Plasma Kallikrein Mediates Angiotensin II Type 1 Receptor–Stimulated Retinal Vascular Permeability

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Abstract—Hypertension is a leading risk factor for the development and progression of diabetic retinopathy and contributes to a variety of other retinal diseases in the absence of diabetes mellitus. Inhibition of the renin-angiotensin system has been shown to provide beneficial effects against diabetic retinopathy, both in the absence and presence of hypertension, suggesting that angiotensin II (Ang II) and the Ang II type 1 receptor may contribute to retinal vascular dysfunction. We investigated the effects of the Ang II type 1 receptor antagonist candesartan on retinal vascular permeability (RVP) in normotensive rats with streptozotocin-induced diabetes mellitus and in rats with Ang II–induced hypertension. We showed that candesartan treatment decreased diabetes mellitus– and Ang II–stimulated RVP by 58% ($P<0.05$) and 79% ($P<0.05$), respectively, compared with untreated controls, suggesting that activation of the Ang II type 1 receptor contributes to blood-retinal barrier dysfunction. We found that plasma kallikrein levels are increased in the retina of rats with Ang II–stimulated hypertension and that intravitreal injection of either plasma kallikrein or bradykinin is sufficient to increase RVP. We showed that a novel small molecule inhibitor of plasma kallikrein, 1-benzyl-1H-pyrazole-4-carboxylic acid 4-carbamimidoyl-benzylamide, delivered systemically via a subcutaneous pump, decreased Ang II–stimulated RVP by 70% ($P<0.05$) and ameliorates Ang II–induced hypertension, measured from the carotid artery by telemetry, but did not reduce Ang II–induced retinal leukostasis. These findings demonstrate that activation of the Ang II type 1 receptor increases RVP and suggest that systemic plasma kallikrein inhibition may provide a new therapeutic approach for ameliorating blood-retinal barrier dysfunction induced by hypertension. **(*Hypertension*. 2009;53:175-181.)**

Key Words: kallikrein ■ retina ■ angiotensin II ■ diabetes ■ AT_1 receptor ■ hypertension

Diabetic macular edema (DME), which can occur at any stage of diabetic retinopathy, is the leading cause of visual impairment associated with both types 1 and 2 diabetes mellitus.[1] The development of DME is thought to be initiated by impaired retinal endothelial cell tight junction integrity and breakdown of the blood-retinal barrier, leading to increased retinal vascular permeability (RVP) and the accumulation of plasma proteins, lipids, and fluid in the neuroretina.[2] Increased RVP is among the earliest retinal changes induced by diabetes mellitus, and further increases in RVP occur in concordance with the severity of diabetic retinopathy.[3] Risk factors for DME include hyperglycemia, dyslipidemia, renal dysfunction, and hypertension.[4] Patients with hypertension are more likely to progress to DME,[5] and increasing diastolic blood pressure (BP) is associated with an increase in its incidence.[6] Moreover, it has been shown that tight BP control reduced the incidence of macular edema by 42% in people with type 2 diabetes mellitus.[7] Although the management of clinical risk factors can reduce the incidence of DME, effective treatments for this condition remain a major unmet clinical need.

The United Kingdom Prospective Diabetes Study demonstrated that BP reduction using either a β-blocker or angiotensin-converting enzyme (ACE) inhibitor in patients with both hypertension and diabetes mellitus can reduce the occurrence of advanced diabetic retinopathy.[7] Although this study and others suggest that high BP exerts adverse effects on the retina, the molecular mechanisms that mediate these effects on retinal vascular function remain poorly understood. A growing body of evidence suggests that renin-angiotensin system inhibition may provide beneficial effects on the retina even in the absence of hypertension. The EURODIAB Controlled Trial of Lisinopril in Insulin-Dependent Diabetes Mellitus Study group has shown that treatment of normotensive type 1 diabetic subjects with an ACE inhibitor reduced the progression of diabetic retinopathy; however, lower BP and hemoglobin A1c levels were also observed in the treated group.[8] The Appropriate Blood Pressure Control in Diabetes Trial examined the effects of intensive versus standard BP control in patients with type 2 diabetes mellitus and found that, even in normotensive patients, intensive BP control decreased the progression of retinopathy.[9] Furthermore, the Diabetic Retinopathy Candesartan Trials Study reported that the angiotensin II (Ang II) type 1 receptor (AT_1R) antagonist candesartan reduced the incidence of diabetic retinopathy in people with type 1 diabetes mellitus without hyperten-

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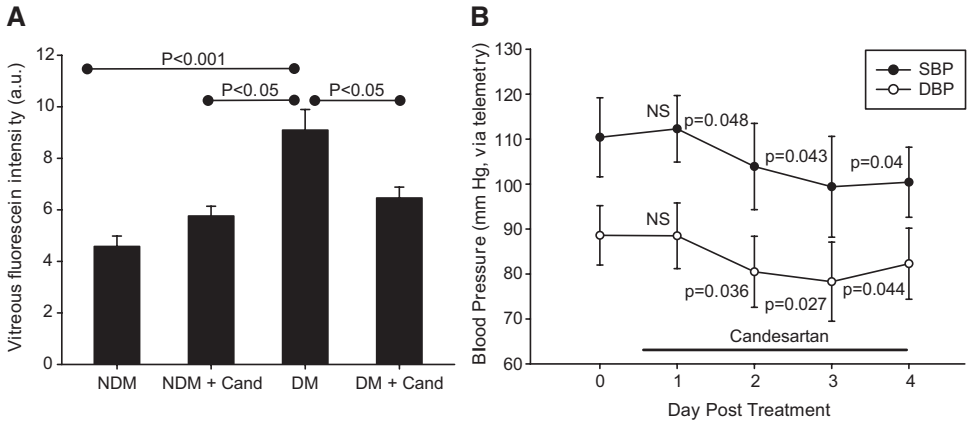

Figure 1. Effect of AT_1R blockade on RVP and BP in diabetic rats. A, NDM (n=8), NDM+Cand (nondiabetic+candesartan; n=5), DM (2-week diabetes mellitus; n=12), and DM+Cand (2-week diabetesmellitus+candesartan; n=11) rats. Bars represent means±SEMs, au. B, SBP and diastolic BP (DBP) measurements (means±SEMs) using telemetry in diabetic rats before and after treatment with candesartan. *P* values indicate comparisons with day 0.

sion. However, this study did not observe a beneficial effect on retinopathy progression.[10] Thus, further understanding of AT_1R-mediated actions on the retina could have relevance to diabetic retinopathy both in the presence or absence of hypertension.

We have investigated the effects of candesartan on RVP in normotensive rats with diabetes mellitus and in rats with Ang II–induced hypertension. In this report we also examined the role of the kallikrein-kinin system (KKS) in mediating the effect of Ang II on RVP using a novel and highly selective small molecule inhibitor of plasma kallikrein. These studies have revealed a role for plasma kallikrein in mediating Ang II–induced RVP and suggest a new approach to treat retinal vascular dysfunction in hypertension.

Methods

Animals

Diabetes mellitus was induced in 8-week-old male Sprague-Dawley rats via IP injection of 55 mg/kg of streptozotocin (Sigma-Aldrich) in 10 mmol/L of sodium citrate (pH 4.5), after overnight fast. After confirmation of hyperglycemia in streptozotocin-injected animals or immediately after the SC implantation of Ang II–loaded osmotic pumps, candesartan-cilexetil in pure powder form (AstraZeneca) was administered ab libitum in drinking water at a concentration of 10 μg/mL. Based on water consumption, this was equivalent to a dosage of 1.1, 2.0, and 4.0 mg/kg per day for saline-treated rats, rats infused with Ang II, and diabetic rats, respectively. Unless specified as being measured by telemetry, all of the BP measurements were obtained by tail-cuff plethysmography using a noninvasive BP/heart rate monitoring system (UR-5000, Ueda Electronic) in conscious animals. BP measurements by telemetry were performed using PA-C40 transmitters (Data Sciences International). Under anesthesia, a telemetric transmitter was fixed to the interscapular area, and the pressure sensing catheter was inserted via the external carotid into the common carotid with the tip ≈3 mm distal to the aortic junction. Rats were housed individually on a receiver pad, and systolic and diastolic pressures were monitored continuously and averaged over 15-second intervals every 15 minutes over a 4-hour period from 9 AM to 1 PM each day. Baseline readings (day 0) were obtained at 48 hours after catheter implantation. Before RVP measurements, all of the animals underwent catheterization with a polyvinyl catheter inserted into the left jugular vein as described previously.[11] All of the experiments were performed in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with approval from the animal care and use committee of the Joslin Diabetes Center.

Ang II, ASP-440, and HOE-140 Treatment

Treatments were achieved by the use of SC implantation of Alzet mini-osmotic pumps (Durect Corporation). Ang II (EMD Chemicals Inc) was delivered at 300 ng/kg per minute, and control rats received saline vehicle. 1-Benzyl-1H-pyrazole-4-carboxylic acid 4-carbamimidoyl-benzylamide (ASP-440) was delivered at 16 μg/kg per hour, with control pumps filled with vehicle (10% polyethylene glycol and 90% PBS). HOE-140 (Sigma-Aldrich) was infused at 1 μg/kg per hour, with control pumps filled with saline.

Retinal Vascular Permeability

Video fluorescein angiography was performed using a scanning laser ophthalmoscope (Rodenstock Instruments) as described previously.[11] Retinal angiograms and first-phase RVP were visualized by video fluorescein angiography immediately after an 80-μL bolus injection of fluorescein in anesthetized animals via left jugular vein catheter. RVP was quantified using vitreous fluorescein photometry, as detailed previously.[12] RVP was examined in rats at 2 weeks of diabetes mellitus with or without candesartan treatment, at 6-days post saline or Ang II infusion with or without candesartan treatment, at 3 days posttreatment with HOE-140 or ASP-440, or 40 minutes after intravitreal injections of either plasma kallikrein (EMD Chemicals Inc) or bradykinin 1-9 (Sigma-Aldrich), with control eyes receiving a 10-μL balanced salt solution (BSS).

Statistical Analysis

Statistical analysis was performed using a 1-way ANOVA or paired Student *t* test (SigmaStat, Systat Software). Values of $P<0.05$ were considered statistically significant. Details on the synthesis, purification, and characteristics of ASP-440, measurement of retinal leukostasis, and Western blot protocol are available in an online data supplement (please see http://hyper.aha.journals.org).

Results

Increased RVP in Diabetes Mellitus Is Attenuated by AT_1R Blockade

We characterized RVP in rats with 2 weeks of diabetes mellitus in the absence or presence of treatment with candesartan. RVP to fluorescein, measured by vitreous fluorescein photometry, was increased 97% from 4.6 arbitrary units (au) in nondiabetic (NDM) rats to 9.1 au in diabetic (DM) rats ($P<0.001$; Figure 1A). We showed that RVP in candesartan-treated NDM and DM rats were 5.76 au and 6.46 au, respectively. This study revealed that candesartan reduced RVP in DM rats by 58% (relative to NDM) and 79% (relative

Table. SBP Measured by Tail-Cuff Plethysmography, Weight, and Blood Glucose Data for NDM, NDM+Candesartan, DM, and DM+Candesartan Animals

Characteristic	NDM	NDM+Cand	DM	DM+Cand
SBP, mm Hg	136.3±3.0	124.7±2.9	141.7±3.3*†	122.0±3.2‡
Weight, g	365.6±4.7	344±10.6	249.6±9.0§†	248.8±6.0‡‖
BG, mg/dL	95.4±2.8	115.3±2.4	444.3±32.1§	459.3±25.2‡

Values represent means±SEMs. BG indicates blood glucose; Cand, candesartan; NDM, n=8; NDM+Cand, n=5; DM, n=12; DM+Cand, n=11 animals.

*P<0.05 DM vs DM+Cand; †P<0.05 DM vs NDM+Cand; ‡P<0.05 NDM vs DM+Cand; §P<0.05 NDM vs DM; ‖P<0.05 NDM+Cand vs DM+Cand.

to candesartan-treated NDM), both with P<0.05. Candesartan treatment also reduced systolic BP (SBP) measured by tail-cuff plethysmography in both NDM and DM groups at 2 weeks posttreatment (P<0.05; Table) and both SBP and diastolic BP in DM rats measured from the carotid artery using telemetry (P<0.05; Figure 1B). Similar decreases in SBP (110.9±6.8 to 96.5±10.2 mm Hg) and diastolic BP (91.4±7.6 to 67.2±9.9 mm Hg; means±SEM) measured by telemetry were observed in NDM rats pretreatment and posttreatment with candesartan for 3 days.

Chronic Ang II Infusion Causes Increased RVP

To further investigate the role of the AT$_1$R on RVP, we examined the effect of chronic Ang II infusion in control and candesartan-treated rats. We infused Sprague-Dawley rats with 300 ng/kg per minute of Ang II via SC pump for 1, 3, or 6 days. This study showed a 2.9-fold increase in RVP was present after 1 day of Ang II infusion, which was sustained at 3 and 6 days (Figure 2A). The effects of Ang II on SBP continued to increase during this time (saline versus Ang II: day 1: 130.4±2.3 versus 157.0±1.8 mm Hg; day 3: 130.6±4.9 versus 190.8±11.1 mm Hg [P<0.001]; day 6: 139±2.4 versus 209±11.4 mm Hg [P<0.001 saline versus Ang II; P<0.001 Ang II day 1 versus Ang II day 6]). First-phase venous RVP was increased in rats infused with Ang II for 6 days compared with saline-treated controls (Figure 2B). Treatment of Ang II–infused rats with candesartan for 6 days prevented the increases in SBP (saline: 134.7±1.7 mm Hg; Ang II: 188.3±6.2 mm Hg; Ang II+candesartan: 126.2±1.7 mm Hg; mean±SEM; P<0.05 saline versus Ang II and Ang II versus candesartan) and decreased RVP by 81% compared with the untreated Ang II group (Figure 2C). Vitreous fluorescein levels were 4.5±0.3, 10.4±1.2, and 5.6±0.4 au for saline, Ang II alone, and Ang II+candesartan groups, respectively (Figure 2C). Candesartan treatment of rats receiving saline vehicle infusion was 5.1±0.8 au (n=3; data not shown), which was similar to untreated rats with a saline pump.

Protein Quantification of Components of the KKS

Because the renin-angiotensin system and KKS interact at multiple levels[13] and both plasma kallikrein and the bradykinin B2 receptor (B2-R) have been implicated in vasogenic edema,[14,15] we investigated the effects of Ang II infusion on components of the KKS in the retina. This study showed that B2-R and B1-receptor levels in the retina were similar in rats receiving Ang II and saline infusion (Figure 3), consistent with studies that have examined B1-receptor and B2-R mRNA levels in the retina of control and diabetic mice.[16] In contrast, we detected an increase in a 25-kDa heavy chain fragment of plasma kallikrein (P<0.05) and a trend for increased cleaved high-molecular-weight kininogen heavy chain (cleaved HK; P=0.064) in Ang II–infused animals (Figure 3), suggesting that active plasma kallikrein and its product, cleaved HK, are present in the retina with their levels increased in Ang II–infused animals.

Intravitreal Injections of Plasma Kallikrein and Bradykinin Increase RVP

To directly investigate the effect of intraocular plasma kallikrein on RVP, we injected 20 ng of activated purified plasma kallikrein into the vitreous chamber of rats and

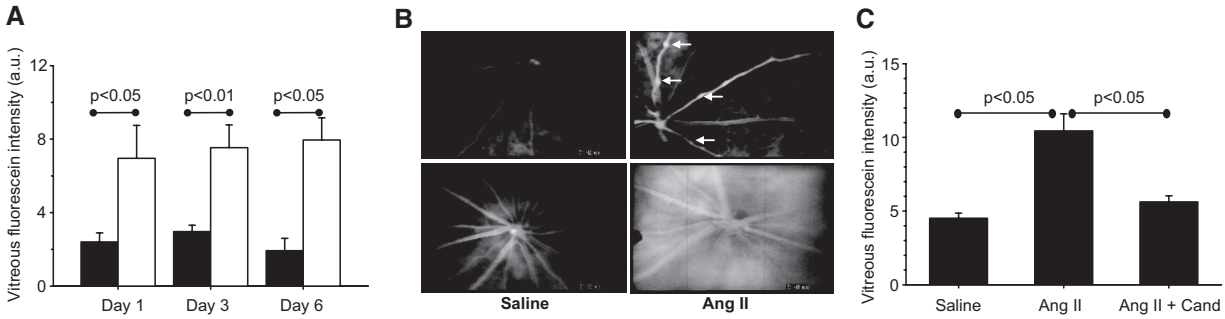

Figure 2. Effects of chronic Ang II infusion and AT$_1$R blockade on RVP. A, The effect of systemic Ang II infusion on RVP measured by vitreous fluorescein intensity at 1, 3, and 6 days posttreatment in Ang II (□, n=4) and saline control animals (■, n=5). B, Representative fluorescein angiograms from 6-day saline- and Ang II–treated rats. Top, Arterial phase, with arrows indicating arterial caliber abnormalities. Bottom, Diffuse vascular leakage of the Ang II animal in the venous phase compared with the saline control. C, Effect on RVP after 6 days of saline (n=19), Ang II (n=16), or Ang II+candesartan (n=10) treatment. Bars represent means±SEMs, au.

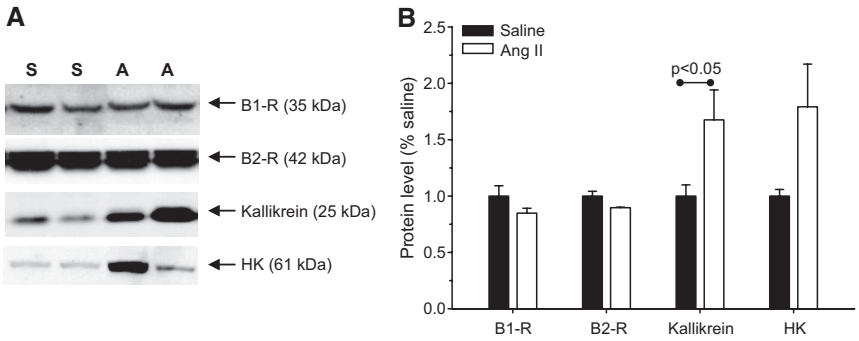

Figure 3. Western blot analysis of KKS components in retinal lysates. A, Representative Western blots showing immunoreactivity of proteins in saline (S)- and Ang II (A)-treated animals. B1-R indicates bradykinin B1-receptor. B, Quantification of Western blot results in saline (n=4 B-1R, n=3 B-2R, n=11 kallikrein, n=12 HK) and Ang II (n=4 B-1R, n=4 B-2R, n=12 kallikrein, n=11 HK) animals infused for 6 days. Bars represent means±SEMs.

measured vitreous fluorescein levels 40 minutes after injection, using the contralateral eye as a control (10-μL injection of BSS). We showed that activated plasma kallikrein increased RVP by 60% compared with BSS-injected eyes (Figure 4A). Similarly, we demonstrated that intravitreal injection of 10 μmol/L of bradykinin (2 μmol/L of final vitreous concentration) increased RVP by 86% ($P<0.01$) compared with BSS-injected control eyes (Figure 4B) and that this bradykinin-induced increase in RVP was decreased in rats infused with HOE-140.

Plasma Kallikrein Inhibitor (ASP-440) Attenuates Retinal Vascular Leakage in Rats With Ang II–Induced Hypertension

We investigated the effects of a novel plasma kallikrein catalytic activity inhibitor, ASP-440 (Figure S1 and Table S1), on Ang II–induced RVP by the infusion of rats with 300 ng/kg per minute of Ang II with or without coinfusion with 16 μg/kg per hour of ASP-440 via separate SC pumps for 3 days. Analysis of RVP by video fluorescein angiography showed that 3-day infusion of Ang II alone increased retinal vascular fluorescein leakage and vessel caliber abnormalities and that coadministration with ASP-440 attenuated these changes (Figure 4C). Quantification by vitreous fluorescein photometry (VFP) showed that treatment with ASP-440 decreased RVP in Ang II–infused rats by 70% ($P<0.001$) compared with saline-treated control animals (Figure 4D). Treatment of control animals with ASP-440 did not alter RVP (Figure 4D). To investigate the possibility that the B2-R may contribute to the increased RVP caused by Ang II, we treated animals with both 300 ng/kg per minute of Ang II and 1 μg/kg per hour of HOE-140. We showed that Ang II–stimulated RVP was reduced in HOE-140–treated rats by 64% compared with animals receiving Ang II alone (Figure 4E). Neither ASP-440

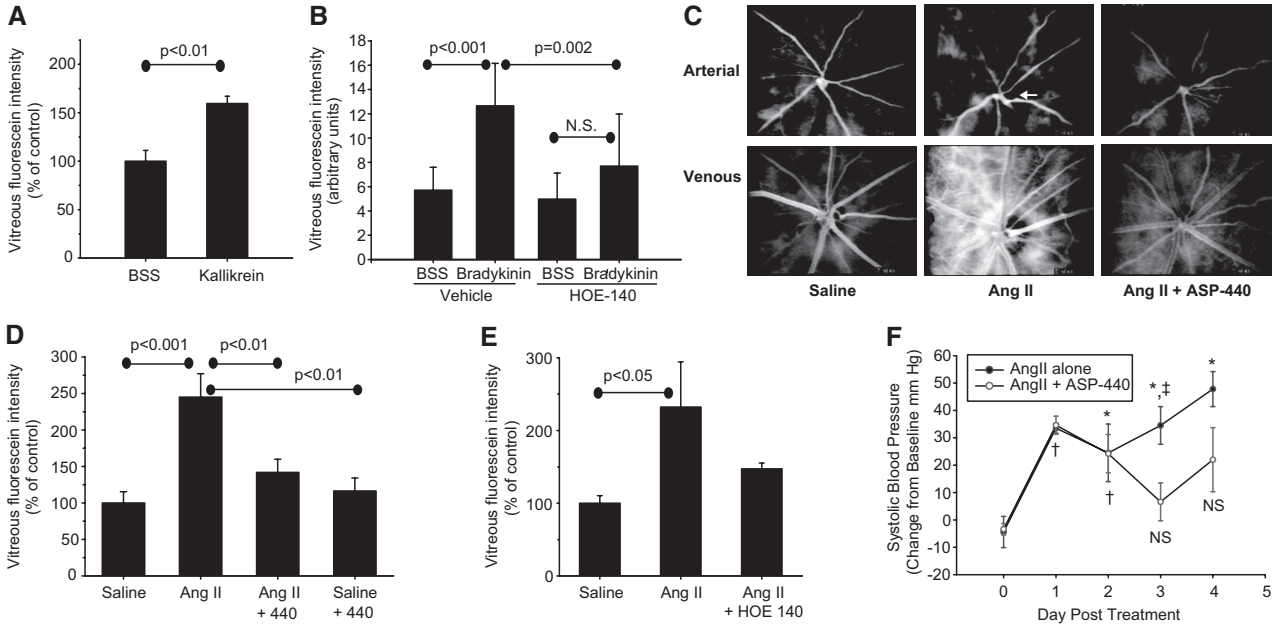

Figure 4. Role of the kallikrein-kinin pathway in RVP and SBP. A, Effect of intravitreal injection of BSS (n=4) or purified activated plasma kallikrein (n=5) on RVP measured by VFP. B, Effect of intravitreal injection of BSS (n=5) or bradykinin (n=8) on RVP in the absence or presence of systemic infusion with HOE-140. C, Fluorescein angiograms from representative animals treated for 3 days with saline, Ang II. and Ang II+ASP-440 in the arterial (top) and venous (bottom) phases. Arrow indicates area of arterial caliber abnormalities in the Ang II–treated animal. D, Effect of 3-day treatment with ASP-440 on Ang II–mediated RVP increases. Saline (n=5), Ang II (n=7), Ang II+ASP-440 (n=7). E, Effect of 3-day treatment with HOE-140 on Ang II–mediated RVP increases. Saline (n=8), Ang II (n=7), Ang II+HOE-140 (n=8). Bars represent means±SEMs. F, Time course of SBP measured using telemetry in rats infused with Ang II in the absence or presence of ASP-440. * and † indicate $P<0.05$ for day 0 vs Ang II alone (n=4) and Ang II+ASP-440 (n=7), respectively. ‡$P<0.05$ for Ang II alone vs Ang II+ASP-440.

(saline+vehicle: 130.2±5.1 mm Hg; Ang II+vehicle: 184.4±8.5 mm Hg; Ang II+ASP-440: 195.8±9.0 mm Hg; $P<0.05$ saline versus Ang II, $P<0.05$ saline versus Ang II+ASP-440) nor HOE-140 treatment (saline: 137.3±3.3 mm Hg; Ang II+vehicle: 183.2±8.5 mm Hg; Ang II+HOE-140: 175.9±9.2 mm Hg; $P<0.05$ saline versus Ang II, $P<0.05$ saline versus Ang II+HOE-140) reduced SBP in Ang II animals when BP was measured via tail cuff at day 3, although animals treated with ASP-440 and Ang II showed a trend for higher SBP compared with Ang II alone using this method. Comparable effects of Ang II on RVP and SBP were observed with Long-Evans rats, and the coinfusion of Ang II+ASP-440 reduced RVP but did not reduce SBP (measured via tail cuff plethysmography) or retinal leukostasis (Figure S2).

Further analysis of BP in Sprague-Dawley rats using telemetry showed an increase in SBP and diastolic BP in the carotid artery at day 1 through day 4 in rats receiving Ang II alone compared with baseline measurements (Figure 4F). Rats receiving Ang II+ASP-440 had an increase in SBP recorded by telemetry that was similar to the Ang II–alone group at days 1 and 2 followed by partial normalization at day 3 ($P<0.05$ for day 3 Ang II alone versus Ang II+ASP-440; Figure 4F). SBP in the Ang II–alone and Ang II+ASP-440 groups, collected from 9:00 PM to 1:00 AM on the end of day 3, was increased by 30.6 and 15.5 mm Hg, respectively, compared with baseline measurements at this time period, changes similar to that observed during the day. SBP of Ang II+ASP-440–infused rats at day 4 measured using telemetry was 122.8±19.7 mm Hg, whereas SBP of these rats measured using tail-cuff plethysmography was 176.4±6.7 mm Hg, which is comparable to 195.8±9.0 mm Hg measured SBP via tail cuff in the RVP group (Figure 4D), confirming the discordance between tail-cuff plethysmography and telemetry SBP measurements in Ang II+ASP-440–treated rats.

Discussion

This report shows that systemic AT$_1$R antagonism and plasma kallikrein inhibition ameliorate retinal vascular hyperpermeability. We show the following: (1) the AT$_1$R antagonist candesartan decreased RVP in diabetic rats; (2) chronic infusion of Ang II in rats increased RVP, and this response was blocked by candesartan; and (3) systemic treatment of rats with a new small molecule inhibitor of plasma kallikrein (ASP-440) reduced Ang II–induced RVP and SBP measured from the carotid artery using telemetry but did not reduce SBP measured via tail-cuff plethysmography or retinal leukocyte adhesion. These findings demonstrate a novel role for plasma kallikrein in mediating the increase in RVP and the sustained elevation of SBP in rats with Ang II–induced hypertension.

Ang II signaling is mediated by 2 receptor subtypes, the AT$_1$R and Ang II type 2 receptor, both of which are expressed in the retina.[17] Although AT$_1$R and Ang II type 2 receptor interact at multiple levels with vascular permeability factors and their receptors, including the bradykinin system (reviewed in References 13,18), the role of Ang II receptors in vascular permeability, particularly in the retina, have not been elucidated. Because the loss of blood-retinal barrier function is thought to play a critical role in the etiology of diabetic retinopathy and macula edema, further understanding of the effects of AT$_1$R antagonists on RVP may have important relevance to their use in the treatment of this disease.

AT$_1$R antagonism has been reported to exert dual vascular effects by reducing AT$_1$R signaling while enhancing Ang II type 2 receptor–mediated responses.[19] Indeed, the improvement in endothelium-dependent vasorelaxation by AT$_1$R blockade has been attributed, in part, to increased bradykinin/B2-R–mediated endothelial NO synthase activation and its generation of cGMP.[20–22] AT$_1$R antagonism has been reported to increase plasma bradykinin levels in people with hypertension[23] and to increase tissue kininogenase activity in mice.[20] However, bradykinin and NO also potently increase vascular hyperpermeability and edema[24,25] and have been implicated in increasing RVP induced by retinal hemorrhage.[12] Therefore, the augmentation of the Ang II type 2 receptor–bradykinin pathway by AT$_1$R blockade could potentially lead to an increase in vascular leakage. We demonstrated that candesartan reduced RVP in normotensive DM rats and in rats with Ang II–induced hypertension, suggesting that, in these experimental models, the AT$_1$R contributes to the increase in RVP. These results extend previous studies, which have shown that ACE inhibition decreased blood-retinal–barrier permeability in hypertensive type 1 diabetic patients and in normotensive diabetic rats.[26,27]

Although a large body of literature suggests that ACE inhibition potentiates endogenous bradykinin, thought to be beneficial in kidney and heart,[28,29] the present study and previous reports suggest that bradykinin has deleterious effects on the retina.[30,31] Indeed, the beneficial effects of ACE inhibition on the retina have also been observed with AT$_1$R antagonism (reviewed in Reference 18), suggesting that inhibition of Ang II production is the critical aspect of ACE inhibition that is important in diabetic retinopathy. Previous reports have shown that Ang II and angiotensinogen are elevated in the vitreous fluid from patients with proliferative diabetic retinopathy compared with NDM subjects[12,32] and that serum concentrations of ACE and renin correlate with the severity of diabetic retinopathy (reviewed in Reference 18), suggesting the involvement of both the local intraocular and systemic renin-angiotensin systems in diabetic retinopathy. Studies that examine the effect of AT$_1$R antagonism on retinal abnormalities in diabetes mellitus, including the present study, have shown a drug-induced decrease in systemic BP,[11] with results from the Diabetic Retinopathy Candesartan Trials-Prevent 1 Study showing a decrease in SBP of 2.6 mm Hg in type 1 diabetic patients with retinopathy when treated with candesartan.[10] Because the efficacious dose of candesartan used in our studies decreased both RVP and SBP in our diabetic and Ang II–infusion rat models, our findings do not characterize the potential individual contributions for the BP-dependent and -independent effects of the AT$_1$R on RVP.

We demonstrated that continuous systemic treatment of rats with the plasma kallikrein inhibitor ASP-440 ameliorated Ang II–induced RVP at both 3 days and 7 days (data not shown). To our knowledge, this is the first report of a small molecule plasma kallikrein–selective inhibitor with in vivo efficacy against vascular hyperpermeability. Because candesartan blocked Ang II–induced RVP, our results suggest that

plasma kallikrein mediates the increase in RVP induced by AT_1R-stimulated hypertension. Moreover, we found that Ang II infusion increased inflammatory cell responses (as measured by retinal leukostasis) that were not normalized by treatment with ASP-440, although RVP was attenuated by ASP-440 in these same animals (Figure S2). This suggests that plasma kallikrein inhibition, although not directly inhibiting leukostasis, does reduce RVP that occurs coincident with inflammatory cell recruitment. Using telemetry, we also observed that ASP-440 decreased SBP in rats exposed to Ang II–induced hypertension; however this effect of ASP-440 was not observed until days 3 and 4 of infusion. The 2-day delay before the appearance of this BP-lowering effect of ASP-440 could suggest that plasma kallikrein contributes to the maintenance of the sustained BP increase in this model. Interestingly, we did not detect a decrease in Ang II–induced SBP by ASP-440 using tail-cuff plethysmography at 3 days postinfusion. Similar discordance between these 2 measures of BP have been reported in the literature,[33] attributed to an exaggerated stress response to the conditions of tail-cuff measurement in the presence of Ang II.

The main effector peptide of the KKS, bradykinin, is generated directly from HK by plasma kallikrein. Bradykinin is also generated from low molecular weight kininogen by the action of an aminopeptidase on the kallidin (Lys-bradykinin) peptide generated by tissue kallikrein. Intravascular delivery of bradykinin in rats has been shown to decrease BP, a response attenuated by HOE-140.[34] Bradykinin-induced vasorelaxation has been attributed to the activation of bradykinin receptors on the endothelium.[19–21] In contrast, bradykinin, via it effects on the central nervous system, has been shown to elevate BP via the B2-R and to induce a cardiac sympathetic response.[34–37] The hypotensive effect of ASP-440 observed here by telemetry suggests that plasma kallikrein-mediated bradykinin formation in the Ang II model leads to a net increase in BP. The absence of an apparent BP-lowering response, using tail-cuff plethysmography, to ASP-440 in Ang II–treated rats shows that plasma kallikrein can exert effects on systemic BP that are not detected by the indirect measurement of BP via tail cuff in heated and restrained animals.

We showed that intravitreal injection of either purified, activated plasma kallikrein or bradykinin increases RVP. These findings are consistent with the well-established effects of the KKS in increasing vascular permeability.[24,38] We found that both a B2-R antagonist and a plasma kallikrein–selective inhibitor reduced Ang II–stimulated RVP. The use of a small molecule plasma kallikrein inhibitor introduces a new strategy to modulate KKS action in vivo. Because this inhibitor is >1000-fold more selective in inhibiting plasma kallikrein compared with tissue kallikrein, this compound provides a new pharmacological opportunity to inhibit bradykinin production mediated by plasma kallikrein. This would suppress pathological effects mediated by increased bradykinin generated by this enzyme rather than pan-antagonizing all actions of bradykinin at the B2-R level, as done by HOE-140. Although the action of increased bradykinin signaling can be deleterious for the eye,[31] bradykinin, likely generated by tissue kallikrein, has been shown to have beneficial effects in several other tissues,[28] particularly the heart.[29] Although we did observe attenuation of increased RVP by treatment with HOE-140, systemic use of B2-R blockers for the treatment of vascular disease may be limited by their ability to indiscriminately inhibit tissue-specific beneficial effects of bradykinin. Because most of the beneficial effects of the KKS have been attributed to tissue kallikrein, in contrast to plasma kallikrein–mediated bradykinin production associated with angioedema,[39] treatment of RVP with a specific plasma kallikrein inhibitor would not be expected to have significant adverse effects. Moreover, the rare occurrence of complete deficiencies in plasma kallikrein in humans is usually only detected in later decades by observation of abnormal prolongation of activated partial thromboplastin clotting time but otherwise with no known clinically significant morbidities.[40]

Although our data show that plasma kallikrein inhibition by ASP-440 decreases retinal hypervasopermeability stimulated by Ang II–induced hypertension, and previous work from our laboratory and others has implicated the KKS in diabetic retinopathy,[12,30] further studies will be needed to characterize the effects of plasma kallikrein inhibitors on other models of hypertensive and diabetic retinopathy. Our results showing efficacy of ASP-440 via systemic delivery suggest that plasma kallikrein inhibition may provide a new therapeutic approach to preserve blood-retinal barrier function and decrease BP.

Perspectives

Recent results from the Diabetic Retinopathy Candesartan Trials Study have found that treatment with an AT_1R blocker has beneficial effects on the incidence of diabetic retinopathy in people with type 1 diabetes mellitus.[10] Our study has found that plasma kallikrein is involved in the biochemical pathways downstream of AT_1R activation and, given that plasma kallikrein, HK, and Factor XII are found in the vitreous of people with diabetic retinopathy,[12] provides additional support for an alternative pathway by which AT_1R blockade may contribute to amelioration of diabetic retinopathy in the absence of ischemia or BP changes. Given the growing prevalence of patients with uncontrolled and resistant hypertension,[41] particularly in people with diabetes mellitus, direct inhibition of plasma kallikrein may provide a new therapeutic approach for the treatment of retinal disorders involving vascular hyperpermeability.

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Disclosures

S.S. and T.J.C., employees of ActiveSite Pharmaceuticals, Inc, are coinventors of the PCT patent application WO/2008/016883. The remaining authors report no conflicts.

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Plasma Kallikrein Mediates Angiotensin II Type 1 Receptor–Stimulated Retinal Vascular Permeability

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Online supplement

Plasma kallikrein mediates angiotensin AT_1 receptor stimulated retinal vascular permeability

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Supplemental methods

Synthesis and purification of ASP-440

ASP-440 was synthesized and purified using methods described elsewhere (S. Sinha and T.J. Chilcote, ActiveSite Pharmaceuticals, "Inhibitors of plasma kallikrein", PCT Publication WO/2008/016883, 2008) and obtained from ActiveSite Pharmaceuticals, Inc. (**Figure S1**). Inhibitory constants towards plasma kallikrein and other serine proteases were calculated from the extent of inhibition of enzyme activity towards the cleavage of peptide para-nitroanilide substrates obtained with 0.1 – 10 μM ASP-440 in 50 mM HEPES, pH 7.5, 0.01% Triton X-100. Purified enzymes were obtained from either Enzyme Research Laboratories (Gary, IN) or Haematologic Technologies (Essex Junction, VT). Synthetic substrates were obtained from Bachem (Torrance, CA) and American Diagnostica (Stamford, CT).

Measurement of retinal leukostasis

Measurement of retinal leukostasis was performed in Long Evans rats using acridine orange (1 mg/ml) and a scanning laser ophthalmoscope (Rodenstock Instruments) as described previously [1]. Briefly, animals were treated for three days via osmotic pump with either Saline, Saline + ASP-440 (16 μg/kg/hr), AngII (300 ng/kg/min) or AngII + ASP-440. Animals were anesthetized with an intramuscular injection of ketamine/xylazine (50 mg/kg, 10 mg/kg), and acridine orange was prepared in sterile saline at 1 mg/ml. Eyes were dilated with 1% mydriacyl, and 4 mg/kg of acridine orange was injected through jugular vein catheter at a rate of 1.5 ml/min via a syringe pump. Twenty minutes following infusion, the number of static leukocytes was observed in the fundus via scanning laser ophthalmoscopy, and images recorded onto videotapes for subsequent analysis.

Western Blot protocol

Anaesthetized animals were perfused with 30 ml of saline and the neural retina removed from the posterior eyecup and stored in ice-cold Tris-lysis buffer (150mM NaCl, 10mM Tris-HCl pH 7.4, 10% glycerol, 1% Triton-X 100, 1mM PMSF, 0.1mg/ml aprotinin). Retina were homogenized and soluble lysates (10 μg protein) were separated by 4-20% gradient SDS-PAGE under reducing conditions and immunoblotted using primary antibodies against the bradykinin receptor 1 (B1-R), the heavy chain of high-molecular weight kininogen (HK) (Santa Cruz Biotechnology, Santa Cruz, CA), the bradykinin receptor 2 (B2-R), and the monoclonal antibody 13G11 directed to plasma kallikrein (Abcam, Cambridge, MA). Results were visualized by enhanced chemiluminescence (Cell Signaling, Danvers, MA). Quantification was achieved using ImageQuant 5.1 (Molecular Dynamics Inc.).

Supplemental results

Characteristics of plasma kallikrein inhibitor ASP-440

ASP-440 is a potent inhibitor of plasma kallikrein's catalytic activity toward D-Pro-Phe-Arg-pNA (S-2302) (**Table S1**), with a K_i = 0.1 μM, and exhibits little inhibitory activity towards the other human serine proteases of the intrinsic and extrinsic blood coagulation pathways. It exhibits selectivity for plasma kallikrein of 160-fold from plasmin, and more than 850-fold from the other proteases tested. Importantly, it has >1000-fold selectivity from tissue kallikrein, and would therefore not be expected to have an inhibitory effect on tissue kallikrein activity *in vivo*.

Chronic Angiotensin II infusion causes increased retinal leukostasis that is not attenuated by treatment with the plasma kallikrein inhibitor ASP-440

In order to investigate whether the increased vascular permeability observed in AngII-treated rats was associated with an increase in inflammatory cells, we evaluated retinal leukostasis and retinal vascular permeability in Long-Evans rats treated for three days with saline, saline + 440, AngII, and AngII + 440. Using acridine orange, we found retinal leukostasis was increased by 1.9-fold in animals treated with AngII compared to saline controls, and increased 2.2-fold in animals treated with Ang II + ASP-440 **(Figure S2A).** In contrast, retinal vascular permeability in the same animals, measured by vitreous fluorophotometry, was increased 2.9-fold in AngII-treated animals, and attenuated in AngII animals treated with ASP-440 ($P<0.01$, **Figure S2B**), in agreement with our data on Sprague-Dawley animals **(Figure 4D)**. This data suggests that ASP-440, although not directly inhibiting leukostasis, does inhibit retinal vascular permeability increases that may be caused by inflammatory changes. No change was seen in retinal leukostasis or retinal vascular permeability in saline animals treated with ASP-440 compared to saline controls **(Figure S2)**. The systolic blood pressure was 152$\pm$3.3, 161$\pm$15, 200$\pm$8.7, and 211+8.6 mmHg (mean$\pm$SEM) for rats infused with saline+vehicle, saline+ASP-440, AngII+vehicle, and AngII+ASP440, respectively.

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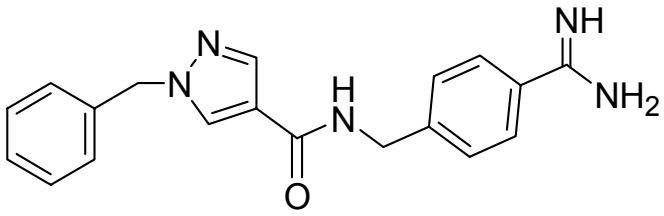
Table S1. Characteristics of kallikrein inhibitor ASP-440. Percentage inhibition at 1 or 10 μM were calculated from the experimentally determined K_i values for each enzyme.

Enzyme	Ki (μM)	% Inhibition at 1 μM	% Inhibition at 10 μM
Plasma kallikrein	0.1	91	99
Factor XIIa	>100	0	1
Factor XIa	120	0	7
Factor IXa	>>100	0	0
Tissue Factor – Factor VIIa	160	0	6
Factor Xa	85	0	11
Thrombin	110	1	9
Plasmin	16	6	38
Tissue Kallikrein	>100	0	1

Figure S1. Structure of the plasma kallikrein inhibitor 1-benzyl-1H-pyrazole-4-carboxylic acid 4-carbamimidoyl-benzylamide (ASP-440).



1-benzyl-1H-pyrazole-4-carboxylic acid 4-carbamimidoyl-benzylamide (ASP-440)

Figure S2. (A) Chronic angiotensin II infusion causes increased retinal leukostasis that is not attenuated by treatment with the plasma kallikrein inhibitor ASP-440. Asterisks indicate statistical significance (PBS vs Angiotensin II $p<0.001$, PBS vs Angiotensin II + ASP-440 $p<0.001$). **(B)** Chronic angiotensin II infusion causes increased retinal vascular permeability in Long-Evans rats that is attenuated by treatment with ASP-440. * $p<0.001$ Angiotensin II vs PBS ** $p<0.01$ Angiotensin II vs Angiotensin II + ASP-440. PBS (phosphate buffered saline). The number of animals in each treatment group is indicated under each panel below each bar.