosis	STZ-induced DN male SD rats	8 g/kg, and 16 g/kg by gavage for 8 weeks	Inhibiting renal Wnt-1/β-catenin pathway	(67)

peroxiredoxins, and glutathione peroxidases (GPxs) (77). There is increasing evidence that oxidative stress contributes to DN progression (78, 79). TWPs up-regulate the levels of catalase in serum and GPxs in kidneys, and down-regulated the levels of malondialdehyde (MDA) in kidneys in the DN (36) (**Table 1**, **Figures 2**, **3**).

Anti-fibrosis Effects

Renal fibrosis is a highly complex process involving a variety of cell types including resident renal cells as well as infiltrating cells, such as macrophages, fibrocytes, and lymphocytes. Intracellular ROS generation in the context of diabetes initiates multiple inflammatory and profibrotic responses (80). Renal fibrosis in

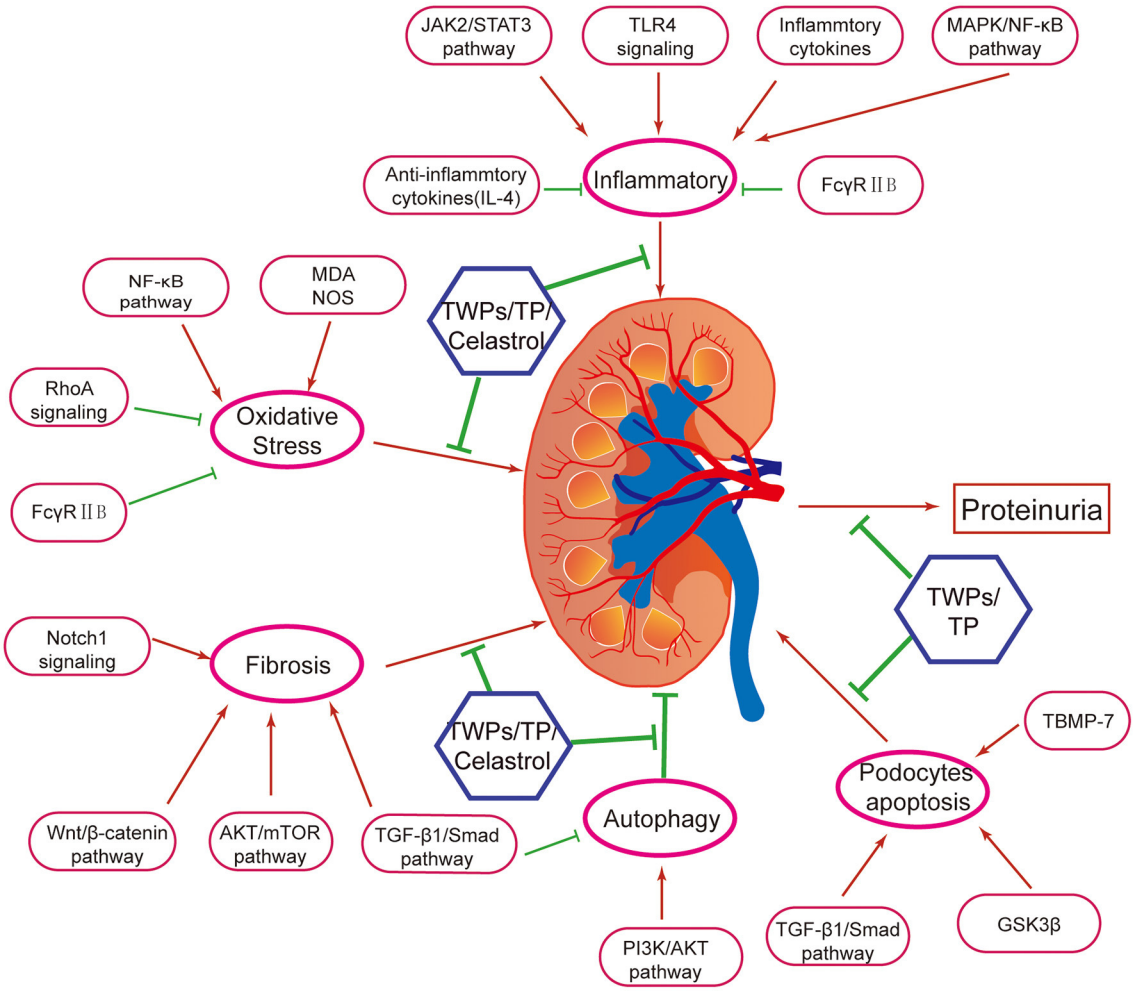

FIGURE 2 | Mechanisms of *Tripterygium wilfordii Hook. f.* and active compounds against proteinuria and kidney injury in DN. TWPs, TP, and Celastrol are the effective medicine against proteinuria and kidney injury in DN. Mechanisms of TWHF, TWPs, TP, and Celastrol are including anti-inflammation, antioxidation, anti-fibrosis, activating autophagy, and anti- podocyte apoptosis, via several mechanisms.

DN is caused by the accumulation of extracellular matrix (ECM) proteins, including predominantly various collagens, fibronectin, and laminin (81). Thickening of the glomerular basement membrane (GBM) is an early histopathological finding in DN (82). Altered GBM remnants contribute to the expansion of the mesangial matrix, but hyperglycemia also stimulates mesangial cells to proliferate and produce matrix by activating transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF), which directly induce the transcriptional activation of matrix collagens (83). It is currently believed that renal fibrosis develops in response to ECM accumulation due to epithelial-mesenchymal transition (EMT), TGF-β signaling, oxidative stress and proteinuria (84, 85).

TGF-β1/Smad signaling pathway plays a critical role in prolonged glomerulosclerosis, which is an important determinant during the progression in DN (86). Bone morphogenetic protein-7 (BMP-7) is a critical developmental and differentiation factor in the kidney, which can inhibit TGF-β signaling to ameliorate renal inflammation, apoptosis, and fibrosis after kidney injury (87, 88). In DN rats, TWPs ameliorate renal fibrosis by down-regulating the expression of TGF-β1 and gremlin (a BMP antagonist), and up-regulating the expression of BMP-7 (37). In db/db mice, TWPs reduce the serum levels of TC, TG, and LDL, glycated serum protein, BUN, Scr, and improve the renal injury by regulating AKT/mTOR pathway (38). And TWPs inhibit the expressions of RhoA and Rock1 to improve renal fibrosis in STZ-induced rats (39).

MicroRNAs (miRNAs) are a class of small non-coding RNAs that regulate gene expression by either downregulating mRNA levels or directly repressing translation of genes. Many miRNAs are corrected with renal injury in DN (89, 90). In unilateral ureteral obstruction rats, TWPs could attenuate renal fibrosis by inhibiting the expression of miR-192 and collagen I (40) (**Table 1**, **Figures 2**, **3**).

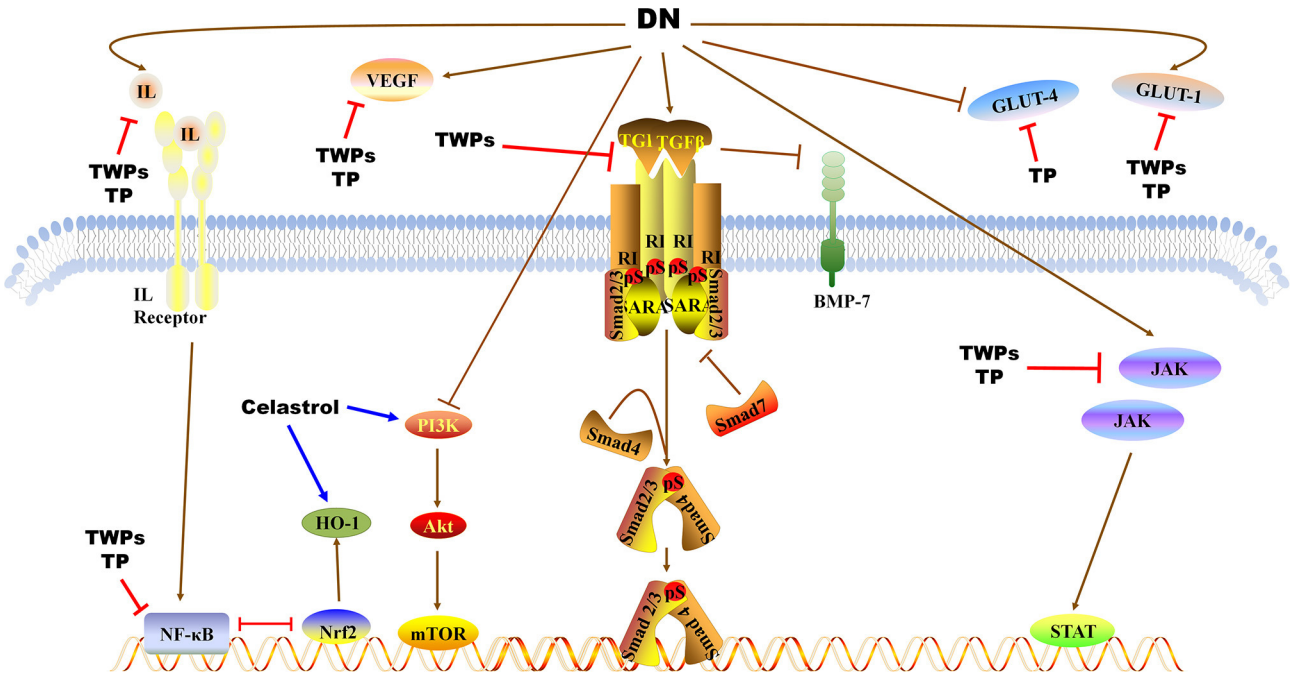

FIGURE 3 | Pathways of TWPs, TP, and Celastrol against proteinuria and kidney injury in DN. TWPs and TP attenuate proteinuria in DN by regulating JAK/STAT pathway, TGF-β1/Smad pathway and NF-κB pathway, and regulating the expressions of IL, VEGF, BMP-7, GLU-1, and GLU-4. Celastrol attenuates proteinuria in DN by regulating PI3K/AKT/mTOR pathway and regulating the expressions of HO-1.

Anti-podocyte Apoptosis Effects

Podocyte injury is a pathological feature in DN. Podocytes are highly specialized, terminally differentiated epithelial cells in the glomerular filtration barrier with interdigitating foot processes (FPs), and play a major role in preventing protein leakage into the Bowman space (91). Structural podocyte injury is central in the pathogenesis of most inherited and acquired glomerular diseases, which are all associated with decreased expression of slit diaphragm (SD) proteins, such as podocin, nephrin, synaptopodin, and CD2-associated protein (CD2AP) (92). These proteins are considered as critical components of epithelial SD and FPs and help maintain the integrity of podocytes in avoiding proteinuria (93). In addition, desmin is a component of the cytoskeleton and considered as a sensitive marker of injury in podocytes (94). DM induces podocytopathy, which is characterized by cellular hypertrophy, foot process effacement, and podocyte loss (6). Li et al. (41) showed using STZ-induced DN rats that TWPs could upregulate the expression of nephrin and podocin and suppress apoptosis in podocytes.

TWPs have also been shown to significantly reduce proteinuria and repair podocyte damage in rats with adriamycin-induced nephropathy, as well as facilitate mixing together of foot processes by upregulating nephrin and CD2AP (42). In addition, TWPs upregulates nephrin and CD2AP in sunitinib-induced podocytes (43) (**Table 1**, **Figures 2**, **3**).

EFFECTS, MECHANISMS, AND THERAPEUTIC TARGETS OF TP AGAINST PROTEINURIA AND KIDNEY INJURY IN DN

Anti-inflammatory Effects

Due to similar structures as hormones, TP can bind to nuclear receptors (95). This unique feature is the reason that triptolide is active to inflammation. Ma et al. (44) have shown that TP markedly attenuated proteinuria and renal injury in DN rats, which may have been correlated with the inhibition of macrophage infiltration and inflammation in the kidneys.

Chronic inflammation is also a common characteristic of membranous nephropathy (MN) and IgAN. Zhou et al. (20) concluded that TP significantly reduces the production of inflammatory cytokines (e.g., IL-1β, TNF-α, and monocyte chemotactic protein 1), and inhibits the NF-κB signaling pathway in MN rats. He et al. (45) declared that TP prevents IgAN progression via by ameliorating of inflammasome-mediated proinflammatory cytokine production by down-regulating Toll-like receptor 4 (TLR4) and nod-like receptor family pyrin domain-containing 3 (NLRP3) expression. In IgAN rats, TP decrease the levels of TNF-α, IL-17A, IFN-γ, and IL-4 in serum, reduce the expression of IL-1β, Caspase-1, IL-18, and NLRP3 in renal tissues (46).

In MRL/lpr lupus mice, TP also inhibition of inflammatory response, ameliorate renal damage, and the mediated by JAK1/STAT1 pathway is a possible molecular mechanism (47).

Zhang et al. (48) have shown that (5R)-5-hydroxytriptolide (LLDT-8, a TP derivative) provides therapeutic benefits to LN by suppressing chemokine expression and inhibiting immune cell infiltration in the kidneys of MRL/lpr mice. Moreover, LLDT-8 inhibits inflammation in the kidneys by downregulating the cytokines IL-6, IL-17, TNF-α, and IFN-γ and upregulating FcγRIIB in the kidneys of a murine anti-glomerular basement membrane (GBM) glomerulonephritis model (49) (**Table 1**, **Figures 2**, **3**).

Antioxidative Stress Effects

TP effectively attenuates the levels of blood glucose, Scr and proteinuria by reducing the expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in renal tissues of DN rats (50). NF-κB is a redox-sensitive transcription factor that responds to ROS at various sites within the signaling pathway such as by activating or inactivating the inhibitory κB kinase complex, which, in turn, affects downstream targets or activates NF-κB via alternative inhibitor κBα phosphorylation (96). TP protects glomerular endotheliocytes of DN by inhibiting the expression of NF-κB, iNOS, endothelial nitric oxide synthase (eNOS), and VEGF (51).

RhoA, a redox sensitive master regulator protein, regulates numerous biological functions (97). Due to lipid peroxidation is a major form of oxidative stress in diabetes, restoring normal RhoA activity levels prevents podocyte loss and consequent proteinuria in DN (98). Zheng et al. (52)concluded that TP ameliorated puromycin amino nucleoside-mediated podocyte injury by suppressing ROS generation and p38 mitogen-activated protein kinase activation while restoring RhoA signaling activity *in vivo* and *in vitro* (**Table 1**, **Figures 2**, **3**).

Anti-fibrosis Effects

The Notch1 signaling plays a core role in the formation of mesangial cells during kidney development, and exacerbates renal tubulointerstitial fibrosis in DN (99). Han et al. (19) declared that TP has anti-glomerulosclerosis effects by suppressing miR-137/Notch1 pathway in DN rats. In addition, renal fibrosis can be regulated through autophagy, a biological regulatory program that maintains homeostasis (100). Phosphatase and tensin homolog deleted on chromosome ten (PTEN) plays an essential role in regulating of AKT/ mammalian target of rapamycin (mTOR) signaling (101). Li et al. (53) found that TP alleviates renal fibrosis by restoring autophagy through the miR-141-3p/PTEN/AKT/mTOR pathway in DN rats (**Table 1**, **Figures 2**, **3**).

Autophagy Regulatory Effects

Autophagy is a highly conserved and lysosome-dependent bulk degradative pathway that participates in the clearance of damaged organelles and proteins, as well as in maintaining homeostasis in tubules and glomeruli (102). Deficiency in autophagy aggravates DN in rodent models. STZ-induced autophagy-deficient mice develop severe microalbuminuria, endothelial lesions, and podocyte damage (103). High-fat diet-induced podocyte-specific autophagy-deficient mice develop hyperglycemia with proteinuria and podocyte damage. Autophagy contributes to the degradation of AGEs and suppresses inflammation in the kidneys (104). Moreover, increased ROS enhances autophagy by controlling the activity of Atg4, a family of cysteine proteases that is essential for autophagy formation (105). ROS promotes autophagy through the activation of AMP-activated protein kinase (AMPK), likely via suppression of mTOR (106). Experimental evidence has shown that autophagy acts as a double-edged sword with regard to cell death and survival because it is accompanied by other forms of cell death such as apoptosis (107). The ratio of LC3 I to LC3 II is closely correlated with the extent of autophagosome formation; therefore, LC3 II could be a marker of autophagic activity (108). In STZ-induced rats, TP decrease the expression of LC3 II, inhibite autophagy by upregulating PI3K/Akt/mTOR pathway (54). In puromycin amino nucleotide-cultured podocytes, TP reduces podocyte injury via the mTOR-autophagy pathway to increase autophagy levels and facilitates podocyte recovery from injury (55). Autophagy may be regulated by mTOR complex 1 (mTORC1) (109). Haploinsufficiency of mTORC1 in podocytes or administration of rapamycin (a mTORC1 inhibitor), resulting in the activation of autophagy, has been shown to prevent progressive DN (106). Conversely, the activation of mTORC1 in podocytes, which results in the inhibition of autophagy, leads to accelerated DN (110). Furthermore, Liang et al. found that TP protects podocyte autophagy by suppressing the mTOR and AKT pathways in IgAN (56) (**Table 1**, **Figures 2**, **3**).

Anti-podocyte Apoptosis Effects

In glucose and TGFβ1-cultured mouse podocytes, TP protected podocytes against diabetic milieu-elicited injury, mitigated cytoskeleton derangement, and preserved podocyte filtration barrier function via inhibiting phosphorylation of GSK3β (57). In glucose-cultured mouse podocytes, TP increases renal synaptopodin, desmin, and nephrin expressions to ameliorate podocyte injury (58, 59). Similarly, TP could significantly decrease proteinuria and upregulate nephrin and podocin mRNA and protein expression in rats with IgAN, suggesting that TP could reduce podocyte injury and repair glomerular filtration membrane barrier damage (60) (**Table 1**, **Figures 2**, **3**).

EFFECTS, MECHANISMS, AND THERAPEUTIC TARGETS OF CELASTROL AGAINST PROTEINURIA AND KIDNEY INJURY IN DN

Anti-inflammatory Effects

As one of triterpenes in TWHF, Celastrol reduces levels of Scr, BUN and proteinuria, inhibits inflammation by regulating MAPK/NF-κB pathway in STZ-induced rats (33). In db/db mice, Celastrol improves insulin resistance and attenuates renal injury by inhibiting the NF-κB-mediated inflammatory (61) (**Table 1**, **Figures 2**, **3**).

Autophagy Regulatory Effects

The PI3K/AKT pathway is one of the most important signaling pathways that regulate autophagy, and phosphorylated AKT can

promote the formation of p-mTOR to inhibit cell autophagy (111). In STZ-induced rats, Celastrol attenuates renal injury by promoting the PI3K/AKT pathway to activate autophagy (62). As a proverbial cytoprotective enzyme, heme oxygenase-1 (HO-1) ameliorates cell injury and inflammation in podocytes via activating autophagy pathway. Celastrol protects against high glucose-induced podocyte injury by restoring HO-1-mediated autophagy pathway (63) (**Table 1**, **Figures 2**, **3**).

OTHER EFFECTS OF TWHF AND ITS MAIN BIOACTIVE INGREDIENTS

Glomerular hypertension and tubulointerstitial hypoxia occur following DN, causing loss of glomerular integrity and tubular damage (112). Hypoxia inducible factor 1 α (HIF-1α) plays a regulatory role in cellular response to renal hypoxia. Chen et al. (64) drew a conclusion that TWPs decreased levels of Scr, BUN, 24-h UAlb, mean glomerular area and mean glomerular volume; improved renal histopathology; and down-regulated the expression of HIF-1α and endothelin-1 mRNA and protein in the kidneys of diabetic rats. HIF-1α activation under hypoxia could upregulate downstream glucose transporter 1 (GLUT-1) gene (113). TWPs and TP significantly reduce proteinuria and GLUT-1 levels in glomerular mesangial and epithelial cells of DN rats (65, 66).

Wnt/β-catenin signaling is an evolutionary conserved signaling pathway, which plays a core role in modulating kidney injury and repair (114). In DN rats, Chang et al. drew a conclusion that TWHF mitigates hyperglycemia-induced upregulated Wnt-1 and β-catenin expression in kidney tissues and ameliorates kidney injury (67) (**Table 1**, **Figures 2**, **3**).

CONCLUSIONS

In this review, we have summarized currently available information on the effects of TWHF on DN. Experimental studies have demonstrated that TWHF interacts with a wide range of cellular processes such as inflammation, oxidative stress, fibrosis, apoptosis, autophagy, and podocytes, indicating that these mechanisms are involved in a variety of cellular signals. Although several genes and proteins involved in the effect of TWHF on cells and tissues have been identified, many of the targets and exact mechanisms participating in these events remain unknown. Further studies regarding the mechanism of DN with TWHF treatment are thus warranted. Its narrow therapeutic window and severe side effects restrict its clinical applications (26, 27). Therefore, hepatotoxicity and sexual inhibition may occur among patients who have used TWHF long term, thus requiring regular monitoring, and if necessary, a reduction in dose or possibly termination of its use.

AUTHOR CONTRIBUTIONS

PL, JZ, D-QC, and XQ mainly drafted the work critical for important intellectual content. YW, ZS, and CW finished the discussion. PL and JZ contributed equally to this work. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by supported by Research Projects of the National Natural Science Foundation of China (No. 81904174), China Postdoctoral Science Foundation (No. 2021M693579), and National Training Program for Innovative Key Talents of Traditional Chinese Medicine (No. 2019-128).

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frontiers in Medicine

REVIEW
published: 25 October 2021
doi: 10.3389/fmed.2021.751529

Lipotoxic Proximal Tubular Injury: A Primary Event in Diabetic Kidney Disease

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The pathogenesis of diabetic nephropathy is a complex process that has a great relationship with lipotoxicity. Since the concept of "nephrotoxicity" was proposed, many studies have confirmed that lipotoxicity plays a significant role in the progression of diabetic nephropathy and causes various renal dysfunction. This review will make a brief summary of renal injury caused by lipotoxicity that occurs primarily and predominantly in renal tubules during diabetic progression, further leading to glomerular dysfunction. The latest research suggests that lipotoxicity-mediated tubular injury may be a major event in diabetic nephropathy.

Keywords: lipotoxicity, tubular injury, diabetic kidney disease, primary event, lipid accumulation

OPEN ACCESS

Edited by:
Qin Zhou,
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Reviewed by:
Li Li,
Thomas Jefferson University, United States
Eliane Pedra Dias,
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Specialty section:
This article was submitted to Pathology, a section of the journal Frontiers in Medicine

Received: *01 August 2021*
Accepted: *27 September 2021*
Published: *25 October 2021*

Citation:
Wang H, Zhang S and Guo J (2021) Lipotoxic Proximal Tubular Injury: A Primary Event in Diabetic Kidney Disease. Front. Med. 8:751529. doi: 10.3389/fmed.2021.751529

INTRODUCTION

Diabetic kidney disease (DKD) is a common complication of diabetes mellitus (DM) and a leading cause of renal failure. Approximately 30–40% of patients with T1DM and T2DM develop DKD, and approximately 50% of them can progress to end-stage renal disease (ESRD) (1). Currently, the prevalence, mortality, and cost of DKD are high (2). According to the 2018 US Renal Data System report, the prevalence of end-stage renal disease due to diabetes continues to increase and is expected to be 44% by 2030 (3). However, understanding of DKD is still insufficient and the effective prevention and treatment rate are poor. In clinical practice, DKD is diagnosed by proteinuria, decreased estimated glomerular filtration rate (GFR), or both (4). However, the precision and prognostic value of these biomarkers in the early stages of the disease are limited, so there is urgent need to find new indicators for the early diagnosis of DKD.

Lipid accumulation is a common phenomenon in patients with DKD (5). Since Moorhead first proposed the "hypothesis of renal toxicity" in 1982 (6), increasing evidence supports the hypothesis that lipotoxicity leads to renal tubular epithelial cell injury and promotes renal disease progression. Lipotoxicity has been found to cause a series of renal injuries, including mitochondrial dysfunction, tubular epithelial cell apoptosis, tubular atrophy, and tubulointerstitial fibrosis. Interestingly, injury occurs preferentially in renal tubules, unlike the traditional concept of diagnosis and treatment of DKD focusing on the glomeruli, and this may provide a new direction for future research in DKD. In this review, we will summarize the early renal injury caused by lipotoxicity and discuss whether tubular lipotoxicity can be used as an indicator for early prediction of DKD.

LIPID METABOLISM IN THE RENAL TUBULE

The kidney is one of the most energy-demanding organs in the human body, and numerous studies have shown that the kidney mainly uses fatty acid oxidation (FAO) as its energy source (7). Because fatty acids (FAs) metabolism produce 3 times more adenosine triphosphate (ATP) than glucose (8). Most renal tubular epithelial cells (TECs) have low metabolic flexibility toward glycolysis and rely on FAs as energy source at baseline (9). This was shown *in vivo* studies measuring ATP synthesis by tracking isotope-labeled FAs with NMR in rat kidney, which indicated that FAs are a preferred fuel (6). Renal FAO occurs mainly in mitochondria, and tubular cells contain a large number of mitochondria (10). For example, the human proximal convoluted tubules contain abundant large mitochondria, which occupy about 16.3% cell volume (7). Thus, renal tubules are the core site of renal energy metabolism (10). In blood, more than 90 % of FAs are esterified and circulate as triglyceride (TAG) within very low-density lipoprotein (VLDL) and chylomicron particles (CM). Esterified FAs are initially catabolized by lipoprotein lipase (LPL) to release non-esterified fatty acids (NEFAs) (11). Then, NEFAs enter the cells with the help of fatty acid transporters and to be metabolized (as shown in **Figure 1**).

Renal Tubular FAs Uptake

The first step in FAs metabolism is the uptake of extracellular FAs, and this involves the participation of a variety of fatty acid transporters, such as cluster of differentiation 36 (CD36) (12) and fatty acid binding proteins (FABPs) (13). Some can also enter cells by simple diffusion.

CD36, also known as scavenger receptor B2, is a membrane protein that is widely expressed (12). In the kidney, CD36 is highly expressed in the epithelial cells of the proximal tubules and distal tubules (14). CD36 mediates the binding and intracellular uptake of long-chain fatty acids (LCFAs), oxidized lipids and phospholipids (ox-LDL), advanced oxidative protein products, thrombospondin, and advanced glycation products (15). FABPs are a family of highly expressed intracellular proteins, with 15 members currently found, and FABP1 is found to be expressed in proximal tubular epithelial cells (16–18). The functions of FABP1 include: facilitating the uptake of intracellular LCFA; transporting LCFA to peroxisomes for beta oxidation; transporting LCFA and long-chain fatty acid acyl-CoA (LCFA-CoA) to mitochondria for oxidation (18).

Beta-Oxidation of FAs

FAs are transported into cells after binding to transporters. FAs that enter the cell are activated by acyl-CoA synthetase (ACS) to fatty acid acyl-CoA (fatty acyl-CoA) (13). Fatty acyl-CoA is transported into the mitochondrial matrix via carnitine shuttles (CPT1, CPT2, CACT) (11). In the mitochondrial matrix, fatty acyl-CoA are degraded *via* β-oxidation, a cyclic process consisting of four enzymatic steps, produces acetyl-CoA (19). Then, acetyl-CoA enters the tricarboxylic acid cycle (TCA) to generate FADH2 and NADH. Finally, ATP is produced by oxidative phosphorylation (OXPHOS) (20). Excess acetyl-CoA can also be transported out of the mitochondria by carnitine acetyltransferase (CACT), which in turn synthesizes new FAs (21). On the other hand, very long chain fatty acids (VLFAs) are initially oxidized in the peroxisome, releasing acetyl CoA until their chain length is shortened to eight carbons and then transported to the mitochondria to complete oxidation (13).

Synthesis of Fatty Acids and Triglycerides

Metabolism of FAs includes catabolism and anabolism. In tubular cells, some of fatty acyl-CoA enters the mitochondria for catabolism and produces the energy required by the kidney; excess fatty acyl-CoA then enters the anabolic pathway and generates TAG for storage. In addition, acetyl-CoA generated by FAs through beta-oxidation in the mitochondria can also be transported out by CACT (21). It is then converted to malonyl-CoA by acetyl-CoA carboxylase (ACC), which re-synthesizes new fatty acids (22, 23).

The intermediates or enzymes related to fatty acid metabolism are regulated by some enzymes or transcription factors, such as AMP protein AMPK), peroxisome proliferator-activated receptor α (PPARα), peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), sterol regulatory element binding proteins (SREBP), and carbohydrate response element binding protein (ChREBP). AMPK is an enzyme that plays a key role in cellular energy homeostasis. AMPK can inhibit ACC activity, hence reducing malonyl-CoA levels, and increase CPT1 activity, thus promoting FAO (9). It is now also found that AMPK is able to activate PPARα to stimulate fatty acid oxidation by increasing PGC-1α activity (24). In addition, SREBP and ChREBP can promote the expression of ACC and FAS, thereby increasing fatty acid synthesis (22, 23).

TUBULAR LIPID METABOLISM IN DIABETES MELLITUS

Chronic kidney disease is associated with altered lipid metabolism and lipid accumulation (25). DKD is a major cause of chronic kidney disease (CKD), and lipid accumulation is a common phenomenon in patients with DKD (5). Metabolic changes associated with diabetes are reported to contribute to early DKD (26), and abnormal metabolism is associated with DM Type 1 or type 2 (27). Under diabetic or continuous high glucose conditions, renal tubular lipid metabolic disorders and lipid accumulation are mainly related to the imbalance between the uptake, metabolism and synthesis of FAs (as shown in **Figure 1**).

Increased FAs Uptake by the Tubules

In tubular cells, the proteins associated with FAs uptake are mainly CD36 and FABPs (15, 18). In the early stages of diabetes, increased levels of CD36 are clearly observed (28). TECs-specific overexpression of CD36 transgenic mice showed an increase of lipid accumulation in TECs (29). Thus, increased expression of CD36 leads to increased uptake of intracellular FAs, and excessive deposition of FAs causes accumulation of tubular lipids.

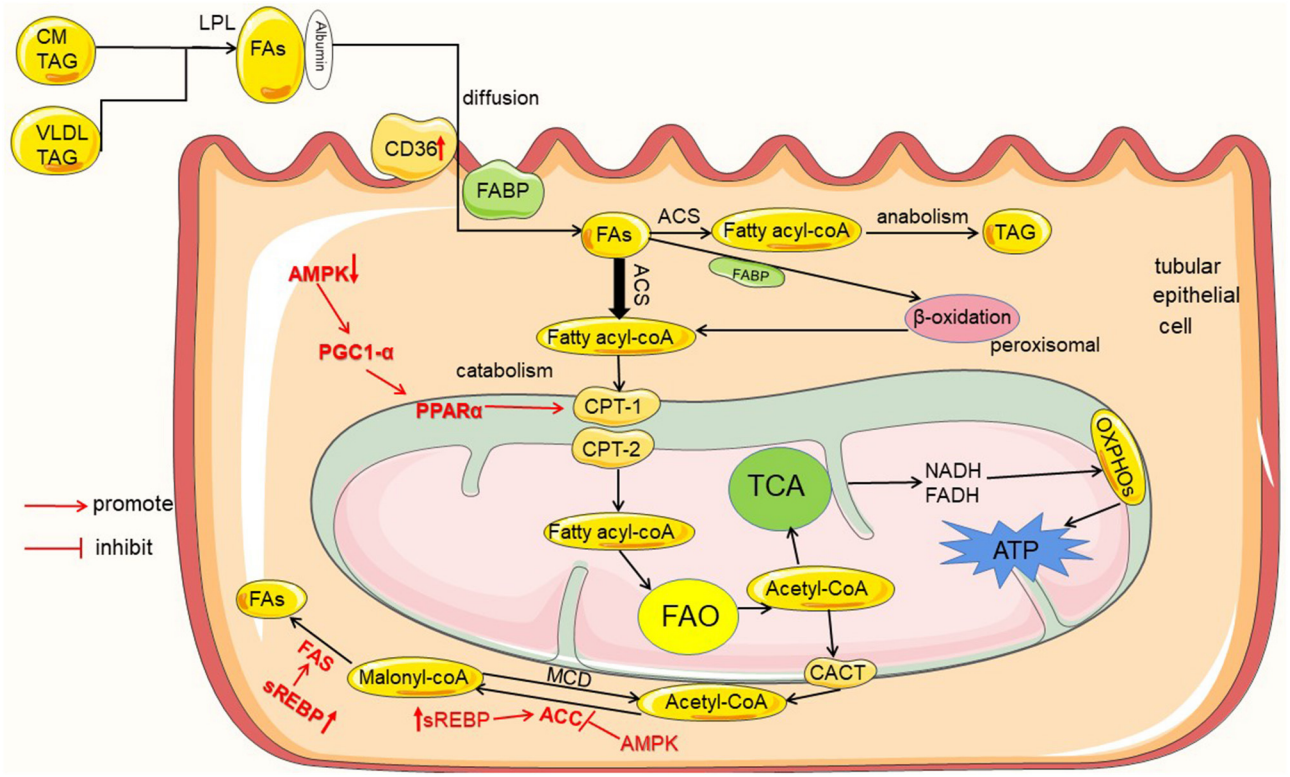

FIGURE 1 | Lipid metabolism in renal tubules and the regulation mechanism in diabetes mellitus. Tubules mainly utilize the metabolism of FAs as an energy source, and the metabolism of FAs includes catabolism and anabolism. FAs entering the renal tubules mainly come from the blood circulation. Initially, FAs are esterified in the form of VLDL and CM-TAG; then, esterified FAs are catabolized by LPL to release FAs. Extracellular FAs enter the cell by autonomous diffusion or transporters as CD36 and FABP. FAs into the cell are activated by ACS to fatty acyl-CoA, and then part of fatty acyl-CoA enters the mitochondria via CPT-1 and CPT-2 for catabolism to produce acetyl-CoA, which enters TCA to produce ATP required by the kidney; and excess fatty acyl-CoA enters the anabolic pathway to generate TAG. In addition, the product of FAO acetyl-CoA can also be transported out of mitochondria by CACT and converted to malonyl-CoA by ACC, and malonyl-CoA resynthesizes new FAs by FAS. In diabetes and high glucose conditions, the proteins are high- or down regulated in the uptake, metabolism and synthesis of FAs, which are marked in red color.

Decreased FAs Beta-Oxidation

PPARα and PGC-1α are key transcription factors for protein (22)expression of CPT-1 (30), which is the rate-limiting enzyme for fatty acid acyl-CoA into mitochondria (23). Under hyperglycemic conditions, it has been demonstrated that down-regulation of FAO genes such as PPARα, PGC-1, and CPT-1 leads to decrease in FAs β-oxidation (31–33). In addition, hyperglycemia caused the reduced activity of AMPK (23), and the expression of downstream of the AMPK pathway PPARα, PGC-1, and CPT-1, is also decreased. Eventually the oxidation of FAs is impaired, causing the accumulation of intracellular lipids.

Increased Synthesis of FAs and Accumulation of TAG

On the one hand, under high glucose conditions, the expression of SREBP and ChREBP is activated, which promotes the expression of ACC, FAS (23) and the synthesis of FAs is increased. On the other hand, surplus FAs due to impaired β-oxidation are restored in the form of TAG inside the renal cells by activating the fatty acid synthesis pathway (23). As a result, the synthesis of FAs are increased in renal tubular cells, and fatty acid transport is impaired and/or FAO is reduced. These cause dysregulation of the metabolism of FAs, as well as excessive intracellular production of FAs and TAG. Finally, there is accumulation of lipids in kidney tubules (22).

Formation of Lipotoxicity

In diabetes, the imbalance of fatty acid uptake, oxidation and synthesis in renal tubular cells causes the over-production of FAs, and when it exceeds the utilization rate of FAs by renal tubules, excessive FAs and TAG are formed and deposited in renal tubules. Recently a study on comprehensive lipidome profiling of the kidney cortex during early stage of DKD showed that there were distinct lipidomic signatures in the kidney. The levels of glyceride lipids, especially cholesteryl esters, lyso-phospholipids and sphingolipids, including ceramide and its derivatives, exhibited a dramatical elevation, while the levels of most phospholipids showed a decline in the DKD kidney cortex. The lipid metabolic disturbance does shed a light on the mechanism of renal dysfunction on the early stage of diabetes (26). Accumulation of large amounts of lipids causes the production of proinflammatory factors, toxic intermediate metabolites, which in turn induce an inflammatory

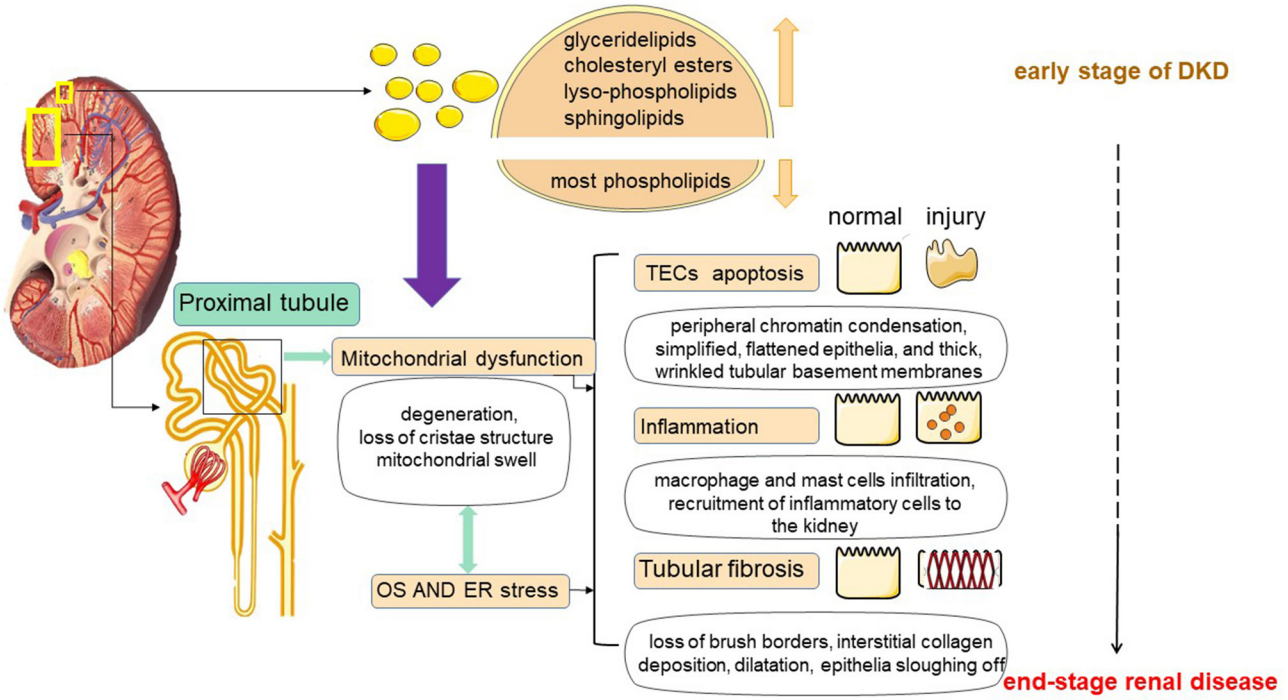

FIGURE 2 | Lipotoxicity-mediated tubular injury. Tubular lipotoxicity leads to the development of a series of renal injuries including tubular epithelial cell apoptosis, inflammation and tubulointerstitial fibrosis. First, in renal tubules, lipotoxicity may cause mitochondrial dysfunction, involving massive ROS production, and inducing oxidative stress (OS) and ER stress. Second, lipotoxicity may cause OS and ER stress, which in turn may cause mitochondrial damage. At the same time, OS and ER stress may also cause damage to mitochondria. In addition, mitochondrial dysfunction, OS and ER stress together do harm to tubular cells.

response, oxidative stress, and ultimately trigger cell damage (34). This abnormal accumulation of lipids in non-adipose tissue leads to dysregulation of intracellular homeostasis, causing the phenomenon of cellular damage known as lipotoxicity (35) (as shown in **Figure 2**).

Analysis on genetic predisposition to diabetic kidney disease implicates genes involving in lipotoxicity. A single nucleotide polymorphism in a noncoding region of the acetyl-CoA carboxylase (ACACB) gene (rs2268388), which plays a critical role in FA oxidation, showed the strongest association with proteinuria in numerous cohorts of individuals from different genetic backgrounds. This genetic risk variants can induce tubular dysfunction by promoting ACACB-mediated inhibition of CPT1 and reducing FA oxidation (36). A case-control study found that the ε2 and ε3 alleles, corresponding coding proteins E2 (Arg158 → Cys), and E3 (parent isoform) of Apolipoprotein E (APOE) influenced lipid profile, and gave rise to independent risk factors of DKD in type 2 diabetes. ApoE2 has the lowest binding ability to apoE receptor, leading to impaired liver uptake and clearance of chylomicrons (CM) or VLDL remnants. Apo ε3, was closely related with significant elevation in total cholesterol and triglyceride levels in DKD patients (37).

Epigenetic factors, including long noncoding RNAs and microRNAs, may act as an important role in lipid metabolism. Farnesoid x receptor (FXR), deficiency of which mediates diabetes acceleration of nephropathy in T1DM, inhibits SREBP-2 and elevates miR-29a, thus relieving renal fibrosis. MTHFR, an enzyme in folate cycle and homocysteine metabolism, indirectly regulates lipid metabolism. *MTHFR* 1298A/C variant is closely associated with DN (38). Moreover, miRNAs may affect the effects of hypolipidemic drugs. Lovastatin reduces miR-33 family members, which in turn suppress SREBP-2 and cholesterol synthesis (39).

LIPOTOXICITY-MEDIATED RENAL TUBULAR INJURY

In 1982, Moorhead first proposed the hypothesis of "renal toxicity" and explained that chronic progressive renal disease may be mediated by abnormal metabolism of lipids, which may contribute to the progression of renal insufficiency (6). Since 1982, more and more studies have demonstrated that abnormal lipid metabolism has a great relationship with kidney injury. Furthermore, the nephrotoxicity by lipids is not only a cause of but also a consequence of renal disease (7, 40). In DKD, renal tubular lipid accumulation is a common phenomenon, and excessive tubular ectopic fat deposition can further trigger lipotoxicity (5, 35). Tubular lipotoxicity leads to the development of a series of renal injuries such as oxidative stress (OS), endoplasmic reticulum stress (ER), tubular epithelial cell apoptosis, tubulointerstitial fibrosis (TIF), mitochondrial

dysfunction and inflammation, etc. (41–43). Lipotoxicity is a mechanism of TECs injury and is associated with a progressive decline in renal function (44) (as shown in **Figure 2**).

Renal Tubular Epithelial Cell Apoptosis by Lipotoxicity

Apoptosis is programmed cell death. Studies have shown that there is apoptosis in TECs in diabetes, and a variety of apoptotic pathways are related to renal tubular atrophy. TECs apoptosis may be a cause of renal tubular atrophy (41). Under persistent apoptotic TECs insults, the resulting pathology exhibited interstitial capillary rarefaction, and tubular atrophy characterized by simplified, flattened epithelia, and thick, wrinkled tubular basement membranes (41). In the presence of albumin, tubular cell apoptosis is often considered to be closely related to albumin (45). However, recent studies have shown that even under physiological conditions, nephrotic amounts of albumin can be normally reabsorbed in tubules (46), which indicate that albumin itself is not toxic to tubules. Christine Ruggiero showed that albumin bound FAs, but not albumin itself mediated apoptosis of TECs. Intracellular FAs and LC-CoA accumulate to levels that exceed the tubular cell metabolic and storage threshold, causing lipotoxicity that can lead to apoptosis (41). Tamaki Iwai also demonstrated in related studies that lipotoxicity caused by accumulation of FAs induces apoptosis in TECs (44).

Lipotoxicity causes mitochondrial damage, leading to dysregulation of tubular lipid metabolism, and massive accumulation of fatty acids induced by incomplete FAO, which promotes ROS production. ROS can further drive tubular epithelial cell apoptosis and affect normal renal function (47).

Tubular Fibrosis by Lipotoxicity

Tubular fibrosis is a powerful predictor of chronic kidney disease progression, which is often accompanied by the phenomenon of tubular lipid accumulation (43), receiving much attention, particularly in DKD. It has been proposed that excess accumulation of triglycerides induces cellular lipotoxicity and has the potential to contribute to the development of fibrosis (5, 48). In unilateral ureteral obstruction (UUO)-induced mice, the accumulation of lipid droplets was found in the kidney on day 7 after surgery. The kidney morphology exhibited degeneration of tubular epithelia with loss of brush borders and dilatation, accompanied by interstitial collagen deposition in UUO groups. It showed tubular epithelial disruption with epithelia sloughing off and shedding of PAS-positive material in the tubular lumina. Treatment with BMS309403, a fatty acid-binding protein 4 (FABP4) inhibitor, alleviated lipid deposition of TECs, as well as interstitial fibrosis by regulating peroxisome proliferator-activated receptor γ (PPARγ) and restoring FAO-related enzyme activities, thus enhancing FAO in TECs (49). In the DKD model, TEC-specific high expression of CD36 caused lipid accumulation, and it was found that the level of triglycerides and long-chain fatty acids alone was not sufficient to induce the development of fibrosis (10). However, when FAO is deficient in PTCs, it contributes to the development of renal fibrosis. Studies suggest that mitochondrial fatty acid oxidation plays a key role in the development of renal fibrosis. Activation of ATF6α, a transcription factor of the unfolded protein response and an upstream regulator of fatty acid metabolism, inhibits PPARα expression and subsequent FAO, followed by apoptosis and further fibrosis of PTCs. Atf6α-/- mice had maintained expression of PPARα and also decreased tubular lipid accumulation, resulting in the amelioration of apoptosis; and less tubulointerstitial fibrosis with reduced expression of α-smooth muscle actin, and collagen I (43).

In addition, lipotoxicity causes mitochondrial damage and produces large amounts of ROS. ROS can induce the expression of pro-fibrogenic factors, such as transforming growth factor-beta (TGF-β) and plasminogen activator inhibitor-1 (PAI-1), and therefore also plays a role in promoting tubular fibrosis (50).

Inflammatory Response by Lipotoxicity

Inflammatory response is an important aspect of tubular injury in DKD. An increasing number of studies have demonstrated that lipotoxicity is an important stimulus for systemic inflammation. Lung-Chih Li has found that the levels of interleukin-1β and interleukin-18 were up-regulated in both diet-fed mice and TECs treated with palmitic acid. In the same conclusion, Xianghui Chen reported palmitic acid could enhance the expression of interleukin-1β and interleukin-18. Besides, FA could increase the mRNA levels of the inflammatory markers F4/80 and MCP-1 (51). These results suggest that accumulation of lipids induces renal tubular inflammation (52). The intensity of adipose differentiation-associated protein (ADRP) and SREBP-1 was markedly upregulated and positively correlated with inflammation. It testified the potential role of ectopic accumulation in renal tubular injury and inflammation in DKD, and confirmed that excess lipids do promote an inflammatory response (53). Macrophages infiltration into the kidney, and monocyte and macrophage recruitment and the circulation cytokine release culminate in inflammatory-related morphological changes. Other cells such as mast cells also infiltrate the tubule-interstitium and releases inflammatory factors and proteolytic enzymes (54).

The Mechanism of Renal Tubular Injury by Lipotoxicity

Mitochondrial Dysfunction by Lipotoxicity

Accumulating evidence suggests that mitochondrial damage and dysfunction are major causes of CKD pathogenesis (55). It has also been shown that abnormalities in tubular mitochondrial structure and dysfunction may be the earliest manifestations of renal disease. In DKD, mitochondrial fission and fragmentation occur more often in proximal tubules. The accumulation of lipid drops in mitochondrial could promote the loss of cristae structure, mitochondrial swelling and degeneration, restraining optimal energetic functioning (35). As early as 4 weeks after experimental DM induction, evidence of impaired mitochondrial ATP production and organelle fragmentation in TECs was found, and these changes preceded increased excretion of proteinuria, abnormal glomerular morphology, and even increased renal injury molecule-1 (KIM-1), suggesting that they may be primary abnormalities (56).

Lipid is not only an important energy source, but also an important part of mitochondrial membrane structure. Unbalanced lipid metabolism can hinder mitochondrial dynamics, leading to changes in mitochondrial lipids and dysfunction (42). Lipids are also substrate of FAO in mitochondria to meet the high energy demand of the kidney (7). The dysregulated metabolism provides more albumin-bound FAs transport to renal cells, leading to mitochondrial overload. Persistent elevated levels of FAs constantly activate mitochondrial dysfunction, leading to the occurrence of incomplete FAO and the production of reactive oxygen species (ROS) (23). In turn, dysregulated mitochondrial oxidation and increased production of ROS further cause impaired mitochondrial aptamer utilization, accumulation and finally renal lipotoxicity (42). Compared with healthy kidneys, the genes and enzymes involved in the renal FAO pathway in patients with DKD have been revealed down-regulation, especially some key transcriptional regulators, such as PPARα, and CPT1 (5). Therefore, lipotoxicity and mitochondrial dysfunction can fall into a vicious cycle.

Oxidative and ER Stress by Lipotoxicity

Oxidative stress (OS) is closely related to ROS generation as the imbalance toward an increasing oxidative environment (57). The production of ROS mainly includes two ways: intercellular ROS in mitochondrial; renal ROS promoted by NADPH oxidase (NOX). Production of ROS by lipotoxicity may cause oxidative damage to the tubule by changing renal pressure and blood pressure (50). Mitochondria are also a key target for the destructive effects of ROS. Oxidative damage leads to mitochondrial dysfunction and loss of mitochondrial membranes, triggering mitochondrial permeability transition (MPT) and/or release of proapoptotic proteins, such as cytochrome c, to induce cell death (58).

The endoplasmic reticulum (ER) is an organelle important for lipid metabolism regulation, protein synthesis, post-translational modification, and trafficking (58). Dysregulation of ER homeostasis is known as "ER stress". Hai-Lu Zhao have demonstrated that excessive ectopic fat deposition in the kidney and lipid overload in intracellular organelles can lead to ER stress (59). In addition, some related experiments have also shown that dietary saturated fatty acids induce ER stress in the kidneys of animal models and in cells cultured from the kidneys (60, 61). ER stress is associated with many pathological conditions, such as inflammation, apoptosis and metabolic disorders (21, 61, 62).

RENAL TUBULAR INJURY: CENTER FOR THE DEVELOPMENT OF DKD

DKD as a common complication of type 1 and 2 DM, is the main cause of CKD and characterized by glomerulosclerosis, tubulointerstitial fibrosis, and renal vascular disease (63). In tradition, the primary clinical symptom of DKD is increased urinary albumin excretion (microalbuminuria: 30 mg/24 h−300 mg/24 h) (64). In addition, studies have shown that absolute ultra-physiological elevation of GFR is observed in the early stage of diabetes in 10–67% and 6–73% of patients with type 1 and type 2 DM, respectively (65), and this hyperfiltration phenomenon is associated with the development and progression of DKD (66). Therefore, in clinical practice, the earliest and obvious features of DKD are microalbuminuria and increased GFR, which are generally used as early diagnostic markers of DKD. Both of them are also considered as markers of glomerular damage, so research on the pathogenesis of DKD has been focused on glomerular injury. Although there is no doubt that glomerular injury is a major factor in DKD, there is recently increasing evidence that renal tubules underlie the early pathogenesis of DKD (67). Urinary neutral gelase-associated lipoprotein (NGAL), a marker of tubulointerstitial damage, has been showed increased expression in DM patients with normal microalbuminuria, suggesting that tubular injury may precede glomerular disease (68). In addition, some studies have suggested that tubular injury may cause abnormal glomerular function (69), implying that tubular lesions may be the center of DKD development.

Tubular Injury Precedes Glomerular Injury

Microalbuminuria Due to Tubular Injury

For a long time, microalbuminuria test has been used as a standardized means of early DKD detection (10). Generally, the mechanisms underlying microalbuminuria in the early stages of DKD are attributed to increased glomerular filtration due to hyperfiltration or glomerular barrier injury, or a combination of both (70). However, Hanet reported that while urinary albumin increased, urinary concentrations of N-acetylβ-D-glucosaminidase (NAG), a marker of tubular injury, also increased (71), suggesting that urinary albumin excretion correlates well with markers of tubular dysfunction. Wagner found that tubular cells could increase absorbtion of albumin after an increase in glomerular endogenous albumin leakage. It showed that tubular cells are able to cope with acute albumin overload. These results suggest that renal tubules can regulate the albumin excretion rate (72). In addition, some researchers have proposed that proteinuria can also occur in nephrotic states with no change in glomerular permeability (73). Study on the glomerular phenotype in 15 mice with congenital nephrotic syndrome, some of them died after 5 weeks and alive mice have shown essentially no change in glomerular permeability, but over 100-fold increase in proteinuria (74). Animal nephrotoxicity studies have also shown that albuminuria is a highly sensitive marker of early tubular toxicity in the absence of glomerular pathology (75). The above results indicate that the glomerular effect on proteinuria may be indirect, and albuminuria may be mainly controlled by renal tubules (74, 76).

Glomerular Hyperfiltration by Tubular Injury

Despite difficulties in precise definition or thresholds, elevated GFR as a marker of glomerular hyperfiltration occurs early in the clinical course of DKD and is considered as an important factor in the development and progression of renal damage (66, 70). Increased GFR is generally thought to be due to increased intraglomerular pressure (causing barotrauma) and renal blood flow, resulting from an imbalance of vasoactive humoral factors that control tension in the pre-post-glomerular

arterioles (65). But now an increasing number of studies have shown that the pathogenesis of glomerular hyperfiltration is complex and proposed that tubular function plays an important role in regulating glomerular filtration in DM (77, 78).

A study exploring the effect of glomerular hyperfiltration on tubular dysfunction reported that two markers of tubular injury, NGAL and KIM-1 were excreted in the urine of patients with glomerular hyperfiltration and positively correlated with GFR (79). The results suggest that glomerular hyperfiltration is associated with changes in tubular function in patients with DM. Besides, similar results have been found. Treatment with empagliflozin, an inhibitor of the sodium-glucose transporter (SGLT2), in T1DM patients under hyperfiltration results in reductions in GFR independent of its effect on plasma glucose levels over 8 weeks (80). The above findings all demonstrate that tubular events may dominate in diabetes.

In recent years, there have also been some views proposing the "tubular theory" of hyperfiltration to describe diabetes-related abnormalities with close interaction between glomeruli and tubules. That is enhanced tubular sodium (and glucose) reabsorption, tubular growth, and up-regulation of tubular sodium-glucose cotransporters (SGLTs) and sodium-hydrogen exchanger (NHE), all of which are associated with tubular factors, and can have some effects on GFR (75). Clues related to this have been proposed in 1994, and a study in diabetic rats indicated that tubular reabsorption may be key to the development of hyperfiltration in DM patients. Besides, K. M Hallow uses a comprehensive mathematical model of the renal vascular system, tubular Na and fluid handling, and systemic blood volume regulation to explore the potential mechanisms of acute and chronic GFR responses to increased tubular Na reabsorption in diabetic patients, which demonstrated that primary tubular hyperabsorption and tubular transport dysregulation determine diabetic glomerular hyperfiltration (81). It was pointed out that tubular cells are able to affect the results and function of glomeruli through alterations in SGLT2, adenosine, ATP, etc. (63). Therefore, tubular function plays an important role in glomerular hyperfiltration, and glomerular hyperfiltration may be secondary to primary tubular injury.

Tubular Injury Leads to Glomerular Damage

For a long time, DKD has been mainly considered as a diabetic glomerulopathy. However, there is increasing evidence that tubular injury is a key cause of chronic kidney injury (82, 83), which is closely related to the progression of DKD and is superior to glomerular injury as a predictor of DKD progression (84, 85).

Recently, many studies have shown that tubular injury may lead to glomerular disease. Studies have demonstrated that nicotinamide mononucleotide (NMN) released from proximal tubular epithelial cells under high glucose conditions is able to spread to the glomeruli and induces podocyte (PCs) foot process effacement (86). Chunmei Xu has found that tubular Bim is able to mediate tubular-podocyte crosstalk and induces cytoskeletal dysfunction in PCs through activated T cell nuclear factor 2 (NFAT2). Bim is a pro-apoptotic factor involved in the crosstalk between TECs and PCs (87).

Studies have demonstrated that tubular injury not only leads to podocyte disease, but also leads to more extensive glomerular injury. A mouse model of renal injury was established using Six2-Cre-LoxP technique to selectively activate the expression of monkey diphtheria toxin (DT) receptor in tubular epithelial cells. By adjusting the time and dose of DT, a highly selective tubular injury model was created, and this was used to observe the consequences of tubular injury. It was found that after repeated administration of DT, the mice developed maladaptive repair with interstitial capillary loss, fibrosis and glomerulosclerosis. And the degree of glomerulosclerosis is closely related to the degree of tubular injury (88). It demonstrated that tubular injury causes glomerular abnormalities.

The role of renal tubular injury in DKD is getting more and more attention. On the one hand, abnormal elevation of tubular injury markers already occurs before the onset of microalbuminuria in DKD patients, indicating that tubular lesions precede glomerular injury in DKD. On the other hand, renal tubules are more accurate than glomeruli in predicting renal function in DKD, and many studies have also indicated that tubular injury can cause secondary glomerular damage. The study of tubular lesions in DKD provides a new direction to investigate the pathogenesis of DKD.

WHETHER TUBULAR LIPOTOXICITY CAN BE USED AS AN INDICATOR FOR EARLY PREDICTION OF DKD?

Researchers have made great efforts in understanding the mechanisms of progressive renal decline and developing prognostic tests in DM patients with impaired renal function (89). However, about early renal impairment with DM patients, little is known about the mechanisms, and it lacks early reliable markers. The test of microalbuminuria has been used as the main way to detect early DKD (*93*), but it has recently been challenged. It has been found that some patients with DM may develop DKD even if the urinary albumin levels are within the normal range (90). Therefore, the accuracy and prognostic value of albuminuria in the early stage of DKD are limited. Therefore, there is need to find biomarkers that more accurately predict DKD and its progression in early stage during course.

Whether metabolic disorders are precursors of renal tubules has been proposed. As early as 1982, Moorhead proposed the hypothesis of "lupus nephrotoxicity", suggesting that abnormal metabolism of lupus may contribute to the progression of renal insufficiency (6). Through metabolic analysis in clinical studies and animal models, it has been shown that alterations in lipid metabolism, TCA cycle, and FAO are the main pathways affecting DKD (91). In addition, the relationship between epithelial transition (EMT) and lipotoxicity in DKD was studied in human proximal tubular cells, and it was found that at 48 h, there was lipid droplet deposit, with more triglycerides, more malonyl-CoA, and lower fatty acid β-oxidation rate, but without morphological change under high glucose conditions. At 96 h,

tubular cells became more elongated, less adherent, and lost their apical to basal polarity accompanying with more lipid accumulation (92). These results suggest that FAs deposition has emerged before the induction of EMT phenotype and morphological changes by high glucose. It demonstrated that the progression of lipotoxicity is involved in the development of early DKD before EMT.

A mouse model lacking carnitine acetyltransferase (CRAT) in the tubule was developed to simulate mitochondrial lipid overload. The results showed that mice developed tubular disease, including tubular dilatation, proteinosis, fibrosis, and secondary glomerulosclerosis. When CRAT-null mice were fed a high fat diet, tubular pathological changes occurred 6 months earlier and were more severe than in mice lacking CRAT alone. These results suggest that lipid metabolism disorders may cause changes in kidney functions by affecting the work of mitochondria and promote the progression of DKD (93). Some studies have indicated that mitochondrial dysfunction may be the earliest manifestation of kidney disease, and mitochondrial injury can be used as a marker of tubular injury (94, 95). We have discussed that lipid toxicity can cause mitochondrial dysfunction, which gives us two hints: tubular lipotoxicity may occur before mitochondrial dysfunction and is an earlier event in DKD; tubular lipotoxicity may be an indicator for early prediction of DKD.

Although there are many biomarkers for early detection of DKD in clinical practice, their specificity and sensitivity need to be improved. Our review provides an idea that tubular lipotoxicity may be a major event occurring early in DKD, and has the potential to serve as a marker for early detection of DKD.

AUTHOR CONTRIBUTIONS

HW devised the conceptual ideas. HW and SZ drafted the original manuscript and drew the figures. JG edited various versions of the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (Grant No. 81803823), and Foundation of The Science and Technology Department of Henan Province (Grant No. 182102310540).

ACKNOWLEDGMENTS

We apologize to all those authors whose work on this subject has not been cited owing to space constraints.

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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.

frontiers in Medicine

ORIGINAL RESEARCH
published: 23 November 2021
doi: 10.3389/fmed.2021.751277

Differential Effects of Insulin-Deficient Diabetes Mellitus on Visceral vs. Subcutaneous Adipose Tissue—Multi-omics Insights From the Munich MIDY Pig Model

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Adipose tissue (AT) is no longer considered to be responsible for energy storage only but is now recognized as a major endocrine organ that is distributed across different parts of the body and is actively involved in regulatory processes controlling energy homeostasis. Moreover, AT plays a crucial role in the development of metabolic disease such as diabetes. Recent evidence has shown that adipokines have the ability to regulate blood glucose levels and improve metabolic homeostasis. While AT has been studied extensively in the context of type 2 diabetes, less is known about how different AT types are affected by absolute insulin deficiency in type 1 or permanent neonatal diabetes mellitus. Here, we analyzed visceral and subcutaneous AT in a diabetic, insulin-deficient pig model (MIDY) and wild-type (WT) littermate controls by RNA sequencing and quantitative proteomics. Multi-omics analysis indicates a depot-specific dysregulation of crucial metabolic pathways in MIDY AT samples. We identified key proteins involved in glucose uptake and downstream signaling, lipogenesis, lipolysis and β-oxidation to be differentially regulated between visceral and subcutaneous AT in response to insulin deficiency. Proteins related to glycogenolysis, pyruvate metabolism, TCA cycle and lipogenesis were increased in subcutaneous AT, whereas β-oxidation-related proteins were increased in visceral AT from MIDY pigs, pointing at a regionally different metabolic adaptation to master energy stress arising from diminished glucose utilization in MIDY AT. Chronic, absolute insulin deficiency and hyperglycemia revealed fat depot-specific signatures using multi-omics analysis. The generated datasets are a valuable resource for further comparative and translational studies in clinical diabetes research.

Keywords: diabetes, insulin deficiency, adipose tissue, proteome, transcriptome, pig model, biobank

OPEN ACCESS

Edited by:
Tingting Zhao, China-Japan Friendship Hospital, China

Reviewed by:
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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *31 July 2021*
Accepted: *25 October 2021*
Published: *23 November 2021*

Citation:
Flenkenthaler F, Ländström E, Shashikadze B, Backman M, Blutke A, Philippou-Massier J, Renner S, Hrabe de Angelis M, Wanke R, Blum H, Arnold GJ, Wolf E and Fröhlich T (2021) Differential Effects of Insulin-Deficient Diabetes Mellitus on Visceral vs. Subcutaneous Adipose Tissue—Multi-omics Insights From the Munich MIDY Pig Model. Front. Med. 8:751277. doi: 10.3389/fmed.2021.751277

INTRODUCTION

Adipose tissue is a major player in whole body energy homeostasis and regulation of metabolic functions. It serves as storage of surplus energy in the form of triglycerides in adipocytes and controls lipid mobilization during fasting by releasing free fatty acids (1, 2). With the discovery of adipocyte-derived factors such as leptin, adiponectin, and resistin, adipose tissue is increasingly recognized as a complex endocrine organ. Via adipokine signaling, adipose tissue is able to communicate with many organs like the liver, pancreas, muscle, and brain, and is therefore able to modulate systemic metabolism (3–6). Thus, adipose tissue dysfunction plays an important role in the pathogenesis of metabolic disorders, such as obesity, cardiovascular disease, insulin resistance, and diabetes mellitus (7–9). How adipose tissue specifically contributes to the pathogenesis of metabolic diseases is however highly complex and varies between different fat depots (10–12). It is thought that visceral adipose tissue is more likely to contribute to the pathogenesis of insulin resistance and type 2 diabetes mellitus (13, 14), while accumulation of subcutaneous fat has even been reported to reduce metabolic disease risk (15–17).

Several recent studies have analyzed adipose tissue proteomes in the context of type 2 diabetes mellitus to get a more global understanding of adipose tissue (dys-)function in states of insulin resistance and hyperinsulinemia (18–22). However, molecular consequences of chronic insulin deficiency on adipose tissue depots remain poorly explored.

As a large animal model for mutant *INS* gene induced diabetes of youth (MIDY), we generated INS^{C94Y} transgenic pigs, which are characterized by reduced body weight and β-cell mass, impaired insulin secretion with resulting hypoinsulinemia, and elevated blood glucose levels (23). Moreover, the model develops diabetes-associated alterations in heart (24), retina (25), immune cells (26), and liver (27). For studying long-term consequences of severe insulin-deficient diabetes mellitus (SIDD) (28), we established a biobank of 2-year-old MIDY pigs and healthy littermate controls following the principles of random systematic sampling (29).

In the current study, we performed label-free quantitative proteomics and RNA-sequencing of mesenteric visceral adipose tissue (MAT) and abdominal subcutaneous adipose tissue (SCAT). Using this holistic multi-omics approach, we report marked adipose tissue depot-specific responses to insulin deficiency and chronic hyperglycemia, and provide new insights into the molecular pathology of insulin-deficient diabetes in adipose tissue depots.

MATERIALS AND METHODS

Biological Samples

Adipose tissue samples were obtained from the Munich MIDY Pig Biobank (29). Mesenteric visceral adipose tissue (MAT) and abdominal subcutaneous adipose tissue (SCAT) samples were collected from 2-year-old female MIDY pigs ($n = 4$) and female WT littermates ($n = 5$) by systematic random sampling (30). Tissue specimen were shock-frozen and stored at −80°C. To minimize variations induced by sample collection, two samples collected from the same animal but from different areas, were pooled separately for MAT and SCAT prior to Omics analysis.

Proteomics

Frozen tissue samples were homogenized in 1% sodium deoxycholate (SDC) and 50 mM ammonium bicarbonate (ABC) using an ART-Miccra D-8 homogenizer (ART Prozess- & Labortechnik) at a speed of 23,500 rpm for two cycles of 1 min. Samples were kept on ice for 30 min and centrifuged at 16,000 × *g* for 5 min. The aqueous phase beneath the top lipid layer was carefully taken and transferred to a new test tube. Protein concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Marshall Scientific) at 280 nm. Fifty microgram of protein was reduced with 4 mM dithiothreitol (DTT) and 2 mM tris(2-carboxyethyl)phosphine (TCEP) at 56°C for 30 min and alkylated with 8 mM iodoacetamide (IAA) at room temperature in the dark. DTT was added to a final concentration of 10 mM to quench residual IAA during 15 min incubation in the dark. Proteins were digested with 1 μg LysC (Wako) for 4 h followed by digestion with 1 μg modified porcine trypsin (Promega) for 16 h at 37°C. SDC was removed by acid precipitation as described elsewhere (31, 32).

Nano-liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis was performed on an Q Exactive HF-X mass spectrometer equipped with an UltiMate 3000 nano LC system (Thermo Scientific) as previously described (33). Briefly, 1.5 μg of peptides were separated on a 50 cm column (PepMap RSLC C18, 75 μm ID, 2 μm; Thermo Scientific) using linear gradients from 5 to 25% solvent B (0.1% formic acid in acetonitrile) in 160 min and from 25 to 40% solvent B in 10 min with a flow rate of 250 nl min^{-1}. Spectra were acquired in data-dependent mode in cycles of one full scan in the range of 300–1,600 *m/z* at a resolution of 60,000, followed by MS/MS scans of the 15 most intense peaks at a resolution of 15,000.

Raw MS data were processed with MaxQuant (v. 1.6.7.0), using the integrated Andromeda search engine (34) and the NCBI RefSeq Sus scrofa database (v. 2020-11-12). Identifications were filtered to 1% false discovery rate (FDR) at peptide and protein level. Statistics and data visualization were performed in R (35). MS-EmpiRe was used to detect differentially abundant proteins (36). Reverse peptides, contaminants and identifications only by site were excluded for quantification. Proteins were quantified with at least two peptides with a minimum of two replicate measurements in each condition. For peptides with measurements in all replicates of one condition and insufficient measurements in the other condition, missing values were imputed from normal distribution (shift = 1.8, scale = 0.3) using the DEP package (37). Proteins were considered as significantly changed in abundance with a Benjamini-Hochberg-adjusted *P*-value < 0.05 and a fold change above 1.3.

Transcriptomics

After weighing the frozen tissue, corresponding volumes of ice-cold Trizol reagent (Invitrogen Life Technologies) were added (1 ml Trizol reagent per 100 mg tissue). Tissue was immediately homogenized using the Heidolph Silents Crusher M. RNA of

homogenized tissue was isolated using the Maxwell RSC miRNA Tissue Kit (Promega). Manufacturer's instructions were followed except for the following changes: the homogenized tissue was in Trizol to which corresponding volumes of 1-thioglycerol were added. Isolated RNA was additionally digested with DNase I (Thermo Scientific) and purified using Agencourt RNAClean XP beads (Beckman Coulter) following manufacturer's instructions. Subsequently, the RNA was quantified (Nanodrop) and quality controlled on an RNA 6000 Nano Chip using a Bioanalyzer (Agilent). Finally, 120 ng high quality total RNA (RIN $>$ 8.0) was used for generating sequencing libraries using the Sense mRNA Seq Library Prep Kit V2 for Illumina platforms (Lexogen) following manufacturer's instructions. Libraries were quantified and quality controlled on the Bioanalyzer (Agilent) and finally sequenced on an Illumina HiSeq1500 machine (single end read, 100 nt).

After demultiplexing obtained FastQ files, a head-crop was performed in order to remove the 12 first bases using the Trimmomatic tool (38). Mapping to the S.scrofa 11.1 reference genome was performed using the short read gapped-mapper STAR (39). Read quantification for each gene was performed with HTSeq (40) using strict intersection mode and a minimum alignment quality of 10. After filtering out low abundant genes (mean counts $<$ 10), DESeq2 (41) with outlier replacement and independent filtering was used on the counts matrix to calculate differential abundance. To remove a hidden technical batch effect, Surrogate Variable Analysis (SVA) (42) was used to estimate a batch variable that was added to the DESeq2 formula.

Bioinformatic Analysis

The STRING preranked enrichment analysis (43) was used to functionally characterize proteome abundance alterations between genotypes (MIDY vs. WT) and tissue types (SCAT vs. MAT). Signed log-transformed *P*-values were used as ranking metric and FDR stringency was set to 0.01. To reduce redundancy, significant Gene Ontology (GO) biological processes were grouped into similar ontological terms with REVIGO (44) at an allowed similarity of 0.5 for the genotype comparison and 0.4 for the tissue type comparison, respectively.

To integrate proteomics and RNA-seq data, protein abundance ratios and DESeq2-normalized mRNA abundance ratios for common identifications were combined and subjected to a statistically controlled 2D annotation enrichment analysis (45). Protein and RNA-seq abundance ratios were separately rank-transformed and are shown as MIDY/WT proteome and transcriptome score, respectively. Statistical enrichment was determined by a two-dimensional generalization of the non-parametric two-sample test. False discovery rate was controlled by correcting for multiple hypothesis testing. The significant cutoff for correlating, non-correlating and anti-correlating GO and KEGG annotations was set to FDR $<$ 0.1.

Leptin RIA

Serum leptin levels were measured using a multi-species leptin radioimmunoassay (Cat. # XL-85K; EMD Millipore Corporation) that has been validated for porcine samples (46). Data were transformed to natural logarithms to approximate normal distribution and analyzed by student's *t*-test.

Histology and Quantitative Morphological Analyses

For histological and quantitative morphological analyses of adipocytes in WT and MIDY pigs, tissue samples were collected from the SCAT and MAT adipose tissue depots, as described previously (29, 30, 47). Isotropic uniform random (IUR) cryo-sections (30, 47, 48) of 10 μm nominal section thickness were prepared and stained with the Periodic acid–Schiff (PAS) reaction. Quantitative morphological analyses were performed, using an automated stereology system with NewCast software (Visiopharm, Denmark). In systematically randomly sampled fields of view, adipocyte cross section profiles were sampled with point-sampled-intercepts and unbiased counting frames at 10x objective magnification (48, 49). The volume weighted mean adipocyte volumes were determined, using the nucleator method (50–52). Per case, 107 ± 6 measurements (mean ± SD) were taken, on the average. For demonstration of the adipocyte histomorphology, additional hematoxylin and eosin-stained sections of paraffin-embedded adipose tissue samples were prepared. The volume weighted mean adipocyte volumes in SCAT and MAT adipose tissue depots were statistically analyzed, using GraphPad PRISM (version 9.1.1., GraphPad Software, USA). Data are presented as means and single values and standard deviations. Data distributions were analyzed, using Shapiro-Wilk tests. Mean adipocyte volumes in SCAT and MAT adipose tissue depots of the same animals were compared, using paired student *t*-tests (normally distributed data). Mean SCAT- and MAT-adipocyte volumes of WT vs. MIDY pigs were compared by student *t*-tests (normally distributed data). *P*-values $<$ 0.05 were considered significant.

RESULTS

Mass Spectrometry-Based Proteome Analysis of Adipose Tissue Depots From Insulin-Deficient Diabetic Pigs

To explore the molecular effects of chronic, absolute insulin deficiency and hyperglycemia on AT depots, we performed a label-free liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS) of MAT and SCAT samples from MIDY ($n = 4$) and WT ($n = 5$) animals.

We identified a total of 23,730 peptides from 2,851 proteins with high confidence (FDR $<$ 0.01). A full list of all identified proteins can be found in **Supplementary Table 1**. Unsupervised hierarchical clustering of normalized protein intensities (**Figure 1A**) and a principal component analysis (**Figure 1B**) show a clear separation of tissue types (MAT and SCAT) and indicate clustering of genotypes (MIDY and WT).

Visceral Mesenteric vs. Subcutaneous Adipose Tissue

We used an MS-EmpiRe workflow (36), to detect quantitative proteome differences between the two adipose tissue types

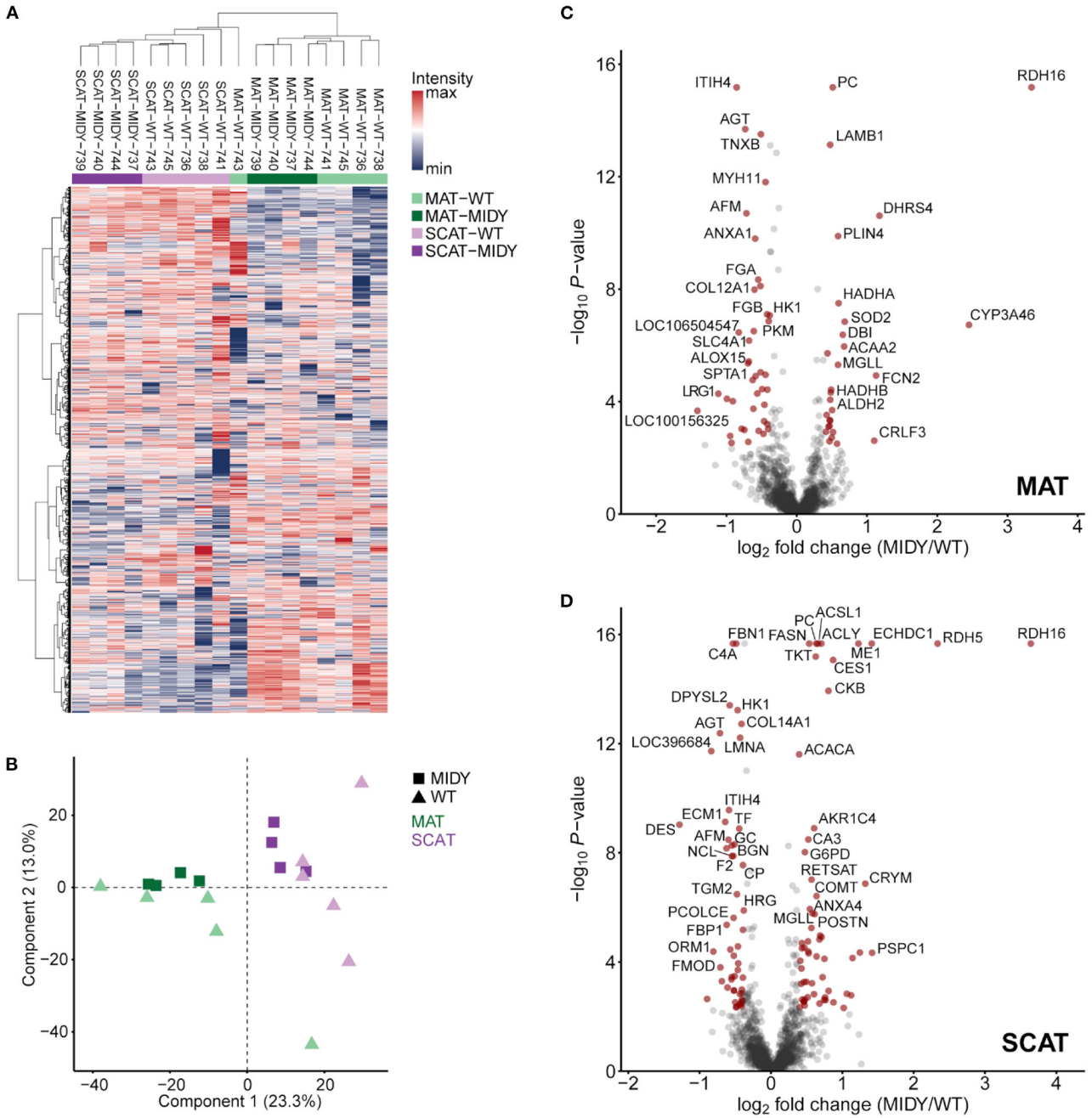

FIGURE 1 | Quantitative proteome analysis of mesenteric (MAT) and subcutaneous (SCAT) adipose tissue from MIDY and WT pigs. **(A)** Unsupervised hierarchical clustering of normalized protein intensities of MAT and SCAT from MIDY ($n = 4$) and WT ($n = 5$) animals. The color code indicates normalized intensity values. **(B)** Principal component analysis (PCA) indicates clustering of individual sample groups. Spots represent individual animals. Green and purple colors indicate MAT and SCAT samples, and squares and triangles indicate MIDY and WT samples, respectively. **(C,D)** Volcano plots visualize the quantitative proteome alterations in MAT **(C)** and SCAT **(D)** from MIDY vs. WT pigs. Red spots indicate differentially abundant proteins (fold-change > 1.3 and Benjamini-Hochberg-adjusted P-value < 0.05).

within genotype. In WT pigs, 331 proteins were found to be differentially abundant (fold-change > 1.3 and Benjamini-Hochberg-adjusted P-value < 0.05) between SCAT and MAT (**Supplementary Figure 1A**, **Supplementary Table 2**). In MIDY pigs, 371 proteins were significantly different in abundance between the fat depots (**Supplementary Figure 1B**, **Supplementary Table 3**).

A STRING pre-ranked functional enrichment analysis (43) of proteome profiles from MAT and SCAT was done to reveal tissue-specific signatures for WT and MIDY animals. From the

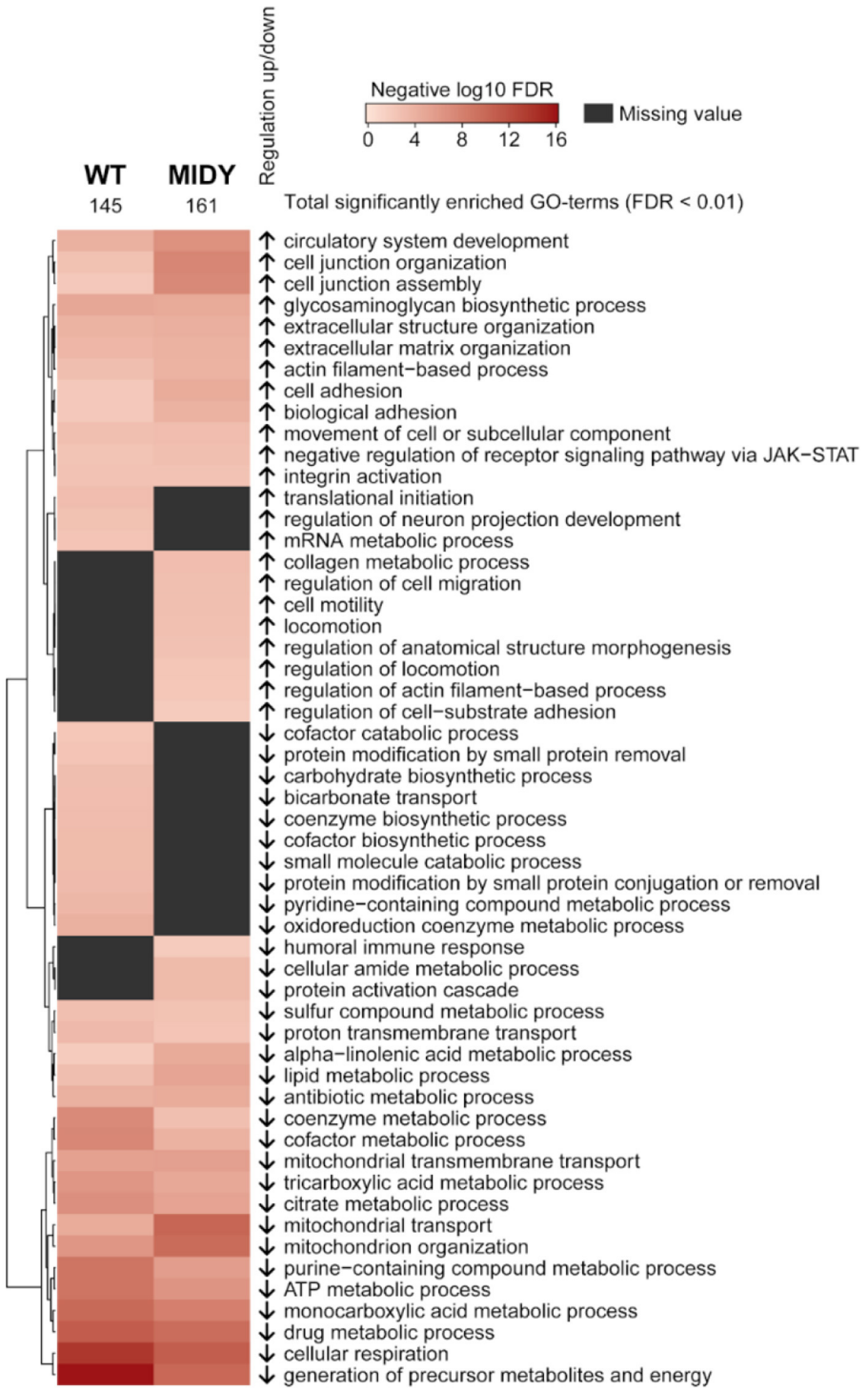

FIGURE 2 | Functional characterization of adipose tissue depot differences between MAT and SCAT in WT and MIDY animals. Heatmap shows GO-term enrichment in SCAT compared to MAT. Enrichment analysis was performed using a pre-ranked STRING analysis and an FDR cutoff of 0.01. Significantly enriched GO biological processes were summarized with REVIGO by grouping semantically similar ontology terms. Arrows indicate regulation in SCAT.

GO biological processes database, 145 and 161 terms were found in WT and MIDY, respectively, to be significantly enriched (FDR < 0.01) in SCAT vs. MAT (**Supplementary Tables 4**, **5**). Similar ontology terms were revealed with REVIGO (44) and the resulting clusters are visualized in **Figure 2**. SCAT from WT and MIDY animals showed, among others, a distinct

enrichment of protein complexes involved in cell junction assembly and organization, of collagens involved in extracellular matrix organization and of actin filaments. On the other hand, proteins related to mitochondrial respiration and to glucose and lipid metabolism were overrepresented in MAT compared to SCAT, the former more pronounced in MIDY and the latter more pronounced in WT. Specifically, proteins involved in pyruvate metabolism, TCA cycle, oxidative phosphorylation (OxPhos), fatty acid biosynthesis and beta oxidation were consistently increased in MAT of both WT and MIDY animals.

Adipose Tissues From Diabetic vs. Non-diabetic Pigs

The quantitative comparison of both fat depot proteomes from diabetic MIDY and non-diabetic WT animals led to 68 differentially abundant proteins (fold-change > 1.3 and Benjamini-Hochberg-adjusted P-value < 0.05) in MAT (**Figure 1C**, **Supplementary Table 6**) and 112 proteins in SCAT (**Figure 1D**, **Supplementary Table 7**). In both AT depots, retinol dehydrogenase 16 (RDH16) was the protein with the highest abundance increase in MIDY, with log2 fold-changes of 3.3 in MAT and 3.6 in SCAT, respectively. Likewise, further proteins involved in retinol metabolism were increased in abundance, namely apolipoprotein A-IV (APOA4) and aldo-keto reductase family 1 (AKR1C4) in both AT depots, dehydrogenase/reductase 4 (DHRS4) exclusively in MAT and retinol dehydrogenase 5 (RDH5) and retinol saturase (RETSAT) exclusively in SCAT. The group of proteins with the strongest decrease in both MIDY AT depots contained, among others, a large number of serpin family A members (e.g., serpin A3-6, serpin A3-8, and SERPING1) as well as leucine-rich repeat (LRR)-containing proteins, e.g., biglycan (BGN), fibromodulin (FMOD), and leucine rich alpha-2-glycoprotein 1 (LRG1) linked to collagen fibril organization and immune response.

To functionally characterize proteome alterations, a STRING pre-ranked annotation enrichment analysis was performed on both AT depot datasets. From the GO biological processes database, 23 terms were significantly enriched (FDR < 0.01) in MAT and 124 in SCAT, respectively (**Figure 3A**). Proteins more abundant in MIDY vs. WT of MAT and SCAT were enriched for terms related to fatty acid and lipid metabolism as well as to mitochondrial respiration, while proteins involved in extracellular matrix organization, immune response and platelet degranulation are more likely to be reduced in MIDY ATs. Terms related to processes relevant for metabolite and energy production, e.g., citrate and purine metabolism were predominantly enriched in the set of proteins more abundant in MIDY vs. WT SCAT, while proteins with reduced abundance were found to be involved in regulation of coagulation and regulation of protein activation cascades. The detailed results of the STRING analysis are provided in **Supplementary Tables 8**, **9**.

A strikingly large part of proteins commonly increased in both tissue depots from MIDY pigs are known to be involved in carbohydrate and lipid metabolism, e.g., pyruvate carboxylase (PC), monoglyceride lipase (MGLL) and acetyl-CoA acyltransferase 1 (ACAA1) (**Figures 4A–C**). Furthermore, well-known metabolic and regulatory enzymes of glucose import were significantly reduced in abundance in MIDY vs. WT pigs, among them hexokinase-1 (HK1) in both depots and solute carrier family 2, facilitated glucose transporter member 4 (SLC2A4/GLUT4) in MAT only. Remarkably, proteins associated with subsequent glucose metabolic pathways (e.g., glycogenolysis, pentose phosphate pathway, pyruvate metabolism, TCA cycle, and mitochondrial oxidative phosphorylation) were consistently more abundant in SCAT from MIDY compared to WT pigs. Although a similar trend was visible for MAT, the changes were less pronounced, and the vast majority was only significant in SCAT. A similar pattern was observed for proteins involved in lipogenesis, the step to provide free fatty acids from acetyl-CoA, where SCAT showed stronger abundance alterations compared to MAT.

Integrative Analysis of Transcriptomics and Proteomics Data

To investigate transcriptional regulation of insulin deficiency and hyperglycemia on AT, we additionally performed RNA-sequencing of the same tissue specimens from MIDY ($n = 4$) and WT ($n = 5$) animals as used for proteomics experiments (**Supplementary Tables 10**, **11**). To detect correlated and uncorrelated functional changes, we performed a 2D annotation enrichment analysis of the transcriptome and proteome data (**Figures 3B,C**) (45). We observed a highly concordant enrichment (FDR < 0.1) of processes related to e.g., fatty acid metabolism, TCA cycle and oxidative phosphorylation in MAT and SCAT of MIDY pigs, and a concordant decrease of terms related to extracellular matrix, the complement system and coagulation cascades in both datasets. Notably, in SCAT from MIDY pigs, we detected a strong enrichment of genes involved in retinol metabolism on both the mRNA and protein level. A discordant pattern could be observed in SCAT, where proteins linked to translational regulation as well as cytosolic large ribosomal subunits showed a decrease in MIDY, while RNA expression levels were slightly elevated in MIDY.

Histology, Quantitative Morphological Analyses and Leptin Measurements

SCAT and MST adipose tissue samples of WT and MIDY pigs displayed a regular histomorphology without evidence of histopathological alterations. In both WT and MIDY pigs, the (volume weighted) mean volumes of adipocytes in the MAT were significantly larger than in the SCAT, whereas the adipocyte volumes of corresponding adipose tissue depots did not significantly differ in WT vs. MIDY pigs (**Supplementary Figures 2A,B**). To address leptin signaling, we measured serum leptin concentrations and found significantly reduced levels ($p = 0.03$) in MIDY pigs (**Supplementary Figure 3**).

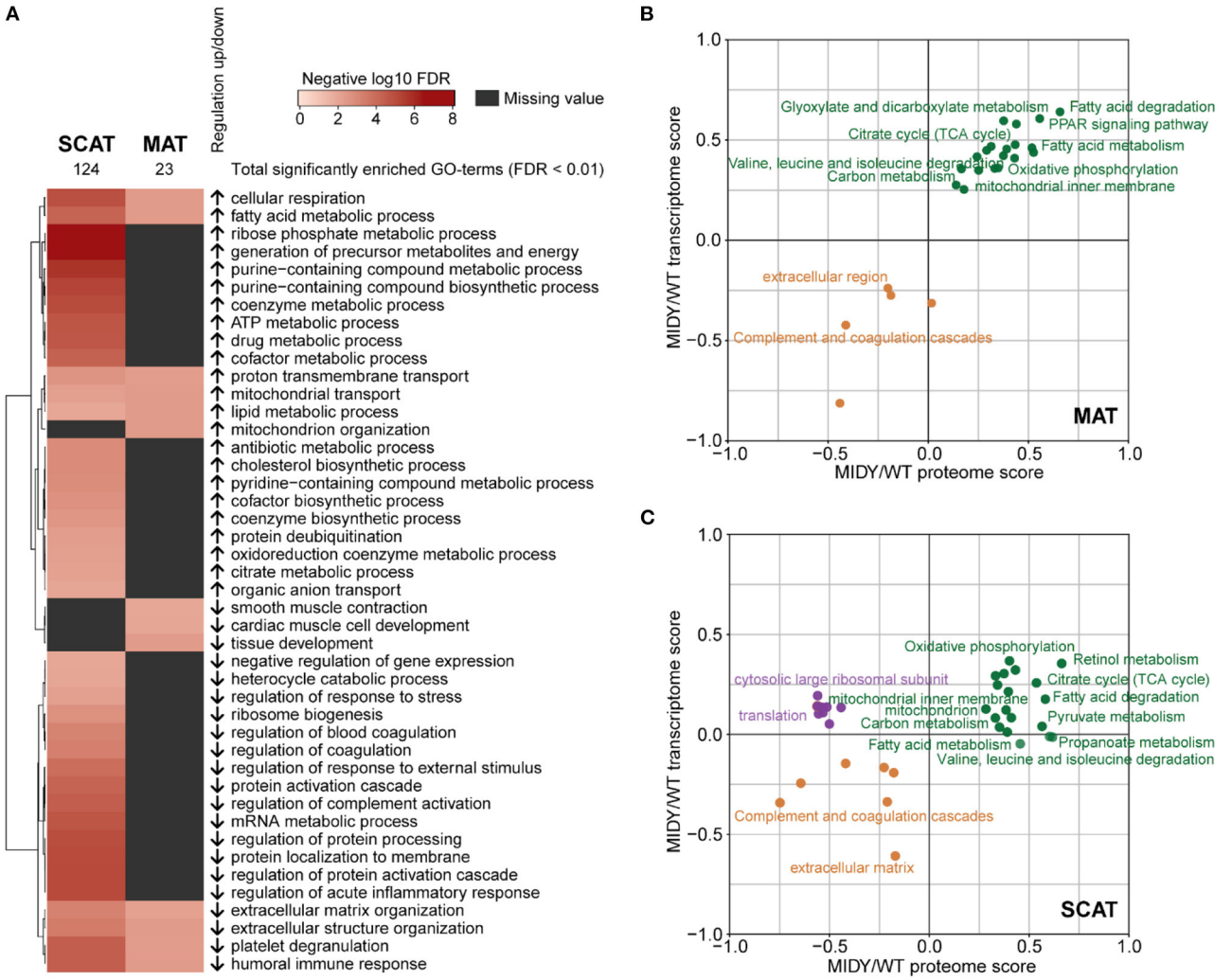

FIGURE 3 | Multi-omic functional characterization of MIDY adipose tissue **(A)** Annotation enrichment analysis of SCAT and MAT proteome alterations in MIDY. Heatmap shows GO-term enrichment in MIDY compared to WT. Enrichment analysis was performed using a pre-ranked STRING analysis and an FDR cutoff of 0.01. Significantly enriched GO biological processes were summarized with REVIGO by grouping semantically similar ontology terms. Arrows indicate regulation in MIDY. **(B,C)** 2D annotation enrichment analysis showing the correlation of proteomics and transcriptomics data. Fold-changes in the proteome (x-axis) and transcriptome (y-axis) are rescaled and shown as scores. Terms depicted in green dots show a common upregulation at the transcriptome and proteome level. Terms in orange show a common downregulation. Purple dots indicate downregulation at the proteome level and upregulation of corresponding mRNA levels. Significant KEGG pathways and Gene Ontology categories with FDR < 0.1 are shown.

DISCUSSION

Adipose tissue plays a central role in energy homeostasis and metabolic function [reviewed in (2, 53)], and its development and functions are regulated by insulin signaling (54). Metabolic and functional characteristics differ between adipose tissue depots and their specific contribution to metabolic health and disease is extensively studied (9, 14, 55–57).

Proteomics provides holistic insights into adipose tissue functions in health and disease. A number of studies investigated proteome profiles of adipose tissue in type 2 diabetes mellitus (18–22), which is characterized by insulin resistance and hyperinsulinemia. However, holistic studies of adipose tissue in insulin-deficient diabetes mellitus are lacking.

Therefore, we analyzed fat tissue samples from long-term diabetic (2 years) *INS*^{C94Y} transgenic pigs, a model for mutant *INS* gene induced diabetes of youth (MIDY) (23), and wildtype (WT) littermates. The samples were taken according to the principles of random systematic sampling and archived in the Munich MIDY pig biobank (29). To explore how chronic, absolute insulin deficiency and hyperglycemia affect transcriptomes and proteomes of different adipose tissue types, a multi-omics analysis of MAT and SCAT from MIDY and WT animals was performed.

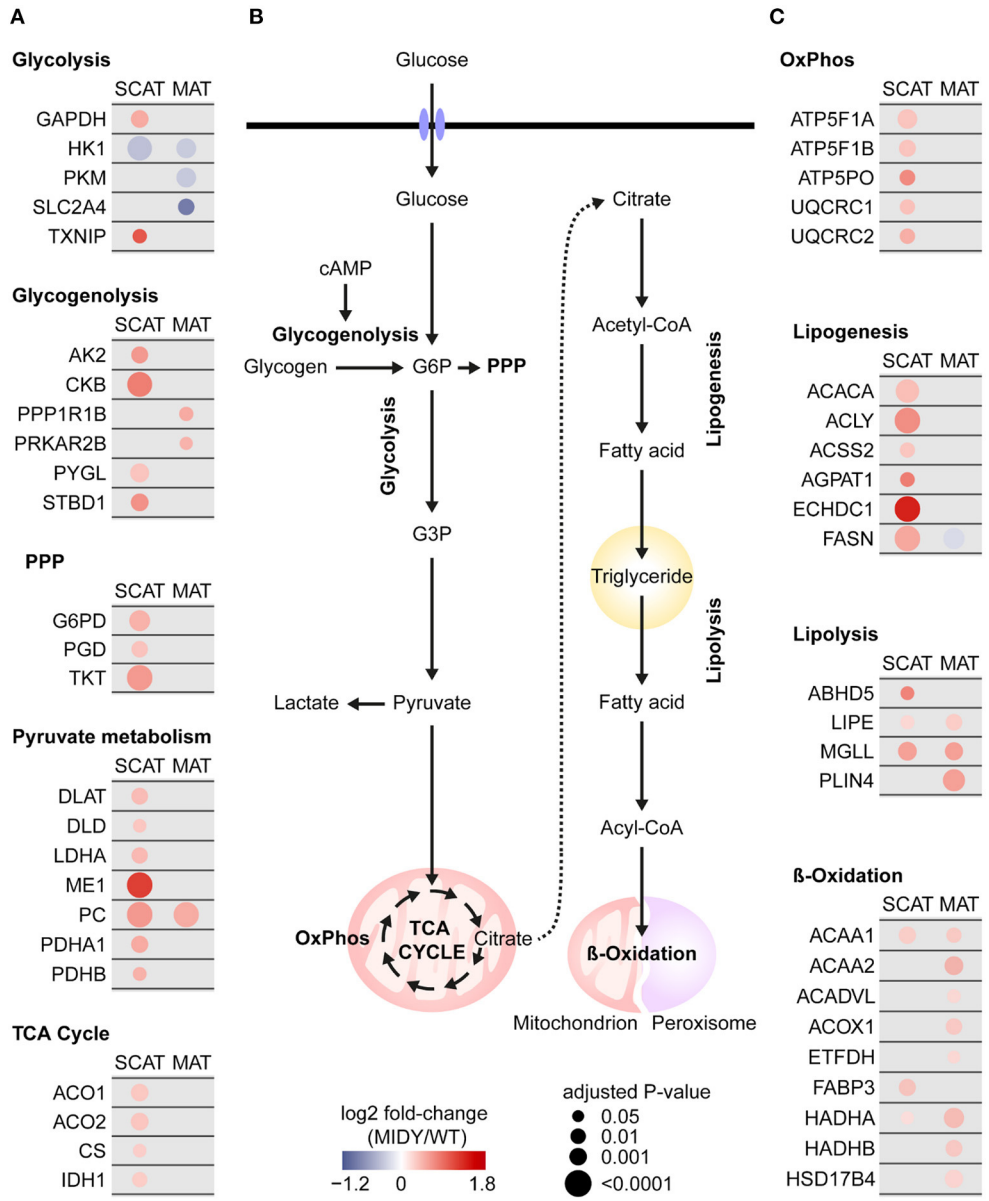

FIGURE 4 | Impaired carbohydrate and fat metabolism in MIDY SCAT and MAT. **(A,C)** Protein abundance alterations in MIDY compared to WT are color coded and shown as log2 fold-change of MIDY/WT. Red circles represent an increased abundance and blue a decreased abundance in MIDY, respectively. Circle size correlates with Benjamini-Hochberg-adjusted *P*-value. Only proteins with an adjusted $P < 0.05$ in at least one tissue depot are shown. Proteins that were not significant or not detected in that tissue depot are grayed out. **(B)** Schematic representation of glucose and fat metabolism in adipose tissue. G6P, glucose-6-phosphate; G3P, glyceraldehyde-3-phosphate; cAMP, cyclic adenosine monophosphate; PPP, pentose phosphate pathway; OxPhos, oxidative phosphorylation.

Proteome Signatures of Subcutaneous and Visceral Adipose Tissue

Adipocytes in different adipose tissue depots have different morphology and fulfill different tasks. They also differ in their reaction toward obesity (adaptive growth patterns)—see Stenkula and Erlanson-Albertsson (58) and recent quantitative morphological analyses of adipose tissue in porcine biomedical models by Theobalt et al. (59). In line with these findings we found major histological differences between SCAT vs. MAT tissue samples of MIDY and WT pigs. Moreover, using a functional enrichment analysis of protein profiles from MAT and SCAT depots from WT and MIDY pigs, we detected distinct proteomic patterns that reflect depot-specific functions. Among the most enriched gene sets in SCAT vs. MAT were clusters related to extracellular matrix (ECM) organization, including significantly higher abundances of multiple subunits of type I, IV, VI, XIV collagens, fibronectin, as well as B2 and A5 subunits of laminin. This agrees with previous DNA microarray

and histological observations, suggesting that the ECM of SCAT maintains a fibrous network connecting dermis and subdermal tissues (60). Similarly, a depot-specific heterogeneity of adipose tissue in ECM composition was reported in previous studies, suggesting ECM as determining factor for adipogenic capacity and a greater adipogenic potential for subcutaneous fat (61, 62). Furthermore, we detected higher abundances of proteins related to cell junction assembly, cell adhesion, and actin cytoskeleton organization in SCAT compared to MAT, which additionally highlights the structural function of SCAT.

The proteome of MAT revealed an enrichment of proteins related to metabolic processes. In particular, glucose and lipid metabolic pathways, including pyruvate metabolism, fatty acid synthesis and degradation were found to be overrepresented. Pertinent metabolic pathways, such as tricarboxylic acid (TCA) cycle, oxidative phosphorylation, and fatty acid oxidation, are localized in the mitochondria (63). In rats and humans, it was shown that mitochondrial content is higher in visceral compared with subcutaneous adipose tissue (64, 65). Accordingly, there was a significantly higher abundance of proteins representing these pathways in MAT. Taken together, our data support the notion that MAT is metabolically more active and more sensitive to mitochondrial substrate supply than SCAT.

Proteome Alterations Between MIDY and WT Reflects Adipose Depot-Specific Response to Insulin Deficiency and Hyperglycemia

The comparison of SCAT and MAT from MIDY pigs with age-matched WT controls revealed significant depot-dependent transcriptome and proteome alterations related to glucose and lipid homeostasis in both AT depots of MIDY pigs. Insulin signaling is fundamental for the regulation of energy and lipid metabolism in adipose tissue [reviewed in (66, 67)]. It promotes glucose uptake into adipocytes by coordinating the translocation of the glucose transporter type 4 (SLC2A4 alias GLUT4) from intracellular sites to the cell surface (68). Impaired GLUT4 translocation is an early sign of developing insulin resistance and type 2 diabetes mellitus (69).

In MAT from MIDY pigs, the abundance of GLUT4 was significantly decreased compared to WT pigs, indicating that not only the membrane translocation but also the absolute expression level of GLUT4 is insulin dependent. Interestingly, GLUT4 was not significantly altered in SCAT from MIDY pigs, suggesting depot-specific regulatory mechanisms of its abundance. Indeed, it was reported that, compared with SCAT, visceral AT has increased insulin-stimulated glucose uptake (70–72) and that insulin signaling was more pronounced in visceral AT than in SCAT (73, 74). This suggests that in MIDY pigs, insulin insufficiency has a stronger impact on GLUT4-mediated glucose uptake in MAT than in SCAT.

The thioredoxin-interacting protein (TXNIP) was strongly increased in abundance in the SCAT samples from MIDY pigs. *TXNIP* transcription is induced by glucose and concurrently, the TXNIP protein suppresses excess cellular glucose uptake. It is therefore described as central regulatory element for acute energy stress response (75). In MIDY pigs, the upregulation of TXNIP can therefore be interpreted as a response of adipose tissue to permanently elevated glucose levels.

After entering the cell, the initial step in glucose metabolism is phosphorylation, catalyzed by hexokinases. Strikingly, hexokinase 1 (HK1), a proposed key regulator of AT glucose uptake (70), was found to be significantly decreased in both MIDY AT depots. Overall, our data suggests an impaired glucose import and a reduced glucose phosphorylation in MIDY MAT and SCAT cells.

The HK1 reaction product glucose-6-phosphate (G6P) can be metabolized in several alternative pathways, namely downstream glycolysis, pentose phosphate pathway (PPP) and glycogen metabolism (76). Surprisingly, despite the presumed reduced glucose import and hexokinase-catalyzed phosphorylation in MIDY AT depots, we detected a consistently higher abundance of key enzymes acting in subsequent glycolytic steps as well as in PPP in MIDY SCAT. This inverse correlation of reduced glucose uptake and phosphorylation to G6P with a simultaneous enhanced glycolytic degradation of G6P was previously described as "hexokinase paradox" (77). A possible cause for a boosted glycolytic degradation could be excess glycogenolysis, which demands glycolytic enzymes for metabolizing glycogen-derived G6P. The rate of glycogen breakdown is critically insulin-dependent, and it was shown that during insulin deficiency, glycogenolysis is increased (78). Indeed, we found elevated levels of glycogen phosphorylase (PYGL), the rate-limiting enzyme in glycogenolysis, as well as of multiple enzymes involved in the activation of glycogenolysis such as creatine kinase B-type (CKB) and adenylate kinase 2 (AK2), advocating a stimulated glycogenolysis in MIDY SCAT.

Glycolysis leads to the generation of pyruvate, which can enter the TCA cycle in mitochondria or can be reduced to lactate by lactate dehydrogenase (79). The abundance of lactate dehydrogenase (LDHA) was significantly increased in MIDY SCAT, and did not change in MIDY MAT compared to WT. Interestingly, Markan et al. also observed an increased lactate production in epididymal adipocytes with elevated glycogenolysis, suggesting a concurrent regulation (80). Since excess lactate can cause intracellular acidosis, it can be released to preserve continuity of glycolysis. An important compensatory mechanism to regulate intracellular pH is the import of HCO_3^-, which can be interconverted to CO_2, through the SLC4 family of transporters. This reversible reaction is catalyzed by carbonic anhydrases [reviewed in (81)]. Strikingly, both the bicarbonate transporter SLC4A1 (alias AE1) as well as multiple carbonic anhydrases (CA1, CA3, CA5B, CA2 as a trend) were detected at increased levels in MIDY SCAT compared to WT, suggesting an active regulation of pH in MIDY SCAT. An alternative use for lactate was proposed recently, namely that it can serve as a rich carbon source and fuel mitochondrial TCA cycle in normal and tumor tissue (82).

Inside mitochondria, the pyruvate dehydrogenase complex (PDC) converts pyruvate into acetyl-CoA to enter TCA cycle for energy production. The consistent increase of PDC enzymes including pyruvate dehydrogenase E1 subunits alpha and beta (PDHA1 and PDHB), dihydrolipoamide S-acetyltransferase

(DLAT) and dihydrolipoamide dehydrogenase (DLD), as well as higher levels of citrate synthase (CS), the rate-limiting enzyme of the TCA cycle, which catalyzes the condensation of acetyl-CoA with oxaloacetate, indicate an enhanced TCA cycle flux in MIDY SCAT compared to WT. As an alternative, pyruvate can be transformed to oxaloacetate by pyruvate carboxylase (PC) to replenish the TCA cycle intermediates (83). Accordingly, PC levels were found to be increased in both AT depots, suggesting an enhanced oxaloacetate generation to cover an increased demand for pathway components.

Adipocytes serve primarily as energy storage for excess nutrients and on the other hand regulate lipid mobilization and distribution in the body. During *de novo* lipogenesis (DNL), excess carbohydrates are converted into fatty acids (FA), which can be stored as triacylglycerides within lipid droplets [reviewed in (1, 84, 85)]. The main substrate for *de novo* synthesis of fatty acids is acetyl-CoA, which can either be generated from citrate by ATP-citrate lyase (ACLY) or from acetate catalyzed by acetyl-CoA synthetase 2 (ACSS2). In the first and rate-limiting step of DNL, acetyl-CoA is transformed into malonyl-CoA by acetyl-CoA carboxylases (ACCs). Malonyl-CoA undergoes a condensation reaction with acetyl-CoA by fatty acid synthase (FASN) in the presence of PPP-produced NADPH to generate triglycerides. In SCAT of MIDY pigs, we found elevated levels of ACLY and ACSS2, which provide metabolic substrates for lipogenesis. Consequently, we observed an increased abundance of G6PD, the rate-limiting PPP enzyme, concomitant with elevated levels of key lipogenic enzymes, such as ACACA/ACC1 and FASN. In contrast, FASN abundance was reduced in MAT of MIDY pigs, suggesting a limited FASN-driven fatty acid synthesis in this fat depot. This might be associated with the significantly reduced levels of the GLUT4/SLC2A4 glucose transporter in MAT of MIDY pigs, as it was shown that adipose tissue lipogenesis strongly correlates with insulin sensitivity (86) and that GLUT4 overexpression in mice led to an elevated AT lipogenesis (87). To compensate the lack of fatty acids from endogenous lipogenesis, MAT might obtain fatty acids from exogenous uptake by passive diffusion or specialized transporters (88–90). Using this route, fatty acids released from lipolysis might be reimported *via* the so-called fatty acid recycling pathway (91). Alternatively, consumption of lipid droplet reserves could help to secure the endogenous FA pool.

Triglyceride turnover is crucial for lipid homeostasis in adipose tissue. Breakdown of triglycerides *via* lipolysis enables release of glycerol and non-esterified fatty acids (NEFAs) which can serve as energy substrates in mitochondrial β-oxidation or can be released and fuel energy metabolism in other organs (92, 93). It is known that insulin suppresses lipolysis and promotes triglyceride storage in adipocytes by diminishing expression of lipolysis-specific enzymes (94–96).

Consequently, in MIDY pigs, we observed an increase of key enzymes involved in lipolysis in both AT depots, among them hormone-sensitive lipase (LIPE alias HSL) and monoglycerol lipase (MGLL alias MGL), which catalyze the stepwise breakdown of triglycerides to glycerol and FAs (92, 97). In this context, it is worth mentioning that enzymes involved in retinol metabolism were increased in both AT depots of MIDY pigs. In particular, retinol dehydrogenase 16 (RDH16), whose expression is negatively regulated by insulin and which catalyzes the first of the two-step reaction from retinol to retinoic acid (98), was among the most significantly increased proteins in MIDY AT. Remarkably, in the liver of MIDY pigs, together with elevated levels of retinal and retinoic acid, RDH16 was also found to be increased in abundance and was suggested as a key driver of stimulated hepatic gluconeogenesis in MIDY pigs (27). Retinoid action has tissue-specific differences and in AT, elevated retinoic acid was shown to suppress adipogenesis (99) and promote lipolysis (100, 101). It is therefore conceivable that the increase in retinol metabolism promotes lipolysis in MIDY AT. Together, in both depots, fat break-down and release potentially predominate accumulation, which is supported by the absence of adipocyte enlargement and by elevated levels of circulating free fatty acids in MIDY pigs (27). β-oxidation is the central pathway for the degradation of long-chain fatty acids and is often discussed in the context of the pathophysiology of insulin resistance, diabetes, and obesity [reviewed in (102)]. While β-oxidation mainly occurs in mitochondria, peroxisomes are indispensable for metabolizing very-long-chain fatty acids and branched-chain fatty acids (103). Acetyl-CoA produced through oxidative degradation of FAs can fuel the TCA cycle and oxidative phosphorylation to push energy production. Our proteomics data showed that a variety of enzymes, transporter and facilitating proteins involved in fatty acid oxidation were increased in MAT and to a lesser extent in SCAT of MIDY pigs. Increased mRNA levels of the two acyltransferases, carnitine O-palmitoyltransferases 1 and 2 (CPT1A and CPT2), and carnitine acyl carnitine translocase (SLC25A20), the key transporters for fatty acid import into mitochondria (102), point toward an increased FA uptake *via* the carnitine cycle in MAT from MIDY pigs. In the β-oxidation cycle, we found increased levels of major enzymes involved in the stepwise shortening of acyl-CoA in MAT of MIDY pigs, namely the very long chain acyl-CoA dehydrogenase (ACADVL) and the medium-chain 3-ketoacyl-CoA thiolase (ACAA2), as well as the mitochondrial trifunctional subunits alpha (HADHA) and beta (HADHB). Taken together, our data indicates that MAT in MIDY pigs has increased capacities for mitochondrial uptake and oxidative degradation of FAs. Following the concept of the Randle cycle (104), the enhanced β-oxidation in MAT of MIDY pigs might therefore be an adaptation to the reduced availability of glucose, with a metabolic switch from glycolysis to fatty acid oxidation. A potential key mediator thereby might be leptin, as it was reported recently that hypoleptinemia promotes a shift from carbohydrate to fat metabolism *via* the hypothalamic-pituitary-adrenal axis, leading to increased AT lipolysis and hepatic ketogenesis, which is necessary to maintain glucose homeostasis and substrate supply during starvation (105). This hypothesis is strongly supported by a reduction of leptin mRNA in MAT and a significant reduction of circulating leptin levels in MIDY pigs.

Collectively, our multi-omics analysis of MAT and SCAT of MIDY pigs revealed severe depot-specific dysregulations in response to insulin deficiency. Our data indicates regionally different metabolic adaptations to overcome energy stress caused by reduced glucose utilization in MIDY adipocytes. This study provides novel pathophysiologic insights and is an important

resource for understanding adipocyte functions in insulin-deficient diabetes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The mass spectrometry proteomics data generated and analyzed during the current study have been deposited to the ProteomeXchange Consortium via the PRIDE (106) partner repository, http://proteomecentral.proteomexchange.org; PXD026910. The transcriptomics results are included in the article/**Supplementary Material**.

ETHICS STATEMENT

All experiments were performed according to the German Animal Welfare Act with permission from the responsible authority (Government of Upper Bavaria), following the ARRIVE guidelines and Directive 2010/63/EU for animal experiments.

AUTHOR CONTRIBUTIONS

FF, EL, BS, and TF: proteomics studies and data analysis. MB, JP-M, and HB: transcriptomics studies and statistics. AB, SR, RW, and EW: generation of animal model and providing samples. MH and GA: investigation. FF, EL, BS, TF, and EW: conceptualization and writing. All authors read and approved the final manuscript prior to submission.

FUNDING

This study was supported by the German Center for Diabetes Research (DZD e.V.) and the German Research Council (Graduate School QBM; TRR127). This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie Grant Agreement No. 812660 (DohART-NET).

ACKNOWLEDGMENTS

We thank Miwako Kösters and Christina Blechinger for excellent technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.751277/full#supplementary-material

Supplementary Figure 1 | Volcano plots visualize the quantitative proteome differences of SCAT vs. MAT in WT **(A)** and MIDY **(B)** animals. Red spots indicate differentially abundant proteins (fold-change > 1.3 and Benjamini-Hochberg-adjusted *P*-value < 0.05).

Supplementary Figure 2 | (A) Histology of MAT and SCAT adipose tissue depots in WT and MIDY animals. Paraffin sections, hematoxylin and eosin staining. Bar = 100 μm. **(B)** Volume weighted mean adipocyte volume in MAT and SCAT adipose tissue depots in WT and MIDY animals. Data are means and standard deviations. Data points corresponding to individual animals are indicated by individual symbols. The mean adipocyte volumes in SCAT and MAT adipose tissue depots of the same animals were compared, using paired student *t*-tests. $^{*}p < 0.05$; $^{**}p < 0.01$. Mean SCAT- and MAT-adipocyte volumes of WT vs. MIDY pigs were compared by student *t*-tests, as indicated. *n.s.*, not significant ($p > 0.05$).

Supplementary Figure 3 | Absolute quantification of leptin levels in sera from WT and MIDY pigs. The difference between the groups was evaluated using a student *t*-test.

Supplementary Table 1 | Proteins Identified and quantified by nano-LC-MS/MS-based proteomics.

Supplementary Table 2 | Results of MS-EmpiRe-based quantitative proteomics of SCAT vs. MAT from WT animals. Proteins showing significant differences in abundance between MIDY and WT animals (FDR < 0.05, fold-change > 1.3) are marked with a "+." Positive log2 fold changes means more abundant in the SCAT group.

Supplementary Table 3 | Results of MS-EmpiRe-based quantitative proteomics of SCAT vs. MAT from MIDY animals. Proteins showing significant differences in abundance between MIDY and WT animals (FDR < 0.05, fold-change > 1.3) are marked with a "+." Positive log2 fold changes means more abundant in the SCAT group.

Supplementary Table 4 | STRING functional enrichment analysis from SCAT vs. MAT from WT animals. Direction "bottom" indicates enrichment in SCAT compared to MAT, "top" indicates enrichment in MAT, and "both ends" indicates enrichment on both sides of the ranked quantification data. Gene Ontology biological processes with FDR < 0.01 are listed.

Supplementary Table 5 | STRING functional enrichment analysis from SCAT vs. MAT from MIDY animals. Direction "bottom" indicates enrichment in SCAT compared to MAT, "top" indicates enrichment in MAT, and "both ends" indicates enrichment on both sides of the ranked quantification data. Gene Ontology biological processes with FDR < 0.01 are listed.

Supplementary Table 6 | Results of MS-EmpiRe-based quantitative proteomics of MIDY vs. WT pig visceral mesenteric adipose tissue. Proteins showing significant differences in abundance between tissue depots of MIDY and WT animals (FDR < 0.05, fold-change > 1.3) are marked with a "+." Positive log2 fold changes means more abundant in the MIDY group.

Supplementary Table 7 | Results of MS-EmpiRe-based quantitative proteomics of MIDY vs. WT pig subcutaneous adipose tissue. Proteins showing significant differences in abundance between tissue depots of MIDY and WT animals (FDR < 0.05, fold-change > 1.3) are marked with a "+." Positive log2 fold changes means more abundant in the MIDY group.

Supplementary Table 8 | STRING functional enrichment analysis from MIDY vs. WT MAT. Direction "bottom" indicates enrichment in MIDY compared to WT, "top" indicates enrichment in WT, respectively. Gene Ontology biological processes with FDR < 0.01 are listed.

Supplementary Table 9 | STRING functional enrichment analysis from MIDY vs. WT SCAT. Direction "bottom" indicates enrichment in MIDY compared to WT, "top" indicates enrichment in WT, respectively. Gene Ontology biological processes with FDR < 0.01 are listed.

Supplementary Table 10 | Transcriptome analysis of MIDY vs. WT pig visceral mesenteric adipose tissue.

Supplementary Table 11 | Transcriptome analysis of MIDY vs. WT pig subcutaneous adipose tissue.

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ORIGINAL RESEARCH
published: 06 December 2021
doi: 10.3389/fmed.2021.732042

Tangshen Formula Improves Diabetes-Associated Myocardial Fibrosis by Inhibiting TGF-β/Smads and Wnt/β-Catenin Pathways

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Cardiovascular disease has become the main cause of death among complications of diabetes. Myocardial fibrosis is a crucial pathological change of cardiovascular disease. Tangshen Formula (TSF) shows a good clinical effect in the treatment of diabetic kidney disease (DKD). However, whether TSF alleviates diabetes-associated myocardial fibrosis is still unknown. In the present research, we studied the effect and mechanism of TSF in the treatment of myocardial fibrosis *in vivo* and *in vitro*. We found that TSF treatment significantly downregulates myocardial fibrosis-related markers, including collagens I and III, and α-SMA. TSF also protects primary mouse cardiac fibroblast (CF) from transforming growth factor-β1- (TGF-β1-) induced damage. Moreover, TSF decreased the expression levels of TGF-β/Smad-related genes (α-SMA, collagens I and III, TGF-β1, and pSmad2/3), and increased Smad7 gene expression. Finally, TSF decreased the expressions of wnt1, active-β-catenin, FN, and MMP7 to regulate the Wnt/β-catenin pathway. Taken together, TSF seems to attenuate myocardial fibrosis in KKAy mice by inhibiting TGF-β/Smad2/3 and Wnt/β-catenin signaling pathways.

Keywords: Tangshen Formula, myocardial fibrosis, TGF-β/Smad, Wnt/β-catenin, KKAy

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *28 June 2021*
Accepted: *05 October 2021*
Published: *06 December 2021*

Citation:
Hu L, Wang Y, Wan Y, Ma L, Zhao T and Li P (2021) Tangshen Formula Improves Diabetes-Associated Myocardial Fibrosis by Inhibiting TGF-β/Smads and Wnt/β-Catenin Pathways. Front. Med. 8:732042. doi: 10.3389/fmed.2021.732042

INTRODUCTION

Diabetes mellitus (DM) is a global epidemic. Nearly half a billion people worldwide have diabetes, and this number is expected to increase by 25% in 2030 and by 51% in 2045 (1). Among the complications of diabetes, diabetic cardiomyopathy is a common cause of death (2). Myocardial fibrosis is a typical pathology in cardiovascular diseases and is marked by profuse deposition and disruption of extracellular matrix (ECM) and the over-proliferation of activated cardiac fibroblasts (CFs) (3). Despite strict blood glucose control, the incidence of diabetic cardiomyopathy remains high. Thus, it is crucial to search for new drugs to treat myocardial fibrosis.

Tangshen Formula (TSF) is a Chinese herbal medicine (CHM) for the treatment of diabetic kidney disease (DKD) (4). In both rat and mouse models of DKD, TSF has been found to effectively treat renal fibrosis by inhibiting the transforming growth factor β (TGF-β)/Smad signaling pathway (5). It is well known that TGF-β/Smad signaling is a key pathway in the development of fibrosis in many organs, including the heart (6). Also, TGF-β1 is proved to play a critical pathogenic role in diabetes-associated myocardial fibrosis. It was potentially found that TGF-β1 could induce the expression of ECM protein in CFs by activating Smads-dependent signals in diabetic mice,

thus leading to pathological fibrosis (7). TGF-β1 binds to its receptor and activates downstream mediators, including Smad2 and Smad3, to exert biologic effects; TGF-β1 is negatively regulated by Smad7 expression. The overexpression of TGF-β1 causes the overproduction of ECM protein and inhibits their degradation, leading to fibrosis (6). Ho et al. reported that TGF-β1 cooperates with Wnt protein signaling to control biologic activities in a variety of cells (8). The Wnt signaling pathway comprises two highly conserved pathways, among which the canonical β-catenin-dependent pathway is involved in myocardial fibrosis (9). β-catenin forms a complex in the nucleus with transcriptional factors of T-cell factor/lymphoid enhancer factor (TCF/LEF) to stimulate the transcription of Wnt target genes, thereby resulting in the deposition of ECM (10).

In this study, we aimed to study the therapeutic effect of TSF on myocardial fibrosis and its underlying mechanism. We found that TSF can alleviate myocardial fibrosis in KKAy mice and fibrotic injury in TGF-β-stimulated myocardial fibroblasts by inhibiting the TGF-β/Smad pathway and the Wnt/β-catenin pathway. This study provided both *in vivo* and *in vitro* evidence for the potential application of TSF in the treatment of myocardial fibrosis.

MATERIALS AND METHODS

Herbal Formulation

Tangshen Formula is made up of seven kinds of herbs comprising astragalus root, burning bush twig, rehmannia root, bitter orange, cornus fruit, rhubarb root and rhizome, and notoginseng root. It was prepared by Beijing Yadong Biopharmaceutical (Beijing, China), and the standardization of the formula was in accordance with the *Pharmacopoeia of The People's Republic of China 2015* (11).

Animals and Experimental Design

Eighteen diabetic male KKAy mice (8 weeks old, 20–25 g) and six male C57BL/6J mice were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). All animals were housed at 23 ± 3°C and 55 ± 15% humidity with 12:12 h light/dark cycle and were allowed access to chow and water *ad libitum*. KKAy mice were treated with a high-fat diet, and their blood glucose was measured once in 2 weeks by One Touch Ultra blood glucose monitoring system (LifeScan, Milpitas, CA, USA). The KKAy mice were randomly divided into the following three groups: (1) KKAy group: KKAy mice-administered distilled water ($n = 6$); (2) KKAy+TSF group: KKAy mice-administered TSF (3.57 g/kg per day) by oral gavage ($n = 6$) (12); and (3) KKAy+Irbesartan group: KKAy mice-administered irbesartan by oral gavage (22.5 mg/kg per day) ($n = 6$). The control group consisted of healthy C57BL/6J mice-administered distilled water ($n = 6$).

All the animals were weighed once a week. After 16 weeks, all mice were sacrificed under anesthesia. Serum was collected for assay. The apex of the heart from each animal was used for a pathologic analysis, and the rest of the heart tissue was used for a molecular biologic analysis. Animal care and treatments were in accordance with the NIH Guiding Principles for the Care and Use of Laboratory Animals, and the protocol was approved by the Beijing National Proteome Science Center Animal Management Ethics Committee.

Heart Histology and Immunohistochemistry

The apex of the heart tissue was fixed with 4% phosphate buffered saline (PBS) buffered paraformaldehyde, embedded in paraffin, and then sectioned into 3 μm thicknesses and stained with Masson trichrome according to the standard procedure. Masson trichrome staining causes muscle fibers to turn red and collagen fibers to turn blue. Immunohistochemistry (IHC) staining was used to detect the distribution and expression of biomarkers for fibrosis. We used a microwave-based heating antigen retrieval and 3% H_2O_2 to block non-specific staining. The antibodies used included: collagen I (1310-01, SouthernBiotech, Birmingham, AL, USA; 1:200 dilution), collagen III (GB111629, Servicebio, Wuhan, China; 1:200 dilution), α-SMA (GM085102, Gene Tech, Shanghai, China; 1:200 dilution), phosphorylated Smad2/3 (sc-11769, Santa Cruz Biotechnology, Dallas, TX, USA; 1:200 dilution), and anti-β-catenin (610154, BD Transduction Laboratories, Shanghai, China; 1:50 dilution). Images were quantitatively analyzed using Image-Pro Plus v 6.0 (Media Cybernetics, Rockville, MD, USA). Briefly, the accumulation of collagens I and III and α-SMA in 10 random areas, except staining around blood vessels, was analyzed under 200× for the percentage of positive areas in the examined field. Numbers of p-Smad2/3+ cells were counted in 10 random areas for each sample under 400×.

Ultrastructure of Myocardium

Heart ultrastructure was detected using transmission electron microscopy (JEOL-100CXII; JEOL, Tokyo, Japan). The heart was sectioned into 1 mm cubes and fixed with 2.5% glutaraldehyde at 4°C for 24 h, and then embedded in an epoxy resin. Ultrathin sections were sliced and stained with uranyl acetate and lead citrate at room temperature, for 30 and 15 min, respectively. Twenty images per sample were randomly selected and observed, and the ultramicrostructure of the myocardium was observed under the magnification ×12,000.

Isolation of Primary Mice Myocardial Fibroblasts

Cardiac fibroblasts were derived from the hearts of neonatal mice. The hearts were removed from the C57BL/6J newborn mice (Beijing Vital River Laboratory Animal Technology, Beijing, China) and, under aseptic conditions, were sectioned into tissue blocks of about 1 mm^3. The tissue blocks were washed with Hanks Balanced Salt solution without Ca^{2+} and Mg^{2+} and digested at 37°C with 0.08% trypsin and 0.06% collagenase. The digested supernatant was collected and neutralized with low glucose (5 mmoL/L) Dulbecco Modified Eagle medium/fatty acid (DMEM/FA) containing 20% serum until the tissue mass became transparent and digestion was terminated. The resulting suspension was filtered with a 200-μm BD Falcon cell strainer (Corning Life Sciences, Corning, NY, USA) and centrifuged from

TABLE 1 | List of primers used for quantificational real-time polymerase chain reaction (qRT-PCR).

Gene	Forward primer	Reverse primer	PCR size (bp)
GAPDH	TGTTTCCTCGTCCCGTAGA	ATCTCCACTTTGCCACTGC	106
TGF-β1	TGGGGACTTCTTGGCACT	ATAGGGGCGTCTGAGGAAC	117
α-SMA	TACTGCCGAGCGTGAGA	TCCAGGGAGGAAGAGGAG	105
ColI	CAGAGGCGAAGGCAACA	GTCCAAGGGAGCCACATC	145

4 to 10 min at 25–300 × g. The filtered solution was then plated onto culture dish.

Cell Viability

Primary mice myocardial fibroblasts were cultured in DMEM with 10% fetal bovine serum (GIBCO, Carlsbad, CA, USA) at 37°C with 5% CO_2. To investigate the role of TGF-β1, the fibroblasts were exposed to TGF-β1 for 6, 16, 24, 36, and 72 h sequentially at 0, 2.5, 5, 10, and 15 μg/ml, respectively. After the TGF-β1-stimulated myocardial fibroblast injury model was successfully obtained, the cells were treated with TSF for 72 h at the concentrations of 100 and 250 μg/ml.

An MTT assay was used to detect whether TSF had an effect on cell viability. Being starved overnight with DMEM/F12 medium without fetal bovine serum (FBS), C57BL/6J mouse primary myocardial fibroblasts were then grown in 96-well plates and incubated with TSF at the concentrations of 0, 100, 250, 500, 750, and 1,000 μg/ml for 72 h. Each well was added with 1/10 volume of MTT solution and incubated at 37°C with 5% CO_2 for 4 h. After adding formazan lysate, the assay was performed at 490 nm wavelength with a microplate analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The experimental methods were completed according to the kit instructions, and cell viability was calculated.

Real-Time Quantitative PCR

Total RNA was isolated from mouse hearts using the TriZol method. RNA concentration was then quantified using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA was reverse transcriptased to the complementary DNA (cDNA) template using the M5 SuperFast qPCR RT kit (Ju Hemei, Beijing, China) quantitative PCR (qPCR) was performed with the QuantStudio 5 Real-Time PCR system (Applied Biosystems, Waltham, MA, USA) using the IQ SYBR Green Supermix reagent (CWBIO, Beijing, China), as previously described (13). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control to normalize the gene expression in the same cDNA. This was followed by applying the $\Delta\Delta C(t)$ threshold cycle method, to analyze the qPCR data (14). The specific primer sequences used are listed in **Table 1**.

Western Blot Analysis

Mouse heart tissues were homogenized in a mixture of radioimmunoprecipitation assay (RIPA) buffer and 1% cocktail (Bimake, Houston, TX, USA) to extract the protein. Protein concentration was measured using the Bicinchoninic acid assay (BCA) Protein Assay kit (Epizyme Biotech, Shanghai, China) and then was denatured. The proteins were separated by 12% sodium dodecyl sulfate (SDS) and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking in 5% skim milk for 1 h, the membranes were incubated with primary antibody at 4°C overnight. The following day, the membranes were washed three times with tris-buffered saline and Tween-20 (TBST) and incubated with secondary antibody. Membrane immunostaining was observed and analyzed using the ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA). Signals were quantified by the Image J program (National Institutes of Health, Bethesda, MD, USA). β-actin normalization detected the protein ratio and was expressed as mean ± SME.

The primary and secondary antibodies were: TGF-β1 (1:1,000 dilution; BS1361, Bioworld Technology, Nanjing, China), smad7 (1:500 dilution; sc-9183, Santa Cruz Biotechnology), anti-rabbit (1:5,000 dilution; S8002, Beijing Guanxingyun Sci and Tech, Beijing, China), anti-mouse (1:5,000 dilution; S8001, Beijing Guanxingyun Sci and Tech, Beijing, China), anti-goat (1:2,000 dilution; ab205723, abcam, Cambridge, UK), FN (1:500 dilution; sc-8422, Santa Cruz Biotechnology), wnt1 (1:1,000 dilution; AF5315, Affinity Biosciences, Cincinnati, OH, USA), β-catenin (1:500 dilution; sc-7963, Santa Cruz Biotechnology), active-β-catenin (1:500 dilution; 05-665, Merck Millipore, Darmstadt, Germany), and MMP7 (1:1,000 dilution; ER1913-08, Huabio, Woburn, MA, USA).

Statistical Analysis

All data collected from this study were expressed as means ± SEM. The statistical analysis was performed and viewed with the GraphPad Prism software (LaJolla, CA, USA), and differences among the means were assessed using a one-way ANOVA.

RESULTS

Effect of TSF on Body Weight and Blood Glucose

During the 16 weeks of treatment with TSF or irbesartan, there were no deaths observed in KKAy mice. Body weights of all animals were measured once a week, and weight gain was recorded (**Figure 1A**). Weight was significantly increased in the KKAy group compared to the C57BL/6J group. However, in the treatment groups, weights were significantly decreased due to TSF or irbesartan therapy. After 16 weeks of treatment, blood glucose levels in KKAy+TSF and KKAy+irbesartan groups were lower than the levels in the KKAy group, but without a significant difference (**Figure 1B**).

Irbesartan ameliorates myocardial fibrosis in diabetic cardiomyopathy (15). It is an angiotensin domain receptor inhibitor and is considered to be a common medication for improving the structure and function of cardiomyocytes and reducing the risk of death from heart failure (16). Therefore, irbesartan was used as a positive control group to investigate the effects of drug therapy.

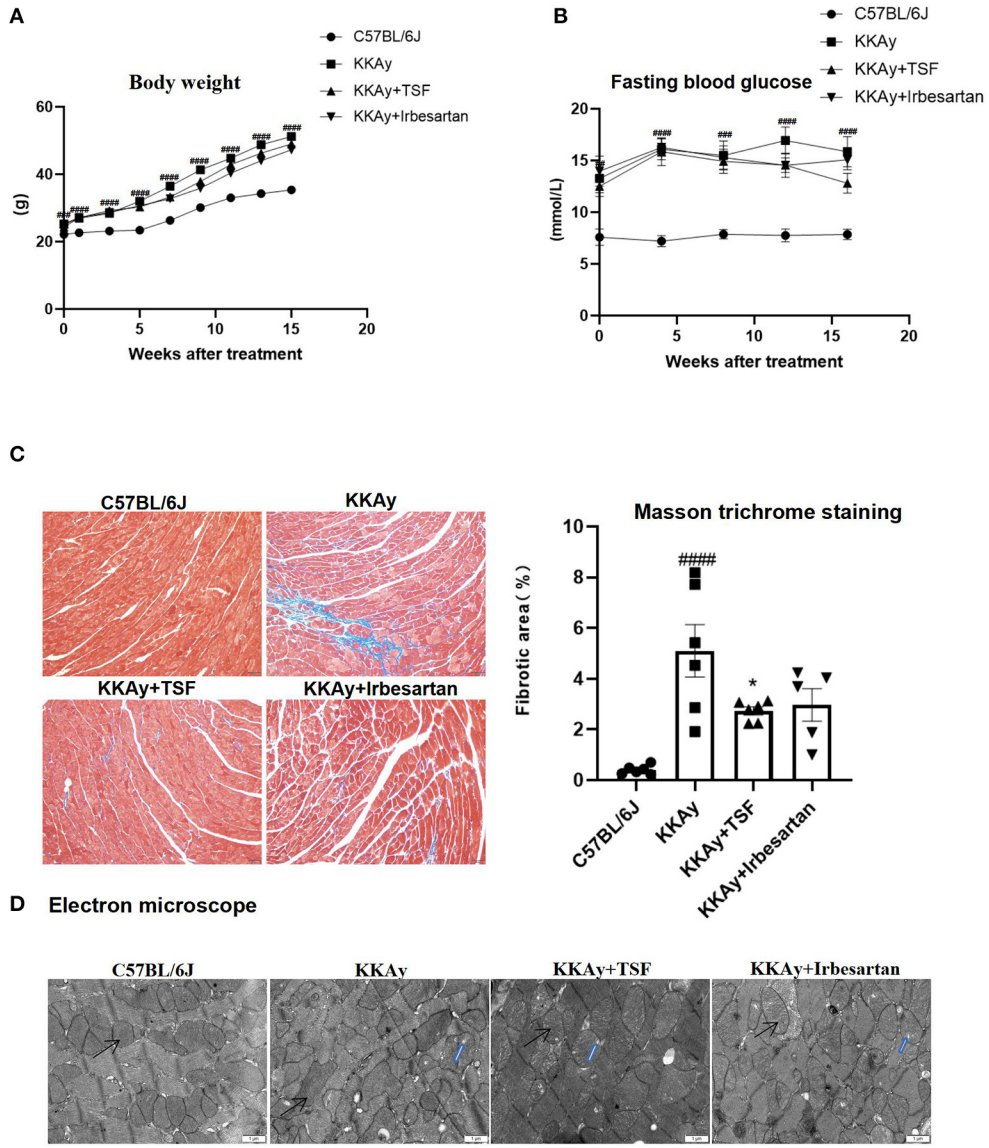

FIGURE 1 | Pathologic changes after Tangshen Formula (TSF) and irbesartan treatments. **(A)** Body weights were recorded once a week. Data are expressed as mean ± SEM. $^{###}p < 0.001$, $^{####}p < 0.0001$ KKAy group vs. C57BL/6J group. **(B)** Fasting blood glucose. After 16 weeks, glucose levels in KKAy mice were significantly increased. TSF-treated KKAy mice exhibited no statistical differences in glucose levels. Data are expressed as mean ± SEM. $^{###}p < 0.001$, $^{####}p < 0.0001$ KKAy group vs. C57BL/6J group. **(C)** Histology (Masson trichrome staining, original magnification, 200×) and semiquantification of the collagen area. Data are expressed as mean ± SEM. $n = 6$. $^{*}p < 0.05$ TSF-treated group vs. KKAy group; $^{###}p < 0.001$ $^{####}p < 0.0001$ KKAy group vs. C57BL/6J group. **(D)** Ultrastructural findings of myocardial tissues in four groups of mice. Magnification ×12,000. Scale bar = 1 μm. Black arrows indicate mitochondria. Blue arrows indicate mitochondria vacuolation.

TSF Treatment Attenuates Cardiac Injury in Diabetic Mice

Masson trichrome staining revealed a large number of blue collagen fibers in the KKAy group compared with the C57BL/6J group. In contrast, in TSF and irbesartan groups, the blue collagen area was significantly reduced (**Figure 1C**). Moreover, quantitative data demonstrated that the treatment with TSF for 16 weeks significantly attenuates these histologic injuries.

Under electron microscopy, the ultrastructure of myocardial cells was observed and the mitochondria remained intact in the C57BL/6J group. In the KKAy group, the myocardial structure was diminished and mitochondrial vacuolization and crest dissolution were evident. In the KKAy+TSF group, the myocardial ultrastructural injury was alleviated though mitochondria did exhibit slight vacuolization. There were no obvious pathologic changes in the KKAy+TSF and

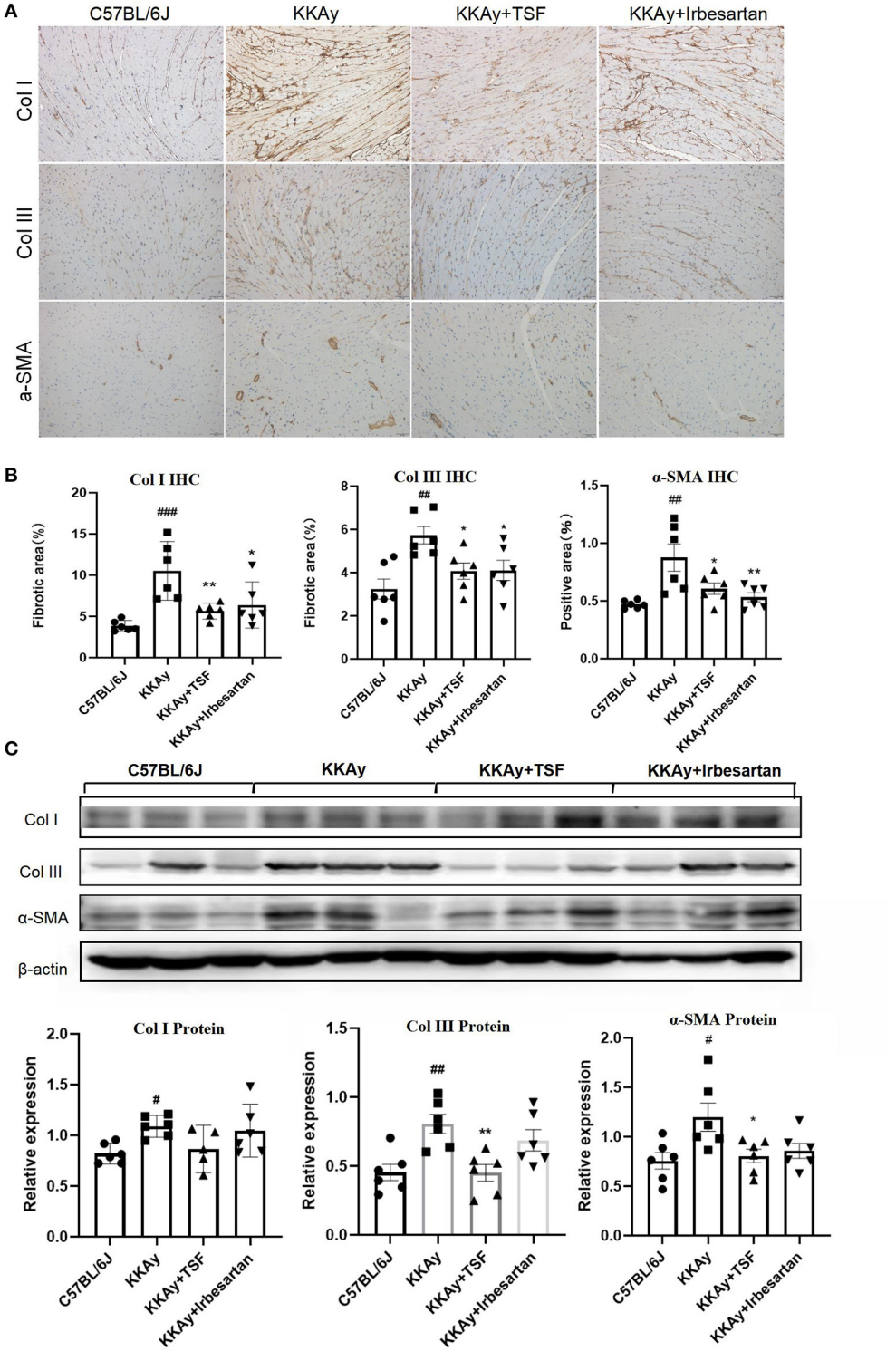

FIGURE 2 | Tangshen Formula inhibited myocardial fibrosis in KKAy mice. **(A)** Immunohistochemistry (IHC) of collagen III (ColIII, original magnification, 200×), collagen I (ColI, original magnification, 200×), and alpha smooth muscle actin (α-SMA, original magnification, 200×). **(B)** IHC and semi-quantitative analyses for collagens I and III, and α-SMA. Data are expressed as mean ± SEM ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$ TSF-treated group vs. KKAy group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ KKAy group vs. C57BL/6J group. **(C)** Western blot and semi-quantitative analyses of ColI, ColIII, and α-SMA expressions.

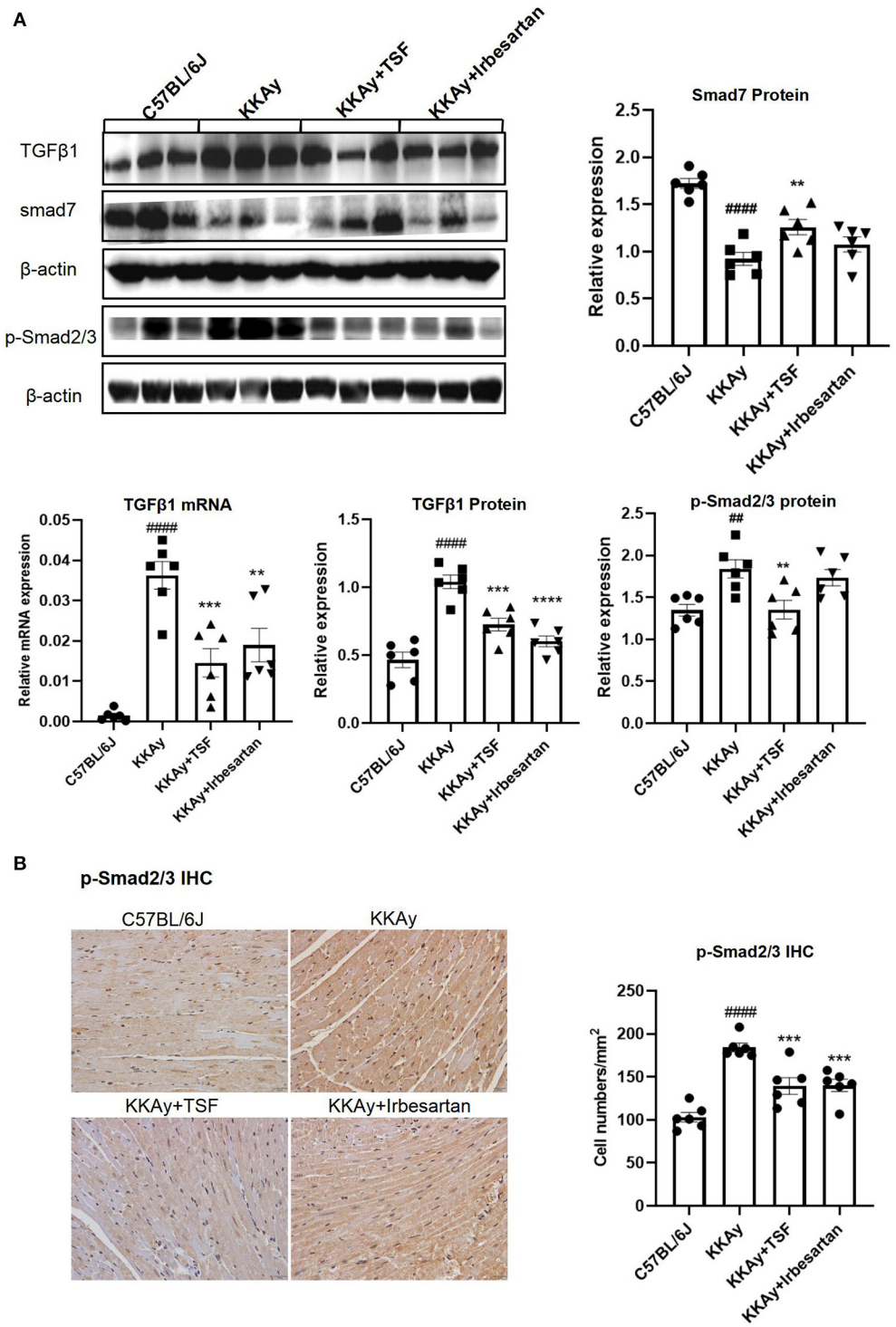

FIGURE 3 | Tangshen Formula treatment inhibited the expression of transforming growth factor-β1 (TGF-β1) and p-Smad2/3 and promoted the expression of smad7 in KKAy mice. **(A)** Western blot and semi-quantitative analyses of TGF-β1, smad7, p-Smad2/3, and Smad2/3 expressions. **(B)** IHC of psmad2/3 (original magnification, 400×) and the number of positive cells in each group. Data are presented as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ TSF-treated group vs. KKAy group; $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ KKAy group vs. C57BL/6J group.

KKAy+ irbesartan group compared with the C57BL/6J group (**Figure 1D**).

TSF Inhibited Myocardial Fibrosis in Diabetic Mice

One of the main symptoms of myocardial fibrosis is the accumulation of collagen in the ECM. Collagen I account for about 80% of the collagen in the heart muscle and increases in myocardial fibrosis. Collagen III is also an important component of the cardiac ECM (17). α-SMA can be assembled into stress fibers to reshape the surrounding ECM. Thus, in our study, we examined the therapeutic effect of TSF on myocardial fibrosis in KKAy diabetic mice. In the heart samples, the immune-positive areas of collagens I and III and α-SMA were yellow-brown (**Figure 2A**). IHC revealed that KKAy mice developed myocardial fibrosis, including a significant upregulation of collagens I and III as well as α-SMA, which were attenuated by the treatment with TSF or irbesartan (**Figure 2B**). Similarly, western blotting revealed that the upregulation of collagens III and α-SMA were significantly decreased after TSF treatment (**Figure 2C**).

TSF Ameliorated Myocardial Fibrosis by Controlling the TGF-β/Smad Pathway in KKAy Mice

Real-time qPCR and western blot revealed that KKAy mice developed myocardial fibrosis, including a significant upregulation of TGF-β1 at the messenger RNA (mRNA) and protein levels as well. This upregulation was inhibited after TSF treatment (**Figure 3A**). IHC results of p-Smad2/3 and western blot analyses of p-Smad2/3 showed that the activation of TGF-β/Smad2/3 signaling in the KKAy group and its effect were reduced after TSF treatment (**Figure 3B**). The protein expression level of smad7 was markedly decreased in KKAy mice compared with the C57BL/6J group ($p < 0.01$), whereas the protein expression was significantly increased in the TSF group compared with the KKAy group (**Figure 3A**). No significant difference was found between C57BL/6J and TSF groups. These results indicated that TSF is effective in inhibiting the TGF-β/Smad pathway in KKAy mice.

Effect of TSF on Wnt/β-Catenin Signaling Pathway in KKAy Mice

To confirm the effect of TSF on Wnt/β-catenin signaling, the western blot analysis was used to detect Wnt1 and β-catenin and their downstream target MMP7, FN. Immunohistochemical analysis of anti-β-catenin showed that the relative expression of anti-β-catenin was upregulated in the KKAy group and was significantly reduced after the treatment with TSF, which demonstrated the activation of Wnt/β-catenin (**Figure 4A**). The presence of the bands only at the target protein sites indicates that the staining is specific. The expression of Wnt1, β-catenin, and active-β-catenin was increased in the KKAy mice compared with the C57BL/6J group, demonstrating that the Wnt/β-catenin pathway was active in the KKAy animal model. TSF decreased the expression of Wnt1, β-catenin, and active-β-catenin proteins in the TSF treatment group compared with the KKAy group. Irbesartan also showed an inhibitory effect on Wnt1 and active-β-catenin expressions. A significant difference in the levels of MMP7 and FN expressions was found between the C57BL/6J group and KKAy group ($p < 0.01$) as well as between the KKAy group and TSF group. In the KKAy group, the expressions of MMP7 and FN significantly increased compared with the C57BL/6J group. In contrast, TSF treatment decreased the expression levels of MMP7 and FN (**Figure 4B**), suggesting that the treatment with TSF attenuates diabetic myocardial fibrosis *via* the Wnt/β-catenin mechanism. Taken together, this study highly suggests that TSF has an inhibitory effect on the Wnt/β-catenin signaling pathway.

TSF Improved CFs Injury, Reduced Expressions of Collagens I and III as Well as α-SMA in C57BL/6J Mice CFs

Combined with protein and mRNA expressions of α-SMA and collagen I, the optimal myocardial fibroblast fibrotic injury model was obtained under the condition of 5 ng/ml TGF-β1 induced for 72 h (**Figures 5A–C**). To determine whether TSF affected cell proliferation, MTT assays were used to investigate the cytotoxicity of TSF (**Figure 5D**). The results showed that the cell survival rate decreased gradually as TSF concentration increased. Thus, the concentrations of 100 and 250 μg/ml TSF were chosen for the *in vitro* experiments.

Cardiac fibroblasts induced by TGF-β1 showed an upregulation of mesenchymal markers, including collagens I and III, and α-SMA (**Figure 5E**), which were all significantly downregulated after 100- and 250-μg/ml TSF treatment.

DISCUSSION

Our study provides evidence that the Chinese traditional herbal medicine TSF markedly alleviated myocardial fibrosis in KKAy mice. The therapeutic impact of TSF on diabetes-associated myocardial fibrosis was related to the inhibitory effect of TGF-β/Smad and Wnt/β-catenin-mediated myocardial fibrosis (**Figure 6**).

In this study, we validated that TSF could relieve fibrosis in the KKAy mice model and in CFs induced by TGF-β1. Compared with C57BL/6J mice, body weight and blood glucose were increased in KKAy mice. After 16 weeks of TSF treatment, body weight was markedly decreased and blood glucose was not statistically different from KKAy mice. Collagen deposition in the heart developed in KKAy mice and was inhibited after 16 weeks of treatment with TSF. *In vitro* experiments also revealed that TSF attenuated fibrosis damage.

Transforming growth factor-β1 is expressed in many tissues and varieties of cells, and its signaling pathway is involved in the expression of fibrogenic factors, including collagens I and III and α-SMA (18). Moreover, TGF-β1 is a crucial mediator of fibroblast activation and diseased heart fibrosis (3), and exerts its biologic effect through Smad signal transduction (19). In the presence of Smad3, TGF-β1 can induce the contraction of collagen lattice and the expression of α-SMA (20). The activation of TGF-β1/Smad3 pathways in cardiac fibrosis can result in myofibroblast

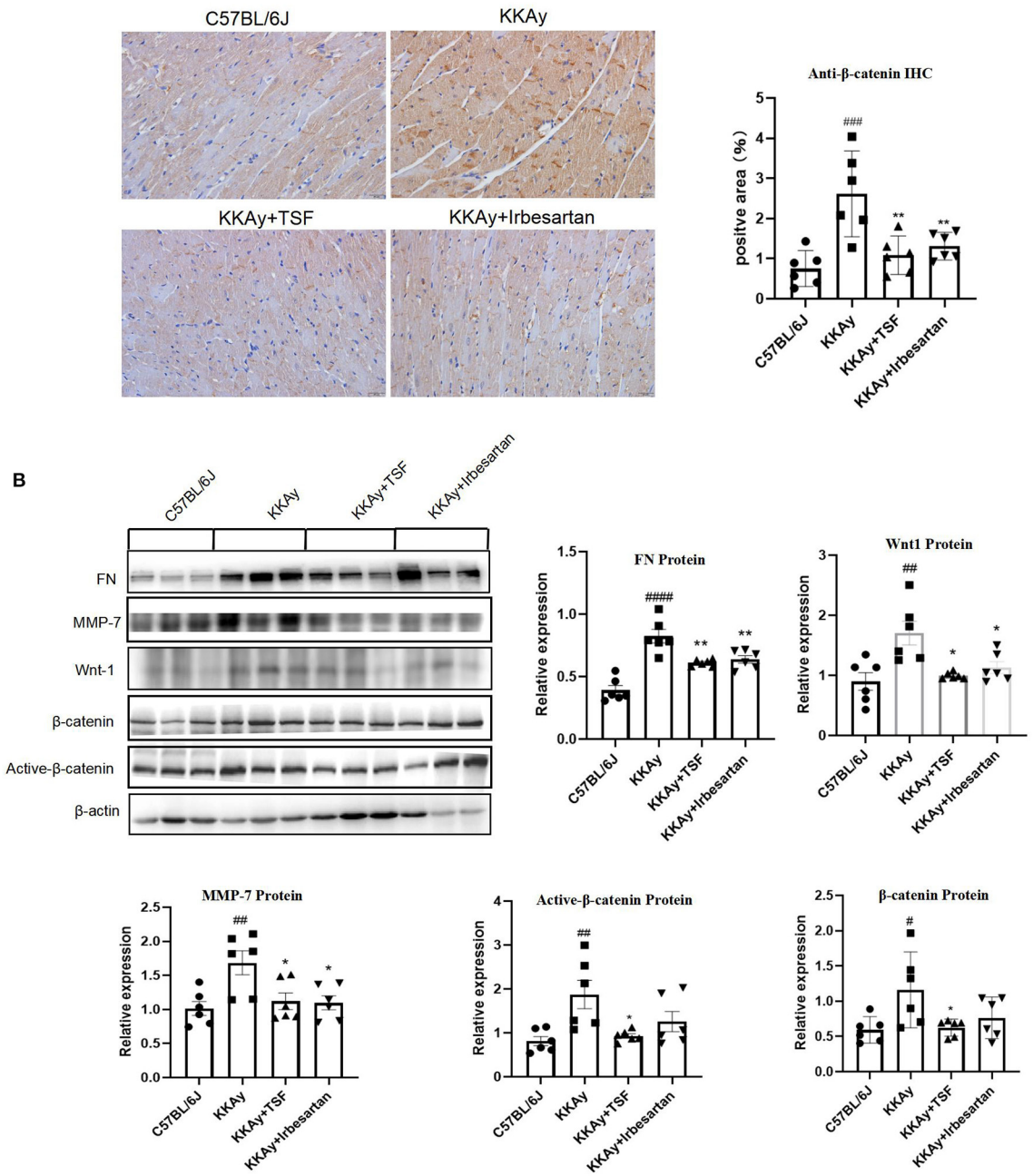

FIGURE 4 | An effect of TSF on the canonical Wnt pathway. **(A)** IHC of anti-β-catenin (original magnification, 200×). Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ TSF-treated group vs. KKAy group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ KKAy group vs. C57BL/6J group. **(B)** Western blot analyses and semi-quantitative analyses revealed expressions of Wnt1, active-β-catenin, β-catenin, FN, and MMP-7 in KKAy mice and the different treatment groups. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ KKAy group vs. C57BL/6J group. $^{*}p < 0.05$, $^{**}p < 0.01$ TSF-treated group vs. KKAy group.

proliferation and a marked upregulation the expressions of collagens I and III (21). In addition, the activation of TGF-β1/Smad3 signaling also leads to the degradation of Smad7. Smad7 is an inhibitory Smad, and the overexpression of Smad7 prevents fibrosis *in vitro* and *in vivo* (5). Our results were consistent with the aforementioned reports. In the KKAy mouse model with myocardial fibrosis, we found the overexpression of collagens I and III and α-SMA, as well as the activation of TGF-β1 and p-Smad2/3 and the reduction of Smad7. TSF is a drug that treats kidney disease, especially renal fibrosis. Previous studies have suggested that TSF alleviates renal fibrosis by regulating TGF-β1/Smad3 (5). TGF-β/Smad is a common signaling pathway in the injury of multiple organs. In this study, we found that TSF decreased the expressions of the target genes of fibrosis markers.

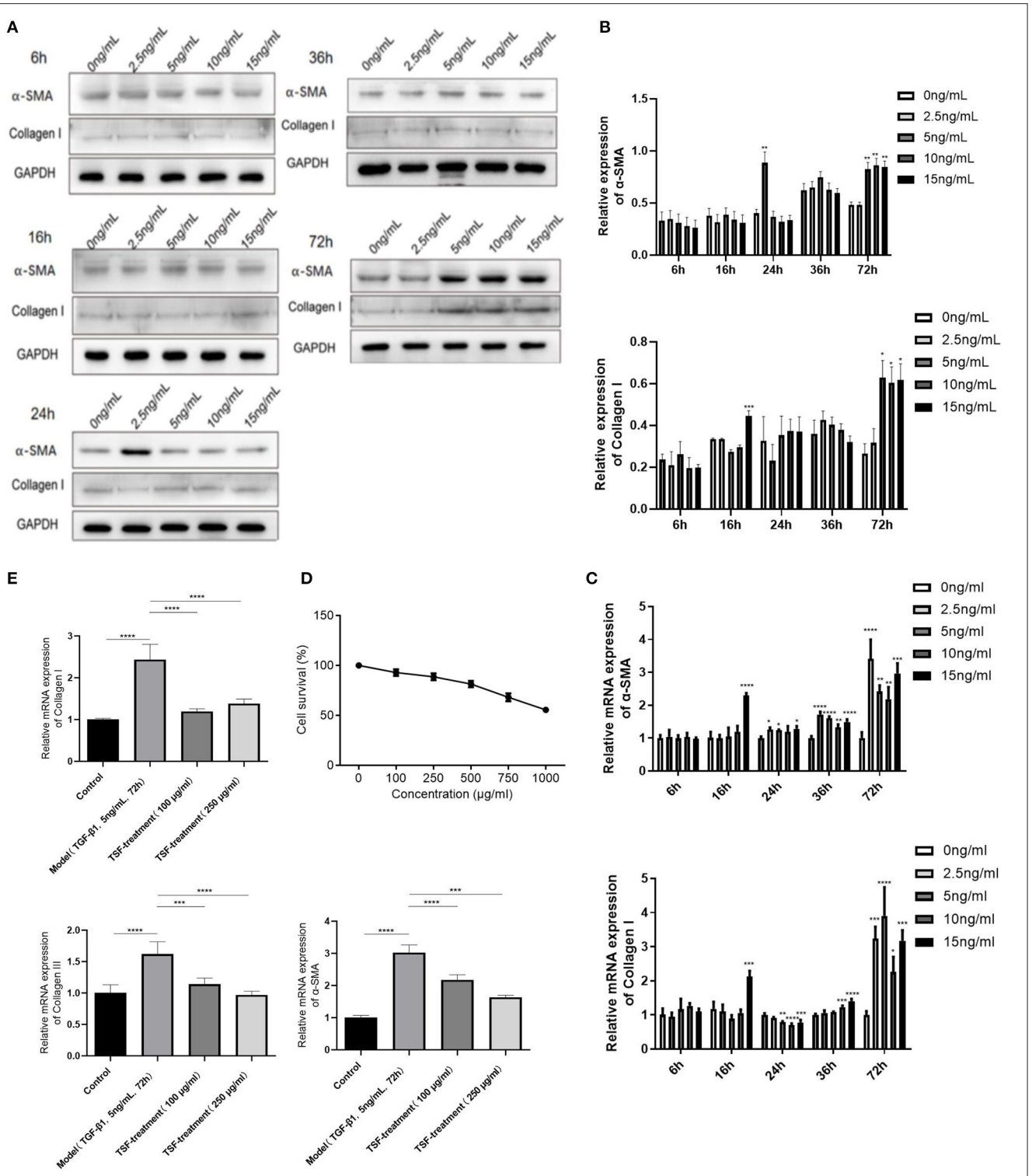

FIGURE 5 | An inhibitory effect of TSF against the activation of the TGF-β/smad pathway *in vitro*. **(A)** The expression of fibrosis index protein in cardiac fibroblast (CF) cells stimulated by TGF-β in different concentrations at different times. **(B)** Quantitative analyses of protein expressions. **(C)** Quantificational real-time polymerase chain reaction (qRT-PCR) of messenger RNA (mRNA) expressions of fibrosis index of CFs induced by TGF-β1. **(D)** Effects of different concentrations of TSF on the survival rate of cells. **(E)** qRT-PCR of mRNA expressions of collagens I and III and α-SMA in CFs induced by TGF-β1 in the different groups. Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

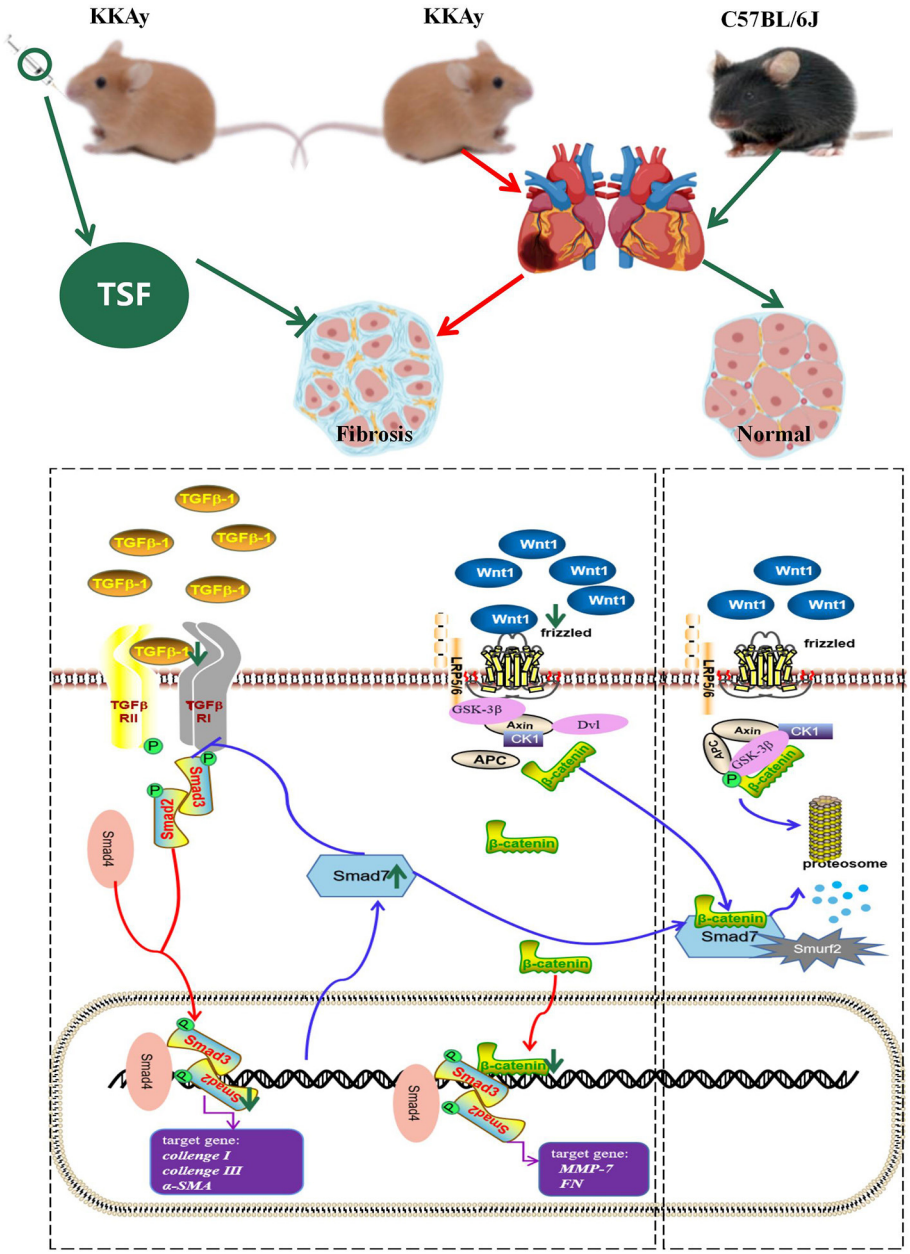

FIGURE 6 | Schema of the possible mechanism of antifibrosis action of TSF. In the TGF-β/smad pathway, TSF decreased the expression of TGF-β1, inhibited the phosphorylation of Smad2/3, and increased the expression of Smad7, which promoted the degradation of catenin. Meanwhile, TSF also inhibited the Wnt pathway and further promoted β-catenin phosphorylation and degradation. The green arrow represents the effect of TSF.

Additionally, TSF decreased the expressions of TGF-β1 and p-Smad2/3, resulting in a significant reduction in both mRNA and protein levels. The expression of smad7 was increased with TSF treatment. Thus, it is conceivable that TSF alleviates myocardial fibrosis by inhibiting the TGF-β1/Smad signaling pathway. Fibroblasts are one of the major cytoeffectors of cardiac fibrosis. They are highly sensitive to TGF-βs, activate Smad-dependent signaling cascades, and are very much involved in regulating fibrosis transcriptional programs (18). In experiments with cultured CFs, TGF-β1 inhibits myocardial fibroblast apoptosis through Smad3 and extracellular signal-regulated kinase 1/2 (22). In our study, we established a TGF-β1-induced myocardial fibroblast fibrosis injury model, and the expression of the Smad downstream fibrosis gene was significantly increased compared with the control group, but after TSF treatment, the fibrosis indexes were significantly decreased, suggesting that TSF can also alleviate fibrosis damage *in vivo*. Taken together, TSF seems to attenuate myocardial fibrosis by regulating the TGF-β/Smad signaling pathway.

Both Wnt/β-catenin and TGF-β1 signaling have been found to stimulate and coordinate with each other, which plays an important role in the process of fibrosis (23). The canonical

Wnt/β-catenin pathway is involved in fibroblast activation and proliferation (24) with Wnt1 being the actual promoter of fibrosis. Under normal conditions, the Wnt ligand is absent, and cytoplasmic β-catenin is phosphorylated by a destruction complex, including Axin adenomatous polyposis coli (APC) protein and glycogen synthase kinase (GSK)-3β, which is then ubiquitinated and destroyed by the proteasome. Therefore, β-catenin is kept at a low level. In contrast, when Wnt is present, its proteins transmit signals through the plasma membrane by interacting with the members of the frizzled (FZD) protein family and LRP5/6; the activation of the messy protein causes a separation of GSK-3β from the complex, which results in an inability to phosphorylate β-catenin. β-catenin is not degraded and translocated to the nucleus where it upregulates a wide range of target genes, such as MMP7 (25–28).

Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, are involved in myocardial interstitial tissue changes in diseased atria (29). Myocardial fibrosis both in human patients and animal models has been found to be accompanied by increased MMP activity (30). Our *in vivo* experiments showed that Wnt1, active-β-catenin, and MMP7 protein expression were increased in KKAy mice. TSF inhibited changes in the expression of Wnt/β-catenin-related proteins that were observed in the myocardia of KKAy mice, indicating that the inhibition of Wnt/β-catenin-driven myocardial fibrosis may be another mechanism associated with the protective effect of TSF on myocardial fibrosis. Furthermore, it has been reported that the loss of the Wnt ligand reduces TGF-β expression and thus reducing the fibrotic response (31). Smad7, Axin, and E3 ubiquitin ligases form complexes after the activation of Wnt ligands, which facilitate Smad7 degradation. Smad7 also induces the ubiquitination and degradation of β-catenin by binding to Smurf 2 (32). In the TGF-β/Smad2/3 pathway, Smad7 blocks the expression of Smad3 and plays a negative feedback regulatory role (33). In our study, TSF promoted the expression of Smad7, thereby inhibiting the TGF-β/Smad2/3 pathway and promoting the phosphorylation of β-catenin. TSF is a Chinese medicine compound and contains monomers, such as calycosin, quercetin, and kaempferol, which have been found to act on the fibrosis pathway. Kaempferol is found in the TSF herbs astragalus root and burning bush twig. It has been shown to inhibit fibroblast collagen synthesis, proliferation, and activation, and inhibit TGF-β/Smads signaling by selectively binding TGF-β RI and significantly downregulating Smad2 and Smad3 phosphorylation in a dose-dependent manner (34). Quercetin has been reported to have protective effects on the heart because of its antioxidant, anti-inflammatory, and other biological properties (35). Albadrani et al. revealed that quercetin upregulates Smad7 and has an anti-fibrosis effect (36). Quercetin is contained in TSF herbs, such as rhubarb root and rhizome, notoginseng root, cornus fruit, burning bush twig, and astragalus root, so the TSF regulation of Smad7 is dependent on quercetin. Astragalus root contains calycosin, which can also upregulate Smad7, thereby inhibiting fibrosis (37). Therefore, perhaps the two monomers in TSF are involved in regulating Smad7. In conclusion, we speculate that Smad7 may have mediated the interaction between TGF-β/Smad2/3 and Wnt/β-catenin in the TSF treatment of myocardial fibrosis (**Figure 6**).

The results of this study indicate that TSF appears to be effective in attenuating myocardial fibrosis both *in vitro* and *in vivo*. Thus, TSF may be a potential clinical treatment for patients with diabetic cardiomyopathy. However, the limitation of our current research is that although we have elucidated the pathway, we have yet to clarify the target. Therefore, one of our future studies will be to demonstrate the specific target of TSF in alleviating myocardial fibrosis.

CONCLUSION

The present study revealed that TSF may alleviate myocardial fibrosis in KKAy mouse models by inhibiting TGF-β/Smad and Wnt/β-catenin signaling pathways. These findings may contribute to our understanding of the protective effect of TSF on myocardial fibrosis and further enrich our understanding of the mechanisms of myocardial fibrosis in diabetes.

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 author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Beijing National Proteome Science Center Animal Management Ethics Committee.

AUTHOR CONTRIBUTIONS

PL, TZ, and LH conceived and designed the experiments. LH and YuzW performed the experiments. LH and YuyW analyzed the data. LM contributed materials and analysis tools. LH and TZ wrote the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation of China [Grant Nos: 81973627, 82074221, and 81620108031] and Beijing Natural Science Foundation [Grant No: 7212195].

ACKNOWLEDGMENTS

The authors thank Nissi S. Wang, M.Sc., for the developmental editing of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.732042/full#supplementary-material

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