# PROCEEDINGS OF KININ2018CLE, CLEVELAND, OHIO, JUNE 18-20, 2018: A COMPENDIUM OF THE PRESENTATIONS

EDITED BY: Alvin H. Schmaier, Marvin T. Nieman, Keith McCrae, Sadashiva Karnik and Joao Bosco Pesquero

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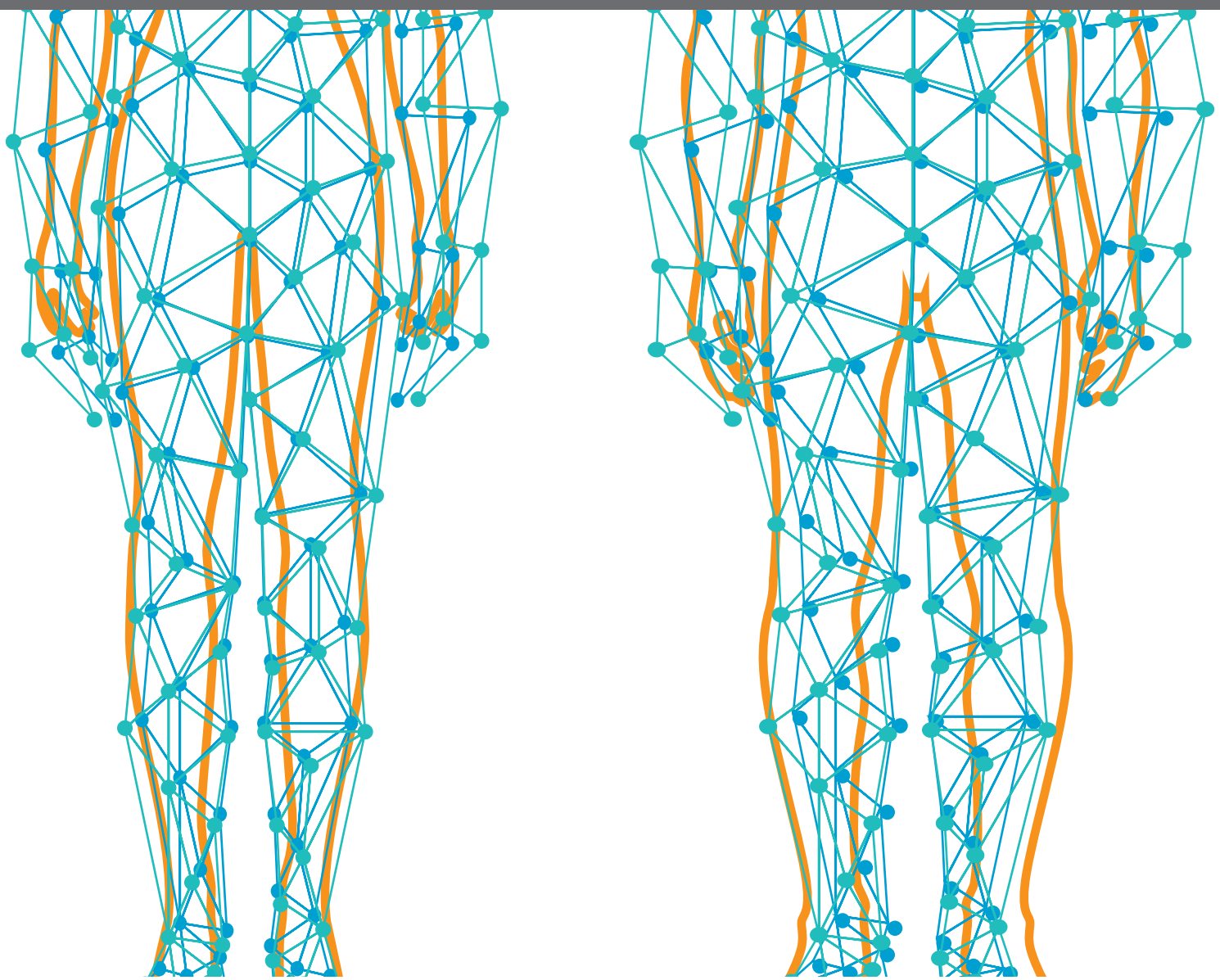

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# PROCEEDINGS OF KININ2018CLE, CLEVELAND, OHIO, JUNE 18-20, 2018: A COMPENDIUM OF THE PRESENTATIONS

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EDITORIAL
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# Editorial: Proceedings of KININ2018CLE, Cleveland, Ohio, June 18-20, 2018: A Compendium of the Presentations

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**Keywords: bradykinin, factor XII, kallikrein, renin, angiotensin**

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**Editorial on the Research Topic**

**Proceedings of KININ2018CLE, Cleveland, Ohio, June 18-20, 2018: A Compendium of the Presentations**

## INTRODUCTION

KININ2018CLE was an international kinin meeting held on the campus of Case Western Reserve University, Cleveland, OH, USA from June 17th to 20th, 2018. This 3-day conference covered the gamut of topics related to the contact activation system, the kallikrein-kinin system, bradykinin biology, and interactions with the renin angiotensin system. This ebook was graciously complied by Frontiers in Medicine (Hematology) and serves as a Proceedings of the meeting. The Proceedings consists of 14 articles submitted by meeting participants. These articles are classified into four categories: contact activation; hereditary angioedema, C1 inhibitor, and bradykinin receptors; kallikreins, kinins, angiotensin converting enzyme; and renin-angiotensin. The papers within each of these four categories will be introduced in this editorial.

## CONTACT ACTIVATION

Mailer et al. presented a paper on polyphosphates (polyP) as a target for interference with inflammation and thrombosis. In their article they give an overview of polyP function, focusing on intra- and extracellular roles of the polymer and discuss open questions that emerge from current knowledge on polyP regulation. Conway discusses his observations of polyP on the complement system. Although polyP promotes factor XII activation, that is regulated by C1 inhibitor. In the complement system polyP potentiates C1 inhibitor and heparin inhibition of C1 activation and contributes to blocking the C5 convertase.

## HEREDITARY ANGIOEDEMA, C1 INHIBITOR, AND BRADYKININ RECEPTOR BIOLOGY

Sanrattana et al. presented a concise review of SERPIN biochemistry and how serpin modification is useful to design new therapeutic tools. Veronez et al. presented a paper examining genetic variability of genes related to bradykinin formation and use to explain phenotypic variability of patient with hereditary angioedema (HAE). She also submitted a novel case report on an association of HAE with acute pancreatitis. Silva et al., presented an important report that bradykinin released by the erythrocytic stages of plasmodium falciparum enhances adhesion of infected red blood cells to endothelium to increase permeability via activation of bradykinin receptors. Perhal et al. from the Quitterer laboratory presented the fascinating finding that deficiency of the bradykinin B2 receptor protects mice from atherosclerosis. Last, Wu et al. from the laboratory of the late Marco Cicardi presented a brief report on a novel device to examine endothelial barrier function as it relates to angioedema and related disorders.

## KALLIKREINS, KININS, AND ANGIOTENSIN CONVERTING ENZYME

Alhenc-Gelas et al., the 2018 Kinin Medal recipient, and his collaborators contributed an important paper on the evolving concept that although bradykinin formation is dependent on local tissue kallikrein and angiotensin converting enzyme (ACE) activity, deficiencies of BK result in detrimental effects of tissue kallikrein and ACE. Barros et al. who is in the Bader laboratory published a related observation that chronic overexpression of bradykinin in the kidney causes polyuria and cardiac hypertrophy. Campbell discussed the mechanisms of neprilysin and its inhibition in the treatment of heart failure and hypertension.

## RENIN ANGIOTENSIN

Jara et al. summarize her work on murine tonin overexpression and how by diminishing sympathetic autonomic modulation by altering angiotensin type 1 receptor responses. Wolf et al. submitted an intriguing paper in which it was observed that sensitization of the angiotensin II AT1 receptor contributes to raf kinase inhibitor protein (RKIP)-induced symptoms of heart failure. Last, Quitterer and AbdAlla presented a brief summary of their seminal work in which pathologic co-localization of G-protein coupled receptors in hetero- or homo-dimers can contribute to disease states like pre-eclampsia.

In conclusion, these 14 articles are just a small sampling of the rich science presented at KININ2018CLE. The success of the meeting was in large part from the support we had from the National Institutes of Health, Division of Heart, Lung, and Blood Institute (R13HL140902), industry (Shire, CSL Behring, Alnylam, Kalvista, Enzyme Research Laboratories, Affinity Biologicals, and Diapharma) and a generous patient donor.

## AUTHOR CONTRIBUTIONS

AS wrote the first draft of the manuscript. All authors reviewed and edited the manuscript.

**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
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# Neprilysin Inhibitors and Bradykinin

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Bradykinin has important physiological actions related to the regulation of blood vessel tone and renal function, and protection from ischemia reperfusion injury. However, bradykinin also contributes to pathological states such as angioedema and inflammation. Bradykinin is metabolized by many different peptidases that play a major role in the control of bradykinin levels. Peptidase inhibitor therapies such as angiotensin converting enzyme (ACE) and neprilysin inhibitors increase bradykinin levels, and the challenge for such therapies is to achieve the beneficial cardiovascular and renal effects without the adverse consequences such as angioedema that may result from increased bradykinin levels. Neprilysin also metabolizes natriuretic peptides. However, despite the potential therapeutic benefit of increased natriuretic peptide and bradykinin levels, neprilysin inhibitor therapy has only modest efficacy in essential hypertension and heart failure. Initial attempts to combine neprilysin inhibition with inhibition of the renin angiotensin system led to the development of omapatrilat, a drug that combines ACE and neprilysin inhibition. However, omapatrilat produced an unacceptably high incidence of angioedema in patients with hypertension (2.17%) in comparison with the ACE inhibitor enalapril (0.68%), although angioedema incidence was less in patients with heart failure with reduced ejection fraction (HFrEF) treated with omapatrilat (0.8%), and not different from that for enalapril therapy (0.5%). More recently, LCZ696, a drug that combines angiotensin receptor blockade and neprilysin inhibition, was approved for the treatment of HFrEF. The approval of LCZ696 therapy for HFrEF represents the first approval of long-term neprilysin inhibitor administration. While angioedema incidence was acceptably low in HFrEF patients receiving LCZ696 therapy (0.45%), it remains to be seen whether LCZ696 therapy for other conditions such as hypertension is also accompanied by an acceptable incidence of angioedema.

**Keywords: neprilysin, bradykinin, neprilysin inhibition, angioedema, ARNI**

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## INTRODUCTION

Despite decreasing incidence, cardiovascular disease remains a major cause of premature morbidity and mortality (1), and there is a continuing search for new therapies for its prevention and treatment. LCZ696 (Entresto) is the first of a new drug class referred to as ARNI (dual acting angiotensin receptor-neprilysin inhibitor) that contains equimolar amounts of valsartan, a type 1 angiotensin II receptor blocker (ARB) and sacubitril, a prodrug that is hydrolyzed to form LBQ657, a potent inhibitor of neprilysin (**Table 1**). The approval of LCZ696 as therapy for heart failure with reduced ejection fraction (HFrEF) represents the first approval of long-term neprilysin inhibitor therapy. Neprilysin is a key enzyme in the degradation of natriuretic peptides, and the primary rationale for neprilysin inhibitor therapy in cardiovascular disease was to increase endogenous

natriuretic peptide levels, and thereby achieve the vasodilatation and natriuresis these peptides produce. However, neprilysin degrades many other peptides, including bradykinin (17). Bradykinin may contribute not only to the benefits of neprilysin inhibitor therapy but also to the adverse effects of this therapy. Of particular concern for drugs that inhibit bradykinin degradation and thereby increase bradykinin levels is the risk of angioedema, with increased bradykinin levels implicated in both hereditary and drug-induced forms of angioedema (18–21). This review will briefly describe neprilysin, the kallikrein kinin system, and the role of neprilysin in bradykinin metabolism, and then discuss the potential role of kinins in mediating the therapeutic benefits and adverse effects of neprilysin inhibitor therapy.

## NEPRILYSIN

Neprilysin, also known as neutral endopeptidase 24.11, common acute lymphoblastic leukemia antigen (CALLA), and cluster of differentiation cell surface molecule 10 (CD10), is a member of the neprilysin (M13) family of metallopeptidases. The neprilysin family also includes the neprilysin homolog membrane metalloendopeptidase-like 1 (NEP2) (22), endothelin converting enzymes 1 and 2 (ECE-1 and ECE-2), endothelin converting enzyme-like 1 (ECEL1), phosphate-regulating neutral endopeptidase (PHEX), and the KELL blood group glycoprotein (23, 24). Neprilysin and several other members of the neprilysin family of metallopeptidases degrade bradykinin (**Table 2**, **Figure 1**). Neprilysin is a predominantly membrane-bound zinc-dependent metallopeptidase with a broad tissue distribution, including the central nervous system, kidney, and vascular endothelium (39). Neprilysin is expressed at a low level on the membrane of mononuclear cells, and at higher levels by neutrophils, lymphocytes, and lymphoid progenitors (40, 41). A soluble form of neprilysin is found in blood plasma, cerebrospinal fluid, amniotic fluid, and seminal plasma. Neprilysin has a broad substrate selectivity (17), preferentially cleaving peptides on the amino side of the hydrophobic residues phenylalanine, leucine, and methionine (39, 42, 43).

## THE KALLIKREIN KININ SYSTEM

The kallikrein kinin system has been reviewed elsewhere (44–46). In humans, plasma kallikrein forms the nonapeptide bradykinin from high molecular weight kininogen, whereas tissue kallikrein forms the decapeptide kallidin ($Lys^0$-bradykinin) from both high and low molecular weight kininogens (**Figure 2**). Bradykinin is also generated by aminopeptidase-mediated cleavage of kallidin. A proportion of high molecular weight kininogen is hydroxylated on the third proline of the bradykinin sequence, leading to the formation of both hydroxylated and non-hydroxylated bradykinin and kallidin peptides. Hydroxylated and non-hydroxylated kinin peptides are of similar abundance (48–50), and hydroxylated kinins have similar biological activity to non-hydroxylated kinins (46). In the rat, both plasma and tissue kallikrein produce bradykinin, which is not hydroxylated (47).

There are two types of kinin receptor, the type 1 ($B_1$) receptor and the type 2 ($B_2$) receptor. The $B_2$ receptor normally predominates, whereas $B_1$ receptors are induced by tissue injury. Bradykinin and kallidin are more potent on the $B_2$ receptor, whereas the carboxypeptidase N (kininase I) metabolites bradykinin-(1-8) and $Lys^0$-bradykinin-(1-8) are also bioactive and more potent on $B_1$ receptors (46). Kinin peptides have a broad spectrum of activities that include the regulation of blood vessel tone and renal function, and protection from ischemia reperfusion injury (45). However, kinins also participate in inflammation, producing vasodilatation, increased vascular permeability, neutrophil chemotaxis and pain (45).

### Tissue Specific Regulation of Kinin Levels

The kallikrein kinin system is primarily a tissue-based system, with tissue kinin levels much higher than blood kinin levels in both humans and in rats (47–49). Evidence for the tissue-specific regulation of the kallikrein kinin system is the marked variation in kinin levels between different tissues of the rat (47). Kinin peptide levels are also higher in atrial tissue than blood of humans (48, 49). Moreover, bradykinin peptide levels are higher than kallidin peptide levels in blood and atrial tissue of humans, whereas kallidin peptide levels are much higher than bradykinin peptide levels in urine (48, 49). Many different enzymes cleave bradykinin and may participate in its metabolism (**Figure 1**), and peptidase activity plays a major role in the tissue-specific regulation of bradykinin levels (51).

## ROLE OF NEPRILYSIN IN BRADYKININ METABOLISM

Several different experimental approaches have been used to study the role of neprilysin in bradykinin metabolism. These include study of the effects of neprilysin gene (*MME*) deletion and mutation, study of the effects of neprilysin inhibitor administration on physiological bradykinin levels, and study of the metabolism of exogenous (supra-physiological) bradykinin levels and the effect of neprilysin and other peptidase inhibitors on the metabolism of exogenous bradykinin. The effect of inhibition of an enzyme on bradykinin levels depends not only on the specific enzyme's contribution to bradykinin metabolism, relative to other enzymes, but also the baseline degradation rate for bradykinin. This is best illustrated by bradykinin metabolism by the pulmonary circulation, where bradykinin is degraded with approximately 99% efficiency (51). Thus, 1% inhibition of pulmonary inactivation will double the amount of bradykinin surviving pulmonary degradation, and may therefore double the level of bradykinin in arterial blood (**Figure 3**).

### Neprilysin Gene Knockout in Mice

Neprilysin gene knockout in mice causes increased basal vascular permeability, hypotension and reduced heart weight/body weight ratio (52). The reduced heart weight/body weight ratio was attributed to the lower blood pressure. The vascular permeability, but not hypotension, was reversed by administration of recombinant neprilysin, and also by separate administration of SR140333, a substance P

**TABLE 1 |** Specificity of neprilysin inhibitors ($K_i$ or $IC_{50}$).

| Inhibitor (units for $K_i$ or $IC_{50}$) | Neprilysin | NEP2 | ACE | ECE-1 | ECE-2 | APP | References |
|---|---|---|---|---|---|---|---|
| Thiorphan (nmol/L) | 4, 4.7 | 120, 129, 250 | 150 | No | >10 μmol/L# | >100 μmol/L | (2–6) |
| Phosphoramidon (nmol/L) | 1.5, 2 | 0.8, 1.0 | 2 | 680*, 675* | 1.2#, 4# | >10 μmol/L | (2, 3, 5–9) |
| Candoxatrilat (nmol/L) | 3.2, 9.5 | 44 | >10 μmol/L | 6.5† | | >10 μmol/L | (10, 11) |
| Omapatrilat (nmol/L) | 0.45, ~2, 3, 5–8 | 8, 17, 25 | 0.64, 0.98, 5 | 10 μmol/L† | | 194, 250, 260 | (3, 10–13) |
| LBQ657 (nmol/L) | 2.3, 5 | Yes | Yes, >10 μmol/L | No | Yes | No | (14–16) |

*Inhibitor concentrations are shown as nmol/L, except where indicated to be μmol/L. *pH: 7.2; †pH 6.5; #pH 5.5. ACE, angiotensin converting enzyme; APP, aminopeptidase P; ECE-1, endothelin converting enzyme 1; ECE-2, endothelin converting enzyme 2; NEP2, neprilysin homolog membrane metalloendopeptidase-like 1.*

**TABLE 2 |** Kinetic parameters of bradykinin hydrolysis by different enzymes.

| Enzyme | $K_m$ (μmol/L) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_m$ ($min^{-1}$ $\mu mol/L^{-1}$) | References |
|---|---|---|---|---|
| ACE (kininase II) | 0.18, 1 | 500, 600 | 500, 3667 | (25, 26) |
| Neprilysin (neutral endopeptidase 24.11) | 34, 92, 120 | 1500, 4771, 6364 | 40, 44, 69 | (3, 26, 27) |
| NEP2 | 2 | 150 | 75 | (3) |
| Aminopeptidase P | 21, 76, 280 | 720, 1560, 2280 | 8, 21, 34, | (5, 28, 29) |
| Carboxypeptidase N (kininase I) | 19 | 58 | 3 | (30) |
| Carboxypeptidase M | 16 | 147 | 9.2 | (31) |
| Neutral endopeptidase 24.15 | 4.9 | 89 | 18 | (32) |
| Endothelin converting enzyme-1* | 340 | 1380 | 4.1 | (33) |
| Endothelin converting enzyme-2† | 27.4 | 348 | 12.7 | (34) |

*ACE, angiotensin converting enzyme; NEP2, neprilysin homolog membrane metalloendopeptidase-like 1. *pH 6.5; †pH 5.5.*

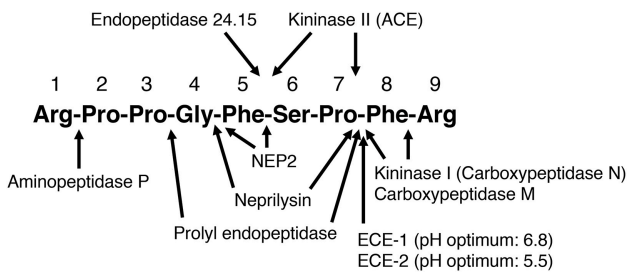


**FIGURE 1 |** Sites of cleavage of bradykinin by different enzymes. ACE, angiotensin converting enzyme; ECE-1, endothelin converting enzyme-1; ECE-2, endothelin converting enzyme-2; NEP2, neprilysin homolog membrane metalloendopeptidase-like 1. (2–5, 7, 25–28, 30, 32–38).

(NK1) receptor antagonist, and the bradykinin $B_2$ receptor antagonist icatibant. The increased basal vascular permeability of neprilysin gene knock out was reproduced by administration of the neprilysin inhibitors thiorphan and phosphoramidon (**Table 1**) to wild-type C57BL/6 mice. These observations indicate an important role for neprilysin in the control of bradykinin- and substance P-mediated regulation of vascular permeability and blood pressure in the mouse. Bradykinin stimulates substance P release from sensory neurons (53), and neprilysin degrades both peptides (54), thereby providing an explanation why neprilysin gene knockout or neprilysin inhibition could increase both bradykinin and substance P levels, and why either a substance P receptor or bradykinin $B_2$ receptor antagonist was able to prevent the increased vascular permeability.

Other consequences of neprilysin gene knockout in the mouse include hyperalgesia and increased susceptibility to inflammation (55, 56), enhanced lethality in response to endotoxin-induced shock (57), shortened ventilatory expiratory time in response to a hypoxic stimulus (58), and improved learning and memory (59). However, apart from hyperalgesia, which was reduced by icatibant (55), the relevance of these consequences of neprilysin gene knockout to bradykinin is unknown.

## Neprilysin Gene Deletion and Neprilysin Gene Mutation in Humans

In contrast to the effects of neprilysin gene knockout in the mouse, five women with total neprilysin deficiency due to homozygous truncating mutations of the neprilysin gene had no reported phenotype, although the absence of neprilysin induced an alloimmunization process against neprilysin present in fetal cells, leading to membranous glomerulopathy in their infants (60).

Loss–of-function and missense mutations in the neprilysin gene are associated with polyneuropathy, and also with decreased tissue availability of neprilysin and reduced neprilysin enzymatic activity (61–63), although the relevance of the polyneuropathy to bradykinin is unknown. However, the association of the rs989692 variant of the neprilysin gene with ACE inhibitor-associated

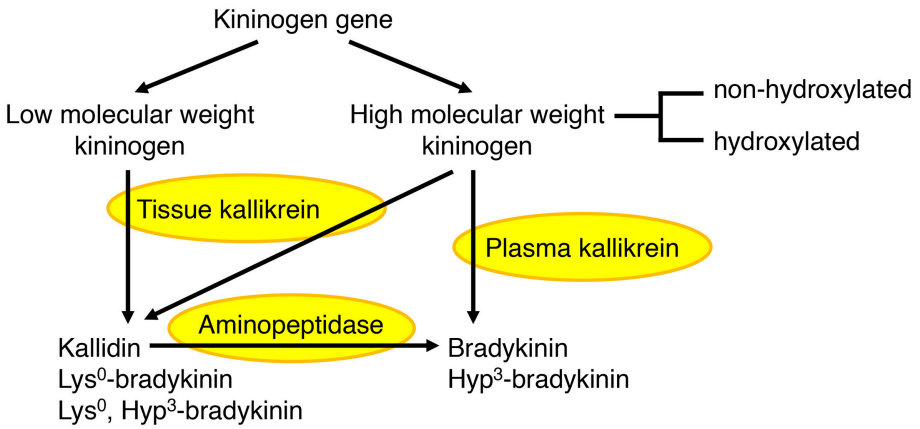


**FIGURE 2 |** Formation of bradykinin and kallidin peptides. In humans, plasma kallikrein cleaves high molecular weight kininogen to produce bradykinin, whereas tissue kallikrein cleaves both high and low molecular weight kininogens to produce kallidin ($Lys^0$-bradykinin). Bradykinin can also be generated by aminopeptidase-mediated cleavage of kallidin. A proportion of high molecular weight kininogen is hydroxylated on the third proline ($Hyp^3$) of the bradykinin sequence, leading to the formation of both hydroxylated and non-hydroxylated bradykinin and kallidin peptides. In the rat, both plasma and tissue kallikrein produce bradykinin, which is not hydroxylated (44–47).

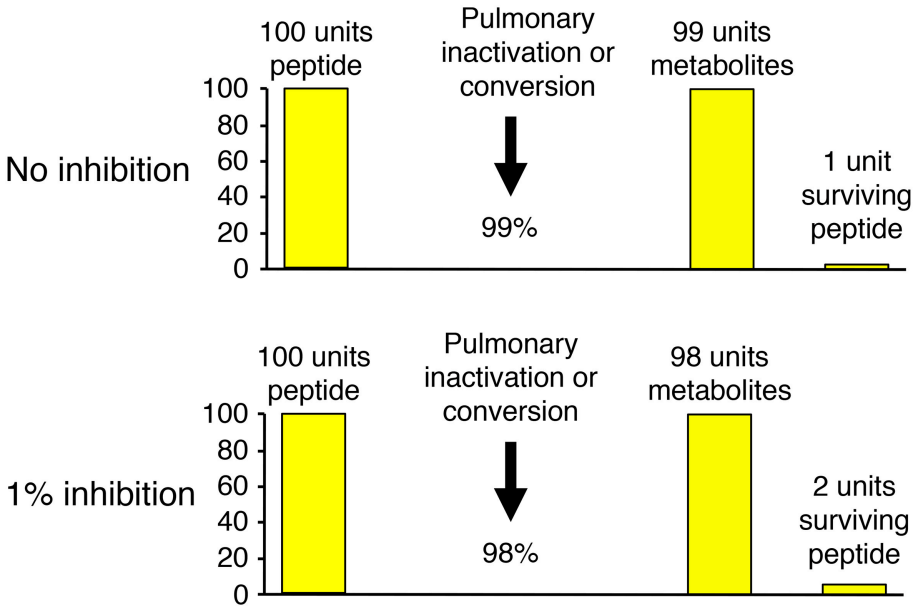


**FIGURE 3 |** Illustration of how the effect of inhibition of an enzyme on bradykinin levels depends not only on the specific enzyme's contribution to bradykinin metabolism, relative to other enzymes, but also the baseline degradation rate for bradykinin. Pulmonary inactivation of bradykinin is approximately 99% (51). Thus, 1% inhibition of pulmonary inactivation will double the amount of bradykinin surviving pulmonary degradation, and may therefore double the level of bradykinin in arterial blood.

angioedema (64) is evidence for a role for neprilysin in the regulation of bradykinin levels in humans.

## Effect of Neprilysin Inhibition on Physiological Bradykinin Levels

We examined the effect of the neprilysin inhibitor ecadotril (acetorphan, an orally active prodrug of (*S*)-thiorphan) on bradykinin levels in Sprague Dawley rats (65). Ecadotril administration produced dose-related occupancy of renal neprilysin, as determined by binding of the neprilysin radioligand $^{125}$I-RB104 to kidney sections, and increased total neprilysin levels in plasma, similar to the induction of plasma ACE levels by ACE inhibitor therapy (66, 67). Ecadotril administration produced diuresis, natriuresis and increased urinary excretion of cyclic GMP and bradykinin, indicating a role for neprilysin in bradykinin degradation in renal tubules and/or in urine. However, ecadotril administration did not increase bradykinin levels in blood or renal tissue, although the ACE inhibitor

perindopril increased bradykinin levels in both blood and kidney. Ecadotril did, however, increase cardiac bradykinin levels by approximately 2-fold; although the increase in cardiac bradykinin levels did not achieve statistical significance, ecadotril produced a statistically significant reduction in the bradykinin-(1-7)/bradykinin-(1-9) ratio in the heart, consistent with reduced formation of bradykinin-(1-7) by neprilysin-mediated cleavage of bradykinin (**Figure 1**), and indicating a role for neprilysin in bradykinin metabolism in the heart.

Neprilysin inhibition also increased urinary bradykinin excretion in deoxycorticosterone acetate (DOCA)-salt hypertensive rats, spontaneously hypertensive rats and renovascular hypertensive rats (68). Together, these studies indicate a role for neprilysin in bradykinin metabolism in renal tubules and/or urine, and also in the heart.

## Role of Neprilysin in the Metabolism of Supra-Physiological Bradykinin Levels

There is need for caution in the interpretation of studies of the degradation of exogenously administered bradykinin where bradykinin levels may be considerably higher than physiological levels. An enzyme's contribution to bradykinin degradation, and the effect of inhibition of that enzyme on bradykinin degradation, depends not only on the concentration of the enzyme but also on the bradykinin concentration and the $K_m$ (Michaelis constant) of the enzyme for bradykinin degradation (**Figure 4**). Thus, depending on an enzyme's abundance and $k_{cat}$ (turnover number), an enzyme with a low $K_m$, such as angiotensin converting enzyme (ACE) (**Table 2**), may have a predominant role in bradykinin metabolism when bradykinin levels are low, whereas an enzyme with a higher $K_m$, such as carboxypeptidase M or N, may play a more dominant role when bradykinin levels are high (35).

Two approaches have been used to investigate the metabolism of exogenously administered bradykinin, either examination of the metabolites of bradykinin, or comparison of the effects of different peptidase inhibitors on bradykinin metabolism. Bradykinin-(1-5) was the predominant bradykinin metabolite when bradykinin was infused into human subjects (69), indicative of cleavage by ACE. Moreover, ACE played a predominant role in bradykinin metabolism by human and rat plasma and serum (35, 70–75), with a lesser contribution by carboxypeptidase (kininase I) and aminopeptidase activities. However, carboxypeptidase, a peptidase with higher $K_m$ than ACE (**Table 2**) played a greater role than ACE in bradykinin metabolism when human or rat serum was incubated with ≥μmol/L bradykinin concentrations (76, 77), thereby illustrating how higher bradykinin concentrations can lead to a greater contribution by an enzyme with higher $K_m$ to bradykinin metabolism (35).

Another example where a higher concentration of bradykinin led to a greater contribution to bradykinin metabolism by a peptidase with higher $K_m$ is the study of bradykinin metabolism by the isolated perfused rat mesenteric arterial bed (78). When bradykinin metabolism was assessed by recovery of bradykinin in the perfusate after injection of ~100 nmol bradykinin, carboxypeptidase inhibition, and to a lesser extent neprilysin inhibition, but not ACE inhibition, reduced bradykinin metabolism (78). These findings were supported by the greater role played by carboxypeptidase B than ACE in the degradation of μmol/L concentrations of bradykinin by mesenteric arterial perfusate (79). However, the opposite result was obtained when bradykinin metabolism was assessed by the vasodilator response of the isolated perfused rat mesenteric arterial bed to ~100 pmol bradykinin, whereby the vasodilator response was potentiated by ACE inhibition, but not by either carboxypeptidase or neprilysin inhibition (78).

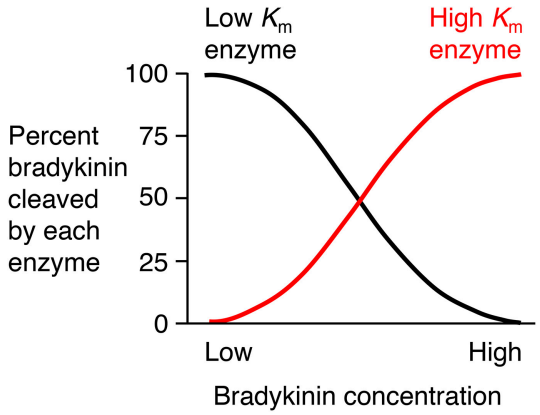


**FIGURE 4 |** Relative contributions of low $K_m$ (Michaelis constant) enzyme and high $K_m$ enzyme to bradykinin degradation by a mixture of low and high $K_m$ enzymes. An enzyme's contribution to bradykinin degradation, and the effect of inhibition of that enzyme on bradykinin degradation, depends not only on the concentration of the enzyme but also on the bradykinin concentration and the $K_m$ of the enzyme for bradykinin degradation. Thus, depending on an enzyme's abundance and $k_{cat}$ (turnover number), an enzyme with a low $K_m$, such as ACE, may have a predominant role in bradykinin metabolism when bradykinin levels are low, whereas an enzyme with a higher $K_m$, such as carboxypeptidase M or N, may play a more dominant role when bradykinin levels are high. Based on data reported by Kuoppala et al. (35).

ACE played a greater role than neprilysin in bradykinin metabolism by isolated human small resistance vessels (80). Additionally, ACE played a dominant role, with a lesser role for aminopeptidase P, carboxypeptidase, and neprilysin, in bradykinin metabolism by the rat pulmonary vascular bed (10, 51, 77, 81), the isolated perfused rat heart (82–86), and isolated porcine coronary arteries (87), and was the predominant kininase in coronary perfusate, with a lesser role for neprilysin and carboxypeptidase (79). ACE was also the dominant peptidase contributing to bradykinin metabolism by the isolated perfused rat kidney, without evidence for contribution by neprilysin, carboxypeptidase, or aminopeptidase P (88).

A key limitation of the studies of the role of neprilysin in bradykinin metabolism so far described is the failure to address how different peptidases may make different contributions to bradykinin metabolism in different tissue compartments. In support of a tissue compartment-specific role for neprilysin in bradykinin metabolism, studies of lung, cardiac and renal bush border membranes, and urine, demonstrated a contribution by neprilysin that was equal to (89), or greater than (89–91), the

contribution of ACE. Further evidence for a tissue compartment-specific role for neprilysin in bradykinin degradation was the metabolism of a bolus of $^{3}$H-bradykinin by the isolated perfused rat heart, which showed a delayed release of $^{3}$H-bradykinin-(1-7) into the perfusate, consistent with $^{3}$H-bradykinin-(1-7) formation in the interstitial compartment of the heart by neprilysin-mediated cleavage of $^{3}$H-bradykinin (84).

## ROLE OF KININS IN MEDIATING THE EFFECTS OF NEPRILYSIN INHIBITION

Many studies have used either bradykinin receptor antagonists, anti-bradykinin antibodies, or serine protease (kallikrein) inhibitors to demonstrate a role for bradykinin in mediating the effects of neprilysin inhibitors. Two different mechanisms may account for the potentiation of bradykinin receptor-mediated actions by neprilysin inhibitors (**Figure 5**). Firstly, neprilysin inhibitors may potentiate bradykinin receptor-mediated actions by inhibiting bradykinin degradation and increasing bradykinin levels in the vicinity of the receptor. Secondly, neprilysin inhibitors may potentiate bradykinin receptor-mediated actions by promoting cross-talk between the neprilysin-inhibitor complex and the bradykinin receptor (92), similar to the cross-talk between the ACE-inhibitor complex and the $B_2$ receptor proposed to mediate ACE inhibitor-induced potentiation of bradykinin receptor-mediated effects (93). Bradykinin receptor antagonists, anti-bradykinin antibodies, and kallikrein inhibitors have different effects on these two mechanisms of neprilysin inhibitor-induced potentiation of bradykinin receptor-mediated actions. A bradykinin receptor antagonist that occupies the bradykinin receptor can prevent both mechanisms of neprilysin inhibitor-induced potentiation of bradykinin receptor-mediated actions. However, bradykinin antibodies that prevent bradykinin binding to its receptor by sequestering bradykinin, and kallikrein inhibitors that prevent bradykinin binding to its receptor by preventing its formation, do not impact on cross-talk between the neprilysin-inhibitor complex and the bradykinin receptor. Therefore, prevention of the effects of neprilysin inhibition by bradykinin antibodies or kallikrein inhibitors indicates that these effects are mediated by increased bradykinin levels consequent to inhibition of neprilysin-mediated bradykinin degradation, and not by cross-talk between the neprilysin-inhibitor complex and the bradykinin receptor.

### Role of Kinins in Mediating the Renal Effects of Neprilysin Inhibition

Icatibant prevented the diuretic and natriuretic effects of neprilysin inhibition in normal Sprague Dawley rats (94–96). Bradykinin receptor antagonism also prevented the neprilysin inhibitor-induced potentiation of atrial natriuretic peptide-induced diuresis and natriuresis in rats (97) and in chronic caval dogs (98). Moreover, anti-bradykinin antibodies prevented neprilysin inhibitor-induced potentiation of diuresis, natriuresis

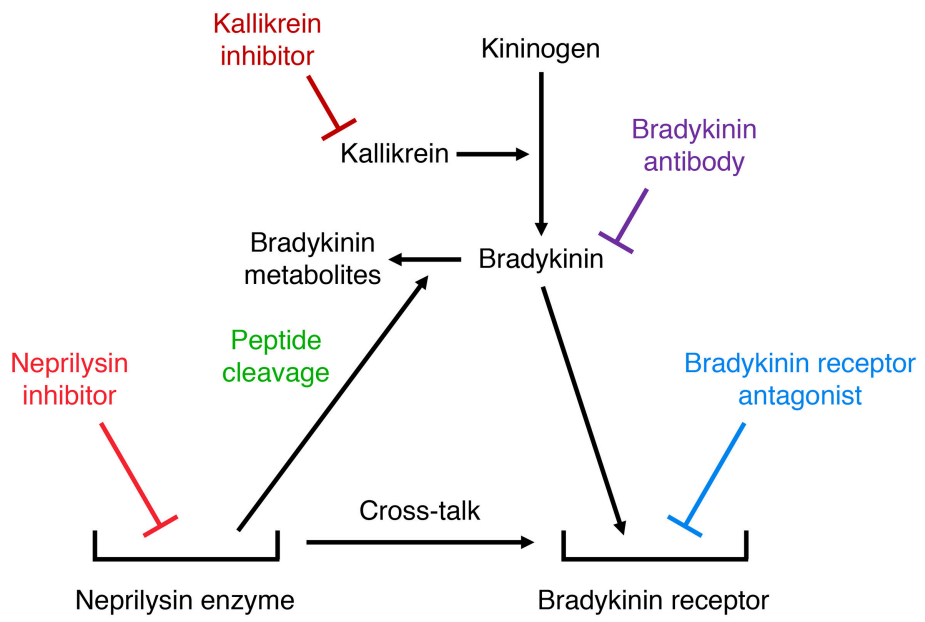


**FIGURE 5 |** Illustration of two different mechanisms by which neprilysin inhibitors may potentiate bradykinin receptor-mediated actions. Firstly, neprilysin inhibitors may increase bradykinin receptor occupancy by inhibiting bradykinin degradation and increasing bradykinin levels in the vicinity of the receptor. Secondly, neprilysin inhibitors may promote cross-talk between the neprilysin-inhibitor complex and the bradykinin receptor. Bradykinin receptor antagonists, anti-bradykinin antibodies, and kallikrein inhibitors have different effects on these two mechanisms of neprilysin inhibitor-induced potentiation of bradykinin receptor-mediated actions. A bradykinin receptor antagonist that occupies the bradykinin receptor can prevent both mechanisms of neprilysin inhibitor-induced potentiation of bradykinin receptor-mediated actions. However, bradykinin antibodies that prevent bradykinin binding to its receptor by sequestering bradykinin, and kallikrein inhibitors that prevent bradykinin binding to its receptor by preventing its formation, do not impact on cross-talk between the neprilysin-inhibitor complex and the bradykinin receptor.

and increase in urinary cyclic GMP excretion in volume-expanded rats (99). However, in contrast to studies in normal Sprague Dawley rats, icatibant did not prevent the natriuretic effects of neprilysin inhibition in DOCA-salt hypertensive rats (96, 100), which suggests that the effects of neprilysin inhibition in DOCA-salt hypertensive rats are primarily mediated by increased natriuretic peptide levels consequent to inhibition of natriuretic peptide metabolism.

### Role of Kinins in Mediating the Cardiac Effects of Neprilysin Inhibition

Icatibant prevented neprilysin inhibitor-induced reduction in ischemia-reperfusion injury in the rat heart (101), and neprilysin inhibitor-induced potentiation of pre-conditioning-induced reduction in infarct size in the rabbit heart (102). In addition, icatibant prevented neprilysin inhibitor-induced reversal of isoproterenol-induced myocardial hypoperfusion in the rat (103), and neprilysin inhibitor-induced nitric oxide production by isolated canine coronary microvessels (104). Neprilysin inhibitor-induced nitric oxide production by isolated canine coronary microvessels was also prevented by the serine protease (kallikrein) inhibitor dichloroisocoumarin (104).

## OMAPATRILAT AND BRADYKININ

Despite the potential therapeutic benefits of increased natriuretic peptide and bradykinin levels, neprilysin inhibitor therapy has only modest efficacy in essential hypertension and heart failure, which might be due in part to the inhibition of neprilysin metabolism of the vasoconstrictors angiotensin II and endothelin 1, and the increased plasma angiotensin II, endothelin 1 and noradrenaline levels that accompany neprilysin inhibitor therapy (17). Therefore, to prevent the renin angiotensin system from countering the therapeutic benefits of neprilysin inhibition, neprilysin inhibitor therapy was combined with inhibition of the renin angiotensin system, leading to the development of omapatrilat. Omapatrilat is a single molecule that inhibits both neprilysin and ACE (**Table 1**). Additionally, omapatrilat inhibits aminopeptidase P, NEP2, and ECE-1 (**Table 1**). There is currently no information on the effects of omapatrilat on bradykinin levels. However, given that both neprilysin and ACE degrade bradykinin, one would predict higher bradykinin levels with omapatrilat than ACE inhibitor therapy, which no doubt accounts for the higher incidence of angioedema with omapatrilat therapy. The incidence of angioedema was higher for omapatrilat therapy (2.17%) than for enalapril therapy (0.68%) in hypertensive patients (105), and omapatrilat failed to achieve regulatory approval because of the angioedema incidence. However, the incidence of angioedema was lower in patients with HFrEF, without statistically significant difference between omapatrilat therapy (0.8%) and enalapril therapy (0.5%) (106).

The potential consequences of combined neprilysin and ACE inhibition were examined in the rat tracheal plasma extravasation assay (**Table 3**). Whereas neither ecadotril, sufficient to produce >90% inhibition of renal neprilysin, nor lisinopril, sufficient to produce 83% inhibition of lung ACE, produced plasma extravasation, their combination produced plasma extravasation, suggesting that their combination increased bradykinin (and substance P) levels sufficient to cause extravasation. It is also possible that omapatrilat-induced inhibition of aminopeptidase P, NEP2, and ECE-1 (**Table 1**) contributed to increased bradykinin (and substance P) levels and the plasma extravasation observed in rats, and angioedema in patients administered this therapy.

## LCZ696 AND BRADYKININ

There is currently no information on the effects of LCZ696, sacubitril or LBQ657 on bradykinin levels. However, several lines of evidence indicate a role for bradykinin in the therapeutic benefits of LCZ696 therapy, and also the angioedema associated with this therapy. Whereas ARBs produce angioedema with an incidence approximately half that of ACE inhibitor therapy

**TABLE 3 |** Effects of combined renin angiotensin system and neprilysin inhibition on tracheal plasma extravasation in the rat.

| Compound | Tracheal plasma extravasation | APP inhibition $K_i$ or $IC_{50}$ (nmol/L) | References |
|---|---|---|---|
| **ACE AND NEPRILYSIN INHIBITION** | | | |
| Ecadotril (99% neprilysin & 23% ACE inhibition) | No | No | (5, 107) |
| Lisinopril (83% ACE inhibition) | No | No | (10, 11, 107, 108) |
| Ecadotril & Lisinopril | Yes | No | (5, 107) |
| Omapatrilat (>90% ACE & 53% neprilysin inhibition) | Yes | 194, 250, 260 | (10, 11, 13) |
| **ARB AND NEPRILYSIN INHIBITION** | | | |
| Valsartan (100 mg/kg) | No | No | (11) |
| Candoxatril (100 mg/kg) | No | No | (11) |
| Valsartan & candoxatril | No | No | (11) |
| Omapatrilat (0.3 mg/kg) | Yes* | 194, 250, 260 | (10, 11, 13) |

**Plasma extravasation caused by omapatrilat was prevented by prior icatibant administration (11). ACE, angiotensin converting enzyme; APP, aminopeptidase P; ARB, type 1 angiotensin II receptor blocker.*

in patients without heart failure (109, 110), LCZ696 produces angioedema with an incidence at least equal to that of ACE inhibitor therapy (111). In the Prospective comparison of Angiotensin Receptor-neprilysin inhibitor with Angiotensin converting enzyme inhibitor to Determine Impact on Global Mortality and morbidity in Heart Failure (PARADIGM-HF) study of patients with HFrEF, angioedema was confirmed in 0.45% of patients receiving LCZ696 therapy and 0.24% of patients receiving enalapril therapy (111), a numerical difference that was not statistically significant ($P = 0.13$). However, the protocol of the PARADIGM–HF study might have resulted in a lower incidence of angioedema in the trial population than might occur in patients naive to LCZ696 therapy. The exclusion criteria for the PARADIGM-HF study included a history of angioedema during treatment with an ACE inhibitor or ARB, and 78 and 22% of participants, respectively, were previously treated with an ACE inhibitor or ARB. Additionally, the study involved a run-in period before randomization during which participants received at least 2 weeks of enalapril therapy, followed by 4–6 weeks of LCZ696 therapy.

## ARBs Increase Bradykinin Levels

Losartan increases bradykinin levels approximately 2-fold in arterial blood of patients with hypertension (50), similar to the increase seen with ACE inhibition (112, 113). Eprosartan produced a similar increase in bradykinin levels in the same patients, although the increase did not achieve statistical significance (50). By contrast, neither losartan nor valsartan increased bradykinin levels in rats (114, 115). There are conflicting data on the role of bradykinin in mediating the effects of ARBs. Both animal and human studies implicate kinin peptides and/or the $B_2$ receptor in the actions of ARBs, possibly mediated by $AT_2$ receptor stimulation by the increased angiotensin II levels that accompany ARB therapy (116–124). However, in contrast to the attenuation of the hypotensive effects of ACE inhibition by concomitant icatibant administration (100 μg/kg/h iv for 1 h) in sodium-deplete normotensive and hypertensive subjects (125), and at a higher dose (10 mg infused iv over 15 min) in sodium replete normotensive subjects (126), a lower dose of icatibant (18 μg/kg/h iv for 6 h) did not attenuate the hypotensive effects of either acute or chronic administration of valsartan in sodium-deplete normotensive and hypertensive subjects (127).

## LBQ657 Inhibits not Only Neprilysin but Also ACE, NEP2, and ECE-2

In contrast to the plasma transudation seen with combined neprilysin and ACE inhibition in the rat tracheal plasma transudation model (**Table 3**), no transudation occurred when candoxatril was combined with valsartan (11), suggesting that combined neprilysin inhibitor and ARB therapy may cause less increase in bradykinin levels than combined neprilysin and ACE inhibition. However, LBQ657 may inhibit enzymes other than neprilysin that degrade bradykinin (**Table 1**). Ksander

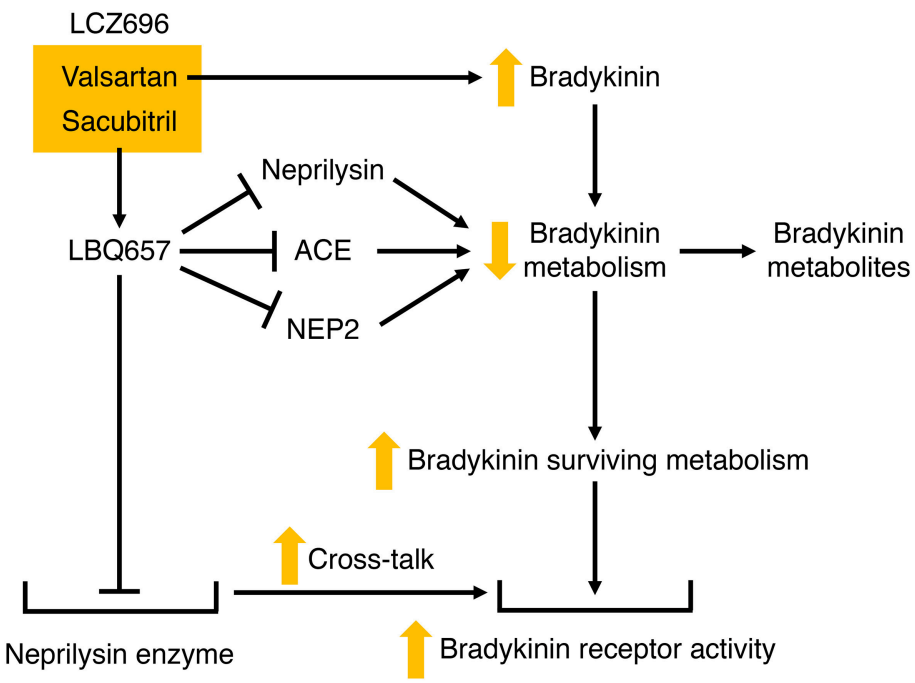


**FIGURE 6 |** Potential mechanisms by which LCZ696 may potentiate bradykinin receptor-mediated actions. Valsartan may increase bradykinin levels, and LBQ657 may also increase bradykinin levels by inhibiting bradykinin degradation by neprilysin, and possibly angiotensin converting enzyme (ACE) and neprilysin homolog membrane metalloendopeptidase-like 1 (NEP2). In addition, LBQ657 may potentiate bradykinin receptor-mediated actions by cross-talk between the LBQ657-inhibitor complex and the bradykinin receptor.

et al. reported that 10 μmol/L LBQ657 produced <50% inhibition of ACE (14). Moreover, based on information provided by Novartis Europharm Ltd, the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency reports that LBQ657 inhibits not only ACE but also NEP2 and ECE-2 (15). It is notable that peak LBQ657 concentrations approximated 37 μmol/L in healthy subjects following 400 mg/day LCZ696, and trough concentrations of LBQ657 (24 h post 400 mg LCZ696) were 4.8 μmol/L. The trough LBQ657 concentration (4.8 μmol/L) is ∼2,000 times the $K_i$ of 2.3 nmol/L for neprilysin inhibition by LBQ657 (16), and the peak LBQ657 concentration is correspondingly higher. Thus, recommended doses of LCZ696 (400 mg/day) may produce LBQ657 concentrations sufficient to inhibit ACE and contribute to increased bradykinin levels, given that, as discussed earlier, as little as 1% inhibition of pulmonary inactivation of bradykinin can double bradykinin levels (**Figure 3**). Furthermore, NEP2 has a much lower $K_m$ for bradykinin than NEP (**Table 2**) and NEP2 inhibition by LBQ657 may also increase bradykinin levels. LBQ657-mediated inhibition of ECE-2 is unlikely to contribute to increased bradykinin levels because ECE-2 is relatively inactive at physiological pH (7, 34).

LCZ696 therapy may therefore potentiate bradykinin-mediated actions by several mechanisms (**Figure 6**). These include the increase in bradykinin levels with ARB therapy (50), the increase in bradykinin levels consequent to LBQ657-mediated inhibition of neprilysin and possibly ACE and NEP2, and cross-talk between the neprilysin-LBQ657 complex and the bradykinin receptor. Bradykinin-mediated actions will likely contribute to not only the renal and cardioprotective effects but also the angioedema associated with LCZ696 therapy. Given that heart failure is associated with suppression of the kallikrein kinin system (48, 128), and resistance to kinin-mediated cutaneous transudation (129), there is concern that LCZ696 therapy for conditions such as hypertension may be associated with a higher angioedema incidence than observed in patients with HFrEF.

## SUMMARY

Tissue levels of bradykinin are higher than circulating levels and the contribution of neprilysin to bradykinin degradation is specific to the tissue and the tissue compartment. Bradykinin is a likely contributor to the therapeutic benefits of neprilysin inhibitor therapy, particularly the renal and cardioprotective effects. However, bradykinin is also an important contributor to angioedema that may result from peptidase inhibitor therapy, including neprilysin inhibitor therapy, particularly when neprilysin inhibition is combined with ACE inhibitor therapy. LBQ657 inhibits not only neprilysin but also ACE, NEP2, and ECE-2. Although angioedema incidence was acceptable, and similar for LCZ696 and enalapril therapy in HFrEF patients, it remains to be seen whether LCZ696 therapy for other conditions such as hypertension is also accompanied by an acceptable incidence of angioedema.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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# Chronic Overexpression of Bradykinin in Kidney Causes Polyuria and Cardiac Hypertrophy

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Acute intra-renal infusion of bradykinin increases diuresis and natriuresis via inhibition of vasopressin activity. However, the consequences of chronically increased bradykinin in the kidneys have not yet been studied. A new transgenic animal model producing an excess of bradykinin by proximal tubular cells (KapBK rats) was generated and submitted to different salt containing diets to analyze changes in blood pressure and other cardiovascular parameters, urine excretion, and composition, as well as levels and expression of renin-angiotensin system components. Despite that KapBK rats excrete more urine and sodium, they have similar blood pressure as controls with the exception of a small increase in systolic blood pressure (SBP). However, they present decreased renal artery blood flow, increased intrarenal expression of angiotensinogen, and decreased mRNA expression of vasopressin V1A receptor (AVPR1A), suggesting a mechanism for the previously described reduction of renal vasopressin sensitivity by bradykinin. Additionally, reduced heart rate variability (HRV), increased cardiac output and frequency, and the development of cardiac hypertrophy are the main chronic effects observed in the cardiovascular system. In conclusion: (1) the transgenic KapBK rat is a useful model for studying chronic effects of bradykinin in kidney; (2) increased renal bradykinin causes changes in renin angiotensin system regulation; (3) decreased renal vasopressin sensitivity in KapBK rats is related to decreased V1A receptor expression; (4) although increased renal levels of bradykinin causes no changes in mean arterial pressure (MAP), it causes reduction in HRV, augmentation in cardiac frequency and output and consequently cardiac hypertrophy in rats after 6 months of age.

**Keywords: kinin, kallikrein-kinin system, blood pressure, polyuria, rats**

## INTRODUCTION

The kallikrein-kinin system (KKS) is present throughout the body. Bradykinin (BK) is its main effective peptide and has two sources: it is released from circulating high molecular weight kininogen by the action of plasma kallikrein; or from low molecular weight kininogen by the action of tissue kallikrein (1). BK is an agonist of the kinin B2 receptor (B2R), and its metabolite Des-Arg$^9$-BK has affinity to the kinin B1 receptor (B1R). First related to vasodilation, inflammation, pain, and edema, the KKS has also been shown to participate in several cellular and physiological events, such as glucose homeostasis (2–4), leptin sensitivity (5), and organ and tissue protection or injury (6). The effects of BK on renal physiology and blood pressure (BP) control are still not completely unraveled.

A number of experiments were carried out to understand the role of BK in kidney and in particular how it is involved in the control of renal homeostasis and BP, as previously reviewed (1, 7). Usual approaches to test intrarenal effects of BK are the renal artery infusion of BK or antagonists of its receptors (8–10), intrarenal tissue BK infusion (11), the use of knockout animals (12, 13), or *in vitro* studies (14, 15). However, all approaches have limitations, mainly due to the complex morphology of the kidney, where the proximal and distal parts of the nephrons are anatomically mixed, and the local circulation has very specific characteristics and morphology. More recent studies have shown that infusion of BK into the kidneys causes increased urinary volume and increased sodium secretion, with no change in BP (16). Studies in dogs showed that infusion of BK in the kidneys increases urinary volume (UV) and sodium excretion (UNaV), without changing glomerular filtration rate (GFR) and these effects are blocked by pretreatment with the B2R antagonist HOE-140 (17). The increase in UV is associated with vasopressin inhibition in the kidney. Schuster et al. has shown that lysyl-BK inhibits the effect of vasopressin with respect to hydraulic conductivity (Lp) in the rabbit cortical collecting tubule perfused *in vitro* (18). The inhibition of vasopressin activity by BK infusion was also confirmed in an *in vivo* study (17). Hebert et al. concluded that this effect is transmitted via B2R and is independent of calcium signaling in the cortical collecting ducts.

Both previous *in vivo* and *in vitro* experiments revealed several acute effects of BK in kidneys, but the models used are not predicting chronic effects of excess BK in this organ. This knowledge is important to evaluate the possibility of using B2R agonists for renal diseases.

Therefore, we generated a transgenic rat overexpressing BK in the proximal convoluted tubules, in order to test the hypothesis that chronic excess of bradykinin may cause changes in BP and physiology of the cardiovascular system. These animals were submitted to diets with different sodium contents. Changes in BP, heart rate variability (HRV), genetic expression of selected components of the renin-angiotensin, vasopressin, and KKSs, as well as cardiac morphology, and physiology of the cardiovascular and urinary systems were evaluated.

## METHODS

### Animals

All studies were performed in accordance with the guidelines for the humane use of laboratory animals by the Max Delbrück Center for Molecular Medicine (Berlin, Germany) and with EU Directive 2010/63/EU for animal experiments. The animals were maintained under standardized conditions with an artificial 12-h dark-light cycle with free access to standard chow (0.25% sodium, SSNIFF Spezialitäten GmbH, Soest, Germany) and water *ad libitum*, except during the experimental diet period when they received special sodium containing diets. Two different diets were fed, low salt diet and high salt diet (E15430-24 sodium deficient; E15431-34, 4% salt, SSNIFF Spezialdiäten GmbH, Germany). If not stated different, male rats at the age of 4 months were used for experiments. All procedures with the animals were analyzed and approved by the Ethical Committee Landesamt für Gesundheit und Soziales (LaGeSo), Berlin, Germany.

### Generation of Transgenic Rats

The basic DNA construct for the generation of transgenic rats codes for an engineered fusion protein consisting of the signal peptide of human renin, the Fc portion of the mouse IgG, a prosegment of the human prorenin, a furin cleavage site followed by the sequence of Tyr$^0$-BK, as previously described (19). The promoter of kidney androgen regulated protein (Kap) was used to direct the expression of this construct specifically in the proximal tubules (20). This peptide delivery system has already been successfully used in several transgenic animal models (21–23). Tyr$^0$-BK, rather than BK itself, had to be selected, since furin would only very inefficiently cut N-terminal to the natural start of the BK peptide, arginine, but will cut in front of tyrosine (19). Tyr$^0$-BK is known to be a full agonist on BK receptors (24). The Kap promoter sequence was cloned into the same vector and the resulting construct (**Figure 1A**) was linearized and microinjected into male pronuclei of Sprague-Dawley rat zygotes as previously described (25). Genomic integration of the transgene DNA construct was determined by PCR analysis using DNA obtained from offspring tail biopsies.

### Assessment of Basic Renal Function

To collect urine, rats were kept in metabolic cages with 1 day of adaptation before collecting samples for analysis. Overnight collected urine samples were centrifuged (4,000 g), the supernatant collected in fresh tubes and stored frozen at −20°C. For measurement of urine concentrations of ions and creatinine, samples were sent and processed by Labor28 (Berlin, Germany).

### Immunohistochemistry

For histochemical analysis, rats were anesthetized with Nembutal (100 mg/Kg body weight), the abdominal cavity opened and kidneys were fixed by retrograde perfusion through the abdominal aorta using 3% paraformaldehyde in PBS as previously described (26). Kidneys were then processed for cryostat sectioning. Cryosections were incubated with primary anti-mouse IgG2b and secondary anti-rabbit Cy2-fluorescence-coupled antibodies and examined in a Leica fluorescence microscope.

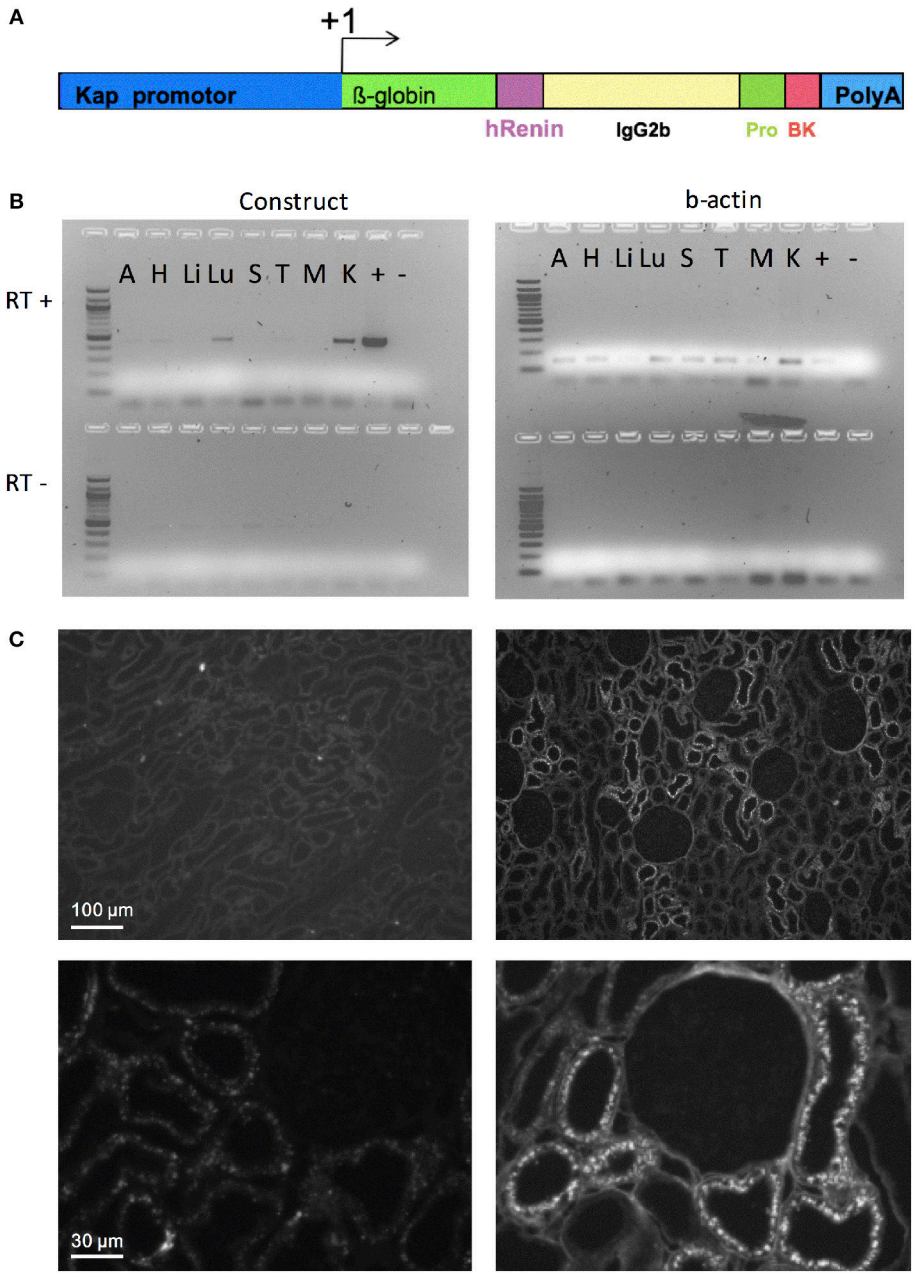


**FIGURE 1 |** Targeted overexpression of BK in proximal convoluted tubules. **(A)** Construct for BK secretion in proximal tubule. The Kap promoter and an intron from β-globin was used to target renal proximal tubule expression of a recombinant protein containing: a signal peptide from human renin gene (hR) to allow the recombinant protein to be secreted; part of mouse IgG2b to provide mass; a human prorenin-derived spacer (Pro) to allow furin activity; a furin cleavage site, the sequence of the $Tyr^0$-BK peptide (BK) followed by a stop codon and a polyadenylation site (polyA). **(B)** RT-PCR analysis of selected tissues of KapBK rats. Image of agarose gels showing the PCR products of β-actin (right) and mouse IgG2b (left) after reverse transcriptase reaction (RT+) of total RNA extract from KapBK rat tissues. As control for DNA contamination the same procedure was done with samples submitted to the same protocol without addition of reverse transcriptase (RT-, bottom of the gels). A, Adrenal gland; H, Heart; Li, Liver; Lu, Lung; S, Spleen; T, Thymus; M, Muscle; K, Kidney; +, positive control; − = $H_2O$.
**(C)** Immunohistochemistry using anti-mouse IgG2b antibodies to show the specific expression of the recombinant protein in kidney proximal tubules. Upper panels show overview of the cortex with immunoreactive signal over proximal tubules exclusively in the KapBK tissue. Lower panels show higher magnification with immunoreactive proximal segments grouped around a single glomerulus; magnification x 150 (top), x 800 (bottom).

## High Frequency Ultrasound Analysis

Animals were anesthetized by inhaling a 2.5% isoflurane oxygen mixture in a heat-controlled chamber to keep the body temperature stable. After reaching full anesthesia, rats were fixed on a heated plate with electrodes on their paws to control heart and respiratory rate, as well as to record the electrocardiogram.

Body temperature was monitored by a rectal sensor and corrected by a heating lamp if necessary. With the ultrasonic detector MS-250 collected data were visualized and analyzed via the VEVO 2,100 high-resolution imaging system (Visualsonics Fujifilm, VisualSonics, Toronto, Ca). Stroke volume and cardiac output was measured by tracing the endocardium in systole and diastole of a parasternal long axis view of the left ventricle.

## Telemetry

The telemetry system for BP and heart rate measurement (Dataquest ART 4.0™, Data Sciences Inc., St. Paul, MN, USA) and the implantation procedure are described in detail by Plehm et al. (27). Briefly, the radiotelemetric pressure transducers (TA11PA-C40) were implanted in the abdominal cavity of the rats, with the pressure-sensing capillary anchored in the lumen of the abdominal aorta. Before the implantation the zero offset was measured and the unit was soaked in 0.9% NaCl solution. Rats were anesthetized with isoflurane. Animals recovered for 10 days before baseline values were recorded. By this time the rats had regained their circadian BP and heart rate rhythm while surgery and anesthesia-induced changes in systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and heart rate (HR) had abated. The data from the implanted transducer were transmitted via radiofrequency signals to a receiver below the home cage and sampled every 5 min (sampling rate 500 Hz).

## Spectral Analysis

Continous telemetric recordings of the BP waveform were stored during resting and activity periods for 1 h. These waveform recordings were used for computing the HRV and the spectral analysis of the HR to analyze the autonomic regulation. For analysis of the HRV and the frequency domain results we used the Kubios HRV analysis software (Kubios, Kuopio, Finland). The HRV spectrum was calculated from artifact-corrected 30 min recordings using Fast Fourier Transformation. The frequency bands were very low frequency (VLF, 0,015–0,250 Hz), low frequency (LF, 0,250–1,000 Hz), and high frequency (HF, 1,000–6,000 Hz).

## Measurement for Renin-Angiotensin System (RAS) Components

The renin activity of plasma samples was calculated from the generation rate of AngI in the samples, for renin concentration a defined amount of angiotensinogen was added before incubation. Plasma angiotensinogen was measured by adding rat kidney extract to the samples and measuring AngI generation. The concentration of AngI was measured by radioimmunoassay. These methods were performed as described before (28, 29).

## Gene Expression Analyses

For quantitative real-time PCR (qPCR) tissues were snap-frozen in liquid nitrogen. Samples were homogenized and total RNA was isolated using a NucleoSpin RNA II purification kit (Macherey-Nagel, Düren-Germany) and then stored at −80°C until use. The RNA integrity was assessed by electrophoresis on an agarose gel. cDNA was synthesized from 1 μg of total RNA with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, USA) using random hexamer nucleotides. Standard curves were made to determine the amplification efficiencies for each primer pair. Quantitative PCR was performed on an ABI Prism 7900 sequence detection system with 100 nM primers, 5 ng of cDNA, and 10 μL SYBRGreen mastermix (Thermo Fisher Scientific, Waltham, USA) in a 20 μL reaction. mRNA expression was normalized to beta-actin mRNA and expressed as a relative value to the control group.

## Statistics

Normality of data was determined using the Shapiro–Wilk test and equality of variance verified using Brown-Forsythe test, and all data presented normal distribution. Statistical analyzes were performed using GraphPad Prism® software (GraphPad Software Inc, La Jolla, USA). Results were considered significant when $p < 0.05$ and data are shown as means ± standard error of the mean (SEM). Differences between two groups were evaluated by using unpaired Student's test, and differences between more than two groups were evaluated by using ANOVA.

# RESULTS

## Basic Characterization of KapBK Rats

The DNA construct coding for an engineered fusion protein controlled by the kidney androgen-regulated protein (Kap) promoter was inserted into the genome of Sprague-Dawley rats (overview see in **Figure 1A**) to elicit BK overproduction specifically in the proximal convoluted tubule. This promoter is androgen dependent and was shown to be specific for proximal convoluted tubules (20). Adult male rats expressed the recombinant protein in the kidney (**Figure 1B**). Transgenic female rats cannot activate the Kap promoter and can be used as control. Low expression in the lung was also detected. Since BK in lung is very quickly inactivated as described before (30), the effect in lung is probably minimal. **Figure 1C** presents an image of the immunohistochemical detection of the fusion protein in kidney sections using an antibody directed against mouse immunoglobulin G2b, which is part of the engineered protein (**Figure 1A**), confirming its specific expression in proximal tubules of KapBK rats.

## KapBK Rats Exhibit Polyuria, Diluted Urine, and Excrete More $Na^+$, but Are Able to Control Urine Volume Under Water Deprivation

The overexpression of BK in renal proximal tubules causes polyuria which is compatible with other experiments infusing BK in anesthetized animals (17). The increase in urine excretion was present when rats were feeding a standard chow as well as with diets containing low and high amounts of salt (**Figure 2A**). The increased diuresis is compensated by reflex polydipsia (**Figure 2A**). In the case of high salt diet, KapBK rats need a smaller adaptive increase in urinary volume in order to excrete the excess of ingested salt. Furthermore, the urine of KapBK rats is diluted with regards to several other components like protein

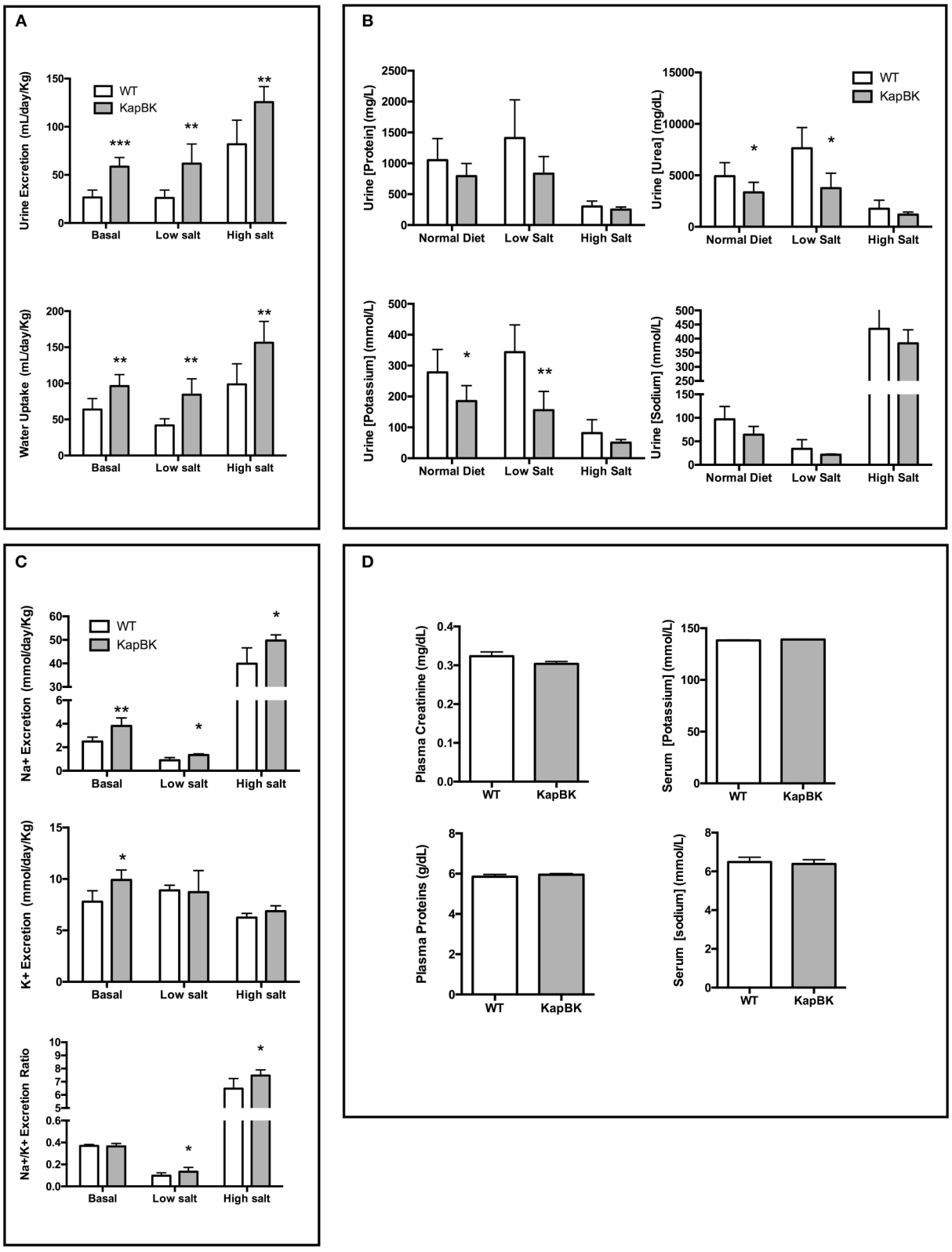


**FIGURE 2 |** Urine and blood parameters. The animals were submitted to diets containing different concentrations of sodium. After 10 days with each diet urine was collected in metabolic cages. KapBK rats secrete more urine and drink more water **(A)** and the urine has lower concentrations of total proteins, urea, potassium and sodium concentrations. **(B)** Total sodium and sodium/potassium ratios are increased. **(C)** Plasma concentrations of total proteins, creatinine, sodium, and potassium were unchanged in rats fed with standard diet. **(D)** Data presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. WT, $n = 6$.

content, sodium, potassium, and urea suggesting reduction of water reabsorption (**Figure 2B**). Analyzing daily sodium and potassium excretion, we noted an increased sodium excretion and an increased $Na^+/K^+$ excretion ratio (**Figure 2C**) in agreement with previous studies (7, 15, 31, 32). No changes were observed regarding plasma levels of proteins, creatinine, sodium or potassium (**Figure 2D**), and blood cell count (**Table S1**) and only body composition analysis (**Figure S1**) showed a trend to increased water content.

Under 20-h water deprivation, KapBK rats present diuresis only in the first 4 h of the experiment. Then they start to produce the same amount of urine as the controls, showing that these rats are able to adapt to water deprivation avoiding severe dehydration (**Figure 3**). These results demonstrate that KapBK rats are a useful model for analyses of the chronic effect of increased renal BK on water/electrolyte homeostasis.

## KapBK Rats Present a Small Increase in Systolic Blood Pressure, Increased Heart Rate, and Decreased Heart Rate Variability

Chronically increased BK in renal proximal tubules of rats did not change MAP but increased SBP (**Figure 4A**) as detected by telemetric measurement (**Figure S2**). However, the cardiovascular system underwent several alterations in the adaptive response to the polyuria. Increased heart rate (**Figure 4B**) was observed, accompanied by decreased HRV (**Figure 4C**) and a decreased low-frequency oscillatory component (that can be a signal of changes in RAS) (**Figure 4D**). The LF/HF ratio (**Figure 4E**) was decreased in KapBK rats indicating decreased sympathovagal balance on the heart contrasting the increased heart rate. The relative and absolute masses of the adrenal glands were reduced in KapBK rats suggesting reduction of adrenergic activation (**Figure 4F**).

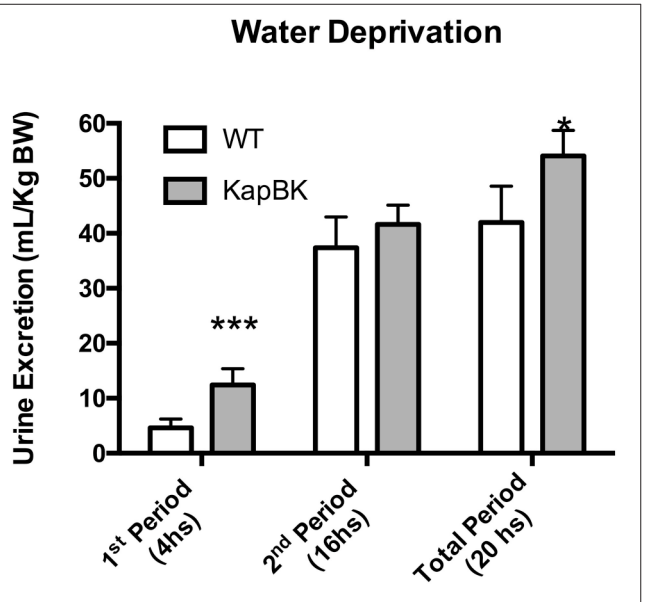


**FIGURE 3 |** Urine excretion under water deprivation. Under normal diet, the animals were submitted to water deprivation and showed adaptation in urine volume production over time. Data presented as mean ± SEM. $^*p < 0.05$, $^{***}p < 0.001$ vs. WT, $n = 6$.

## Chronic BK Excess in Kidney Causes Cardiac Hypertrophy and Increased Cardiac Output, Contrasting With Smaller Renal Artery Blood Flow, and Normal Glomerular Filtration Rate

Probably as a consequence of the increased heart rate, cardiac hypertrophy develops in older KapBK rats (6 months old) as evidenced by increased left ventricular fractional shortening, left ventricular mass, and cardiac output (**Figures 5A–C**). Despite the increased cardiac output, KapBK rats have reduced renal artery blood flow (**Figure 5D**) suggesting high pressure in the glomerular capillary system. The activity of the systemic RAS is changed (**Figures 5F–H**) with decreased plasma renin activity and concentration (2-fold) and increased concentration of plasma angiotensinogen (3-fold). Connecting the low blood flow in the renal artery with the polyuria, we found normal GFR as estimated by creatinine clearance (**Figure 5E**).

## Chronic Overexpression of BK in Kidney Increases Local Angiotensinogen and Decreased Epithelial Sodium Channel (SCNN1, Alpha, and Gamma Subunits) and AVPR1A mRNA Expression

We analyzed the mRNA expression of selected genes by qPCR in the following regions of the kidney: Fragments of outer cortex (OC), inner cortex (IC), and medulla (M) (**Figure 6**). Increased angiotensinogen expression was observed in all parts of kidney (**Figure 6A**), showing that the local RAS is activated in KapBK rats. We also found increased expression of B2R in OC (**Figure 6B**), probably due to the increased BK levels that were shown to stimulate the expression of B2R by Ricciardolo et al. in other tissues (33). The reduced expression of vasopressin V1A receptor (AVPR1A, **Figure 6C**) can explain the diluted urine although vasopressin V2 receptor (AVPR2) expression remained unchanged (**Figure 6D**). Increased sodium excretion can be related to reduced expression of alpha and gamma subunits of the epithelial sodium channel (ENaC, SCNN1, **Figures 6E–G**). The increased aquaporin 2 (AQP2) expression in medulla after water deprivation is part of the mechanism involved in the adaptation against severe dehydration (**Figure 6H**). No changes were observed in renal mRNA expression of renin (**Figure 6I**).

# DISCUSSION

Acute infusion of bradykinin in the kidneys causes polyuria inhibiting vasopressin activity in collecting tubules (17, 18, 34) and increasing sodium excretion (1, 7, 9, 10, 15, 32, 35). However, the consequences of chronic BK excess in the kidneys are still incompletely studied (36, 37). In the present study a new animal model producing an excess of BK in the proximal tubules was generated. The overproduction of BK in these rats

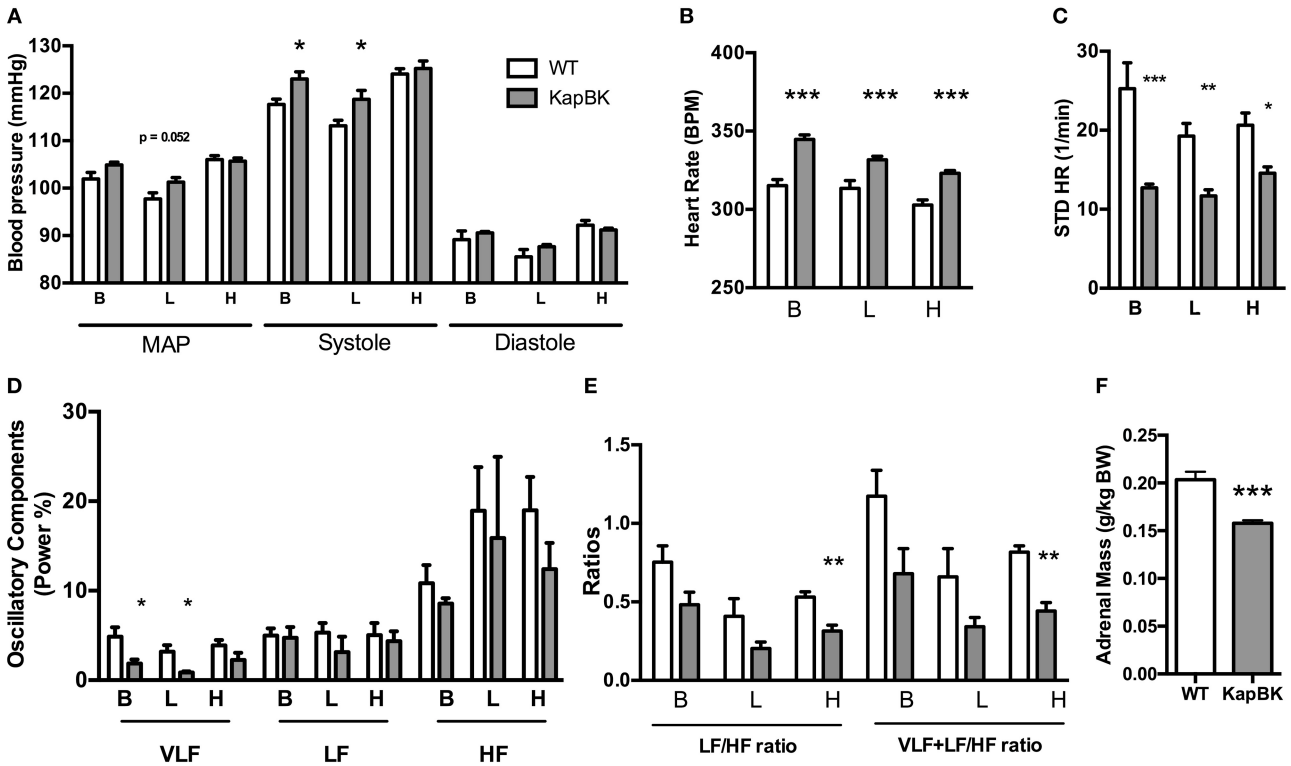


**FIGURE 4 |** Cardiovascular and autonomic system. The blood pressure parameters were measured by telemetry after 10 days of normal (B), low salt (L), or high salt (H) containing diet. **(A)** Mean arterial pressure (MAP), systolic blood pressure (systole) and diastolic blood pressure (diastole). **(B)** Heart rate under normal diet (basal, B) and low (L) and high (H) salt diets. **(C)** Heart rate variability (HRV; STD HR = standard deviation of Heart Rate). **(D)** Power (%) of oscillatory components (VLF, very low frequency; LF, low frequency and HF, high frequency). **(E)** Ratios of oscillatory components. **(F)** Adrenal gland mass normalized to body weight. Data presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. WT, $n = 6$.

starts from puberty and is maintained during the remainder of life, allowing studies about long-term effects of BK in kidney. For better understanding of these chronic effects, the animals were submitted to three diets with different salt concentrations. Changes in renal and cardiovascular functions as well as in the renin-angiotensin and KKSs were assessed. Additionally, the expression of several genes was evaluated in different parts of the kidney. The main results showed that the chronic BK excess in the kidneys causes cardiac hypertrophy, changes in the regulation of the local, and systemic renin angiotensin systems, increased heart rate and cardiac output, decreased heart rate variability, maintenance of GFR despite of reduced blood flow in the renal artery, and changes in gene expression in kidney, mainly increasing angiotensinogen and decreasing vasopressin V1A receptor mRNA levels. Moreover, there was no change in MAP, in agreement with the described acute models of BK excess in the kidney (17, 36).

The results confirmed that the overexpression of BK in the kidney of the animals was successfully achieved. Although we could not measure BK levels directly, this conclusion is supported by genotyping, RT-PCR, immunohistochemistry and also by the concordance of physiological changes observed in the present study with previous literature, mainly polyuria, increased sodium excretion and the diluted urine of the transgenic animals. These effects were observed after acute BK treatment in kidney (17) but also in studies searching for chronic effects (36). Furthermore, the DNA construct was designed to be expressed in proximal tubules and to secrete BK into the extracellular space, based on the signal peptide present in the expressed protein and the ubiquitous expression of furin, and it is well-established that intraluminally generated kinins in kidney exert autocrine and paracrine actions on distal tubular cells, favoring natriuresis and diuresis (32) as observed in the present study. In addition, interstitially released BK contributes to the regulation of cortical and medullary blood flow (38), as was also observed in KapBK rats and will be discussed below. These results confirm that the novel rat model is appropriate to study chronic excess of BK in kidney.

Although BK is known to dilate vessels and promote sodium excretion and polyuria, very few experiments show a sustained alteration in MAP by BK. Different methods were used to test the potential hypotensive effect by infusing BK in kidney, but BP normally returned to normal values after an acute decline (37, 39, 40). Moreover, it was also observed that knockout mice for either kinin receptor, B1 and B2, as well as double knockout mice are normotensive (41–45), although they can react differently than controls in relation to BP control when submitted to challenges such as high-salt diet. Only general overexpression of

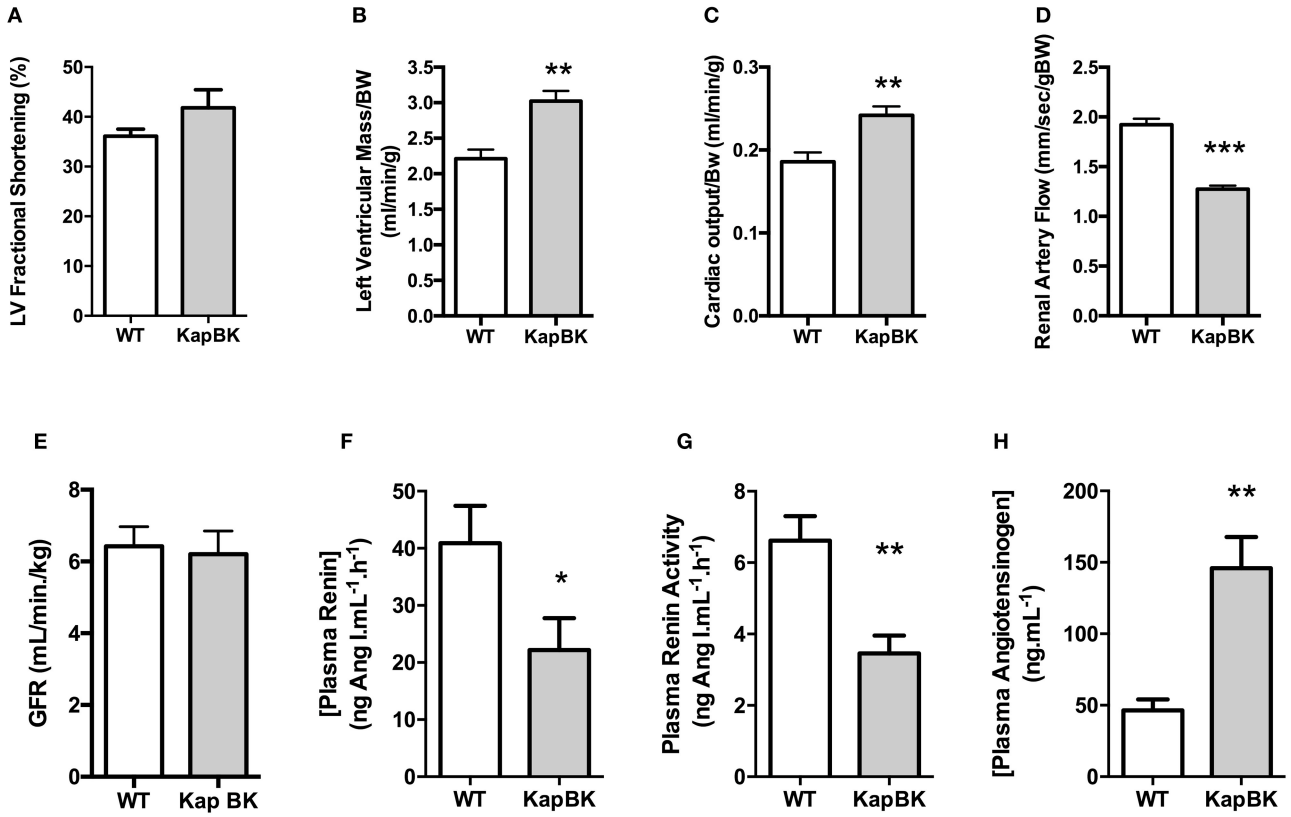


**FIGURE 5 |** Cardiac and renal function, renin-angiotensin system. **(A)** left ventricular fractional shortening; **(B)** left ventricular mass; **(C)** normalized cardiac output; **(D)** renal artery flow; **(E)** glomerular filtration rate (GFR) as estimated by creatinine clearance **(F)** plasma renin concentration; **(G)** plasma renin activity; **(H)** plasma angiotensinogen concentration. Data presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. WT, $n = 6$.

tissue-kallikrein in transgenic rats leads to a small but significant reduction in MAP probably by the permanent rise of BK in several tissues (42). Although KapBK rats presented similar MAP as the controls, we observed several changes in the cardiovascular system concerning BP regulation. The local renal RAS seems to be activated with increased expression of angiotensinogen. This increased intrarenal RAS activity is also evidenced by the diminished renal artery blood flow since AngII is known to cause vasoconstriction of both afferent and efferent glomerular arterioles increasing intraglomerular pressure (46). On the other hand, the observed reduction of plasma renin concentration and activity suggests a reduction in systemic RAS activity. Despite the severe reduction in renal artery blood flow in KapBK rats, GFR does not change. The reduction in glomerular blood flow seems to be a mechanism to maintain normal GFR which is also observed in AVPR1A knockout mice (47).

It is well established that BK inhibits vasopressin-induced water flow in the collecting duct *in vitro* (18, 34) and *in vivo* via B2R (17). The present study shows that after several months of chronic overexpression of BK in kidney the rats still present polyuria. The dilution of all urinary components indicates increased water content suggesting decreased water reabsorption. This is compatible with the inhibition of vasopressin activity as previously described both *in vitro* and *in vivo* in acute models of BK infusion in the kidney (17, 18, 34). However, the present study shows a marked decrease in the expression of vasopressin V1A receptors in the parts of the kidney that are rich in distal nephron structures, but no change in V2 receptor expression, showing that vasopressin resistance can be regulated directly via gene expression of V1A receptors. Previous studies showed that the effect of BK on inhibition of vasopressin activity is dependent on prostaglandin formation (and can be blocked with indomethacin) and independent of increased calcium concentration (17). This suggests that the increase in endogenous prostaglandin synthesis by B2R activation antagonizes vasopressin-stimulated cAMP generation and reduces vasopressin-induced water flow in the collecting duct (18, 34). The new observation that decreased expression of vasopressin V1A receptors is related to these effects opens new doors to understand the underlying mechanisms. Further gene expression studies are warranted using acute models of excessive BK supply to elucidate whether the changed AVPR1A expression found here is indeed a chronic effect, or is also part of acute BK effects on vasopressin-induced water flow in the kidney. Favoring that vasopressin receptors mediate the effects of chronic BK excess, AVPR1A knockout mice exhibited a similar phenotype as KapBK rats concerning urine volume and composition (48). Accordingly, polyuria in KapBK rats seems to

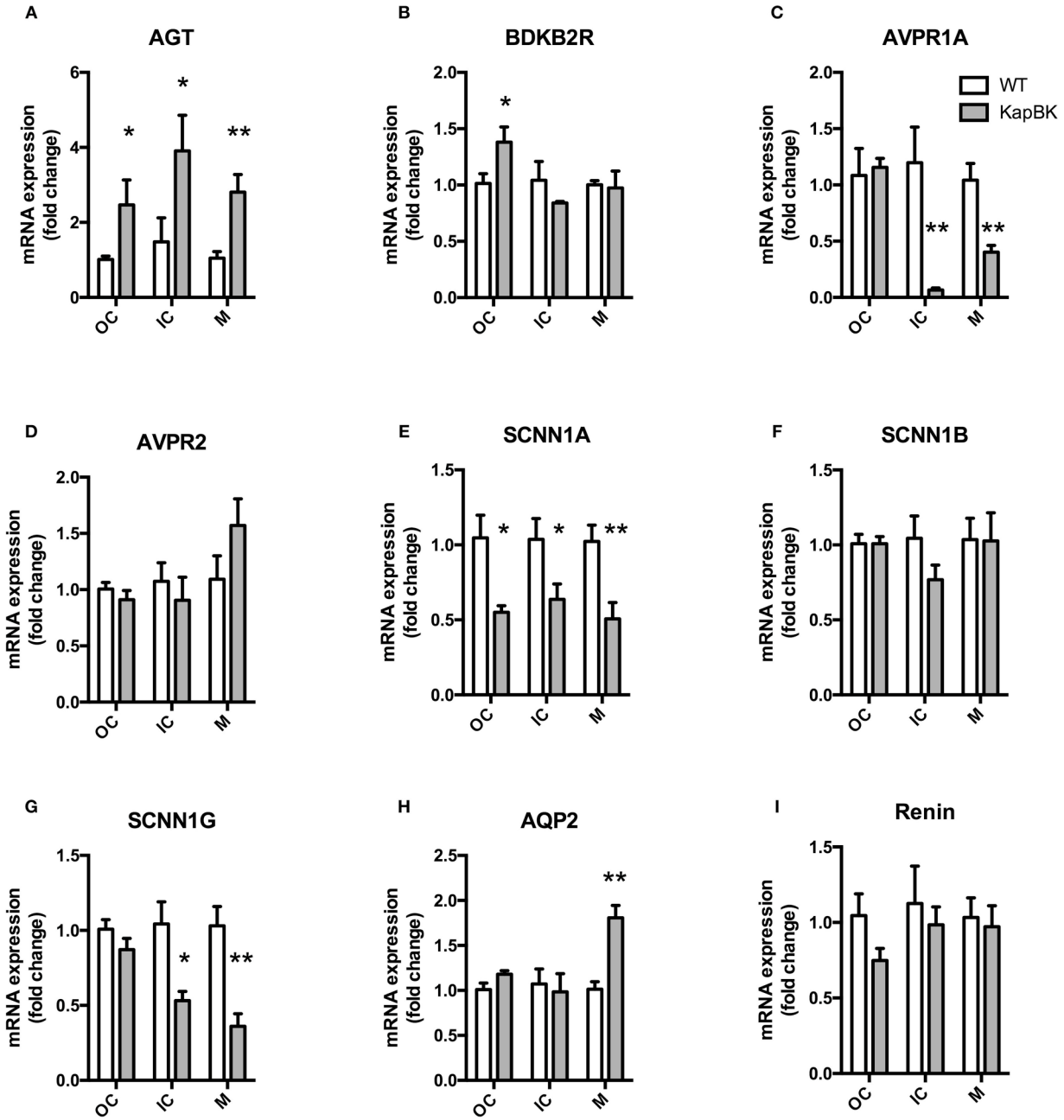


**FIGURE 6 |** Renal gene expression: Total RNA was extracted from outer (OC) and inner (IC) cortex, and medulla (M) of kidney. **(A)** AGT, angiotensinogen; **(B)** BDKB2R, bradykinin B2 receptor; **(C)** AVPR1A, vasopressin V1A receptor; **(D)** AVPR2, vasopressin V2 receptor; **(E–G)** SCNN1-A, B, and G, subunits alpha, beta, and gamma of the Epithelial Sodium Channel (ENaC); **(H)** AQP2, aquaporin 2; **(I)** renin. All data were normalized for WT OC group and are shown as relative fold change. Data presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. WT, $n = 5$.

be an effect caused in the distal nephron by reduction of AVPR1A receptors as evidenced in the mRNA expression analysis. Aoyagi et al. showed that polyuria observed in AVPR1A knockout mice was associated with decreased water reabsorption and not with increased GFR, as observed in KapBK rats. They also described a lower renin concentration in plasma of AVPR1A knockout mice again agreeing with KapBK rats (14). Furthermore, Yasuoka et al. noted that in water restriction conditions AVPR1A knockout mice also increase renal AQP2 expression as observed in KapBK rats (47). All these similarities suggest that the consequences of increased BK in kidney are due to the altered regulation of AVPR1A expression in KapBK rats.

To localize gene expression to certain structures of the kidney, we used RNA originating from three different regions of the

kidney, called here: the "outer cortex," the more external part of the kidney cortex containing mainly the proximal structures of the nephrons; the "inner cortex," containing all parts of the nephrons including small fractions of the distal nephrons; and the "medulla," containing mainly collecting ducts and loops of Henle. One main result of this expression study was the reduction of alpha and gamma subunits expression of ENaC mainly in parts of the kidneys containing distal nephron structures. Again, these new data suggest that the inhibition of ENaC activity by BK infusion can be induced by gene expression regulation. It is well-established that the increase in UNaV by BK is due to the inhibition of ENaC (7, 15, 32). Other authors have shown inhibition of sodium reabsorption by BK (7, 31, 49). Zaika et al. presented a related effect on SCNN1 in an experiment *in vitro* (15), showing that BK decreases the probability of SCNN1 channel opening. In the present study, the ENaC activity was not measured since it was well-demonstrated previously (7, 17). However, the measurements of $Na^+$, $Cl^-$, and $K^+$ in urine indicated that only $Na^+$ showed increased excretion in KapBK rats, suggesting that the inhibition of $Na^+$ reabsorption is not due to cotransporters such as Na/K-ATPase, thiazide-sensitive sodium chloride co-transporter (NCCT) or sodium-potassium-chloride co-transporter (NKCC2), all of them important sodium transporters in the apical membrane of different regions of the nephrons (50). Together with previous studies demonstrating that BK inhibits only ENaC (7, 15, 17, 32), the here presented reduction of the gene expression of ENaC subunits, and the urine concentration of the excreted ions together indicate that the observed natriuresis in KapBK rats is caused by reduction of ENaC, at least partially on the level of gene expression.

Although vasopressin sensitivity is reduced in KapBK rats by decreased AVPR1A expression, expression of AVPR2 is similar to that of control animals. Accordingly, AQP2 mRNA expression is increased in renal medulla of KapBK rats after water deprivation. AVPR2 is the main regulator of AQP2 translocation to the apical membrane providing a strong mechanism of water reabsorption in the presence of increased vasopressin levels. Both, the induction of AQP2 expression and normal expression of AVPR2 explain why KapBK rats can concentrate urine under water deprivation, and fit with observations in AVPR1A knockout mice, which also can concentrate urine under this condition (14). Therefore, we speculate that KapBK rats are equipped with a reduced sensitivity to basal vasopressin levels, due to low AVPR1A expression, but they respond adequately to increased level of vasopressin, since there is a normal AVPR2 expression and an inducible AQP2 expression in kidney.

The increased renal BK expression affects the heart as evidenced by increased heart rate and contractility in KapBK rats, leading to an augmented cardiac output and even cardiac hypertrophy when KapBK rats get older. These are typical phenomena of cardiac sympathetic stimulation, but we found decreased LF/HF ratio and a smaller mass of the adrenal gland suggesting a reduction in the sympathovagal balance. This discrepancy may at least partially be related to the differential regulation of the sympathetic innervation of different organs.

Taken together, our results show that chronic intrarenal overexpression of BK exerts similar alterations in renal physiology as known from acute models of intrarenal BK infusion. However, also unexpected effects appeared in the chronic model showing that the new transgenic KapBK rats are suitable for studying chronic actions of BK in the kidney. These new effects of intrarenal BK include changes in RAS regulation, decreased renal vasopressin sensitivity based on decreased V1A receptor expression and reductions in cardiac rhythm variability, augmentation in heart rate and cardiac output and consequently cardiac hypertrophy without changes in MAP. These data have implications for the concept that B2 receptor agonists may have a beneficial effect in controlling hypertension. It is already well-established that ACE inhibitors exert their main effects by reducing angiotensin II, but in addition, much of the protective effects on the kidneys are associated with the increase of the BK concentrations in this organ (51–54). In the present study, the chronic increase of renal BK happened in the presence of an active RAS, since there is no ACE inhibition. The present data suggest even an increased local RAS activity, based on increased angiotensinogen expression and decreased renal artery blood flow. The systemic RAS activity, on the other hand, seems to be decreased based on decreased plasma renin concentration and activity. Under these conditions, novel adaptations and physiological effects of BK have been reported here, which should be followed up in the case of administration of B2 receptor agonists as experimental treatment in patients without ACE inhibitor treatment.

## AUTHOR CONTRIBUTIONS

CB conducted the physiological analyses, collecting, organizing and analyzing all data, and writing of manuscript. IS performed the molecular analyses. GS conducted urine analyses. FR genotyped the rats. PX cloned the DNA vector to generate the animals. EP made the microinjections to generate the animals. IL performed the radioimmunoassay for RAS measurements. RP analyzed telemetric data. AH conducted echocardiography and its analysis. MT conducted surgeries and telemetric data analysis. SB performed immunohistochemistry of the kidneys. NA helped to design the experiments, and with the drafting and revision of the manuscript. RA helped with data interpretation, drafting, and revision of the manuscript. JP helped with data interpretation, drafting, and revision of the manuscript. MB conceived and designed the work, drafted, and revised the manuscript.

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# Sensitization of the Angiotensin II AT1 Receptor Contributes to RKIP-Induced Symptoms of Heart Failure

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Inhibition of the G-protein-coupled receptor kinase 2 (GRK2) is an emerging treatment approach for heart failure. Therefore, cardio-protective mechanisms induced by GRK2 inhibition are under investigation. We compared two different GRK2 inhibitors, i.e., (i) the dual-specific GRK2 and raf kinase inhibitor protein, RKIP, and (ii) the dominant-negative GRK2-K220R mutant. We found that RKIP induced a strong sensitization of Gq/11-dependent, heart failure-promoting angiotensin II AT1 receptor signaling. The AT1-sensitizing function of RKIP was mediated by the RKIP-GRK2 interaction because the RKIP-S153V mutant, which does not interact with GRK2, had no effect on AT1-stimulated signaling. In contrast, GRK2-K220R significantly inhibited the AT1-stimulated signal. The *in vivo* relevance of these major differences between two different approaches of GRK2 inhibition was analyzed by generation of transgenic mice with myocardium-specific expression of RKIP and GRK2-K220R. Our results showed that a moderately increased cardiac protein level of RKIP was sufficient to induce major symptoms of heart failure in aged, 8-months-old RKIP-transgenic mice in two different genetic backgrounds. In contrast, GRK2-K220R protected against chronic pressure overload-induced cardiac dysfunction. The AT1 receptor contributed to RKIP-induced heart failure because treatment with the AT1 receptor antagonist, losartan, retarded symptoms of heart failure in RKIP-transgenic mice. Thus, sensitization of the heart failure-promoting AT1 receptor by the RKIP-GRK2 interaction contributes to heart failure whereas dominant-negative GRK2-K220R is cardioprotective. Because RKIP is up-regulated on cardiac biopsy specimens of heart failure patients, the deduced heart failure-promoting mechanism of RKIP could also be relevant for the human disease.

**Keywords: GRK2, ADRBK1, RKIP, PEBP1, AGTR1, angiotensin II, losartan, heart failure**

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## INTRODUCTION

The family of G-protein-coupled receptor kinases (GRKs) initiates the process of signal desensitization by phosphorylation of G-protein-coupled receptors (1, 2). Among different GRKs, the G-protein-coupled receptor kinase 2 (GRK2) is the most important member of the GRK family because functions of GRK2 are indispensable, and complete loss of GRK2 in GRK2-knockout mice is lethal (3). On the other hand, there are multiple lines of evidence, which show that inhibition

of exaggerated GRK2 activity in experimental models of heart failure is cardio-protective (4–8). Based on these data, inhibition of GRK2 appears as a promising treatment approach of heart failure (9, 10).

Experimental evidence indicates that a major cardio-protective mechanism induced by GRK2 inhibition relies on re-sensitization of desensitized beta-adrenoceptors in heart failure (9, 10). In addition, several other cardio-protective mechanisms of GRK2 inhibition were deduced, which include improvement of mitochondrial function and promotion of cardiomyocyte survival (11, 12). Prevention of cardiomyocyte death by GRK2 inhibition was partially attributed to enhancement of the pro-survival Raf-Erk pathway (8, 12).

Apart from inhibition of kinase-dependent functions of GRK2, recent data show that kinase-independent functions of GRK2 are also important for cardio-protection. In this respect, the focus lies on the amino-terminal domain of GRK2, which contains a regulator of G-protein signaling (RGS) domain (13, 14). The RGS domain of GRK2 is functional and inhibits Gq/11-mediated signaling (13, 14), which is an established causative factor of myocardial hypertrophy (15). Consequently, by inhibition of pro-hypertrophic Gq/11-mediated signaling (15), the RGS domain of GRK2 has documented cardioprotective activity (16). It is noteworthy that most approaches of GRK2 inhibition leave this cardioprotective RGS domain of GRK2 intact, i.e., the betaARKct, which inhibits GRK2-mediated receptor phosphorylation by scavenging of Gβγ subunits (4, 5), or ATP-site directed kinase inhibitors such as paroxetine (7, 17). Also, the kinase-inactive GRK2-K220R mutant, which acts as a dominant negative GRK2 mutant, has a preserved amino-terminal RGS domain (18), and shows effective RGS domain-mediated inhibition of Gq/11-stimulated signaling (12). But other approaches of GRK2 inhibition target specifically the amino-terminal domain of GRK2 such as the raf kinase inhibitor protein, RKIP, which is the alias for phosphatidylethanolamine-binding protein 1, *PEBP1* (19). Consequently, RKIP could also inhibit the cardioprotective RGS domain of GRK2.

RKIP is an inhibitor of GRK2, which switches from Raf1 to GRK2 by PKC-mediated phosphorylation on serine 153 (19). The serine-153-phosphorylated RKIP interacts with the amino-terminal domain of GRK2 and thereby blunts GRK2-mediated phosphorylation of receptor substrates such as the beta-adrenoceptor (19). On the other hand, by interaction with the RGS domain-containing amino-terminus of GRK2, serine-153-phosphorylated RKIP could also interfere with the cardioprotective Gq/11-inhibitory function of the RGS domain (19). As a consequence, the RKIP-GRK2 interaction, would sensitize signaling stimulated by major Gq/11-coupled, heart failure-promoting GPCRs such as the angiotensin II AT1 receptor (20) by a dual mechanism, which involves GRK2 and RGS domain inhibition.

Because RKIP is up-regulated on cardiac biopsy specimens of failing human hearts (21), these potentially detrimental functions of RKIP are of pathophysiological relevance. We addressed this issue and found that RKIP strongly enhanced signaling stimulated by the Gq/11-coupled AT1 receptor in cells. *In vivo*, a moderately increased cardiac RKIP level induced by transgenic RKIP expression under control of the myocardium-specific alpha-MHC promoter was a sufficient cause for development of major symptoms of heart failure. For comparison, targeting of GRK2 by the dominant-negative GRK2-K220R showed RGS-domain-mediated inhibition of AT1-stimulated signaling in cells, and cardioprotective activity against chronic pressure-overload-induced cardiac dysfunction *in vivo*. In agreement with a causative role of AT1 receptor sensitization in the cardiac phenotype triggered by RKIP, inhibition of the AT1 receptor by the AT1-specific antagonist, losartan, retarded signs of heart failure in RKIP-transgenic mice. Together our data strongly suggest that sensitization of AT1 receptor signaling contributes to RKIP-induced cardiac dysfunction.

# MATERIALS AND METHODS

## Generation of Transgenic Mice

Different transgenic mouse lines were generated and/or characterized in frame of this study. For transgenic expression of RKIP, the cDNA encoding *PEBP1* (phosphatidylethanolamine-binding protein 1, RKIP) was placed under control of the alpha-myosin heavy chain (alpha-MHC) promoter (8). The cDNA encoding GRK2-K220R (*ADRBK1K220R*) was also inserted into the alpha-MHC plasmid. Plasmid backbone was removed by *Not1* digestion. After pronuclear injection of transgenic DNA (2 ng/μL) into fertilized oocytes isolated from super-ovulated B6 (C57BL/6J) or FVB (FVB/NJ) mice, embryo transfer of 2-cell embryos was performed into 0.5-day pseudo-pregnant CD-1 foster mice. PCR genotyping of offspring was performed at an age of 3 to 4 weeks with ear-punch biopsies. Founder mice of the FO generation with stable integration of the transgenic DNA into the genomic mouse DNA were used for further breeding. Different RKIP-transgenic mouse lines were generated in the B6 (C57BL/6J) background (C57BL/6Tg(MHCPEBP1)1Sjaa; JAX strain ID 911818), and FVB (FVB/NJ) background (FVB/NTg(MHCPEBP1)1Sjaa, JAX strain ID 911819). Tg-GRK2K220R mice were generated in the B6 background (C57BL/6Tg(MHCADRBK1K220R)1Sjaa; JAX strain ID 911825). As indicated, phenotyping of male transgenic mice was performed at an age of 8 months. In addition, 8-week-old male B6 mice were subjected to 2 months of chronic pressure overload imposed by abdominal aortic constriction, AAC (22). At the end of the observation period, AAC-induced chronic pressure overload was controlled and confirmed by an increased systolic aortic pressure (>150 mmHg) as determined by invasive hemodynamic measurement (Micro-Tip® Catheter Pressure Transducer 1F, Millar Instruments). The left ventricular ejection fraction was determined under tribromoethanol anesthesia (250 mg/kg body weight; i.p.; freshly prepared and protected from light) by M-mode echocardiography in the parasternal long-axis view with a Vivid 7 echocardiography equipment and a 12 MHz linear array ultrasound transducer (GE Healthcare). Data were evaluated offline with the EchoPac Pc 3.0 software (GE Healthcare) using the formula of Teichholz to calculate the left ventricular ejection fraction (22). Systolic blood pressure was measured with a PowerLab data acquisition system coupled to a pulse transducer/cuff (AD Instruments). All animal experiments

were conducted in agreement with the NIH guidelines, and reviewed and approved by the local committee on animal care and use (Cantonal Veterinary office, Zurich).

## Whole Genome Microarray Gene Expression Profiling

For whole genome microarray gene expression profiling, anesthetized mice (ketamine/xylazine, 100 mg/10 mg/kg) were perfused with PBS, hearts from transgenic mice (Tg-RKIP, Tg-GRK2K220R), and non-transgenic B6 controls were isolated, pulverized under liquid nitrogen, and total RNA was isolated by the RNeasy Midi kit according to the protocol of the manufacturer (Qiagen). RNA purity was confirmed by an absorbance ratio A260/280 of ∼2.0. The absence of RNA degradation and RNA quality were further controlled by the presence of bright bands of 18S and 28S ribosomal RNA in denaturing RNA electrophoresis. The RNA was reverse transcribed and processed for whole genome microarray gene expression profiling following the Affymetrix protocol (GeneChip Expression Analysis Technical Manual, rev. 5, Affymetrix Inc., Santa Clara, CA, USA). Fragmented and biotin-labeled cRNA (15 μg/gene chip) in 200 μl of hybridization solution was hybridized to the microarray gene chip (Mouse Genome MG430 2.0 Array, Affymetrix) in a hybridization oven 640 (Affymetrix) for 16 h at 45°C. After washing and staining of gene chips with a Fluidics Station 450 (Affymetrix) according to the GeneChip Expression Analysis Technical Manual, microarrays were scanned with the GeneChip Scanner 7G (Affymetrix), and signals were processed with a target value of 300 using GCOS software version 1.4 (Affymetrix). Cardiac RNA from three mice was pooled for one gene chip, and two gene chips are presented for each group. Such an approach is feasible with inbred mouse lines due to negligible intra-individual variability (23). Probe sets with significantly different signal intensities were identified by TIGR MeV ($p < 0.01$, just alpha, ≥2-fold difference between probe sets with call present and/or signal intensity ≥100). These selection criteria were specifically validated for treatment effects (23) and follow the guidelines of the MicroArray Quality Control (MAQC) project for the identification of reproducible gene lists (24, 25). Results were similarly obtained with GCOS/RMA-processed data using GeneSpring GX software (Agilent Technologies Inc., Santa Clara, CA, USA). Selected transcripts were analyzed after reverse transcription by quantitative real time qRT-PCR using a LightCycler 480 Instrument (Roche). Primers used for qRT-PCR of PEBP1 expression in Tg-RKIP mice did not amplify the mouse *Pebp1* (PEBP1 forward 5′-GCA GCA CCC GCT GCA TGT CAC-3′; PEBP1 reverse 5′-CTC GTC ACA CTT TAG CGG CCT G-3′). Microarray gene expression data were deposited to the NCBI GEO database (accession number GSE 120020).

## Immunoblot Detection and Immunohistology

Cardiac protein levels of RKIP (PEBP1, Pebp1), GRK2, and Pparg were determined by immunoblot analysis of hearts isolated from Tg-RKIP and Tg-GRK2K220R mice. The respective non-transgenic mice (B6 or FVB) were used as controls. Anesthetized mice (ketamine/xylazine, 100 mg/10 mg/kg) were perfused with PBS, hearts were isolated and immediately frozen in liquid nitrogen. For protein detection in immunoblot, hearts were pulverized under liquid nitrogen, and proteins were extracted with RIPA buffer supplemented with protease/phosphatase inhibitors. Pellets from AT1 receptor-expressing HEK cells with transfection of expressing plasmids encoding RKIP and GRK2 or mock-transfected cells as indicated were similarly extracted. Particulate material was removed by centrifugation followed by protein precipitation/delipidation with acetone/methanol (12:2; final concentration 83%) for 90 min at 4°C. The precipitate was collected by centrifugation and washed 3 times with 0.2 ml of ice-cold acetone. The pellet was dissolved in SDS sample buffer containing 2% SDS, 0.1 M DTT and 6 M urea by incubation for 90 min at room temperature. Samples were stored at −70°C for further use. Immunoblot detection of proteins was performed after separation of proteins by SDS-PAGE and electrophoretic protein transfer to PVDF membranes. For immunoblot detection of proteins, we used affinity-purified antibodies or $F(ab)_2$ fragments of the respective antibodies pre-absorbed on mouse/human serum proteins. Bound antibody was visualized with $F(ab)_2$ fragments of peroxidase-coupled secondary antibodies (pre-absorbed on mouse/human serum proteins) or peroxidase-coupled protein A followed by chemiluminescent western blot detection (ECL Plus or ECL Prime; Amersham). Histological analyses were performed with paraffin-embedded cardiac sections of the different transgenic mouse lines and compared to non-transgenic controls (8). Myocyte cross-sectional diameter was determined by histomorphometrical analysis of hematoxylin-eosin-stained longitudinal sections of 5 μm thickness. The mean cardiomyocyte cross-sectional diameter (CSD) in the left ventricular free wall was determined by computerized image analysis (Image J) by an observer, who was blinded to the mouse genotype. Cardiomyocyte diameter quantification used 6 different hearts/group with five separate fields of cells on each heart. A total of 100 cardiomyocytes with centered nuclei were evaluated per heart. Immunohistological detection of RKIP was performed on longitudinal cardiac sections after antigen retrieval with affinity-purified polyclonal antibodies raised against full-length, recombinant RKIP. Antibody incubation was performed for 60 min at 37°C in blocking buffer (PBS, pH 7.4, supplemented with 5% bovine serum albumin, 0.05% Tween-20), and unbound antibodies were removed by three washing steps with PBS supplemented with 0.05% Tween 20. After incubation with peroxidase-conjugated secondary antibody (dilution 1:500, Dianova, Hamburg) and removal of unbound antibodies by washing steps, bound antibody was visualized by an enzyme substrate reaction (DAB Enhanced Liquid Substrate System, Sigma). Myocardial necrosis was determined by von Kossa staining (Calcium stain kit, modified Von Kossa No. KT028, Diagnostic Biosystems Pleasanton, CA, USA). A Leica DMI6000 microscope equipped with a DFC 420 camera was used for imaging of (immuno)-histological sections.

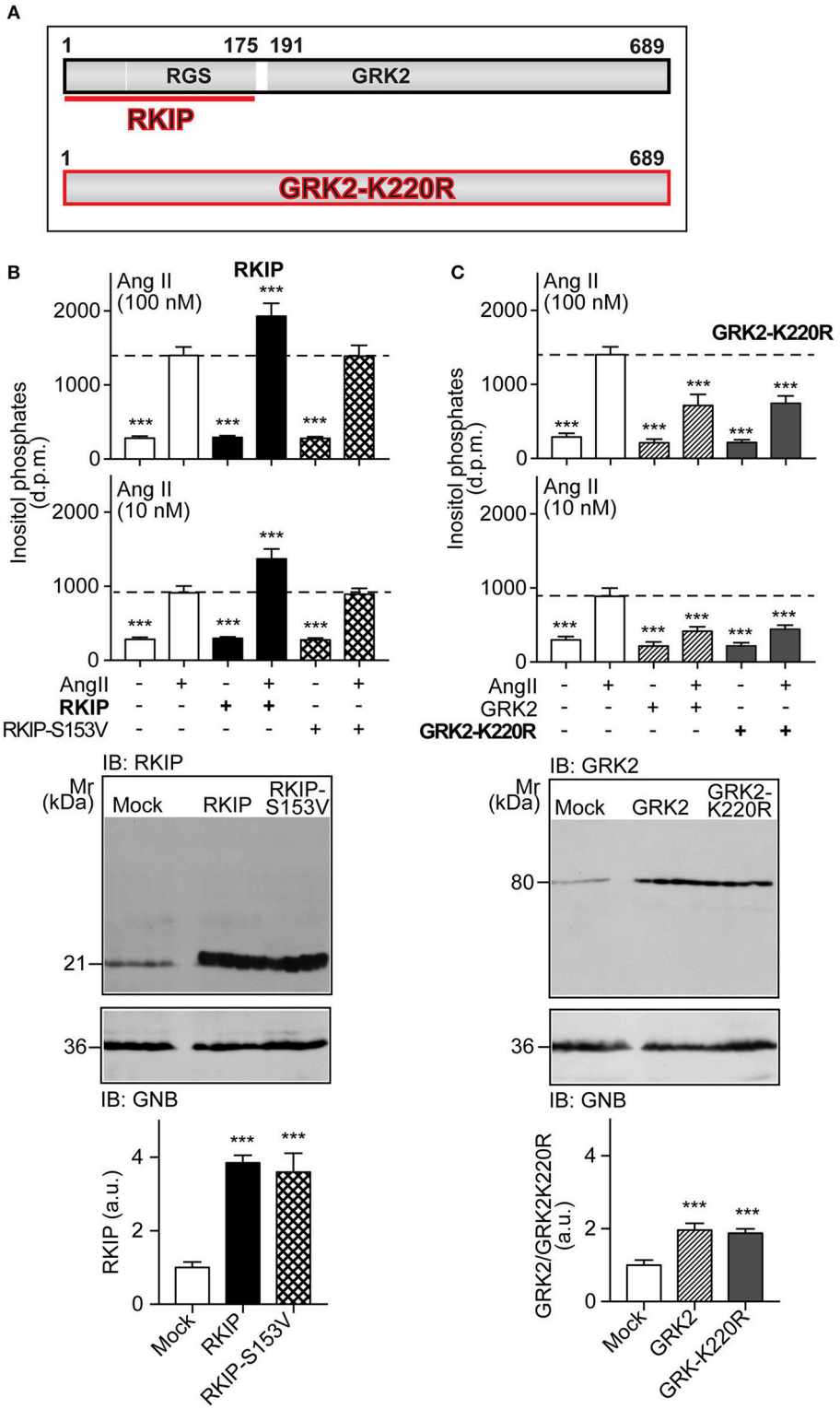


**FIGURE 1 |** RKIP promotes AT1 receptor sensitization whereas GRK2-K220R inhibits AT1-stimulated signaling. **(A)** Scheme of GRK2 and the amino-terminal RKIP interaction site (upper panel) compared to the dominant-negative GRK2-K220R mutant (lower panel). **(B,C)** Total inositol phosphate levels of AT1 receptor-expressing HEK cells stimulated without (–) or with (+) angiotensin II (100 and 10 nM) and transfected without (–) or with (+) RKIP and RKIP-S153V **(B)**, or GRK2 and GRK2-K220R **(C)** as indicated (upper panels). Data are expressed as mean ±s.d. ($n = 8$; ***$p < 0.001$ vs. column 2; Tukey's test).The lower panels show immunoblots, which detect RKIP **(B)** and GRK2 **(C)** proteins of HEK cells expressing the indicated proteins Immunoblot detection of GNB was used as a loading control (±s.d.; $n = 4$; ***$p < 0.001$ vs. mock; Tukey's test). See also **Supplementary Figures 1A,B**.

## Antibodies

The study used the following antibodies for immunoblotting and immunohistology: polyclonal anti-RKIP antibodies were raised in rabbit against full-length, recombinant RKIP (8); polyclonal anti-phospho-S153-RKIP antibodies were raised in rabbit against an epitope in RKIP encompassing phospho-serine-153 (sc-32623; Santa Cruz Biotechnoloy Inc.); polyclonal anti-GRK2 antibodies were raised in rabbit against full-length, recombinant GRK2 (ADRBK1) protein expressed in and purified from Sf9 cells (12); polyclonal anti-Gnb/GNB antibodies were raised in rabbit against purified GNB (8); polyclonal anti-Pparg antibodies were raised in rabbit against a peptide encompassing amino acids 8-106 of PPARG (Santa Cruz Biotechnology Inc.); polyclonal antibodies against phospho-Ser-273 PPARG were raised against a synthetic phosphopeptide encompassing the phosphorylation site of serine-273 of PPARG (bs-4888R; BIOSS antibodies).

## Biochemical Assays

For lipid analysis, frozen hearts were pulverized under liquid nitrogen, and cardiac lipids were extracted (26). Gas chromatography (GC) analysis of cardiac lipids was performed on a gas chromatograph (Focus, Thermo Scientific) equipped with a DB-23 column (Agilent J&W). After transesterification of cardiac lipids, fatty acid methyl esters (FAMEs) were detected by a flame ionization detector and identified by comparison with a mixture of commercial FAME reference standards (Supelco 37 component FAME mix, Sigma Aldrich). For quantitative lipid analysis, an internal standard was included. Cardiac TAG (triacylglycerol) contents were determined by a commercial kit (TR0100; Sigma). Cardiac DAG (diacylglycerol) and ceramide contents were quantified by the DAG kinase method (8, 27). The urinary albumin to creatinine ratio (ACR) was determined by commercial kits (BCG albumin assay kit MAK124; creatinine assay kit MAK080; Sigma). Number of cardiac AT1 receptor binding sites was determined with cardiac membranes by radioligand binding assay with $Sar^1$,[$^{125}$I]$Tyr^4$,$Ile^8$-angiotensin II (2200 Ci/mmol) in the presence and absence of a 1,000-fold molar excess of losartan to determine non-specific binding. HEK cells were cultured and transfected with expression plasmids encoding the AT1 receptor (*AGTR1*), GRK2 (*ADRBK1*), GRK2-K220R, RKIP (*PEBP1*), and RKIP-S153V as described (12, 28). Total cellular inositol phosphate levels were determined of HEK cells with stable AT1 receptor expression, which were transfected with the indicated expression plasmids (28). Endogenously expressed human RKIP (*PEBP1*) of AT1 receptor-expressing HEK cells, and transgenic RKIP (*PEBP1*) of Tg-RKIP mice were down-regulated by transfection/transduction of a plasmid/lentivirus with an engineered pre-miRNA, which targets *PEBP1* by RNA interference (RNAi). The following double-stranded oligonucleotides, which encode an engineered pre-miRNA targeting human RKIP (*PEBP1*) by RNAi were inserted into pcDNA6.2-GW/miR for down-regulation of endogenous RKIP in HEK cells and pLenti6/V5-Dest Gateway (Invitrogen) for generation of a lentiviral expression plasmid to down-regulate human RKIP in Tg-RKIP mice: miPEBP1-top 5′-TGC TGT GTA GAG CTT CCC TGA ATC AAG TTT TGG CCA CTG ACT GAC TTG ATT CAG AAG CTC TAC A−3′; and miPEBP1-bottom 5′-CCT GTG TAG AGC TTC TGA ATC AAG TCA GTC AGT GGC CAA AAC TTG ATT CAG GGA AGC TCT ACA C−3′. For lentiviral transduction of mice, a pseudotyped lentivirus was generated as described (8). By a similar RNAi-mediated approach, endogenously expressed GRK2 of HEK cells was down-regulated (12). Neonatal mouse cardiomyocytes were isolated from Tg-RKIP mice, Tg-GRK2-K220R mice, and non-transgenic B6 controls as described (8). Cellular cAMP levels of isolated neonatal cardiomyocytes (without and with stimulation by 100 nM isoproterenol) were determined using a commercial kit (CA200, Sigma Aldrich).

## Statistical Analysis

Data are presented as mean ± s.d. Statistical significance between two groups was calculated by the unpaired, two-tailed Student's *t*-test. For comparisons between more than two groups, analysis of variance followed by a Post-test as indicated was performed. Statistical significance was set at a $p$-value of $<0.05$ unless otherwise stated. Statistical evaluation was performed with GraphPad PRISM 7.0a. Whole genome gene expression data were analyzed by TIGR Multi Experiment Viewer MeV.

# RESULTS

## RKIP Promotes AT1 Receptor Sensitization Whereas GRK2-K220R Inhibits AT1-Stimulated Signaling

RKIP interacts with the amino-terminal domain of GRK2 and thereby blunts the interaction of GRK2 with receptor substrates, with subsequent inhibition of receptor phosphorylation and desensitization [**Figure 1A** and (19)]. The amino-terminal domain of GRK2 also contains an intact RGS domain (**Figure 1A**), which blunts Gq/11-dependent signal transduction (13, 14). We investigated the impact of RKIP on signaling mediated by the heart failure-promoting, Gq/11-coupled angiotensin II AT1 receptor. For *in vitro* experiments, we used HEK cells as a model system because these cells are widely used to study mechanisms of signal transduction, notably signaling events triggered by the AT1 receptor, GRK2 and RKIP (12, 19, 28). We found that RKIP led to a significantly enhanced angiotensin II AT1-stimulated inositol phosphate generation in HEK cells (**Figure 1B**). This signal sensitization of the AT1 receptor was mediated by the RKIP-GRK2 interaction because the RKIP-S153V mutant, which cannot switch from Raf1 to GRK2 (19), had no effect on the AT1 signal (**Figure 1B**). As a control, RKIP and RKIP-S153V protein levels were comparable (**Figure 1B**).

In contrast to RKIP, the GRK2-K220R mutant, which acts as a dominant-negative mutant of GRK2 (**Figure 1A**), led to a strong inhibition of the AT1-stimulated signal (**Figure 1C**). Inhibition of the Gq/11-coupled AT1 receptor was similarly observed with wild-type GRK2 (**Figure 1C**). This experiment confirms that the Gq/11-inhibitory activity of GRK2 is a kinase-independent effect.

## Physiological RKIP Levels Are Sufficient to Sensitize AT1-Stimulated Signaling in Cells

We asked whether physiological protein levels of RKIP are sufficient to sensitize the AT1-stimulated response, and down-regulated endogenously expressed RKIP by transfection of a pre-miRNA targeting RKIP (*PEBP1*) by RNAi. Upon RNAi-mediated down-regulation of endogenously expressed RKIP, the AT1-stimulated inositol phosphate generation was significantly decreased (**Figure 2A**). As a control, down-regulation of endogenously expressed RKIP was confirmed by immunoblot analysis (**Figure 2A**). This experiment shows that physiological RKIP levels are sufficient to sensitize the angiotensin II AT1 receptor because down-regulation of RKIP led to a decreased AT1 response.

We also down-regulated the endogenously expressed GRK2 by RNAi (**Figure 2B**). In contrast to RKIP, down-regulation of GRK2 led to a significantly increased AT1-stimulated signal (**Figure 2B**). This finding shows that endogenously expressed GRK2 inhibits the Gq/11-coupled AT1 receptor. Together these experiments reveal that RKIP sensitizes the heart failure-promoting AT1 receptor whereas endogenously expressed GRK2 and the kinase-deficient GRK2-K220R mutant inhibit AT1-stimulated signaling.

## Myocardium-Specific Expression of RKIP Induces Cardiac Dysfunction Whereas GRK2-K220R Improves Cardiac Function

In view of these major differences between two different approaches of GRK2 inhibition with RKIP and GRK2-K220R, we compared the two inhibitors *in vivo*. We generated transgenic mice with myocardium-specific expression of RKIP and GRK2-K220R under control of the alpha-MHC promoter in B6 background (**Figures 3A–C**). The cardiac RKIP protein levels of two different RKIP-transgenic mouse lines, Tg-RKIP2 and Tg-RKIP3, were increased 3.5-fold and 2.9-fold, respectively, over non-transgenic B6 controls (**Figure 3A**). RKIP-transgenic mice were compared with Tg-GRK2K220R mice, which showed a 3.4 ± 0.46-fold increased cardiac GRK2-K220R protein level compared to non-transgenic B6 controls (**Figure 3B**).

We analyzed the cardiac phenotype of 8-months-old transgenic mice with myocardium-specific expression of RKIP and GRK2-K220R, respectively (**Figure 3C**). Eight-months-old Tg-RKIP mice developed signs of heart failure in the absence of additional stressors (**Figures 3D–I**). Heart failure in Tg-RKIP mice was documented by a significantly decreased left ventricular ejection fraction of 28.2 ± 4.2% and 37.0 ± 3.6% in Tg-RKIP2 and Tg-RKIP3 mouse lines, respectively (**Figure 3D**). In addition to cardiac dysfunction, echocardiographic data showed that Tg-RKIP hearts were enlarged with significantly increased left ventricular end-diastolic (LVEDD) and end-systolic diameters (LVESD) compared to non-transgenic B6 mice while interventricular septal thickness at diastole (IVSD) was not significantly different (**Figure 3E**). The phenotype of cardiac hypertrophy in Tg-RKIP mice compared to non-transgenic B6 controls was further documented by an increased heart-to-body weight ratio (**Figure 3F**). Histological analysis of

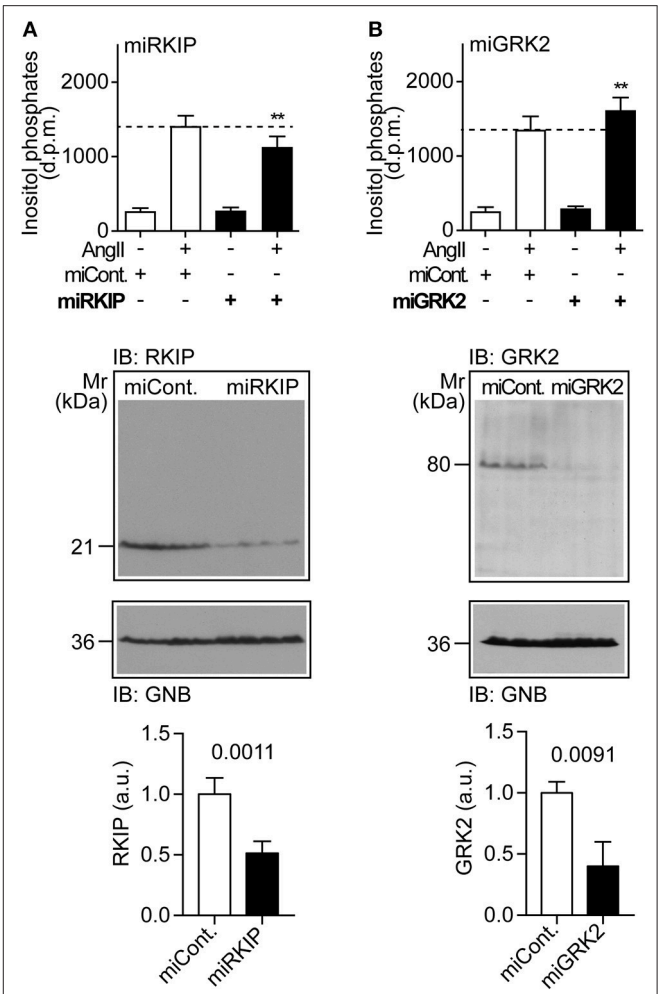


**FIGURE 2 |** Physiological RKIP levels are sufficient to sensitize AT1-stimulated signaling in cells. **(A,B)** Total inositol phosphate levels of AT1 receptor-expressing HEK cells stimulated without (–) or with (+) angiotensin II (100 nM) and transfected without (–) or with (+) a control pre-miRNA (miCont) or a pre-miRNA targeting endogenously expressed RKIP (miRKIP) or GRK2 (miGRK2) by RNAi as indicated. Data represent mean ± s.d. ($n = 8$; $^{**}p < 0.01$ vs. column 2; Dunnett's test). The lower panels confirm RNAi-mediated down-regulation of endogenously expressed proteins by immunoblot detection of RKIP **(A)** and GRK2 **(B)**. Data represent mean ± s.d. ($n = 4$). See also **Supplementary Figures 2A,B**.

Tg-RKIP2 hearts confirmed these data and showed a phenotype of cardiac hypertrophy with dilatation (**Figure 3G**). Histological analysis further documented significant cardiac fibrosis and cardiomyocyte necrosis in Tg-RKIP2 hearts (**Figures 3H,I**). This histological characterization of Tg-RKIP mice complements functional data and shows that cardiac dysfunction in Tg-RKIP mice was accompanied by severe myocardial damage, which develops during the pathogenesis of heart failure as a consequence of maladaptive cardiac remodeling.

In contrast to Tg-RKIP mice, transgenic mice with myocardium-specific expression of the kinase-inactive GRK2-K220R mutant had a slightly improved cardiac function and showed no signs of cardiac hypertrophy (**Figures 3D–F**).

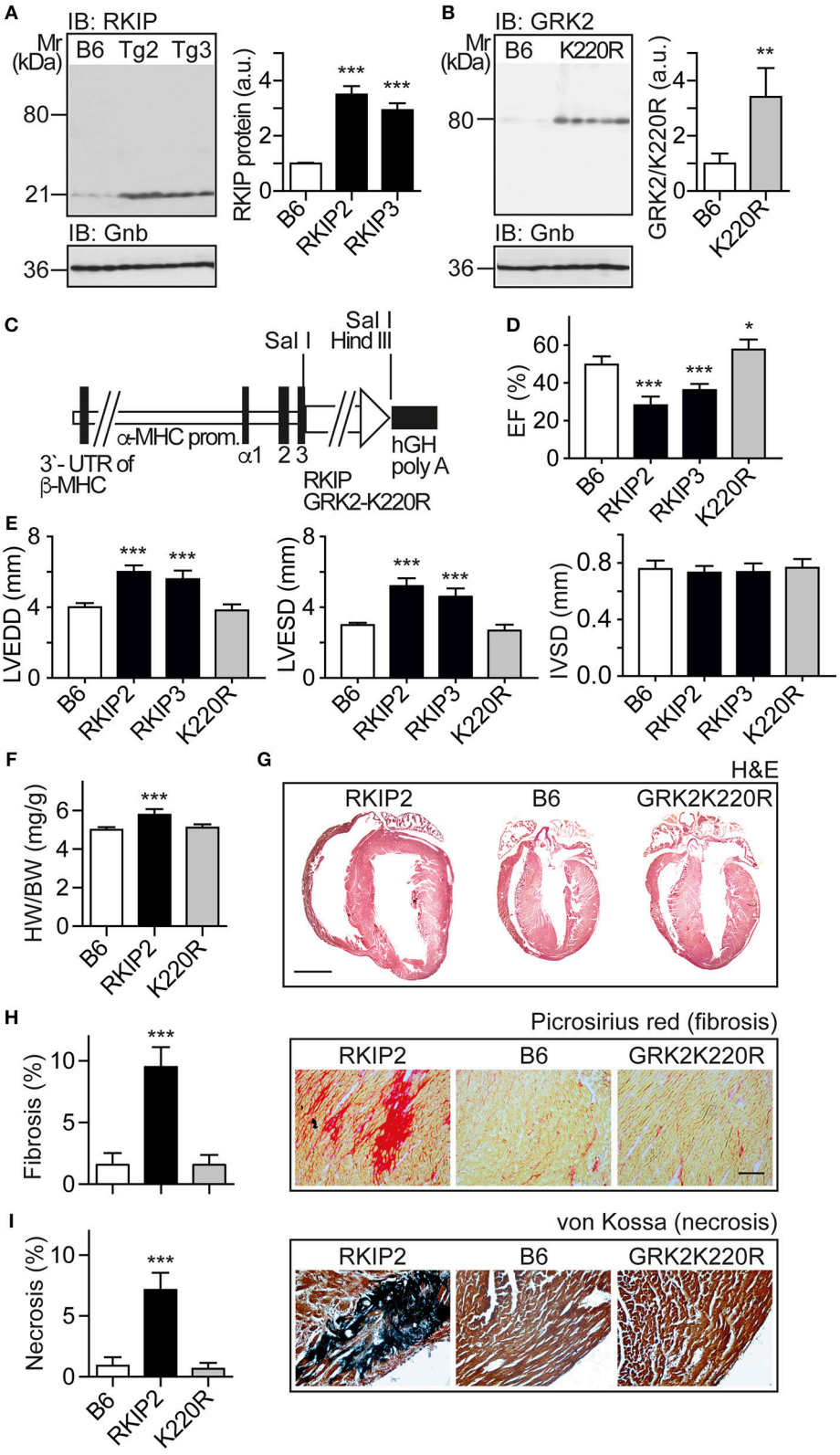


**FIGURE 3 |** Myocardium-specific expression of RKIP induces cardiac dysfunction whereas GRK2-K220R improves cardiac function. **(A)** Immunoblot detection of RKIP in non-transgenic B6 and two different Tg-RKIP mouse lines, Tg-RKIP2 and Tg-RKIP3 (±s.d.; $n = 3$; $^{***}p < 0.001$ vs. B6; Dunnett's test). **(B)** Immunoblot

*(Continued)*

**FIGURE 3 |** detection of GRK2/GRK2-K220R in non-transgenic B6 and Tg-GRK2K220R mice [±s.d., $n = 3$ (B6) and $n = 5$ (K220R); $^{**}p = 0.0097$]. **(C)** Scheme of expression plasmid used for generation of Tg-RKIP and Tg-GRK2K220R mice with myocardium-specific expression of RKIP and GRK2-K220R under control of the alpha-MHC promoter. **(D)** Left ventricular ejection fraction (EF) of different eight-months-old transgenic mouse lines compared to age-matched, non-transgenic B6 controls (±s.d., $n = 5$; $^{***}p < 0.001$, and $^{*}p < 0.05$ vs. B6; Dunnett's test). **(E)** Left ventricular end-diastolic diameter (left), left ventricular end-systolic diameter (middle), and interventricular septal thickness (right) of B6, Tg-RKIP2, Tg-RKIP3, and Tg-GRK2K220R mice (±s.d., $n = 5$; $^{***}p < 0.001$ vs. B6; Dunnett's test). **(F)** Heart-to-body weight ratio of different transgenic lines compared to non-transgenic B6 controls (±s.d.; $n = 6$; $^{***}p < 0.001$ vs. B6; Dunnett's test). **(G)** Histological assessment of cardiac sections from a Tg-RKIP2 mouse, a non-transgenic B6 mouse and a Tg-GRK2K220R mouse. Sections were stained with hematoxylin-eosin (H&E) and are representative of six mice/group (bar: 2 mm). (H,I) Cardiac fibrosis was determined by picrosirius red staining **(H)**, and myocardial necrosis was assessed by von Kossa stain **(I)** of Tg-RKIP2 and Tg-GRK2K220R mice compared to non-transgenic B6 mice. Left panels show quantitative data evaluation (±s.d.; $n = 6$ mice/group; $^{***}p < 0.001$ vs. B6; Dunnett's test), and right panels show representative histological sections (bar: 40 μm). See also **Supplementary Figures 3A,B**.

The histological analysis confirmed the normal phenotype of Tg-GRK2K220R hearts, which was not different from non-transgenic B6 controls (**Figures 3G–I**). Notably, there was no evidence of cardiac hypertrophy and cardiac dilatation in Tg-GRK2K220R mice (**Figures 3E–G**).

## Major Symptoms of Heart Failure With Pulmonary Congestion and Renal Dysfunction in Tg-RKIP Mice

Cardiac dysfunction of Tg-RKIP mice was accompanied by major symptoms of heart failure. RKIP-mediated sensitization of pro-hypertrophic AT1-stimulated signaling in Tg-RKIP2 hearts could contribute to the significantly enlarged cardiomyocyte cross-sectional diameter (**Figures 4A,B**), which is a major factor accounting for maladaptive remodeling in heart failure. In agreement with excessive AT1-stimulated signaling and complementary to human heart biopsy specimens from heart failure patients (29, 30), the AT1 receptor was down-regulated in Tg-RKIP2 hearts (**Figure 4C**). This decrease in cardiac AT1 receptor content could be a direct consequence of RKIP-induced sensitization of AT1-stimulated signaling (cf. **Figure 1**), because AT1-stimulated signaling is a causative factor of AT1 down-regulation (31, 32). In contrast, Tg-GRK2K220R mice did not show cardiomyocyte hypertrophy (**Figures 4A,B**). Moreover, transgenic GRK2-K220R expression did not lead to decreased cardiac AT1 receptor levels (**Figure 4C**).

Symptoms of heart failure with severe cardiac dysfunction in Tg-RKIP2 mice were accompanied by an increased systolic blood pressure (**Figure 4D**). Concomitantly, histological lung sections of Tg-RKIP2 mice showed signs of pulmonary congestion with alveolar septal thickening, which was absent in healthy, non-transgenic B6 controls, and Tg-GRK2K220R mice (**Figure 4E**). Pulmonary congestion was confirmed by an increased lung weight and lung-to-body weight ratio of Tg-RKIP2 mice compared to B6 and Tg-GRK2-K220R mice (**Figure 4F**, **Supplementary Figure 4**). Congestive heart failure was further documented by a significantly increased body weight of Tg-RKIP2 mice (**Figure 4G**). In addition, heart failure in Tg-RKIP2 mice led to symptoms of renal insufficiency with significant proteinuria (**Figure 4H**). Taken together, a moderately increased cardiac RKIP level is a sufficient cause for the development of major systemic symptoms of chronic congestive heart failure in aged Tg-RKIP mice.

## Up-Regulation of Heart Failure-Related *Pparg* Target Genes in Tg-RKIP Hearts

We performed cardiac whole genome microarray gene expression profiling to analyze mechanisms underlying the different phenotype caused by transgenic expression of two GRK2 inhibitors, RKIP and GRK2-K220R (**Figure 5A**). Microarray gene expression analysis identified highly up-regulated genes (≥6-fold) in Tg-RKIP hearts compared to non-transgenic B6 controls (**Figure 5B**). Most of these highly up-regulated genes are targets of the adipogenic and heart failure-promoting transcription factor, peroxisome proliferator-activated receptor-gamma, *Pparg*, and have documented relationship to heart failure (**Figure 5B**). In contrast to Tg-RKIP hearts, transgenic expression of GRK2-K220R did not up-regulate any of these heart failure-promoting genes (**Figure 5B**).

## Activation of *Pparg*, and Cardiotoxic Lipid Load in Tg-RKIP but Not in Tg-GRK2K220R Hearts

The up-regulation of *Pparg* targets was accompanied by a significantly decreased phosphorylation of Pparg on serine-273 in Tg-RKIP hearts (**Figure 6A**). This finding is relevant because dephosphorylation of Pparg on serine-273 leads to an enhanced *Pparg* transcriptional activity (8, 33). In contrast, *Pparg* was not activated in Tg-GRK2K220R hearts, and the content of serine-273-phosphorylated Pparg in Tg-GRK2K220R hearts was comparable to B6 control level (**Figure 6A**).

Together with an enhanced activation of the adipogenic *Pparg*, Tg-RKIP hearts had an increased cardiac protein content of the major palmitate-synthesizing enzyme and *Pparg* target, fatty acid synthase, Fasn (**Figures 6B,C**).

Lipid analysis showed that cardiac lipid contents of palmitate, TAG (triacylglycerol), DAG (diacylglycerol), and ceramide were also significantly higher in Tg-RKIP hearts compared to non-transgenic B6 control hearts and Tg-GRK2K220R hearts (**Figures 6D–H**). In addition to palmitate, DAG and ceramide are also cardiotoxic lipid species, which could contribute to cardiac degeneration and cardiomyocyte loss in Tg-RKIP hearts (8, 27, 34).

## RKIP Induces Symptoms of Heart Failure in FVB Background

Because cardiac effects induced by RKIP in mice could depend on the genetic background (35), we generated transgenic

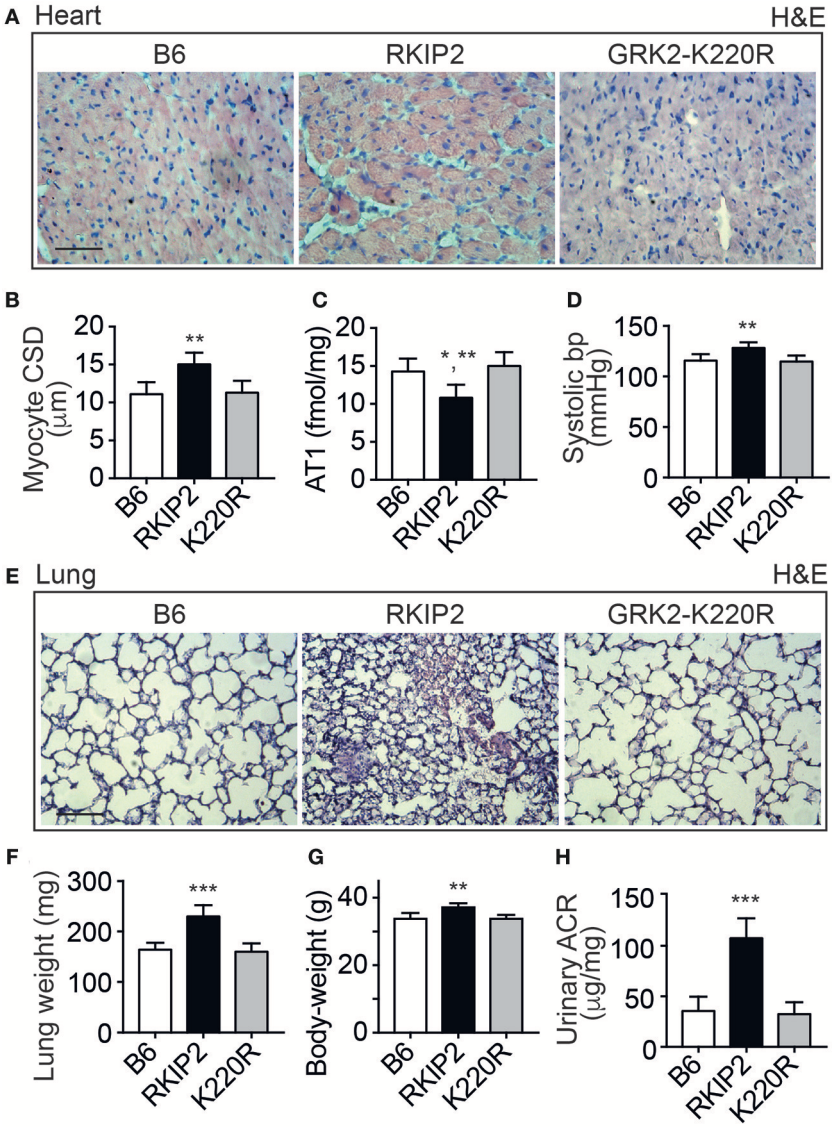


**FIGURE 4 |** Major symptoms of heart failure with pulmonary congestion and renal dysfunction in Tg-RKIP mice. **(A,B)** Cardiomyocyte cross-sectional diameter was determined with histological cardiac sections of B6, Tg-RKIP2, and Tg-GRK2K220R mice **(A)**. **(B)** Shows quantitative data evaluation. Sections were stained with hematoxylin and eosin (H&E) and are representative of six mice each (bar: 20 μm). **(C,D)** Cardiac AT1 receptor content **(C)**, and systolic blood pressure **(D)** in B6, Tg-RKIP2 and Tg-GRK2K220R mice. **(E)** Histological lung sections show heart failure-related lung remodeling with chronic pulmonary congestion and thickening of alveolar septa in Tg-RKIP2 mice compared to healthy non-transgenic B6 and Tg-GRK2K220R mice. Sections were stained with hematoxylin and eosin (H&E) and are representative of six mice each (bar: 40 μm). **(F)** Increased lung weight of Tg-RKIP2 compared to B6 and Tg-GRK2K220R mice. **(G,H)** Increased body weight **(G)**, and renal insufficiency with proteinuria **(H)** of Tg-RKIP2 mice compared to non-transgenic B6 and Tg-GRK2K220R mice. Data represent the mean ±s.d. [$n = 6$; $^{*}p < 0.05$ vs. B6; $^{**}p < 0.01$ vs. B6 **(B,D,G)** and K220R **(B,C,D,G)**; $^{***}p < 0.001$ vs. B6 and K220R; Tukey's test]. See also **Supplementary Figure 4**.

mice with a moderately increased cardiac RKIP protein level in the FVB background (**Figure 7A**). Two different transgenic lines were generated, which showed 2.7 ± 0.3-fold and 3.3 ± 0.4-fold increased cardiac RKIP protein levels over the non-transgenic FVB control (**Figure 7A**). Immunohistological analysis confirmed the increased cardiac RKIP protein content in an 8-months-old Tg-RKIP heart compared to the non-transgenic FVB control (**Figure 7B**).

Tg-RKIP mice with FVB background developed cardiac hypertrophy, which was detected by an increased heart-to-body weight ratio of 8-months-old mice (**Figure 7C**). Concomitantly, Tg-RKIP mice with FVB background showed symptoms of heart failure, which was documented by a severely decreased left ventricular ejection fraction of 30.4 ± 3.5% and 25.1 ± 4.7% in Tg-RKIP1 and Tg-RKIP2 mice (**Figure 7D**). Echocardiographic data confirmed that Tg-RKIP mice with FVB background had significant cardiac hypertrophy at an age of

**A**

| | Number present (%) | 3`/5`ratio *Gapdh* | Average signal | Average signal (scaled) |
|---|---|---|---|---|
| Tg-RKIP1 | 47.7 | 0.83 | 301 | 516 |
| Tg-RKIP2 | 47.4 | 0.82 | 292 | 516 |
| Tg-GRK2K220R-1 | 46.9 | 0.83 | 313 | 516 |
| Tg-GRK2K220R-2 | 46.0 | 0.86 | 289 | 525 |
| B6-1 | 44.6 | 0.94 | 258 | 533 |
| B6-2 | 46.3 | 0.87 | 272 | 530 |

**B**

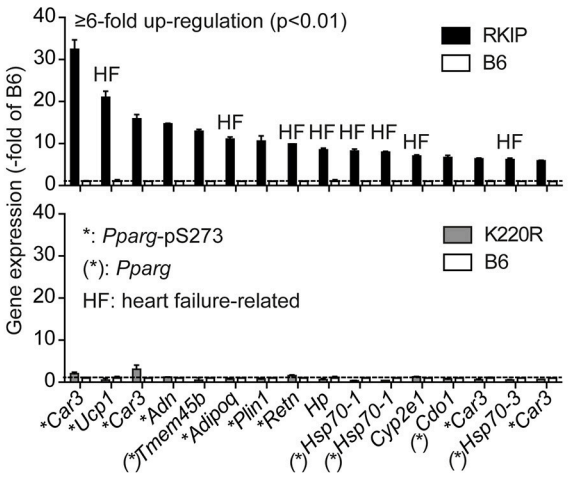


**FIGURE 5 |** Up-regulation of heart failure-related *Pparg* target genes in Tg-RKIP hearts. **(A,B)** Cardiac whole genome microarray gene expression profiling **(A)** identified highly up-regulated probe sets (≥6-fold up-regulation compared to B6 with $p < 0.01$) in Tg-RKIP hearts (**B**, upper panel). None of these probe sets was significantly regulated in Tg-GRK2K220R hearts (**B**, lower panel). Gene expression data are expressed as -fold of B6 (±s.d., $n = 2$ gene chips/group with total RNA isolated from $n = 3$ hearts/gene chip). Highly up-regulated genes (≥6-fold up-regulation in Tg-RKIP hearts compared to B6 hearts; $p < 0.01$; with call present and/or signal intensity ≥100) in Tg-RKIP hearts have documented relationship to heart failure (HF), are *Pparg* targets [(*): *Pparg*)], and/or are induced by *Pparg* serine-273 phosphorylation (*:*Pparg*-pS273).

8 months (**Figure 7E**). Cardiac enlargement was documented by significantly increased left ventricular end-diastolic and end-systolic diameters of Tg-RKIP compared to non-transgenic FVB mice (**Figure 7E**). As a control, interventricular septal thickness was not significantly different between study groups (**Figure 7E**).

Histological assessment of hearts from two different RKIP-transgenic mouse lines in FVB background complemented this phenotype and showed massive cardiac hypertrophy with dilatation (**Figure 7F**). Histomorphological analysis confirmed the phenotype of cardiomyocyte hypertrophy by a significantly increased cardiomyocyte cross-sectional diameter in Tg-RKIP mice compared to non-transgenic FVB mice (**Figure 7G**). Similarly to Tg-RKIP mice with B6 background (cf. **Figure 4**), aged Tg-RKIP mice with FVB background progressed to chronic congestive heart failure with a significantly elevated systolic blood pressure, proteinuria and an increased body weight with pulmonary congestion, which was documented by an increased lung weight and lung-to-body weight ratio (**Figures 7H–J**).

The RKIP-induced cardiac hypertrophy and cardiac dysfunction were (partially) prevented by down-regulation of RKIP with lentiviral transduction of an miRNA targeting the RKIP (*PEBP1*) by RNA interference (**Figures 7K,L**). These experiments confirm that the heart failure phenotype in Tg-RKIP mice was caused by transgenic expression of RKIP. The data also indicate that heart failure symptoms induced by an increased cardiac RKIP level can be counteracted by RKIP down-regulation. Because RKIP levels are increased in failing human hearts (21), this finding could be relevant to the human disease.

## RKIP Promotes Cardiac Lipid Overload in FVB Background

In addition to symptoms of chronic congestive heart failure, Tg-RKIP mice with FVB background also developed cardiac lipid overload (**Figures 8A–E**). Analysis of cardiac fatty acid composition by gas chromatography (GC) indicated that palmitate was the major lipid in Tg-RKIP hearts (**Figures 8A,B**). Other cardiotoxic lipid species such as ceramide and DAG were also significantly increased in Tg-RKIP hearts with FVB background (**Figures 8D,E**). Together these findings show that a moderately increased cardiac RKIP protein level is sufficient to promote symptoms of congestive heart failure, cardiac hypertrophy with dilatation and cardiotoxic lipid load in two different genetic backgrounds, i.e., B6 and FVB.

## RKIP and GRK2-K220R Act as GRK2 Inhibitors *in vivo*

RKIP is a dual-specific inhibitor of GRK2 and the Raf-Erk1/2 axis (19). By PKC-mediated phosphorylation on serine-153, RKIP switches from Raf1 to GRK2 (19). Because the PKC-activating DAG was strongly increased in Tg-RKIP hearts, we investigated whether RKIP acted as GRK2 inhibitor *in vivo*, in Tg-RKIP mice with signs of heart failure. In agreement with an increased DAG load, immunoblot detection revealed a strongly increased content of serine-153-phosporylated RKIP in Tg-RKIP hearts compared to non-transgenic B6 controls (**Figure 9A**).

The serine-153 phosphorylation-dependent transformation of RKIP into a GRK2 inhibitor was accompanied by sensitization of cAMP signaling in isolated cardiomyocytes upon stimulation by the beta-adrenoceptor agonist, isoproterenol, (**Figure 9B**). In addition, sensitized cAMP signaling was also detected *in vivo* by a significant up-regulation of the cAMP-inducible gene, *Ttc14* [tetratricopeptide repeat protein 14; (36)], (**Figure 9C**). RKIP-mediated sensitization of cAMP signaling in Tg-RKIP cardiomyocytes and hearts was comparable to effects seen with the dominant-negative GRK2-K220R mutant in Tg-GRK2K220R cardiomyocytes and hearts (**Figures 9B,C**). Taken together, RKIP acts as GRK2 inhibitor *in vivo* and mediates sensitization of cAMP signaling similarly as the dominant-negative GRK2-K220R mutant.

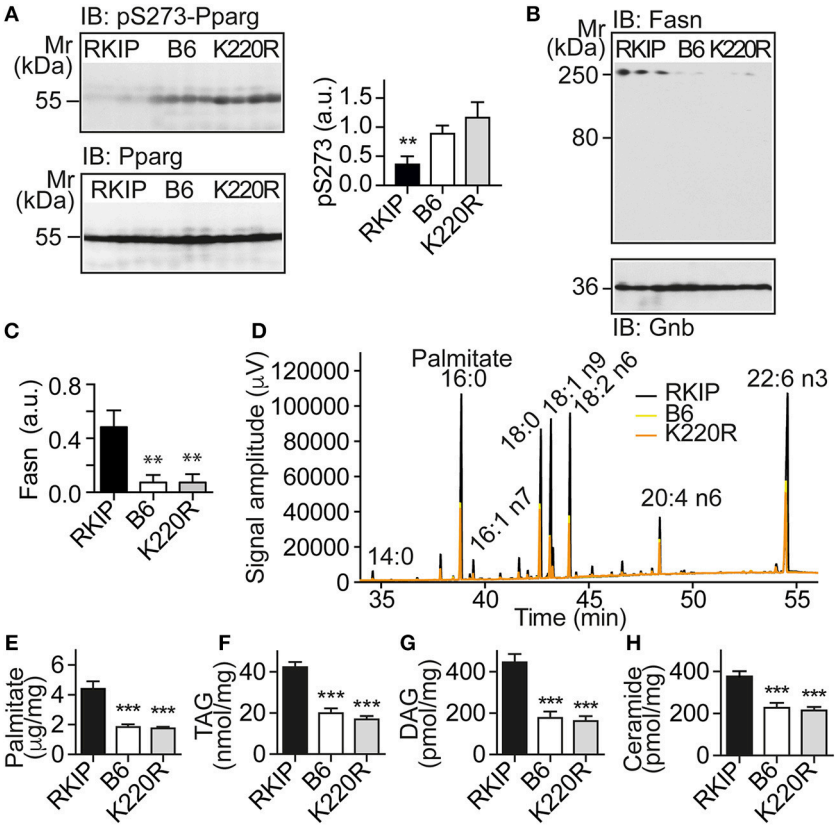


**FIGURE 6 |** Activation of *Pparg*, and cardiotoxic lipid load in Tg-RKIP but not in Tg-GRK2K220R hearts. **(A)** Immunoblot detection of Pparg phosphorylated on serine-273 (pS273-Pparg) in Tg-RKIP, non-transgenic B6 and Tg-GRK2K220R hearts (±s.d.; $n = 4$ hearts/group; $^{**}p < 0.01$ vs. B6 and K220R; Tukey's test). The lower panel shows the total cardiac Pparg protein content. **(B,C)** Immunoblot detection of Fasn in Tg-RKIP, non-transgenic B6 and Tg-GRK2K220R hearts (±s.d.; $n = 3$ hearts/group; $^{**}p < 0.01$ vs. RKIP; Tukey's test). **(D)** GC analysis of cardiac lipids of Tg-RKIP, Tg-GRK2K220R, and non-transgenic B6 hearts. **(E-H)** Cardiac lipid analysis detects increased cardiac contents of palmitate **(E)**, TAG **(F)**, DAG **(G)**, and ceramide **(H)** in Tg-RKIP hearts (±s.d.; $n = 7$; $^{***}p < 0.001$ vs. RKIP; Dunnett's test). See also **Supplementary Figures 5A–C**.

## Concordant Gene Regulation in Tg-RKIP and Tg-GRK2K220R Hearts

We analyzed the impact of GRK2 inhibition by RKIP and GRK2-K220R on cardiac gene expression. Whole genome microarray gene expression profiling data showed concordant gene regulation in Tg-RKIP and Tg-GRK2K220R hearts (**Figures 10A,B**). Notably, 46% of all genes regulated by the dominant-negative GRK2-K220R mutant in Tg-GRK2K220R hearts were also concordantly regulated by RKIP in Tg-RKIP hearts (**Figure 10A**). These data further support the notion that moderate transgenic expression levels of RKIP and GRK2-K220R induce comparable GRK2 inhibition in transgenic mice.

## Dominant-Negative GRK2-K220R Retards Chronic Pressure Overload-Induced Cardiac Dysfunction

We asked whether GRK2-K220R was cardio-protective in a model of heart failure induced by chronic pressure overload imposed by abdominal aortic constriction, AAC. As a control for cardioprotective GRK2 inhibition, we used Tg-GRKInh mice with myocardium-specific expression of a cardioprotective GRK2-inhibitory peptide derived from the first intracellular loop of the beta2-adrenoceptor, which is known to retard AAC-induced cardiac dysfunction (8, 12). Systolic aortic pressure of B6, Tg-GRK2K220R and Tg-GRKInh mice was comparable under basal conditions (**Figure 11A**). At the end of the observation period, AAC-induced chronic pressure overload was controlled and confirmed by a significantly increased systolic aortic pressure (>150 mmHg), which was not different between study groups (**Figure 11A**). The transgenic expression of dominant-negative GRK2-K220R in Tg-GRK2K220R mice significantly retarded the development of cardiac dysfunction induced by 8 weeks of AAC compared to non-transgenic B6 controls (**Figures 11B,C**). The cardioprotective effect of transgenic GRK2-K220R expression was comparable to GRK2 inhibition by transgenic expression of GRKInh (**Figures 11B,C**). Echocardiographic data further documented that inhibition of GRK2 by GRK2-K220R and GRKInh significantly inhibited the development of AAC-induced dilative cardiac hypertrophy, i.e., the left ventricular end-diastolic and end-systolic dimensions were significantly

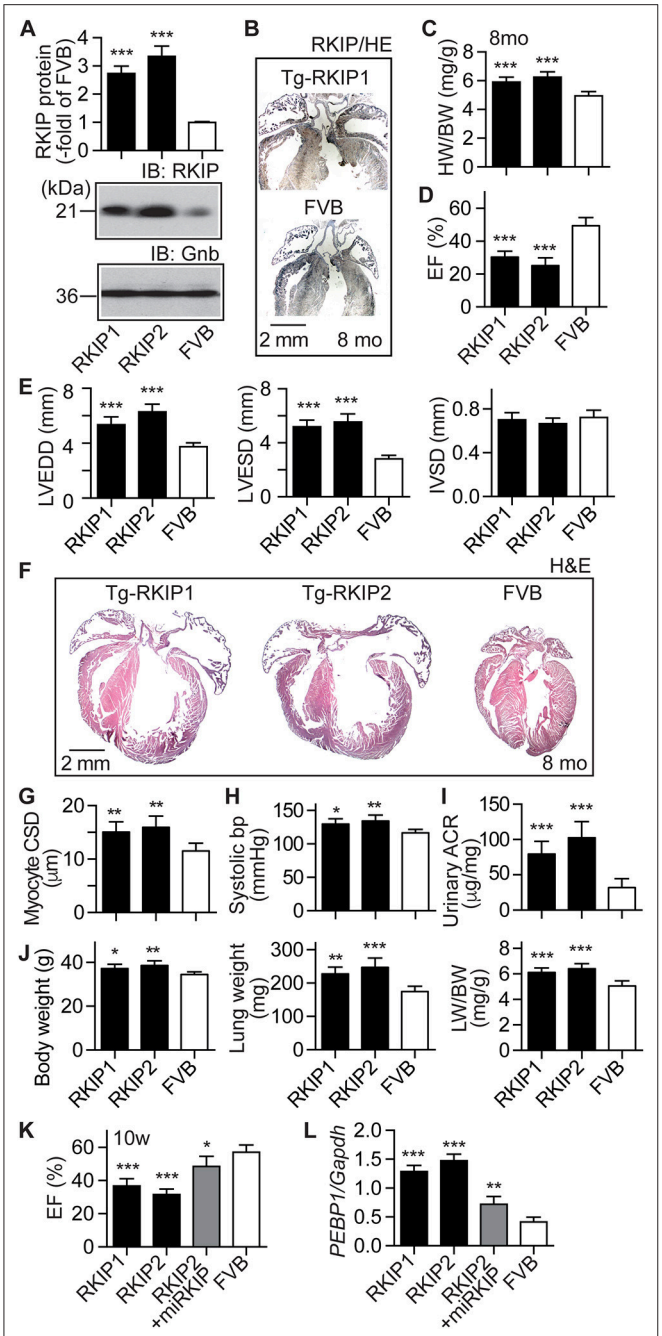


**FIGURE 7 |** RKIP induces symptoms of heart failure in FVB background. **(A)** Cardiac RKIP protein levels were determined in Tg-RKIP1, Tg-RKIP2 and non-transgenic FVB mice by immunoblot detection (±s.d.; $n = 8$; $^{***}p < 0.001$ vs. FVB; Dunnett's test). **(B)** Immunohistological localization of RKIP on cardiac sections from an 8-months-old Tg-RKIP1 mouse and an age-matched non-transgenic FVB mouse (bar: 2 mm; nuclei were stained with hematoxylin, HE). Immunohistology is representative of four hearts/group. **(C,D)** Heart-to-body weight ratio **(C)**, and cardiac left ventricular ejection fraction **(D)** of 8-months-old Tg-RKIP1, Tg-RKIP2 and non-transgenic FVB mice [±s.d.; $n = 6$ **(C)**, and $n = 5$ **(D)**; $^{***}p < 0.001$ vs. FVB; Dunnett's test]. **(E)** Echocardiographic determination of left ventricular end-diastolic diameter (left), left ventricular end-systolic diameter (middle), and interventricular septal thickness (right) in 8-months-old Tg-RKIP1, Tg-RKIP2 and non-transgenic

*(Continued)*

**FIGURE 7 |** FVB control mice (±s.d.; $n = 5$; $^{***}p < 0.001$ vs. FVB; Dunnett's test). **(F)** Histological analysis of cardiac sections shows cardiac hypertrophy with dilatation in Tg-RKIP1 and Tg-RKIP2 hearts. **(G)**. Cardiomyocyte cross-sectional diameter **(G)**, systolic blood pressure **(H)**, and urinary albumin to creatinine ratio, ACR **(I)** in Tg-RKIP1, Tg-RKIP2, and non-transgenic FVB mice. **(J)** Increased body weight (left), lung weight (middle), and lung-to-body weight ratio (right) in Tg-RKIP1 and Tg-RKIP2 mice compared to non-transgenic FVB mice (±s.d.; $n = 6$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. FVB; Dunnett's test). **(K,L)** 4 weeks of RKIP down-regulation by lentiviral transduction of an miRNA targeting RKIP by RNAi retards the development of cardiac dysfunction in 10-week-old (10 w) Tg-RKIP2 mice **(K)**. Panel **(L)** documents the down-regulation of cardiac RKIP (*PEBP1*) expression in miRKIP-transduced Tg-RKIP2 mice by qRT-PCR (±s.d.; $n = 5$; $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ vs. FVB; Dunnett's test). See also **Supplementary Figure 6**.

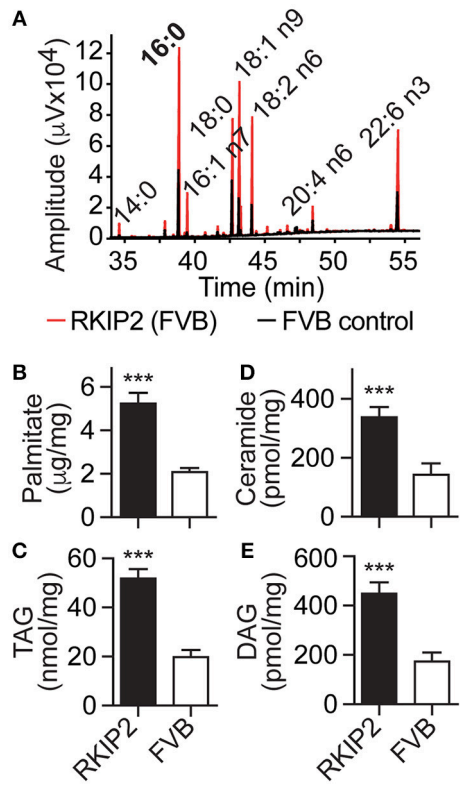


**FIGURE 8 |** RKIP promotes cardiac lipid overload in FVB background. **(A–E)** GC analysis of cardiac lipids **(A)**, cardiac palmitate **(B)**, TAG **(C)**, ceramide **(D)**, and DAG **(E)** contents in 8-months-old Tg-RKIP2 mice with FVB background compared to age-matched non-transgenic FVB control mice (±s.d.; $n = 6$; $^{***}p < 0.001$). See also **Supplementary Figure 7**.

smaller in Tg-GRK2K220R and Tg-GRKInh mice compared to non-transgenic B6 mice whereas the interventricular septal thickness was not significantly different between study groups (**Figures 11D–F**). Together these findings present strong evidence that inhibition of GRK2 by dominant-negative GRK2-K220R exerts protection against cardiac dysfunction induced by chronic pressure overload.

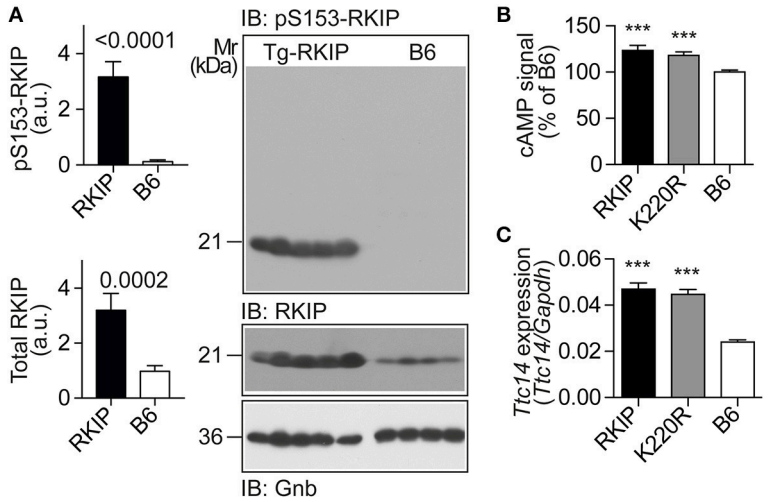


**FIGURE 9 |** RKIP and GRK2-K220R act as GRK2 inhibitors *in vivo*. **(A)** Immunoblot detection of RKIP phosphorylation on serine-153 and total cardiac RKIP content in 8-months-old Tg-RKIP compared to non-transgenic B6 hearts (±s.d.; $n = 5$ Tg-RKIP; $n = 4$, B6). **(B,C)** Sensitization of the isoproterenol-stimulated cAMP response in neonatal cardiomyocytes isolated from Tg-RKIP and Tg-GRK2K220R mice compared to non-transgenic B6 cardiomyocytes **(B)**, and increased cardiac expression of the cAMP-inducible gene, *Ttc14,* in Tg-RKIP, and Tg-GRK2K220R hearts **(C)**. Data represent mean ±s.d. ($n = 6$, **B**; $n = 3$, **C**; ***$p < 0.001$ vs. B6; Dunnett's test). See also **Supplementary Figure 8**.

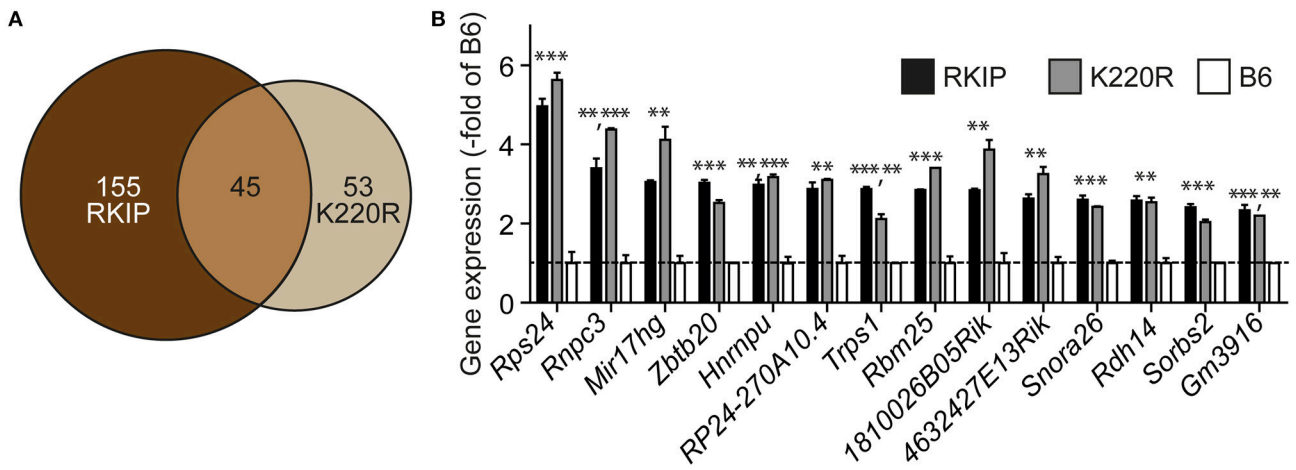


**FIGURE 10 |** Concordant gene regulation in Tg-RKIP and Tg-GRK2K220R hearts. **(A,B)** Whole genome microarray gene expression profiling was performed with 8-months-old Tg-RKIP2 and Tg-GRK2-K220R hearts compared to non-transgenic B6 hearts. Probe sets with significantly different signal intensity (with call present and/or signal intensity >100) were identified with $p < 0.01$, and ≥2-fold difference compared to B6. The Venn diagram illustrates the number of concordantly regulated genes between Tg-RKIP and Tg-GRK2K220R hearts **(A)**. Representative probe sets **(B)** show comparable up-regulation of concordantly regulated genes in Tg-RKIP and Tg-GRK2K220R hearts (±s.d.; $n = 2$ gene chips/group with $n = 3$ hearts pooled for one gene chip; ***$p < 0.001$, **$p < 0.01$ vs. B6; Dunnett's test).

## Inhibition of the AT1 Receptor Retards RKIP-Induced Symptoms of Heart Failure

We finally investigated whether the AT1 receptor contributed to RKIP-induced symptoms of heart failure *in vivo*. To address this question, we treated Tg-RKIP mice for 3 months with the AT1-specific antagonist, losartan, at a dose of 5 mg/kg/day. The applied dose of losartan does not alter systolic blood pressure in mice (37). Treatment with losartan, significantly retarded the development of RKIP-induced cardiac hypertrophy as evidenced by a significantly decreased heart-to-body weight ratio, the histological assessment, and the histomorphological analysis of cardiomyocyte size (**Figures 12A–C**). Concomitantly, losartan treatment led to an improved cardiac function, which was documented by a significantly increased left ventricular ejection fraction (**Figure 12D**). Echocardiographic data further documented that inhibition of the pro-hypertrophic AT1 receptor by losartan treatment counteracted the phenotype of cardiac hypertrophy in Tg-RKIP mice (**Figure 12D**). Together these data provide strong evidence that sensitization of AT1 receptor signaling contributes to RKIP-induced cardiac hypertrophy and cardiac dysfunction.

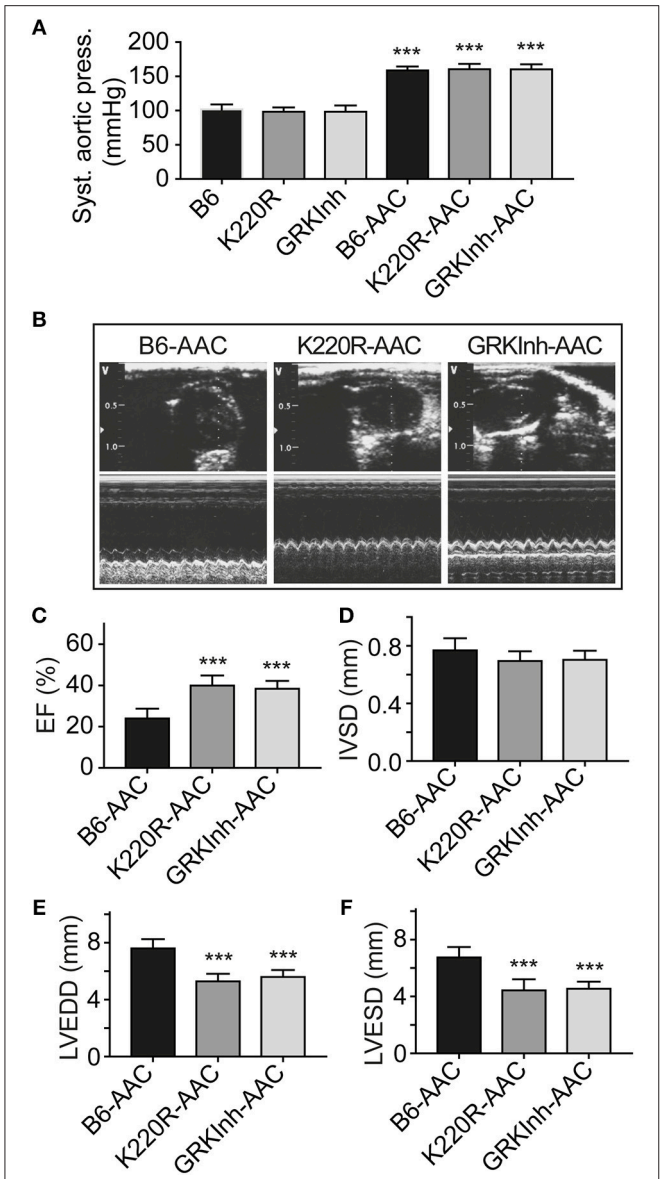


**FIGURE 11 |** Dominant-negative GRK2-K220R retards chronic pressure overload-induced cardiac dysfunction. **(A)** Systolic aortic pressure of B6, Tg-K220R and Tg-GRKInh mice under basal conditions and at the end of the observation period after 2 months of AAC (±s.d.; $n = 6$; ***$p < 0.001$ vs. B6; Dunnett's test). **(B)** Representative echocardiographic images in 2D mode (upper) and M-mode (lower) of B6, Tg-GRK2K220R and Tg-GRKInh mice with 2 months of chronic pressure overload imposed by AAC. **(C–F)** Left ventricular ejection fraction **(C)**, interventricular septal thickness **(D)**, LVEDD **(E)** and LVESD **(F)** were determined by echocardiography of B6, Tg-GRK2K220R and Tg-GRKInh mice at the end of the observation period after 8 weeks of AAC (±s.d.; $n = 6$; ***$p < 0.001$; Dunnett's test).

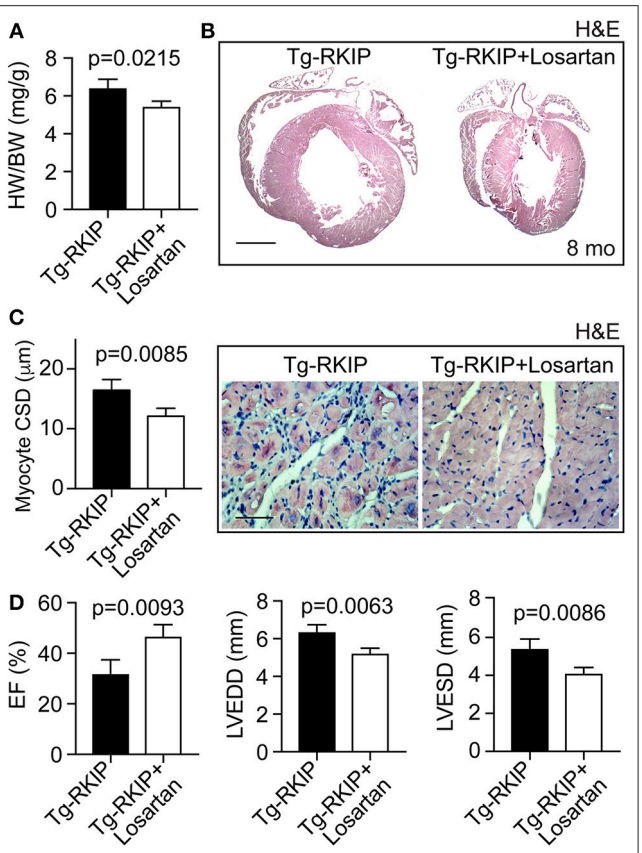


**FIGURE 12 |** Inhibition of the AT1 receptor retards RKIP-induced symptoms of heart failure. **(A–C)** Treatment for 3 months with the AT1 antagonist, losartan, retards the development of RKIP-induced cardiac hypertrophy in 8-month-old Tg-RKIP mice with FVB background as evidenced by a decreased heart-to-body weight ratio **(A)**, histological analysis (**B**; bar: 2 mm), and cardiomyocyte cross-sectional diameter (**C**; bar 20 μm). Data represent mean ± s.d.; $n = 4$ **(A,C)**. Sections were stained with hematoxylin-eosin (H&E) and are representative of four mice/group **(B,C)**. **(D)** Left ventricular ejection fraction (left), left ventricular end-diastolic diameter (middle), and left ventricular end-systolic diameter (right) of Tg-RKIP mice without and with losartan treatment were determined by echocardiography (±s.d.; $n = 4$).

## DISCUSSION

In this study, we found that kinase-inactive GRK2K220R improves cardiac performance under basal conditions and exerts protection against chronic pressure overload-induced cardiac dysfunction. These findings with the GRK2-K220R mutant reflect the *in vivo* effects of a kinase-inhibited GRK2, which can be similarly achieved by a small molecule GRK2 inhibitor. In this respect our data with the kinase-inactive GRK2-K220R extend previous studies on the cardioprotective function of GRK2 inhibition, which is documented by different approaches of GRK2 inactivation, e.g., GRK2 deficiency (6), transgenic expression of the Gβγ-scavenging betaARKct (5, 9), the beta2-adrenoceptor-derived peptide, GRKInh (8, 12), and inhibition of GRK2 by an ATP-site-directed inhibitor (7).

Our study also shows that not all approaches of GRK2 inhibition are cardioprotective. In contrast to cardioprotective GRK2 inhibition with kinase-inactive GRK2-K220R, transgenic expression of the dual-specific GRK2 and Raf-Erk1/2 axis inhibitor, RKIP, promoted signs of heart failure, i.e., cardiac hypertrophy, cardiac dilatation and cardiotoxic lipid load. In addition, symptoms of chronic congestive

heart failure were evident in Tg-RKIP mice as documented by pulmonary congestion with increased lung and body weight, kidney insufficiency with proteinuria, and elevated systolic pressure. RKIP-induced signs of heart failure were similarly observed in two different genetic backgrounds, i.e., B6 and FVB.

Signs of heart failure developed in RKIP-transgenic mice despite of significant GRK2 inhibition. Experimental data showed that GRK2 inhibition-induced sensitization of the cAMP signal was comparable between Tg-RKIP and Tg-GRK2K220R cardiomyocytes and hearts. Also, the phosphorylation of RKIP on serine-153 was significant in Tg-RKIP hearts, which is required for RKIP to act as GRK2 inhibitor. Nevertheless, the GRK2-inhibitory activity of RKIP was insufficient to protect against RKIP-induced heart failure.

In this study, several lines of evidence are presented, which show that sensitization of the AT1 receptor contributes to the observed RKIP-induced symptoms of heart failure. (I) RKIP led to increased AT1 receptor-stimulated inositol phosphate levels in cells. This AT1-sensitization was mediated by the RKIP-GRK2 interaction because the RKIP-S153V mutant, which cannot switch from Raf1 to GRK2, did not sensitize the AT1 receptor response. (II) RNAi-mediated down-regulation of RKIP demonstrated that endogenously expressed RKIP levels were sufficient to sensitize the AT1-stimulated signal. (III) Tg-RKIP mice with symptoms of heart failure showed down-regulation of the cardiac AT1 receptor content, which could be triggered by RKIP-mediated AT1 receptor sensitization because excessive AT1-mediated signaling down-regulates AT1 (31, 32). In this respect, Tg-RKIP mice resemble human patients with heart failure (29, 30). (IV) Tg-RKIP mice with myocardium-specific RKIP expression developed cardiac hypertrophy with dilatation, and cardiac dysfunction, and these symptoms of heart failure were retarded by treatment with the AT1-specific antagonist, losartan.

In addition to the heart failure-promoting effect of RKIP-triggered AT1 receptor sensitization, which was attributed to the RKIP-GRK2 interaction, symptoms of RKIP-induced heart failure could be aggravated by additional functions of RKIP. Notably, the severity of RKIP-induced symptoms of heart failure could be a consequence of the dual activity of RKIP as GRK2 and Raf1-Erk1/2 axis inhibitor. While AT1 receptor sensitization is largely mediated by the RKIP-GRK2 interaction, the development of cardiotoxic lipid load is attributed to Raf-Erk1/2 pathway inhibition, which leads to heart failure-promoting and adipogenic *Pparg* activation by inhibition of Pparg phosphorylation on serine-273 (8, 33). As a consequence, cardiotoxic lipid load occurs with accumulation of the PKC-activating DAG, which accounts for RKIP serine-153 phosphorylation and the RKIP-GRK2 interaction. The ensuing RKIP-GRK2 interaction not only restores the beta-adrenoceptor responsiveness but also triggers cardiotoxic AT1 receptor signaling. In addition, DAG-mediated activation of PKC is *per se* an independent contributor to heart failure (38).

Exaggerated cardiotoxic Gαq/11-stimulated calcium signaling seems to be a specific feature of RKIP because RKIP shields the amino-terminal domain of GRK2, which contains a cardioprotective and Gαq/11-inhibitory RGS domain (13, 14). In contrast to RKIP, established cardioprotective GRK2 inhibitors do not interfere with this RGS domain of GRK2. ATP-site-directed GRK2 inhibitors such as paroxetine, inhibit the kinase activity of GRK2 but leave kinase-independent functions of GRK2 intact, e.g., mediated by the Gαq/11-inhibitory RGS domain. Also, the betaARKct inhibits GRK2 by Gβγ subunit scavenging but retains the activity of the RGS domain (4).

The heart failure-promoting activity of RKIP in transgenic mouse models could also be relevant for the human disease because cardiac biopsy specimens from heart failure patients have an increased cardiac RKIP content (21). Notably, RKIP expression levels in several transgenic mouse models with two different genetic backgrounds, were comparable to the increased RKIP level found on human heart biopsy specimens of heart failure patients (21). Because down-regulation of an increased RKIP level in transgenic mice retarded the development of heart failure, RKIP inhibition could be considered as a potential target for development of a pharmacological treatment approach of heart failure. The recently elucidated beneficial and cell-protective side-effect profile of RKIP deficiency and/or RKIP inhibition further supports such a concept (39).

## DATA AVAILABILITY STATEMENT

Whole genome expression data generated and analyzed for this study can be found in the NCBI GEO database (accession number GSE 120020).

## AUTHOR CONTRIBUTIONS

SW and JA performed experiments. JA generated transgenic mice. UQ: conducted the study, designed experiments, and wrote the manuscript. All authors evaluated data, read and approved the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2018.00359/full#supplementary-material

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# Tonin Overexpression in Mice Diminishes Sympathetic Autonomic Modulation and Alters Angiotensin Type 1 Receptor Response

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**Background:** Tonin, a serine-protease that forms Angiotensin II (AngII) from angiotensinogen, is increased in failing human heart samples. Increased blood pressure (BP) and decreased heart rate (HR) variabilities are associated with higher risk of cardiovascular morbidity. Losartan has been used to reduce hypertension and, therefore, lowers the risk of fatal and non-fatal cardiovascular events. Determination of tonin's impact on BP and HR variabilities as well as the impact of losartan remain questions to be elucidated.

**Aim:** Evaluation of cardiovascular autonomic profile in transgenic mice overexpressing the rat tonin enzyme TGM'(rton) and the impact of AT1 receptor blocker, losartan.

**Methods:** Male C57BL/6 (WT) and TGM'(rTon) mice were cannulated for recording BP (Windaq, 4 MHz) for 30 min at baseline and 30 min after losartan injection (20 mg/kg). BP and HR variabilities were analyzed in time and frequency domain method. Low-frequency (LF) and high-frequency (HF) components were identified for sympathetic and parasympathetic modulations analysis. Ang I, AngII, and Ang1-7 were quantified by high performance liquid chromatography method. The total enzymatic activity for AngI, AngII, and Ang1-7 formation was evaluated in the heart and plasma by Liquid chromatography mass spectrometry (LC-MS/MS).

**Results:** At the baseline TGM'(rTon) exhibited higher BP, lower cardiac LF, higher cardiac HF, lower LF/HF, and lower alpha index than wild type (WT). After losartan injection, TGM'(rTon) mice presented an additional decrease in cardiac LF and increase in HF in relation to baseline and WT. In the vasculature, losartan caused decreased in BP and LF of systolic BP in WT mice in relation to its baseline. A similar effect was observed in the BP of TGM'(rTon) mice; however, LF of systolic BP increased compared to baseline.

Our data also indicates that AT1R receptor signaling has been altered in TGM'(rTon) mice. Interestingly, the dynamics of the renin-angiotensin system kinetics change, favoring production of Ang1-7.

**Conclusion:** Autonomic evaluation of TGM'(rTon) mice indicates an unclear prognosis for diseases that affect the heart. HR variability in TGM'(rTon) mice indicates high risk of morbidity, and sympathetic and parasympathetic modulation indicate low risk of morbidity. The low risk of morbidity could be the biased production of Ang1-7 in the heart and circulation; however, the altered response of AT1R in the TGM'(rTon) remains to be elucidated, as well aswhether that signaling is pro-protection or pro-pathology.

**Keywords: tonin, serino protease, AT1R, losartan, renin angiotensin system, autonomic control, angiotensin II, angiotensin 1-7**

## INTRODUCTION

Angiotensin II (Ang II), the main agonist of the renin-angiotensin system, plays an important role in the maintenance of a healthy heart. Inotropism and chronotropism are a few of its direct roles in the heart (1–3), and as part of the integrated system it participates in hemodynamic stability (4). Deleterious effects of overstimulation by Ang II contribute to an array of cardiovascular diseases. More specifically, this hormone is implicated in a chain of events that contribute to the initiation and progression of cardiovascular disease. Ang II has been implicated in risk factors such hypertension, hyperlipidemia, and diabetes, in development processes such as atherosclerosis and left ventricular dysfunction, leading to ventricular hypertrophy and fibrosis followed by congestive heart failure. Thus, Ang II is a key target for the treatment of cardiovascular diseases (3, 5, 6). This powerful peptide can be formed by many enzymatic pathways within the renin-angiotensin system, including renin and angiotensin converting enzyme (ACE), via tonin, cathepsin, and chymase (7–9). However, the main routes to formation of Ang II are tissue specific. A systematic study reported that 80% of the total AngII-forming activity in heart was inhibited by serine protease inhibitor (10). This observation explained production of Ang II in heart failure patients treated with ACE inhibitors. Tonin is a serine protease that can form Ang II from Ang I and directly from angiotensinogen (Agt) (11–13). Therefore, this enzyme could be another source of Ang-II, independent of that produced via ACE. We have advanced only a few steps toward a more precise definition of the role of tonin-angiotensin II in the puzzling system of angiotensin II-generating enzymes. This lack of clarity may be due to the unique characteristics of that differ from other converting enzyme(s). A closer relationship between tonin-angiotensin II system and norepinephrine (14–16) system could be part of an integrated system by which myriad of complex mechanisms maintains homeostasis of the cardiovascular autonomic system. Taken together, tonin, a serine protease could play a role in the development of the cardiovascular diseases. Thus, our goal is to evaluate how the upregulation of tonin affects the risk of cardiovascular morbidity, its impact on the other angiotensins and on the main receptor of the renin-angiotensin system, the angiotensin type 1 receptor (AT1R).

## MATERIALS AND METHODS

### Mice

Wild type C57BL/6 (WT) and homozygous transgenic mice overexpressing the rat tonin enzyme [TGM'(rton)] were used in this study. Mice were housed in a temperature-controlled room (21 ± 1°C) under a 12:12-h dark light cycle, received standard laboratory chow and water *ad libitum*. The research ethics committee of the Federal University of São Paulo approved experimental protocols used in this study.

### Generation of Transgenic Mice

Transgenic TGM'(rTon) mice were generated by microinjection of the rat tonin transgene into zygotes as described by Ribeiro et al. (17, 18). TGM'(rTon) mice exhibited higher tonin activity in the brain, heart, kidney, liver, and bladder compared to WT (17).

### Catheter Implantation and Hemodynamic Recording

Catheters filled with heparinized saline were implanted into carotid artery and jugular vein of the anesthetized mice (80 mg/kg ketamine and 12 mg/kg xylazine, i.p.) Animals were placed in individual clean cages with free access to food and water. A heating lamp was used to keep the animals warm until their full recovery. Forty-eight hours later if a mouse presented signs of decreased activity, reluctance to move, hunched posture, and ungroomed fur, it was withdrawn from the study. The animals that recovered well were connected to computerized acquisition systems by connecting the arterial cannula to an electromagnetic transducer (Kent Instruments) by a polyethylene tube. This transducer was linked to an amplifier (General Purpose Amplifier-Stemtech, Inc.) and connected to a computer with an analog to digital converter board (Windaq/DataQ). The venous catheter was tethered to a 25 cm polyethylene tube filled with heparinized saline. To allow for stabilization of their blood pressure, the animals remained connected for at least half an hour to the system. After the adaptation period, 30 min of real-time

blood pressure and heart rate signals were recorded before and after a single injection of losartan (20 mg/Kg, iv) in conscious animals who were free to move. Losartan was injected through the venous catheter.

## Heart Rate and Blood Pressure Variability

Analysis of blood pressure signals was performed using an algorithm implemented in acquisition system Windaq/DataQ. This software allowed the detection of the maximal value in the blood pressure curve, beat to beat, providing the values of systolic blood pressure (SBP). Heart rate was determined from the pulse interval (PI) between two systolic peaks. Heart rate variability was measured by linear methods in time and frequency domains. Temporal series of PI and SBP (30 min of baseline recording) were analyzed in time domain, obtaining the total variance of PI (VAR PI) and the variance of SBP (VAR SBP). Mean square root of differences between consecutives PI (RMSSD) was also evaluated. For the frequency domain, one spectrum was obtained for each segment and the oscillatory components were quantified in two different frequencies: low frequency (LF) from 0.1 to 1 Hz and high frequency (HF) from 1 to 5 Hz. The results are represented by absolute values ($ms^2$ and $mmHg^2$), the percentage of total spectrum (%) and normalized units (nu) (percentage of LF and HF bands only).

## Sample Collection

Animals were euthanized by decapitation at 12–14 weeks old. Blood samples were collected and plasma was immediately separated from the whole blood by centrifugation at 1,500 rpm for 10 min and stored at −80°C. Hearts were rapidly removed, snap frozen in liquid nitrogen and stored at −80°C until experimental procedures were performed.

## Sample Preparation and Quantification of Angiotensins by High Performance Liquid Chromatography

Atria and right and left ventricles were dissected for homogenization in 100 mM sodium phosphate, 240 mM sucrose, 300 mM NaCl, and the protease inhibitors: 1 mM EDTA (metalloprotease inhibitor), 1 μM o-phenanthroline (metalloprotease inhibitor), 120 μM pepstatin A (aspartyl protease inhibitor), 1 μM PMSF (serine protease inhibitor), and 1 mM 4-chloromercuribenzoic acid (cysteine protease inhibitor) at a ratio of 5 ml of buffer per gram of tissue. Homogenates were centrifuged at 20,000x g for 1 h at 4°C. In each resulting supernatant, 1 nmol of $Sar^1$-AngII was introduced as an internal standard. Samples were purified and concentrated using C18 Sep-Pak columns previously activated with methanol (5 ml), tetrahydrofuran (5 ml), hexane (5 ml), methanol (5 ml), and water (10 ml). After loading the samples into the column, purification was performed by washing the column with ultrapure water. Samples were eluted from the column using 5 ml of ethanol/acetic acid/water in the proportion (90:4:6). The eluates were lyophilized then rehydrated in 500 ul of mobile phase A (5% acetonitrile in 0.1% orthophosphoric acid. Each sample was filtered (0.22 um pore size filter) and injected into the HPLC. The peptides were separated on the reverse phase column Aquapore 300 ODS (250 x 4.6 mm 7 μ) connected to a high-performance liquid chromatography system (Shimatzu) with 214 nm UV detection. The peptide separation was performed with 20 min of linear gradient from 5 to 35% mobile phase B (95% acetonitrile in 0.1% orthophosphoric acid) after 5 min of isocratic gradient over, at constant flow rate of 1.5 ml/min. $Sar^1$-AngII, AngI, AngII, and Ang1-7 synthetic peptides (Sigma) were used as standards and the angiotensins in each sample were identified according to the angiotensin standards retention time. The concentrations were expressed in picomol per gram of tissue.

## Sample Preparation for Enzymatic Activity

Hearts were dissected into atria, right ventricle, and left ventricle. These chambers were homogenized in 50 mM Tris/HCl, 0.5 mM $ZnCl_2$ buffer, pH 7.5, followed by centrifugation at 10,000 rpm at 4°C for 10 min. The resulting supernatants were separated from the pellet. The protein concentration of the supernatants was determined by Bradford (Biorad) method as recommended by the manufacture. Protein quantification was performed on the supernatants for further normalization of each peptide chromatographic-peak-area.

## Total Angiotensin II and Angiotensin 1-7 Forming Enzymes Activities

Tetradecapeptide (TDP) (10 nmol), a synthetic analog of angiotensinogen, was incubated in 1 ml of buffer (50 mM Tris/HCl, 0.5 mM $ZnCl_2$, pH 7.5) containing either 50 ul of plasma, 100 ul of atria or right or left ventricle homogenates at 37°C for 24 h. Aliquots were collected at 10, 20, 40, 60, 120, 240, 360, and 1,440 min into a tube containing 10% formic acid. Angiotensin I, Angiotensin II, and Angiotensin 1-7 were quantified by liquid chromatography/mass spectrometry.

### Chromatographic Conditions

LC experiments were carried out with an Agilent1290 System with a Luna C18 100 × 2 mm, 3 μm column at 40°C with a 9 min gradient of eluent water and acetonitrile with 0.1% formic acid at a flow rate of 400 μL/min. The injection volume was set to 5 μL.

### Mass Spectrometry Conditions

The AB SCIEX QTRAP® 5500 system was operated with Turbo V Electrospray Ionization probe. The 3 angiotensins were confirmed by comparison with angiotensin standard retention time, 3 MRM transitions per peptide to allow quantitation and identification based on the ratio of quantifier and qualifier transitions and an exclusive scan mode, the Enhanced Product Ion (EPI) which is at least 100 times more intense than a common MS/MS scan. The method used triggers an ion trap MS/MS scan on the ion of interest once the MRM intensity exceeds a certain threshold. The MS/MS data obtained confirms the identity of the angiotensins with the entire MS/MS spectrum rather than just with the presence of two diagnostic fragments.

## Statistics

All data are expressed as mean and standard error of mean. Significance level was determined by using two-way ANOVA

with Bonferroni *post-hoc* test or with multiple *t*-test comparison. 95% confidence of interval was used and *P*-value $< 0.05$ was considered statistically significant.

## RESULTS

### Tonin Enzyme Overexpression *in vivo* Increased Blood Pressure

Overexpression of tonin caused an increase in diastolic and systolic BP ($p = 0.0464$) without altering heart rate. Pharmacological blockade of AT1R reduced systolic and diastolic BP in both strains ($p = 0.0036$; **Figure 1**). However, when the delta value of BP was evaluated before and after losartan injection, it was observed that losartan induced greater reduction of BP in TGM'(rTon) than WT (21.46 vs. 11. 86 mmHg, respectively).

### Losartan, Angiotensin Type 1 Receptor Blocker, Further Decreases Sympathetic and Increases Parasympathetic Modulation on the Cardiac Autonomic Control in TGM'(rTon) Mice

At baseline the TGM'(rTon) mice showed a reduction in total HR variability, represented by variance of pulse interval (VAR PI). Together a clear reduction in the sympathetic specific spectrum band (LF) was observed when analyzed the total ($ms^2$), percentage (%), and normalized (nu). Interestingly, an elevation of parasympathetic modulation was observed when analyzing its specific spectrum band (HF). Consequently, a decreased sympathovagal balance was observed when compared to WT group (**Figure 2**). In the TGM'(rTon), blockade of AT1R caused further decrease and increase in the sympathetic and parasympathetic modulation, respectively. Both effects were greater in the TGM'(rTon) when compared to the WT group.

### Losartan, Angiotensin Type 1 Receptor Blocker, did not Affect Blood Pressure Variability Even Though the Sympathetic Modulation of the Vasculature Was Altered in TGM'(rTon) Mice

TGM'(rTon) at baseline and after angiotensin receptor blockage did not significantly alter the systolic BP variability represented by VAR SBP. Pharmacological blockade of AT1R caused reduction of sympathetic modulation in the controls, but it increased in the TGM'(rTon) (**Figure 3**). After losartan injection, TGM'(rTon) exhibited lower baroreflex sensitivity measured by the alpha index compared to WT group.

### Tonin Overexpression Alters the AngI, AngII, and Ang1-7 Content in the Heart

Within each WT and TGM'(rTon) group, levels of AngII and Ang1-7 did not differ among heart compartments, indicating that the steady-state levels of AngII and Ang1-7 levels follow similar regulation. Levels of Ang I in the atria were 2 times higher than in the right and left ventricle in WT group and 1 time higher in TGM'(rTon). When levels of angiotensins are compared between groups, TGM'(rTon) mice showed decreased levels of AngI in atria and higher levels of AngI in both right ventricle and left ventricle. Contrastingly, AngII levels were uniformly decreased in TGM'(rTon) heart chambers compared with WT group (**Figure 4**). This observation was accompanied by a large increase in Ang1-7. These results indicate changes in angiotensin processing, resulting in production of Ang1-7 instead of production of Ang II in TGM'(rTon) mice.

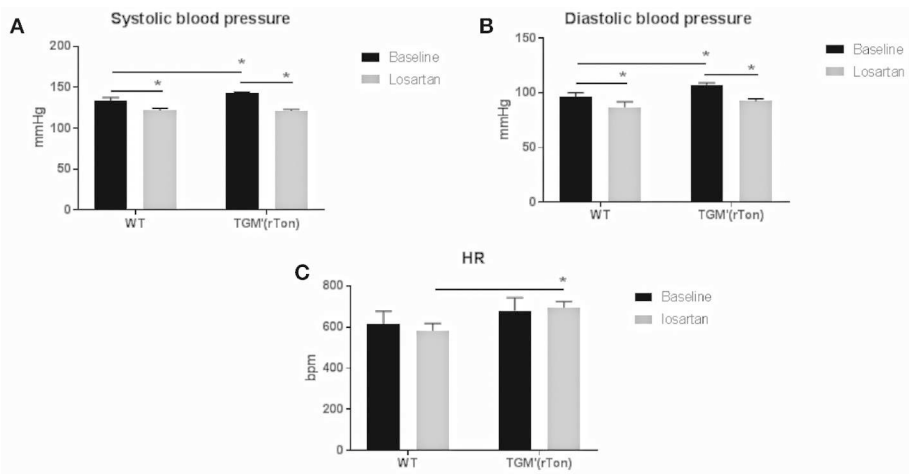


**FIGURE 1 |** Hemodynamic evaluation in wild type (WT) and transgenic mice overexpressing rat tonin [TGM'(rTon)] at baseline and after angiotensin type 1 receptor blocker, losartan: Overexpression of tonin enzyme increased the systolic **(A)** and diastolic **(B)** blood pressure (BP, $p = 0.0464$) without significant changes in the heart rate (HR, **C)**. Blockage of AT1R reduced systolic and diastolic BP in both strains ($p = 0.0036$). Mice given losartan maintained similar HR to baseline. $N = 4$, Two-way ANOVA $^*p < 0.05$, followed by bonferroni *post hoc*, bar graphs are means ±SEM.

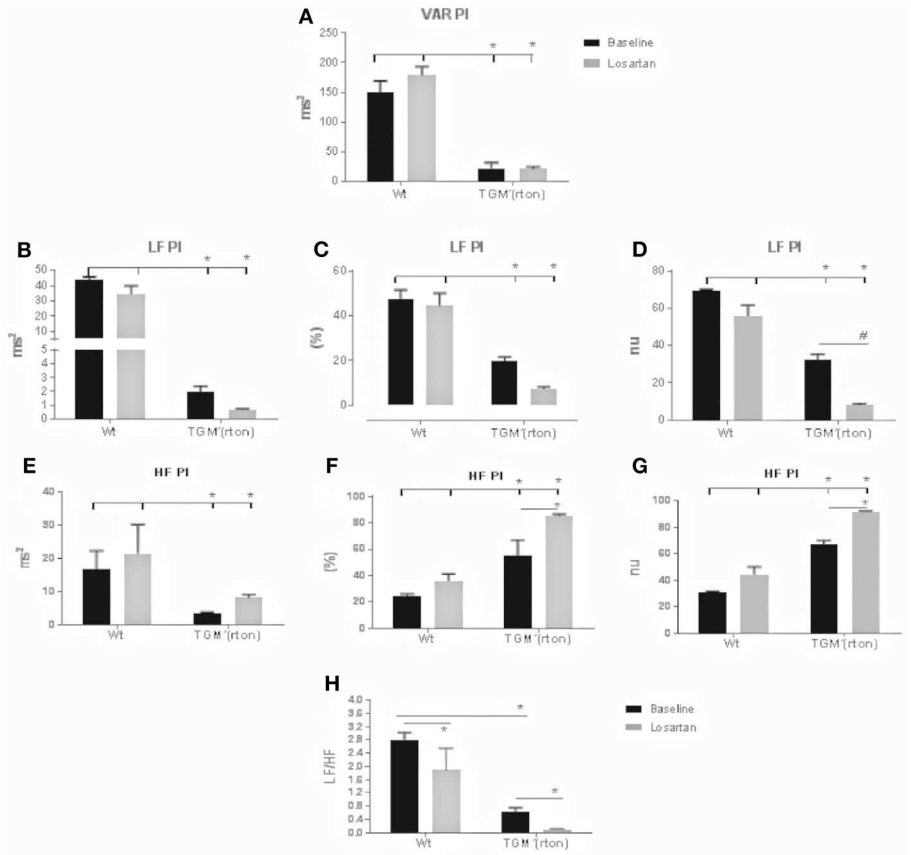


**FIGURE 2 |** Time and frequency domain evaluation of heart rate (HR) variability in wild type (WT) and transgenic mice overexpressing rat tonin [TGM'(rTon)] at baseline and after angiotensin type 1 receptor blocker, losartan: Variance of pulse interval (VAR PI) **(A)** low frequency band of pulse interval (LF PI), including absolute **(B)**, percent **(C),** and normalized units **(D)**, high frequency of pulse interval (HF PI) including absolute **(E)**, percent **(F),** and normalized units **(G)**, sympathovagal balance (LF/HF) **(H)**. LF bands represent the sympathetic modulation whereas HF parasympathetic. $N = 4$, Two-way ANOVA followed by bonferroni *post hoc* $^*p < 0.05$, bar graphs are means ±SEM.

## Tonin Overexpression Alters the Angiotensins Formation Dynamic in the Heart

The proteolytic capacity of each compartment generating angiotensins were tested *in vitro* by a mass spectrometry-based assay. Extracts of atria, right ventricle and left ventricle of both WT and TGM'(rTon) mice were preincubated with tetradecapeptide and levels of Ang I, Ang II, and Ang1-7 were quantified by LC-MSMS in a time-dependent manner. Total atria extract were capable of rapidly consuming the TDP, generating Ang I in the first minute of incubation until a maximum concentration was reached between the 120 and 240 min for WT group and 360 min for TGM'(rTon) mice (**Figure 5A**). Corroborating the observation made using HPLC assay, atrial production of Ang I was shown to be higher in WT than TGM'(rTon)mice.

Levels of angiotensins generated by right ventricle extracts from tetradecapetide precursor were also measured. There was a rapid formation (0–40 min) of Ang I, AngII, and Ang1-7 in the TGM'(rTon) group compared to the WT group (120–360 min), contrasting with the delay in formation observed in the WT extracts. Interestingly, the highest peak of Ang I production corresponds to 20 min in TGM'(rTon) group; in contrast, the highest peak of Ang I formation was reached at 4 h in the WT mice (**Figure 5B**). A similar difference was obtained for Ang II (40 min for TGM'(rTon) group vs. 4 h for WT group; **Figure 5C**) and for Ang1-7 (60 min for TGM'(rTon) and 360 min for WT group, **Figure 5D**). It is noteworthy that the total formation of Ang1-7 was 2 times higher than WT group. These data suggest that RV extracts of TGM'(rTon) produce all angiotensins faster than WT mice.

The levels of Ang1-7 were also measured using left ventricle extracts using Ang II as precursor (**Figure 5E**). The levels of Ang1-7 was significantly higher for TGM'(rTon) compared to WT mice. Total Ang1-7 formation in the atria, RV and LV corroborate the observation of Ang1-7 content by HPLC, i.e., higher levels of Ang1-7. Similarly, total AngII formation in the RV corroborate the observation found by HLPC, lower levels of AngII in the heart.

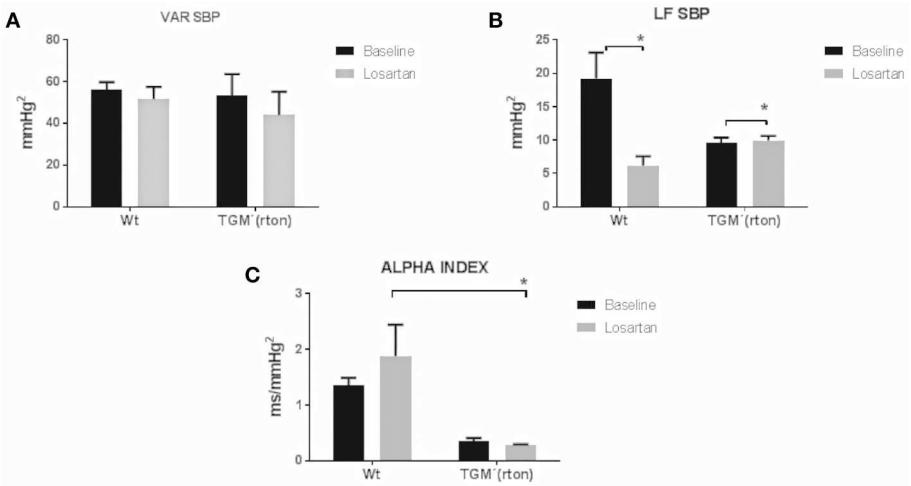


**FIGURE 3 |** Time and frequency domain evaluation of blood pressure variability in wild type (WT) and transgenic mice overexpressing rat tonin [TGM'(rTon)] at baseline and after angiotensin type 1 receptor blocker, losartan: Variance of SBP (VAR SBP) **(A)**, low frequency band of SBP (LF SBP) **(B)** and alpha index **(C)**. LF bands represent sympathetic modulation and alpha index represents baroreflex sensitivity. $N = 4$, Two-way ANOVA followed by bonferroni *post hoc* $^*p < 0.05$, bar graphs are means ±SEM.

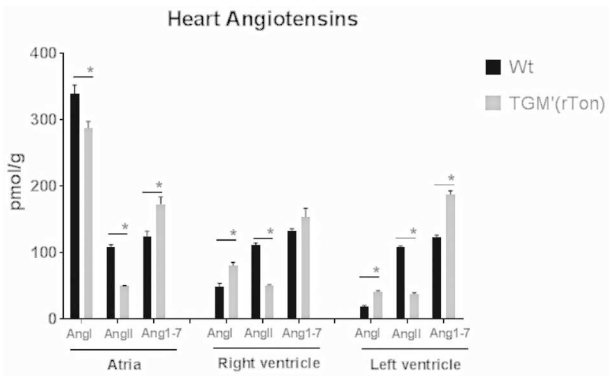


**FIGURE 4 |** Angiotensin content in three chambers of the heart in wild type (WT) and transgenic mice overexpressing rat tonin [TGM'(rTon)]: Heart angiotensin concentration of WT and TGM'(rTon) mice were quantified by High Performance Liquid Chromatography. AngI, AngII, and Ang1-7 were measured in each chamber. $N = 4$, Two-way ANOVA followed by multiple *t*-tests $^*p < 0.05$, bar graphs are means ±SEM.

## Tonin Impacts the Circulating Renin Angiotensin System Homeostasis

The proteolytic capacity of plasmatic proteases generating angiotensins from tetradecapeptide were also analyzed. Plasmatic proteases were capable of rapidly consuming tetradecapeptide, generating high concentrations of Ang I in the first 10 min of incubation for both TGM'(rton) and WT mice (**Figure 6A**). However, the amounts of plasmatic Ang I produced by TGM'(rTon) group was 10 times lower than the amounts obtained by WT mice, but still higher than the amounts obtained using atrial and RV extracts. The amounts of AngII formed in the plasma from TDP as substrate were similar in both mice (**Figure 6B**). Again, the amount of Ang1-7 was higher in the TGM'(rTon) compared to WT group (**Figure 6C**).

The amounts of Ang II and Ang1-7 generated by plasmatic proteases using Ang I and Ang II as precursors were also measured. When the tested precursor was Ang I (**Figures 7A,B**), the level of Ang II and Ang 1-7 levels were significantly higher in TGM'(rTon) compared to control mice, reaching a maximum concentration after 40–60 min of incubation. Again, higher Ang 1-7 levels were obtained using Ang II as precursor in the TGM'(rTon) group when compared to the WT mice (**Figure 7C**).

## DISCUSSION

In the present study TGM'(rTon) mice exhibited an increase in BP at pre-hypertensive levels; similar results were published by Cardoso et al. (19). One of the mechanisms underlying this phenotype is an increase in circulatory concentrations of Ang II, which was two-fold higher than the increased concentration of Ang 1-7 observed. However, it is worth noting that when we evaluated the proteolytic capacity of the plasmatic proteases to the tetradecapeptide, a synthetic analog of angiotensinogen which mimics the peptide initiator of the renin angiotensin cascade, similar levels of Ang II were produced when compared to WT group. Only when incubated with Ang I was it possible to see increased levels of Ang II. This finding indicates that in circulation, the slight increase of AngII levels observed in the TGM'(rTon) mice are derived from Ang I only. Tonin synthesized in the brain most likely is being released into the circulation. Once released into the circulation, tonin is immediately trapped by α1-Macroglobulin (α1-M) to form a tonin-α1-M (20). Hence, previous attempts to detect tonin activity in plasma (human and rat) were unsuccessful (21–24). Only using the immunoimmobilization technique was it possible

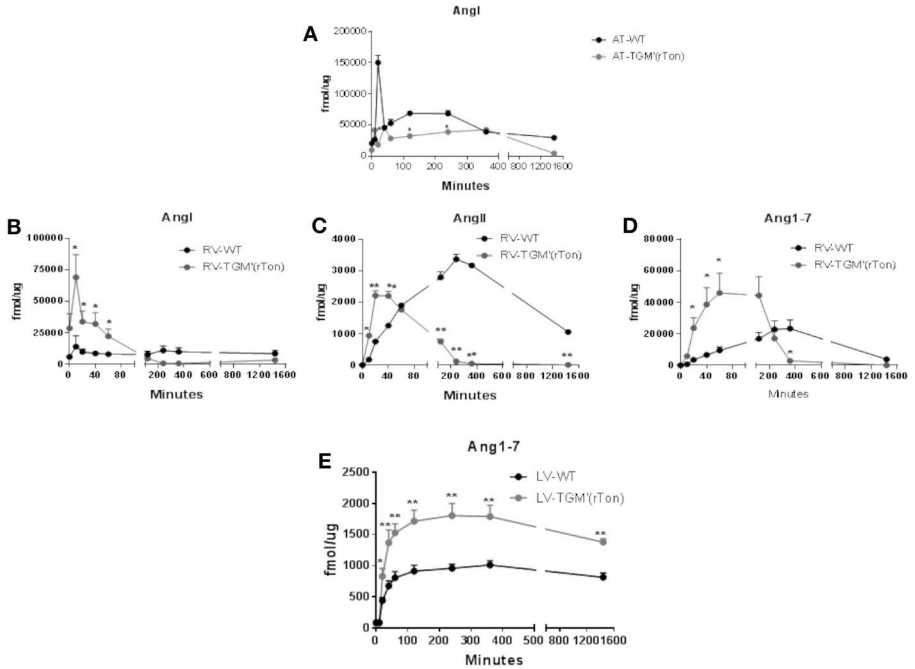


**FIGURE 5 |** Total AngI, AngII, and Ang1-7 formation activity in atria, right ventricle and left ventricle of wild type (WT) and transgenic mice overexpressing rat tonin [TGM'(rTon)]: AngI levels in the atria after incubation with tetradecapeptide as substrate at 0, 10, 20, 40, 60, 120, 240, 360, and 1,440 min ($n = 2$) **(A)**. AngI **(B)**, AngII **(C)**, and Ang1-7 **(D)** in the right ventricle (RV) after incubation with tetradecapeptide as substrate at 0, 10, 20, 40, 60, 120, 240, 360, and 1,440 min by LC-MS/MS ($n = 4$). Ang1-7 **(E)** levels in the left ventricle (LV) after incubation with AngII as substrate at 0, 10, 20, 40, 60, 120, 240, 360, and 1,440 min by LC-MS/MS ($n = 4$). Multiple *t*-tests were performed, $^*p < 0.05$, $^{**}p < 0.001$ time points are represented by means ± SEM.

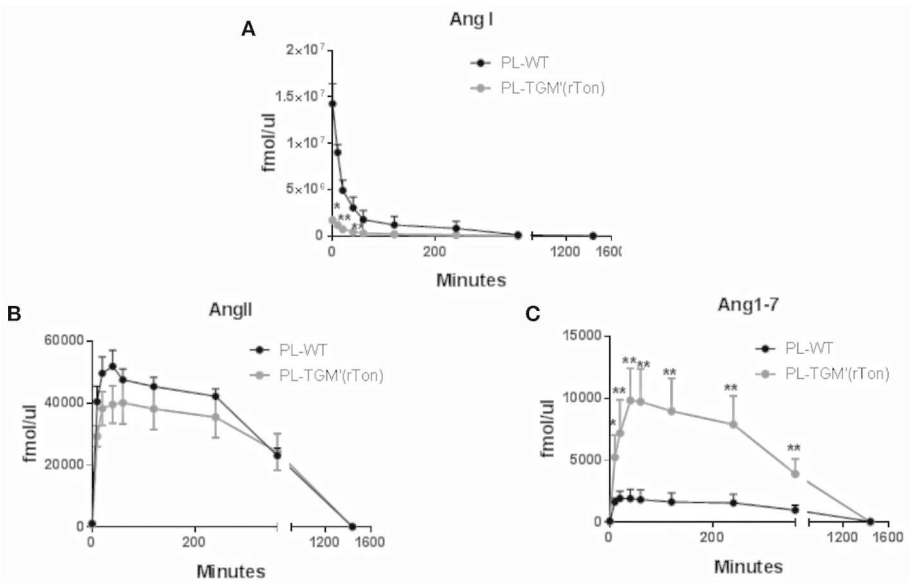


**FIGURE 6 |** Total Ang I, Ang II, and Ang1-7 formation in the plasma of wild type (WT) and transgenic mice overexpressing rat tonin [TGM'(rTon)]: AngI **(A)**, AngII **(B),** and Ang1-7 **(C)** in the plasma (PL) after incubation with tetradecapeptide as substrate at 0, 10, 20, 40, 60, 120, 240, 360, and 1,440 min by LC-MS/MS ($n = 4$). Multiple *t*-test were performed $^*p < 0.05$, time points are represented by means ±SEM.

to detect this enzyme in the blood (20). The tonin-α1-M complex forms Ang II solely from Ang I and not from angiotensinogen, due a lack of fit in the catalytic site now partly occupied by the inhibitor. Ikeda et al. (20) observed that when tonin was intravenously administered as a bolus injection, tonin's fate followed two paths. First, fast clearance of 125 I-labeled tonin

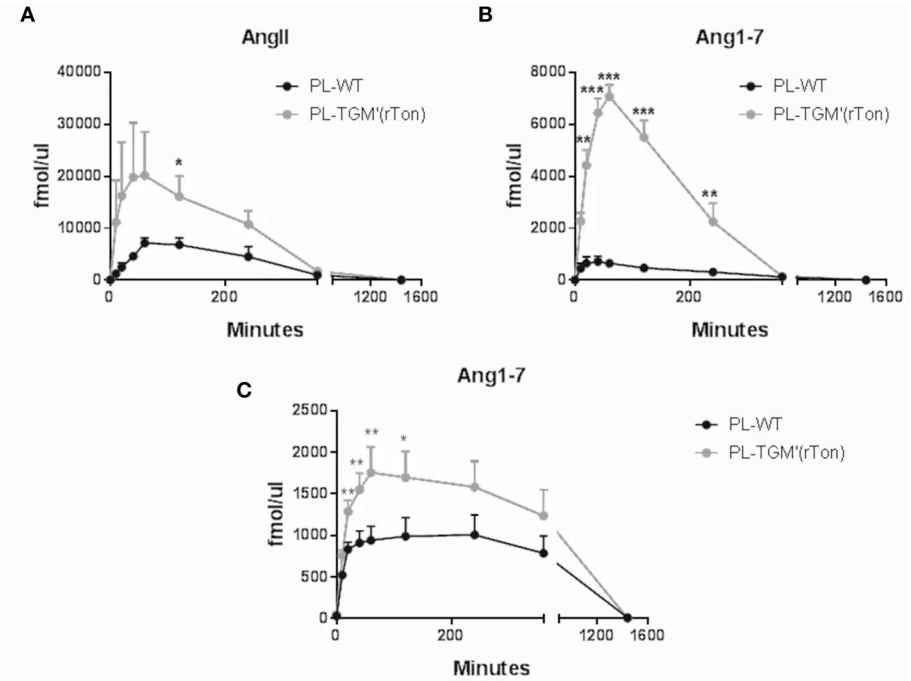


**FIGURE 7 |** Total Ang II and Ang1-7 formation in the plasma of wild type (WT) and transgenic mice overexpressing rat tonin [TGM'(rTon)]: AngII **(A)** and Ang1-7 **(B)** in the plasma (PL) after incubation with AngI as substrate at 0, 10, 20, 40, 60, 120, 240, 360, and 1,440 min by LC-MS/MS ($n = 4$). Ang1-7 **(C)** in the plasma after incubation with AngII ($n = 4$–5). Multiple *t*-test were performed $^*p < 0.05$, time points are represented by means ± SEM.

from the circulation was observed. Secondly, when tonin is in complex with-α1-M, Ang II-forming activity by solid-phase antibody-bound tonin-α1-M complex persisted for over 1 h and even showed an increase after rapid clearance in the early phase. The formation of Ang II was demonstrated despite the complete inhibition of converting enzyme (25).

Blockade of AT1R by losartan resulted in greater BP reduction in the TGM'(rTon) group than the in the WT group; this observation indicates that AT1R signaling was preponderant in the acute control of BP in TGM'(rTon) group. Taken together, increased Ang II due to increased circulating pathway of Ang I-tonin-AngII-AT1R is one of the mechanisms of a net result of higher BP in the TGM'(rTon) mice; however, we cannot be certain that the only route for higher Ang II formation in the circulation is via tonin in the TGM'(rTon) mouse, because tonin can affect the expression of other enzymes such as ACE as we have previously shown (17).

When evaluating the autonomic control of the heart, we found contradictory observations. On the one hand, reduction of HR variability indicates risk for developing cardiovascular morbidity associated with decreased baroreflex sensitivity (alpha index). HR variability is an indirect estimator of autonomic modulation of heart rate and is considered a risk marker in critical illness, particularly in heart failure, and severe sepsis. A reduced HR variability has been found in critically ill patients and has been associated with neuro-autonomic uncoupling or decreased baroreflex sensitivity. On the other hand, reduction of sympathetic modulation together with increase in parasympathetic modulation indicates a cardioprotective state. Increase in sympathetic activity has been positively associated with hypertension in humans and animal models (26, 27). Furthermore, acute injection of tonin *in vivo* caused imbalance of the autonomic control reflected by an increase in the sympathetic tone (28). The question remains: why do TGM'(rTon) mice decrease sympathetic modulation with higher Ang II plasmatic environment? We hypothesize that this surprising observation could be explained by a defective tonin-AngII-catecholamines axis produced in a chronic tonin mileu. Interestingly, parasympathetic tone was increased when we evaluated autonomic control of the heart. This observation can be explained by the increased levels of Ang 1-7 in the heart and in the circulation. The parasympathetic excitatory effect of Ang 1-7 was consistently reported in the literature (29, 30). After losartan injection, sympathetic decrease and parasympathetic increase was observed in both groups; however, statistical significance is only seen in the TGM'(rTon) mice. This finding indicates that AT1R control of the autonomic function in the heart was maintained or even exacerbated. In other words, this observation again indicates that AT1R signaling is predominant in the TGM'(rTon) mice compared to WT group. Sensitization of AT1R could be one of the mechanisms to increase responsiveness of AT1R. Surprisingly, the Ang II levels in the TGM'(rTon) heart were lower compared to the WT group, even though increased tonin activity was observed in the atria and left ventricle as we previously described (18). The lower Ang II levels could be the result of changes in the dynamics of the

renin-angiotensin system kinetics, favoring the formation of Ang 1-7 from Ang II. Therefore, Ang II formation is rapidly broken down to Ang1-7. This observation is consistent with our previous report, where we observed increased ACE2 expression in a mileu of increased tonin activity. Thus, it seems that super activation of ACE2, an Ang1-7 forming enzyme, overrides activation of tonin and other Ang II forming enzymes. This pathway could be one of the mechanisms for a decrease in AngII and an increase in Ang1-7 (31, 32).

When we evaluated the autonomic control of the vasculature neither tonin nor losartan had an impact on the BP variability in both groups. As for the sympathetic modulation at baseline, the TGM'(rTon) group showed similar sympathetic modulation to what was observed in the WT group. Furthermore, in contrast to what we observed in the autonomic modulation of the heart, the TGM'(rTon) group responded to AT1R blockade in a opposite fashion than the WT group; in other words, the AT1R was not as responsive as in the WT group for the autonomic control of the vasculature. This observation could be explained by the observation of AT1R desensitization by Cardoso et al. (19). In their work, injection of Ang II was not able to increase BP in TGM(rTon) when compared to WT. Therefore, a desensitized AT1R was overridden by the other receptors that work in a opposite manner to control the autonomic function of the vasculature; thus a net result of increase sympathetic modulation was observed in the TGM'(rTon).

In summary, TGM'(rTon) mice presented indicators of lower risk for vascular morbidity compared to WT mice. However, our findings presented an unclear prognosis for diseases that affect the heart. HR variability in TGM'(rTon) mice indicates high risk of morbidity, and sympathetic and parasympathetic modulation indicate low risk of morbidity. One of the underlying mechanisms for the protective prognosis may rely on the Ang1-7 effect. AT1R signaling properties have changed in different fashion in the heart compared to the vasculature in the TGM'(rTon) mice. This observation needs further study as well as understanding the mechanism of impairment that may have occurred in the tonin-AngII-catecholamines axis.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of UNIFESP CEDEME Committee of Ethics in Research. The protocol was approved by the CEP committee of Ethics in Research.

## AUTHOR CONTRIBUTIONS

ZJ, RY, IW, AR, and LS performed experiments. ZJ, FdS, MYI, and MF evaluated data. ZJ and MYI wrote the manuscript. DC, MCI, JP, and ZJ conducted the study. All authors read and approved the final version of the manuscript.

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REVIEW
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# Discovery of Pathologic GPCR Aggregation

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The family of G-protein-coupled receptors (GPCRs) is one of the most important drug targets. Mechanisms underlying GPCR activation and signaling are therefore of great pharmacologic interest. It was long thought that GPCRs exist and function as monomers. This feature was considered to distinguish GPCRs from other membrane receptors such as receptor tyrosine kinases or cytokine receptors, which signal from dimeric receptor complexes. But during the last two decades it was increasingly recognized that GPCRs can undergo aggregation to form dimers and higher order oligomers, resulting in homomeric and/or heteromeric protein complexes with different stoichiometries. Moreover, this protein complex formation could modify GPCR signaling and function. We contributed to this paradigm shift in GPCR pharmacology by the discovery of the first pathologic GPCR aggregation, which is the protein complex formation between the angiotensin II AT1 receptor and the bradykinin B2 receptor. Increased AT1-B2 heteromerization accounts for the angiotensin II hypersensitivity of pregnant women with preeclampsia hypertension. Since the discovery of AT1-B2, other pathologic GPCR aggregates were found, which contribute to atherosclerosis, neurodegeneration and Alzheimer's disease. As a result of our findings, pathologic GPCR aggregation appears as an independent and disease-specific process, which is increasingly considered as a novel target for pharmacologic intervention.

**Keywords: G-protein-coupled receptor, oligomerization, preeclampsia, atherosclerosis, Alzheimer's disease, neurodegeneration, biased agonist, beta-arrestin**

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## INTRODUCTION

G-protein-coupled receptors (GPCRs) constitute one of the largest gene families in the human genome, and provide the target for about 20–30% of all drugs currently on the market. Since the discovery of rhodopsin and the beta-adrenergic receptor as first GPCRs (1, 2), currently more than 800 different genes are classified as members of the GPCR family. The pharmacologic and biologic importance of this huge receptor family led to enormous efforts world-wide to delineate activation and signaling mechanisms of GPCRs. A common theme of GPCRs is their seven membrane-spanning domain structure (3), which makes this type of protein a versatile platform for sensing of a panoply of different signals and stimuli including light, stress, hormones, peptides, proteins, ions, volatile odorants, tastants, and mechanical forces.

The seven membrane-spanning domain structure distinguishes GPCRs from other membrane receptors, which are, e.g., single-pass membrane proteins such as tyrosine kinase receptors or cytokine receptors (4). Consequently, major differences between GPCRs and these non-GPCR membrane receptors were found. The major distinguishing feature is that GPCRs signal through activation of heterotrimeric G-proteins, which gives the name to this class of receptors (5, 6).

Mechanistic studies of GPCR proteins focused on the identification of features, which account for GPCR-mediated G-protein activation. A major understanding came from experiments with receptor-derived peptides, which encompass segments of the second, third or fourth cytoplasmic domain of a GPCR and are capable to activate G-proteins independently of the presence of the entire transmembrane-spanning GPCR (7, 8). Other studies with purified receptor preparations found that interaction of a single GPCR with a single G-protein was a sufficient cause for G-protein activation (9, 10). Consequently, based on a 1:1 stoichiometry between a GPCR and a heterotrimeric G-protein, it was concluded that GPCRs function as monomers, and this feature distinguishes GPCRs from other membrane receptors such as tyrosine-kinase receptors or other monotopic transmembrane receptors, which require dimerization for receptor activation and signaling (4).

In contrast to these apparently conclusive data, many researchers, who worked with purified GPCRs, found protein aggregation of GPCRs *in vitro*, by gel filtration and on SDS-PAGE, and cooperative effects in various experimental settings. All these data could be interpreted as evidence for a dimeric/oligomeric assembly of GPCRs under certain experimental conditions. The first thematic study on GPCR dimerization was published in 1996 by the group of Michel Bouvier, who reported the functional characterization of GPCR dimers formed of beta-2-adrenergic receptors expressed in Sf9 cells (11). This was the beginning of a new era, and many other researchers started to report their "forbidden" findings of GPCR dimerization with transfected and endogenous receptors. But the question of relevance of GPCR dimerization still remained because most studies were performed with overexpressed receptors. There was the identification of the GABA(B) receptor (GABA-B; GABBR) heterodimer composed of GABBR1 and GABBR2, which as a class C GPCR relies on receptor dimerization for protein folding and function (12, 13). According to the A-F classification scheme of GPCRs, class C receptors contain a huge N-terminal clam-shaped domain with almost 600 amino acid residues (14, 15). This extracellular region of GABA-B receptors forms a dimer even in the absence of the transmembrane domain-containing protein core (15). Therefore, it was concluded that GABBR is a unique example of a GPCR that requires heterodimerization for functionality whereas for most other GPCRs, dimerization is a redundant process and has no obvious *in vivo* relevance.

Beginning from 1999, we initiated an independent research project on GPCR homo- and heterodimerization, which was based on our previous unpublished data in the early nineties. And we discovered the first pathologically relevant GPCR hetero(di)mer, which is the protein complex formed between the vasopressor angiotensin II AT1 receptor and the vasodepressor bradykinin B2 receptor (16, 17). We found that increased protein complex formation between AT1 and B2 on circulating blood cells and vessels of patients with preeclampsia hypertension contributes to the angiotensin II hypersensitivity of this severe pregnancy-specific complication (17, 18). Our findings were intensely disputed (19), and final acceptance of our findings only came after more than a decade of scientific discussions (20). Meanwhile, our findings contributed to a paradigm shift in GPCR pharmacology, which is now on the verge to lead to new pharmacologic treatment approaches (21). The following article briefly overviews the timeline of the discovery of pathologic GPCR aggregation (**Figure 1**).

## FIRST EVIDENCE OF AN INTERACTION BETWEEN THE BRADYKININ B2 RECEPTOR AND THE ANGIOTENSIN II AT1 RECEPTOR IN THE EARLY 1990s (1990-1994)

Our discovery of a functional AT1-B2 receptor protein complex began in the early 1990s at the Institute of Pathobiochemistry of the University of Mainz (Germany), where our key findings were made during a project on the protein purification of the bradykinin B2 receptor from native human skin fibroblasts applying ligand and antibody affinity chromatography. This work was performed in frame of a common project between U. Quitterer and S. AbdAlla, who led the protein purification group. He found by amino-terminal protein sequencing that enrichment of the bradykinin B2 receptor protein, yielded co-enrichment of the angiotensin II AT1 receptor. At the same time, these data were complemented by U. Quitterer's functional studies, which showed that angiotensin II could induce a conformational change at the bradykinin B2 receptor with a shift of the B2 receptor from a high affinity to a low affinity binding state for the agonist bradykinin. This affinity shift could reflect a conformational change imposed by the activated AT1 receptor on the associated B2 receptor with subsequent G-protein activation and uncoupling of the B2 receptor, as a process which is known of other GPCRs to decrease agonist affinity (22, 23). "We discovered receptor heterodimers," was the immediate interpretation of S. AbdAlla, who came from the Heinrich-Pette-Institute, Hamburg (Germany), where he had worked on dimerization of interleukin-2 and interleukin-2 receptors. But when we presented our data and the idea of AT1-B2 receptor dimerization to the Head of the Institute, he opposed this concept because in the GPCR field, protein dimerization does not exist. This was the end of our project on AT1-B2 receptor dimerization in Mainz (**Figure 1**).

## DISCOVERY OF AT1-B2 AS THE FIRST PATHOLOGIC GPCR HETEROMER (1999-2001)

After completion of the doctorate at the University of Mainz in 1994, and a postdoctorate at Roche Bioscience (Palo Alto, CA, USA) in 1995, U. Quitterer moved to the Institute of Pharmacology at the University of Wuerzburg (Germany) to continue research on GPCRs in 1996. Only 1 year later, in 1997, she re-established contacts with S. AbdAlla, who at that time became professor of biochemistry at the newly founded Genetic

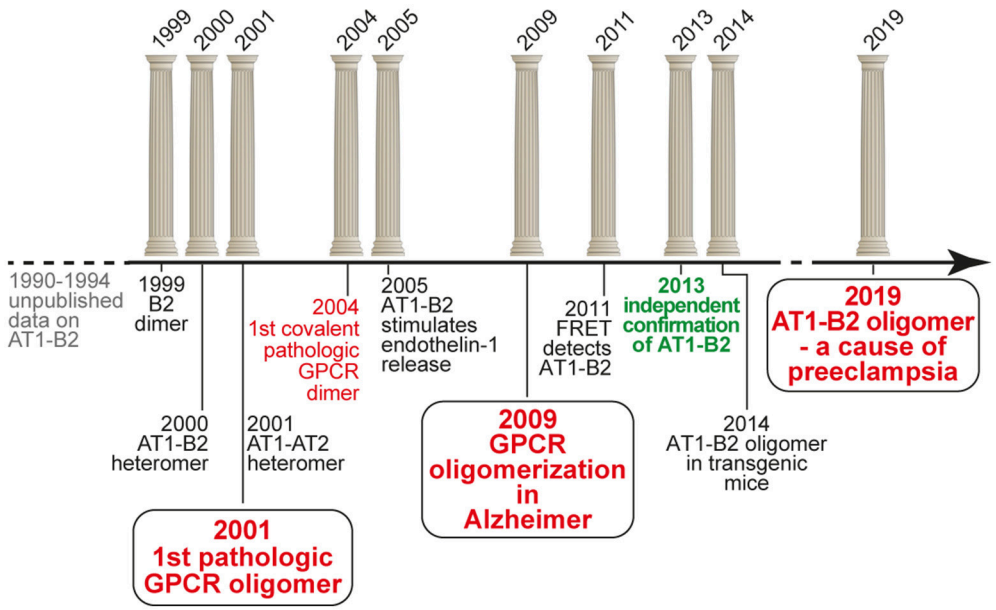


**FIGURE 1 |** Timeline of the discovery of pathologic GPCR aggregation.

Engineering and Biotechnology Research Institute (GEBRI) in Alexandria, Egypt. We continued our work on AT1-B2 receptor heterodimerization, and during our studies, we discovered and characterized functional bradykinin B2 receptor homodimers in 1999 (24). The project on GPCR dimerization was conducted together with Dr. Heinz Lother, who invited S. AbdAlla as a visiting scientist into his labs at the Heinrich-Pette-Institute, Hamburg (Germany). After we had opened the path for the concept of receptor dimerization in the kinin field in 1999, the study on AT1-B2 receptor heteromerization was finished in a short time, and in the year 2000, we published the discovery of AT1-B2 receptor heteromerization in Nature [**Figure 1**; (16)].

The identified heteromeric protein complex between AT1 and B2 receptors was functional, and led to enhanced angiotensin II AT1-mediated G-protein activation and signaling. The B2-mediated sensitization of the AT1 receptor-mediated response was independent of B2 receptor stimulation with the agonist bradykinin because a B2 receptor mutant with defective bradykinin binding still enhanced the AT1-stimulated G-protein activation and signaling. In contrast, a B2 receptor mutant with defective G-protein activation due to a mutation in the DRY motif in the 2nd intracellular loop of the B2 receptor was incapable to enhance AT1-stimulated signaling (16). These data are compatible with the notion that activation of the AT1 receptor by angiotensin II imposes a conformational change onto the associated unstimulated B2 receptor, which in turn adopts an active conformation capable to promote GDP-GTP exchange of the B2-coupled G-protein with subsequently enhanced signaling (**Figure 2**). The heteromeric AT1-B2 protein complex thus could constitute a platform, which enables enhanced G-protein activation (**Figure 2**).

The study of the AT1-B2 heteromer was complemented by our discovery of the AT1-AT2 receptor complex, which is a prototype of an inhibitory GPCR interaction [**Figure 1;** (25)]. In contrast to the B2 receptor, which enhances the angiotensin II AT1-stimulated G-protein activation (16, 17), the angiotensin II AT2 receptor inhibits the activation of the AT1 receptor by direct protein interaction (25). Thus, GPCR dimerization is capable to modulate the activation state of the dimerizing partner by direct interaction. As a consequence, dimerization could enhance or dampen the guanine nucleotide exchange factor-like function of a GPCR toward the heterotrimeric G-protein.

A major point in the field of GPCR dimerization and oligomerization always was the question about physiologic and pathophysiologic relevance. Most experiments on GPCR dimerization were made with transfected receptors and/or with cultured cells. Therefore, our focus immediately shifted to the question: Does AT1-B2 heteromerization occur *in vivo*? We knew that angiotensin II hypersensitivity was a major feature of preeclampsia hypertension (26, 27), which is the most frequent pregnancy-related complication with no cure. To study the role of AT1-B2 heteromerization in preeclampsia, S. AbdAlla initiated a collaboration with the Medical Research Center (MRC) at Ain Shams University, Cairo. The head of MRC, Prof. Adel el Missiery, strongly supported the project because preeclampsia has a high frequency among women in Egypt. Our study found that AT1-B2 contributes to the angiotensin II hypersensitivity of women with preeclampsia (17). This publication of "Receptor double-trouble in preeclampsia" (17, 18) marks the discovery of the first pathologic GPCR oligomer (**Figure 1**).

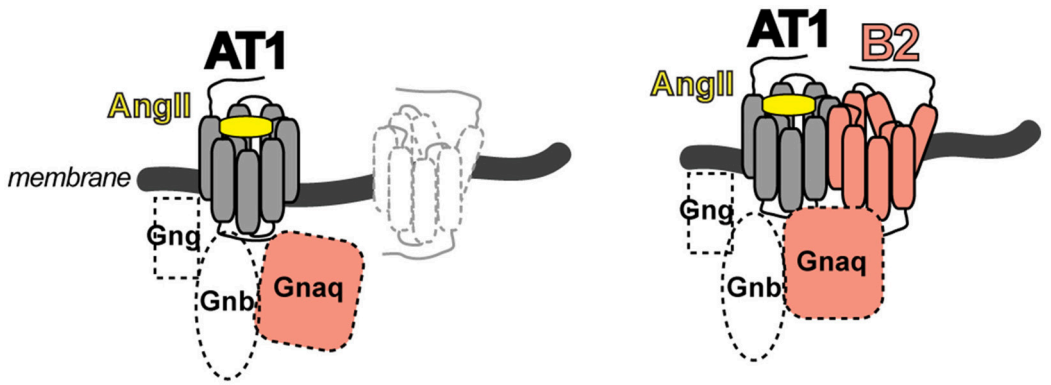


**FIGURE 2 |** Scheme of the AT1-B2 heteromer, which forms a platform for enhanced G-protein activation **(right)** compared to a monomeric AT1 receptor **(left)**.

In this study, the AT1-B2 receptor heteromer was identified *in vivo*, after covalent stabilization with a cleavable cross-linker followed by affinity enrichment with AT1 receptor-specific antibodies and subsequent immunoblot detection of the co-enriched B2 receptor. This approach detected a significantly higher content of AT1-B2 heteromeric complexes on platelets and omental vessels from preeclampsia patients compared to biopsy specimens from uncomplicated pregnancies (17). Reciprocal experiments with enrichment of B2 and detection of co-enriched AT1 receptor gave similar results, and confirmed the disease-related AT1-B2 receptor aggregation in preeclampsia (17).

Our study also shows that augmented AT1-B2 receptor protein complex formation is disease-relevant and contributes to the well-established angiotensin II hypersensitivity in preeclampsia with enhanced angiotensin II-stimulated calcium signaling of platelets isolated from preeclamptic women (17, 26, 27). Sensitization of the angiotensin II-stimulated response is mediated by the AT1-B2 receptor heteromer not only in transfected cells but also in patients with preeclampsia (17). This conclusion was proved by domain-specific antibodies, which shielded the connecting loop between membrane domains III-IV of the bradykinin B2 receptor. With these antibodies, we found that the bradykinin B2 receptor protein is involved in angiotensin II-stimulated AT1-B2-mediated G-protein activation on omental vessels isolated from preeclamptic patients. Notably, the angiotensin II-stimulated response was largely blocked by these domain-specific antibodies to the connecting loop between membrane domains III-IV of the bradykinin B2 receptor whereas control antibodies had no effect (17). Together these findings complement our previous data on AT1-B2, which show that mutation of the DRY motif in the same connecting loop between membrane domains III-IV of the B2 receptor abolishes the B2-mediated sensitization of the angiotensin II-stimulated response triggered by the AT1-B2 heteromer (16).

In subsequent years, the pathophysiologic importance of aberrant AT1-B2 aggregation was supported by additional studies. Notably, AT1-B2 receptor heteromerization was found to enhance endothelin-1 release [**Figure 1**; (28)], which now is known as a major factor contributing to symptoms of preeclampsia (29).

## DISCOVERY OF COVALENTLY STABILIZED AT1 RECEPTOR DIMERS WITH PATHOLOGIC RELEVANCE FOR CARDIOVASCULAR DISEASE AND ATHEROSCLEROSIS IN 2004

After elucidation of the pathologic relevance of AT1-B2 receptor heteromerization, we continued our search for aberrant GPCR dimerization as contributor to pathomechanisms of disease. In view of our previous studies, we focused on disease states with angiotensin AT1 receptor hypersensitivity and found an increased content of covalently stabilized AT1 receptor dimers on monocytes isolated from the peripheral blood of patients with hypertension (30). This finding of stable AT1 dimerization on monocytes of hypertensive patients in the year 2004 marks the discovery of the first covalently stabilized GPCR dimer in human pathology [**Figure 1**; (30)]. The covalent stabilization of AT1 receptor dimers was unprecedented and mediated by an exaggerated intracellular transglutaminase factor XIIIA activity in patients with increased cardiovascular risk factors (30).

The crosslinking activity of the intracellular factor XIIIA does not rely on thrombin, which activates circulating factor XIIIA in plasma by proteolytic cleavage as part of the blood clotting cascade. Instead, the transglutaminase activity of intracellular factor XIIIA could be activated by an altered cellular ion homeostasis, increased cytosolic calcium and/or oxidative stress (30). All these causes of intracellular factor XIIIA transglutaminase activation are promoted by cardiovascular risk factors such as hypertension, hypercholesterolemia, and atherosclerosis. In concert with induction of factor XIIIA gene expression and protein in monocytes by cardiovascular risk factors (30), the consecutively exaggerated intracellular factor XIIIA activity accounts for covalent stabilization of AT1 receptor dimers, which are triggered by increased circulating angiotensin II levels in patients "at the onset of atherosclerosis" (30–32).

Our functional studies show that covalent stabilization of AT1 receptor dimers accounts for angiotensin II hypersensitivity *in vivo* and contributes to the atherogenic function of the AT1 receptor on monocytes of patients with cardiovascular disease and hypertension (30). Apart from disease relevance, our studies also confirm our previously established concept of modulation of G-protein activation by receptor dimerization: covalent stabilization of AT1 receptor homodimers creates a receptor platform, which enables efficient G-protein activation compared to AT1 receptor monomers and/or dissociable AT1 receptor complexes (30–32).

## MOVE TO ETH ZURICH AND DISCOVERY OF PATHOLOGIC GPCR AGGREGATION IN ALZHEIMER'S DISEASE (2006–2009)

Our work on the discovery of pathologic GPCR aggregation was highly recognized by the international scientific community. The initial publication in Nature in the year 2000 (16) was ranked top 1% in the scientific field according to ISI web of science. Meanwhile this publication is cited more than 500 times. The discovery of AT1-B2 heteromerization as the first pathologic GPCR aggregation also became international textbook knowledge and was included in the "Blue Bible," which is the nickname of the 12th edition of Goodman Gilman's The Pharmacological Basis of Therapeutics (33). Another highlight was the Wenner Grenn Symposium on GPCR dimerization in Stockholm in 2004. Kjell Fuxe from Karolinska Institute invited major players in the field of GPCR dimerization to Stockholm. During the meeting, U. Quitterer had the chance to dispute the existence and pathologic relevance of GPCR aggregation on stage with Robert Lefkowitz, who later received the Nobel Prize in Chemistry, in the year 2012, for studies on G-protein-coupled receptors. The discussion was moderated by Michel Bouvier, who supported U. Quitterer's statements and conclusions. With a high international reputation, the President of ETH Zurich, Prof. Olaf Kübler, offered U. Quitterer at the age of 39, the Chair of Molecular Pharmacology at ETH Zurich. She accepted the offer in summer 2005, and was nominated full professor and Chair of Molecular Pharmacology by the ETH Counsil at the end of 2005. We moved into newly refurbished ETH labs at the beginning of 2006.

At ETH Zurich, we continued our work on pathologic GPCR aggregation. Stimulated by ongoing discussions on existence and mechanisms of GPCR aggregation, our focus shifted to Alzheimer's disease (AD). We reasoned that GPCR aggregation should readily be detectable in a typical protein aggregation disease. We searched for covalently stabilized GPCR aggregates as a consequence or cause of Alzheimer senile plaques, because in a previous study, we found covalently stabilized GPCR aggregates formed of the angiotensin II AT1 receptor in atherosclerosis (30), which is also a "plaque-forming" disease. With this concept in mind, we discovered SDS-stable aggregates of the angiotensin II AT2 receptor as a characteristic feature of AD patient biopsy specimens isolated from prefrontal cortex (34, 35). Covalent aggregation of AT2 receptor oligomers is disease-specific for AD because related GPCRs such as the AT1 receptor are not aggregated in AD brains (34). The identification of covalently cross-linked AT2 receptor oligomers in brains of AD patients extends the pathologic relevance of aberrant GPCR aggregation to neurodegenerative diseases (**Figure 1**).

Why does the AT2 receptor form high molecular weight aggregates in brains of AD patients? We investigated the underlying mechanism and found that AT2 receptor aggregation in AD is a disease-specific process with two consecutive cross-linking steps, i.e., mediated by (i) reactive oxygen species (ROS), and (ii) transglutaminase. Initially, we aimed to deduce the AD-specific mechanism of AT2 aggregation in the transgenic Tg2576 AD model with neuron-specific expression of $APP^{Swe}$, which is a mutant of the amyloid precursor protein (APP) isolated from a Swedish family with familial AD (36). Tg2576 AD mice reproduce major AD features such as symptoms of neuronal degeneration, cognitive impairment and beta-amyloid (Abeta) plaques in the hippocampus starting at an age of 12 months. However, Tg2576 mice did not develop pathologic AT2 oligomers but only showed the initial ROS-dependent AT2 crosslinking step, which leads to stable AT2 dimers (34). In search for the mechanism of pathologic AT2 aggregation in AD, we realized that aged Tg2576 AD mice are largely devoid of neuronal loss, which is a major characteristic of AD patients (34). To enhance the process of neurodegeneration, we subjected aged Tg2576 AD mice to chronic unpredictable mild stress (CUMS), which mimics psychosocial stress as an established risk factor for AD in patients (37). Chronic unpredictable mild stress augmented the hippocampal transglutaminase activity of Tg2576 mice, and triggered the final transglutaminase-mediated crosslinking of oxidized AT2 dimers to AD-related and pathologic AT2 oligomers. Concomitantly, chronic unpredictable mild stress increased hippocampal Abeta accumulation, promoted neurodegeneration-enhancing PHF tau phosphorylation and induced overt neuronal loss in the hippocampus of Tg2576 mice.

Cellular accumulation of covalently cross-linked AT2 receptor oligomers is pathologic and contributes to neurodegeneration and neuronal loss, which are hallmarks of AD brains. The pathologic AT2 receptor aggregates promote neuronal deterioration in AD by sequestration and inhibition of cognition-enhancing Gq/11-mediated-signaling stimulated, e.g., by the M1-cholinoceptor (34, 35). In agreement with this notion, inhibition of AT2 receptor oligomerization retarded symptoms of neurodegeneration in the experimental AD model (35). Our ongoing studies aim to translate these experimental findings into a therapeutic strategy to restore the function of the neuroprotective AT2 receptor and halt disease progression in AD models and patients (34, 35).

## DIFFICULTIES TO DETECT AT1-B2 RECEPTOR HETEROMERIZATION

Immediately after the discovery of covalent AT2 receptor aggregation in AD (34, 35), the research on neurodegenerative GPCR aggregation in Alzheimer's disease was interrupted by

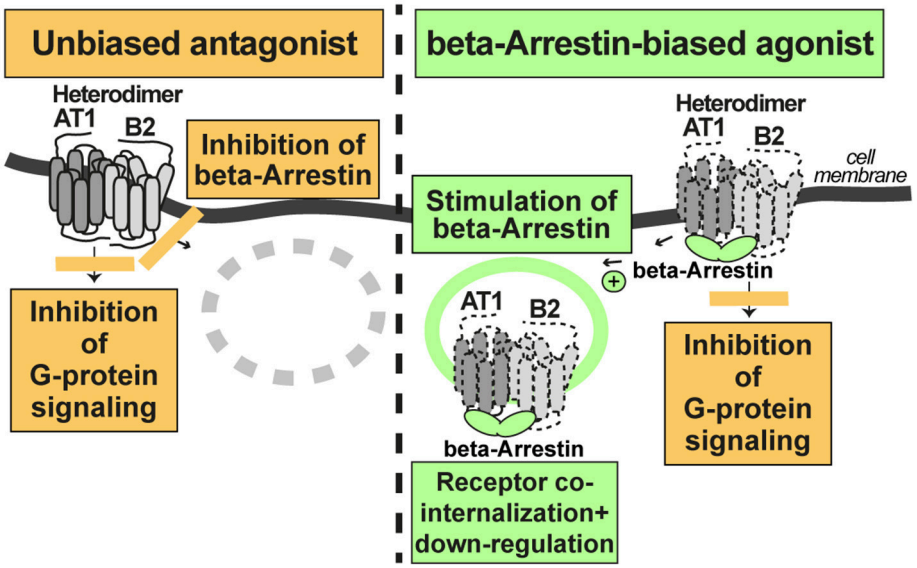


**FIGURE 3 |** A beta-arretin-biased agonist targets the AT1-B2 heteromer and promotes AT1-B2 down-regulation **(right)**. The unbiased AT1 antagonist does not promote AT1-B2 down-regulation **(left)**.

a publication, which reported "lack of evidence for AT1R/B2R heterodimerization" (19). When we saw this and similar other publications (19, 38), we realized that we had to redirect our research toward the AT1-B2 heteromer.

In search for causes, which could account for the failure to detect the AT1-B2 receptor heteromer in transfected cells, we focused on the role of chaperones because chaperones are critically influenced by variable cell culture conditions (39). Whole genome microarray gene expression profiling identified the chaperone, calreticulin, as an important factor required for efficient B2 receptor maturation and AT1-B2 heteromerization (39). Loss of essential chaperones during *in vitro* cell culture could be a cause for failure to detect AT1-B2 (39). To overcome limitations of *in vitro* cell culture, we established cell expansion conditions, which mimic the *in vivo* environment because our studies mainly focus on GPCR aggregation under pathologic conditions *in vivo*. The *in vivo* cell expansion model applies NOD.Scid mice for expansion of cultured cells. Microarray gene expression analysis documented that the expression of essential chaperones is restored upon *in vivo* expansion of cultured cells in NOD.Scid mice (40).

Another critical factor is the intrinsic pathologic nature of the AT1-B2 heteromer, which could contribute to further difficulties to detect AT1-B2 in transfected cells. Expression of functional AT1-B2 heteromers leads to exaggerated calcium signaling with consecutively reduced cellular growth rates, premature senescence, and ultimately calcium-induced cell death (41, 42). Consequently, only slow-growing cells with low AT1-B2 expression level will survive. Under these conditions, there is a high risk that the cell culture dish is rapidly overgrown by fast dividing, non-transfected cells and/or cells expressing either AT1 or B2. This scenario is particularly relevant for *in vitro* cultured cells due to the loss of essential pro-survival factors, e.g., components of the pro-survival ERK pathway (43). Control experiments, which control for co-expression of AT1-B2 at the single cell level are necessary to identify and overcome such problems.

At present, it is not clear, why several groups failed to detect AT1-B2 receptor heteromerization in transfected cells. While we addressed some possible issues, a definite answer is not possible because experiments in these publications were performed with batches of cells, and precise biochemical and/or histological control experiments are lacking, e.g., the control for co-expression of AT1 and B2 at the single cell level, co-immunoenrichment studies and immune-fluorescent microscopy of receptor proteins (19, 38).

Apart from the above-mentioned causes, a high cellular expression level of the transfected GPCRs, notably the AT1 receptor, could become another factor of failure to detect AT1-B2 heteromerization. The documented reporting of constitutive AT1 receptor homomerization could be indicative of high AT1 receptor expression at the single cell level (19). High AT1 receptor expression at the single cell level could be attributed, e.g., to the SV40 large T-antigen-driven DNA replication at high copy number in COS cells (19). A high cellular GPCR expression level could be a critical factor because it could favor the formation of receptor homomers over receptor heteromers (44), i.e., the homomeric interaction between AT1-AT1 could become dominant and prevent the heteromeric interaction of AT1 with B2. As mentioned above, essential control experiments were not performed, e.g., the visualization of AT1 and B2 receptor proteins by immunoblot to detect protein aggregation and/or immaturely folded proteins (19, 38).

Concomitantly with the identification of critical parameters required for AT1-B2 heteromer formation in cells, we established a versatile method for detection of the AT1-B2 receptor heteromer by confocal FRET imaging, which is capable to detect protein-protein interactions in close proximity at a distance of <10 nm (45). We added a signal peptide to the AT1 receptor-cerulean fusion protein, which otherwise barely reached the cell surface under standard cell culture conditions. With this approach, we demonstrated a high FRET efficiency of 24.7% for the interaction between AT1-Cerulean and B2-eYFP (45). Thus, we had proven that the AT1-B2 receptor heteromer exists in cultured cells, and can readily be detected by a standard FRET-based method, which is usually applied for detection of GPCR dimerization in cells (**Figure 1**). In addition, we documented functionality of AT1-B2 receptor heteromerization, which accounts for receptor co-internalization upon stimulation with angiotensin II (45).

Taken together, the question, why several groups were unable to detect AT1-B2 receptor heteromerization in cells, cannot be answered because specific control experiments were not performed (19, 38). In contrast, different groups in different laboratories worldwide demonstrated that AT1-B2 receptor heteromerization can be detected by different biochemical and biophysical methods (16, 17, 20, 45).

## INDEPENDENT CONFIRMATION OF AT1-B2 HETEROMERIZATION IN THE YEAR 2013, AFTER MORE THAN A DECADE OF SCIENTIFIC DISCUSSIONS

While our own work established the methodology for detection of functional AT1-B2 heteromerization, final acceptance of AT1-B2 by the scientific community occurred in 2013, after more than a decade of scientific discussions (**Figure 1**). In 2013, the group of Louis Luttrell, a former member of the Nobel Prize-winning Lefkowitz lab, reproduced our findings of AT1-B2 receptor heteromerization (20). With this publication, years of ongoing scientific discussions were finally resolved. The publication not only confirmed our initial findings but also opened the path to a new pharmacologic targeting approach of the AT1-B2 heteromer by the beta-arrestin-biased AT1 agonist, SII (Sar1,Ile4,Ile8-AngII) (20).

According to the concept of beta-arrestin-biased agonism, stimulation of the AT1 receptor by a beta-arrestin-biased agonist inhibits G-protein-stimulated signaling but promotes the recruitment of beta-arrestin to the activated AT1-B2 receptor complex. As a consequence of beta-arrestin recruitment, the AT1-B2 receptor complex undergoes co-internalization and subsequent down-regulation (**Figure 3**).

In contrast, a classic, unbiased AT1 receptor antagonist such as losartan, blocks both, AT1-stimulated signaling, and beta-arrestin recruitment to the receptor. Consequently, the AT1-B2 receptor complex is not down-regulated (**Figure 3**). All currently approved AT1 antagonists are unbiased and contraindicated in preeclampsia because they cross the placental barrier and promote fetal malformations in pregnancy (46). Experimental beta-arrestin-biased agonists such as SII or TRV027 are peptides (47, 48) and not expected to cross the placental barrier. Therefore, such beta-arrestin-biased AT1 agonists could be envisaged as a future therapy for preeclampsia. Notably, beta-arrestin-biased AT1 agonists target the AT1-B2 receptor heteromer and promote AT1-B2 heteromer co-internalization and subsequent down-regulation (20, 49, 50).

## OUTLOOK

Our ongoing studies at ETH Zurich currently address the feasibility to target the AT1-B2 heteromer and other pathologic GPCR aggregates by beta-arrestin-mediated down-regulation. To study such an approach, we generated transgenic models with increased AT1-B2 heteromerization. With these transgenic models, the *in vivo* relevance of AT1-B2 receptor heteromerization was demonstrated (50). Our data show that increased vascular AT1-B2 levels are a sufficient cause for preeclampsia symptoms in pregnant mice [**Figure 1**; (50)]. Moreover, symptoms of preeclampsia in AT1-B2-transgenic mice can be prevented by beta-arrestin-mediated down-regulation of AT1-B2 (50). Thus, targeting of the AT1-B2 heteromer by beta-arrestin-mediated down-regulation is feasible (50). And the initial identification of pathologic GPCR aggregation more than a decade ago is currently being translated into a new pharmacologic treatment approach.

## AUTHOR CONTRIBUTIONS

UQ and SA wrote, read, and approved the final version of the manuscript.

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# SERPINs—From Trap to Treatment

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Excessive enzyme activity often has pathological consequences. This for example is the case in thrombosis and hereditary angioedema, where serine proteases of the coagulation system and kallikrein-kinin system are excessively active. Serine proteases are controlled by SERPINs (serine protease inhibitors). We here describe the basic biochemical mechanisms behind SERPIN activity and identify key determinants that influence their function. We explore the clinical phenotypes of several SERPIN deficiencies and review studies where SERPINs are being used beyond replacement therapy. Excitingly, rare human SERPIN mutations have led us and others to believe that it is possible to refine SERPINs toward desired behavior for the treatment of enzyme-driven pathology.

**Keywords: SERPIN (serine proteinase inhibitor), protein engineering, bradykinin (BK), hemostasis, therapy**

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## INTRODUCTION

Serine proteases are the "workhorses" of the human body. This enzyme family is conserved throughout evolution. There are 1,121 putative proteases in the human body, and about 180 of these are serine proteases (1, 2). They are involved in diverse physiological processes, ranging from blood coagulation, fibrinolysis, and inflammation to immunity (**Figure 1A**). The activity of serine proteases is amongst others regulated by a dedicated class of inhibitory proteins called SERPINs (serine protease inhibitors). So far, 37 SERPINs have been identified in the human body. Thirty of these are functional protease inhibitors (7, 8). Human SERPINs are subdivided into 9 subgroups (clade A to I) based on their phylogenetic relationship (9). It is noteworthy that SERPINs are generally capable of inhibiting multiple enzymes. Rather than being considered promiscuous, they appear selective in the sense that the targeted enzymes are often part of a conserved biological mechanism. This for instance is the case for antithrombin (AT), that inhibits multiple enzymes all involved in the coagulation system.

### Structure

SERPINs generally consist of ∼ 350–400 amino acid residues, for example, α1-antitrypsin (α1AT) has 394 amino acids. Their molecular weight varies between 40 and 100 kDa due to differences in their glycosylation profile. They are highly expressed in the liver, but are expressed ubiquitously throughout the body (10). SERPINs fold into 7–9 α helices and 3 β-sheets (11). The core structure of SERPINs is highly conserved, which is important for their function. **Figure 1B** shows the structure of a native SERPIN. Native SERPINs have two main features; (1) five-stranded β-sheet A (s1A, s2A, s3A, s5A, and s6A) are positioned in the middle of the molecule and (2) a flexible reactive center loop (RCL) is positioned on top of the molecule. The RCL contains an enzyme cleavage site (P1-P1′), denoted accordingly to the nomenclature of Schecter and Berger (12), which is located near the C-terminus of the protein sequence.

## Mechanism of Action

SERPINs inhibit target enzymes through a conserved mechanism (13), which involves a unique dramatic conformational change. The nature of native (uncleaved) SERPINs is that they are metastable; i.e., not (yet) in their most stable form. When executing their function, SERPINs act as molecular "mousetraps", where the RCL is a "bait" and target proteases are "mice" (14). The inhibition process starts when a protease recognizes the bait and binds to the SERPIN by forming a reversible Michaelis-Menten complex (**Figure 1C**). When the

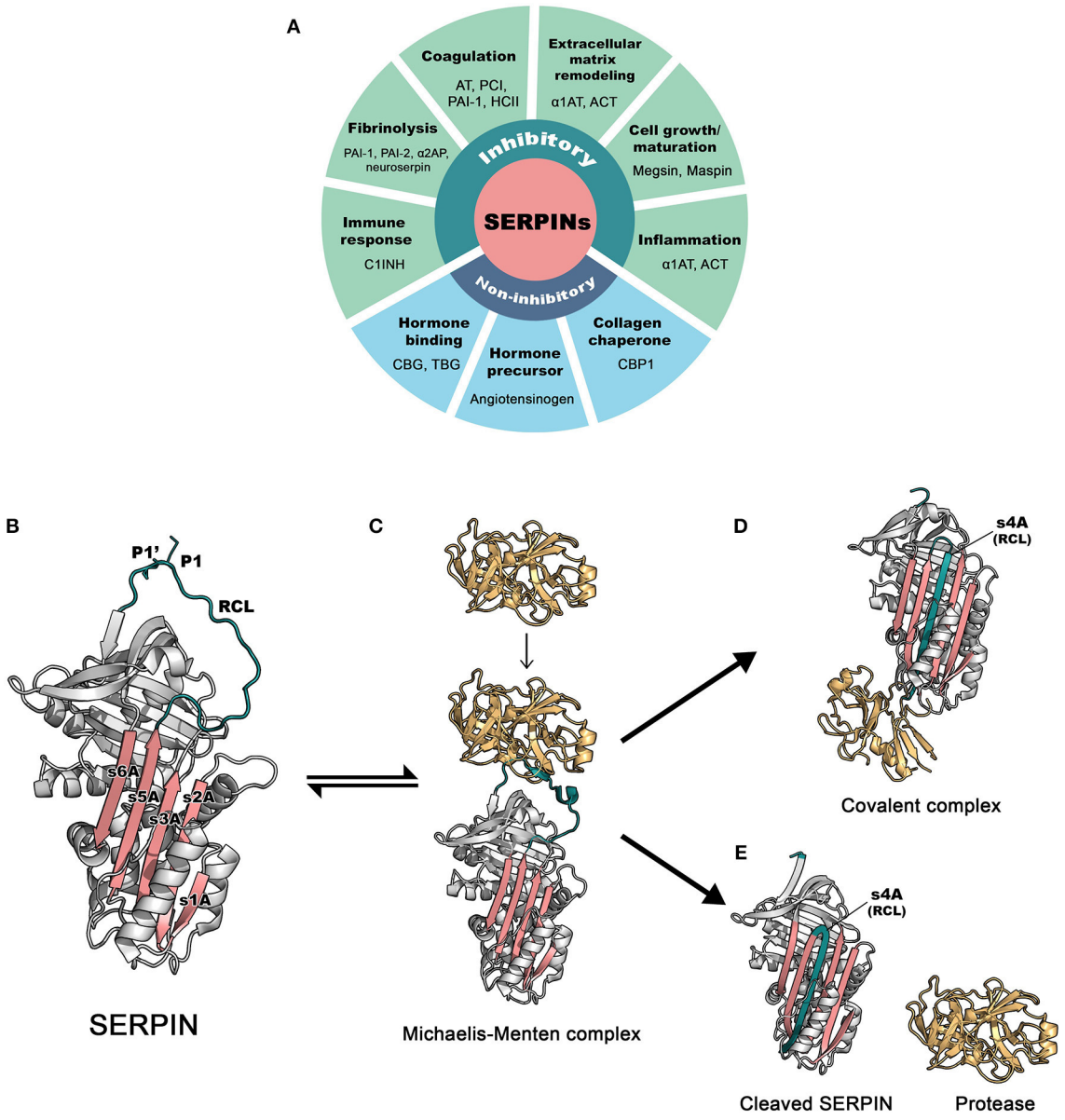


**FIGURE 1 |** The basic functions and inhibitory mechanism of SERPINs. **(A)** Regulatory functions of SERPINs **(B)** The structure of archetype native α1-antitrypsin. The reactive center loop (RCL) is in green, containing a protease cleavage site (P1-P1′). β-sheet A, comprising of 5 strands (s1A, s2A, s3A, s5A, and s6A) is in pink. These two regions serve as main features, which play an important role in the dramatic conformational change that SERPINs undergo during inhibition. The image was made in PyMol using the PDB file code: 1QLP (3). **(C)** Initially, a target protease docks and binds the recognition site, exposed on the RCL. This step leads to formation of the non-covalent Michaelis-Menten complex [PDB code: 1OPH (4)]. **(D)** Upon cleavage at P1-P1′, the SERPIN spontaneously refolds into a hyperstable conformation, where the N-terminal portion of cleaved RCL is inserted between central β-sheet A. This conformational change of the SERPIN results in "trapping" the covalently linked protease into an inactive form [PDB code: 1EZX (5)]. This SERPIN-protease complex will subsequently be eliminated from the circulation. **(E)** In some cases, a SERPIN can act as a substrate, where protease and SERPIN do not remain covalently linked. This results in an active protease that disassociates from the SERPIN, which leaves the SERPIN in a cleaved form [PDB code: cleaved 7API (6)]. AT: antithrombin; PCI, protein C inhibitor; PAI-1, plasminogen activator inhibitor 1; PAI-2, plasminogen activator inhibitor 2; HCII, heparin cofactor II; α1AT, α1-antitrypsin; ACT, antichymotrypsin; α2AP, α2-antiplasmin; C1INH, C1 esterase inhibitor; CBG, Corticosteroid-binding globulin; TBG, Thyroxine-binding globulin; CBP1, collagen-binding protein 1.

docking protease cleaves the bond between P1 and P1′ residues of the SERPIN, it becomes covalently bound to the main chain carbonyl carbon of the P1 residue of the SERPIN. This cleavage event releases the SERPIN from its metastable conformation (i.e., springing the mousetrap). Hereafter, either the SERPIN remains a stable covalent complex with the enzyme or is used as a "substrate". In this latter case, the active enzyme dissociates. In 2000, the first SERPIN-protease complex crystallography structure was unveiled (5), confirming the mousetrap-like mechanism of SERPINs. Upon cleavage of the P1-P1′ bond, the C-terminal loop of the SERPIN RCL inserts into the SERPIN body, between its β-sheet A. This leads to the formation of the s4A strand and to a complete antiparallel β-sheet A. When loop insertion is rapid enough, the enzyme active site becomes distorted and inactivated, leaving the enzyme-SERPIN complex covalently bound (**Figure 1D**) (15, 16). When loop insertion is too slow, the covalent bond is already disrupted before the enzyme active site can be inactivate (4, 17). Now, the SERPIN becomes consumed as a substrate (**Figure 1E**). The ratio between the two possible pathways is expressed as the stoichiometry of inhibition and should be close to 1 (17) for SERPINs to become powerful inhibitors.

## KEY DETERMINANTS FOR SERPIN FUNCTIONALITY

Four features are important for proper SERPIN functionality. Two of these are structural, the other two are sequence-based motifs.

### Reactive Center Loop Mobility

Mobility of the RCL enables loop insertion into β-sheet A after protease cleavage, which is critical for SERPIN stabilization and enzyme inhibition. The N-terminal sequence that precedes the cleavage site (P15-P9), the so-called hinge region, facilitates RCL mobility, and loop insertion (18). Amino acid sequences of alanine-rich hinge region are considerably conserved among inhibitory SERPINs (**Table 1**).

Lawrence et al. created a plasminogen activator inhibitor 1 (PAI-1) mutant library, which contains 15 different amino acid substitutions at P14 of PAI-1. Results demonstrate that substitutions with a charged residue at P14, which is normally a small uncharged residue in most of inhibitory SERPINs, significantly retard the inhibitory function of PAI-1 and convert it to a substrate (17). However, the mutations at P14 do not affect protease recognition (19). Therefore, it demonstrates that an uncharged residue is preferable in the hinge region for a proper loop insertion. Remarkably, hinge regions are less conserved among non-inhibitory SERPINs. This suggests that the conserved sequence of the hinge region is important to SERPINs in order to function as inhibitors.

### Reactive Center Loop Length

The length of the N-terminal portion of the RCL is conserved among the members of SERPIN family (**Table 1**). It has been shown that the length of the RCL critically impacts the kinetic stability of the serpin-protease complex. The length of the RCL, especially the N-terminal portion, should fit the length of β-sheet A to insert in between the sheets during enzyme inhibition. A study by Zhou et al. showed that modifying the RCL length by adding one or two residues dramatically reduced the stability of the complex by up to 1,000,000-fold (20). In contrast, shortening the RCL length by deletion of one or two residues lowered the efficiency of inhibition, but doubled the stability of the complex. Finally, the deletion of more than two residues completely converted the serpin into a substrate.

### Protease Recognition Sequence

In order for a SERPIN to acts as a bait, its RCL contains a sequence motif that is specifically recognized by target enzymes. Interestingly, amino acid sequences adjacent to the cleavage site are highly variable between different SERPINs (**Table 1**). This variation partially explains their different specificities.

Anderson et al. successfully shifted the target specificity of one of the SERPINs, α1AT through mutagenesis from an inhibitor of neutrophil elastase (an extracellular enzyme) into an inhibitor of furin (an intracellular enzyme). The minimal P4-P1 peptide sequence that is required for recognition and an efficient cleavage by furin is -Arg (R)-X-X-R- (21). Hence, Anderson and co-workers replaced the P4 and P1 residues of the RCL of α1AT, changing it from $^{355}$AIPM$^{358}$ to $^{355}$RIPR$^{358}$, and named this variant α1AT-Portland. *In vitro*, the engineered α1AT-Portland exhibited a potent inhibition toward furin and no longer inhibited neutrophil elastase (22).

### Exosites

The specificity of SERPINs is not only determined by their RCL sequences, but also by exosites (23). Exosites are secondary binding sites that are remote from the RCL cleavage site (24). Exosites refine SERPIN specificity in three ways. Firstly, an exosite facilitates a temporary docking site for a target protease, to improve protease binding at P1 residue of SERPIN. For example, when replacing RCL of α1-antichymotrypsin from P6-P3′ with that of α1AT, the inhibition rate toward neutrophil elastase, was greatly reduced by 1,500-fold compared to wild-type α1AT (25). This suggests that the SERPIN body selectively contributes to its inhibitory function. Secondly, exosites on extended N- and C-termini assist the binding of target proteases or to specific sites to increase inhibition locally. Alpha 2-antiplasmin (α2AP) uses its C-terminal extension to bind to plasmin, but at the same time uses its N-terminal extension to cross-link to fibrin surface. As a result, α2AP that is cross-linked to fibrin, protects it from degradation. A human single nucleotide polymorphism affects this behavior of α2AP, with functional consequences for the cross-linking of α2AP to fibrin (26). Thirdly, exosites enable interaction with cofactors. The interaction of SERPIN with a cofactor tremendously boosts the inhibition rate of SERPINs and also refines their target specificity. A classic example is the contribution of heparin to the inhibition of coagulation enzymes by antithrombin (AT). The inhibition rate of thrombin by AT is increased by 10,000-fold in the presence of heparin (27). Heparin contains a pentasaccharide sequence that is recognized by the exosites in AT. This induces a conformational change in AT that increases its inhibitory capacity. Furthermore, the bound

**TABLE 1 |** Amino acid sequence alignments of human SERPIN reactive center loop.

| SERPIN | N-terminal ← P4 | P3 | P2 | P1 | P1′ | P2′ | P3′ | P4′ → C-terminal |
|---|---|---|---|---|---|---|---|---|
| **INHIBITORY** | | | | | | | | |
| SERPINA1 | G T E A A G A M | F | L | E A | I | P | M | S I P P E V – – – – K F N K P F |
| SERPINA2 | G T E A T G A P | H | L | E E | K | A | W | S K Y Q T V – – – – M F N R P F |
| SERPINA3 | G T E A S A A T | A | V | K | I | T | L | L S A L V E T R T I V R F N R P F |
| SERPINA4 | G T E A A A A T | T | F | A | I | K | F | F S A Q T T N R H I L R F N R P F |
| SERPINA5 | G T R A A A A T | G | T | I | F | T | F | R S A R L N S Q R L V – F N R P F |
| SERPINA9 | G T E A T A A T | T | T | K | F | I | V | R S K D G S Y F T V S – F N R T F |
| SERPINA10 | G T E A V A G I | L | S | E | I | T | A | Y S M P P V I – – – – K V D R P F |
| SERPINA11 | G T E A G A A S | G | L | L | S | Q | P | P S L N T M S D P H A H F N R P F |
| SERPINA12 | G T E G A A G T | G | A | Q | T | L | P | M E T P L V V K I – – – – D K P Y |
| SERPINB1 | G T E A A A A T | A | G | I | A | T | F | C M L M P E E N – F T A – D H P F |
| SERPINB2 | G T E A A A G T | G | G | V | M | T | G | R T G H G G P Q – F V A – D H P F |
| SERPINB3 | G A E A A A A T | A | V | V | G | F | G | S S P T S T N E E F H C – N H P F |
| SERPINB4 | G V E A A A A T | A | V | V | V | V | E | L S S P S T N E E F C C – N H P F |
| SERPINB6 | G T E A A A A T | A | A | I | M | M | M | R C A R F V P R – F C A – D H P F |
| SERPINB7 | G T E A T A A T | G | S | N | I | V | E | K Q L P Q S T L – F R A – D H P F |
| SERPINB8 | G T E A A A A T | A | V | V | R | N | S | R C S R M E P R – F C A – D H P F |
| SERPINB9 | G T E A A A A S | S | C | F | V | V | A | E C C M E S G P R F C A – D H P F |
| SERPINB10 | G T E A A A G S | G | S | E | I | D | I | R I R V P S I E – F N A – N H P F |
| SERPINB11 | G T E A A A A T | G | D | S | I | A | V | K S L P M R A Q – F K A – N H P F |
| SERPINB12 | G T Q A A A A T | G | A | V | V | S | E | R S L R S W V E – F N A – N H P F |
| SERPINB13 | G T E A A A A T | G | I | G | F | T | V | T S A P G H E N V H C – – N H P F |
| SERPINC1 | G S E A A A S T | A | V | V | I | A | G | R S L N P N R V T F K A – N R P F |
| SERPIND1 | G T Q A T T V T | T | V | G | F | M | P | L S T Q V R – – – F T V – D R P F |
| SERPINE1 | G T V A S S S T | A | V | I | V | S | A | R M A P E E I I M – – – – D R P F |
| SERPINE2 | G T K A S A A T | T | A | I | L | I | A | R S S P P W – – – F I V – D R P F |
| SERPINE3 | G T K A S G A T | A | L | L | L | K | R | S R I P I – – – F K A – D R P F |
| SERPINF2 | G V E A A A A T | S | – | I | A | M | S | R M S L S S – – – F S V – N R P F |
| SERPING1 | G V E A A A A S | A | – | I | S | V | A | R T L L V – – – – F E V – Q Q P F |
| SERPINI1 | G S E A A A V S | G | M | I | A | I | S | R M A V L Y P Q V I V - – D H P F |
| SERPINI2 | G S E A A T S T | G | I | H | I | P | V | I M S L A Q S Q - F I A – N H P F |
| **NON-INHIBITORY** | | | | | | | | |
| SERPINA6 | G V D T A G S T | G | V | T | L | N | L | T S K P I I L R N Q – – – – – P F |
| SERPINA7 | G T E A A A V P | E | V | E | L | S | D | Q P E N T F L H P I I Q I D R S F |
| SERPINA8 | E R E P T E S T | Q | Q | L | N | K | P | E V L E V T L N R – – – – – – P F |
| SERPINB5 | G G D S I E V P | G | A | R | I | L | Q | H K D E – – L N A D H – – – – P F |
| SERPINF1 | G A G T T P S P | G | L | Q | P | A | H | L T F P – – L D Y H L N Q – – P F |
| SERPINH1 | G N P F D Q D I | Y | G | R | E | E | L | R S P K – – L F Y A D H – – – P F |

heparin polysaccharide molecule forms a scaffold that facilitates interaction between thrombin and AT.

## LESSONS FROM HUMAN SERPIN DEFICIENCIES

SERPIN deficiencies show us how SERPINs are involved in physiology.

### Alpha 1-Antitrypsin (α1AT)

Alpha 1-antitrypin is a 52 kDa glycoprotein that strongly inhibits neutrophil elastase. It is encoded by the SERPINA1 gene (28) and is abundantly present in plasma (150–300 mg/dL). Its levels can increase during acute phase reactions. α1AT has a relatively long circulating half-life of 4.5–6 days. By comparison, it is about 3 days, 2.6 days, 1 day, and only 1–2 h for AT, α2AP, C1INH, and plasminogen activator inhibitor-1 (PAI-1), respectively (29, 30). Alpha 1-antitrypin is a powerful protease inhibitor. It inhibits neutrophil elastase at the association rate constant of $6.5 \times 10^7$ $M^{-1}.s^{-1}$ (31). Functional α1AT deficiency associates with increases risk of pulmonary emphysema and chronic obstructive pulmonary disease (COPD). In this condition, uncontrolled neutrophil elastase activity destructs extracellular matrix components such as collagen and elastin in lung alveolar that leads to remodeling of the lung architecture (32). Replacement therapy

is indicated for the treatment of pulmonary disease due to severe α1AT deficiency, along with other pharmacologic therapies such as bronchodilator and anti-inflammatory drugs (33). Alpha 1-antitrypsin is susceptible to pathologic intracellular aggregation as a result of mutations. As α1AT is mainly expressed in the liver, it forms aggregates that accumulate intracellularly in hepatocytes, which consequently leads to liver diseases such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma (34).

## C1 Esterase Inhibitor (C1INH)

C1INH is encoded by the SERPING1 gene. It is a heavily glycosylated glycoprotein (105 kDa; six N- and ten O-glycosylation sites) (35). C1INH inhibits C1s and C1r of the classical complement pathway. It is also a major inhibitor of enzymes in the plasma contact system, i.e., plasma kallikrein (PKa) and activated factor XII (FXIIa) (36).

The clinical phenotype of C1INH deficiency is surprising. Rather than a complement-related disorder, C1INH deficiency causes an overproduction of bradykinin because of an under-regulated contact system. This subsequently leads to hereditary angioedema (37); a disorder characterized by tissue swelling (38). Surprisingly, there is little evidence for excessive intrinsic coagulation, resulting in thrombosis. Plasma-derived C1INH and recombinant C1INH are a treatment of choice for patients with angioedema.

Compared to other SERPINs, C1INH is a relatively poor protease inhibitor, which generally inhibits its targets at the rate constants of about $10^5$ $M^{-1}.s^{-1}$. By comparison, other SERPINs such as α1AT, AT, PAI-1, and α2AP have rate constants of about $10^7$ $M^{-1}.s^{-1}$ (39). Due to its poor inhibitory capability and short circulation time, high dose infusion is required for C1INH replacement therapy in HAE patients.

Similar to α1AT, some mutations can cause C1INH polymerization and subsequent hepatocellular accumulation (40). In heterozygous patients, the resulting aggregates contain both mutant and wildtype C1INH, as a result of protein-protein interactions. This explains why patients can have plasma C1INH levels below 50%.

## Antithrombin

Antithrombin is a broad inhibitor of blood coagulation proteases. It inhibits thrombin and factor Xa (FXa) and to a lesser extent, factor IXa, XIa, XIIa, PKa, tissue plasminogen activator, urokinase, and plasmin (41–43). AT is encoded by SERPINC1 gene. Low plasma AT levels increases the risk of deep vein thrombosis, pulmonary embolism and ischemic stroke (44). Pharmacological prophylaxis management is only recommended for AT deficient individuals with some clinical circumstances that provoke thrombosis (e.g., surgery, immobility, pregnancy). Current treatment and prophylaxis include low-molecular weight heparin, vitamin K antagonists, plasma-derived or recombinant human AT replacement therapy (45).

# SERPINS AS THERAPEUTIC AGENTS BEYOND REPLACEMENT THERAPY

SERPIN replacement therapies are valuable to restore deficiencies. However, SERPINs have also been studied in animal studies for their therapeutic potential beyond this application. For example, C1INH has been investigated for its therapeutic benefit toward a number of inflammation-related complications. In a porcine model for hemorrhage, a bolus injection of recombinant human C1INH, decreased tissue complement activation and attenuated metabolic acidosis. Furthermore, it reduced circulating tumor necrosis factor α and attenuated renal, intestinal, and lung injury in a dose-dependent manner (46). Pretreatment of Wistar rats with human plasma-derived C1INH exhibited protective effects in ischemia/reperfusion injury of lower extremities and associated lung damage. After 3 h of hind limb ischemia and 24-h reperfusion, C1INH significantly reduced edema formation in the reperfused muscle as well as in the lung, improved muscle viability, and decreased plasma levels of pro-inflammatory cytokines (47).

In clinical studies, administration of C1INH was found to attenuate renal function, but not overall mortality in septic patients (48). In capillary leak syndrome, which may occur secondary to bone marrow transplantation, systemically increased capillary permeability leads to hypertension. Administration of C1INH concentrate improves the overall outcome from 14 (placebo) to 57% over a mean observation period of 9 months after the symptoms (49). Finally, C1INH treatment appeared to confer a benefit in reducing the need for dialysis post-transplant and improved renal function at 12 months post-transplant compared to controls in kidney transplant recipients (50).

## Lessons From α1AT-Pittsburgh

Alpha 1 antitrypsin-Pittsburgh is a rare mutation within the RCL of α1AT. It was first reported in 1,978 and caused severe bleeding episodes in a boy who carried the mutation (51). This single substitution mutation from methionine (M) to arginine (R) at position 358 (M358R), causes a dramatic change in the target specificity of α1AT. α1AT-Pittsburgh is a strong inhibitor of PKa, FXIIa, thrombin, plasmin, and activated protein C (APC), but no longer inhibits neutrophil elastase (52–57).

Alpha 1 antitrypsin-Pittsburgh has been investigated as a therapy for sepsis. In this setting, thrombin and APC, are thought to contribute to cardinal manifestations of gram-negative septicemia, including hypotensive shock and disseminated intravascular coagulation. Recombinant α1AT-Pittsburgh was investigated in a piglet *Pseudomonas aeruginosa* sepsis model (58). Pretreatment with low doses of recombinant α1AT-Pittsburgh attenuates the characteristic decreases in the functional concentrations of AT, FXI, and fibrinogen. In addition, α1AT-Pittsburgh-pretreated group had higher survival rate compared to control. In contrast, in a primate model of *Escherichia coli* sepsis, treatment with recombinant α1AT-Pittsburgh showed no benefit and even exacerbated the associated coagulopathy (59). This unfavorable outcome may have been caused by an overly broad inhibitory spectrum of

α1AT-Pittsburgh, which includes inhibition of APC (60, 61). There is little to no experience with the application of α1AT-Pittsburgh in human clinical studies. Most probably, its apparent lack of specificity makes its development as a therapeutic agent unfavorable.

## DESIGNER SERPINS

### Refined Versions of α1AT-Pittsburgh

In order to narrow down the specificity of α1AT-Pittsburgh to FXIIa and PKa, Schapira et al. (53) mutated the RCL of α1AT-Pittsburgh by replacing proline (P) with alanine (A) at the P2 position. As a result, the RCL now imitates the RCL of C1INH, where P2-P1 residues are A and R. This results in a $^{357}$AR$^{358}$ α1AT mutant. The second-order rate constants show that $^{357}$AR$^{358}$ α1AT mutant inhibits PKa better than α1AT-Pittsburgh and C1INH by 5.2- and 21.2-fold, respectively. However, this $^{357}$AR$^{358}$ α1AT mutant inhibits β-FXIIa and thrombin less efficiently (3.8 and 4.9-fold, respectively). This can be explained by the peptide sequence substrate preference of thrombin, which prefers P at P2 position (62). As a consequence, the $^{357}$AR$^{358}$ α1AT mutant does not prolong the thrombin time in plasma of Wistar rats. Moreover, rats that were pretreated with $^{357}$AR$^{358}$ α1AT mutant (0.7 mg) were protected from β-FXIIa-induced hypotensive reaction, which is driven by PKa-mediated bradykinin production. A patent application on this invention was filed (US4973668A) in 1990 as a PKa inhibitor. However, to our knowledge, this variant has never been further evaluated for its therapeutic value.

In 2002, Sulikowski et al. (39) developed α1AT-Pittsburgh variants. For this design, they used information from synthetic peptide substrate studies to target PKa and C1s, but not APC. Based on this information, they changed P3-P2 residues of α1AT-Pittsburgh from isoleucine-proline to either leucine-glycine ($^{356}$LGR$^{358}$) or proline-phenylalanine ($^{356}$PFR$^{358}$). The investigators found that the first mutant $^{356}$LGR$^{358}$ remained a broad-spectrum inhibitor of C1s, PKa, FXIIa, and also APC. However, the second mutant $^{356}$PFR$^{358}$ showed increased specificity toward PKa, but inhibited all other enzymes less efficiently than mutant $^{356}$LGR$^{358}$. In other experiments, an additional mutant was developed, based on $^{56}$LGR$^{358}$ in which the RCL's P4′ residue was changed from a P to glutamine (E; $^{356}$LGRSIPE$^{362}$). However, this additional change did not show beneficial effects.

Another interesting, more recent example of therapeutic SERPIN development is found in the field of hemophilia. In this bleeding disorder, Polderdijk et al. (57) sought to restore the hemostatic balance by developing a strong inhibitor of APC. The investigators chose α1AT-Pittsburgh as a template for the development of a strong APC inhibitor. Hereto, they replaced the P2 and P1′ residues of α1AT-Pittsburgh with a bulky lysine (K) to avoid interaction with thrombin, resulting in a $^{357}$KRK$^{359}$ α1AT mutant. This mutant specifically inhibits APC, over other coagulation proteases. The $^{357}$KRK$^{359}$ α1AT mutant has no effects on the PT, aPTT assays or thrombin generation in normal pooled plasma. However, it promotes thrombin generation in plasma from patients with hemophilia A or hemophilia B, indicating its specificity and procoagulant properties. Moreover, the mutant demonstrated efficacy in two different mouse models for hemophilia.

All in all, these studies demonstrate the possible applications of SERPINs beyond replacement therapy. Presently, SERPIN therapies are very costly. For example, the cost of a single-used vial of plasma-derived C1INH is up to $2,300/500 units (63) and the cost of a 2-day treatment with a recombinant AT is approximately $23,000/patient (64). However, these molecules are native protein sequences. The possibility to fine-tune SERPIN specificity and efficacy may reduce the required dosing, thereby potentially lowering the cost. In addition, the use of alternative SERPIN back bones may have dramatic consequences for therapeutic half-life. For example, α1AT has a much longer half-life than C1INH (29, 30).

We expect that an α1AT variant with the inhibitory profile of C1INH will retain this favorable property, enabling cost-effective prophylactic therapy. Finally, liver-specific expression of (designer) SERPINs through gene therapy holds great promise for long-term treatment of enzyme-driven disorders.

## CONCLUSION

Together, these studies show that engineered SERPINs hold promise for the treatment of a wide variety of diseases. This motivates researchers to find ways to improve this unique class of molecules and extend their application well-beyond disorders in the hemostatic system.

## AUTHOR CONTRIBUTIONS

WS, CM, and SdM performed literature searches and wrote the manuscript.

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frontiers in Medicine

BRIEF RESEARCH REPORT
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# Genetic Variation of Kallikrein-Kinin System and Related Genes in Patients With Hereditary Angioedema

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Hereditary angioedema (HAE) is an autosomal dominant disease caused by C1-INH deficiency due to mutations in *SERPING1* (C1-INH-HAE) in most of the cases, or by specific mutations in factor XII gene, *F12* (F12-HAE). Identification of polymorphisms in the genes encoding proteins from key pathways driving HAE can help to understand how genetic diversity contributes to its phenotypic variability. Here, 15 genes related to the Kallikrein-Kinin System (KKS) were analyzed by next generation sequencing in 59 patients with C1-INH-HAE or F12-HAE from Brazil, Denmark and Spain, and 19 healthy relatives in a total of 31 families. We identified 211 variants, from which 23 occurred only in Danish subjects and 79 were found only in Brazilian individuals, resulting in 109/211 variations in common between European and Brazilian population in the HAE families analyzed. *BDKRB2* and CPM presented a large number of variants in untranslated regions, 46/49 and 19/24, respectively; whereas *ACE* ($n = 26$), *SERPING1* ($n = 26$), *CPM* ($n = 24$), and *NOS3* ($n = 16$) genes presented the higher number of variants directly affecting amino acid sequence. Despite the large amount of variants identified, the lack of association between genotype and phenotype indicates that the modulation of HAE symptom requires a more complex regulation, probably involving pathways beyond the KKS, epigenetics and environmental factors. Considering the new HAE types recently described, molecules involved in the regulation of vasculature and in plasminogen activation become promising targets for future genetic studies.

**Keywords: hereditary angioedema, Kallikrein-Kinin System, genetic variation, genotype-phenotype correlation, C1 inhibitor deficiency, *F12* mutation**

## INTRODUCTION

Hereditary angioedema (HAE) is a rare autosomal dominant disease caused by deleterious mutations in *SERPING1*, leading to quantitative or functional C1-inhibitor deficiency (C1-INH-HAE) (1). Less frequently, specific mutations in *F12* gene (F12-HAE) can be detected in patients with HAE with normal C1-inhibitor (2, 3). In addition, two new forms of HAE have been

reported, HAE with mutations in angiopoietin-1 gene (*ANGPT1*) (4) and HAE with mutations in plasminogen gene (*PLG*) (5). A non-determined but substantial proportion of patients with HAE and normal C1-inhibitor cannot be explained by mutational findings at the moment and therefore is described as HAE of unknown cause (U-HAE) (1, 6). C1-INH-HAE and F12-HAE are related to the Kallikrein-Kinin System (KKS) activation and augmented production of bradykinin (BK), leading to vasodilation and angioedema episodes (1). In the KKS, factor XII (encoded by *F12* gene) activates plasma kallikrein (KK; *KLKB1* gene), which hydrolyzes high molecular weight kininogen (HMWK; *KNG1* gene) releasing BK (7). BK binds the B2-receptor (*BDKRB2* gene) leading to endothelial nitric oxide synthase activation (*NOS3* gene). Carboxypeptidases N and M (genes *CPN* and *CPM*) metabolize BK into des-Arg9-BK, which preferentially activates B1-receptor (*BDKRB1*). Deleterious variations in genes responsible for the control of BK release, as *SERPING1*, or its metabolization, as *CPN, CPM,* angiotensin-converting enzyme (*ACE*), and neprilysin (*MME*), are able to increase the amount of BK released or its half-life, leading to or intensifying angioedema episodes. Whereas, deleterious variations in *F12, KLKB1,* and *KNG1* could impair BK formation, working as a protective factor in BK-mediated angioedema (7). Although a small phenotype distinction can be noticed in HAE subtypes (2), there is a huge variation in clinical characteristics even among carriers of the same disease-causing mutation (1, 6).

A typical genome differs from the reference human genome at 4.1–5.0 million sites with >99.9% being single nucleotide polymorphisms and short indels (8). This large number of variants can possibly be disease-causing or may contribute to phenotypic variation and drug-response susceptibility. Indeed, a cumulative effect of multiple polymorphisms may lead to complex-trait genetic diseases (8). Here, we hypothesize that variants in the KKS could explain the phenotypic variation observed in HAE.

# MATERIALS AND METHODS

## Patients

Seventy-eight subjects analyzed by targeted next-generation sequencing (NGS) belonged to 26 C1-INH-HAE families (45 mutation carriers, 15 healthy relatives) and 5 F12-HAE families (14 mutation carriers, 4 healthy relatives) (**Table 1**). Additional 56 HAE patients were included in the study and had single gene mutations sequenced by Sanger. Patients were previously diagnosed by biochemical C1-INH and C4 measurements and *SERPING1* and/or *F12* genotyping by Sanger sequencing.

This study was carried out in accordance with the recommendations of ethical standards of the 1964 Declaration of Helsinki with written informed consent from all subjects. The protocol was approved by the Ethics Committee in Research of Universidade Federal de São Paulo (n°56522).

## Genetic Analysis

Since only a few possible genetic variants have been suggested to be associated to the HAE phenotype (9–13), we explored 15 genes related to KKS in individuals from Brazil, Denmark, and Spain, by NGS as previously described (14). Briefly, the targeted genes (*ACE, MME, KNG1, KLKB1, KLK1, F12, ENPEP, BDKRB1, BDKRB2, NOS3, TAC1, CPN1, CPM, SERPING1, PRCP*) were amplified from patients' DNA extracted from blood samples by using Ion AmpliSeq™ Library Kit-2.0V (Life Technologies, Carlsbad, CA, USA). NGS was performed with the Ion 316™ Chip-v2 in Ion PGM™ Sequencer, which determined the amount of sequence data analyzed. To guarantee a high-quality coverage (minimum 20X) we limited the panel to the 15 selected genes, in a total of 366 amplicons. Torrent Suit v3.2.1 and Ion Reporter 4.0 (Life Technologies) were used to data analysis and interpretation of variants. Probably pathogenic variants, low coverage regions and *SERPING1* and *F12* coding regions were validated by Sanger sequencing. Variants annotation was performed using Ingenuity® Variant Analysis (Qiagen) and Metacore™ (Thomson Reuters).

In addition to the 78 subjects evaluated by NGS, in 56 HAE patients the exons 2 and 3 of *BDKRB2* were also sequenced by Sanger method (11 C1-INH-HAE and 45 U-HAE). U-HAE patients were diagnosed according to Cicardi et al.'s guidelines (1). Distribution of variants between groups was calculated by Chi-square test.

All datasets generated and analyzed in this study are included in the manuscript and the Supplementary Files (**Tables S1**, **S2**).

## Clinical Characteristics

Patients were grouped and compared according to nationality (Brazilian vs. European), HAE type (C1-INH-HAE vs. F12-HAE), edema localization (face, extremities, abdominal pain, upper respiratory tract, and genitals), mean frequency of attacks (per year) and duration of episodes (days). Patients were categorized in three groups according to the disease onset: (1) <12-year-old, (2) from 12 to 25-year-old, and (3) >25-year-old. Mean frequency and duration of swelling episodes data were considered at baseline without prophylaxis or acute treatment. The frequency of attacks was divided in low (<13 attacks per year), medium (from 13 to 24), and high (>24). Patients were also grouped according to the mean duration of swelling episodes in short duration (<2 days), medium (2–3 days), and long duration (>3 days). Distribution of variants was compared within the selected groups.

# RESULTS

## NGS Analysis

In 26 C1-INH-HAE families, 23 mutations were considered responsible for HAE, and all F12-HAE patients carried p.Thr328Lys (**Table 1**). A total of 211 different variants were identified in the 15 genes analyzed. Nine alterations occurred in the 5′-UTR (4.3%) and 89 in the 3′-UTR (42.2%). Missense

**Abbreviations:** ACE, angiotensin-converting enzyme; C1-INH-HAE, hereditary angioedema with C1 inhibitor deficiency; F12-HAE, hereditary angioedema with mutation in *F12* gene; HMWK, high molecular weight kininogen; KK, Plasma Kallikrein; KKS, Kallikrein-Kinin System; U-HAE, hereditary angioedema of unknown cause.

**TABLE 1 |** Families analyzed by NGS.

| HAE type | Fam ID | Nat | Mutation leading to HAE | | Mutation type | N° symptomatic/ asymptomatic | Healthy relatives (no mutation) |
|---|---|---|---|---|---|---|---|
| | | | cDNA change | Protein change | | | |
| C1-INH-HAE | 1 | D | c.-22-2A>G | None | Regulatory region | 6/0 | 1 |
| | 2 | D | c.23insT | p.(Thr9fs) | Frameshift | 1/0 | 0 |
| | 3 | B | c.51+1G>T | ? | Splicing defect | 1/0 | 0 |
| | 4 | B | c. 51+2T>C | ? | Splicing defect | 2/0 | 0 |
| | 5 | B | c.97_115del15 | p.(Asp33fs) | Frameshift | 1/0 | 0 |
| | 6 | D | c.143_144delCA | p.(Thr48fs) | Frameshift | 2/0 | 0 |
| | 7 | D | c.143_144delCA | p.(Thr48fs) | Frameshift | 1/0 | 0 |
| | 8 | D | c.143_144delCA | p.(Thr48fs) | Frameshift | 1/0 | 0 |
| | 9 | B | c.550G>C | p.Gly184Arg | Missense | 1/0 | 0 |
| | 10 | D | c.597C>G | p.Tyr199Ter | Nonsense | 1/0 | 0 |
| | 11 | D | c.668delA | p.(Gln223fs) | Frameshift | 1/0 | 0 |
| | 12 | B | c.752T>C | p.Leu251Pro | Missense | 1/0 | 0 |
| | 13 | D | c.762_763delCA | p.(Asn254fs) | Frameshift | 2/0 | 0 |
| | 14 | D | c.795G>A | p.Trp265Ter | Nonsense | 1/0 | 0 |
| | 15 | D | c.838_846del9 | p.Ser280_Pro282del | Inframe | 1/0 | 0 |
| | 16 | D | c.878T>C | p.Ile293Thr | Missense | 1/0 | 0 |
| | 17 | B | c.889G>A | p.Ala297Thr | Missense | 2/0 | 0 |
| | 18 | D | c.1029+4delA | ? | Splicing defect | 1/0 | 0 |
| | 19 | B | c.1104delA | p.(Asp369fs) | Frameshift | 2/0 | 0 |
| | 20 | B | c.1353_1354delGA | p.(Glu451fs) | Frameshift | 1/0 | 1 |
| | 21 | B | c.1369G>C | p.Ala457Pro | Missense | 8/0 | 13 |
| | 22 | D | c.1397G>A | p.Arg466His | Missense | 1/0 | 0 |
| | 23 | D | c.1417G>A | p.Val473Met | Missense | 2/0 | 0 |
| | 24 | B | c.1431C>A | p.Phe477Leu | Missense | 1/0 | 0 |
| | 25 | B | c.1480C>T | p.Arg494Ter | Nonsense | 2/0 | 0 |
| | 26 | D | c.1480C>T | p.Arg494Ter | Nonsense | 1/0 | 0 |
| F12-HAE | 27 | B | c.983C>A | p.Thr328Lys | Missense | 4/2 | 0 |
| | 28 | B | c.983C>A | p.Thr328Lys | Missense | 3/0 | 3 |
| | 29 | B | c.983C>A | p.Thr328Lys | Missense | 2/0 | 0 |
| | 30 | B | c.983C>A | p.Thr328Lys | Missense | 1/0 | 1 |
| | 31 | S | c.983C>A | p.Thr328Lys | Missense | 2/0 | 0 |

*The mutations in SERPING1 and F12 identified as causative of HAE in each family are described. The number of symptomatic and asymptomatic mutation carriers, and healthy relatives (without mutation) analyzed by NGS are shown in the last columns. B, Brazilian; D, Danish; Fam ID, family identification; Nat, nationality; S, Spanish; ?, not known.*

mutations corresponded to 22.7% of the total, followed by synonymous (21.8%), small insertions/deletions leading to frameshift (3.8%), non-sense (1.9%), splice site (1.4%), and intronic alterations (1.9%). A list containing all variants is found in **Table S1** (Supplementary Material). *BDKRB2* presented the highest number of variants ($n = 49$), followed by *ACE* ($n = 26$) and *SERPING1* ($n = 26$), and *CPM* ($n = 24$). The distribution of variants per type or per gene was not significantly between C1-INH-HAE and F12-HAE groups (Chi-square test with Yates' correction). Considering only variants leading to change in the structure of the protein (missense, nonsense, indels, and predicted splicing sites), the genes in which more mutations were found were *SERPING1* ($n = 23$) and *ACE* ($n = 10$). No probably damaging variants were identified in *BDKRB1*, *CPN/M*, *KNG1*, *KLK1, ENPEP, PRCP,* or *TAC1* genes. Few studies suggest tissue kallikrein (*KLK1* gene) may be involved in HAE manifestations (15). Tissue kallikrein releases Lys-BK from low molecular weight kininogen (also encoded by *KNG1*), which is converted into BK by aminopeptidases (16) or into Lys-des-Arg9-BK by carboxypeptidases.

## *BDKBR2* Variants

The change p.Arg14Cys in *BDKRB2* was found 15 individuals, and plGly354Glu in 16, without significant difference in distribution among HAE subtypes ($p = 0.1203$ and $p = 0.1900$, respectively, Chi-square test). The alterations p.Val376Met (rs141958164) and p.Val208Ile (rs139203012) were found in one U-HAE patient each.

## Clinical Features and Phenotype-Genotype Association

We found 23 mutations exclusively in Danish individuals and 79 only in Brazilians, resulting in 109/211 mutations in common between European and Brazilian HAE families analyzed.

We compared the symptomatic patients regarding the presence of facial swellings (43/57), swellings of extremities

(41/57), abdominal swellings (45/57), upper airways (28/57), and edema of the genitalia (21/57). The first attacks occurred before the age of 12 in 26 patients (24 C1-INH-INH, 2 F12-HAE) (mean = 6.7 years); between 12 and 25 years of age in 21 patients (20 C1-INH-HAE, 5 F12-HAE) (mean = 17), and after 25 years of age in eight (3 C1-INH-HAE, 5 F12-HAE) (mean = 33.8). Most of the patients reported to experience up to 12 swelling episodes/year without prophylaxis (31/47; mean = 4.6). Nine C1-INH-HAE patients reported 13–24 attacks/year (mean = 19.8), and seven reported more than 24 attacks/year (mean = 43.2).

Distribution of variants was compared within the selected groups, but no specific mutation was significantly associated to any of the clinical characteristic selected. However, five missense mutations were classified as probably damaging by all *in silico* predictive software (excluding *SERPING1* variations) (**Table 2**).

## DISCUSSION

HAE can be caused by C1-INH deficiency, in which more than 300 different mutations have already been considered responsible for the disease (17). A smaller proportion of HAE patients present specific mutations affecting a proline-rich region of factor XII that leads to loss of glycosylation and enhanced autoactivation mechanism of the zymogen activating the KKS (18). In spite of the large mutational spectrum of *SERPING1* in HAE, the variation in the presentation of symptoms seems not to be related to the type of the disease-causing mutation or even with functional levels of C1-INH (19). Therefore, the search for new genetic biomarkers could not only explain the variation in HAE symptomatology, but also be used in diagnosis, prognosis, and treatment response. Proteins of the KKS are strong candidates to be regulating the severity and frequency of HAE attacks, since they are directly involved in the BK-release pathway.

In this study, a large number of variants was found in a regulatory portion of the 3′-untranslated *BDKRB2* region (20). Also, 40/78 individuals presented the 9 bp deletion in the exon 1 of *BDKRB2*, described to increase B2-receptor expression (21). However, no correlation was found with these variants and HAE clinical characteristics suggesting that the regulation of B2-receptor expression through these regions is not strong enough to modulate HAE attacks itself, in agreement with previous data (22).

Variants classified as probably pathogenic in *ACE* (p.Gly354Arg, p.Tyr244Cys, p.Thr916Met) were found in Brazilian and Danish symptomatic patients, and p.Arg324Trp was found only in one non-affected relative (family 21). Since ACE degrades BK, deleterious alterations could increase BK half-life, favoring longer edema episodes. It has been demonstrated that ACE activity inversely correlates with severity scores in F12-HAE (23); in contrast, C1-INH-HAE severity is not depending on ACE, but highly depends on aminopeptidase P activity (24). However, the C1-INH-HAE patients carrying these mutations had distinguished clinical features from each other (**Table 2**).

The mutation p.Cys548Tyr in *KLKB1*, found in two patients from family 1 and one asymptomatic mutation carrier from family 27 (F12-HAE), has been associated with KK deficiency in a compound heterozygosity with the change p.Trp402Ter (25). KK deficiency is a rare autosomal recessive condition which causes an augmented activated partial thromboplastin time, but no coagulation anomalies. Therefore, coexistence of KK deficiency could attenuate or even abolish the symptoms of HAE, since it is the key enzyme responsible for BK release. The only missense mutation found together with p.Cys548Tyr was the high frequent polymorphism p.Ser143Asn (66/78). This polymorphism was shown to cause KK deficiency with the change p.Gly125Arg, both in homozygosity (26) and lying in the apple domain 2 of the heavy chain of KK, through which kallikrein interacts with HMWK domain 6 (27). However, p.Ser143Asn was found in heterozygosity in the three patients carrying the p.Cys548Tyr, discarding the hypothesis that this association could be responsible by itself for the lack of symptoms observed in the F12-HAE carrier. Furthermore, the Cys548 lies in the catalytic domain of KK, far from the apple domain 2.

**TABLE 2 |** Probably pathogenic variants found in C1-INH-HAE and F12-HAE patients by NGS.

| Gene | Protein change | Nat | Patient/ family | Age (years) | Onset (years) | Frequency (per year) | Duration (days) | Face | Extremities | Abdominal | Upper airways | Genitalia |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *ACE* | p.G354R | B | 1/17 | 49 | 16 | 12 | 3 | – | × | – | × | – |
| | | B | 2/17 | 25 | NI | <1 | <1 | – | – | × | – | – |
| | | D | 1/14 | 54 | 10 | 4 | 3 | × | × | × | × | × |
| | p.Y244C | D | 6/1 | 73 | 15 | <1 | 2–3 | × | × | × | – | – |
| | | B | 1/24 | 42 | 7 | 2 | 3 | × | × | × | × | × |
| | p.T916M | D | 3/1 | 71 | 21 | 3 | 2–3 | × | × | × | – | – |
| | | D | 7/1 | 36 | 10 | 18 | 3 | × | × | × | × | – |
| *NOS3* | p.D287N | D | 5/1 | 16 | 3 | 2 | NI | – | – | × | – | – |
| *KLKB1* | p.C548Y | D | 3/1 | 71 | 21 | 3 | 2–3 | × | × | × | – | – |
| | | D | 6/1 | 73 | 15 | <1 | 2–3 | × | × | × | – | – |
| | | B | 4/27 | 51 | Asympt. | Asympt. | Asympt. | Asympt. | Asympt. | Asympt. | Asympt. | Asympt. |

*The mutations were analyzed as probably pathogenic when classified as damaging for all the in silico software assessed (SIFT, PolyPhen-2, PROVEAN, and CADD). Asympt, asymptomatic; NI, not identified.*

Pathogenicity level of most of the new variants cannot be confirmed only by *in silico* analysis (28). For example, the variant p.Thr328Lys in *F12* is responsible for most of F12-HAE cases (3, 18), but is classified as benign by many pathogenicity predictors. Therefore, the discovery of new and rare variants possibly involved in HAE pathogenesis requires not only experimental evidences to assure the pathogenicity degree of each variant but also the analysis of the overlap effect of different variations and genes. Thus, despite the large number of variants identified in KKS and related genes in this work, the small number of subjects included and the lack of biochemical and functional analysis on the variants are some limitations.

The studies of modulating factors in HAE must combine the discovery of new mutations, genotype-phenotype association including a large number of patients, and consider epigenetic and environmental factors. To our knowledge, this is the largest study exploring different genes in many HAE families. We expect that the disclosure of the variants here presented in HAE patients and the new recent findings in U-HAE will encourage further studies correlating the KKS and the regulation of vasculature, as well as larger international multicentric studies.

## AUTHOR CONTRIBUTIONS

CV coordinated the analysis, and drafted the manuscript. AA and AB designed data collection. CV, PN, and AM performed all the experiments. CV, RM, and RF-S performed the bioinformatics analysis. AA, RG, JD, MG, AG, EM, and AB performed the data collection instruments, and coordinated data collection. AB conceptualized the study and supervised data collection. JP conceptualized and designed the study, coordinated and supervised all the experiments and analysis and revised the manuscript. All the authors drafted, reviewed and approved the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2019.00028/full#supplementary-material

**Table S1 |** Genetic variants found by NGS in HAE families**.** All the 211 variants found in the 15 genes analyzed in the 78 subjects are listed in the table. Pathogenicity prediction from SIFT (D, damaging; T, tolerated), PolyPhen-2 (B, benign; P, possibly damaging; D, damaging), PROVEAN (D, damaging; N, non-damaging) and CADD (threshold > 20) indicate the *in silico* analysis results. † Found only in Danish individuals.

**Table S2 |** Full dataset of genetic variants and clinical data. The sheet entitled Genetic Variants contains all the variants found by NGS before validation and patients filtering (raw data). Variants with the annotation ARTIFACT in the column Functional Class correspond to the variations not confirmed by Sanger sequencing validation and discarded in the final evaluation. The sheet Patients Raw contains the chip and barcode identification, and clinical data from all the patients initially sequenced, including U-HAE patients posteriorly excluded from NGS analysis.

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# Increased Reactive Oxygen Species Generation Contributes to the Atherogenic Activity of the B2 Bradykinin Receptor

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Atherosclerosis and ensuing cardiovascular disease are major causes of death with insufficient treatment options. In search for pathomechanisms of atherosclerosis, we investigated the impact of the B2 bradykinin receptor, *Bdkrb2*, on atherosclerotic lesion formation, because to date it is not clear whether the B2 bradykinin receptor is atheroprotective or atherogenic. As a model of atherosclerosis, we used hypercholesterolemic *ApoE*-deficient (apolipoprotein E-deficient) mice, which develop atherosclerotic lesions in the aorta with increasing age. The role of *Bdkrb2* in atherosclerosis was studied in *ApoE*-deficient mice, which were either *Bdkrb2*-deficient, or had moderately increased aortic B2 bradykinin receptor protein levels induced by transgenic *BDKRB2* expression under control of the ubiquitous CMV promoter. We found that *Bdkrb2* deficiency led to a significantly decreased atherosclerotic plaque area whereas transgenic *BDKRB2* expression enhanced atherosclerotic lesion formation in the aorta of *ApoE*-deficient mice at an age of 8 months. Concomitantly, the aortic content of reactive oxygen species (ROS) was higher in *BDKRB2*-expressing mice whereas *Bdkrb2* deficiency decreased aortic ROS levels of *ApoE*-deficient mice. In addition, aortic nitrate as a marker of nitric oxide activity and the endothelial nitric oxide synthase (eNOS) co-factor, tetrahydrobiopterin (BH4) were reduced in *BDKRB2*-expressing *ApoE*-deficient mice. The decreased aortic BH4 content could be a consequence of increased ROS generation and down-regulated aortic expression of the BH4-synthesizing enzyme, *Gch1* (GTP cyclohydrolase 1). In agreement with a causal involvement of decreased BH4 levels in the atherogenic function of *BDKRB2*, we found that treatment with the BH4 analog, sapropterin, significantly retarded atherosclerotic plaque formation in *BDKRB2*-expressing *ApoE*-deficient mice. Together our data show that the B2 bradykinin receptor is atherogenic, and the atherosclerosis-promoting function of *BDKRB2* is partially caused by decreased aortic BH4 levels, which could account for eNOS uncoupling and further enhancement of ROS generation.

**Keywords: BDKRB2, reactive oxygen species, atherosclerosis, hypercholesterolemia, GCH1, Apoe, AGTR1, ACE**

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## INTRODUCTION

Atherosclerosis and cardiovascular disease are leading causes of death worldwide (1, 2). The high morbidity and mortality of atherosclerotic vascular disease is in part attributed to limited treatment options (1, 2). Elucidation of pathomechanisms with the identification of potential new targets to improve the pharmacotherapy of atherosclerosis therefore is of great interest (1–3). To study pathomechanisms of atherosclerosis, hypercholesterolemic, apolipoprotein E (*ApoE*)-deficient mice are often used as a model because these mice reproduce major features of atherosclerotic vascular disease such as hypercholesterolemia-induced atherosclerotic lesion formation in the vascular system with increasing age (4–8).

In this study, we investigated the role of the B2 bradykinin receptor (*BDKRB2*) in atherosclerotic lesion formation. To date it is not known whether *BDKRB2* is atheroprotective or atherogenic. Due to the blood pressure-lowering and nitric oxide- (NO)-generating activity, the B2 bradykinin receptor is considered to exert cardioprotection (9, 10). This cardioprotective potential of B2 bradykinin receptor stimulation by the agonist bradykinin and related kinins of the kinin-kallikrein system is exploited therapeutically with ACE (angiotensin-converting enzyme) inhibitors. ACE inhibition not only blunts the generation of the vasopressor angiotensin II but also prevents the proteolytic degradation of bradykinin (9, 10). The contribution of bradykinin to the antihypertensive activity of ACE inhibitors is well-documented in experimental models and patients (11, 12). On the other hand, the atherosclerosis-decreasing potential of ACE-inhibition is reportedly independent from bradykinin and B2 bradykinin receptor stimulation (13). Moreover, bradykinin and related kinins are pro-inflammatory peptides, and inflammation is an established risk factor of atherosclerosis (14, 15). In addition, beneficial B2 bradykinin receptor-stimulated nitric oxide (NO) generation and vasodilation are impaired in atherosclerosis (16). Hypercholesterolemia and atherosclerosis are known to cause endothelial dysfunction with concomitant uncoupling of endothelial nitric oxide synthase (eNOS), which then generates atherosclerosis-promoting reactive oxygen species (ROS) instead of atheroprotective NO (17–19).

In view of this scenario, we investigated the impact of the B2 bradykinin receptor on atherosclerotic lesion development. To study the role of the B2 bradykinin receptor (*Bdkrb2*) in atherosclerosis, we used (i) *ApoE*–/– mice with endogenously expressed *Bdkrb2*, (ii) *ApoE*–/– mice with *Bdkrb2* deficiency, and (iii) *ApoE*–/– mice with moderately increased transgenic *BDKRB2* expression level. We found that transgenic B2 receptor expression enhanced atherosclerotic plaque formation in the aorta of *ApoE*–/– mice whereas *Bdkrb2* deficiency retarded the development of atherosclerosis.

## MATERIALS AND METHODS

### Experimental Model of Atherosclerosis, and Generation of Transgenic Mice

The study was performed with three groups of male mice, i.e., (i) apolipoprotein E-deficient (*ApoE*–/–) mice in B6 (C57Bl/6J) background (4–8), (ii) double-deficient *Bdkrb2*–/–*ApoE*–/– mice (B2–/–*ApoE*–/–), and (iii) *ApoE*–/– mice with transgenic expression of *BDKRB2* under control of the ubiquitous CMV immediate-early promoter/enhancer (derived from plasmid pcDNA3, Invitrogen AG - Thermo Fisher Scientific). *Bdkrb2*-deficient mice in B6 background were obtained by backcross breeding of *Bdkrb2*–/– mice (20) for ten generations into B6 background. Double-deficient, *Bdkrb2*–/– and *ApoE*–/– mice, were subsequently generated by cross-breeding, and identified by genotyping PCR. For transgenic expression of *BDKRB2*, the *BDKRB2* transgene (2 ng/μL) was injected into the pronucleus of fertilized oocytes isolated from super-ovulated *ApoE*–/– mice with B6 background followed by transfer of 2-cell embryos into pseudo-pregnant CD-1 foster mice similarly as described (21). After weaning at an age of 3–4 weeks, PCR genotyping was performed with ear-punch biopsies. Founder mice of the FO generation were identified with stable integration of the transgenic DNA into the genomic mouse DNA and used for further breeding. Due to the pathologic phenotype in *ApoE*–/– background, Tg-CMVBDKRB2 (Tg-B2++) mice with *ApoE*-deficiency only were used for phenotyping but otherwise, the colony of Tg-CMVBDKRB2 mice was maintained on the B6 background without *ApoE*-deficiency (C57BL/6-Tg-(CMVBDKRB2)Sjaa; Janvier No. 181.281 ETH Zurich). Phenotyping was determined with 8-month-old mice. Mice were kept on a 12 h light/12 h dark cycle, had free access to food and water, and were fed a standard rodent chow diet (Ain-93-based diet without addition of tocopherol acetate) containing 7% fat and 0.15% cholesterol. As indicated, 3-month-old mice received sapropterin (10 mg/kg/d, in drinking water, freshly prepared, every day) for 5 months. Treatment of Tg-B2++*ApoE*–/– mice and *ApoE*–/– mice with the ACE inhibitor, captopril (20 mg/kg/d, in drinking water, freshly prepared, every day) was started at an age of 3 months and continued for 5 months until the end of the observation period at 8 months. At the end of the study, anesthetized mice (ketamine/xylazine 100 mg/10 mg per kg body-weight) were perfused intracardially with ice-cold, sterile PBS, the aorta was rapidly dissected on ice and processed for further analysis. All animal experiments were performed according to NIH guidelines, and reviewed and approved by the local committee on animal care and use (Cantonal Veterinary office, Zurich).

### Biochemical Assays and Aortic Atherosclerotic Lesion Determination

The atherosclerotic lesion area was determined in the aorta by quantitative image analysis of oil red O-stained aortas opened longitudinally (7, 8). The area of the aortic intima with pathologic intimal atherosclerotic lesions was determined with hematoxylin-eosin-stained, aortic paraffin sections of the aortic arch. The aortic content of BH4 was determined as described (22). The content of reactive oxygen species (ROS) was determined by quantitative fluorescence evaluation of dihydroethidium- (DHE)-stained aortic cryosections (8, 23). The aortic expression of *Gch1* (GTP cyclohydrolase 1) was assessed after reverse transcription of mRNA into cDNA followed by quantitative real-time qRT-PCR using a LightCycler 480 Instrument (Roche). For quantitative real-time qRT-PCR, total

aortic RNA was isolated by the RNeasy Mini kit according to the protocol of the manufacturer (Qiagen). RNA purity was confirmed by an absorbance ratio A260/280 of ∼2.0. The absence of RNA degradation and RNA quality were further controlled by the presence of bright bands of 18S and 28S ribosomal RNA in denaturing RNA electrophoresis. RNA was reverse transcribed into cDNA by the Transcriptor High Fidelity cDNA Synthesis Kit and subjected to qRT-PCR using the LightCycler® 480 System with the LightCycler® 480 SYBR Green I Master reaction mix according to the protocol of the manufacturer (Roche Molecular Systems). Primer sequences used for determination of *Gch1* expression by qRT-PCR were as follows: Gch1 forward 5′-GCC GCTTACTCGTCCATTCT-3′, and Gch1 reverse 5-CCACCG CAATCTGTTTGGTG-3′. Specific amplification of the *Gch1* fragment of 358 bp was controlled by agarose gel electrophoresis. Total number of B2 bradykinin receptor binding sites was determined with aortic smooth muscle cells in HEPES-buffered DMEM (supplemented with 1% BSA, protease inhibitors and enalaprilat) by saturation radioligand binding (for 2 h at 4°C) with increasing concentrations (0.1–10 nM) of [2,3-prolyl-3,4-$^{3}$H(N)]bradykinin (79-96 Ci/mmol; Perkin Elmer) in the absence and presence of 10 μM HOE140 to determine non-specific binding. Likewise, the number AT1 receptor binding sites was determined with Sar$^{1}$,[$^{125}$I]Tyr$^{4}$,Ile$^{8}$-angiotensin II (2200 Ci/mmol; Perkin Elmer) in the absence and presence of 10 μM losartan. Aortic vascular smooth muscle cells were isolated from aortas of *ApoE*–/–, *Bdkrb2*–/–*ApoE*–/–, Tg-B2++*ApoE*–/– and non-transgenic B6 control mice at an age of 6–10 days. For vascular smooth muscle cell (VSMC) isolation, aortas from 10 to 15 mice were dissected. Aortas were endothelium-denuded and minced with a scalpel followed by a washing step with HEPES-buffered DMEM (supplemented with 1 mM L-glutamine, 100 I.U./ml penicillin and 100 μg/ml streptomycin) and digestion at 37°C with collagenase (2 mg/ml of collagenase type 2) under constant agitation with a magnetic stirrer. The first digestion step was discarded and in subsequent steps, the enzyme solution was exchanged every 5 min. Cells were filtered through a nylon mesh (pore size 40 μm) and collected in DMEM supplemented with 10% FCS. After the aortic tissue was digested, cell fractions were pooled. Thereafter, cells were collected by centrifugation (300 x g), plated on cell culture plates and cultured in DMEM supplemented with 10% FCS as described (24). Total inositol phosphates of aortic smooth muscle cells were determined as described (25). The specific B2 bradykinin receptor-stimulated signal is given and was determined by bradykinin stimulation (100 nM) in the absence and presence of 10 μM of the B2-specific antagonist, HOE140. Aortic Gch1 protein contents were determined by immunoblot detection with GCH1/Gch1-specific antibody (GCH1 monoclonal antibody M01, clone 4A12, Cat. No. H00002643-M01, Abnova) similarly as detailed previously (25). Aortic tissue nitrate content was determined with a fluorometric assay kit according to the protocol of the manufacturer (Cayman Chemical).

## Statistical Analysis

All data are presented as mean ± s.d. Analysis of variance followed by a Post-test as indicated was performed to determine statistical significance between more than two groups. Statistical significance was set at a $p$-value of $< 0.05$. Statistical evaluation was performed with GraphPad PRISM 7.0.

# RESULTS

## Increased Number of Aortic B2 Bradykinin Receptors in Tg-B2++*ApoE*–/– Mice

To investigate the role of the B2 bradykinin receptor in atherosclerosis, we used hypercholesterolemic *ApoE*–/– mice as an experimental model of atherosclerosis. These mice develop atherosclerotic lesions in the vascular system and the aorta with increasing age (4–8). In frame of our study, we compared three groups of *ApoE*–/– mice with different expression levels of the B2 bradykinin receptor, i.e., (i) *ApoE*–/– mice in B6 (C57Bl/6J) background with endogenous *Bdkrb2* expression level, (ii) *ApoE*–/– mice in B6 background, which are deficient in the B2 bradykinin receptor gene, *Bdkrb2*–/–*ApoE*–/ (B2–/–*ApoE*–/–), and (iii) *ApoE*–/– mice in B6 background with transgenic expression of *BDKRB2* under control of the ubiquitous CMV promoter, Tg-B2++*ApoE*–/– mice (**Figure 1A**). Because the genetic background has a strong influence on the atherosclerotic phenotype of *ApoE*–/– mice (26), all groups of transgenic mice used in our study had identical B6 background. To obtain *Bdkrb2*–/– mice with pure B6 (C57Bl/6J) background, we performed 2 years of backcrossing of *Bdkrb2*–/– mice into the B6 background for more than 10 generations. These *Bdkrb2*–/– mice with B6 background were then used for cross-breeding with *ApoE*–/– mice, to finally obtain homozygous mice, which were double-deficient in *Bdkrb2* and *ApoE* (*Bdkrb2*–/– and *ApoE*–/–). To generate mice with moderately increased *BDKRB2* level, we generated *BDKRB2*-transgenic mice in *ApoE*–/– background with expression of *BDKRB2* under control of the ubiquitous CMV promoter. We used the CMV promoter, because the endogenous B2 bradykinin receptor is also ubiquitously expressed. Male offspring of these three study groups of mice with different expression levels of the B2 bradykinin receptor were used to analyze the impact of this receptor on the pathogenesis of atherosclerosis (**Figure 1A**).

We initially characterized the vascular B2 bradykinin receptor content of study groups. B2 bradykinin receptor levels on aortic vascular smooth muscle cells (VSMC) isolated from different transgenic mouse lines were determined by radioligand binding. Our data show that B2 bradykinin receptor levels were moderately increased on VSMC of Tg-B2++*ApoE*–/– mice compared to *ApoE*–/– mice, i.e., the total number of B2 receptor bindings sites was 72.9 ± 10.5 fmol/mg on Tg-B2++*ApoE*–/– VSMC compared to 43.7 ± 8.2 fmol/mg in *ApoE*–/– mice with endogenous *Bdkrb2* level and 44.1 ± 7.7 fmol/mg in non-transgenic B6 mice (**Figure 1B**). As a control, the B2 bradykinin receptor was absent in VSMC from double-deficient, B2–/–*ApoE*–/– mice (**Figure 1B**). In contrast to the B2 bradykinin receptor, numbers of angiotensin II AT1 receptor binding sites were not significantly different between all study groups (**Figure 1B**). Endogenously expressed and transgenic

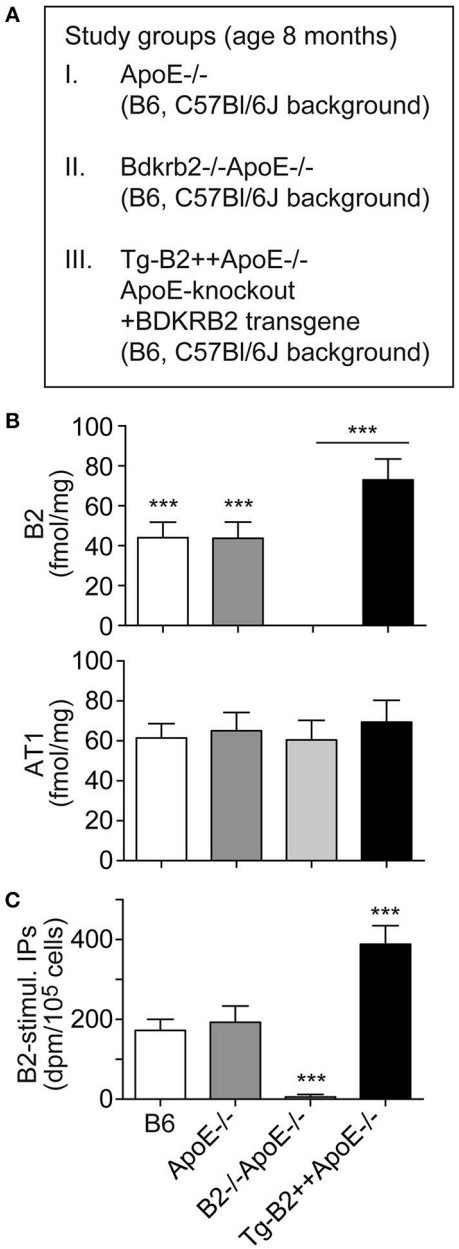


**FIGURE 1 |** Increased number of aortic B2 bradykinin receptors in Tg-B2++*ApoE*–/– mice. **(A)** Overview of three study groups of *ApoE*–/– mice. **(B)** B2 bradykinin receptor binding sites (upper) and angiotensin II AT1 receptor binding sites (lower) were determined with aortic vascular smooth muscle cells isolated from three study groups of *ApoE*–/– mice and non-transgenic B6 mice (mean ± s.d.; $n = 6$; ***$p < 0.001$ (B6 and *ApoE*–/– vs. B2–/–*ApoE*–/– and Tg-B2++*ApoE*–/–); Tukey's test). **(C)** The specific B2 bradykinin receptor-stimulated increase in total inositol phosphate levels (B2-stimul. IPs) was determined with aortic vascular smooth muscle cells isolated from different study groups (±s.d.; $n = 3$ biological replicates; ***$p <$ 0.001 (Tg-B2++*ApoE*–/–) vs. B6 and *ApoE*–/–; ***$p < 0.001$ (B2–/–*ApoE*–/–) vs. B6, *ApoE*–/–, and Tg-B2++*ApoE*–/–; Tukey's test).

B2 bradykinin receptors of aortic smooth muscle cells were functional and mediated an increase in total inositol phosphate levels upon bradykinin stimulation (**Figure 1C**).

Together these data show that transgenic *BDKRB2* expression under control of the ubiquitous CMV promoter led to a moderately increased number of B2 bradykinin receptor binding sites on aortic smooth muscle cells of Tg-B2++*ApoE*–/– mice whereas the B2 receptor was absent in B2–/–ApoE–/– mice with deficiency of the B2 bradykinin receptor gene, *Bdkrb2*.

## Transgenic *BDKRB2* Expression Enhances Atherosclerotic Lesion Formation Whereas Bdkrb2 Deficiency Leads to a Decreased Atherosclerotic Plaque Area in the Aorta of *ApoE*–/– Mice

To investigate the impact of the B2 bradykinin receptor on atheroma formation, we determined the atherosclerotic lesion area in the aorta of 8-month-old *ApoE*–/– mice. As outlined before, the study compared three different groups of mice, i.e., (i) *ApoE*–/– mice with endogenous *Bdkrb2* expression level, (ii) B2–/–A*poE*–/– mice with *Bdrkb2* deficiency, and (iii) Tg-B2++*ApoE*–/– mice with moderately increased B2 receptor level due to transgenic *BDKRB2* expression. All mice had B6 background. Atherosclerotic lesion area in the aorta was quantified by oil red O staining (**Figure 2A**). We found that transgenic *BDKRB2* expression led to a significantly enhanced atherosclerotic plaque formation in the aorta of Tg-B2++*ApoE*–/– mice, i.e., the atherosclerotic lesion area was increased 2.2 ± 0.4-fold in Tg-B2++*ApoE*–/– mice compared to *ApoE*–/– mice with endogenous *Bdkrb2* level (**Figure 2B**). In contrast, B2 bradykinin receptor deficiency led to a significantly decreased atherosclerotic plaque area in double-deficient, *B2*–/–*ApoE*–/– mice compared to ApoE–/– mice with endogenously expressed *Bdkrb2* (**Figure 2B**). These data provide strong evidence that the B2 bradykinin receptor enhances the progression of atherosclerotic plaque formation in the aorta of 8-month-old *ApoE*–/– mice as an experimental model of atherosclerosis.

## Transgenic *BDKRB2* Expression Leads to an Increased Aortic ROS Content in Tg-B2++*ApoE*–/– Mice

Enhanced generation of reactive oxygen species (ROS) is a major contributor to atherosclerotic plaque formation in experimental models of atherosclerosis and involved in the pathogenesis of atherosclerosis in patients (8, 18, 27). We asked whether the B2 bradykinin receptor led to an increased aortic ROS content of Tg-B2++*ApoE*–/– mice. Aortic ROS was detected *in situ* by dihydroethidium (DHE) staining (8, 23). Quantitative image analysis shows that the ROS production in the aorta of *BDKRB2*-expressing Tg-B2++*ApoE*–/– mice was significantly increased compared to *ApoE*–/– mice with endogenous B2 receptor expression level, i.e., aortic ROS levels of Tg-B2++*ApoE*–/– mice were 1.6 ± 0.3-fold higher than those of *ApoE*–/– mice (**Figures 3A,B**). Vice versa, there was a significant decrease in the aortic ROS content of double-deficient *B2*–/–*ApoE*–/– mice compared to *ApoE*–/– mice with intact *Bdkrb2* gene (**Figures 3A,B**). Taken together, the B2 bradykinin receptor mediates an enhanced production of ROS

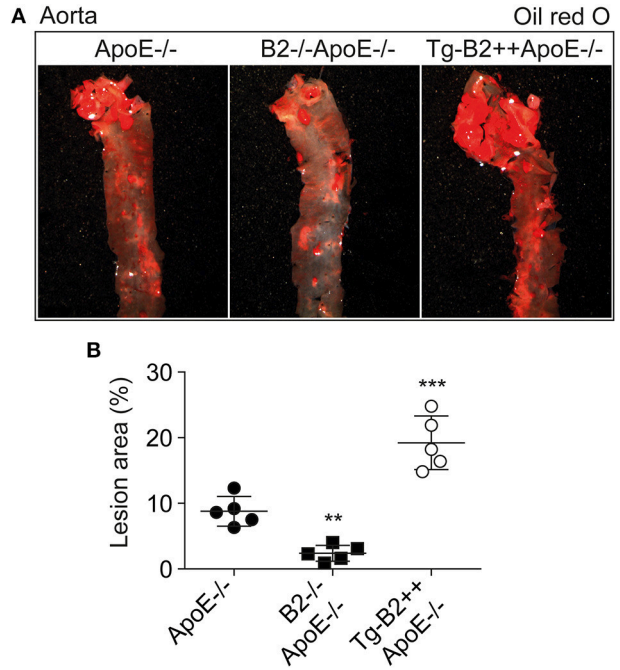


**FIGURE 2 |** Transgenic *BDKRB2* expression enhances atherosclerotic lesion formation whereas *Bdkrb2* deficiency leads to a decreased atherosclerotic plaque area in the aorta of *ApoE*–/– mice. **(A,B)** Atherosclerotic lesion area was determined with oil red O-stained aortas by quantitative image analysis. Representative aortic images are shown in **(A)**, and **(B)** shows quantitative data evaluation (mean ± s.d.; $n = 5$; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. *ApoE*–/–; Dunnett's test).

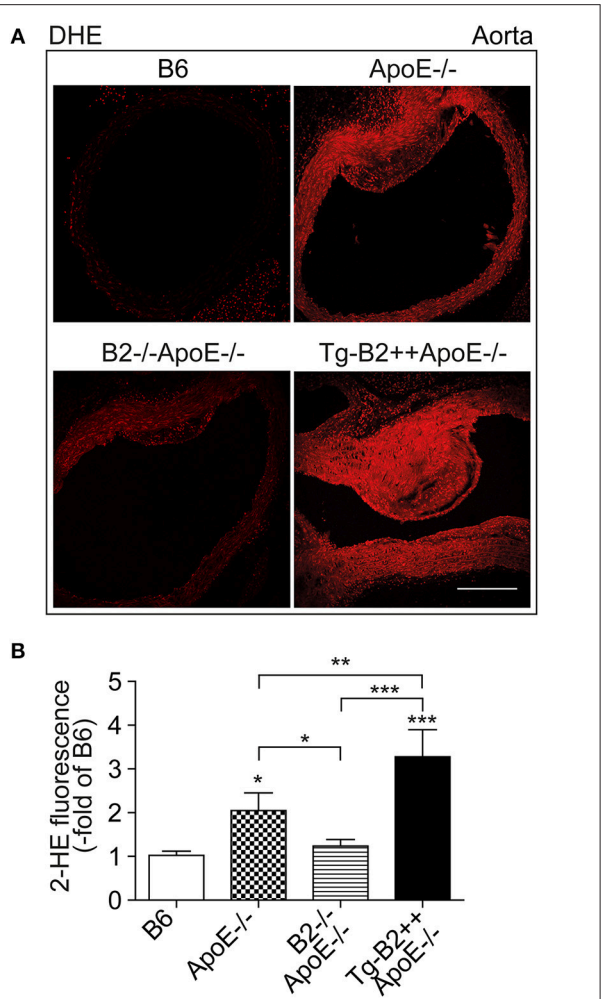


**FIGURE 3 |** Transgenic BDKRB2 expression leads to an increased aortic ROS content in Tg-B2++ApoE–/– mice. **(A,B)** The aortic ROS content was determined by DHE staining of aortas from different study groups. Representative images are shown in **(A)**, and **(B)** shows quantitative data (mean ± s.d.; $n = 4$; $^{*}p < 0.05$ (ApoE–/–) and $^{***}p < 0.001$ (Tg-B2++ApoE–/–) vs. B6; $^{**}p < 0.01$; other comparisons are indicated; Tukey's test; bar: 100 μm).

in the aorta of atherosclerosis-prone Tg-B2++*ApoE*–/– mice with hypercholesterolemia.

## The Aortic Nitrate Content as a Marker of NO Activity and the Nitric Oxide Synthase (NOS) Cofactor Tetrahydrobiopterin (BH4) Are Decreased in Tg-B2++*ApoE*–/– Mice

On vascular endothelial cells, the B2 bradykinin receptor is known for its nitric oxide (NO)-stimulating function (10, 28, 29). Nitric oxide is considered as a major atheroprotective factor released from intact endothelium (30, 31). On the other hand, cardiovascular risk factors such as hypercholesterolemia and atherosclerosis trigger endothelial dysfunction (29, 30), which leads to endothelial nitric oxide synthase (eNOS) uncoupling (19). Uncoupled eNOS produces atherogenic superoxide instead of atheroprotective NO (19). In agreement with previous data on defective NO generation in hypercholesterolemic *ApoE*–/– mice (32), the aortic tissue nitrate content as a marker of NO activity *in vivo* (33), was significantly decreased in *ApoE*–/– mice compared to non-transgenic B6 mice, i.e the aortic nitrate of ApoE–/– mice was 539 ± 89 nmol/g compared to 1031 ± 158 nmol/g in B6 mice (**Figure 4A**). Transgenic expression of *BDKRB2* further decreased the aortic nitrate content in Tg-B2++*ApoE*–/– mice compared to *ApoE*–/–mice with endogenous *Bdkrb2* expression (**Figure 4A**). Vice versa, deficiency of *Bdkrb2* led to a significantly increased aortic nitrate content of *Bdkrb2*–/–*ApoE*–/– mice compared to *ApoE*–/– mice (**Figure 4A**). This finding is complementary to previous data, which show that deficiency of *Bdkrb2* increases the serum nitrate level (34). Nevertheless, the aortic nitrate content of *Bdkrb2*–/–*ApoE*–/– mice was not normalized to B6 control level by B2 bradykinin receptor deficiency (**Figure 4A**), most likely because defective NO generation in atherosclerotic *Bdkrb2*–/–*ApoE*–/– mice has additional causes, which are independent of *Bdkrb2*, e.g., *ApoE* deficiency-induced hypercholesterolemia. Together these data show that the B2 bradykinin receptor mediates a decrease in aortic nitrate content as a marker of dysfunctional aortic (e)NOS activity in Tg-B2++*ApoE*–/– mice whereas deficiency of *Bdkrb2* increases the aortic nitrate level in *Bdkrb2*–/–*ApoE*–/– mice.

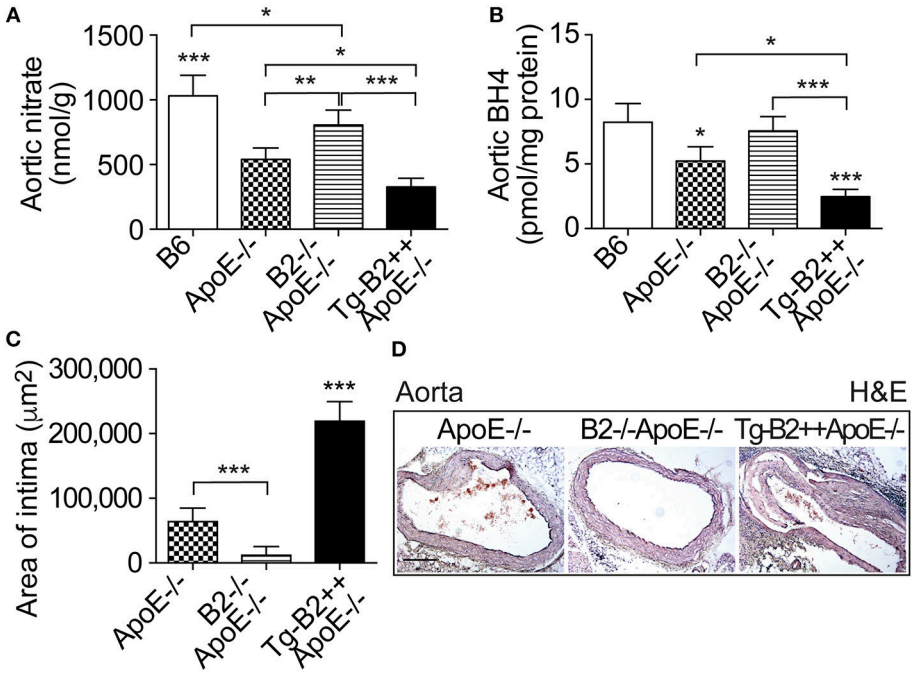


**FIGURE 4 |** The aortic nitrate content as a marker of NO activity and the nitric oxide synthase (NOS) cofactor tetrahydrobiopterin (BH4) are decreased in Tg-B2++*ApoE*–/– mice. **(A,B)** Aortic nitrate **(A)** and aortic BH4 **(B)** contents of study groups (mean ± s.d.; $n = 5$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. *ApoE*–/– and TgB2++*ApoE*–/– **(A)**; mean ± s.d.; $n = 4$; *$p < 0.05$ vs. B6 and B2–/–*ApoE*–/–, and ***$p < 0.001$ vs. B6 **(B)**; other comparisons are indicated; Tukey's test). **(C,D)** Intimal atherosclerotic lesion area was determined on hematoxylin and eosin- (H&E)-stained paraffin sections of the aortic arch. Quantitative data evaluation is shown in **(C)**, and **(D)** shows representative sections (mean ± s.d.; $n = 3$; ***$p < 0.001$ vs. *ApoE*–/– and B2–/–*ApoE*–/–; other comparisons are indicated; Tukey's test).

The eNOS uncoupling during the pathogenesis of atherosclerosis was partially attributed to down-regulation of the (e)NOS cofactor, tetrahydrobiopterin, BH4 (35, 36). We asked whether the decreased aortic nitrate content of *BDKRB2*-transgenic Tg-B2++*ApoE*–/– mice was accompanied by changes in aortic BH4 levels. We measured the aortic BH4 content and found that transgenic *BDKRB2* expression led to a decrease in the aortic BH4 content of Tg-B2++*ApoE*–/– mice, which was 2.48 ± 0.56 pmol/mg in Tg-B2++*ApoE*–/– mice compared to 5.23 ± 1.09 pmol/mg in *ApoE*–/– mice with endogenous *Bdkrb2* expression level (**Figure 4B**). For comparison, aortic BH4 levels were not significantly different between *ApoE*–/– mice with *Bdkrb2*-deficiency and non-transgenic B6 mice (**Figure 4B**). This observation could be due to the fact that there are two opposing mechanisms acting on the aortic BH4 level in *Bdkrb2*–/–*ApoE*–/– mice, i.e., (i) *ApoE* deficiency, which decreases the aortic BH4 content, and (ii) *Bdkrb2* deficiency, which counteracts the *ApoE* deficiency-induced BH4 decrease. The net result is a normalization of aortic BH4 content in *Bdkrb2*–/–*ApoE*–/– mice toward the level of non-transgenic B6 mice (**Figure 4B**).

Histologic evaluation of hematoxylin-eosin-stained aortic specimens from the different groups of *ApoE*–/– mice confirmed the enhanced atherosclerosis progression of Tg-B2++/*ApoE*–/– mice by an increased intimal atherosclerotic lesion area in the aortic arch of Tg-B2++*ApoE*–/– mice with transgenic *BDKRB2* expression compared to *ApoE*–/– mice with endogenous *Bdkrb2* expression, and double-deficient *B2*–/–*ApoE*–/– mice (**Figures 4C,D**). Together these data show a decrease in (e)NOS activity as documented by a reduced aortic content of nitrate and depletion of the (e)NOS co-factor, BH4, by the B2 bradykinin receptor in hypercholesterolemic Tg-B2++*ApoE*–/– mice with concomitantly enhanced atherosclerotic lesion development.

## The Major BH4-Synthesizing Enzyme, *Gch1* (GTP Cyclohydrolase 1) Is Down-Regulated by *BDKRB2* in Tg-B2++*ApoE*–/– Mice

We searched for the mechanism underlying the aortic decrease in the (e)NOS cofactor, BH4, which was triggered by the B2 bradykinin receptor. BH4 deficiency in atherosclerosis could be caused by at least two different mechanisms, i.e., by (i) BH4 oxidation as a consequence of increased ROS levels (cf. **Figure 3**), and (ii) impaired BH4 synthesis due to decreased levels/activity of the GTP cyclohydrolase 1 (*Gch1*), which is the rate-limiting enzyme in tetrahydrobiopterin (BH4) biosynthesis (36, 37). Previous data have shown that endothelial *Gch1* activity and expression are decreased by various cardiovascular risk factors (37). Therefore, we determined the aortic expression level of *Gch1* in Tg-B2++*ApoE*–/– mice. Our data show that aortic *Gch1* expression levels were significantly decreased in *BDKRB2*-expressing Tg-B2++*ApoE*–/– mice compared to *ApoE*–/– mice with endogenous *Bdkrb2* levels, i.e., aortic *Gch1* levels were 2.1-fold lower in Tg-B2++*ApoE*–/– mice compared to *ApoE*–/– mice (**Figure 5A**). For comparison, the aortic *Gch1* expression level of B2–/–*ApoE*–/– mice with *Bdkrb2* deficiency was not significantly different from non-transgenic B6 mice (**Figure 5A**). As a control,

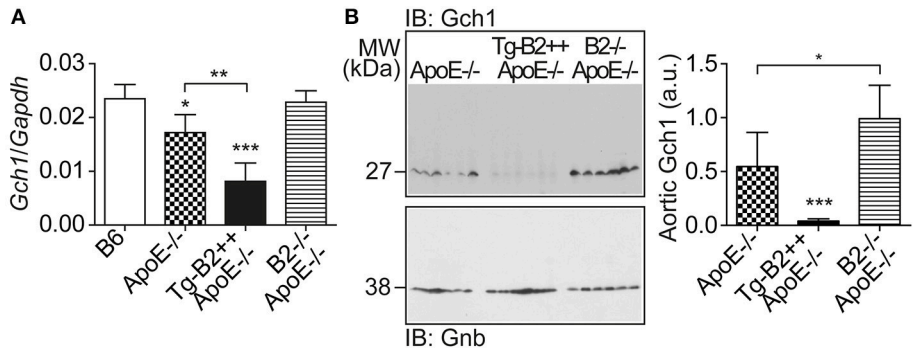


**FIGURE 5 |** The major BH4-synthesizing enzyme, *Gch1* is down-regulated by *BDKRB2* in Tg-B2++*ApoE*–/– mice. **(A)** Aortic gene expression level of *Gch1* was quantified of indicated study groups (mean ± s.d.; $n = 4$; *$p < 0.05$ vs. B6; **$p < 0.01$; ***$p < 0.001$ vs. B6 and B2–/–*ApoE*–/–; other comparisons are indicated; Tukey's test). **(B)** The aortic protein content of Gch1 was determined by immunoblot with Gch1-specific antibodies. The left panel shows immunoblot detection of aortic Gch1 of indicated study groups, and the right panel shows quantitative data (mean ± s.d.; $n = 6$; *$p < 0.05$; ***$p < 0.001$ vs. *ApoE*–/– and B2–/–*ApoE*–/–; other comparisons are indicated; Tukey's test).

immunoblot detection of the aortic Gch1 protein confirmed the significant decrease of the aortic Gch1 content in *BDKRB2*-expressing Tg-B2++ApoE–/– mice compared to *ApoE*–/– mice with and without endogenously expressed *Bdkrb2* (**Figure 5B**).

## Treatment With the BH4 Analog, Sapropterin, Retards Atherosclerotic Plaque Formation and Decreases the Aortic ROS Content of Tg-B2++*ApoE*–/– Mice

Is there a causal relationship between decreased aortic BH4 levels and enhanced atherosclerotic plaque formation in *BDKRB2*-expressing, Tg-B2++*ApoE*–/– mice? To address this question, we treated Tg-B2++*ApoE*–/– mice with the BH4 analog, sapropterin. Treatment outcome of 8-month-old mice was evaluated after 5 months of treatment. Our experiments show that BH4 supplementation retards the enhanced atherosclerotic plaque formation of Tg-B2++*ApoE*–/– mice (**Figures 6A,B**). In contrast, the effect of BH4 in *ApoE*–/– mice with endogenously expressed *Bdkrb2* was not significant (**Figure 6B**). Concomitantly, supplementation of BH4 also decreased the exaggerated aortic ROS content of Tg-B2++*ApoE*–/– mice (**Figures 6A,C**). As a control, saproterin treatment led to increased aortic BH4 levels in Tg-B2++*ApoE*–/– mice and *ApoE*–/– mice with endogenous *Bdkrb2* levels (**Figure 6D**). These experiments show that BH4 supplementation is capable to counteract the atherosclerosis-promoting ROS generation in Tg-B2++*ApoE*–/– mice with transgenic *BDKRB2* expression.

## ACE Inhibition With Captopril Inhibits Atherosclerotic Plaque Accumulation in Tg-B2++*ApoE*–/– Mice

The angiotensin II AT1 receptor is a major contributor to atherosclerosis-promoting ROS generation in *ApoE*–/– mice (8, 38–40). Atherogenic functions of the AT1 receptor are enhanced by *BDKRB2* (25, 41), and atherosclerosis-related endothelial dysfunction can be prevented by inhibition of angiotensin II AT1 receptor stimulation with an inhibitor of the angiotensin II-generating ACE (42, 43). Because *BDKRB2* enhanced the endothelial dysfunction of Tg-B2++*ApoE*–/– mice, we asked whether inhibition of ACE-dependent angiotensin II generation in Tg-B2++*ApoE*–/– mice could retard the *BDKRB2*-enhanced atherosclerosis progression. To address this question, we treated Tg-B2++*ApoE*–/– mice with the ACE inhibitor, captopril (20 mg/kg/d) for 5 months. Atherosclerotic lesion area was evaluated of oil red O-stained aortas and revealed that captopril largely prevented the accumulation of atherosclerotic plaques in 8 month-old Tg-B2++*ApoE*–/– mice (**Figures 7A,B**). In agreement with previous studies (7, 8), captopril also inhibited the formation of atherosclerotic lesions in *ApoE*–/– control mice with endogenous *Bdkrb2* expression (**Figures 7A,B**). Taken together, inhibition of ACE-dependent angiotensin II AT1 receptor activation is capable to prevent the *BDKRB2*-enhanced atherosclerotic lesion development in Tg-B2++*ApoE*–/– mice. Consequently, the atherogenic function of the B2 bradykinin receptor in *ApoE*–/– mice involves a synergistic interplay between the B2 bradykinin receptor and angiotensin II-stimulated AT1 receptor activation.

# DISCUSSION

In this study, we investigated the impact of the B2 bradykinin receptor on atherosclerotic lesion formation in hypercholesterolemic *ApoE*–/– mice as a model of atherosclerosis. We found that a moderately increased *BDKRB2* level in Tg-B2++*ApoE*–/– mice led to a significantly enhanced progression of atherosclerotic lesion development whereas deficiency of *Bdkrb2* retarded the accumulation of atherosclerotic plaques in *Bdkrb2*–/–*ApoE*–/– mice.

In search for pathomechanisms underlying the enhanced atherogenesis triggered by *BDKRB2*, we detected an increased aortic ROS content in Tg-B2++*ApoE*–/– mice compared to

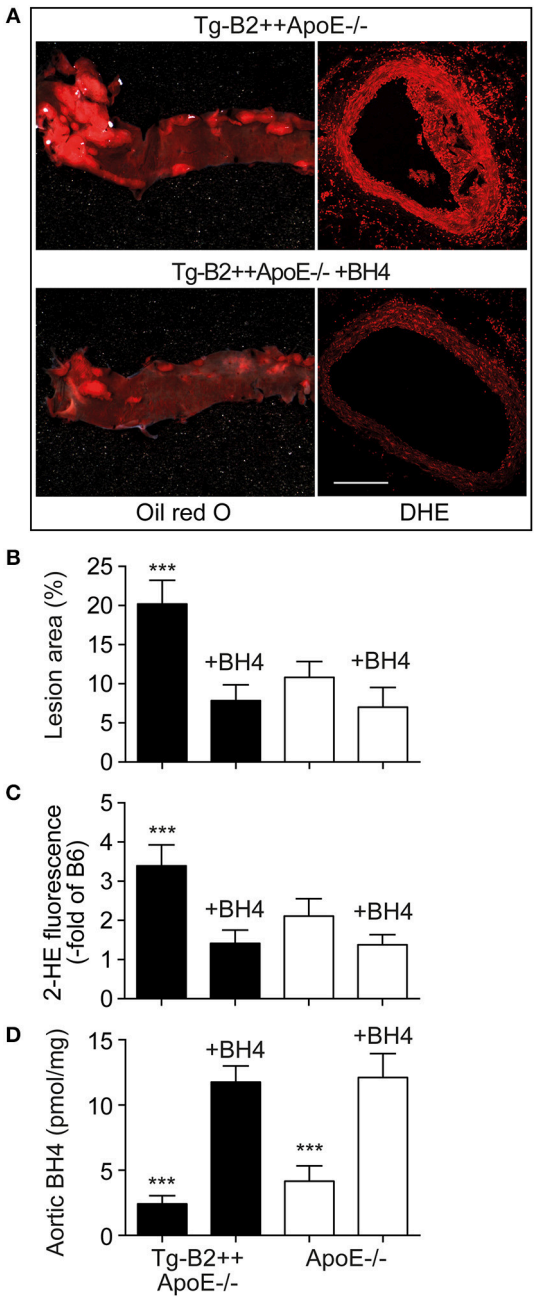


**FIGURE 6 |** Treatment with the BH4 analog, sapropterin, retards atherosclerotic plaque formation and decreases the aortic ROS content of Tg-B2++*ApoE*–/– mice. **(A–C)** Enhanced atherosclerotic plaque formation of Tg-B2++*ApoE*–/– mice is retarded, and aortic ROS content is decreased by treatment with sapropterin. In **(A)**, representative oil red O-stained aortas (left panels) and DHE-stained aortic sections (right panels) from TgB2++*ApoE*–/– mice are shown (bar: 100 μm; right panels). Panels **(B,C)** present quantitative data (mean ± s.d.; $n = 4$; ***$p < 0.001$ vs. all other groups of mice; Tukey's test). **(D)** Aortic BH4 contents of study groups (mean ± s.d.; $n = 4$; ***$p <$ 0.001 vs. BH4-treated mice; Tukey's test).

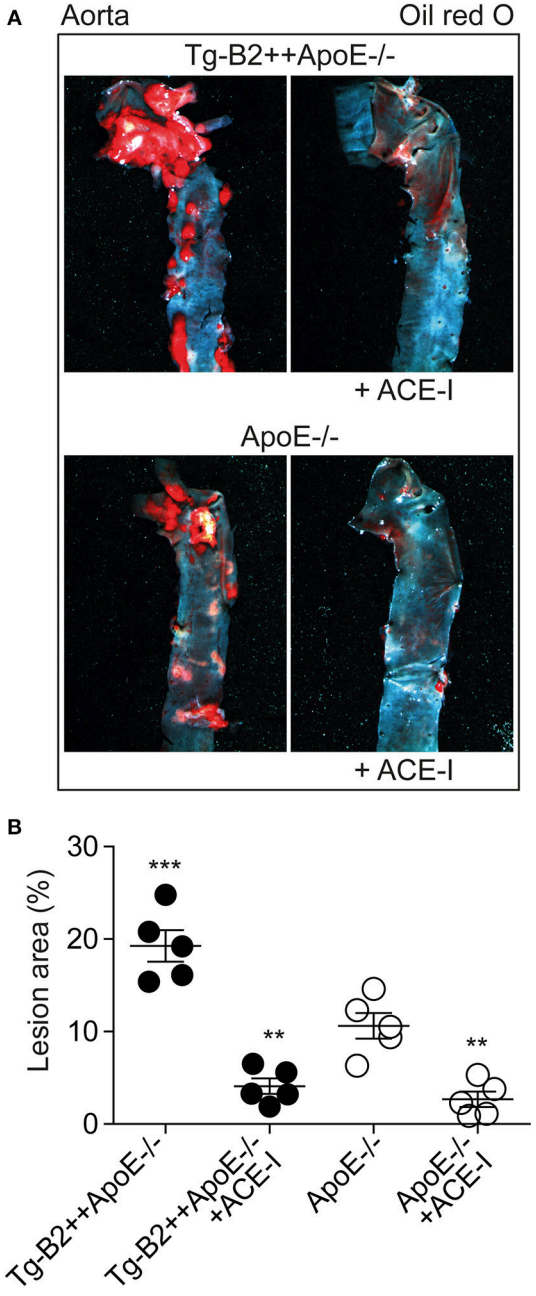


**FIGURE 7 |** ACE inhibition with captopril inhibits atherosclerotic plaque accumulation in Tg-B2++*ApoE*–/– mice. **(A,B)**. Atherosclerotic plaque area was determined of 8 months-old Tg-B2++*ApoE*–/– and *ApoE*–/– mice after treatment without and with the ACE-inhibitor (+ACE-I), captopril. Panels in **(A)** show representative oil red O-stained aortas, and **(B)** shows quantitative data (mean ± s.d.; $n = 5$; **$p < 0.01$ vs. *ApoE*–/–; ***$p < 0.001$ vs. Tg-B2++*ApoE*–/–+ACE-I, *ApoE*–/– and *ApoE*–/–+ACE-I; Tukey's test).

*ApoE*–/– mice with endogenous *Bdkrb2* expression level. In addition, the endogenous *Bdkrb2* contributes to enhanced ROS generation in *ApoE*–/– mice because the aortic ROS content was significantly lower in double-deficient *Bdkrb2*–/–*ApoE*–/– mice compared to *ApoE*–/– mice with intact endogenous *Bdkrb2* gene. The increased vascular ROS level could directly contribute to enhanced atherosclerotic lesion formation because ROS is known to accelerate atherogenesis in animal models

of atherosclerosis and patients with atherosclerotic vascular disease (8, 18, 27). Aortic ROS generation in *ApoE*–/– mice was previously attributed to the atherosclerosis-enhancing function of the angiotensin II AT1 receptor, which generates ROS by activation of NADPH oxidases (8, 38). Activation of the AT1 receptor in experimental models of atherosclerosis and patients with cardiovascular disease is increased due to hypercholesterolemia-induced up-regulation of the systemic renin angiotensin system (39, 40). Our study identifies the B2 bradykinin receptor as another player involved in enhanced ROS generation in *ApoE*–/– mice. B2 bradykinin receptor-enhanced ROS generation could as well be mediated by the AT1 receptor, which becomes hyperactive by protein complex formation with the B2 bradykinin receptor (25, 41). Notably, endogenous *Bdkrb2* levels are sufficient to sensitize the AT1 receptor-stimulated response, and the AT1-sensitizing function of the B2 bradykinin receptor does not require bradykinin (25, 41). In agreement with a bradykinin-independent function of the B2 bradykinin receptor in atherosclerosis, treatment of *ApoE*–/– mice with the B2 bradykinin receptor-specific antagonist, HOE140, did not alter atherosclerosis progression (13). However, the (brady)kinin-generating kallikrein system was not active in this study, because treatment with the B2-specific antagonist, HOE140, had no effect on blood pressure, neither under basal conditions nor upon blood pressure lowering with the ACE inhibitor, ramipril (13). Thus, bradykinin-dependent and bradykinin-independent effects need to be considered in the atherosclerosis-promoting activity of the B2 bradykinin receptor depending on the activation state of the kinin-kallikrein system.

As a consequence of the AT1-receptor sensitizing activity, the B2 bradykinin receptor could mediate a decrease in the nitric oxide synthase (NOS) cofactor, BH4, which is inactivated by ROS (37). Decreased BH4 leads to uncoupled eNOS, which in turn generates more ROS. The decrease in aortic BH4 content could synergistically be aggravated by downregulation of *Gch1*, which is the rate-limiting enzyme in BH4 synthesis. In concert with hypercholesterolemia, *BDKRB2* and *Bdkrb2* could decrease vascular *Gch1* expression and protein level by activation of Gi-coupled signaling because hypercholesterolemia and Gi-coupled signaling are both known to down-regulate *Gch1* (44–46). The ensuing depletion of vascular BH4 could contribute to eNOS uncoupling in the pathogenesis of atherosclerosis (35). The uncoupled eNOS generates detrimental ROS instead of atheroprotective NO (**Figure 8**). Transgenic animal models support that *Gch1* deficiency and reduced vascular BH4 accelerate atherosclerosis whereas supplementation of BH4 or *Gch1* expression reverse these deficits and retard atherosclerosis (19, 35, 47). In agreement with these findings, we found that treatment with BH4 decreased aortic ROS levels and dampened the atherosclerosis-enhancing effect of *BDKRB2*.

It is well-established that the bradykinin-potentiating effect of ACE inhibitors contributes to cardio-protection [**Figure 8A**, (9, 10)]. However, the atherosclerosis-lowering potential of ACE inhibition does not necessarily rely on bradykinin-stimulated *Bdkrb2* activation, e.g., when there is no activation of the (brady)kinin-generating kallikrein system (13). A B2-specific antagonist, HOE140, also had no effect on atherosclerosis

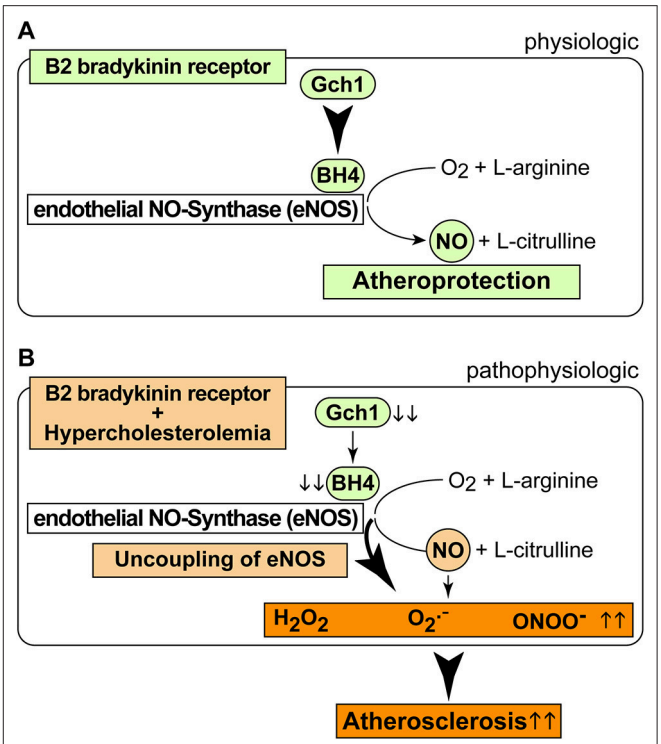


**FIGURE 8 |** Scheme of physiologic and pathophysiologic functions of the B2 bradykinin receptor. **(A,B)** Physiologic functions of the B2 bradykinin receptor in endothelial cells are atheroprotective **(A)**, and pathophysiologic functions **(B)** of the B2 bradykinin receptor in hypercholesterolemia enhance atherogenesis.

progression in *ApoE*–/– mice without ACE inhibition (13), most likely because the beneficial NO-generating capacity of endothelial B2 bradykinin receptor stimulation is impaired in atherosclerosis and cardiovascular disease [**Figure 8B**, (42, 43, 48)]. Because atherosclerosis progression in *ApoE*–/– mice is not affected by the B2-specific antagonist, HOE140 (13), atherosclerosis promotion by *Bdkrb2* in *ApoE*–/– mice could largely be mediated by the angiotensin II AT1 receptor-sensitizing function of *Bdkrb2*, which is bradykinin-independent (25, 41). In agreement with this conclusion, our study shows that inhibition of angiotensin II AT1 receptor stimulation by an ACE inhibitor completely prevents atherosclerotic lesion formation in Tg-B2++*ApoE*–/– mice. Thus, under hypercholesterolemia, the B2 bradykinin receptor gene is atherogenic, leads to increased vascular ROS, mediates a decrease in (e)NOS activity as evidenced by a reduced aortic nitrate content, and enhances aortic BH4 deficiency (**Figure 8B**). BH4 depletion contributes to the atherogenic activity of the B2 bradykinin receptor because supplementation with BH4 was sufficient to inhibit aortic ROS and retard atherosclerosis progression triggered by transgenic *BDKRB2* expression (**Figure 8B**).

Enhanced ROS generation in hypercholesterolemia and atherosclerosis is largely attributed to angiotensin II AT1 receptor stimulation (8, 38). Consequently, inhibition of detrimental AT1 receptor-stimulated ROS generation by an ACE inhibitor or AT1 antagonist in animal models and patients could exert a

dual protective function regarding the atherogenic activity of *BDKRB2*, i.e., (i) restoration of protective B2 bradykinin receptor signaling, and (ii) neutralization of the AT1-sensitizing function of *BDKRB2*. Symptoms of the herein deduced atherogenic activity of the B2 bradykinin receptor can be prevented by ACE inhibition, which inhibits sensitized AT1 receptor signaling, blunts exaggerated ROS generation, and heals uncoupled eNOS in atherosclerosis and cardiovascular disease (42, 43, 47). Consequently, the agonist-stimulated B2 bradykinin receptor could become anti-atherogenic upon treatment with an ACE inhibitor, which prevents endothelial dysfunction triggered by oxidized low-density lipoproteins in a B2 bradykinin receptor stimulation-dependent manner (49).

Taken together, our study shows that the atherosclerosis-enhancing function of the ubiquitously expressed B2 bradykinin receptor involves a synergistic interplay between enhanced ROS generation, dysfunctional NO activity and AT1 receptor activation. Pathomechanisms identified in this study could act in concert with already established B2 and/or AT1 receptor-dependent atherogenic activities. Notably, the following mechanisms mediated by the B2 and/or AT1 receptor could play a direct or indirect role in B2 bradykinin receptor-enhanced atherosclerosis: (I) The B2 bradykinin receptor could promote atherosclerosis by the reduced activation of atheroprotective AT1-inhibitory receptors, *AGTR2* and *MAS1* (34, 50–52), which are directly down-regulated by the B2 bradykinin receptor and angiotensin II AT1 receptor (34, 53) and indirectly dampened via angiotensin II AT1-mediated *ACE2* down-regulation (54). (II) In addition, the B2 bradykinin receptor could enhance atherogenesis by decreasing the expression of the vasculoprotective endothelial Kruppel-like factor 4, *KLF4* (55), which is directly downregulated by angiotensin II AT1 stimulation [(56); NCBI GEO dataset GSE19286 in Abd Alla et al. (7)], and indirectly down-regulated by reduced Mas receptor activation (57). (III) And finally, the B2 bradykinin receptor-stimulated atherogenesis could involve the enhanced aortic infiltration with macrophages and inflammatory immune cells, which is promoted by angiotensin II AT1 receptor signaling directly (7, 58), and indirectly by B2 bradykinin and angiotensin II AT1 receptor-mediated downregulation of Mas, which accounts for a decreased expression of atheroprotective sirtuin 1, *SIRT1* (34, 57, 59). In view of this panoply of different atherogenic functions, specific targeting of the detrimental B2 - AT1 receptor axis could be envisaged as a potential approach to treat not only atherosclerosis but also related pro-thrombotic activities triggered by the B2 bradykinin and angiotensin II AT1 receptors (34, 60, 61).

## AUTHOR CONTRIBUTIONS

AP, SW, YJ, AL, and JA performed experiments. JA generated transgenic mice. All authors evaluated data. UQ conducted the study, designed experiments and wrote the manuscript. All authors read and approved the final version of the manuscript.

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# Polyphosphates and Complement Activation

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To sustain life in environments that are fraught with risks of life-threatening injury, organisms have developed innate protective strategies such that the response to wounds is rapid and localized, with the simultaneous recruitment of molecular, biochemical, and cellular pathways that limit bleeding and eliminate pathogens and damaged host cells, while promoting effective healing. These pathways are both coordinated and tightly regulated, as their over- or under-activation may lead to inadequate healing, disease, and/or demise of the host. Recent advances in our understanding of coagulation and complement, a key component of innate immunity, have revealed an intriguing linkage of the two systems. Cell-secreted polyphosphate promotes coagulation, while dampening complement activation, discoveries that are providing insights into disease mechanisms and suggesting novel therapeutic strategies.

**Keywords: innate immunity, thrombosis, inflammation, coagulation, age-related macular degeneration, mouse models, C1-esterase inhibitor, membrane attack complex**

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## INTRODUCTION

Two major blood-borne proteolytic cascades, complement and coagulation, are fully integrated to cooperatively fight infections and prevent excessive bleeding from wounds. Interplay between these systems is evolutionarily conserved, as evident in the horseshoe crab, a "living fossil" representing arthropods from 500 million years ago (1–3). The released contents of hemocytes induce clotting and destroy invading pathogens and toxins. Complement and coagulation pathways in mammals now appear more distinct, but interactions are increasingly being recognized. Elucidation of the links is yielding novel treatments, including, eculizumab, an effective anti-complement antibody that prevents thrombosis in paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS) (4, 5). More therapies for other common diseases will undoubtedly enter the clinic in coming years (6).

In this report, I review the complement system, highlighting some key mechanisms by which it is regulated, and how it interfaces with coagulation [Readers are referred to excellent reviews of the coagulation cascade (7–10)]. I then focus on recently uncovered insights into the role of the polyanion polyphosphate (polyP)—known to promote coagulation—in dampening complement activation. This will be followed by a discussion of how such an apparent dichotomy in the function of polyP is physiologically relevant.

## COMPLEMENT ACTIVATION

Comprising over 30 soluble and membrane-bound proteins, complement contributes to innate immunity and provides a bridge to adaptive immunity (11–13). Complement activation is triggered by exposure of blood to damage-associated molecular patterns that include, for example, pathogens, host DNA from damaged cells, lipids and oligosaccharides (**Figure 1**). Three pathways—lectin (LP), classical (CP), and alternative (AP)—converge with generation of C3 convertases that proteolyse C3 into C3b and release the anaphylatoxin C3a. The CP is triggered by C1q recognition of antibodies bound to antigens or microbial surfaces. C1q may also recognize other targets, such as C-reactive protein, apoptotic cells, and microbes. It circulates in complex with zymogens of serine proteases C1r and C1s ($C1qr^2s^2$). When C1q binds to its target, C1r autoactivates and activates C1s (15) which in turn cleaves C4, releasing C4a, and C4b, the latter which covalently binds to target surfaces. C2 binds to immobilized C4b and is cleaved by C1s into C2b and C2a, allowing C2a to complex with C4b and form the CP C3 convertase, C4b2a. The LP is similar to the CP (16), but pathogen recognition comprises mannose binding lectin (MBL), ficolins and/or collectin-11. These circulate bound to MBL-associated zymogens of serine proteases MASP1/MASP3 and MASP2 and bind to sugars or N-acetylated groups on micro-organisms. MASP1 autoactivates and cleaves C2 and activates MASP2, while MASP2 cleaves C2 and C4, yielding the C4b2a LP C3 convertase (17).

The AP is constitutively active, sustained by a "tick-over" mechanism in which small amounts of C3 are hydrolyzed to $C3(H_2O)$ (18), exposing a binding site for factor B (FB). Circulating factor D (FD) cleaves FB into Ba and Bb, the latter which binds to $C3(H_2O)$ to form a fluid-phase C3 convertase which cleaves C3 to C3b and C3a. With exposure to a pathogen or damaged cell, more C3a and C3b are generated (19), resulting in formation of the AP C3 convertase, C3bBb. Complement is amplified via the AP as the pathways converge and form cell bound C3 convertases. The additional C3b binds to C4b2b and C3bBb, yielding $C4bBb(C3b)_n$ and $C3bBb(C3b)_n$, which thus become C5 convertases, cleaving C5 into C5b and C5a. C5b is the trigger for the terminal pathway, which spontaneously proceeds with sequential assembly of C6, C7, C8, and multiple C9 subunits, forming the C5b-9 pore-like, lytic membrane attack complex (MAC) that targets invading pathogens and promotes prothrombinase assembly and tissue factor (TF) activation (20).

C5a is a pleiotropic biologically active peptide, exhibiting potent anaphylatoxin properties. C5a also triggers coagulation and inflammation via TF by endothelial cells and monocytes, release/exposure of VWF and P-selectin by endothelial cells and platelets, secretion of inflammatory cytokines, expression of leukocyte adhesion molecules, and release of platelet granule

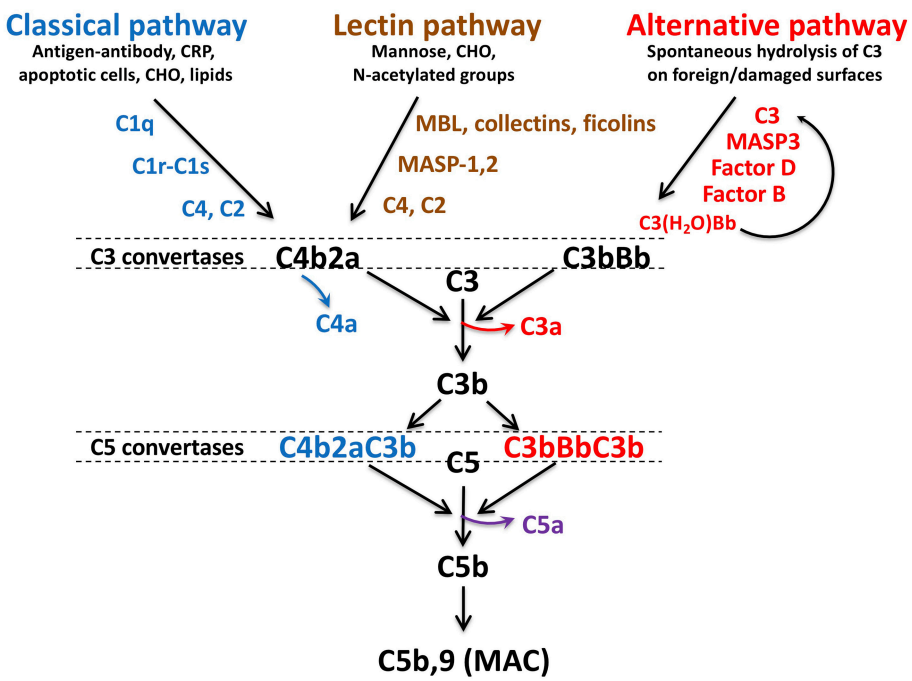


**FIGURE 1 |** Schematic of complement activation pathways. Complement activation proceeds via the classical, lectin, or alternative pathways, triggered by exposure of surveillance molecules, C1q, MBL, collectins and ficolins, of specific danger signals. The alternative pathway is constitutively "on," due to spontaneous hydrolysis of C3. The pathways converge to form C3 convertases: C4b2a for the classical and lectin pathways, C3bBb for the alternative pathway. C4a and C3a, are released with cleavage of C4 and C3, respectively. As C3b is further generated, C5 convertases C4bBbC3b and C3bBbC3b are formed, resulting in liberation of the most potent anaphylatoxin C5a, in conjunction with C5b. C5b is the initial component required for spontaneous assembly of the C5b-9 membrane attack complex (MAC) which polymerizes and induces lysis of the cellular target. MBL, mannose binding lectin, CRP, C-reactive protein, CHO, carbohydrate, MASP, MBL associated serine protease. Figure and legend from Conway (14).

contents that further promote coagulation and complement activation (21–24).

## REGULATION OF COMPLEMENT

Complement activation is down-regulated at numerous steps. This ensures a highly localized and temporally appropriate response that spares the host from undesired damage. Acquired or genetic alterations in factors that regulate complement are commonly associated with disease, often featuring varying degrees of vascular-thrombosis. Characterization of these pathways is revealing novel strategies for drug development (6, 25, 26). In the following, I describe a few of the mechanisms by which complement activation is dampened. References for more comprehensive reviews are provided (13, 25, 27–29).

C1-esterase inhibitor (C1-INH) is a serine protease inhibitor that highlights the coordinated regulation of coagulation and complement (10). In coagulation, C1-INH interferes with the proteolytic activities of factor XIa, factor XIIa, and kallikrein, suppressing the contact/intrinsic pathways of coagulation and inflammation. In complement, C1-INH interferes with C1r, C1s, MASP1, and MASP2, preventing formation of the CP/LP convertases. The inhibitory activity of C1-INH is variably potentiated by polyanions, such as heparin (15, 30). Thus, heparin augments C1-INH inhibition of factor XIa and MASP2 (31), but actually dampens C1-INH neutralization of factor XIIa (32), and has almost no effect on C1-INH inhibition of kallikrein, C1r or MASP1 (32, 33). These differential effects of C1-INH that are partly dependent on the cofactor activity of the polyanion, heparin, may help explain why functional deficiencies of C1-INH are not associated clinically with thrombosis.

The major fluid-phase negative regulator of the AP is factor H (FH) (34). Synthesized primarily by the liver, but also by endothelial cells and platelets, FH binds to C3b and glycosaminoglycans of host cells where it suppresses complement by acting as a cofactor for protease factor I (FI) mediated inactivation of C3b to iC3b, accelerating decay of the AP C3 convertase, and competing with FB binding to C3b. Patients with FH mutations are at increased risk of developing aHUS and cardiovascular disease (35–37). FH also binds to VWF in Weibel-Palade bodies and/or facilitates ADAMTS13-mediated proteolysis of ultra large VWF (38–41), providing protection against thrombosis.

The membrane-bound complement receptor (CR)1, glycosylphosphatidylinositol (GPI)-linked CD55, and CD46 are also decay accelerating factors for C3b-containing convertases. Moreover, CR1 and CD46 promote FI-mediated proteolysis of C3b to iC3b (42). Interesting for their distinct clinical presentations, CD46 deficiency is implicated in aHUS (43), while CD55 deficiency is associated with PNH (44).

Assembly and function of the MAC are also regulated to limit host cell damage. The GPI-linked CD59, deficiency of which is also associated with PNH (45), binds to C8 and C9 and prevents C9 polymerization (46). Clusterin binds to C7, C8, and C9, inducing structural changes that reduce integration of C5b-9 into the membrane (47). Vitronectin also prevents C5b-9 membrane binding by promoting formation of a soluble C5b-7 complex (48).

The activities of C3a and C5a are reduced via several coagulation-related enzymes. C5a is proteolysed by plasmin and matrix metalloproteinase 12 (49), while carboxypeptidase B2 (CPB2) [also referred to as activated thrombin-activatable fibrinolysis inhibitor (TAFIa)] (50), reduces the activity of C3a and C5a by cleaving their C-terminal basic amino acids.

### PolyP, Coagulation, and Complement

PolyP is a ubiquitously expressed, linear, anionic polymer of monophosphate units, linked by phosphoanhydride bonds (51). Polymer lengths vary from ∼25 to 1,000 units in mammalian cells (52), extending to thousands of units in some bacteria (53). It is abundant in the dense granules of platelets (54, 55), released to the cell surface and/or into the circulation upon activation (55, 56), likely in a charge-neutral form, bound to divalent cations ($Ca^{2+}$, $Mg^{2+}$) and amines (54). Rather than acting as a calcium ion chelator and anticoagulant, polyP promotes coagulation at multiple steps in the cascade (57–61). Long chain polyP is believed to provide a template for autoactivation of factor XII, thereby also triggering inflammation via factor XIIa-mediated activation of the kallikrein-kinin system (62). Shorter forms of polyP released particularly from platelets, bind directly to thrombin (63), fibrinogen, (64), factors XI and XII, pre/kallikrein, high molecular weight kininogen and VWF (56), amplifying generation of factor XIa and thrombin, enhancing the activation of factor V, inactivating tissue factor pathway inhibitor (TFPI), and integrating into the fibrin clot, rendering it more resistant to fibrinolysis (57). The physiologic relevance of several of these and other polyP-coagulation/fibrinolysis protein interactions remain incompletely understood, but targeting polyP is gaining wide interest as a safe anti-thrombotic (65).

In view of its profound pro-coagulant effects, we predicted that polyP would similarly activate complement. Somewhat surprising, polyP did exactly the opposite (**Figure 2**). In a C1-INH-dependent manner, polyP dampened C1s-mediated cleavage of C4 and C2 in gel-based assays and cell systems (67). Binding studies revealed that C1-INH directly interacts with the serine protease domain of C1s at a rate that is augmented ∼90-fold by the presence of polyP—an effect similar to that seen with heparin. Not formally tested, the data suggested that polyP similarly potentiates C1-INH interactions with MASP2. Interestingly, like heparin, polyP had little potentiating effect on C1r (33). However, these parallels with heparin are limited, since heparin accelerates neutralization of thrombin by antithrombin (AT) >2,000-fold (68), whereas polyP has no effect on the thrombin-AT interaction (57).

PolyP also significantly interferes with activation of the terminal pathway of complement (69) in a size and concentration-dependent manner. It destabilizes C5b-6, reducing the ability of C5b-7 and C5b-8 to bind to and integrate into the target membrane. Other pathways by which polyP might modulate complement activation have not yet been explored, however, given its highly anionic charge,

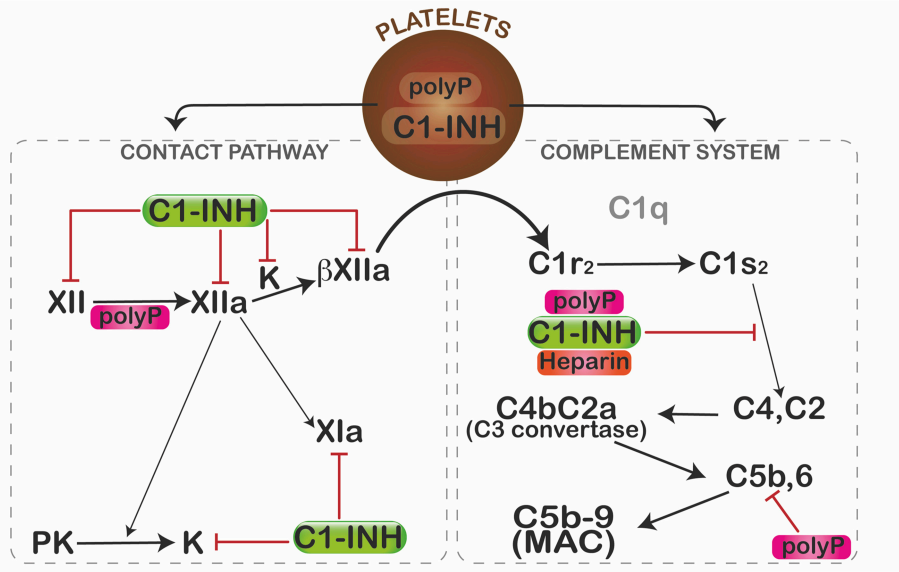


**FIGURE 2 |** Mechanisms by which polyP regulates complement activation. In resting platelets, polyP and C1-INH are housed in different organelles. After activation, polyP and C1-INH coalesce toward the center of the platelets where they colocalize and are subsequently secreted (66). PolyP triggers a conformational change in factor XII, resulting in generation of XIIa, which can activate prekallikrein (PK) and/or factor XI to XIa. Kallikrein (K) or plasmin (not shown) can further cleave XIIa to generate βXIIa which may activate C1r and thus promote complement activation. C1-INH dampens that pathway by inhibiting factor XII, XIIa, βXIIa, and kallikrein (K). C1s cleaves C4 and C2 to generate the C4b2a C3 convertase, which ultimately leads to formation of the C5b,6 complex, and assembly of the C5b-9 membrane attack complex (MAC). PolyP or heparin potentiate the inhibitory function of C1-INH via direct interactions with C1-INH and the target protease, C1s. PolyP also destabilizes C5b,6, thereby dampening formation of the MAC. Interestingly, in spite of binding to factors XIa and XIIa, reduced levels or function of C1-INH do not cause thrombosis, possibly due in part to differential effects of polyanions (polyP, heparin) on the function of the target enzymes. The over-riding effect of polyP in a serum-based endothelial cell culture system is to suppress complement activation. Figure and legend from Wijeyewickrema et al. (66).

its stability in a calcium-nanoparticle form (62), and its wide expression profile, it is likely that polyP has multiple effects on this innate immune pathway, analogous to its role in coagulation.

## RELEVANCE OF polyP IN COMPLEMENT

In the face of its pro-coagulant and pro-inflammatory properties, what might be the physiologic relevance of polyP in complement activation? The complement-dampening effect of polyP does not entirely conflict with the defined role of polyP in other biological systems. PolyP is prominently expressed in several cellular compartments of prokaryotes, where it exhibits pro-survival properties as an energy source, a metal ion chelator, a molecular chaperone, in enhancing pathogenicity (70–72), and in some cases, protecting against complement mediated death (73).

One can speculate on how polyP and C1-INH might co-operate in host protection. C1-INH is synthesized by and found on the surface of endothelial cells (74). Endothelial cells also display abundant glycosaminoglycans on their surface as heparan sulfate. PolyP, released by activated cells and found at low concentrations in the blood of healthy individuals (75, 76) would be available to bind to C1-INH on the endothelium, where it could potentiate the function of C1-INH, allowing the C1-INH:polyanion complex to recruit and neutralize target proteases, such as C1s and/or MASP2. Binding of the polyanion first to C1-INH is required for optimal neutralization of C1s. Such an order of events would best keep complement activation in check. PolyP would also be positioned to dampen generation of MAC on the host cell surface. In such a scenario, the activated endothelial cell would be protected against host-mediated destruction, while retaining its prothrombotic and pro-inflammatory properties.

This model may also apply to other cells. As mentioned, polyP is abundant in platelets and released upon activation (54, 55, 77). C1-INH is also found in platelets, secreted and deposited on the activated platelet membrane (78). Although initially housed in separate granules, platelet activation results in colocalization of polyP and C1-INH in and on the platelet (66). High levels of polyP on activated platelets would therefore readily dampen complement activation by potentiating the inhibitory properties of C1-INH and interfering with the terminal pathway (69), overall protecting the underlying host cells from innate destruction, while allowing the platelets to promote hemostasis-thrombosis and inflammation. Interestingly, polyP also binds to FH (69) and may similar to C1-INH, coat and protect host cells (34) from complement activation, convertase assembly and MAC binding/integration. Disturbances in the release of adequate polyP might therefore be predicted to result in disease. This

is in fact evident in patients with dense granule storage pool diseases (79) who have low platelet polyP (80) and exhibit a bleeding diathesis and organ dysfunction secondary to excessive inflammation.

### Taking Advantage of the Complement-Dampening Properties of polyP for Therapeutic Purposes

Immediate clinical application of the finding that polyP, a naturally occurring and easily synthesized polyanion that suppresses complement activation, is enticing but not without challenges. Depending on the length, dose and formulation, systemic delivery of polyP may entail risk of thrombosis. However, polyP has been administered safely *in vivo*, providing protection against endotoxin-induced sepsis in a mouse model (81). If validated, one could envisage using polyP for a wide range of disorders with excess complement activation. Our group has limited *in vivo* testing of polyP to study its role in protecting against age-related macular degeneration (AMD). AMD is a common cause of blindness where over-activation of complement is a major pathogenic driver (82). In a mouse model of laser-induced AMD, intravitreal administration of polyP dampened the pathologic neovascularization and complement deposition to a similar extent as currently used anti-VEGF targeted therapies (83). No adverse effects of polyP were observed, providing strong rationale for further exploration.

## CONCLUSION

In the last 20–30 years, major inroads have been made in delineating the molecular mechanisms by which complement, coagulation and inflammation intersect. The preceding discussion underlines the unique role that polyP plays in suppressing complement, while promoting coagulation and inflammation. Further understanding of how polyP modulates complement activation through the induction of structural changes in key factors in these different proteolytic cascades, and/or interactions with other proteins and cells, will reveal novel sites for therapeutic intervention for a range of thrombotic and inflammatory disorders.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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# Polyphosphate as a Target for Interference With Inflammation and Thrombosis

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Activated platelets and mast cells expose the inorganic polymer, polyphosphate (polyP) on their surfaces. PolyP initiates procoagulant and proinflammatory reactions and the polymer has been recognized as a therapeutic target for interference with blood coagulation and vascular hyperpermeability. PolyP content and chain length depend on the specific cell type and energy status, which may affect cellular functions. PolyP metabolism has mainly been studied in bacteria and yeast, but its roles in eukaryotic cells and mammalian systems have remained enigmatic. In this review, we will present an overview of polyP functions, focusing on intra- and extracellular roles of the polymer and discuss open questions that emerge from the current knowledge on polyP regulation.

**Keywords: polyphosphate, factor XII, hereditary angioedema, thrombosis, inflammation, coagulation, immune activation, vascular permeability**

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## POLYPHOSPHATE METABOLISM

Inorganic polyphosphate (polyP) is abundantly found in every cell in nature, however previous studies had mainly focused on prokaryotes to investigate polyP metabolism. PolyP is a polyanion consisting of up to several hundreds of phosphate units ($P_i$) linked by energy-rich phosphoanhydride bonds. PolyP is formed in bacteria by polyP kinase through transfer of ATP/GTP's γ-phosphate residues onto the nascent polymer chain (1, 2), while depolymerization of polyP is catalyzed by exopolyphosphatase (ppx) (3). Dependency of polyP for bacterial growth and survival initiated efforts to develop drugs that target polyP metabolism [reviewed in (4–6)].

Eukaryotic polyP metabolism is poorly understood with exception for *S. cerevisiae* cells. Yeast expresses a polyP polymerase, vacuolar transporter chaperone, and polyP phosphatases including exopolyphosphatase (Ppx1), endopolyphosphatases (Ppn1) and diadenosine and diphosphoinositol phosphohydrolase (Ddp1) (7–10). Despite intensive investigations, mammalian homologs for these polyP-related enzymes have not been identified, however diphosphoinositol polyP phosphohydrolases (DIPPs) have been shown to degrade polyP in alkaline conditions (10). The same report also demonstrated that intracellular polyP concentrations were dependent on enzymes regulating inositol phosphorylation, such as phospholipase C, inositol polyP multikinase and inositol hexakisphosphate kinase (Ip6k1) in yeast (10). Notably, genetic ablation of *Ip6k1*, a gene coding for the kinase that generates diphospho-moieties through phosphorylation of the 5-position of inositol penta- and hexakisphosphate, has been shown to reduce platelet polyP levels in mice (11). Together, the data suggest that polyP is intertwined with polyphosphorylated inositol metabolism. Multiple cellular processes are regulated by the (poly)phosphorylated inositol, including signal transduction, $Ca^{2+}$ channel permeability and gene expression [reviewed in (12)]. Particularly, immune cell activation depends on membrane-bound phosphoinositides and soluble inositol phosphates [reviewed in (13)].

In yeast, intracellular concentrations of $P_i$ are sensed through $P_i$-responsive signaling (Pho-regulon) mediated by both, polyP and diphosphoinositol pentakisphosphate (14, 15). Under phosphate-limiting growth conditions, the transcription factor Pho4 controls expression of high affinity $P_i$ transporters and secreted acid phosphatases to replenish intracellular $P_i$. Orthologs of the yeast Pho-regulon seem not to exist in multicellular eukaryotes. However, $P_i$ sensor domains have been identified as part of $P_i$ transporters that are conserved among various organisms and share a SPX domain (16). This common SPX domain, named after yeast Syg1 and Pho81 and human XPR1 (xenotropic and polytropic retrovirus receptor 1), binds to diphosphoinositol pentakisphosphate and increases polyP synthesis in yeast and plants. Mice with conditional deficiency of Xpr1 in renal tubular cells develop proximal tubular dysfunction (17). In humans, impaired XPR1 function due to mutations in the $P_i$ exporter is associated with primary familial brain calcification (18). These findings indicate a link between $P_i$ sensing, intracellular $P_i$ levels and polyP, although further studies in eukaryotes are required to elucidate polyP regulation *in vivo*.

## INTRACELLULAR ROLES OF POLYPHOSPHATE

High cytoplasmic polyP levels have been found in various cell lines, including NIH3T3 fibroblasts, Vero epithelial kidney cells and Jurkat CD4+ T cells (19). Tissues with high energy demand and high regeneration or proliferation capacity (e.g., brain, heart, liver, and cancer cells) are rich in polyP (19–21). In line with polyP's function as energy storage pool, defective polyP synthesis confers disadvantages for growth and survival of bacteria, fungi and protozoa (9, 22, 23). In bacteria, polyP is bound to $Ca^{2+}$ ions and complexed with poly-β-hydroxybutyrate forming membrane channels utilized for DNA uptake (24, 25).

In eukaryotes, polyP has functions in mitochondria and the polymer is enriched within the intermembrane compartment and uncouplers of oxidative phosphorylation decrease mitochondrial polyP levels in *S. cerevisiae* (26, 27). Degradation of polyP using Ppx1 polyphosphatase, that is localized to the mitochondrial intermembrane space by a cytochrome c-derived targeting sequence, decreases the membrane potential and increases NADH levels in mitochondria (28, 29). Moreover, polyP appears to contribute to opening of the mitochondrial permeability transition pore in cardiac myocytes. In these cells in culture, Ppx1-mediated polyP degradation inhibits mitochondrial $Ca^{2+}$ ion accumulation and interferes with $Ca^{2+}$-induced cell death that is associated with myocardial infarction and ischemia reperfusion injury (30).

Based on the function of the polymer for growth and survival, polyP is believed to have a role in malignant diseases. In cancer cells, polyP has been detected in epithelial tumor cell lines derived from prostate and mammary gland as well as in primary myeloma B cells (21, 31, 32). In addition to cancer-associated pro-coagulant activities of extracellular polyP (see below), the polymer facilitates various intracellular functions in cancer cells. PolyP increases the kinase activity of mammalian target of rapamycin (mTOR) in MCF-7 tumor cells and accumulates at nucleolar transcription sites in myeloma cells. In contrast, Ppx1-mediated polyP degradation and actinomycin D-induced transcription inhibition abrogate these polyP effects (31, 32). Together, the data suggest, that polyP may act as metabolic driving force in different cellular compartments, thereby promoting tumorigenesis.

As a negatively charged polymer, polyP exerts chaperone activity in bacteria such as *E. coli* (33, 34). Short and long chain polyP regulate ribosomal translation efficiency and ribosomal protein degradation, indicating a differential role of polyP dependent on chain lengths in bacterial protein biosynthesis (35, 36). Similarly to the polymer starch and its monomeric form glucose, polyP is condensed phosphate and reduces the concentration of $P_i$ and thus the intracellular osmotic pressure conferred by the ion. The polymer acts as $P_i$ storage pool and serves as non-enzymatic protein (pyro-)phosphorylation mediator (37, 38) [as does inositol pyrophosphate (39, 40)] in eukaryotes. PolyP also activates the cytoplasmic portion of transient receptor potential (TRP) A1 and melastatin 8 in neurons (41, 42). Moreover, a recent report identified a glucokinase that relies on polyP as phosphoryl donor in the liver (43), indicating that future research on tissue-specific polyP functions may broaden our view of its physiological roles in various cell types. In this context, $Ca^{2+}$-dependent, mTOR- and TRP-regulated immune responses could be a potential target for polyP.

Besides its established mitochondrial and nuclear distribution, polyP accumulates in intracellular vesicles. Yeast, parasites, mast cells and platelets store polyP in vacuoles, acidocalcisomes, heparin-containing granules and dense granules, respectively (44–48). $Ca^{2+}$-complexed polyP derived from these acidic organelles is insoluble, retained on the plasma membrane in nanoparticle form upon release and provides the pro-coagulant surface for factor XII (FXII) contact activation (49). PolyP-containing vesicles are also found in lysosome-related organelles derived from fibroblasts and astrocytes (50, 51). The latter cell type releases polyP as a neurotransmitter from lysosomes expressing vesicular nucleotide transporter in response to pH changes, exogenous polyP and $Ca^{2+}$ ion signaling (52). Taken together, these studies indicate that polyP have fundamental functions for secretion and possibly stability of lysosome-related vesicles in other cell types, such as immune cells. However, quantification of $P_i$ via malachite green assay and $^{32}P_i$ via radiodetection obtained from hydrolysed polyP has to be interpreted with caution, since microbial contaminations interfered with these assays to detect polyP in human granulocytes in a previous report [update to (53)]. Analysis of *in vivo* polyP functions is an area of ongoing research and recently a flow cytometry-based assay has been established to quantify the polymer on cell surfaces (54). **Table 1** provides a summary of polyP functions in different organisms, cell populations and intracellular compartments.

## EXTRACELLULAR POLYPHOSPHATES

While intracellular polyP activities are established only recent data have revealed a role of extracellular polyP in mammalian and human cardiovascular biology. The *in vivo* activation of FXII in the initiating steps of the intrinsic blood coagulation

**TABLE 1 |** Overview on polyP-mediated activities in various cell types.

| Organism | Location | PolyP-function | Effect | References |
|---|---|---|---|---|
| Bacteria | Various phyla | PolyP metabolism | Increased virulence | (22) |
| | | Membrane pore formation | Vector uptake | (25) |
| | | Protein biosynthesis | Expression control, chaperone activity | (33–36) |
| Fungi | *S. cerevisiae* | $P_i$ sensing | $P_i$ regulon | (16) |
| | | $P_i$ metabolism | $P_i$ reservoir | (37) |
| | | Mitochondrial energy storage | Supported oxidative phosphorylation | (27) |
| | *D. discoideum* | PolyP secretion | Growth inhibition | (55, 56) |
| Animals | Amoeba histolyticum | PolyP metabolism | Increased biological fitness | (23) |
| | Osteoblasts | Mineralization inhibition | Apatite binding | (57) |
| | Myocytes | Mitochondrial permeability transition pore activation | $Ca^{2+}$ ion accumulation | (30) |
| | Hepatocytes | Metabolic contribution | Metabolic control | (28, 43) |
| | Neurons | TRPA1, TRPM8 signaling | Stimulating co-factor | (41, 42) |
| | Astrocytes | Vesicular release | Neurotransmitter | (51, 52) |
| | Fibroblasts | Fibroblast growth factor binding | Unknown | (50, 58, 59) |
| | Epithelial cells | mTOR pathway | Proliferation | (31) |
| | Endothelial cells | mTOR, $P2Y_{1,}$ and Wnt pathways | Induced apoptosis, permeability, cell adhesion | (60–63) |
| | Platelets | PolyP secretion | Bradykinin formation | (48) |
| | Platelets | PolyP secretion | FXIIa-mediated coagulation | (48, 64) |
| | Platelets | Extracellular nuclear protein binding | Increased vascular permeability | (62) |
| | Mast cells, basophils | PolyP secretion | Bradykinin formation | (46) |
| | Neutrophils | mTOR inhibition, autophagy induction | NET formation | (65) |
| | Plasma B cells | Unknown mechanism | Apoptosis | (66) |
| | Plasma | Increased C1 esterase inhibitor activity | Matrix for C1 esterase inhibitor regulation | (67) |
| | Plasma | Complement system | Inhibition | (68) |
| | Plasma | Platelet factor 4 binding | Autoimmune-induced thrombocytopenia | (69) |

system has been an enigma for many years [reviewed in (70)]. FXII activation by binding to negatively charged kaolin or ellagic acid ("contact activation") provides the mechanistic basis for activated partial thromboplastin time (aPTT), the clinical coagulation test (with an estimated 4–5 billion tests annually). However, the natural surface that induces FXII contact activation *in vivo* had been unknown. Activated platelets induce plasma clotting in a FXII-dependent manner, indicating that platelets release FXII-activating structures. Studies in mice and human plasma revealed that polyP serves as the long sought FXII-activating surface on activated platelets linking primary and secondary hemostasis. *Vice versa,* humans with polyP deficiency (Hermansky Pudlak Syndrome) have defective platelet-driven FXII activation and clotting [(48) and reviewed in (71) and (72)]. In addition to FXII activation, polyP has been reported to modulate other coagulation reactions *in vitro*, however a potential role of these pathways *in vivo* remains to be demonstrated (64, 73–75). The chain length of synthetic polyP determines its solubility and FXII activation capacity in plasma (76). However, natural platelet polyP forms insoluble $Ca^{2+}$ ion-rich nanoparticles independently of the chain length of the polyP molecules that are maintained on the surfaces of pro-coagulant platelets and thus activate FXII (49). Hence size of polyP does not matter for FXII-activating potency of the physiologically occurring $Ca^{2+}$-saturated polymer. Similarly to polyP, also synthetic polyI:C, a dsRNA analog that activates Toll-like receptor 3, has been shown to have pro-coagulant activity (77).

PolyP is a chelator for positive metal ions and dense granules of platelets contain polyP bound to $Ca^{2+}$ and possible $Zn^{2+}$ ions at high concentrations (47). In plasma, $Ca^{2+}$-saturated polyP has a half-life of about 90 min (76), due to degradation by polyphosphatases, such as alkaline phosphatase that has exopolyphosphatase activity (48, 78). Synthetic polyP derived from melted phosphoric acid is mostly complexed to $Na^{+}$ ions. Counterions regulate structure, biophysical properties and biological activities of polyP (49). Most of the *in vitro* studies have been performed with synthetic (often $Ca^{2+}$-free) polyP in the absence of phosphatases. Physiologically occurring $Ca^{2+}$-polyP is not soluble and operates on the cell surface. The low solubility in plasma challenges suggested functions for the extracellular polymer in solution. In contrast $Ca^{2+}$-free synthetic polyP of short and medium sized chain length (50–150 $P_i$ moieties) depletes free $Ca^{2+}$ ions leading to anti-coagulant effects similarly to EDTA (76).

Most cell culture experiments have been performed with medium sized synthetic polyP (10–50 μM, based on monophosphate units). In buffer or plasma environments *in vitro*, synthetic polyP is associated with an array of binding partners, including von Willebrand factor, coagulation factors XII, V and XI, complement factors C1s, C5b6, C6, and C7

(64, 68, 73, 74). Medium chain polyP and heparin have been reported to bind C1 esterase inhibitor (C1INH), thereby increasing function of the inhibitor (67, 79). However, FXII is readily activated by platelet-derived polyP to initiate the intrinsic coagulation cascade *in vivo*, indicating that increased activity of physiologic C1INH levels following polyanion binding is not sufficient to prevent FXIIa-driven coagulation. In contrast, high dosages of C1INH (15 IU/kg) but not low dosages (7.5 IU/kg) decrease ischemia/reperfusion injuries (80), suggesting that polyP binding to supra-physiological C1INH levels has the capacity for regulating thrombosis. The amount of polyP exposed on activated platelet surface is not precisely known, and it remains to be analyzed whether low amounts of platelet-derived C1INH that is retained on the surface of activated platelets and is possibly bound to polyP have a role in regulating serine proteases *in vivo* (81).

PolyP increases endothelial cell permeability *in vivo*, stimulates expression of cell adhesion molecules and induces apoptosis in culture (60). Follow-up studies, have elucidated the pathways involved in these endothelial reactions. PolyP application activates NF-κb and β-catenin pathways via receptor for advanced glycation end products, $P2Y_1$ receptor signaling and Wnt signaling (61, 62). *Vice versa*, the anti-thyroid drug methylthiouracil interferes with polyP-mediated inflammation of the endothelium *in vivo* (82). On the other hand, probiotic-derived polyP improves epithelial barrier function and may contribute to immunomodulatory mechanisms against commensal bacteria in the gut via NF-κb inhibition (83).

High extracellular polyP levels (>500 μM) elicit scaffolding effects that control amyloidogenic processes (84). In addition, polyP binds to fibroblast growth factor (FGF) (58). However, possible effects of polyP-FGF complexes on FGF-receptor signaling have remained controversial, as both increased and inhibited mitogenic activities have been reported (58, 59). A possible explanation for the dual activities might be that FXII, which initially promotes angiogenesis via urokinase plasminogen activator receptor (uPAR) signaling (85), gets rapidly depleted following polyP application via binding of FXIIa to C1INH. In the presence of serum-derived C1INH, FXIIa gets rapidly cleared and might limit FXII(a)/uPAR-driven pathways. Supporting this idea, tumor cells secrete exosomes that expose polyP and are associated with worse outcome involving immune evasion possibly through reduced FXII(a)/uPAR-driven differentiation of tumor-reactive T cell subsets (21, 86, 87). Moreover, human plasma cells induce apoptosis following polyP treatment, whereas other lymphocyte populations do not (66).

Presentation of polyP on activated platelets allows for contact of polyP with immune cells. Activation of platelets increases the intracellular $Ca^{2+}$ ion concentration via stromal interaction molecule 1 to release $Ca^{2+}$ ions from the sarcoplasmatic reticulum (88), which opens store-operated calcium entry channels in the plasma membrane (89). $Ca^{2+}$-mediated platelet activation mirrors the process of T cell stimulation (90). Moreover, conjugates of platelet-CD4+ T cells have been reported (91) and T-cell proliferation and differentiation is modulated by platelet-released molecules, such as platelet factor 4 (92), soluble CD40 ligand (93), ADP/ATP (94) and transforming growth factor-β (95). Recent findings also suggest that platelets inhibit pro-inflammatory interleukin (IL)-17 secreting effector T helper (Th17) cells in a tumor model (96). Taken together, these studies suggest that polyP modulates immune responses via direct or indirect pathways.

Neutrophils express FXII that is translocated to the plasma membrane upon activation and facilitates uPAR signaling via an autocrine mechanism (97). FXII signaling in neutrophils is independent of FXII cleavage and promotes adhesion, migration and formation of neutrophil extracellular traps (NET). In transfer experiments, neutrophils derived from FXII-deficient bone marrow show reduced tissue infiltration and better wound healing in the host, compared to neutrophils derived from wild-type bone marrow. PolyP treatment of neutrophils *in vitro* is independent on uPAR signaling and promotes the generation of NET through decreased mTOR acitivity and autophagy induction (65). Supporting a link between polyP and neutrophils, neutrophil-mediated inflammation is reduced in polyP-deficient mice in two independent mouse models with (i) genetic *IP6K1* ablation or (ii) pharmacological IP6K1 inhibition (98). The data suggest that both neutrophil FXII and platelet-derived polyP contribute to NET formation.

Both polyP and heparin are negatively charged polymers and polyP has been shown to substitute for heparin. Both heparin and polyP derived from mast cells drive the activation of the kallikrein-kinin system (46, 99). Antibodies that recognize complexes of platelet factor 4 (PF4) bound to heparin induce platelet aggregation in heparin-induced thrombocytopenia (HIT). These antibodies also cross-react with PF4/polyP complexes (69), indicating that polyP might contribute to HIT offering a rational for the recently described heparin-independent HIT forms (100).

## POLYPHOSPHATE-MEDIATED INFLAMMATION

PolyP promotes the autocatalytic activation of FXII to the serine protease FXIIa and FXIIa production is amplified by the protease plasma kallikrein (PKa) through a feed-forward mechanism of FXIIa-cleaved plasma prekallikrein (PK, fluid phase activation). Deficiency in FXII is not associated with obvious abnormalities in humans and mice. However, FXII-deficient plasma (Hageman trait) has a prolonged aPTT. Mice lacking FXII ($F12^{-/-}$) have largely defective arterial and venous thrombus formation (101) and are protected from thromboembolic diseases without showing hemostatic abnormalities (48, 102). The impact of FXII on thrombosis recently steered the World Health Organization to establish an international standard for human plasma-derived FXII (103). The fact that FXII deficiency is not associated with bleeding, suggests that FXII-mediated intrinsic blood coagulation contributes to other vascular pathways rather than hemostasis [reviewed in (104)].

FXII and FXIIa are both ligands of uPAR that is expressed on antigen-presenting cells and promotes differentiation of pro-inflammatory Th17 cells (85, 87). Consistently, $F12^{-/-}$ mice were shown to be protected from Th17-driven experimental

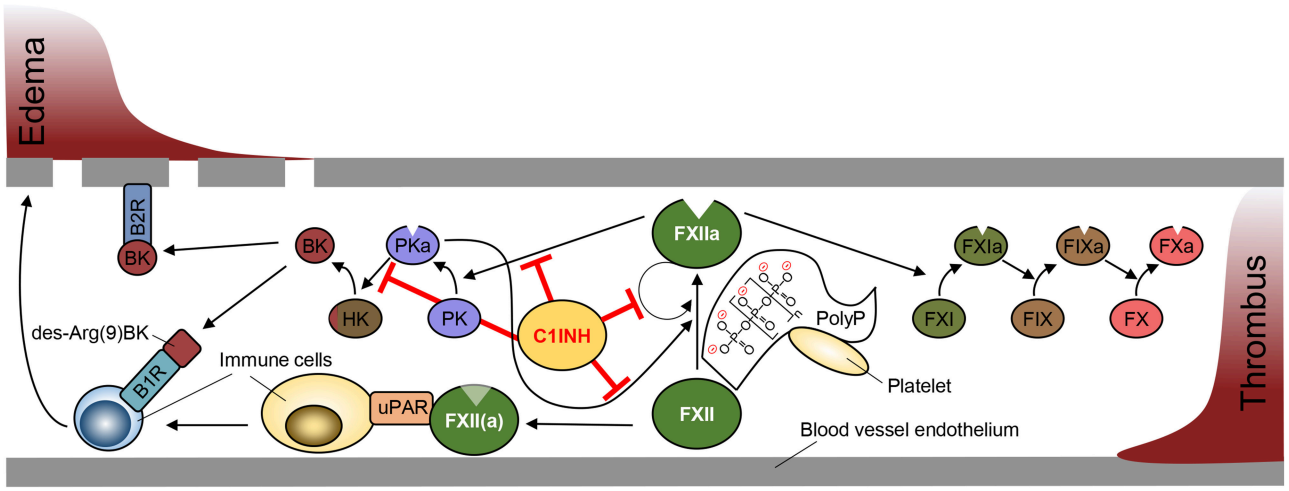


**FIGURE 1 |** Schematic overview of Factor XII-mediated pathways in edema and thrombosis formation. Factor XII (FXII) activation is driven by autocatalytic activation promoted by binding to negatively charged surfaces (contact activation), such as polyphosphate (PolyP) retained on platelet surface. FXIIa production is amplified by plasma kallikrein (PKa) that is produced from FXIIa-cleaved plasma prekallikrein (PK). C1 esterase inhibitor (C1INH) blocks the activity of FXIIa and PKa to prevent PKa-driven edema formation via cleavage of high molecular weight kininogen (HK) to release bradykinin (BK). Binding of BK and its metabolite des-Arg(9)BK to bradykinin receptors (B1R, B2R) promotes vascular permeability and immune cell activation. FXII and FXIIa bind urokinase plasminogen activator receptor (uPAR) to stimulate immune responses (left). FXIIa initiates the activation of the intrinsic coagulation cascade via activation of factor XI (FXI), factor IX (FIX) and factor X (FX) to form a thrombus (right).

autoimmune encephalomyelitis (EAE) development (87). Autoimmune diseases, such as EAE, require the extravasation of auto-reactive Th1 and Th17 cells to infiltrate and attack target cell structures. Furthermore, FXIIa increases vascular permeability via the kallikrein-kinin system, in which bradykinin (BK) is produced by PKa-mediated high molecular weight kininogen cleavage. BK is a peptide hormone and BK signal transduction is mediated by G-coupled kinin B1 and B2 receptors on endothelial cells as well as leukocytes [reviewed in (105)]. Kinin B1 receptor expression is induced by pro-inflammatory cytokines (e.g., IL-1β) and stimulated by BK and the C-terminal truncated BK derivate des-Arg(9)BK, whereas constitutively expressed kinin B2 receptors recognize BK [reviewed in (106)]. Deficiency in kinin B1 receptors affects Th-cell migration and outcome in murine EAE models and possibly humans with multiple sclerosis (107), suggesting that several FXII-dependent pathways can interact with T-cell homeostasis. In line with this notion, increased populations of Th17 cells have been reported in patients with defective FXIIa regulation (108).

Proteolytic activity of FXIIa is regulated by C1INH, a serpin that irreversibly inhibits FXIIa through covalent binding into its reactive center. C1INH also has the capacity to inhibit several other serine proteases, including PKa, active factor XI (FXIa) and complement factors C1r and C1s. Impaired FXIIa inhibition augments BK formation by the kallikrein-kinin system and is associated with a BK-mediated life-threatening inherited swelling disorder, hereditary angioedema (HAE) [reviewed in (109)]. C1INH deficiency has no impact on thrombosis and HAE patients have a normal thromboembolic risk [reviewed in (110)]. HAE is a rare disease that is mainly autosomal dominant inherited and characterized by reduced C1INH levels (HAE type I) or function (HAE type II). In addition HAE has been reported in patients with normal C1 esterase inhibitor activity (HAE type III). Disease-causing HAE type III mutations have been mapped to the *F12* gene. Analysis of mutant FXII T309K and T309R (positions refer to the mature protein) revealed that mutant FXII is defective in a single O-linked glycosylation, which promotes contact-driven FXIIa formation (111). Additionally, the mutations create new serine protease cleavage sites and thus increase FXIIa- and plasmin-mediated mutant FXII zymogen activation (112). Other HAE type III associated mutations have been linked to plasminogen and angiopoeitin (113, 114). While BK is short-lived and degraded within seconds, the activity of FXIIa or activation of FXII is increased in HAE leading to sustained and prolonged BK formation (115, 116). A schematic overview depicting PolyP/FXII-mediated pathways is shown in **Figure 1**.

In addition to the kallikrein-kinin system, FXIIa triggers the intrinsic blood coagulation pathway that has a role in thrombus formation [reviewed in (117)]. Thrombi occlude vessels causing tissue ischemia but also function in host defense in a concept termed "immunothrombosis." A recent report on bacterial sepsis-associated pro-coagulant mechanisms showed that inflammation-driven thrombosis occurs earlier in the spleen compared to liver despite similar bacterial burden in both organs, suggesting that organ-specific environment rather than bacterial components contribute to microthrombus formation (118). Bacteria-derived polyP is highly pro-coagulant *in vitro*, however, it is unknown whether bacteria have the capacity to expose or secrete their long chain polyP (>1,000 $P_i$ moieties).

Intracellular polyP gets released during necrosis and may act as a damage associated molecular pattern inducing immune

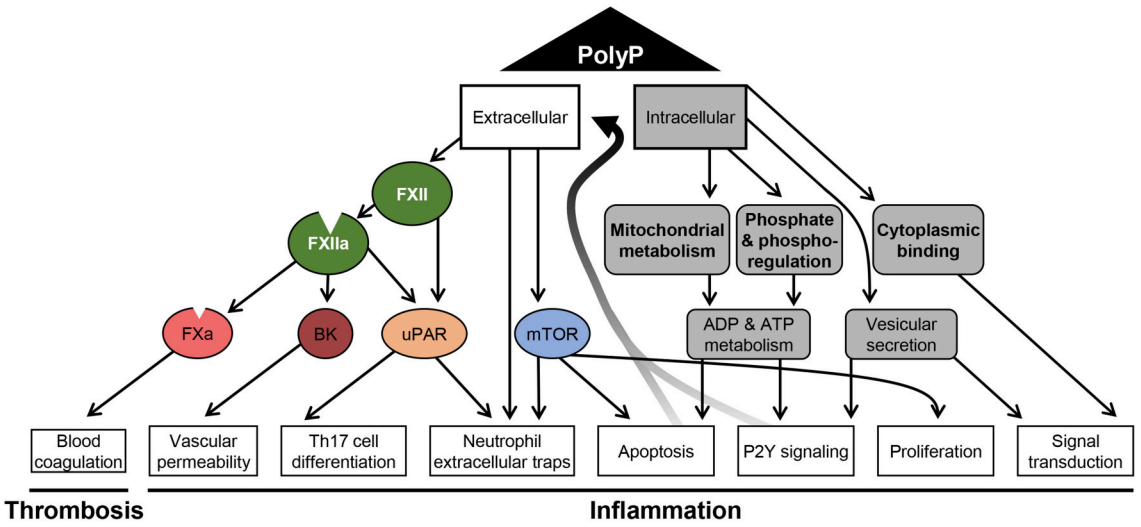


**FIGURE 2 |** Schematic overview of polyphosphate-mediated pathways. A summary of the diverse mechanisms that are affected by polyP and have been reported for distinct cell types is shown. Polyphosphate (polyP) regulates intracellular mechanisms (gray) related to metabolism, signaling and apoptosis. The effect of polyP on phoshoregulation has been shown in yeast, the impact on mitochondrial activity in yeast and cardiomyocytes. Protein binding and vesicular secretion of polyP was reported for neurons and polyP-driven proliferation has been shown for tumor cells. Active and passive release of polyP promotes extracellular pathways to activate coagulation factors (FXIIa and FXa), to generate bradykinin (BK) and to stimulate cells via urokinase plasminogen activator receptor (uPAR)- and mammalian target of rapamycin (mTOR)-driven mechanisms. Signaling via these mediators were reported to affect endothelial cells and immune cells.

responses through P2Y signaling (62). Indeed, long chain polyP induces thrombosis via FXII activation but also leads to platelet activation and consumptive coagulopathy (119). Moreover, polyP serves as phosphoryl donor for adenylate kinase in the extracellular space (120). Thus, polyP may regulate ADP/ATP ratio and signaling via purinergic receptors in immune cells. An overview of the complex network by which polyP impacts on cellular mechanisms in different cell types to facilitate coagulation and inflammation is depicted in **Figure 2**.

## INTERFERENCE WITH POLYPHOSPHATE FUNCTIONS

Intracellular polyP levels have been modulated in *S. cerevisiae* through phosphate starvation and abrogation of the mitochondrial membrane potential by uncouplers (27), indicating that intracellular polyP broadly influences cell metabolism. Moreover, Ppx1-mediated degradation of polyP regulates mitochondrial $Ca^{2+}$ ion efflux (30).

Mammalian enzymes that control formation and degradation of polyP have remained unknown. To interfere with polyP/FXIIa-driven inflammation plasma derived C1INH (Berinert™) and B2R antagonist (Icatibant™) dampen bradykinin formation and signaling and have been used for treatment of excessive bradykinin-mediated swellings in HAE patients (121, 122). Neutralizing FXIIa antibody 3F7 prevents thromboembolism and bradykinin generation with minimal therapy-associated adverse effects such as increased bleeding (123). Interference with the extracellular polyP/FXII pathway is a promising strategy to dampen coagulation and inflammation [reviewed in (124)]. To directly target extracellular polyP, neutralization and degradation approaches have been tested. Structural homology of polyP and heparin suggests that the polycation protamine sulfat, the heparin antidote, can also neutralize polyP. Indeed, polyP inhibitor screening identified various polycation substrates, such as spermidine, histone H1, polymyxin B and cationic polymers as possible agents for interference with polyP-mediated coagulation and inflammation (125). Moreover, the recombinant polyP-binding domains of *E. coli* ppx (PPX_Δ12) bind to polyP with high affinity and block polyP-mediated FXII activation. Similarly, degradation of polyP with recombinant ppx inhibits arterial and venous thrombus formation without interfering with hemostasis, indicating that polyP operates via FXII in *in vivo* coagulation (126).

Interference with polyP developed evolutionary in blood-sucking insects. Sand flies express salivary proteins that enable the insects to feed on mammalian blood without triggering BK-mediated itching or FXIIa-mediated clotting. These proteins, termed PdSP15a and PdSP15b, provide a positively charged helix that efficiently binds to and neutralizes polyP (127). Taken together, polyP inhibition has been shown to block thrombosis and inflammation and provides an opportunity for efficient and safe future treatment.

## OUTLOOK

Recent advantages to visualize, measure and detect polyP and to modulate polyP metabolism offers the possibility to analyze cell-specific roles for polyP (11, 28, 126, 128). Open research questions include regulation of polyP in mammalians

and eukaryotic cells, and possibilities to target polyP-mediated activities in therapeutic settings.

## AUTHOR CONTRIBUTIONS

RM wrote the manuscript and created the figures. LH, MA, and TR reviewed the manuscript.

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# Kinins Released by Erythrocytic Stages of *Plasmodium falciparum* Enhance Adhesion of Infected Erythrocytes to Endothelial Cells and Increase Blood Brain Barrier Permeability via Activation of Bradykinin Receptors

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**Background:** *Plasmodium falciparum,* the etiologic agent of malaria, is a major cause of infant death in Africa. Although research on the contact system has been revitalized by recent discoveries in the field of thrombosis, limited efforts were done to investigate the role of its proinflammatory arm, the kallikrein kinin system (KKS), in the pathogenesis of neglected parasitic diseases, such as malaria. Owing to the lack of animal models, the dynamics of central nervous system (CNS) pathology caused by the sequestration of erythrocytic stages of *P. falciparum* is not fully understood. Given the precedent that kinins destabilize the blood brain barrier (BBB) in ischemic stroke, here we sought to determine whether *Plasmodium falciparum* infected erythrocytes (*Pf*-iRBC) conditioned medium enhances parasite sequestration and impairs BBB integrity via activation of the kallikrein kinin system (KKS).

**Methods:** Monolayers of human brain endothelial cell line (BMECs) are preincubated with the conditioned medium from *Pf*-iRBCs or RBCs (controls) in the presence or absence of HOE-140 or DALBK, antagonists of bradykinin receptor B2 (B2R) and bradykinin receptor B1 (B1R), respectively. Following washing, the treated monolayers are incubated with erythrocytes, infected or not with *P. falciparum* mature forms, to examine whether the above treatment (i) has impact on the adhesion of *Pf*-iRBC to BMEC monolayer, (ii) increases the macromolecular permeability of the tracer BSA-FITC, and (iii) modifies the staining pattern of junctional proteins (ZO-1 and β-catenin).

**Results:** We found that kinins generated in the parasite conditioned medium, acting via bradykinin B2 and/or B1 receptors (i) enhanced *Pf*-iRBC adhesion to the endothelium monolayer and (ii) impaired the endothelial junctions formed by ZO-1 and β-catenin, consequently disrupting the integrity of the BBB.

**Conclusions:** Our studies raise the possibility that therapeutic targeting of kinin forming enzymes and/or endothelial bradykinin receptors might reduce extent of *Pf*-iRBC sequestration and help to preserve BBB integrity in cerebral malaria (CM).

**Keywords: malaria, *Plasmodium falciparum*, KKS, bradykinin, endothelial barrier**

## INTRODUCTION

Still recognized as major cause of death in Africa, severe malaria is a complex multi-system disorder caused by infection with *Plasmodium falciparum*. Afflicting millions of people per year [219 million cases worldwide in WHO (1)], the major complications of acute infection with *P. falciparum* are cerebral malaria (CM), pulmonary edema, acute renal failure, severe anemia, and/or bleeding (2). Although the number of patients that develop CM is relatively low (incidence of 1,120/100,000 infected children/year in the endemic areas of Africa), the lethality in children under 5 years old is high (3–5).

The human phases of the malaria life cycle include a silent liver stage which produces infective merozoytes that, subsequently, establish the blood phase of the disease, by invading erythrocytes. Within red blood cells, several cycles of asexual reproduction occur resulting in elevated number of parasites and human disease (6).

In *Plasmodium falciparum* malaria, erythrocytes containing mature parasites are sequestered in the brain vascular bed, consequently causing obstruction of microvessels, reduced blood flow, and cerebral hypoxia (7). Proinflammatory cytokines are thought to aggravate the infection-associated microvasculopathy that characterizes severe disease (8). This observation is followed by the accumulation of activated platelets and leukocytes, including $CD8^+$ T cells, within the brain microvasculature (9). Besides the classical components required for the development of effector T lymphocytes, there are indications in the literature that the renin angiotensin system (RAS) is involved in this process (10). Accordingly, our group demonstrated that angiotensin II (Ang II) acts as a co-stimulatory molecule during activation and development of effector function of $CD8^+$ T cells *in vitro* and *in vivo*, by using Ag-specific transgenic mice lacking the AT1 receptor (11–13). Moreover, we have also demonstrated that cerebral edema as well as the infiltration of T cells into the brain of infected mice were attenuated by captopril, the inhibitor of angiotensin-converting enzyme (ACE) (14). The dual-role of ACE connects two distinct proteolytic pathways: RAS and the kallikrein kinin system (KKS). Besides to be responsible for the formation of Ang II, ACE is also able to degrade kinins (15). KKS is an inflammatory mechanism that proteolytically generates proinflammatory kinins, such as the proinflammatory bradykinin (BK). In infectious diseases, the unbalance between pro and anti-coagulant responses may influence infectious-associated vasculopathies (16). In the past years, progress in the understanding of the role of the KKS in the pathogenesis of experimental Chagas disease revealed that BK-induced microvascular leakage translates into mutual benefits to the host/parasite relationship (17, 18).

In the malaria field, although the notion that sequestration of infected erythrocytes to the microvascular brain endothelium is required for the development of CM is well-accepted (19, 20), there have been reports that patients infected with *Plasmodium vivax* develop CNS pathology without obvious signs of parasite sequestration in the brain (21, 22). Experimental models of cerebral malaria (ECM) have unveiled a number of common pathogenic features with the human CM. For example, in both cases the infection-associated vasculopathy includes platelet activation, coagulopathy, vascular leakage, edema, microhemorrages, vascular occlusion, and adhesion of activated leukocytes (23–25). Also, it has been proposed that *Pf*-iRBC might activate the KKS through contact activation by surface-exposed phophatidylserine (26–29). Relying on falcipains, *Pf*-iRBC directly cleaves internalized kininogens, to release proinflammatory kinins, such as BK Bagnaresi et al. (30). More recently, we demonstrated that the short-lived BK is detectable (mass spectrometry) in culture supernatants of *Pf*-iRBCs treated with ACE inhibitors (31). Using monolayers of BMECs as a model of BBB (32, 33), here we provide *in vitro* evidence that BK accumulating in the supernatant of *Pf*-iRBC cultures (i) enhance the adhesion of *Pf*-iRBC adhesion to BMECs (ii) impair the integrity of the brain blood barrier.

## MATERIALS AND METHODS

### Drugs

D-sorbitol, HEPES, glucose, sodium bicarbonate, hypoxanthine, bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), B2 receptor (B2R) antagonist, HOE-140, B1 receptor (B1R) antagonist des-$Arg^9$-[$Leu^8$]-BK (DALBK) were purchased from Sigma-Aldrich.

### Ethics Statement

Healthy volunteers were randomly selected for collection of $A^+$ blood samples. All procedures were approved by the Research Ethics Committee of the Hospital Universitário Clementino Fraga Filho from the Federal University of Rio de Janeiro (Permit Number 074/10). All volunteers provided written informed consent for the collection and subsequent use of the samples to maintain parasite cultures.

### Parasite Culture

*Plasmodium falciparum* from the W2 strain (chloroquine resistant, mefloquine sensitive), were cultured in RPMI 1,640 medium (Invitrogen) supplemented with 50 μg/mL gentamicin (Invitrogen) and 10% $A^+$-type human plasma at 5% $A^+$-hematocrit, obtained from healthy donors, using citrate as anti-coagulant agent. Parasite cultures were maintained under a gas-controlled atmosphere (5% $CO_2$, 5% $O_2$, and 90% $N_2$) as

described by Trager and Jansen (34). Parasitemia was assessed by light microscopy in thin blood smears stained with hematologic staining by analyzing at least 10 random microscopic fields. Parasitemia was calculated as a percentage of the number of infected cells in 100 erythrocytes.

## Culture Synchronization and Generation of the Conditioned Medium

Erythrocytic stages of *P. falciparum* were synchronized by treatment with 5% D-sorbitol (10 min). Mature forms of malaria parasites are known to have osmotic fragility and are sensitive to 5% D-sorbitol (35). After discarding mature forms, the suspension of parasitized erythrocytes, enriched in young trophozoites, was washed and reintroduced in the above described culture medium to allow for schizont formation. Conditioned medium was obtained by incubating schizont cultures (3–5% parasitemia) for 24 h, a timepoint in which schizonts give rise to young trophozoites. After centrifugation of *Pf*-iRBC (2,500 rpm, for 10 min), the supernatant (conditioned medium) was collected and freshly applied to BMEC monolayers. As control, the supernatant of non-infected erythrocytes suspensions, maintained in the same culture conditions of infected erythrocytes, for 24 h, was used.

## Brain Microvascular Endothelial Cell (BMEC) Culture

The brain microvascular endothelial cells (BMEC) are an immortalized cell line that has been previously described and used as a BBB model in studies of the trans-migration of African trypanosomes (32, 33). The BMECs were cultured in medium 199 (M199, Sigma Aldrich) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA) and antibiotics (Sigma Chem Co; St. Louis, MO) (complete medium), at 37°C in a humidified atmosphere containing 5% $CO_2$.

## Adhesion Assay

To assess *Pf*-iRBC adhesion to endothelial cells, BMEC were plated in 24-well culture chambers (Nunc, New York, USA) ($5 \times 10^4$ cells/well) and cultured for 24 h. After that, BMECs were treated or not overnight with *Pf*-iRBC conditioned medium (20%) or control conditioned medium (normal RBC). Where indicated, the BMEC treatment with conditioned medium was performed in medium supplemented with the B2R or B1R antagonists ($10^{-7}$ M HOE-140 or DALBK). Next, the BMEC-treated monolayers were incubated with *Pf*-iRBCs ($4 \times 10^5$ cells/well, 5% parasitemia) or control RBCs for 1 h. Non-adherent erythrocytes were gently washed away with PBS, and the remaining cells were fixed and stained with hematologic staining (commercial kit from Laborclin, Brazil, BR). The number of adhered erythrocytes per BMEC was determined by direct counting in light microscopy, considering at least 10 random microscopic field. The data are expressed as Adhesion index calculated according to Souza et al. (36): Adhesion Index (AI) = {[(BMEC with bound erythrocytes)/total BMEC number] × [(erythrocytes bound to BMEC)/total BMEC number]} × 100.

## Permeability Assay

Permeability was accessed through BSA-FITC transendothelial transport (37). Briefly, BMEC cell line was grown until confluence on Transwell chamber inserts of 6.5 mm diameter and 8 μm pore (Corning Costar). The endothelial monolayer culture in the upper compartment was exposed to RPMI control medium, *Pf*-iRBC ($4 \times 10^5$ cells), 20% conditioned medium, $10^{-7}$ bradykinin (BK), $10^{-7}$ HOE 140. BSA-FITC (15 μg/mL) was simultaneously added to the upper compartment of each Transwell unit. After 14 h of incubation times, endothelial monolayer permeability of BSA-FITC flux across intact monolayers to the lower compartment was measured through fluorimetry (SpectraMax M2, Molecular Devices) at emission/excitation wavelengths of 495/520 nm.

## Immunofluorescence

The immunofluorescence experiments were carried out as before (38, 39). Briefly, BMEC cells were grown in coverslips and treated as described above. After treatment, cells were fixed in paraformaldehyde 4% for 15 min, followed by membrane permeabilization with PBS–Triton X-100 0.2% for 15 min. Cells were blocked with PBS-BSA 5%, and antibody against ZO-1 (617,300, Invitrogen) and β-catenin (sc-7963, Santa Cruz Biotechnology) was incubated for 1 h at room temperature. Anti-rabbit Alexa-Fluor 488 and Anti-mouse Alexa-Fluor 546 (Life Technology) was incubated to detect ZO-1 and β-catenin, respectively. Nuclei were stained with DAPI. Cells were mounted with anti-fading mounting medium (Vectashield, Vector Laboratories). Images were acquired with a confocal microscope Leica TCS SP8 (Leica) and software LAS X, and the final images were analyzed with Fiji software. Images were acquired at 630x and the scale bar represents 20 μm.

## HK-Alexa Fluor 488 Uptake

The HK-Alexa Fluor 488 uptake was measured as described by Bagnaresi et al. (30). Briefly, schizont-enriched *P. falciparum* cultures were incubated overnight with 70 μg/mL high molecular weight kininogen conjugated to Alexa Fluor 488 (HK-Alexa Fluor 488) at 37°C. The cells were harvested, washed with PBS 1x and plated in poly-lysine-coated microscope dishes. Parasites nuclei were stained with DAPI. Images were acquired with a confocal microscope Leica TCS SP8 (Leica) and software LAS X, and the final images were analyzed with Fiji software. Images were acquired at 630x and the scale bar represents 5 μm.

## Statistical Analysis

The results are expressed as means ± standard error of at least three independent experiments. GraphPad Prism 7 (version 7.0, GraphPad Software, San Diego California, U. S. A., www.graphpad.com) was used for statistical analysis. Differences between groups were compared by one-way analysis of variance (ANOVA), followed by the Tukey post-test. Significance was determined as $P < 0.05$.

## RESULTS

### *P. falciparum* Conditioned Medium Increases Sequestration of Infected Erythrocytes to BMEC Monolayers

Although it is well-established that iRBCs are recognized and sequestered by the endothelium, the influence of soluble compounds produced during the erythrocytic cycle in this process is still poorly known. Thus, in the first experimental group, we analyzed the influence of *P. falciparum* conditioned medium in the sequestration of *Pf*-iRBCs to BMEC monolayers. Conditioned medium was originated from a 24 h schizont culture (3–5% parasitemia) as described in the Material and methods section. For controls, we obtained supernatants from 24 h cultures of non-infected erythrocytes. Sub-confluent BMEC cultures were treated with the respective supernatant, overnight, and subsequently exposed to fresh *Pf*-iRBCs, for 1 h. We observed that the pre-incubation of BMEC with increasing proportions of the conditioned medium enhanced *Pf*-iRBC adhesion at 20% (**Figures 1A,B**). At this concentration,

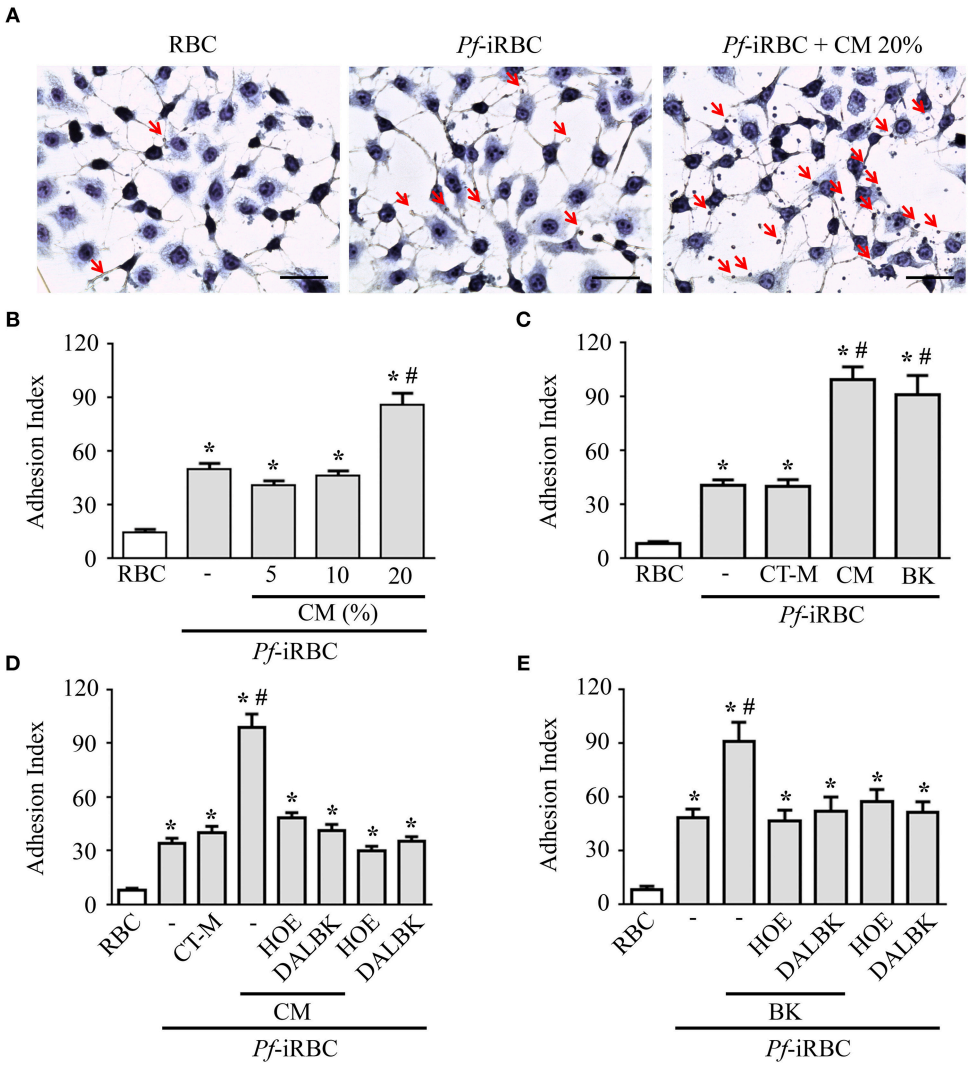


**FIGURE 1 |** Adhesion of *Pf*-iRBCs to BMEC monolayers is increased by the supernatant from *P. falciparum* culture. BMEC were cultured in 24-well plates ($4 \times 10^5$ cells/well) for 24 h and incubated with *Pf*-iRBCs ($5 \times 10^4$ erythrocytes/well, 5% parasitemia). When indicated, BMEC were treated with different compounds before incubation with *Pf*-iRBCs. The adhesion index was determined by direct counting of adhered erythrocytes per BMEC, as described in the Materials and methods. **(A)** Representative images of adhesion of *Pf*-iRBCs to BMEC monolayers. Arrows indicates adhered *Pf*-iRBCs. Scale bar = 50 μm. **(B)** Adhesion index was determined in BMEC preincubated or not with increasing concentrations of *Pf*-iRBC conditioned medium (CM), *overnight*, before incubation with *Pf*-iRBCs. **(C)** Adhesion index was determined in BMECs preincubated or not with 20% control medium (CT-M), 20% *Pf*-iRBC conditioned medium (CM) or $10^{-7}$ M BK before incubation with *Pf*-iRBCs. **(D,E)** Effect of $10^{-7}$ M DALBK or $10^{-7}$ M HOE-140 on the adhesion of *Pf*-iRBCs to BMEC. BMECs were pretreated with drugs for 30 min prior to overnight incubation with conditioned medium **(D)** or BK **(E)**. CT-M, control medium, obtained from a suspension of non-infected erythrocytes; CM, *P. falciparum* conditioned medium; RBC, basal adherence of non-infected erythrocytes; *Pf*-iRBC, *P. falciparum*-infected erythrocytes. Results are expressed as the mean ± SEM from three different experiments. *Statistically significant differences compared with RBCs, #statistically significant different compared with iRBCs ($P < 0.05$).

the conditioned medium produced 2-fold increase in *Pf*-iRBCs binding to endothelial cells. The stimulatory effect of the conditioned medium was comparable to the effect of addition of $10^{-7}$ M BK alone (**Figure 1C**). To characterize the receptors activated by the parasite conditioned medium, we preincubated the monolayer of BMECs with $10^{-7}$ M DALBK (B1R antagonist) or HOE-140 (B2R antagonist) before adding the *Pf*-iRBC conditioned medium (**Figure 1D**) or BK (**Figure 1E**). Notably, both antagonists abolished the subsequent adhesion of *Pf*-iRBC to BMECs. Of further interest, none of these GPCR blockers changed the basal levels of adhesion of infected erythrocytes. These results suggest that the adhesion of erythrocytic stages of *P. falciparum* to BMECs was enhanced by kinins generated in the *Pf*-iRBC conditioned medium (30). Accordingly, when infected cultures enriched with mature forms of the parasite were incubated with 70 μg/mL HK-Alexa Fluor 488, we observed the fluorescent substrate inside infected cells, revealed by immunofluorescence, but not in non-infected erythrocytes (**Figure 2**).

## *P. falciparum* Conditioned Medium Directly Induces Permeability of the BMEC Monolayer

Using fluorimetry and album-FITC as a macromolecular tracer, we sought to measure the permeability of a confluent BMEC monolayer prepared in a transwell system. The permeability measurements were made after incubating the BMEC monolayer overnight with 20% *P. falciparum* conditioned medium vs. 20% control medium (CT-M) obtained from 24 h culture of non-infected erythrocytes. Our results showed that the conditioned medium induced a 10-fold increase in BMEC permeability over the effect of the control (**Figure 3**). Of note, the intensity of the permeability response induced by *Pf*-iRBC conditioned medium was equivalent to responses induced by $10^{-7}$ M BK alone (**Figure 3**). Importantly, addition of the B2R antagonist HOE-140 ($10^{-7}$ M) stabilized the barrier function in BMECs incubated either with BK or parasite-conditioned medium. As observed in the results obtained in the endothelial cell adhesion assay, we found that the B1R blocker DALBK ($10^{-7}$ M) partially protected the barrier from the permeability-inducing signals generated in the parasite-conditioned medium or BK (**Figure 3**). Hence, these pharmacological studies linked

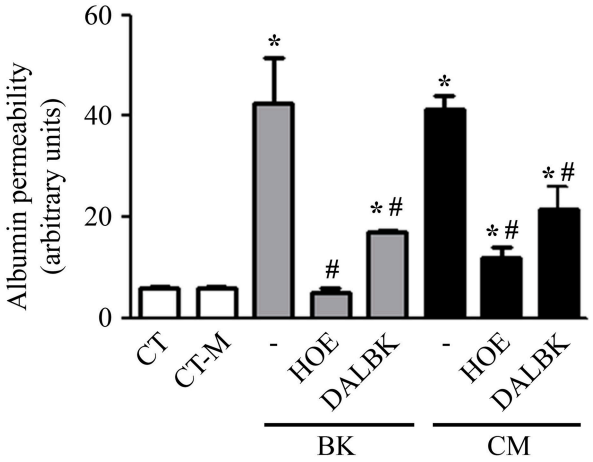


**FIGURE 3 |** *P. falciparum* conditioned medium induces endothelial barrier disruption in a BKR-dependent manner. Confluent BMEC monolayers, cultivated in the upper chamber of a transwell system, were pretreated or not with $10^{-7}$ M HOE140 or $10^{-7}$ M DALBK, for 30 min, before overnight incubation with 20% conditioned medium or $10^{-7}$ M BK. The next day, cells were washed and incubated with 15 μg/mL BSA-FITC, for 15 min. The supernatant from the lower chamber was collected for fluorimetry analysis. Bar graph representing the amount of fluorescence detected in the lower chamber for each experimental setup. Albumin permeability was expressed in arbitrary units as the mean ± SE of at least three independent experiments. Statistical significance compared with *control; #BK or conditioned medium ($P < 0.05$).

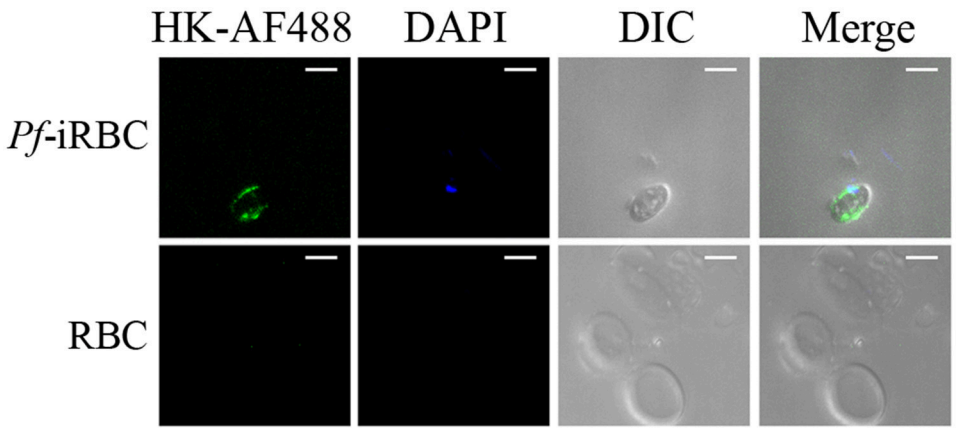


**FIGURE 2 |** *P. falciparum* infected erythrocytes uptake high molecular weight kininogen from the extracellular medium. Schizont-enriched *P. falciparum* cultures were incubated overnight with 70 μg/mL high molecular weight kininogen conjugated to fluorescein isothiocyanate (HK-AF488) at 37°C. The cells were harvested, plated in poly-lysine-coated microscope dishes, and analyzed by fluorescence microscopy. Images were analyzed using green (internalized HK-AF488) and blue (parasite nucleus) filters or DIC (erythrocytes). RBC, non-infected erythrocytes; *Pf*-iRBC, infected erythrocytes. Scale bar = 5 μm.

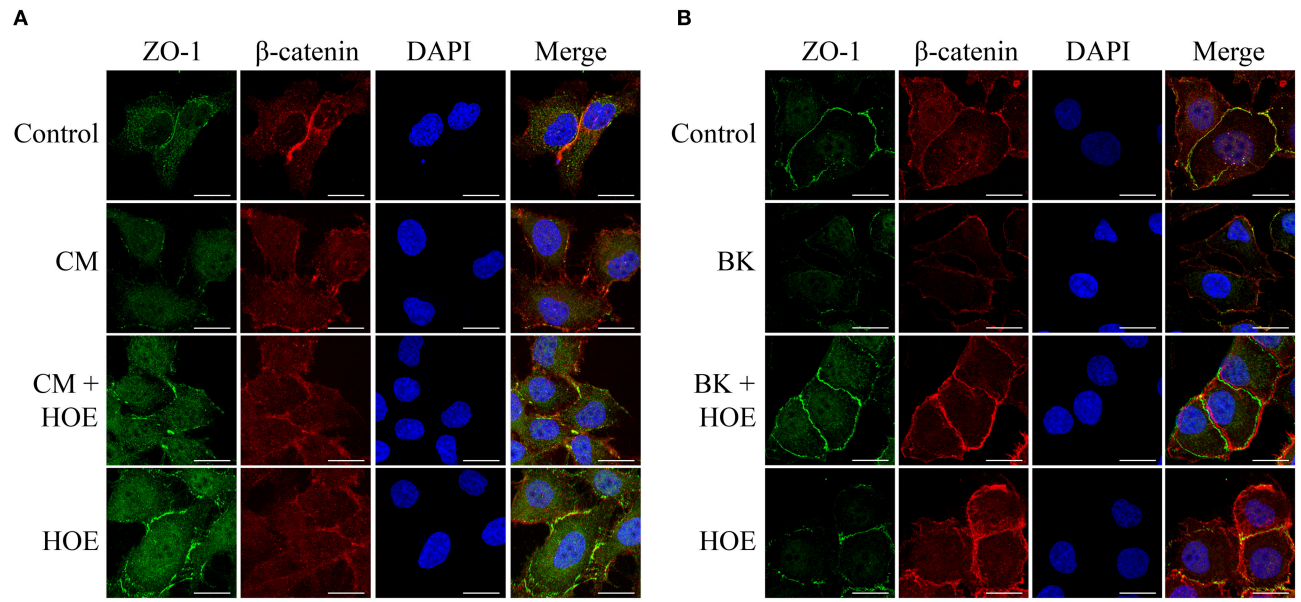


**FIGURE 4 |** *P. falciparum* conditioned medium induces disruption of the endothelial barrier by reducing β-catenin and ZO-1 expression. Sub-confluent BMEC monolayers, cultivated in coverslips, were pretreated or not with $10^{-7}$ M HOE140, for 30 min, before overnight incubation with 20% *Plasmodium falciparum* conditioned medium (CM) **(A)** or $10^{-7}$ M BK **(B)**. The next day, cells were washed and prepared for immunofluorescence as described in Materials and Methods section. Representative photomicrographs of ZO-1 (green), β-catenin (red), DAPI (Blue), and merged channels are shown. Magnification = 630x, Scale bar = 20 μm. The figures are representative of three independent experiments with different cell suspensions. At least three different slides were prepared for each experimental condition and at least five fields per slide, randomly chosen, were analyzed.

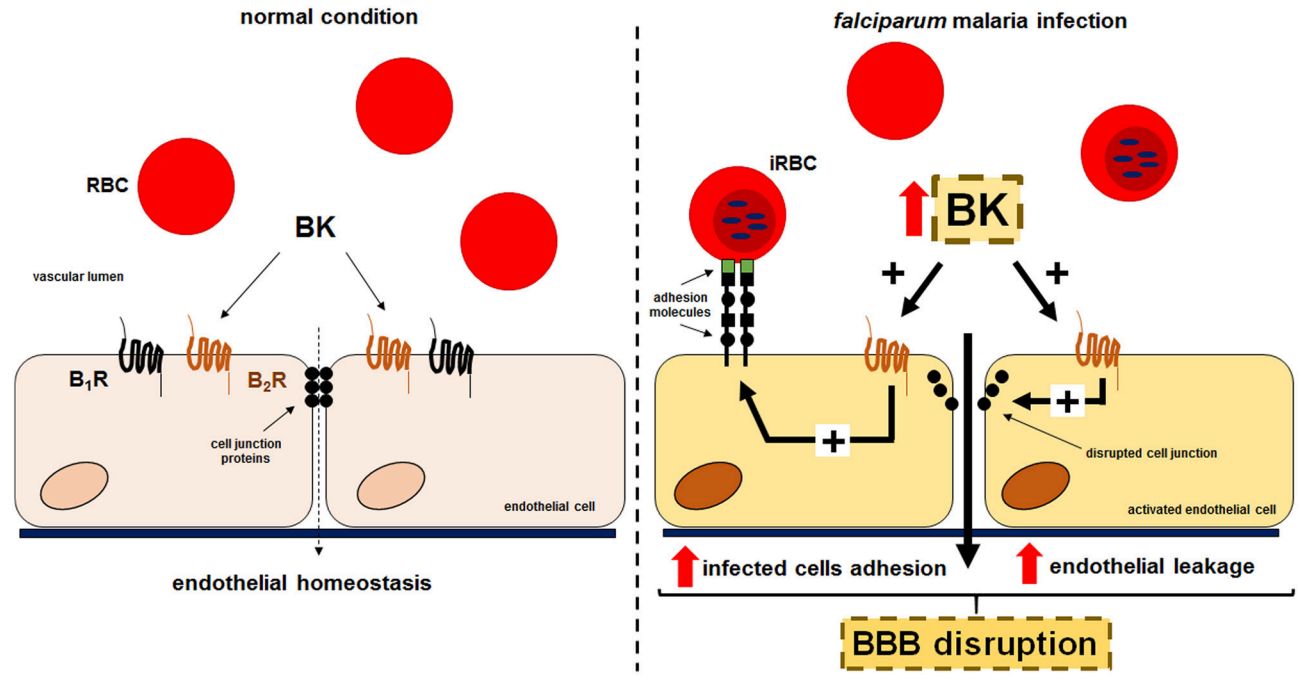


**FIGURE 5 |** Proposed model for the effect of kinins liberated in the supernatant of cultures of *P. falciparum*-infected erythrocytes in the sequestration of iRBCs and endothelial permeability. Erythrocytes that freely traffic in the blood flow acquire adherent properties during *P. falciparum* malaria infection. PfEMP-1 expressed in the surface of infected erythrocytes is recognized by receptors in the endothelium promoting parasite sequestration. Kinins accumulated in the plasma, through B2R activation, enhances *Pf*-iRBC sequestration within the microvasculature and induce disruption of the endothelial junctions culminating in leakage.

the increased permeability response induced by the *Pf*-iRBC conditioned medium to BMEC activation via the kinin/BKRs pathways.

### Kinins/B2R Axis Induces Permeability by Changing Morphological Distribution of Proteins From the Intercellular Junction

To further investigate the impact of the conditioned medium in BMEC permeability, we examined the morphological distribution of the components involved in the formation of interendothelial junction, e.g., ZO-1 and β-catenin. Typically, these proteins clearly co-localize in the periphery of the cell maintaining the integrity of the endothelial monolayer. However, in the presence of the parasite-conditioned medium (but not erythrocytes control medium) ZO-1 and β-catenin staining pattern was not clearly visualized. Consistent with the opening of intercellular gaps, these results suggested that the barrier function of the BMECs was disrupted (**Figure 4A**). Importantly, the steady-state localization of ZO-1 and β-catenin were restored by $10^{-7}$ M HOE-140, hence reinforcing the conclusion that kinins, acting via B2R, are responsible for the permeability-inducing properties of the *Pf*-iRBC conditioned medium (**Figure 4A**). In parallel, the direct effect of $10^{-7}$M BK on the structural assembly of the intercellular junction was evaluated. BK, through B2R activation, reproduced similar results compared with the addition of *Pf*-iRBC supernatant (**Figure 4B**).

Collectively, our results suggest that kinins generated during cultivation of *P. falciparum* erythrocytic stages might contribute to the pathogenesis of CM via two distinct, but not mutually exclusive activation pathways. The released BK (i) might favor *Pf*-iRBC sequestration within the microvasculature and (ii) disrupt the integrity of the endothelial junctions, inducing interstitial edema (**Figure 5**). Both mechanisms seem to be dependent on B2R activation.

## DISCUSSION

Although sequestration of erythrocytic stages of *P. falciparum* is thought to be a crucial event in the pathogenesis of CM (7), the hemostatic derangements are thought to be aggravated by formation of microthrombi, local or systemic production of proinflammatory cytokines. Despite progress in the molecular characterization of the parasite factors that promote adhesive interactions of *Pf*-iRBC with the endothelium (40, 41), the lack of experimental models to investigate the dynamics of infection by human species of *Plasmodium* has limited progress in this field. *In vitro* experiments using mature forms (schizonts) of parasitized erythrocytes showed upregulated endothelial expression of tissue factor, a trigger of fibrin-formation via the extrinsic pathway of coagulation (27). Multiple mechanisms may promote intravascular activation of the contact system (intrinsic pathway) following the sequestration of *Pf*-iRBC in the cerebral microvessels. For example, it is well-established that formation of microthrombi is potentiated by fibrin as a result of contact system activation by negatively charged platforms (26–29). Along similar lines, DNA associated to neutrophil extracellular traps (NETs) was also identified as a trigger of Factor XII, the serine protease that generates plasma kallikrein, the main BK-forming serine protease in the blood (15, 42).

Early *in vitro* studies performed by our group revealed that parasitized erythrocytes might modulate immunity and vascular homeostasis via activation of the renin-angiotensin system (RAS) (11–13). Given awareness that the hypertensive ACE efficiently degrades kinins, follow up studies performed with ACE inhibitors identified the presence of this nanopeptide in the parasite conditioned medium (31). Interestingly, in this context, independent studies by Bagnaresi et al. (30) demonstrated that high molecular weight kininogen is internalized and processed by E-64-sensitive kinin releasing cysteine proteases in different species of *Plasmodium*.

Although we did not investigate the mechanisms by which *P. falciparum* might generate kinins in the conditioned medium, the levels of this short-lived nanopeptide were sufficiently high to destabilize endothelial junctions and promote enhanced diffusion of the albumin-FITC tracer through the BMECs. B1R antagonist (DALBK) inhibited the adhesion of *Pf*-iRBCs to the endothelial cells as efficiently as B2R antagonist (HOE-140). Also, both antagonists efficiently rescued the barrier function of BMECs, along with the restoration of the peripheral staining of β-catenin and ZO-1 in the BMECs. In summary, our results suggest that kinins generated by erythrocytic stages of *P. falciparum* might disrupt BBB integrity following parasite sequestration in microvessels.

## ETHICS STATEMENT

Healthy volunteers were randomly selected for collection of A+ blood samples. All procedures were approved by the Research Ethics Committee of the Hospital Universitário Clementino Fraga Filho from the Federal University of Rio de Janeiro (Permit Number 074/10). All volunteers provided written informed consent for the collection and subsequent use of the samples to maintain parasite cultures.

## AUTHOR CONTRIBUTIONS

LS performed all experiments, collected, organized and analyzed all data. ASP and DT performed all cell cultures, parasite synchronization, and adhesion experiments. RS-A performed immunofluorescence experiments and helped with data organization. DP helped with drafting and revision of the manuscript. JS and CC-N helped with data interpretation, drafting, and revision of the manuscript;

AASP conceived and designed the work, drafted, and revised the manuscript.

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CASE REPORT
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# Hereditary Angioedema-Associated Acute Pancreatitis in C1-Inhibitor Deficient and Normal C1-Inhibitor Patients: Case Reports and Literature Review

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Abdominal pain due to intestinal swellings is one of the most common manifestations in hereditary angioedema (HAE). Bowel swellings can cause severe abdominal pain, nausea, vomiting, and diarrhea, which may lead to misdiagnosis of gastrointestinal disorders. In rare cases, HAE abdominal attacks can be accompanied by acute pancreatitis. Here, we report 3 patients with HAE and acute pancreatitis and present a literature review of similar cases. Patients with confirmed diagnosis of HAE secondary to C1-inhibitor (C1-INH) deficiency ($n = 2$) and HAE with normal C1-INH and *F12* mutation (F12-HAE) ($n = 1$) were included. Pancreatitis was diagnosed based on clinical symptoms and high lipase and amylase levels. Three HAE patients were diagnosed with acute pancreatitis based on increased amylase levels during severe abdominal swelling episodes. Two were previously diagnosed with HAE type I and one with F12-HAE. Pancreatitis was efficiently treated in two patients using Icatibant, with pain relief within hours. When conservatively treated, pancreatitis pain took longer time to resolve. Eighteen pancreatitis cases in HAE with C1-INH deficiency were previously reported and none in F12-HAE. Most patients (12/18) underwent invasive procedures and/or diagnostic methods. Although rare, severe abdominal HAE attacks could cause pancreatitis; HAE-specific treatments may be efficient for HAE-associated pancreatitis. HAE should be considered as a differential diagnosis of acute idiopathic pancreatitis. To our knowledge, this is the first report of HAE-associated pancreatitis in a F12-HAE patient treated with Icatibant.

**Keywords: hereditary angioedema, acute pancreatitis, abdominal swelling, C1-inhibitor deficiency, *F12* mutation**

## BACKGROUND

Hereditary angioedema (HAE) is an autosomal dominant disease caused mostly by deleterious mutations in the gene encoding the C1-inhibitor (C1-INH) (C1-INH-HAE). It occurs in quantitative and functional C1-INH deficiency (type I) or functional C1-INH deficiency (type II) (OMIM #106100) (1). Another group of patients present with clinical characteristics of HAE with normal C1-INH (2). In these cases, HAE can be caused by specific mutations in *F12* gene (F12-HAE) (OMIM #610618) (2). Recently, HAE with normal C1-INH has been linked to mutations in genes encoding plasminogen (3) and angiopoietin 1 (4), however, there are still patients with unknown genetic cause.

The characteristic symptoms are nonpruritic and nonpitting swelling of submucosal or subcutaneous tissues involving the face, hands, feet, arms, legs, intestines, genitourinary tract, and upper airways, which can be life threatening (5). HAE symptoms generally last 2–5 days before resolving spontaneously without treatment; the most common trigger factors include emotional stress, infections, trauma, medical and surgical procedures, and estrogens (1, 5).

Abdominal pain is one of the most common manifestations of HAE (5, 6). Bowel swellings can cause severe abdominal pain, nausea, vomiting, and diarrhea. These symptoms may lead to misdiagnosis of gastrointestinal disorders; consequently, patients are frequently submitted to invasive procedures and unnecessary surgeries (5–7). In rare cases, HAE abdominal attacks can be accompanied by acute pancreatitis; this association is not fully understood and/or documented. Herein, we described 3 patients with HAE who presented with pancreatitis and abdominal attacks concomitantly and a review of other cases previously described in the literature.

This study was carried out in accordance with the recommendations of ethical standards of the 1964 Declaration of Helsinki. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee in Research of Universidade Federal de São Paulo (n°56522). Written informed consent for publication was obtained from all the participants of this case report.

## CASE PRESENTATION

### Case 1

A 21-year-old male patient presented to the emergency with severe abdominal pain of 7 h and 8 vomiting episodes. The first test showed normal amylase (93 U/L; normal 30-110 U/L) and slightly augmented lipase levels (332 U/L; normal 23-300 U/L). Analgesics were administered with partial improvement of pain. The second test performed 8 h after patient's admission revealed increase in levels of amylase to 292 U/L and lipase to 1,159 U/L, indicating acute pancreatitis. An increased volume of pancreatic tail, but no gallbladder, was observed through endoscopic retrograde cholangiopancreatography (ERCP) and magnetic resonance imaging (MRI) showed intestinal swelling. Two years before this episode of pancreatitis, he had been diagnosed with HAE type I, characterized by low C1-INH and C4 levels. The onset of HAE occurred at 1 year of age and consisted of facial edema triggered by trauma. Since then, he has been presenting with intermittent and irregular swelling episodes of the hands and feet, abdominal pain, and 3 episodes of upper airway edema. Due to HAE diagnosis, Icatibant (30 mg) was administered 19 h after admission, and the pain significantly reduced within 3 h. Amylase (69 U/L; normal 30–110 U/L) and lipase (165 U/L; normal 23–300 U/L) normalized 18 h after Icatibant injection and the patient was discharged the next day.

### Case 2

A 47-year-old female patient with C1-INH-HAE diagnosed 8 years earlier, presented to the emergency department with distended abdomen and severe abdominal pain lasting 24 h . The first test revealed increased amylase 210 U/L (normal 28–100 U/L), which considering a longer duration of abdominal pain indicated the development of pancreatitis. Since the hospital located in the North of Brazil had no vacancy, a single dose of Icatibant (30 mg) provided by the patient was administered and she was subsequently discharged. The next day, she presented with almost complete relief from the abdominal pain; in a total of 7 days, amylase and lipase reduced to normal levels. This patient has been presenting with recurrent angioedema attacks in the abdomen, face, limbs, and a few episodes in the upper airways, since she was 28-year-old. At that age, she underwent appendectomy and was misdiagnosed with Familial Mediterranean Fever. Only after 11 years, was she correctly diagnosed with C1-INH-HAE, confirmed using low C4 (6 mg/dL; normal 10–40 mg/dL) and C1-INH plasma levels (2 mg/dL; normal 19–40 mg/dL). She was treated with a prophylactic use of plasma-derived C1-INH and Icatibant during the attacks.

### Case 3

A 52-year-old female patient with F12-HAE (mutation p.Thr328Lys) had the onset of angioedema attacks at 16 years of age, during her first pregnancy. Symptoms were edema affecting the face, hands, and feet and abdominal pain. Currently, angioedema episodes occur monthly despite tranexamic acid prophylaxis (500 mg/day), mostly affecting gastrointestinal tract. Recently, one abdominal attack required 4 days of hospitalization. Pancreatitis was diagnosed using acute abdominal pain, high serum amylase levels (391 U/L; normal 25–125 U/L), and pathological signs at abdominal ultrasonography (US). She had normal leucocyte and platelet counts, total bilirubin, and aspartate aminotransferase. She was conservatively treated for pancreatitis due to the lack of the specific medication for HAE.

## DISCUSSION

Acute pancreatitis is diagnosed if a patient presents with at least two of the following characteristics: (a) severe and persistent acute abdominal pain, (b) high serum lipase and/or amylase (3 times the normal upper limit), (c) and/or characteristic findings of pancreatitis on imaging tests (computed tomography, MRI, or US) (8). In addition, if the major and minor risk factors for acute pancreatitis is absent, like gallstones and alcohol misuse,

**TABLE 1 |** Case reports of pancreatitis in HAE patients.

| References | N°/gender | Age,y | HAE type | Amylase (IU/L) (normal range) | Lipase (IU/L) (normal range) | Treatment for attacks / Prophylactic treatment | Effect | Invasive procedures |
|---|---|---|---|---|---|---|---|---|
| Case 1 | 1/M | 21 | I | 292 (30–110) | 1,159 (23–300) | Icatibant (30 mg) | Significant abdominal pain relief within 3 h. | ERCP. |
| Case 2 | 1/F | 47 | I | 210 (28–100) | ND | Icatibant (30 mg) | Alleviation of symptoms in hour and resolution in 48 h. | Appendectomy. |
| Case 3 | 1/F | 52 | F12-HAE | 391 (25-125) | ND | Withholding food intake, intravenous hydration, analgesics. | Improvement in a few days. | ND |
| Brickman et al. (10) | 3/ ND | ND | I | ND | ND | ND | ND | Abdominal surgery in 2 patients. Marsupialization. |
| Cutler et al. (11) | 1/F | 44 | I | 95–100; 150 (<85) | 400 (0–210) | Danazol, diphenhydramine, corticotropin, propranolol, hydrocortisone. | Unsuccessful | Esophagogastroduodenoscopy, ERCP, appendectomy, tonsillectomy. |
| Matesic et al. (12) | 1/M | 40 | I | 1,117 (0–130) | 11,485 (0–95) | Intravenous hydration, antibiotic prophylaxis, analgesics, nasogastric decompression, proton pump inhibitor.<br>Prophylactic danazol (200 mg/day). | Marked improvement in 3 days. Resolution in 7 days. | Esophagogastroduodenoscopy, ERCP. |
| Chung et al. (22) | 1/F | 51 | ND | 369 | 1,735 | Withholding food intake, intravenous hydration, analgesics.<br>Prophylactic danazol. | Improvement in a few days. | ND |
| Majoni and Smith (13) | 1/M | 43 | I | 340 (35–110) | ND | C1-INH concentrate.<br>Discharged of danazol due to renal impairment. | Abdominal pain resolved in 24 h. Normalization of amylase in 3 days. Resolution in 7 days. | Tonsillectomy, appendectomy. |
| Marín Garcia et al. (14) | 1/M | 49 | II | 445 (25–115) | 2,342 (114–286) | ND<br>Prophylactic tranexamic acid (20 mg/day) after discharge. | ND | Tonsillectomy, endoscopy. |
| Cancian et al. (15) | 1/F | 32 | I | ND | ND | C1-INH concentrate (18 U/kg). | Alleviation of symptoms in 30 min and resolution in 16 h. Normalization of amylase in 24 h. | ND |
| Czaller et al. (16) | 1/F | 29 | I | 2,615 (28–100 U/L) | 1,452 (13–60 U/L) | C1-INH concentrate (500 IU) twice, methimazole sodium, drotaverine.<br>Prophylactic danazol. | Alleviation of symptoms in 4 h and resolution in 24 h. | Appendectomy. |
| Ben Maamer et al. (17) | 1/F | 73 | I | 869 U/L | 1,235 U/L | Intravenous fluids, antibiotic prophylaxis, analgesics, nasogastric decompression, proton pump inhibitor.<br>Prophylactic danazol (200 mg/day). | Alleviation of symptoms in 5 days. Discharge after 14 days. | Esophagogastroduodenoscopy. |

*(Continued)*

**TABLE 1** | Continued

| References | N°/gender | Age,y | HAE type | Amylase (IU/L) (normal range) | Lipase (IU/L) (normal range) | Treatment for attacks / Prophylactic treatment | Effect | Invasive procedures |
|---|---|---|---|---|---|---|---|---|
| Berger et al. (18) | 1/M | 6 | I | 1,440 (10–100 U/L) | 180 (16–65 U/L) | Fluids, papaverin, low-fat diet (before HAE diagnostic).<br>C1-INH concentrate (after HAE diagnostic). | Alleviation of symptoms, normalization of amylase and lipase levels.<br>Disappearance of symptoms within minutes. | Cholecystectomy. |
| Loudin et al. (19) | 1/F | 56 | I | ND | 663 IU/L | C1-INH concentrate (20 mg/kg). | Disappearance of symptoms after 30 min.<br>Lipase decreased to 142 IU/L. | Cholecystectomy, endoscopic ultrasound. |
| Aksoy et al. (20) | 1/M | 55 | I | ND | ND | ND<br>Prophylactic danazol (200 mg/day). | ND<br>Free of symptoms (3-month follow-up). | Laparotomy, partial omentectomy. |
| Hirose et al. (21) | 1/ND | ND | I or II | ND | ND | ND | ND | ND |

*ERCP, endoscopic retrograde cholangiopancreatography; ND, not described; F, female; M, male.*

and hereditary or drug-induced pancreatitis, other uncommon causes must be considered for an effective management (9).

The first report of pancreatitis associated with HAE is from 1986, which describes 3 C1-INH-HAE patients with recurrent abdominal attacks found to develop idiopathic chronic pancreatitis (10). In the sequence, more 12 independent cases have been reported, all are C1-INH-HAE (11–22) (**Table 1**). Although abdominal attacks are quite frequent among the different types of HAE, monitoring pancreatic enzymes is not routinely performed. Aksoy et al. (20) described a male patient diagnosed with HAE type I only after presenting with 4–5 episodes of abdominal pain and pancreatitis, despite that swelling of the face, lips, and tongue had occurred in the first attack (20). In another report, a 6-year-old boy with acute pancreatitis was diagnosed with HAE only after 10 hospitalizations in a period of 18 months. Within this period, this patient underwent cholecystectomy, however, no gallstones were found (16).

Not only is pancreatitis underdiagnosed, HAE patients frequently undergo invasive or improper diagnostic methods and treatments (5, 23), especially when they present with isolated or predominantly abdominal swellings. Patient 2 underwent appendectomy and spent years relying on a wrong diagnosis. In Case 1, the patient was submitted to an ERCP during the pancreatitis investigation. Likewise, in the other 15 published cases, 7 invasive diagnostic procedures and 12 surgeries possibly related to HAE had been done (**Table 1**). These include 3 appendectomies and 3 tonsillectomies, 2 cholecystectomies, 2 non-specified abdominal surgeries, 1 laparotomy, 1 omentectomy, and 1 marsupialization (**Table 1**). In a prospective study, one Japanese patient presented with acute abdomen with intestinal edema; acute pancreatitis was newly diagnosed with C1-INH-HAE (21).

Patients with HAE and normal C1-INH present with similar clinical manifestations as HAE-C1-INH ones. Some authors described facial edema as the most frequent event among F12-HAE (24), nevertheless, we observed that more than 60% of these patients had recurrent abdominal swellings (6). Here, we report the first patient with pancreatitis associated with HAE attack.

During HAE attacks, the obstruction of pancreatic duct or ampulla of Vater can result from duodenal edema, the most probable cause of pancreatitis in this situation. Pancreatic edema itself is not reported in HAE. Aksoy et al. (20) reported mild pancreatic duct dilation and side duct ectasia using magnetic resonance cholangiopancreatography during an HAE-associated pancreatitis episode, supporting the hypothesis that obstruction of pancreatic duct is responsible for pancreatitis in HAE (20); however, in other case reports, pancreatic duct presented normal (12, 17, 19). The obstruction of pancreatic duct causes the blockage of pancreatic secretion, leading to zymogen granules fusion with lysosomes, trypsinogen activation, and autodigestive injury of acinar cells, stimulating inflammatory response (9).

Since angioedema attacks resolve spontaneously in 2–5 days, HAE-associated acute pancreatitis should also disappear within a few days. This was observed in Case 3, and in the reviewed cases in which pancreatitis was treated conservatively (11, 12, 17, 18). However, we cannot establish if the episode described in Case 3 is a pancreatitis associated with an HAE swelling,

or a single acute pancreatitis, since no specific medication was administered. Indeed, as well as HAE abdominal attacks are frequently misdiagnosed and mistreated, a comprehensive evaluation of abdominal pain in HAE patients unsuccessfully treated with specific medication should be performed.

When specific treatment for acute HAE attacks was administered (C1-INH replacement), the alleviation and resolution of symptoms was remarkably faster, within hours to a maximum of 1 day (13, 15, 16, 18, 19). C1-INH administration was beneficial in different experimental animal models of pancreatitis (25–27), but ineffective in others (28). Although the benefits of C1-INH therapy are questionable in general pancreatitis, the cases here reviewed indicate good efficacy in HAE-associated pancreatitis.

For the first time, we observed improvement of clinical symptoms caused by abdominal pain and pancreatitis with the administration of Icatibant, a specific antagonist of bradykinin B2 receptor, in Cases 1 and 2. B2 receptor antagonists were shown to diminish pain associated to pancreatitis (29), to reduce pancreatic plasma extravasation, and pancreatic neutrophil influx (30), and to reduce serum amylase and lipase (31) in different pancreatitis-induced models. Although a direct activation of B2 receptors in pancreatitis pathogenesis can be speculated (by both circulating or local bradykinin released by tissue kallikrein), the role of the kinin system in pancreatitis is still unknown.

To our knowledge, other treatments available for acute HAE attacks have not reported for pancreatitis yet. In addition to patient from Case 1, six of the reported patients were under prophylactic treatment with danazol, an attenuated androgen which increases the production of hepatic C1-INH. On the other side, danazol can also be associated with pancreatitis development (9, 32), but in this case, pancreatitis should not resolve spontaneously. Moreover, elevated liver enzymes, a common sign of steroid injury, had been described in only one of the patients presenting pancreatitis and taking danazol (12). One of our patients (case 3) presented with high gamma-glutamyl transferase and slightly high alanine aminotransferase, but she was under long-term prophylaxis with tranexamic acid, a plasmin inhibitor, and not steroids. Moreover, we could not find a causative association between danazol prophylaxis and HAE-associated pancreatitis in any of the 18 cases described.

In conclusion, we present here the first description of HAE-associated acute pancreatitis in a F12-HAE patient and the first cases treated with icatibant. Although HAE attacks are rarely associated with pancreatitis, our findings and the review of literature suggest to investigate HAE in idiopathic pancreatitis. Likewise, pancreatitis signs should be searched for in severe abdominal attacks in HAE patients to avoid further complications. The efficient response to C1-INH replacement and B2 receptor antagonist (Icatibant) indicates that specific drugs for HAE attacks can be useful options for HAE-associated pancreatitis in addition to the conservative treatment.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of ethical standards of the 1964 Declaration of Helsinki with written informed consent from all subjects. The protocol was approved by the Ethics Committee in Research of Universidade Federal de São Paulo (n°56522).

## AUTHOR CONTRIBUTIONS

CV, RC-S, and PN performed the analysis and reviewed the literature. CV conceptualized and drafted the manuscript. RAC and AG performed the data collection instruments, and coordinated data collection. AG and JP conceptualized the study, coordinated and supervised data collection. All the authors drafted, reviewed and approved the manuscript.

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PERSPECTIVE
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# Paroxysmal Permeability Disorders: Development of a Microfluidic Device to Assess Endothelial Barrier Function

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**Background:** Paroxysmal Permeability Disorders (PPDs) are pathological conditions caused by periodic short lasting increase of endothelial permeability, in the absence of inflammatory, degenerative, ischemic vascular injury. PPDs include primary angioedema, idiopathic systemic capillary leak syndrome and some rare forms of localized retroperitoneal-mediastinal edema.

**Aim:** to validate a microfluidic device to study endothelial permeability in flow conditions.

**Materials and Methods:** we designed a microchannel network (the smallest channel is 30μm square section). Human Umbilical Vein Endothelial Cells (HUVECs) were cultured under constant shear stress in the networks. Endothelial permeability assessment was based on interaction of biotinylated fibronectin used as a matrix for HUVECs and FITC-conjugated avidin. The increase in endothelial permeability was identified as changes in fluorescence intensity detected by confocal fluorescent microscopy.

**Results:** The microchannels were constantly perfused with a steady flow of culture medium, ensuring a physiologically relevant level of shear stress at the wall of ∼0.2 Pa. Our preliminary results demonstrated that circulation of culture medium or plasma from healthy volunteers was associated with low fluorescence of fibronectin matrix. When bradykinin diluted in culture medium was perfused, an increase in average fluorescence was detected.

**Conclusion:** Our microvasculature model is suitable to study endothelial functions in physiological flow conditions and in the presence of factors like bradykinin known as mediator of several PPDs. Therefore, it can be a promising tool to better understand the mechanisms underlying disorders of endothelial permeability.

**Keywords: endothelial permeability, endothelial function, Paroxysmal Permeability Disorders, microfluidic device, microchip, shear stress, angioedema, idiopathic systemic capillary leak syndrome**

## BACKGROUND

The vascular endothelium is considered as a complex organ, which is responsible for the dynamic control of vessel functions, such as the transport of fluids and proteins from the intra- to the extravascular space (and vice versa), the modulation of the immune response, the regulation of the balance between procoagulant and anticoagulant factors, nutrients' trafficking, angiogenesis and the orchestration of organ development (1). The endothelium is continuously exposed to shear stress and changes in pressure, including rhythmic fluctuations due to heart beating. Endothelial cells (ECs) are physiologically separated by an intercellular space of 6–8 nm. ECs' membrane proteins allow tightly controlled and regulated passage of water, gases, electrolytes and small molecules through this intercellular space. Fluid exchange between intravascular and extravascular space occurs via diffusion and filtration. Proteins cross the vascular wall via either paracellular and transcellular pathways. The first one is controlled by the dynamic opening and closing of interendothelial junctions (2), the latter includes vesicular transport systems, fenestrae, and biochemical transporters (3).

The sieving properties and permeability of the endothelium depend on the function of specialized selective junctional regions which link ECs one to another. These junction regions are organized mainly by two types of contacts with different functions: adherens junctions (AJs) initiate cell-to-cell contacts and promote their maturation and maintenance; tight junctions (TJs) regulate the passage of ions and solutes through the paracellular route (4–6). ECs express cell-type-specific transmembrane adhesion proteins such as VE-cadherin (also known as CD144) as a core protein of AJs and claudin-5 at TJs (7).

VE-cadherin is expressed in essentially all types of vessels. Different mediators including histamine, tumor necrosis factor, platelet activating factor, and vascular endothelial growth factor as well as changes in fluid shear stress are able to induce phosphorylation of VE-cadherin, β-catenin, and p120. This phosphorylation results in an increased permeability of the endothelial monolayer, changes in flow sensing, and vascular remodeling (2–5, 8–10). VE-cadherin may also be phosphorylated through inhibition of associated phosphatases, e.g., the VE-PTP phosphatase, which is endothelial-specific (11). Other pathways that may modify endothelial permeability are VE-cadherin cleavage and regulation of its expression (12).

Phenotypic heterogeneity is a key feature of the vascular endothelium, which displays different morphology, behavior, biosynthetic repertoire according to different sites (13–15).

Over the past decade there has been an explosion of interest in the thin (∼500 nm), gel-like endothelial glycocalyx layer that coats the luminal surface of blood vessels and participates in the regulation of a wide range of vascular functions (16). It is currently known that its main functions include modulation of vessel permeability to water and macromolecules; mechanotransduction of fluid shear stress and pressure to the endothelial cytoskeleton and regulation of shear stress-mediated NO production; regulation of red and white blood cells' adhesion as well as modulation of the inflammatory response via binding of inflammatory cytokines (16). Alterations and shedding of the glycocalyx can contribute to the pathophysiology of very severe conditions such as septic or hemorrhagic shock (17, 18).

Since ECs are exposed to signals from both surrounding tissues and flowing blood, their regulatory pathways are numerous and cross interacting. It is known that several soluble mediators can influence ECs via many receptors, activating a number of intracellular pathways, which influence each other and result in multiple feedback mechanisms, able to modify endothelial permeability either directly or indirectly, rapidly or through long-term processes (19, 20). Moreover, endothelial cells' behavior might be influenced not only by hemodynamic forces but also by factors such as changes in blood oxygenation, temperature and pH. There is a huge variety of mediators, receptors, junctions and pathways, which are constantly exposed to hemodynamic forces and, as the musical instruments of an orchestra, interact one with another, each one with its specific role and timing, in order to generate a complex harmony, namely the fine modulation of endothelial permeability.

## PAROXYSMAL PERMEABILITY DISORDERS

Over the decades the term "endothelial dysfunction" has been applied to a huge variety of conditions associated with alterations of the endothelial morphology and functions.

For instance, thickening of the intima, proliferation of smooth muscle cells, formation of the fibrous plaques, changes of the vasa vasorum, loss of endothelium-derived nitric oxide, alterations of the endothelial glycocalyx, hyper-adhesiveness of the vascular lining toward platelets especially in complex hemodynamic shear stress regions are the processes lying at the basis of atherosclerosis, which is responsible for coronary, cerebral, peripheral artery and aortic diseases (21, 22).

The term "endothelial dysfunction" has been also used with reference to the loss of the ability of the endothelium to regulate vascular resistance. This alteration might imply chronic structural changes of the endothelial cell barrier as it happens in pulmonary hypertension (23). However, in some conditions the alterations of the endothelial morphology and functions are transient followed by a restoration almost to normal after the resolution of the acute phase. Among the latter conditions is for instance sepsis, which is characterized by increased leukocyte adhesion and trafficking, altered vasomotor tone, loss of endothelial barrier function, shifts in hemostatic balance and programmed cell death, even though endothelial structural changes can also occur—nuclear vacuolization, cytoplasmic swelling and fragmentation, ECs detachment (24).

It is possible to distinguish a variety of diseases which are due to recurrent alterations of endothelial permeability, with no inflammatory, degenerative, ischemic vascular injury and complete *restitutio ad integrum* after each episode. For these cases we would like to propose a sort of a new nosological entity, namely the Paroxysmal Permeability Disorders (PPDs) in the effort of grouping conditions that are due to periodic dysfunction of endothelial permeability and probably share some common

**TABLE 1 |** Paroxysmal Permeability Disorders: features for inclusion/exclusion together with currently identifiable clinical phenotypes.

| PAROXYSMAL PERMEABILITY DISORDERS |
|---|
| **CRITERIA** |
| **Features for inclusion** |
| - Recurrent self-limiting local or systemic interstitial edema |
| - For non-lethal episodes, complete healing max 1 week, mostly 1–3 days |
| **Features for exclusion** |
| - Any underlying clinical condition causing increase in endothelial permeability (infections, systemic inflammation, allergy, malignancy, injury, autoimmune disease etc.) |
| - Local signs of inflammation, ischemia-necrosis, tissue degeneration |
| **CLINICAL PHENOTYPES** |
| **Primary Angioedema** |
| - Idiopathic histaminergic angioedema |
| - Hereditary/acquired angioedema due to C1 inhibitor deficiency |
| - Hereditary angioedema with normal C1 inhibitor |
| - Idiopathic non-histaminergic angioedema |
| **Idiopathic Systemic Capillary Leak Syndrome** |
| **Yet poorly defined forms of periodic edema** |
| - Recurrent retroperitoneal edema |
| - Recurrent female periodic edema of unknown origin |
| - Recurrent edema in patients with hypereosinophilia (Gleich's syndrome) |

pathophysiological mechanisms, although they are characterized by different clinical pictures and differ in therapeutic approaches (**Table 1**).

Among such diseases we include clinical conditions which can be rapidly lethal, due to a localized process (e.g., laryngeal edema in primary angioedema) or to a systemic derangement (e.g., hypovolemic shock due to massive plasma and protein extravasation in idiopathic systemic capillary leak syndrome). PPDs also include some yet poorly defined forms of periodic edema. Scanty data are available about the pathogenesis of some of these conditions, possible triggers and means to correctly treat them, avoiding acute and long-term complications.

## Primary Angioedema

Hereditary angioedema due to C1 inhibitor deficiency or dysfunction (C1-INH-HAE) is a rare autosomal dominant disorder (prevalence around 1:50,000 people in the general population) characterized by localized, non-pitting edema of the skin and submucosal tissues of the upper respiratory and gastrointestinal tracts, without significant wheals or pruritus, due to a temporary increase in vascular permeability. It represents the best characterized form of primary angioedema, a group of conditions where angioedema occurs in the absence of wheals and of an identified causative factor. The same group of pathological conditions include also the forms of hereditary angioedema related to Factor XII / Plasminogen /Angiopoietin 1 mutations and the forms with unknown etiology.

In these angioedema chronically recurrent symptoms cause significant personal, domestic, social, and occupational disability and exposes patients to the risk of death (due to asphyxia when the respiratory tract is involved) (25–27).

## Idiopathic Systemic Capillary Leak Syndrome

Idiopathic Systemic Capillary Leak Syndrome (ISCLS), also known as Clarkson's Disease (28), is a rare disorder (about 260 cases described) with recurrent potentially life-threatening episodes of distributive shock with hemoconcentration and hypoalbuminemia (29–32). During attacks, endothelial hyperpermeability results in leakage of water, solutes and plasma proteins (up to the size of 300kDa) into the interstitial space. The management of this condition is extremely challenging not only because of the intrinsic severity of the attacks but also because of the high risk of inducing iatrogenic damage both in the acute and post-acute phases, especially when a strategy of aggressive fluid replacement is erroneously chosen (33, 34).

## Yet Poorly Defined Forms of Periodic Edema

Among PPDs there are also conditions characterized by recurrent edema with no known trigger and/or etiology. We recently reported cases of significant isolated retroperitoneal edema (with no thickening of omentum/intestinal walls or ascites), with complete resolution after the acute crisis (35).

# ASSESSMENT OF ENDOTHELIAL PERMEABILITY

When investigating alterations of endothelial permeability, different kinds of strategies can be chosen. Depending on the specific clinical conditions under investigation, different parameters can be evaluated (e.g., cleaved high molecular weight kininogen in primary angioedema, hematocrit and albumin values in idiopathic systemic capillary leak syndrome). This strategy is certainly useful from the diagnosis point of view and can give some important clues to unveil some of the pathophysiological mechanisms underlying PPDs. The chase for a permeability increasing factor led Xie et al. to show that circulating angiopoietin 2 (Angpt2) and vascular endothelial growth factor (VEGF) are increased in acute but not in convalescent sera from ISCLS patients and both of them are able to induce endothelial hyperpermeability *in vitro* by disrupting endothelial adherent junctions (36). Angpt2 and VEGF cause endothelial cells' retraction without inducing cell death, with attenuation of membrane VE-cadherin and actin stress fiber formation (36). Likewise, research is ongoing to assess the role of the monoclonal component which can be found in the majority of ISCLS patients (32).

In order to investigate endothelial function, a variety of static *in vitro* models has been proposed and used in recent years and provided some relevant information to the understanding of B2 and B1 types of bradykinin receptor and gC1q receptor in the vascular leakage induced by plasma from C1 inhibitor deficient patients (37).

Microfluidic technology highly developed in physics is now widely used to create tools for cell biology (38). A variety of bioassays and investigations can be carried on in microfluidic systems where living cells can be cultured: cell migration and interaction, cancer cell invasion, drug delivery assays, wound healing, angiogenesis, thrombosis, studies of blood flow and shear stress etc. (38). The insights derived from this kind of research have potential implications to get some clues in clinical settings, both for a better understanding of some pathophysiological mechanisms (such as wound healing and cancer progression) and for searching of therapeutic approach (e.g., study of the blood brain barrier in order to achieve a better delivery of drugs).

Recently, different types of endothelial cells have been used in *in vitro* models to obtain organ-specific vascular models (39) and this is what we are also interested in.

## An Innovative Tool: The "Microvasculature-on-a-chip" Model

In order to test endothelial cells' behavior in a three dimensional dynamic model reproducing the influence of physiological flow and shear stress as an important part of "everyday life" of the endothelium, we developed and tested a "microvasculature-on-a-chip" microfluidic device (40).

Briefly, the model consists of 30μm-high microchannels organized in a branching/converging network (**Figure 1A**). At each branching point the width of each channel is divided by two, reaching 30 × 30 μm (height × width, square section) in the middle part of the chip. Circuits were fabricated from PDMS and sealed with a glass coverslip at the bottom to allow high-resolution microscopy. Channel walls were coated with biotin-conjugated fibronectin (Cytoskeleton Inc, USA) as a matrix before seeding the circuit with Human Umbilical Vein Endothelial Cells (HUVECs, PromoCell, Germany), chosen as a commonly used human model to study endothelial functions and physiology. HUVECs were cultured within the networks, in the presence of a steady flow of culture medium, ensuring a physiologically relevant level of fluid shear stress at the wall of ∼0.2 Pa. In the present condition HUVECs were able to adhere to all four walls of each channel and to form a confluent monolayer within a few days after seeding (**Figure 1A**).

After reaching confluence, HUVECs' cytoplasm was stained with CellTracker Red (Molecular Probes$^{TM}$) and the cells were exposed for 15 min to a constant flow of one of the following media: (i) usual endothelial cells culture medium (PromoCell, Germany) as a control, (ii) plasma from healthy volunteer—blood was withdrawn in Sodium Citrate tubes, plasma was separated from cells by centrifugation and diluted 1:1 with medium for ECs culturing, (iii) bradykinin (Sigma, USA) diluted in endothelial cells culture medium at a concentration of 25 μM. FITC-conjugated avidin (Molecular Probes$^{TM}$) was finally added to the perfusion solution (final concentration is 25 μg/ml for 5 min) before cells were fixed with 4% paraformaldehyde and their nuclei stained with Hoechst 33342 (Molecular Probes$^{TM}$).

Laser Scanning Confocal Fluorescence Microscope (Zeiss, Germany) was used for cell imaging. 3D image stacks (425 × 425 × 35 μm in X, Y, and Z) of various parts of the microchannels were acquired with a 20x objective, with a sampling of 0.140 μm/pixel in the X/Y and 1.5 μm/pixel in Z direction. The acquired image stacks were processed and analyzed using the Fiji open-source platform (41). 3D stacks were first transformed into 2D images by projecting (summing) the pixel intensities along the Z direction, for the 3 detection channels corresponding to nuclei, cytoplasm and avidin (see **Figure 1B**).

Under our experimental conditions, high-affinity interactions between FITC-avidin and biotinylated-fibronectin can take place only when avidin molecules have crossed the endothelial layer. By monitoring the fluorescence level associated with the presence of FITC-avidin bound to the walls of the microchannels, we can therefore assess the permeability of the endothelial monolayer. This was done by computing intensity histograms from the Z-projected 2D images (**Figure 1C**). In order to quantitatively compare such histograms for the three different media to which HUVECs were exposed, we took care to acquire all stacks using the same imaging parameters, and to compute histograms from sections of the microchannels spanning the same surface (namely 49,000 $\mu m^2$ here). Doing so, we observe that (**Figure 1C**):

(i) no major difference in histogram peak or width can be seen between the control and the 50%-plasma from healthy volunteer conditions,
(ii) in the circuit exposed to 25 μM solution of bradykinin the histogram is clearly shifted to larger intensity values compared to that obtained for the control.

Moreover, such a trend is confirmed when comparing the average intensity values computed from histograms measured over 3–6 different regions of the microchannel networks (**Figure 1D**): we observe a significantly higher average value ($I_{Brad} = 230 \pm 76$) for the bradykinin-treated channels as compared to the control circuit ($I_{Control} = 68 \pm 18$), whereas no statistical difference is found between control and plasma ($I_{Plasma} = 77 \pm 23$) conditions. Since the intensity of the fluorescent signal depends on the quantity of FITC-avidin bound to the surfaces of the channels, this result can be considered as a sign of increased endothelial permeability after its exposure to bradykinin.

This was further confirmed by negative controls (images not shown) where we have imaged channels coated with non-biotinylated fibronectin after exposure to FITC-avidin. When no cells were present in the channels (i.e., fibronectin freely accessible to avidin), we measure an average fluorescence intensity of 15 ± 8, barely different from the detection noise ($I_{noise} = 10 \pm 4$), showing the very low level of non-specific binding of FITC-avidin to non-biotinylated fibronectin. With HUVECs cultured in such channels, we find an average intensity of 38 ± 6, with fluorescence being detected within the cytoplasm of cells but not at the channel walls, which suggests that a large fraction of the intensity detected in the control and plasma conditions above correspond to FITC-avidin internalized by the cells without reaching the underlying walls.

## DISCUSSION

Under the term Paroxysmal Permeability Disorders, we would like to gather pathological conditions which probably share some common pathophysiological mechanisms and whose humanistic

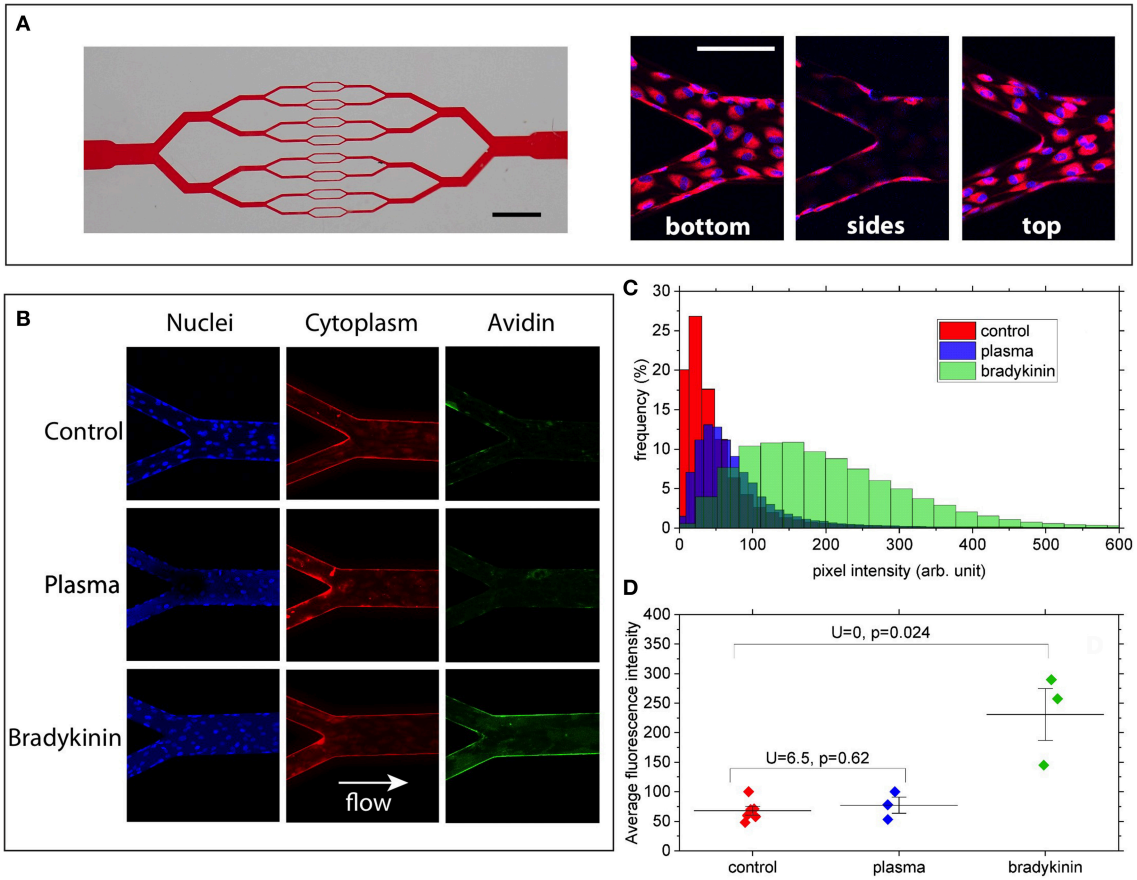


**FIGURE 1 | (A)** Left: picture of the channel network illustrating the branching/converging geometry used (scale bar: 2 mm). Right: merged images showing cell nuclei (blue) and cytoplasm (red) at the bottom, on the lateral walls and at the top of the channels (scale bar: 100 μm). **(B)** Z-summed images obtained from 3D-stacks showing nuclei (blue), cytoplasm (red), and wall-bound avidin (green) for the 3 conditions: control, 50%-plasma, and bradykinin. Image size is 425 × 425 μm. Imaged regions correspond to a merging point between two 60 μm-wide channels and one 120 μm-wide. **(C)** FITC intensity histograms (relative frequency as a function of pixel intensity) computed for the 3 conditions from the images shown in **(A)**. **(D)** Average fluorescence intensity values measured for the various conditions over 3–6 different bifurcation areas of the networks. U and *P*-values correspond to two-samples Mann–Whitney *U*-tests showing that, at the 5% threshold, Bradykinin and Control data are different whereas Plasma and Control are not.

burden is remarkable not only because of possible lethality, but also because they can significantly affect patients' quality of life for long-term complication, social impact and anxiety related to fear of upcoming events. Increased knowledge and awareness of the pathophysiological mechanisms underlying these conditions has been the cornerstone for the development of new drugs in some cases (e.g., hereditary angioedema) and getting deeper insights is of pivotal importance in order to correctly treat these conditions and avoid acute and long-term complications.

In our study we propose to focus not only on soluble factors, mediators and receptors when investigating PPDs, but also on the endothelium itself as an active dynamic player with high impact on appropriate functioning of the vascular system. Our "microvasculature-on-a-chip" model can reproduce the size of real capillary beds and allows performing experiments in tightly controlled conditions: shear stress, specific fluid composition, varying humoral factors and mediators. Endothelial cells can be seeded into the systems, reaching a physiological confluent monolayer, expressing the main markers typical for endothelium including a glycocalyx lining the entire lumen of the channels (40). The microchannel networks are suitable to investigate normal and abnormal interactions between blood cells and vessel walls. In the next future endothelial cells from specific vessels' beds or from vessels with specific characteristics (e.g., microvascular endothelial cells) could be seeded into the system. It is also possible to conceive the elaboration of gene study assays, thanks to the possibility to collect cells from the device after challenge with different kinds of "stressors." Even though our results are very preliminary, our study confirms that the proposed three dimensional dynamic model is able to detect changes in endothelial permeability. Since our previous work (40) demonstrated HUVECs' longevity in the channels under constant culture medium flow, restoration of the endothelial barrier after exposure to the agents increasing intercellular junctions permeability is expected. Therefore, our microfluidic device could be a promising powerful tool to get deeper insights into the mechanisms underlying PPDs.

It should be noted that our "microvasculature-on-a-chip" model still has some limitations. First of all, the stiffness of polydimethylsiloxane is different from that of the glass coverslip at the bottom of the circuit, so that the extravascular "microenvironment" is still not very close to physiology and changes of some endothelial functions have to be carefully interpreted between the sides of the circuit. However, new strategies involving the use of soft hydrogels and materials mimicking the interstitial matrix may allow investigations which are more consistent with the conditions *in vivo*, with a possibility to evaluate not only endothelial permeability to fluids, but also the transport of molecules and flowing cells. Further refinement of the fabrication and cell seeding procedures will allow higher automation and reproducibility which is of high importance when dealing with cells exposure to different kinds of fluids (e.g., RBC suspension with varying hematocrit levels), circulating factors and mediators. The design of a micro-chamber surrounding the microchannels network and filled with interstitial-like matrix will open the way to test in more details the response of the system to plasma (and eventually blood) from angioedema and ISCLS-patients (collected both during attacks and in intercritical periods) as well as cells' migration through the endothelial monolayer in both directions.

Despite the above-mentioned limitations, we believe that the presented system may be suitable to investigate new therapeutic approaches, including, for instance, the delivery of drugs via innovative nanotechnologies. When investigating new treatments for ISCLS, we would like to evaluate the effect of agonist and antagonist of Tie 2 as AKB-9778 and Angt2. We hypothesize that AKB-9778 might be a valuable strategy not only to treat or prevent diabetic retinopathy, but also to treat systemic conditions characterized by increased permeability, such as ISCLS. Finally, we speculate that angioedema and ISCLS should be regarded as "simple disease models" in the huge scenario of conditions characterized by hyperpermeability and that our "microvasculature-on-a-chip" model can be a powerful tool to advance in study and better understand alterations underlying this type of disorders.

Ethical approval was not required for this study in accordance with the local legislation and institutional requirements. Written informed consent was obtained from all participants.

## ETHICS STATEMENT

Involvement of human subjects was limited to blood withdrawals from healthy volunteers. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

## AUTHOR CONTRIBUTIONS

MW, DT, LiB, CM, and MC were responsible for the conceptualization of the study. DT, LiB, AD, and CM designed the microvasculature on a chip model. MW, IB-G, LaB collected blood samples from healthy volunteers; MW, DT, and MI developed the protocol to test endothelial permeability in the microfluidic device. MW, DT, and MI performed the experiments (from fabrication of the microchip and seeding of HUVECs to cell imaging with confocal fluorescence microscopy). MW, DT, and LiB were responsible for image processing and analysis. MW wrote the first draft of the manuscript. MW, DT, LiB, IB-G, AD, LaB, CM, and MC revised the original draft and helped in manuscript editing. CM and MC were in charge of supervision of all the steps of the study. CM and MC were responsible for project administration. CM, LiB, and MC provided the resources to develop the project.

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REVIEW
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# Kallikrein/K1, Kinins, and ACE/Kininase II in Homeostasis and in Disease Insight From Human and Experimental Genetic Studies, Therapeutic Implication

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Kallikrein-K1 is the main kinin-forming enzyme in organs in resting condition and in several pathological situations whereas angiotensin I-converting enzyme/kininase II (ACE) is the main kinin-inactivating enzyme in the circulation. Both ACE and K1 activity levels are genetic traits in man. Recent research based mainly on human genetic studies and study of genetically modified mice has documented the physiological role of K1 in the circulation, and also refined understanding of the role of ACE. Kallikrein-K1 is synthesized in arteries and involved in flow-induced vasodilatation. Endothelial ACE synthesis displays strong vessel and organ specificity modulating bioavailability of angiotensins and kinins locally. In pathological situations resulting from hemodynamic, ischemic, or metabolic insult to the cardiovascular system and the kidney K1 and kinins exert critical end-organ protective action and K1 deficiency results in severe worsening of the conditions, at least in the mouse. On the opposite, genetically high ACE level is associated with increased risk of developing ischemic and diabetic cardiac or renal diseases and worsened prognosis of these diseases. The association has been well-documented clinically while causality was established by ACE gene titration in mice. Studies suggest that reduced bioavailability of kinins is prominently involved in the detrimental effect of K1 deficiency or high ACE activity in diseases. Kinins are involved in the therapeutic effect of both ACE inhibitors and angiotensin II AT1 receptor blockers. Based on these findings, a new therapeutic hypothesis focused on selective pharmacological activation of kinin receptors has been launched. Proof of concept was obtained by using prototypic agonists in experimental ischemic and diabetic diseases in mice.

**Keywords: angiotensin-converting enzyme, kallikrein (tissue), kinins, vasodilation, genetic human, genetic mouse models, Ischemic heart disease, diabetic nephropathy**

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Kallikrein-K1 is the main kinin-forming enzyme in organs (including blood vessels) in resting condition whereas angiotensin I-converting enzyme/kininase II (ACE) is the main kinin-inactivating enzyme in the circulation (being present in both endothelium and plasma) (1–3). Both ACE and kallikrein have other, non-kinin related physiological actions, which are especially well-documented for ACE, first known as the angiotensin I-activating/angiotensin II-generating enzyme [(4), **Figure 1**].

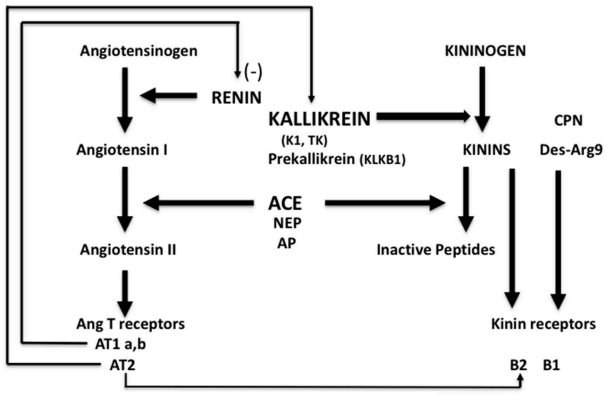


**FIGURE 1 |** Schematic representation of the renin-angiotensin and kallikrein-kinin systems with physiological interrelation. ACE, angiotensin-converting enzyme/kininase II; NEP, neutral-endopeptidase; AP, aminopeptidase P; CPN, carboxypeptidase N.

Bioavailability of kinins in organs depends mainly on the balance between activities of K1 and ACE, locally. Bioavailability of angiotensin II depends on renin but also on ACE which is the only enzyme, in the human species, releasing angiotensin II from angiotensin I in the circulation. While early conception of regulation of the circulation was based on endocrine action of the renin-angiotensin system governed by renal renin secretion and autocrine/paracrine activity of the kallikrein-kinin system in organs where K1 is synthesized, subsequent research corrected, and expanded this concept. Indeed, ACE is not uniformly synthesized in the vasculature and its abundance displays strong vessel and organ specificity. Vascular ACE content is high in the lung and very low in the kidney and to a lesser extent the heart (5–7). Accordingly, bioavailability of angiotensin II is reduced in heart and kidney compared to other organs while action of locally produced kinins is potentiated in these organs. Both contribute to limiting vasoconstriction and maintaining high blood flow, especially in the kidney (5–8). This is the anatomical and physiological basis of the role of ACE in homeostasis, and of its role in disease (see below). The action of ACE adds a paracrine component to the renin-angiotensin system, especially active in the lung but downregulated in the heart and kidney.

Conversely, the kallikrein-kinin system has been shown to have an endocrine component based, in man, on renal (and perhaps also arterial) secretion of K1 (9). In rodents, circulating K1 comes mainly from salivary glands, which are well-developed in these animals (10). Endocrine action of K1 remains however poorly documented compared to local paracrine action of the enzyme in kidney, arteries, and exocrine glands.

Recent research, based mainly on human and animal genetic studies has documented the largely unsuspected physiological role of K1 in the circulation and has also refined understanding of the role of ACE/kininase II.

## THE PHYSIOLOGICAL ROLE OF K1

Kallikrein-K1 belongs to a large family of genetically and structurally related proteases but is the sole enzyme in the family able to catalyze the hydrolysis of kininogens and release kinins (10, 11). In absence of specific inhibitors of K1 suitable for *in vivo* studies the issue of the physiological role of the enzyme was addressed through inactivation of the *Klk1* gene in the mouse and discovery of a loss of function polymorphism of K1 in man (12).

The K1 deficient mice grow and reproduce normally. They have normal blood pressure in resting condition but display arterial functional abnormalities (13–15). The mice have severe impairment of flow dependent vasodilatation, a prominent feature of arterial physiology ensuring proper delivery of blood to organs during variation in cardiac output [(13, 15), **Figure 2**]. Flow dependent dilatation is partly a kinin and kinin-B2 receptor mediated process with kinins released locally by arterial K1 from circulating and endothelial-bound kininogen acting in a paracrine/autocrine manner (15). This occurs in both buffer and resistance arteries (15, 17). The vasodilator effect of the angiotensin II AT2 receptor is also impaired in K1 deficient mice, extending to arteries observation of functional coupling between AT2 and B2 receptors originally made in the kidney and establishing role of K1 and kinins in this coupling (18–20). Overall, arterial functional abnormalities in K1 deficient mice are consistent with endothelial dysfunction, secondary to kinin deficiency (15, 17, 18).

Interestingly, heterozygote deficient mice with roughly 50% K1 activity also display a defective arterial phenotype, indicating that K1 activity level in arteries (where K1 is synthesized in low abundance compared to several other organs) is a critical factor for arterial function (15). This has been further documented in man (see below).

Despite having functional arterial abnormalities, K1 deficient mice have normal blood pressure regulation and conserved circadian variation in resting condition (13, 21). While K1 was initially discovered as a hypotensive agent, the enzyme, at physiological level, either does not significantly influences vascular resistance or its action is offset by compensatory regulations. However, K1 has anti-hypertensive action in at least one pathological setting. The K1 deficient mice, when challenged with salt and aldosterone, a treatment increasing K1 synthesis in wild-type mice, develop hypertension (see below).

While K1 is synthesized in high abundance in epithelial cells in the kidney (in the distal tubule) and in other exocrine glands, K1 deficient mice display only minor renal epithelial abnormalities related to calcium and potassium handling, with mild hypercalciuria and delayed response to dietary potassium load (13, 22, 23).

Interestingly, it has been possible to extent to man the observations made in K1 deficient mice. Indeed K1 activity level is genetically determined in man and this is caused, at least in part, by a loss of function polymorphism of the enzyme substituting an histidine for an arginine in a substrate binding subsite of the active site (R53H) (24–26). The mutation has an allele frequency of 0.03 and only heterozygote subjects (7% of white population) could be studied (26). But these partially deficient K1 subjects display functional arterial abnormalities evidenced by vascular echotracking study. These abnormalities, increase in sheer stress with paradoxical inward arterial remodeling, are, like in K1 deficient mice, suggestive of arterial endothelial dysfunction [(16), **Figure 2**].

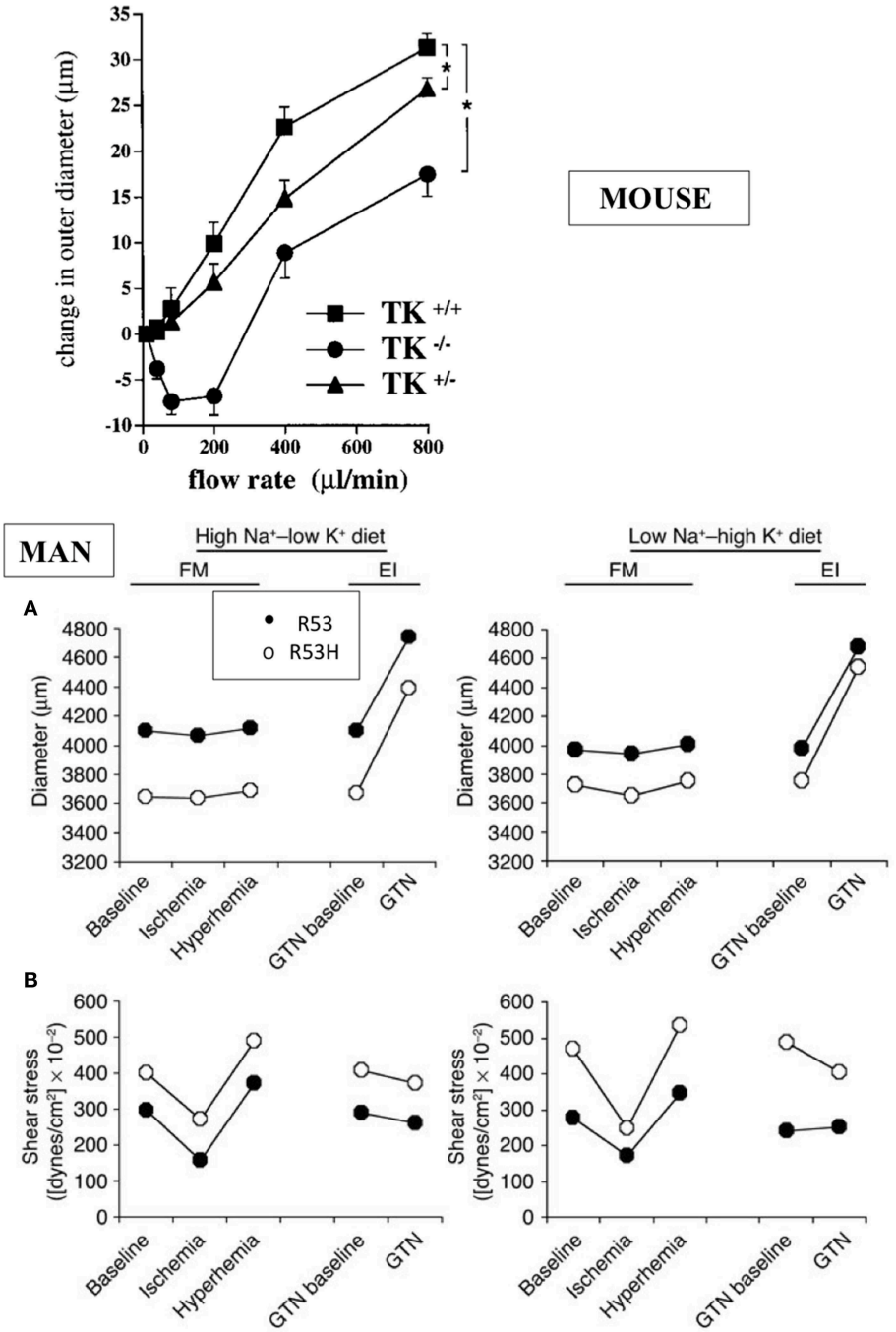


**FIGURE 2 |** Arterial dysfunction in K1 deficient mice and human subjects partially deficient in K1 activity. Upper graph: impairment of flow-induced vasodilatation in carotid artery of K1 deficient mice. Homozygote ($TK^{-/-}$) and heterozygote ($TK^{+/-}$) mice with inactivated K1 gene compared to littermate wild type animals ($TK^{+/+}$). *$p < 0.05$ compared to littermate $TK^{+/+}$. Note paradoxical vasoconstrictor response at low flow rate in $TK^{-/-}$ and partial defective phenotype of $TK^{+/-}$ mice (see text for discussion). Reproduced from Bergaya et al. (15). Lower panels: arterial dysfunction in subjects carrying the defective R53H mutation of K1 (heterozygote) and having roughly 50% K1 activity level of non-mutated, homozygous R53 subjects. Subjects were studied by vascular echotracking of brachial artery in basal condition, during hand ischemia and reactive hyperhemia and after nitroglycerin (GTN) administration. Study was repeated at contrasted dietary Na/K intake. Low Na-High K stimulates K1 synthesis in the kidney. Note increase in sheer stress **(A)** in R53H subjects with paradoxical reduction of arterial diameter **(B)**. Reproduced from Azizi et al. (16). Observations in both man and mouse are indicative of endothelial dysfunction (see text).

The R53H subjects also display minor epithelial abnormalities related to calcium or potassium handling, similar to the mouse (27, 28).

Mouse and human genetic studies thus allowed recognizing, consistently in both species, the physiological role of K1 in the circulation and its role in the kidney.

Overall, in resting condition, K1 deficiency induces only minor vascular and renal abnormalities in young individuals, albeit it is not known whether these defects can eventually translate into altered life expectancy. But, in pathological situations resulting from hemodynamic, ischemic, or metabolic insult to the cardiovascular system and the kidney K1 exerts critical end-organ protective action and K1 deficiency results in severe worsening of the conditions, at least in the mouse. This is being discussed below.

## PHYSIOLOGICAL ROLE OF ACE/KININASE II IN THE CIRCULATION AND IN THE KIDNEY

The ACE is an endothelial ectoenzyme, also secreted as a so-called soluble form in plasma by enzymatic cleavage separating the core molecule from its C-terminal transmembrane anchor (5, 29). Conversion of angiotensin I and inactivation of kinins are believed to occur mainly on the endothelial surface albeit, as said above, there is strong heterogeneity in endothelial ACE content along the vascular tree, with physiological consequence.

In man, ACE is mainly synthesized in small muscular arteries and in arterioles. Immunoreactive ACE is low or undetectable in large arteries and in veins. Only a fraction of capillaries in organs contains ACE, with two divergent exceptions, the lung and the kidney [(7), **Figure 3**].

In the pulmonary capillary bed high and homogeneous ACE content ensures conversion of angiotensin I and inactivation of bradykinin, both produced in the renal circulation and post-renal venous system and conveyed to the lung (7). In the renal circulation on the opposite, low or undetectable endothelial ACE content in face of high renin and angiotensin I concentrations limits local angiotensin II formation and consequently renal vasoconstriction (6, 7, 30). This is consistent with early studies documenting reduced angiotensin I conversion in the renal circulation (8). Kinins are also formed in abundance in the kidney interstitium from K1 synthesized in the distal tubule and released in both blood and urine. Lack of ACE in renal vessels potentiates the vascular action of kinin. Plasma ACE contributes to some extent to angiotensin I conversion and kinin inactivation in the renal circulation but cannot fully substitute for endothelial ACE in these functions (30, 31).

Bradykinin is the best substrate for ACE, i.e., the substrate with the most favorable kinetic parameters. Somatic ACE, which results from duplication of an ancestral gene, has two active sites and bradykinin is the best substrate for both ACE active sites (5, 32–36). ACE is not the only enzyme able of inactivating kinins in the circulation but because of its kinetic parameters it plays a major role, physiologically. Accordingly, kinins produced in organ interstitium by K1 in physiological or pathological conditions or in plasma by the other kallikrein, (pre)kallikrein (KLK1) when activated in pathological situations are quickly inactivated locally, except in the kidney (37, 38).

Interestingly, both theoretical and experimental evidences suggest that *in vivo* variations in ACE level influence largely bradykinin concentration but have little effect on angiotensin II concentration (39–41). This is because of a superimposed negative regulatory loop in the renin-angiotensin system between renal angiotensin II concentration and renin secretion, offsetting any ACE-related increase in angiotensin II, at least in the kidney.

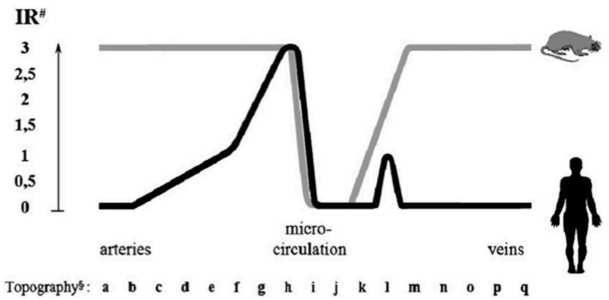


**FIGURE 3 |** Endothelial ACE distribution along the human and rat vascular tree. Endothelial ACE is heterogeneously distributed in humans (black line). Large arteries contain no or little ACE while maximal ACE content is observed in small muscular arteries and arterioles. Only a fraction of capillaries network contains ACE. Venules and veins contain no or little ACE except some muscular veins in upper and lower extremities. Exceptions to this general scheme are the renal circulation and the pulmonary capillary network (not shown, see text for discussion). In the rat (gray line) ACE is more homogeneously distributed in arteries and veins, while only a fraction of capillaries contains ACE, like in man. ACE is also dowregulated in renal arterioles (not shown). #Immunoreactivity (IR) shown is scored from 0 (no reactivity detectable) to 3 (maximal reactivity) and refers to defined mAb concentrations. §Localization of endothelial cells: (a) aorta ascendens, arcus aortae, aorta descendens/thoracalis/abdominalis; (b) a. brachiocephalica, a. pulmonalis, a. iliaca communis, a. carotis communis; (c) a. iliaca interna/externa, a. carotis interna/externa, a. subclavia; (d) truncus coeliacus, a. renalis, a. mesenterica sup./inf., a. axillaris, a. femoralis; (e) a. brachialis, a. poplitea, a. tibialis ant./posterior, a. ulnaris, a. radialis, a. peronea; (f) a. dorsalis pedis, a. digitalis plantaris/palmaris; (g) small peripheral arteries; (h) pre-arterioles, arterioles; (i) capillaries; (j) venules; (k) small peripheral veins; (l) v. digitalis plantaris, v. dorsalis pedis, v. saphena parva, v. saphena magna; (m) v. digitalis palmaris, v. tibialis, v. poplitea; (n) v. axillaris, v. femoralis, v. jugularis ext./int; (o) v. iliaca int./ext., v. subclavia; (p) v. iliaca communis, v. brachiocephalica, v. renalis; (q) v. cava sup./inf. Reproduced from Metzger et al. (7).

Angiotensin I-converting enzyme has other substrates, at least *in vitro*, for which the enzyme displays less favorable kinetic parameters than bradykinin or angiotensin II, like substance P and LHRH (35). It is not clear whether substance P and LHRH are metabolized by ACE physiologically (42). But hydrolysis of the hematopoietic peptide N-AcSDKP by the N-terminal active site of ACE has been documented both *in vitro* and *in vivo* (43). This peptide has been reported to have cardiac and renal anti-fibrotic action in experimental models (44).

Angiotensin I-converting enzyme, especially its N-terminal active site, can hydrolyse *in vitro* the Alzheimer amyloid Aβ-peptide that is believed to be causally involved in Alzheimer disease (45, 46). But, while some evidence for a role of ACE in limiting progression of experimental neurodegenerative diseases has been obtained in animals the role of ACE in Alzheimer disease remains controversial, despite being suggested by some clinical studies (45–48).

Angiotensin I-converting enzyme has also been shown to behave as a signaling molecule in cells, independently of its

enzymatic activity, through phosphorylation of a serine in the short intracellular domain. Signaling is triggered by ACE inhibitor binding, likely as a result of ACE conformational change and dimerization, involves CK2 kinase, mitogen-activated protein kinase kinase 7, and c-Jun N-terminal kinase and induces expression of some endothelial genes, including the *ACE* gene (49, 50). However, no physiological agonist of this pathway has been identified and its physiological role remains undocumented. The ACE signaling is however probably involved in the well-documented phenomenon of counter-regulatory increase in ACE synthesis during ACE inhibitor treatment (51). It has also been reported that ACE is involved in angiotensin II signaling *in vitro* in cultured cells but this observation remains of unknown physiological relevance (52).

Angiotensin I-converting enzyme levels are genetically determined in man. The above anatomical and physiological considerations are relevant to mechanistic issues pertaining to the well-documented role of this genetic variation in cardiovascular and renal diseases (see below).

## ROLE OF K1, KININS, AND ACE/KININASE II IN CARDIOVASCULAR AND RENAL DISEASES

Through pharmacological or genetic inactivation of K1 and kinin receptors in animals, manipulation of ACE/kininase II gene expression or activity as well as clinical studies it was shown that kinin release during hemodynamic, metabolic, or ischemic insult reduces organ damage, especially in the heart and kidney. This has been reviewed previously (37, 53, 54).

### Experimental Studies, Cardiac and Peripheral Ischemia, Diabetes, Hypertension

In cardiac ischemia-reperfusion in mice, genetic deficiency in K1 suppresses cardioprotective mechanisms limiting necrosis, like ischemic preconditioning [(55), **Figure 4**]. Moreover, deficiency in K1 abolishes the infarct-size reducing effect of an ACE inhibitor or an angiotensin II AT1 blocker and also the effect of the mitochondrial pore opening inhibitory drug cyclosporin A (55, 57, 58). Loss of the cardioprotective effect of ACE inhibitors in cardiac ischemia in K1 and kinin deficient mice is explained by the well-documented role of kinins in the beneficial effect of the drugs in this experimental model (59). Loss of the cardioprotective effect of AT1 blockers is also explained by kinin deficiency, because AT1 receptor inhibition increases renin secretion and angiotensin II production resulting in activation of the angiotensin II AT2 receptor. As discussed above, the AT 2 receptor is functionally coupled to K1 and kinins. This physiological pathway, inactivated in K1 or B2 receptor deficient mice, is shown here to play a prominent role in the effect of AT1 blockers in experimental cardiac ischemia (57, 60). For cyclosporin A, the mechanism of the permissive effect of K1 is less understood but may be related to direct mitochondrial action of the enzyme (58).

The effect of *Klk1* gene inactivation in acute cardiac ischemia is mimicked by duplication of the ACE gene, suggesting that kinin depletion plays a prominent role in organ damage in both genetic mouse models [(56, 61), **Figure 4**]. However, the deleterious cardiac effects of ACE gene duplication can be partially prevented by renin inhibition, indicating that both kinin depletion and enhanced local angiotensin II formation are involved in ACE-mediated cardiac damage in ischemia (56).

Cardiac or renal protective effect of kinins in ischemia has also been documented by studies in kininogen or kinin receptor deficient animals and in animals treated with kinin receptor antagonists (62). Kinins are potent endothelial activators promoting vasodilatation of collateral arteries thus increasing distal blood flow delivery and limiting thrombus extension by releasing endothelial mediators inhibiting platelet aggregation and triggering fibrinolysis (15, 63–66). Moreover, K1 and kinins exert cytoplasmic and mitochondrial actions decreasing oxidative stress in tissues (58, 67).

In chronic ischemic and post-ischemic diseases, vascular and cellular actions of kinins also prevent end-organ damage. In the ischemic hindlimb of diabetic mice or rats submitted to femoral ligation, an experimental situation where post-ischemic angiogenesis is impaired by diabetes, K1 and kinins exert vasodilatory and proangiogenic effects restoring distal blood flow. Deficiency in K1, kinins, or kinin receptors worsens hindlimb ischemia (68–70). Kinins exert proangiogenic effect through mobilization of progenitor cells with endothelial potentiality and also through mobilization and activation of macrophages (a so-called inflammatory response) (71–73).

In post-ischemic heart failure secondary to irreversible coronary occlusion, kinins prevent excess ventricular remodeling, which has deleterious electrophysiological and hemodynamic consequences. Deficiency in K1 or kininogen exaggerates remodeling and decreases long-term survival (74, 75). K1 is synthesized in the heart, although it is not clear whether synthesis occurs only in coronary vessels or also in cardiomyocytes. Kinins are involved in the cardioprotective effect of ACE inhibitors in post-ischemic heart failure and also in the effect of the renin inhibitor Aliskiren (74, 76). For Aliskiren, the mechanism proposed involves drug-induced increase in cardiac K1 synthesis, kinin release, and kinin B2 receptor activation, independently of renin inhibition (76).

### Diabetes

Diabetes is a disease where the role of K1 and kinins in limiting organ damage has been especially well-documented, experimentally. In mice rendered diabetic, the synthesis of K1, kininogen, and kinin receptors increases rapidly in the kidney after the onset of hyperglycemia. Inactivation of the *Klk1* gene aggravates diabetic nephropathy [(77), **Figure 5**]. The same aggravating effect is observed by inactivating the kinin B2 receptor gene (79). Diabetic nephropathy is both a vascular and a renal disease secondary to chronic hyperglycemia and in humans it is strongly linked, already at its early stage, to enhanced risk of coronary heart disease. Diabetic nephropathy can also

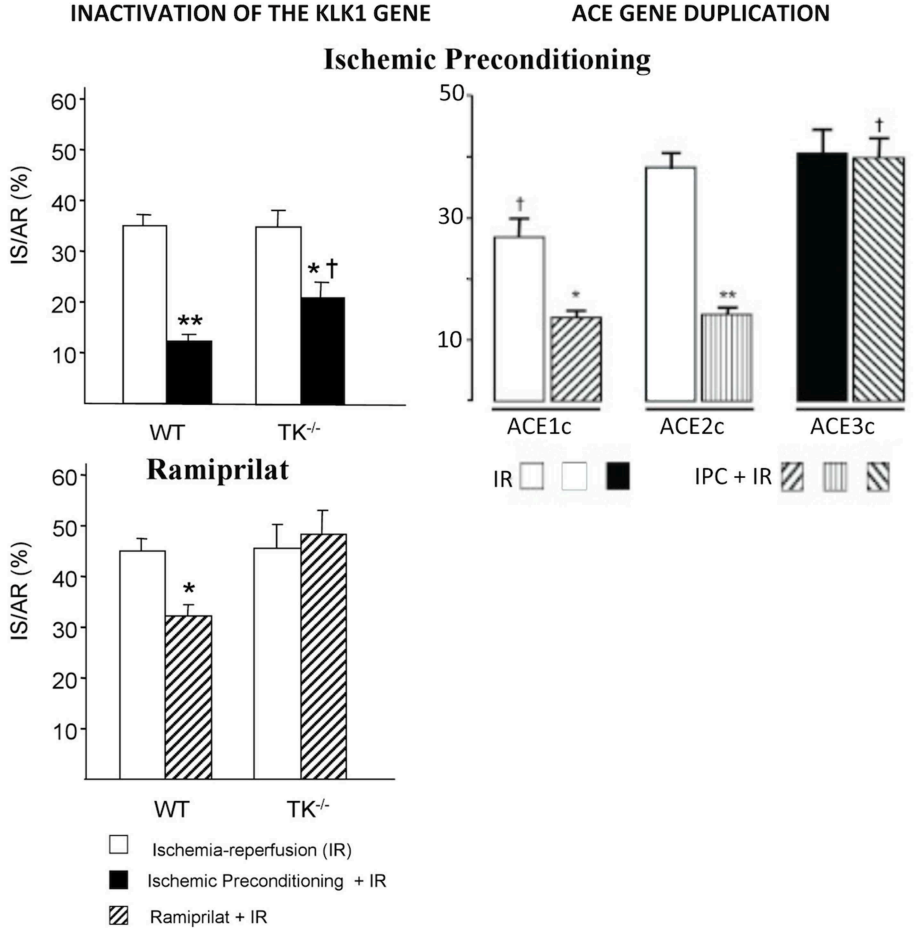


**FIGURE 4 |** Loss of the cardioprotective effect of ischemic pre-conditioning in mice with an inactivated *Klk1* gene or mice carrying a duplication of the *ACE* gene in cardiac ischemia-reperfusion. IS/AR: infarct size relative to area at risk. IR, ischemia-reperfusion; IPC, ischemic pre-conditioning followed by IR; WT, wild type; $TK^{-/-}$, K1 deficient mice; ACE1c, mice with a single ACE gene copy; ACE2c, mice with two ACE gene copies (wild type); ACE3c, mice with three ACE gene copies having roughly 140% ACE activity level of wild-type mice. Note also loss of cardioprotective effect of the ACE inhibitor Ramiprilat in K1 deficient mice. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to IR; $^{\dagger}p < 0.05$ compared to littermate WT or ACE2c (WT). Adapted from Griol-Charhbili et al. (55) and Messadi et al. (56).

evolve into renal insufficiency, thus carrying dual cardiac and renal risk. Interestingly, in the Akita mouse with early onset insulinopenic diabetes, deficiency in kinin B2 receptor induces not only renal damage but also a generalized, pro-senescent phenotype of organs (80).

Synthesis and secretion of endothelial ACE increases in diabetes. Duplication of the ACE gene in insulinopenic mice accelerates development of diabetic nephropathy [(78), **Figure 5**]. As discussed above for cardiac ischemia, induction of similar, deleterious renal phenotypes in diabetes by either deficiency in K1 or overexpression of the *ACE* gene is consistent with kinin bioavailability having a major role in renal protection against hyperglycemia (77, 78). However, genetic increase in ACE can also potentiate angiotensin II formation locally and, for less understood reasons, angiotensin II action (56, 81). Angiotensin II is involved together with kinin depletion in the effect of ACE gene duplication, at least in the heart (56).

## Hypertension

An antihypertensive role of K1 has been repeatedly suggested after K1 was discovered by its hypotensive property (82). This role seems to be mainly exerted in the setting of salt and aldosterone excess, where renal K1 synthesis is stimulated, and may not be, or entirely be, kinin-mediated (83). K1, like other tubular proteases, may regulate sodium transporter activity in the kidney (53, 83). Deficiency in K1 has no effect in renovascular hypertension (one clip, one kidney), a renin rather than volume (salt) dependent type of hypertension (21).

Taken together, mice studies document that the physiological actions of K1 have, overall, beneficial consequence for preservation of end-organ trophicity and function in the settings of acute or chronic ischemia, chronic hyperglycemia and mineralocorticoid, and salt excess. The role of K1 is kinin-mediated in ischemia and in diabetes. ACE activity has, on the opposite, deleterious end-organ effect in ischemia or diabetes, through both local angiotensin II formation and kinin depletion.

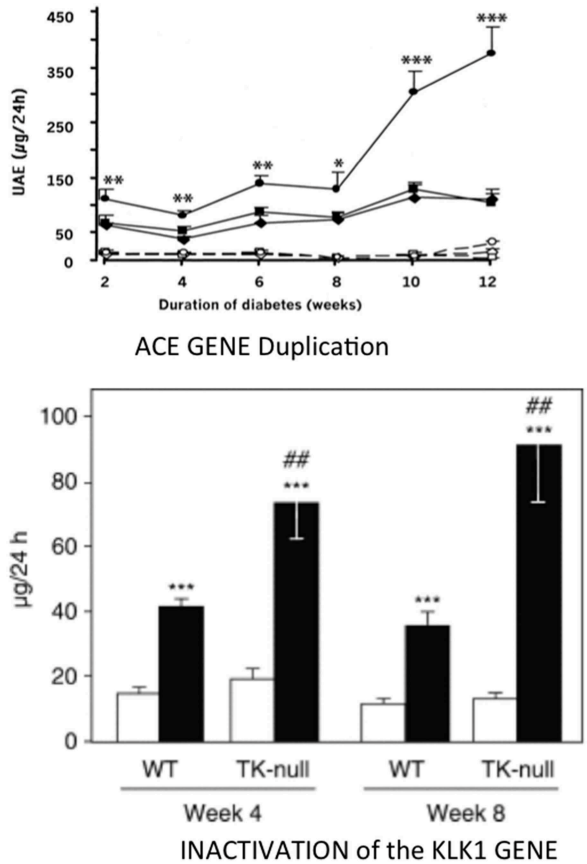


**FIGURE 5 |** Aggravating effect of inactivating the *KLK1* gene or duplicating the *ACE* gene on development of diabetic nephropathy in diabetic mice**.** Ordinate in both graphs: urinary albumin excretion. Upper graph, ACE gene titration; open symbols, non-diabetic mice; filled symbols, diabetic mice. Diamonds, one-copy mice; squares, two-copy mice (wild-type); circles, three-copy mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to diabetic two-copy (WT) or one-copy mice. Lower graph: K1 gene inactivation. open bars control, non-diabetic mice; filled bars diabetic mice. $^{***}p < 0.001$ compared to non-diabetic mice. $^{\#\#}p < 0.01$ compared to corresponding WT mice. Adapted from Bodin et al. (77) and Huang et al. (78).

## Clinical Studies, Genetic Variation in ACE/Kininase II Level, and Risk of Cardiovascular and Renal Diseases

Both ACE and K1 activity level are well-established quantitative genetic traits in man. This was first documented in familial transmission studies (24, 25, 84, 85). Genomic markers for these traits have been then identified. For K1, a frequent mutation in the active site (R53H, discussed above) is causally involved in the genetic variability of K1 activity (26). For ACE, an intronic insertion/deletion polymorphism is strongly associated with plasma and tissue ACE levels, although it is not clear whether this genomic variation is causally involved in the phenotype (through modulating mRNA stability and splicing) or is only a neutral marker in linkage disequilibrium with another, causal mutation (86–88). Despite extensive investigation of the ACE gene the putative causal mutation has however not been identified (5, 89).

In any case, the genetic variation in ACE level has been, quickly after its discovery, associated with susceptibility to and prognosis of cardiovascular and renal diseases. First observation was made for myocardial infarction in a landmark European multicenter study where the subjects homozygote for the ACE D allele, the allele which is associated with higher ACE levels, were found to be at increased risk (90, 91). The association has been reproduced in several other studies but not in all adequately powered studies, although it was consistently observed in the setting of diabetes (5, 92). ACE is probably a weak genetic risk factor for myocardial infarction in the general population, but its effect is potentiated by diabetes and reciprocally. Interestingly, mice carrying a duplication of the ACE gene and having a modest genetic increase in ACE level similar to that observed in humans homozygote for the D allele display reduced myocardial tolerance to cardiac ischemia, thus, documenting causality and mechanism behind the proposed clinical association (56) (**Figure 4**).

But, genetic variation in ACE level has been well-established as a risk factor for diabetic nephropathy, in type 1 diabetes. The association, originally found independently in the GENEDIAB study in Europe and at the Joslin clinic in the US, has been confirmed in the major cohort studies of type 1 diabetic patients, including the landmark DCTT/EDIC study [(92–100), **Figure 6**]. Hyperglycemic diabetic patients with genetically high ACE levels are at increased risk of developing nephropathy and the disease evolves more severely in these patients (92–100). Diabetic nephropathy is both a vascular and a renal disease and affected patients display, even at early stage of nephropathy clinically marked by elevated urinary albumin excretion and conserved renal function, increased incidence of cardiovascular events, especially myocardial infarction (101). Diabetic nephropathy was known to have a strong genetic determinism in type 1 diabetes (102). The ACE gene is the first recognized and so far best documented gene involved in the disease. Causality between genetically determined ACE level and renal involvement in diabetes was established by ACE gene titration (1 to 3 copies) in the mouse, as discussed above. Nephropathy develops faster in mice having three ACE gene copies and a moderate increase in ACE level, when the mice are rendered diabetic (78) (**Figure 5**).

In summary, through combined human genetic association studies and causality study in genetically modified mice ACE was established as a risk/prognosis factor for diabetic nephropathy in insulinopenic diabetes. As discussed above, both theoretical and experimental evidences suggest that reduced bioavailability of kinins is involved in the deleterious effect of ACE in diabetes. This is also supported by clinical physiology studies (103, 104).

On the other hand the defective K1 mutation was not found to be associated with cardiovascular and renal diseases so far, including in studies where ACE was associated. It can however be noted that because of the low allele frequency of the mutation only heterozygote subjects with partial deficiency in K1 activity were included in these studies.

## THERAPEUTIC DEVELOPMENT, SELECTIVE AGONISM OF KALLIKREIN-KININ

Based on the organ-protective action of K1 and kinins in ischemia and diabetes, a new therapeutic approach to

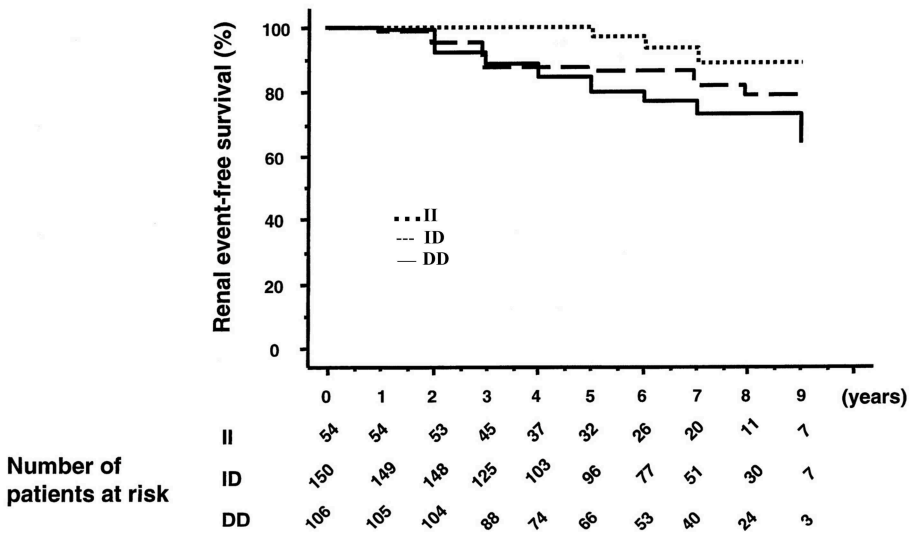


**FIGURE 6 |** Association of the ACE gene ID polymorphism with development of diabetic nephropathy in patients with type 1 diabetes. Graph shows cumulative incidence of renal events (progression from physiological to pathological microalbuminuria or from a given stage of nephropathy to a higher stage) according to ACE genotype. D allele is co-dominantly associated with higher ACE levels. Reproduced from Hadjadj et al. (97). This study documents the role of the genetic variation in ACE level in susceptibility to and progression of diabetic nephropathy. Same observation was made in other patient populations, before and after this study. See text and Marre et al. (93) through Hadjadj et al. (100).

cardiovascular and renal diseases has been proposed, selective agonism of the kallikrein-kinin system. The topic has been reviewed previously (37, 105).

Rationale for developing pharmacological agonists of kallikrein-kinin is further based on the well-documented role of kinins in the therapeutic effect of both ACE inhibitors and angiotensin II AT1 receptor blockers, as discussed above. But, as K1 activity level is low in the cardiovascular system, kinins are only produced at slow rate in the circulation and ACE inhibitors, or angiotensin II AT1 blockers can only potentiate endogenously formed kinins. Low K1 activity and slow kinin formation might limit the therapeutic efficacy of current kinin-potentiating drugs (15, 37, 105).

Kallikrein cannot be easily targeted for pharmacological activation [albeit Aliskiren, a clinically approved compound originally designed as a renin inhibitor has been reported to stimulate K1 synthesis in the failing rat heart as discussed above, Koid et al. (76)]. Kallikrein gene therapy and additive transgenesis with the human *klk1* gene have been attempted in animals, with report of beneficial effects in cardiac or peripheral ischemic and diabetic diseases (68, 106–110). However, kallikrein gene therapy is difficult evaluating experimentally, in part because of species specificity in K1 activity and it is unlikely to be ever developed in clinical practice (105, 111). Bradykinin and other natural kinins, such as des- $Arg^9$-bradykinin, the endogenous B1 receptor agonist, cannot be used pharmacologically as therapeutic agents as they are readily destroyed by peptidases, especially ACE or carboxypeptidase N, in the circulation (3).

Pseudo-peptide analogs of kinins have been synthesized. These compounds are potent kinin receptor agonists, selective for either the B1 or the B2 receptor and are resistant, by design, to peptidases action (112, 113). The agonists can be administered intravenously or chronically through osmotic micropumps (73, 114). The B2 agonist dose-dependently decreases blood pressure during acute administration but has no sustained hypotensive effect in chronic administration, probably as a consequence of counter-regulations. The B1 agonist has no effect on blood pressure (73, 114).

These prototypic agonists allowed establishing proof of concept for therapeutic efficacy of pharmacological kinin receptor activation in animals. They have also helped documenting the functions of the B1 and the B2 receptor in diseases because of their selectivity. Indeed, while the B2 receptor (B2R) is constitutively synthesized in the cardiovascular system and the kidney the B1 receptor (B1R) is considered as being only synthesized in pathological situations (115). Situations where B1R synthesis is induced include ischemia, chronic hyperglycemia, and also, interestingly, ACE inhibitor therapy (77, 115–118). Use of selective agonists in experimental diseases has further documented the physiological balance between the two receptor-subtypes and shown that the balance is strongly influenced by diabetes, especially in the heart.

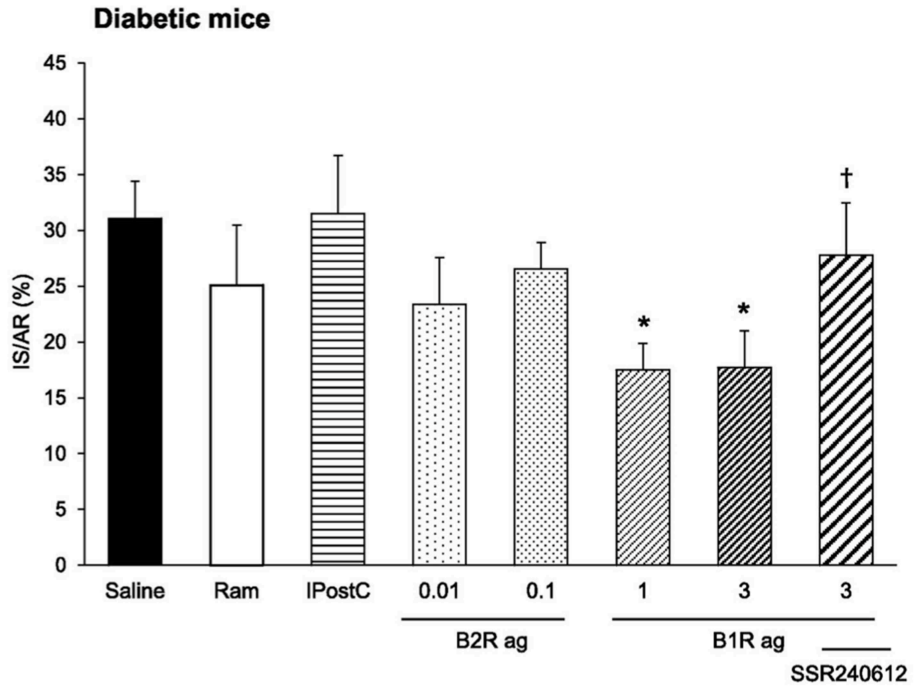


**FIGURE 7 |** Cardioprotective effect of a pharmacological kinin B1 receptor agonist in cardiac ischemia-reperfusion in diabetic mice. IS/AR, infarct size relative to area at risk; B1R ag, kinin B1 receptor agonist; B2R ag, kinin B2 receptor agonist; Ram, ramiprilat; IpostC, ischemic post-conditioning; SSR240612, kinin B1 receptor antagonist *$p < 005$ compared to saline, †$p < 0.05$ compared to B1R ag. Note resistance of the diabetic heart to cardioprotective treatments otherwise efficient in non-diabetic mice (Ram, B2R ag and IpostC). Reproduced from Potier et al. (114).

The kinin receptor agonists have been tested in experimental ischemic and/or diabetic diseases in mice. Cardiac ischemia-reperfusion is an experimental model for human ischemic heart disease. A B2R agonist administered at reperfusion largely reduces infarct size (by 47%). A B1R agonist has no effect. However, if the mice are rendered diabetic prior to inducing ischemia the B2R agonist loses its cardioprotective effect. But in these diabetic mice, the B1R agonist becomes efficient and largely reduces infarct size (by 44%). Cardioprotective signalization involves, for both receptors, phosphoinositide three kinase/Akt and extracellular signal-regulated kinase 1/2, GSK-3b inactivation and inhibition of mitochondrial pore opening [(114), **Figure 7**]. These observations suggest that B2R signaling is suppressed in the diabetic heart with compensatory induction of B1R, taking over cardioprotective signaling. Infarct size reducing effect of the B2R agonist is consistent with the role of the B2R in cardiac ischemia previously documented by using loss of function approaches. Interestingly, in this study, the B1R agonist was the only pharmacological agent tested displaying therapeutic effect in diabetic animals, as an ACE inhibitor, like the B2R agonist had no effect on infarct size (114) (**Figure 7**). Ischemic post-conditioning was also ineffective in diabetic animals. The mouse or rat diabetic heart is known to be resistant to established cardioprotective treatments. Interestingly, pharmacological B1R agonism is able to overcome this resistance (114).

Pharmacological kinin receptor agonism also displays therapeutic efficacy in chronic hindlimb ischemia in diabetic mice. Mechanisms involved here pertain to angiogenesis rather than tissue tolerance to ischemia. While, in non-diabetic mice femoral artery ligation does not induce sustained hindlimb ischemia and distal perfusion is recovered in a few days through neovascularization, in diabetic mice with defective neovascularization capacity the hindlimb remains ischemic. Treatment of diabetic mice with either a B1R or a B2R agonist for 2 weeks following artery ligation enhances neovascularization and restores distal blood flow. Monocyte/macrophage mobilization, VEGF synthesis, and mobilization of progenitor cells are involved in the angiogenic effect of the agonists post-ischemia (73).

But B1R or B2R activation in diseases may not always be beneficial. Indeed, in cerebral ischemia-reperfusion in mice B2R activation increases mortality, perhaps through peripheral hemodynamic effects. The B1R agonist has no effect in non-diabetic mice. But interestingly, in diabetic mice, B1R agonist given at reperfusion decreases brain infarct size and reduces neurological deficits, further documenting efficiency of B1R agonism in improving tolerance to ischemia of diabetic organs (116). In a study of skin wound healing, which can be an important clinical issue in diabetic patients, pharmacological B2R activation delayed healing in non-diabetic or diabetic mice, while B2R inhibition corrected the healing defect observed in diabetic mice. Activation of B1R had no effect on wound healing in either non-diabetic or diabetic mice, despite induction of B1R synthesis in the wounded diabetic skin (117).

Overall, mice studies document beneficial effects of B1 receptor agonism in ischemia in the setting of diabetes, consistently in the heart, brain, or hindlimb. B2R agonism has cardioprotective effect in cardiac ischemia and proangiogenic action in peripheral ischemia but deleterious effects in brain ischemia. Studies in diabetic nephropathy are still awaited. For retinopathy, role of kinins is controversial. Studies have suggested that some retinal vascular effects of B2R may be deleterious (119, 120). The issue might be clarified by gain of function studies with selective pharmacological agonists (37).

Results of experimental therapeutic studies should always be translated with caution to clinical situations, regarding both efficiency and tolerance of compounds tested. The present studies provide at least proof of concept for therapeutic action of selective pharmacological kinin receptor agonism. Kinin receptor agonists might have enhanced therapeutic efficacy compared to ACE inhibitors, especially in the diabetic heart. No unwanted effects, like hypotension, oedema, or abnormal psychomotor behavior suggestive of excessive pain suffering were observed during up to 2 weeks agonist treatment in the mice (73, 117). However, in the clinical setting, pharmacological B2R agonism might have serious unwanted side effects, including angioedema, pain or tumor development (37, 105, 121–123). This remains speculative. On the other hand, B1R agonism was consistently efficient in diabetic animals and had no detrimental or off-target effects in the experimental diseases studied so far. The B1R may not be involved in angioedema, contrary to the B2R. Indeed, selective blockade of the B2R, presumably resulting in enhanced kinin-induced B1R activation, is considered to improve outcome of angioedema crises. Clinical development of kinin receptor agonists, especially B1R, is worth considering.

## AUTHOR CONTRIBUTIONS

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

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