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Plasma Kallikrein Contributes to Intracerebral Hemorrhage and Hypertension in Stroke-Prone Spontaneously Hypertensive Rats

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Abstract

Plasma kallikrein (PKa) has been implicated in contributing to hemorrhage following thrombolytic therapy; however, its role in spontaneous intracerebral hemorrhage is currently not available. This report investigates the role of PKa on hemorrhage and hypertension in stroke-prone spontaneously hypertensive rats (SHRSP). SHRSP were fed with a high salt–containing stroke-prone diet to increase blood pressure and induce intracerebral hemorrhage. The roles of PKa on blood pressure, hemorrhage, and survival in SHRSP were examined in rats receiving a PKa inhibitor or plasma prekallikrein antisense oligonucleotide (PK ASO) compared with rats receiving control ASO. Effects on PKa on the proteolytic cleavage of atrial natriuretic peptide (ANP) were analyzed by tandem mass spectrometry. We show that SHRSP on high-salt diet displayed increased levels of PKa activity compared with control rats. Cleaved kininogen was increased in plasma during stroke compared to SHRSP without stroke. Systemic administration of a PKa inhibitor or PK ASO to SHRSP reduced hemorrhage and blood pressure, and improved neurological function and survival compared with SHRSP receiving control ASO. Since PKa inhibition was associated with reduced blood pressure in hypertensive rats, we investigated the effects of PKa on the cleavage of ANP. Incubation of PKa with ANP resulted in the generation fragment $ANP_{5\text{-}28}$, which displayed reduced effects on blood pressure lowering compared with full length ANP. PKa contributes to increased blood pressure in SHRSP, which is associated with hemorrhage and reduced survival. PKa-mediated cleavage of ANP reduces its blood pressure lowering effects and thereby may contribute to hypertension-induced intracerebral hemorrhage.

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Conflict of Interest

J. G., L. D. P., T. U., and F. S. declare no competing financial interests. A. S. R. and A. R. M. are employees of Ionis Pharmaceuticals Inc., Carlsbad, CA. A. C. C. and E. P. F. are employees of KalVista Pharmaceuticals Inc., Cambridge, MA.

Ethical Approval

Experiments were performed in accordance with the guidelines of National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the Joslin Diabetes Center. This article does not contain any studies with human participants performed by any of the authors.

Keywords

Plasma kallikrein; Stroke; Intracerebral hemorrhage; Hypertension; Atrial natriuretic peptide

Introduction

Hypertension is a leading risk factor for spontaneous intracerebral hemorrhage and is associated with hematoma expansion, neurological deterioration, and poor outcome [1,2,3]. Elevated blood pressure is present in approximately 80% of patients with acute intracerebral hemorrhage on admission, even without a history of previous hypertension [3,4,5]. Patients with transient or sustained high blood pressure are a risk factor for recurrent hemorrhage within the first 24 h after the onset of intracerebral hemorrhage [6]. In a systematic review, systolic blood pressure elevations of 10 mmHg were associated with near 40% increased risk of hemorrhagic stroke [6,7]. Treatments to reduce the risk of hemorrhage in hypertensive patients are needed to minimize the high morbidity and mortality associated with this disease.

Plasma kallikrein (PKa) is a serine protease activated from plasma prekallikrein (PK) by factor XIIa (FXIIa). PKa exerts effects on vascular function, inflammation, the intrinsic coagulation cascade, and complement [8,9]. While deficiencies in FXII and PK do not significantly affect bleeding associated with peripheral vascular injury, recent reports have demonstrated that these proteins can promote hemorrhage in the retina and brain [10]. Intravitreal injection of PKa induces retinal microvascular hemorrhages, and intracerebral injection of PKa increases intracranial bleeding [9,11]. PK deficiency and PKa inhibition ameliorates hematoma formation following cerebrovascular injury in diabetic animals [9]. Moreover, both PK and FXII deficiency are protective against hemorrhage caused by tissue plasminogen activator–mediated thrombolytic therapy in mice with thrombotic middle cerebral artery occlusion [12]. These findings have implicated the role of PKa in hemorrhage following acute cerebrovascular injury; however, the role of PKa in spontaneous intracerebral hemorrhage is not yet available.

Stroke-prone spontaneously hypertensive rats (SHRSP) are widely used as model of hypertension-induced spontaneous intracerebral hemorrhage. While PK deficiency is not associated with blood pressure abnormalities in normotensive animal models [13], we have previously reported that PKa inhibition reduced blood pressure in rats with angiotensin II (Ang II)–induced hypertension [14]. Jaffa and colleagues [15] have found that augmented PK levels in type I diabetic patients are associated with increased blood pressure and macroalbuminuria. The mechanisms that contribute to the blood pressure–lowering effects through PKa inhibition in hypertensive animals are unknown. In this report, we investigate the effects of PKa on blood pressure and spontaneous intracerebral hemorrhage in SHRSP.

Methods

Experimental Animals

Male Wistar Kyoto (WKY) rats and stroke-prone spontaneously hypertensive rats (SHRSP; Strain Code: 324; SHR/A3NCrl) were purchased from Charles River Laboratories (Wilmington, MA). Plasma prekallikrein gene-deficient mice ($Klkb1^{-/-}$) were described previously [9]. Littermate $Klkb1^{+/+}$ were used as wild type (WT). Rodents were maintained under specific pathogen-free conditions in a temperature-controlled room with a 12-h light/dark cycle. Experiments were performed in accordance with the guidelines of National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the animal care and use committee of the Joslin Diabetes Center. All experimental assessments were performed by investigators who were blind to the experimental groups.

Treatment Protocols

SHRSP were fed a Japanese-style stroke prone diet (#39–288, Zeigler Bros., Gardners, PA) along with 1% salt in water beginning at 8 weeks of age and maintained until the end of the study. At 14 weeks of age, the rats were randomized to 3 groups, including control antisense oligonucleotide (Control ASO; Ionis Pharmaceuticals, Inc.), treatment with plasma prekallikrein antisense oligonucleotide (PK ASO; Ionis Pharmaceuticals, Inc.) [13,16], and treatment with PKa inhibitor (1-benzyl-1H-pyrazole-4-carboxylic acid 4-carbamimidoyl-benzylamide) (BPCCB), which has been characterized previously [14]. The Rat PK ASO was 20 nucleotides connected by phosphorothioate internucleotide linkages. The 5 nucleotides at both the 5′ and 3′ ends have 2′-O-methoxyethyl modifications, which confer an increased affinity to the target mRNA. The central portion is composed of 10 deoxynucleotides, enabling RNase H1 to recognize and cleave the target mRNAs in the ASO:RNA duplex. ASOs were designed and tested by Ionis Pharmaceuticals to avoid any off-target effects [13,16]. PK ASO or control ASO (40 mg/kg s.c) was injected twice per week for 3 weeks. Subcutaneous injections twice per week for 3 weeks of 40 mg/kg of PK ASO showed to reduce the PK mRNA levels to more than 80% [16]. BPCCB (12 μg/kg/h) or vehicle (10% polyethylene glycol 400 in saline) was administered using subcutaneously implanted osmotic pump for 4 weeks (0.25 μl/h; Model 2004, Alzet Corp, CA) as described previously [9,14]. Angiotensin II (Ang II)–induced hypertension was induced in SHRSP rats and WT and $Klkb1^{-/-}$ mice as follows. Ang II (EMD Millipore, Billerica, MA) in sterile phosphate-buffered saline (PBS) was infused using an Alzet mini-osmotic pump for 4 weeks (0.11 μl/h; Model 1004, Alzet Corp, CA) as described previously [9]. Rats were infused as 800 ng/kg and mice were infused as 1,000 ng/kg. Equimolar doses of ANP, $ANP_{5\text{-}28}$, or $ANP_{5\text{-}19}$ (mini-ANP) (Bachem, Torrance, CA) were administered by continuous intravenous infusion for 45 min for rat (33 pmol/kg per minute), as well as a bolus of infusion for mice (1485 pmol/kg).

Onset of Stroke

Stroke was assessed by the appearance of neurological symptoms and physiological changes as described previously [17]. The neurological findings were scored on a 5-point scale: score 0—indicated no neurologic deficit; score 1—(failure to extend left forepaw fully) indicated a mild focal neurologic deficit; score 2—(circling to the left) indicated a moderate focal

neurological deficit; score 3—(falling to the left) indicated a severe focal deficit; score 4—rats could not walk spontaneously and had a depressed level of consciousness. Each rat was carefully monitored every day for 6 weeks for 1 or more signs occurred in SHRSP, they were regarded as stroke sign-positive. Plasma samples were collected from SHRSP upon developing a neurological sign scored > 3, or at the end of study if the rat did not develop apparent neurological abnormalities.

Kallikrein-Like Activity

Citrated rat plasma was diluted 1:100 in 100 mmol/L Tris, 0.5 mmol/L EDTA, and 0.1% BSA (pH 8.0) at 25 °C in a microtube. Kallikrein-like activity was determined by measurement of the changes in fluorescence at 410 nm due to hydrolysis of 100 mmol/L D-Pro-Phe-Arg-7-Amino-4-Trifluoromethylcoumarin (MP Biomedicals, Santa Ana, CA) for 5 min using a Synergy MX microplate reader (Biotek, Winooski, VT) and expressed as fluorescence intensity.

Blood Pressure Monitoring

Blood pressure was measured in conscious animals by tail-cuff plethysmography (UR-5000, Ueda Electronics, Tokyo, JP) as previously described [14]. Rats were maintained at 37 °C on a heated platform and systolic artery blood pressure (SBP) and diastolic blood pressure (DBP) values were obtained from the average of 10 measurements. Blood pressure was measured on rats and mice for 5 consecutive days as acclimation before recording measurement final data.

Quantitative RT-PCR

Total RNAs were isolated from liver rat tissues using Trizol reagent (Invitrogen, Carlsbad, CA) and were used to synthesize the first-strand cDNAs by using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, CA). cDNA samples were amplified by using iTaq Fast SYBR Green Supermix with ROX Kit and analyzed on an ABI PRISM 7000 sequence detection system (Applied Biosystems). Amplification was performed by using the following primers: rat Klkb1, 5′-ACCAACCTCAGTGCCATATTC-3′ (forward) and 5′-AGGAAACTGTAGCGAACAAGG-3′ (reverse); rat Gadph 5′-TCCAGTATGACTCTACCCACG-3′ (forward) and 5′-CACGACATACTCAGCACCAG-3′ (reverse).

Western Blot

Proteins were isolated from the whole cortex rat tissue and plasma using T-PER tissue protein extraction reagent (Thermo Scientific). The protein concentration was determined by using the Protein Assay Dye Reagent Concentrate (Bio-Rad). Thirty micrograms of protein from each sample was analyzed by 4–20% SDS–polyacrylamide gel electrophoresis and immune blotted using primary antibodies against PK (1:1000, R&D system), HK (1:1000, Santa Cruz), ZO-1 (1:1000), Occludin (1:1000), Claudin-5 (1:1000), GFAP (1:1000), and CD11b (1:1000, Thermo Fisher).

Mass Spectrometry

ANP (50 μmol/L) was cleaved with 100 nmol/L purified PKa (#420,307, Millipore, Billerica, MA) in 50 μl PBS buffer containing 50 μmol/L zinc sulfate. After incubation at 37 °C, 0.2 μmol/L BPCCB was used to terminate the reaction. ANP and its proteolytic peptides fragments were analyzed by selective reaction monitoring by nanospray liquid chromatography-tandem mass spectrometry (LC–MS/MS) using a LTQ linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA) as described previously [18].

Statistical Analyses

Data were presented as mean ± s.e.m. GraphPad Prism 7 software was used for statistical analyses. Single parameters measured in groups of rats were analyzed using a one-way ANOVA followed by Tukey's test. Time series of systolic blood pressure were analyzed by repeated measures one-way ANOVA. Data from two groups were compared by unpaired Student's t test. For stroke onset and survival curves, a Kaplan–Meier curve was obtained, and the comparison of groups was performed using the Log-Rank test. $P < 0.05$ was considered statistically significant.

Results

Stroke-Prone Spontaneously Hypertensive Rats Display Increased Plasma Kallikrein Activity

PKa activity was measured in plasma from SHRSP after 6 and 12 weeks of high-salt diet and control WKY rats. PKa activity in plasma was increased by 29% in SHRSP plasma compared with WKY rat plasma ($P < 0.01$, Fig. 1a). PK protein in plasma and PK mRNA in liver were also increased in SHRSP compared with WKY rats (Fig. 1b, c). Furthermore, PKa activity in plasma from SHRSP during stroke was increased during symptomatic stroke onset and compared with plasma collected from SHRSP at 6 weeks on the high-salt diet ($P < 0.001$, Fig. 1d) and age-matched SHRSP without neurologically apparent stroke symptoms. Exogenous addition of the PKa inhibitor (BPCCB) to plasma from SHRSP with signs of stroke decreased PKa by 85% ($P < 0.001$, Fig. 1e) in a dose-dependent manner, confirming that substrate hydrolysis was mediated, in large part, by PKa. Cleaved high molecular weight kininogen (cHK) in plasma was approximately twofold greater in SHRSP during stroke compared with SHRSP without stroke symptoms ($P < 0.001$; Fig. 1f).

Effect of Plasma Kallikrein Blockade on Neurological Function and Survival Rate

To explore the effects of blocking PK on ICH outcomes in SHRSP, we first measured PK protein levels in plasma of SHRSP treated with PK ASO and PKa inhibitor (BPCCB) (Fig. 2a). Administration of PK ASO to SHRSP decreased PK protein levels in plasma to < 5% of PK levels detected in rats receiving control ASO ($P < 0.001$; Fig. 1g). We did not see changes in PK protein levels in plasma between rats treated with BPCCB and vehicle (Fig. 1h). The effects of PKa inhibition on neurologic function were evaluated using neurological severity score. The neurological severity score in the BPCCB and PK ASO groups was decreased when compared with that in the control ASO group (Fig. 2b). During the treatment period, all the rats in the PK ASO group survived until the termination of the

study at 12 weeks ($P < 0.001$ vs. control ASO; Fig. 2c). The BPCCB group also improved the survival rate of SHRSP compared with the control group ($P < 0.01$; Fig. 2c). Rats that did not die during the stroke induction protocol were euthanized after 12 weeks.

Effect of Plasma Kallikrein Inhibition and Plasma Prekallikrein Knockdown on Intracerebral Hemorrhage in SHRSP

The effects of PKa blockade on intracerebral hemorrhage in SHRSP were investigated in rats administered with PK ASO or BPCCB. Ten of the SHRSP receiving control ASO ($n = 11$) displayed hemorrhage during the administration of the high-salt and stroke-prone diet (Fig. 3a, b), which are consistent with the reported phenotype for untreated SHRSP. Intracerebral hemorrhage was confirmed in images of both the superior brain surface and coronal sections. Hemorrhage was not detected in brains from SHRSP treated with the PKa inhibitor BPCCB ($n = 5$) or SHRSP injected with PK ASO ($n = 8$) (Fig. 3a, b).

Effect of Plasma Kallikrein Inhibition on Tight Junction and Glial Activation in SHRSP

Hypertension has been associated with changes in tight junction linked to damage in cerebral endothelial cells, consequently promoting blood–brain barrier (BBB) leakage. To explore whether the PKa blockage in SHRSP had any impact on tight junction proteins, we performed western blots in cerebral cortex tissue of all groups to evaluate the levels of ZO-1, occludin, and claudin-5. As shown in Fig. 3c, SHRSP receiving control ASO decreased occludin ($P < 0.05$) and claudin-5 ($P < 0.01$) protein levels in brain cortex compared with WKY rats. PK ASO significantly prevented the reduction of occludin ($P < 0.05$) and claudin-5 ($P < 0.01$) compared with SHRSP receiving control ASO (Fig. 3c). The BPCCB treatment group also prevented the reduction of occludin ($P < 0.05$) and claudin-5 ($P < 0.01$) compared with control ASO (Fig. 3c). We did not see changes in ZO-1 between SHRSP and WKY rats. Complex interactions between glial cells and BBB-endothelial cells are essential to the barrier function and its maintenance. To investigate whether PKa blockage could protect from glial activation in SHRSP, we performed western blot for markers of astrocyte (GFAP) and microglia (CD11b) activation. We found that SHRSP receiving control ASO induced high levels of GFAP ($P < 0.01$) and CD11b ($P < 0.01$) protein expression compared with WKY. The increased GFAP and CD11b levels in cerebral cortex of SHRSP suggest increased activation of astrocyte and microglia. PK ASO significantly ameliorated the increases in GFAP ($P < 0.05$) and CD11b ($P < 0.05$) compared with control ASO (Fig. 3d). BPCCB treatment group also ameliorated the increases in GFAP ($P < 0.05$) and CD11b ($P < 0.05$) compared with control ASO (Fig. 3d).

Plasma Kallikrein Knockdown Decreases Blood Pressure

The systolic blood pressure and diastolic systolic blood pressure from each group of rats were measured at 6 weeks after initiation of the high-salt diet (baseline), and 2 and 4 weeks after the treatment with either PK ASO or BPCCB and compared with control ASO ($P < 0.001$, $P < 0.05$, respectively; Fig. 4a). Furthermore, PK ASO–treated SHRSP showed a lower systolic blood pressure early after 2 weeks (week 8) of treatment compared with control ASO group ($P < 0.01$). BPCCB-treated SHRSP did not show changes in systolic blood pressure when compared with control ASO after 2 weeks of treatment. We found a significant decrease in diastolic blood pressure on SHRSP after 4 weeks (week 10) of

treatment with PK ASO when compared with control ASO-treated SHRSP ($P < 0.05$; Fig. 4b). BPCCB-treated SHRSP did not show changes in diastolic blood pressure during the treatment period.

PKa-Mediated Cleavage of Atrial Natriuretic Peptide In Vitro

Previous studies indicated that SHRSP have elevated concentrations of ANP in plasma and heart tissue [19]. ANP is a cardiac hormone that lowers blood pressure, regulates electrolyte homeostasis [20], and has been implicated for hypertension in humans [21]. Chronic treatment with ANP in SHRSP results in decreased systolic blood pressure and increased sodium and water excretion [22]. Previous studies have implicated PKa in the inactivation of ANP [23,24]. The amino acid sequence of ANP includes basic residues that are potential sites for PKa cleavage (Fig. 5a). To determine whether ANP is a direct substrate for PKa, we incubated purified PKa with purified ANP and utilized mass spectrometry to measure intact ANP and the appearance of ANP fragments. Full-length ANP was detected as an ion at m/z 766.5 (+) and incubation with PKa reduced the appearance of ANP. Following incubation with PKa, we detected a fragment of ANP with m/z 851.1(+3) consistent with c-terminal cleavage at Arg4 and the generation of $ANP_{5\text{-}28}$ (Fig. 5b). The ion $ANP_{5\text{-}28}$ is absent in pure ANP with PBS as shown in Fig. 5d. The abundance of $ANP_{5\text{-}28}$ increased following incubation of ANP with PKa in a dose- and time-dependent manner (Fig. 5c, d). We quantified the cleavage of ANP by PKa up to 40 min ($P < 0.001$ vs 0 min; Fig. 5e).

Effect of ANP and $ANP_{5\text{-}28}$ on Blood Pressure

To further investigate the potential effect of the fragment ion $ANP_{5\text{-}28}$, we compared the effects of ANP and $ANP_{5\text{-}28}$ on blood pressure lowering in SHRSP. Infusion of 33 pmol/kg/min full-length ANP in SHRSP decreased systolic blood pressure by 40 mmHg at 30 min ($P < 0.001$) compared with baseline (Fig. 6a). In contrast, infusion of the fragment $ANP_{5\text{-}28}$ at 33 pmol/kg/min did not change systolic blood pressure in SHRSP.

Enhanced Actions of ANP by PKa Inhibition in Angiotensin II–Induced Hypertension

To investigate whether PKa could modulate ANP function in a hypertensive rat model, we induced hypertension in SHRSP with Ang II (800 ng/kg/min) continuously delivered for 4 weeks by osmotic pumps. Hypertensive SHRSP were randomized and treated with BPCCB (10 μg/kg/h in osmotic pumps for 4 weeks), PK ASO (0.5 mg/kg s.c. for 14 days), and control ASO. Rats were infused with ANP (33 pmol/kg/min) for 45 min and the effects on blood pressure were monitored. We found that systolic blood pressure in control rats infused with ANP decreased by 40 mmHg at 30 min ($P < 0.001$ vs baseline, Fig. 6b). Interestingly, we found that the blood pressure–lowering effect of ANP was larger in both BPCCB (− 120 mmHg, $P < 0.001$) and PK ASO (− 110 mmHg, $P < 0.05$) groups compared with control ASO (− 40 mmHg) rats receiving ANP at 30 min (Fig. 6b). To further characterize the role of PKa on ANP function, we measured blood pressure in Ang II–induced hypertensive $Klkb1^{-/-}$ mice (27 mmHg, $P < 0.001$) when compared with WT (10 mmHg) at 5 min (Fig. 6c). Next, we determine whether $ANP_{5\text{-}19}$ amide (mini-ANP), which conserves high activity [25], plays a role on blood pressure into WT and PK deficiency mice. Infusion of mini-ANP into Ang II–induced hypertension mice reduced systolic blood pressure in both $Klkb1^{-/-}$ and

WT mice (Fig. 6d). Mini-ANP cleavage was not observed following incubation with PKa in vitro (data not shown).

Discussion

The present study demonstrated that PKa inhibition and PK deficiency reduced the incidence of intracerebral hemorrhage and improved neurological function and survival in SHRSP. These beneficial effects of PKa blockade were associated with a reduction in blood pressure. We show that PKa cleaves ANP resulting in an $ANP_{5\text{-}28}$ fragment with markedly reduced effects on blood pressure lowering. These results suggest that PKa activation in SHRSP contribute to the development of spontaneous intracerebral hemorrhage and hypertension.

SHRSP fed a high-salt diet for 12 weeks develop chronic hypertension and stroke. It has been demonstrated that prolonged severe hypertension (> 225 mmHg) in rats was sufficient to cause cerebral injury independent of a genetic deficiency [26]. Chronic hypertension in high-salt SHRSP induces vascular changes, such as smooth muscle cell disarray, reduced contractibility, structural remodeling, and vascular permeability [27]. The structural and functional alterations seen in high-salt SHRSP could represent regions of weakness in the arterial architecture and develop foci of increased vascular permeability [28]. In many cases, these lesions were predictive of subsequent intracerebral hemorrhage both spatially and temporally [29]. Interestingly, SHRSP receiving antihypertensive treatment (i.e., losartan, amlodipine) and a low-salt diet showed delayed intracerebral hemorrhage development and prolonged longevity [30,31]. Thus, the fact that stroke is less common during stable hypertension suggests that modified vasculature provides at least partial protection from hemorrhage and appears to be hypertension-related [32]. We have found that treatment of SHRSP with PKa inhibitor or PK knockdown reduced systolic blood pressure and improved survival when compared with vehicle group. It is well accepted that treatments leading to reduction of blood pressure levels counteract the appearance of spontaneous death in SHRSP [33]. It may be possible that PKa inhibitor produce vascular protective effects in SHRSP by lowering blood pressure levels below 200 mmHg. We previously demonstrated that PKa inhibition decreases Ang II–stimulated retinal vascular permeability and ameliorates Ang II–induced hypertension [14]. In clinical trials, antihypertensive therapy has been associated with reductions in stroke incidence, with an average 41% reduction in stroke risk with systolic blood pressure reductions of 10 mmHg [34]. Although anti-hypertensive drugs angiotensin-converting enzyme (ACE) inhibitors and angiotensin type-1 receptor (AT1R) antagonists block the same system by different mechanisms of actions, both drugs increase plasma bradykinin and may contribute to the therapeutic effects of the these drugs [35,36]. Since PKa drives multiple proteolytic reaction cascades and is responsible for the formation of bradykinin, we provide an alternative pathway by which PKa blockage can decreases high blood pressure. Given the growing prevalence of patients with uncontrolled and resistant hypertension [37], direct inhibition of PKa may provide a new therapeutic approach for the treatment of hemorrhage involving high blood pressure.

Pathological changes, such as fibrinoid necrosis, observed in the cerebral arteries of SHRSP and of lacunar infarction are thought to be triggered by plasma components leaked through damaged arterioles [38]. Braun and colleagues demonstrated that an early

microvascular dysfunction was associated with a local restricted thrombus formation, diapedesis of erythrocytes into the parenchyma and node like constrictions of arterioles leading to blood–brain barrier damage [39]. When arterial injury occurs, the activated endothelial cells and subendothelial material such as collagen may serve as surfaces for the assembly and activation of the contact coagulation system, leading to FXIa-driven thrombin generation and subsequent platelet activation. PKa can activate FXII, the origin of the intrinsic coagulation cascade, and PK deficiency has been shown to ameliorate thrombus formation in models of vessel wall injury [13]. Moreover, PKa inhibition caused reduction in inflammation and thrombus formation after stroke without intracerebral bleeding risk [40]. In this study, SHRSP showed higher levels of cHK in the plasma followed by increased PKa activation in stroke onset. By blocking PKa, we may reduce contact coagulation system activation and decrease thrombus formation. Degradation of extracellular matrix could disrupt microvascular function and thereby increase hemorrhage [11,41]. We have found multiple sites of hemorrhage in control ASO-treated SHRSP, and PKa inhibition or PK knockdown prevented the development of hemorrhagic stroke. We previously have demonstrated that PKa can interfere with collagen-induced platelet activation leading to intracerebral hematoma expansion in rodent models [9]. These reports have revealed that PKa has collagen-binding and collagenase-like activities that might facilitate hemorrhage under certain pathological conditions.

Cerebrovascular permeability induced by chronic hypertension may enable components of the KKS to leak into the subendothelial space and gain contact with the basement membrane. Previous work had found cerebral endothelial structural changes in SHRSP as early as 5 weeks of age, predating hypertension [42]. Lee and colleagues have found that high-salt SHRSP have increased vascular permeability up to 2 weeks before intracerebral hemorrhage, suggesting that hypertensive hemorrhage is preceded by focal vasculopathy detectable by Gadolinium leak [43]. The integrity of the BBB dependents in part on the formation and maintenance of tight junction proteins complexes between endothelial cells that limit and control the passage of cells and molecules [44]. We found that SHRSP expressed lower levels of occludin and claudin-5, and PKa inhibition or PK knockdown prevented the reduction of these proteins. Rajani and colleagues found a decreased in the number of blood vessels expressing claudin-5, the key tight junction protein of the BBB, indicative of an altered BBB in SHRSP [45]. Decreased expression of tight junctions proteins indicates dysfunctional endothelial cells and leads to decreased barrier integrity and microbleeds [44]. Abnormal cerebrovascular permeability may cause greater vulnerability to stroke. Supporting this idea, several studies pointed out leakiness of BBB in SHRSP as well as in white-matter lesions in humans [46,47,48]. Moreover, we found that hypertension in SHRSP causes activation of microglia and astrocyte cells. However, PK knockdown or PKa inhibition prevented the microglia and astrocyte activation. Microglia are the resident immune cells in the brain and are essential in immune response, but are also an important component of the neurovascular unit and therefore are involved in BBB disruption [49,50]. Previous studies have demonstrated the presence of microglia activation in the brains of SHRSP even before stroke onset [51]. Our result is consistent with previous finding on increased expression of microglia and astrocyte observed in SHRSP [50, 52]. These changes in glial cells may reflect an increase in neuro-inflammation in tandem with

increased vascular inflammation. Therefore, the protective effects of PKa blockage against stroke in SHRSP seem to be at least partially mediated by the improvement of endothelial dysfunctional and glial activation.

Plasma concentrations of ANP are increased in SHRSP compared with WKY rats [53]. ANP is produced mainly in the cardiac atria and released into the circulation in response to volume expansion and increased atrial distension [19]. Transgenic animal studies have shown that mice completely lacking ANP gene were hypertensive with blood pressure elevated by 23 mmHg compared with controls [54]. However, over expression of the ANP gene lowers systemic blood pressure [55] and attenuates hypoxia-induced pulmonary hypertension [56]. These studies demonstrate that ANP modulates the blood pressure response to dietary salt, and that impaired ANP production would be associated with development of hypertension. Interestingly, a 5-day low-dose ANP infusion in patients with essential hypertension lowered blood pressure and this effect persisted for 3 days after the infusion [57]. We showed that ANP infusion in Ang II–induced hypertension in SHRSP decreases blood pressure. Remarkably, blocking PKa with either BPCCB or PK ASO resulted in sustained systolic blood pressure reduction in Ang II–induced hypertension in SHRSP. These changes were also demonstrated in Ang II–induced hypertension in Klkb1$^{-/-}$ mice when compared with WT mice after infusion of ANP. These findings are supported by the fact that PKa cleaves ANP in vitro. These data suggested that PKa promotes ANP degradation and PKa inhibition can prolong and enhance the effects of ANP on blood pressure lowering in hypertensive rodents.

To further clarify whether cleaved ANP has effect on systolic blood pressure, administration of the major fragment $ANP_{5\text{-}28}$ in Ang II–induced SHRSP did not promote changes on systolic blood pressure. Moreover, infusion of mini-ANP, a fragment of active ANP without a site for PKa cleavage, into Ang II-induced hypertensive mice displayed similar effects on systolic blood pressure in Klkb1$^{-/-}$ and WT mice. Our findings suggest that hypertension might be the result of a deficiency in biologically active natriuretic peptides. A reduction of ANP secretion could result in Na^+ retention and salt-sensitive hypertension. This possibility is supported by studies showing that a disruption of the proANP gene in mice causes salt-sensitive hypertension [54]. Our data suggest that ANP cleavage by PKa induce a diminished systemic biologic action in the vasculature.

Conclusions

The present study suggests that inhibition of PKa can improve neurological function outcome in stroke-prone spontaneously hypertensive rats. The beneficial effect may be due to attenuation of blood pressure and consequently suppression of intracerebral hemorrhage. The potential mechanism may be associated with impaired ANP activity caused by PKa cleavage. PKa inhibition may reduce the damaging processes that promote intracerebral hemorrhage caused by hypertension.

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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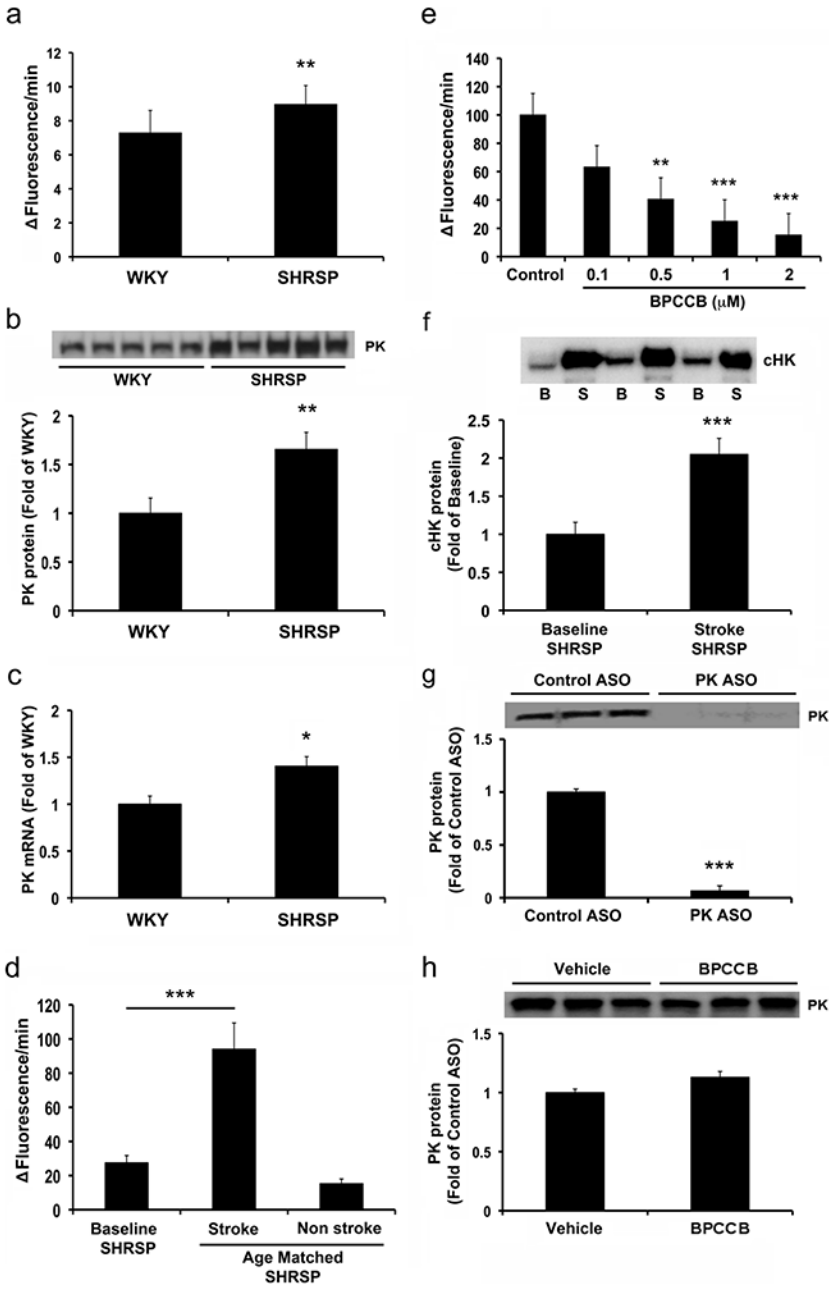

Fig. 1.

Plasma kallikrein is activated in stroke-prone spontaneously hypertensive rats. **a** Plasma kallikrein activity in WKY and SHRSP plasma at 12 weeks of high-salt diet. Kallikrein-like activity was determined by measuring changes in fluorescence at 410 nm due to hydrolysis of D-Pro-Phe-Arg-7-Amino-4-Trifluoromethyl coumarin. Data are expressed as mean ± s.e.m of Δfluorescence/min, $n = 6–8$; $^{**}P < 0.01$. **b** Plasma prekallikrein protein levels in WKY and SHRSP plasma at 12 weeks of high-salt diet. Data are expressed as mean ± s.e.m of fold change from WKY rats, $n = 9$; $^{**}P < 0.01$. **c** Plasma prekallikrein mRNA expression in WKY and SHRSP liver at 12 weeks of high-salt diet. Data are expressed as mean ± s.e.m of fold change from WKY rats, $n = 9$; $^{*}P < 0.05$. **d** Plasma kallikrein activity in stroke

or non stroke SHRSP. Data are expressed as mean ± s.e.m of Δfluorescence/min, $n = 6–8$; ***$P < 0.001$. **e** Dose response of inhibition of kallikrein-like activity from plasma of stroke SHRSP by BPCCB (0.1–2 μM; **$P < 0.01$, ***$P < 0.001$ vs control; $n = 5$). **f** Western blot analysis of cleaved high molecular weight kininogen (cHK) in plasma. Representative western blot on top showing immunoreactivity of cHK in SHRSP on 6 weeks of high-salt diet (B, baseline) and on 12 week of high-salt diet with stroke signs (S, stroke). Bar graph displays the quantification for cleaved HK. Data are presented as mean ± s.e.m, $n = 6$. ***P < 0.001. **g** Representative western blots on top showing immunoreactivity of PK in control ASO and PK ASO-treated rats. Bar graph displays the expression of PK in SHRSP plasma after 4 weeks of treatment. Data are presented as mean ± s.e.m, $n = 3$. ***$P < 0.001$. **h** Representative western blots on top showing immunoreactivity of PK in vehicle and BPCCB-treated rats. Bar graph displays the expression of PK in SHRSP plasma after 4 weeks of treatment. Data are presented as mean ± s.e.m, $n = 3$

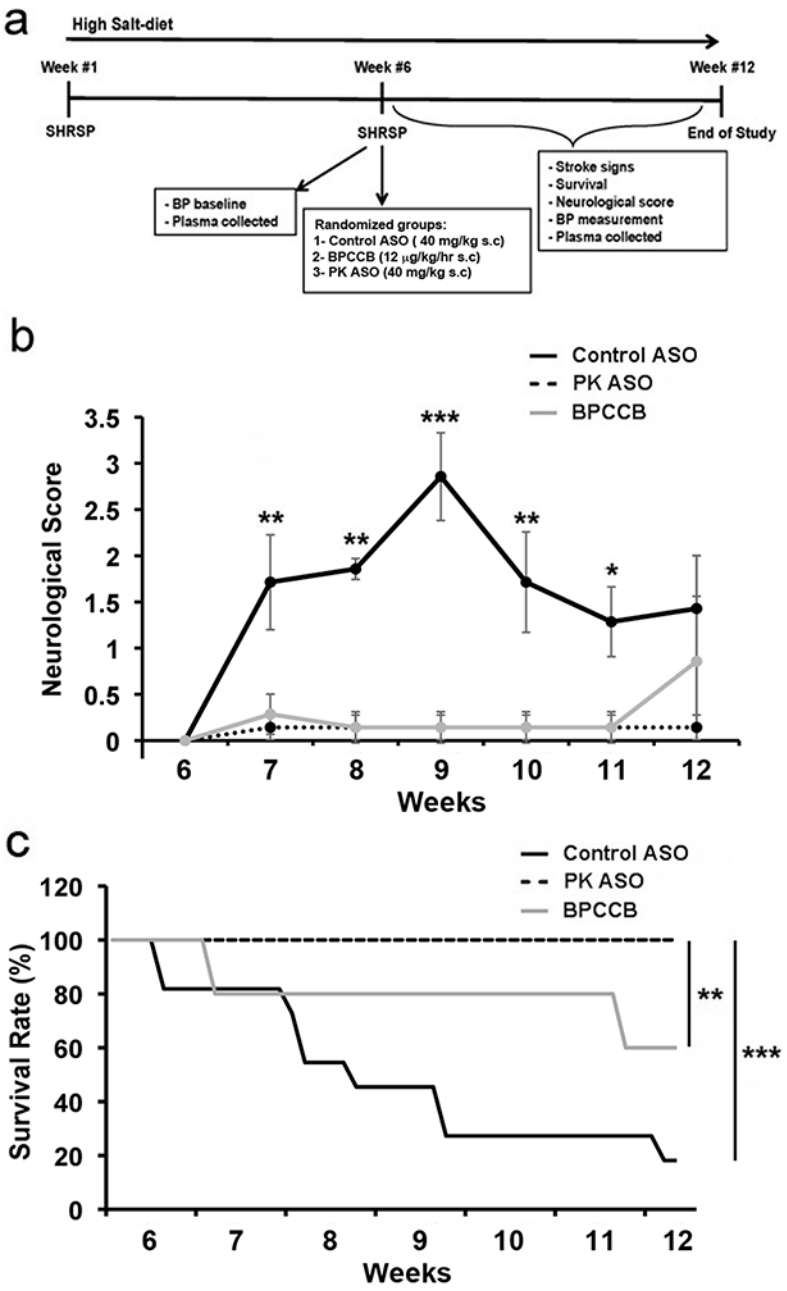

Fig. 2.

Effect of plasma kallikrein inhibition on neurological function and survival rate. **a** Schematic illustration of the experimental design. **b** Neurological score was higher in the control ASO group than in the PK ASO and BPCCB groups. Data are presented as mean ± s.e.m, n = 5–11; ***P < 0.001. **c** Kaplan–Meier plot of survival rate. Survival is longer in SHRSP with PKa inhibition and PK knockdown compared with control. Data are presented as mean ± s.e.m, n = 5–11; **P < 0.01, ***P < 0.001

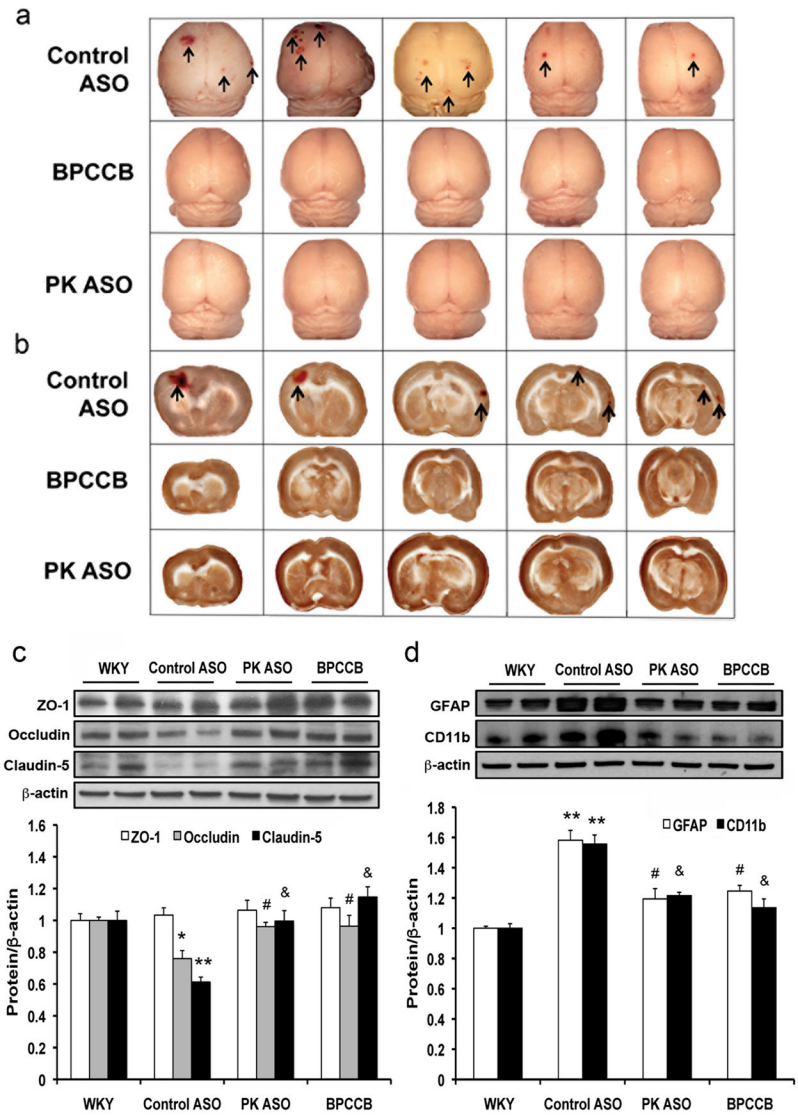

Fig. 3.

Effect of PKa blockage on intracerebral hemorrhage, tight junction proteins, and glial activation. **a** Representative pictures of brains from SHRSP showing intracerebral hemorrhages as indicated with arrows. ICH was detected in 10 SHRSP receiving control ASO ($n = 11$). Rats treated with PK ASO ($n = 8$) or BPCCB ($n = 5$) did not develop hemorrhage. **b** Coronal section through brain of SHRSP treated with control ASO, PK ASO, and BPCCB. Arrows indicate intracerebral hemorrhage. **c** Representative western blots on top showing immunoreactivity of ZO-1, occludin, and claudin-5. Bar graph displays the expression of ZO-1, occludin, and claudin-5 in cerebral cortex of SHRSP after 4 weeks of treatment. Data are presented as mean ± s.e.m, $n = 5$. *$P < 0.05$, **$P < 0.01$ vs WKY; #$P < 0.05$, &$P < 0.05$ vs control ASO. **d** Representative western blots on top showing immunoreactivity of GFAP and CD11b. Bar graph displays the expression of GFAP and CD11b in cerebral cortex of SHRSP after 4 weeks of treatment. Data are presented as mean ± s.e.m, $n = 5$. **$P < 0.01$ vs WKY; #$P < 0.05$, &$P < 0.05$ vs Control ASO

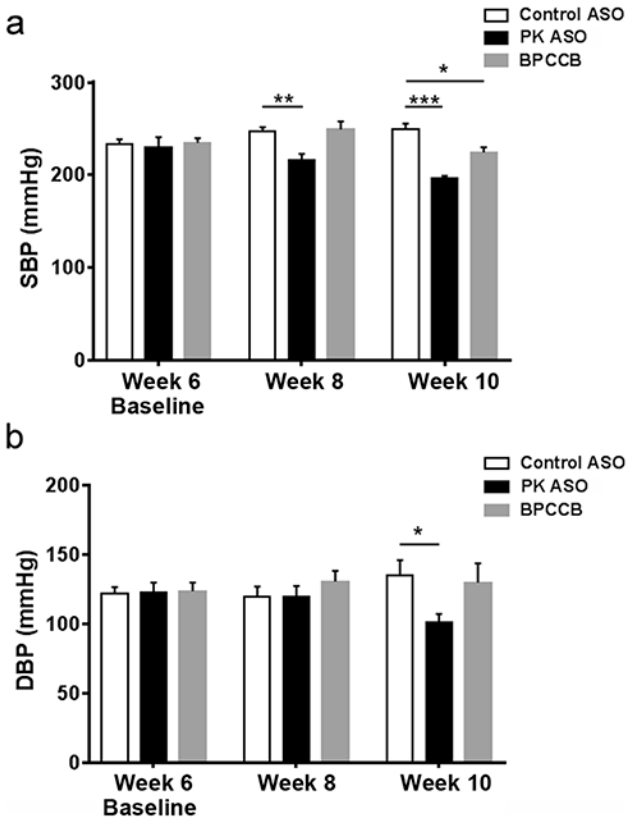

Fig. 4.

Effects of plasma kallikrein inhibition on blood pressure of SHRSP. **a** Systolic blood pressure (SBP) and **b** diastolic blood pressure (DBP) of rats from each group were determined at 2 and 4 weeks during treatment. Data are presented as mean ± s.e.m, n = 8–10; *P < 0.05, **P < 0.01, ***P < 0.001.

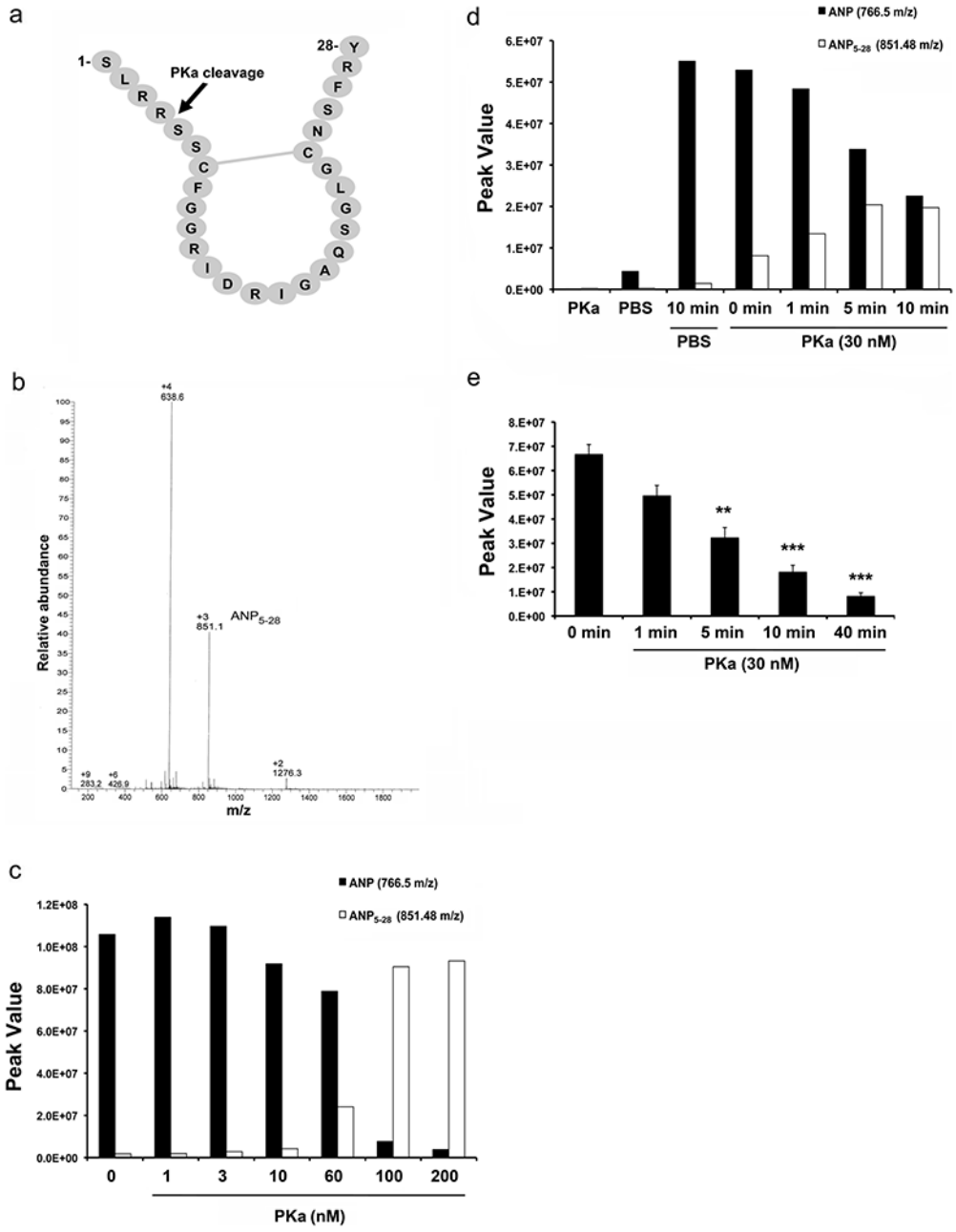

Fig. 5.

Identification of ANP fragments and cleavage by PKa. **a** ANP structure and Arg4-Ser5 cleavage site by PKa indicated by black arrow. **b** MS/MS of a concentration curve of cleaved ANP fragment detected by mass spectrometry. **c** Concentration response of PKa on ANP cleavage. Bar graph represents the peak value of ion fragments (ANP and ANP_{5-28}) versus PKa concentrations. **d** Time course of ANP cleavage and ANP_{5-28} generation by PKa. Bar graph represents the peak value of ANP cleavage and ANP_{5-28} generation during 10 min. **e** Time course of ANP cleavage by PKa during 40 min. Data are presented as mean ± s.e.m, $n = 3$; $^{**}P < 0.01$, $^{***}P < 0.001$

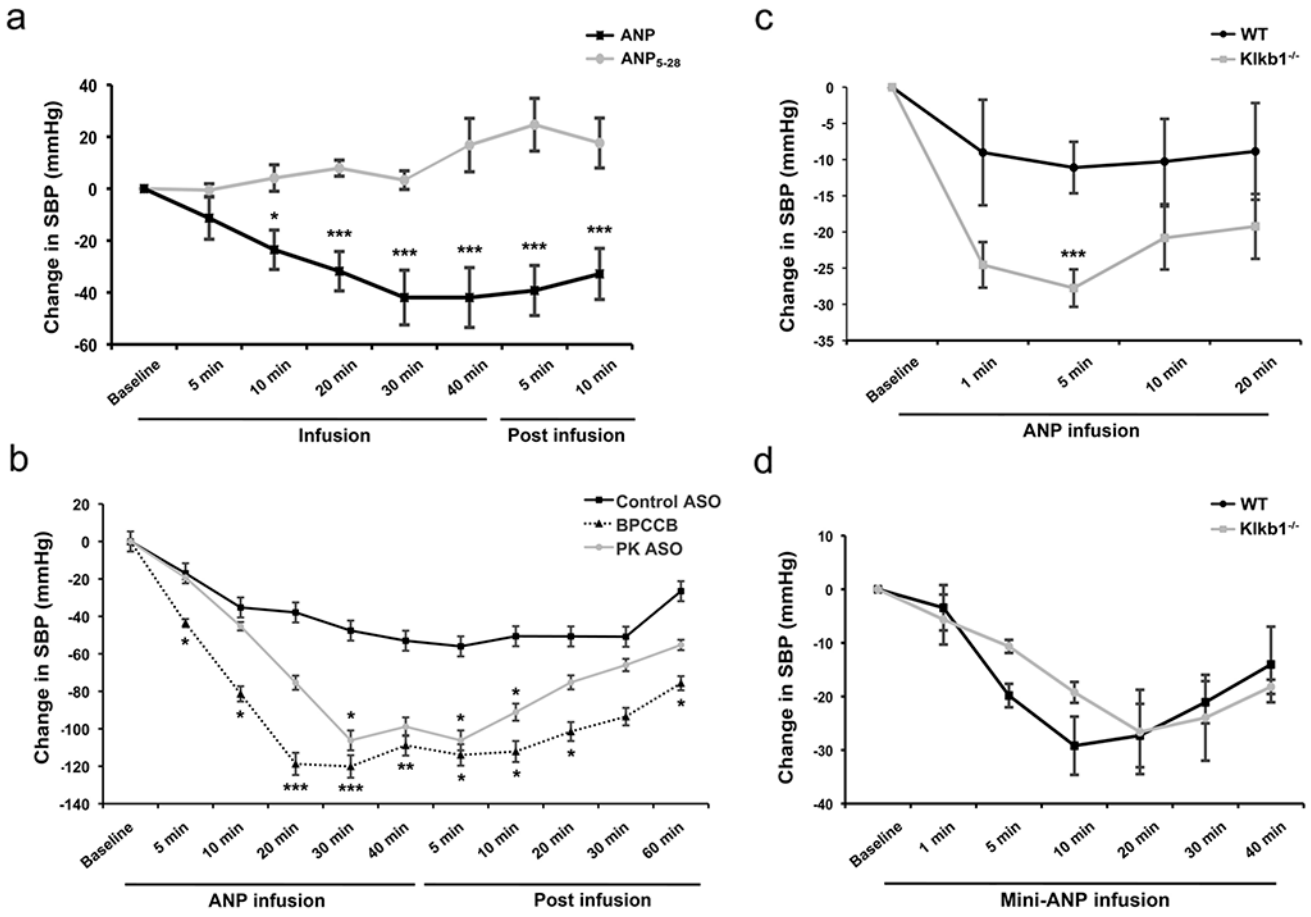

Fig. 6.

Effect of ANP on systolic blood pressure in vivo. **a** Change in systolic blood pressure following infusion of ANP and ANP_{5-28} via jugular vein in SHRSP with Ang II–induced hypertension. Data are presented as mean ± s.e.m, $n = 5$; $^{*}P < 0.05$, $^{***}P < 0.001$. **b** Effects of ANP infusion on the change in SBP in Ang II–induced hypertensive rats treated with BPCCB, PK ASO, or control ASO. Data are presented as mean ± s.e.m, $n = 5–6$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control ASO. **c** Effects of ANP infusion on the change in SBP in WT and $Klkb1^{-/-}$ mice with Ang II–induced hypertension. Data are presented as mean ± s.e.m, $n = 4–5$; $^{***}P < 0.001$. **d** Effects of mini-ANP infusion on the change in SBP in WT and $Klkb1^{-/-}$ mice with Ang II–induced hypertension. Data are presented as mean ± s.e.m., $n = 3$.