Calcimimetic R568 reduced the blood pressure and improved aortic remodeling in spontaneously hypertensive rats by inhibiting local renin-angiotensin system activity

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Abstract. Hypertension is a cardiovascular disease that seriously affects human health. Activation of the calcium-sensing receptor (CaSR) inhibits cyclic adenosine monophosphate (cAMP) formation by increasing $[Ca^{2+}]_i$ and subsequently inhibiting renin release. The renin-angiotensin system (RAS) plays an important role in the development of essential hypertension (EH). The purpose of this study was to determine the effects of NPSR568 (R568)-activated CaSR on blood pressure (BP), proliferation, and remodeling of vascular smooth muscle cells, and the activity of the RAS in spontaneously hypertensive rats (SHRs). In this study, we treated SHR and Wistar-Kyoto rats with R568 for 8 weeks. The tail-cuff method was used to assess rat BP weekly. Morphological changes in the thoracic aorta were evaluated with hematoxylin-eosin and Masson staining. Western blotting and immunohistochemistry were used to detect the expression of RAS-related proteins and proliferative remodeling proteins in the thoracic aorta. An enzyme-linked immunosorbent assay was used to detect the content of cAMP, the RAS, and the CaSR in plasma and the thoracic aorta. Finally, we found that treatment with R568 for 8 weeks reduced the BP and inhibited arterial vascular proliferation remodeling in SHRs. R568 administration significantly suppressed the activity of local RAS in the thoracic aortas of SHRs. Moreover, R568 treatment reversed the low expression of CaSR in SHRs. R568 may serve as an effective strategy against EH.

Introduction

Hypertension is one of the most important risk factors for cardiovascular disease. Epidemiologic studies have shown that patients with essential hypertension (EH) accounted for 90-95% of patients with hypertension (1). Although the etiology and pathogenesis of EH has not been determined, factors such as genetics and environment may have some impact on the occurrence and development of EH (2). Among the factors, the renin-angiotensin system (RAS) plays a crucial role in the pathogenesis of EH (3). Renin is the first rate-limiting enzyme in the RAS (4). Angiotensin II (Ang II) is a peptide hormone that mediates vasoconstriction and increases blood pressure (BP) by binding to the Ang II type 1 receptor (AT1R) and is involved in the development of hypertension (5).

The extracellular calcium-sensing receptor (CaSR) is a class C G protein-coupled receptor (GPCR), which is part of a superfamily of seven transmembrane domain receptors, the primary function of which is to regulate calcium homeostasis. The CaSR is mainly expressed in the thyroid, parathyroid, kidneys, and bone (6,7). There is also functional expression in the vascular wall, including vascular smooth muscle and endothelial cells (8-10). Increased extracellular Ca^{2+} $(Ca^{2+}]_o)$ induces binding to the CaSR and activates the G-protein-phospholipase C (PLC)-inositol 1,4,5-trisphophate (IP_3) receptor pathway, triggers an elevation in intracellular Ca^{2+} $([Ca^{2+}]_i)$, and is involved in the occurrence and development of hypertension (6). Studies on the relationship between calcium and BP have shown that proper intake of Ca^{2+} can effectively lower BP (11). This conclusion has been confirmed in animal experiments (12). Some researchers have also shown that elevated Ca^{2+} leads

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to vasodilation *in vitro* (13). Unfortunately, despite these findings, the molecular mechanisms underlying this association are not fully understood.

NPSR568 (R568), a calcimimetic, is an allosteric agonist of the CaSR. The mechanism of action of R568 is to increase the sensitivity of the CaSR to $[Ca^{2+}]_o$, and shift the calcium concentration response curve to the left. It is currently believed that the binding site of R568 to the CaSR is at the 7th transmembrane region of the receptor. The CaSR may be involved in BP regulation, and has become a current focus of research. Ogata *et al* (14) argued that R568 reduces BP in uremic rats and SHRs, but has no effect on normotensive rats. Rybczynska *et al* (15,16) reported that administration of NPS2143, an allosteric inhibitor of the CaSR, increased BP in normotensive rats; however, in rats with surgically removed parathyroid glands or an AT1R (e.g., losartan) in the presence of a calcium channel blocker or antagonist, no effect of elevated BP was observed. Atchison *et al* (11) and Ortiz-Capisano *et al* (17) suggest that the CaSR is expressed in juxtaglomerular cells, and that activation of the CaSR activates the ryanodine receptor (RyR) via the PLC/IP$_3$ pathway to increase $[Ca^{2+}]_i$ and inhibit cAMP formation, thereby inhibiting renin release; meanwhile, Maillard *et al* (18) also proved that the calcimimetic R568 can regulate renin release via CaSR. Our previous research also proved that reduced expression of the CaSR is associated with increased proliferation and remodeling of vascular smooth muscle cells (VSMCs) and promotes the development of EH via activation of the cAMP-RAS pathway (9).

The goal of this study was to determine the effect of CaSR activation on BP in spontaneously hypertensive rats (SHRs) and to partially elucidate the mechanism of action of the CaSR in regulating BP from the perspective of the RAS. Thus, we hypothesized that a R568-activated CaSR may lower BP and improve thoracic aortic proliferation and remodeling through the RAS pathway.

Materials and methods

Animals. For this study, 42 male SHRs and age-matched male WKY (8-week old, 180-220 g, purchased from Vital River Laboratory Animal Science and Technology Co., Ltd, Beijing. License Number: SCXK2012-0001) were randomly divided into 4 groups: Group 1, WKY+NS (n=21); group 2, WKY+R568 (n=21); group 3, SHR+NS (n=21); and group 4, SHR+R568 group (n=21). R568 was dissolved in normal saline (NS) at a dose of 1.2 mg/kg/day in group 2/4, and was administered by intraperitoneal (i.p.) injection. At R568 treatment at 0, 4, and 8 weeks, rats were equivalent to 8, 12, and 16 weeks of age. Seven rats in each group were randomly selected and sacrificed to detect the corresponding indexes. In group 1/3, the rats received NS i.p., and treated as mentioned above. All animals were housed at a constant room temperature, humidity, and light cycle (alternating 12 h light/dark cycle), and had access to food and drinking water *ad libitum*. All experimental procedures in this study were approved by the Institutional Animal Research Committee of Shihezi Medical University, and all animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication 86-23, revised 1986).

Evaluation of EH. In the experiment, systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure [MAP; (MAP=DBP+1/3 {SBP-DBP})] were measured once a week. At the same time of the week, the BP of rats was measured by tail-cuff method [BP-98A-L; Softron, Tokyo, Japan] (19), the room temperature was kept at a controlled temperature of 30°C, and repeated measurements were taken 3 times and averaged. At 0, 4, and 8 weeks with this treatment and after SBP, DBP, and MAP measurements, the rats were anesthetized, blood was extracted from the abdominal aorta and isolated plasma, then stored at -80°C for an enzyme-linked immunosorbent assay (ELISA). Rats were sacrificed and the thoracic aorta was isolated, 0.5 cm of the thoracic aorta was cut, placed in 10% neutral buffered formalin, and paraffin-embedded for histopathologic evaluation (hematoxylin and eosin and Masson staining) and immunohistochemistry analysis. The remaining part of the thoracic aorta tissue were stored at -80°C for subsequent protein extracted for western blotting and aorta homogenate for ELISA assay.

Histologic evaluation. Thoracic aorta tissue was fixed in 10% formalin for more than 48 h, then dehydrated, embedded, sectioned, and stained with hematoxylin and eosin or Masson's trichrome. We then randomly selected three fields in each slice, calculated the parameters of the thoracic aorta vascular cross-sections by measuring the wall thickness, cross-sectional vessel area and wall area, total vessel wall thickness, and the percentages of medial wall thickness-to-the external diameter (WT%), cross-sectional vessel wall area-to-the total area (WA%), and collagen area-to-total vessel wall area (CA%). We captured the thoracic aorta images with an Olympus BX40 microscope and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics Inc., Buckinghamshire, UK).

Immunohistochemistry analysis. Tissue sections were deparaffinized, washed, and antigen was retrieved with 0.01 M sodium citrate buffer (pH=6.0) in a pressure cooker. The sections were incubated with primary mouse polyclonal antibodies against CaSR (1:50; Abcam, MA, USA), calponin (1:600; Boster, Wuhan, China), smooth muscle actin α (α-SMA, 1:400; Boster), proliferating cell nuclear antigen (PCNA, 1:200; Boster), and primary rabbit monoclonal antibody against osteopontin (OPN, 1:100; Abcam) overnight at 4°C. Slides were washed three times in PBS (pH 7.4-7.6) and incubated with the corresponding secondary horseradish peroxidase-conjugated antibody (Invitrogen, Beijing, China) for 30 min at 37°C. A diaminobenzidine/peroxidase substrate was used to generate a brown signal. Negative controls were omitted with primary antibodies. To quantify protein levels, positive staining in tissue sections was further analyzed by Integral Optical Density (IOD)/vascular wall area using Image-Pro Plus 6.0 software. The same microscope and camera set was used for all images.

Western blot analysis. Total protein was extracted from the thoracic aorta and quantified for protein concentration. The

extracted protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred proteins to 0.45-mm SequiBlot PVDF membranes. After incubation with 5% non-fat milk, then incubated overnight at 4°C with primary antibodies against CaSR (1:1,000; Abcam), PCNA (1:250; Boster), α-SMA (1:400; Boster), calponin (1:300; Boster), OPN (1:1,000; Abcam), renin (1:250; Bioss, Beijing, China), AT1R (1:1,000; Abcam,), and β-actin (1:1,000; Boster). After being washed with TBST, the membranes were incubated with fluorescence conjugated goat anti-mouse or anti-rabbit IgGs (1:20,000; Boster) for approximately 2 h at room temperature. Protein was visualized using an enhanced chemiluminescence system (ECL, Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA USA). The intensity of protein bands was quantified using Bio-Rad Quantity One software (Bio-Rad, Hercules, CA, USA) and the levels of protein detected were normalized to β-actin levels.

Enzyme-linked immunosorbent assay. An ELISA kit (Wuhan Elabscience Biotechnology, Wuhan, China) was used to determine the levels of CaSR, cAMP, renin, and Ang II from plasma and thoracic aortic tissue homogenate, and the specific measurement method was according to the manufacturer's instructions. A microplate reader was used to read the absorbance at 450 nm (Bio-Rad Model 3550-UV).

Drugs. R-568 has a chiral carbon atom and acts stereospecifically on CaSR. R568 was purchased from Tocris Bioscience Co., Ltd. (Minneapolis, MN, USA). The hydrophobic compounds were dissolved in normal saline at a dose of 1.2 mg/kg/day as a stock solution.

Statistical analysis. Quantitative data were expressed as the mean ± SEM. For multiple experimental groups, we used one way analysis of variance (ANOVA) or Kruskal-Wallis test, then a Bonferroni post hoc test was used after ANOVA. For all analysis results, $P<0.05$ was considered statistically significant. Statistical analysis was performed using the SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Effects of R568 on SBP, DBP, and mean arterial pressure (MAP) in SHRs. To investigate the effect of R568 on BP in SHRs, we measured the changes in SBP, DBP, and MAP using the tail-cuff method. Results showed that the BP of SHRs at different ages was higher than age-matched WKY rats ($P<0.05$), and the BP of the SHR16 group was significantly higher than the SHR8w group ($P<0.05$; Fig. 1). During 8 weeks of treatment, R568 reduced the SBP of SHRs at 16 weeks ($P<0.05$; Fig 1A), while R568 had little effect on the DBP and MAP (Fig. 1B and C).

R568 attenuates aortic media remodeling in SHRs. The morphology of the thoracic aorta was determined with HE and Masson staining (Fig. 2). Compared with WKY rats, thoracic aorta endothelial cells were injured and the aortic media was thickened (increased WT% and WA%, $P<0.05$; Fig. 2A, C and D), and abnormal collagen accumulated (increase in
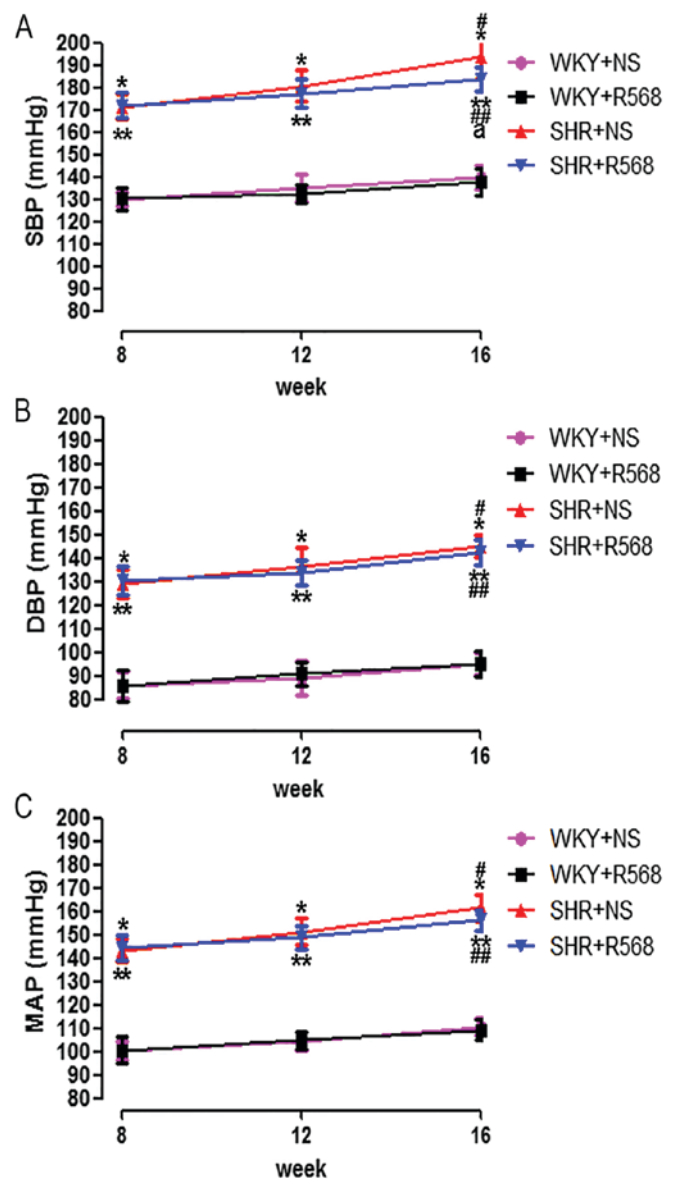

Figure 1. Comparison of SBP, DBP, and MAP between different groups of rats. (A) Systolic blood pressure; (B) diastolic blood pressure; (C) mean arterial pressure. Data are means ± standard deviation (n=7). SHR+NS groups vs. the age-matched WKY+NS groups, *P<0.05; SHR16w+NS groups vs. SHR8w+NS groups, #P<0.05; SHR+R568 groups vs. the age-matched WKY+R568 groups, **P<0.05; SHR16w+R568 groups vs. SHR8w+R568 groups, ##P<0.05; SHR+R568 groups vs. SHR+NS groups, aP<0.05. WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; NS, normal saline; R568, NPSR568. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

CA%, $P<0.05$; Fig. 2B and E) as the BP increased. The above indicators in the SHR-16 group changes were more apparent than SHR-8 group ($P<0.05$; Fig. 2). After R568 treatment, 16w SHRs can reduce thoracic aortic proliferation and remodeling ($P<0.05$; Fig. 2).

R568 inhibits proliferation and remodeling in thoracic aortas of SHRs. Immunohistochemistry and western blotting were used to analyze marker protein changes in hypertensive aortas. Compared with the age-matched WKY rats, two contractile/differentiated phenotype marker proteins (calponin and α-SMA) were decreased in the media of
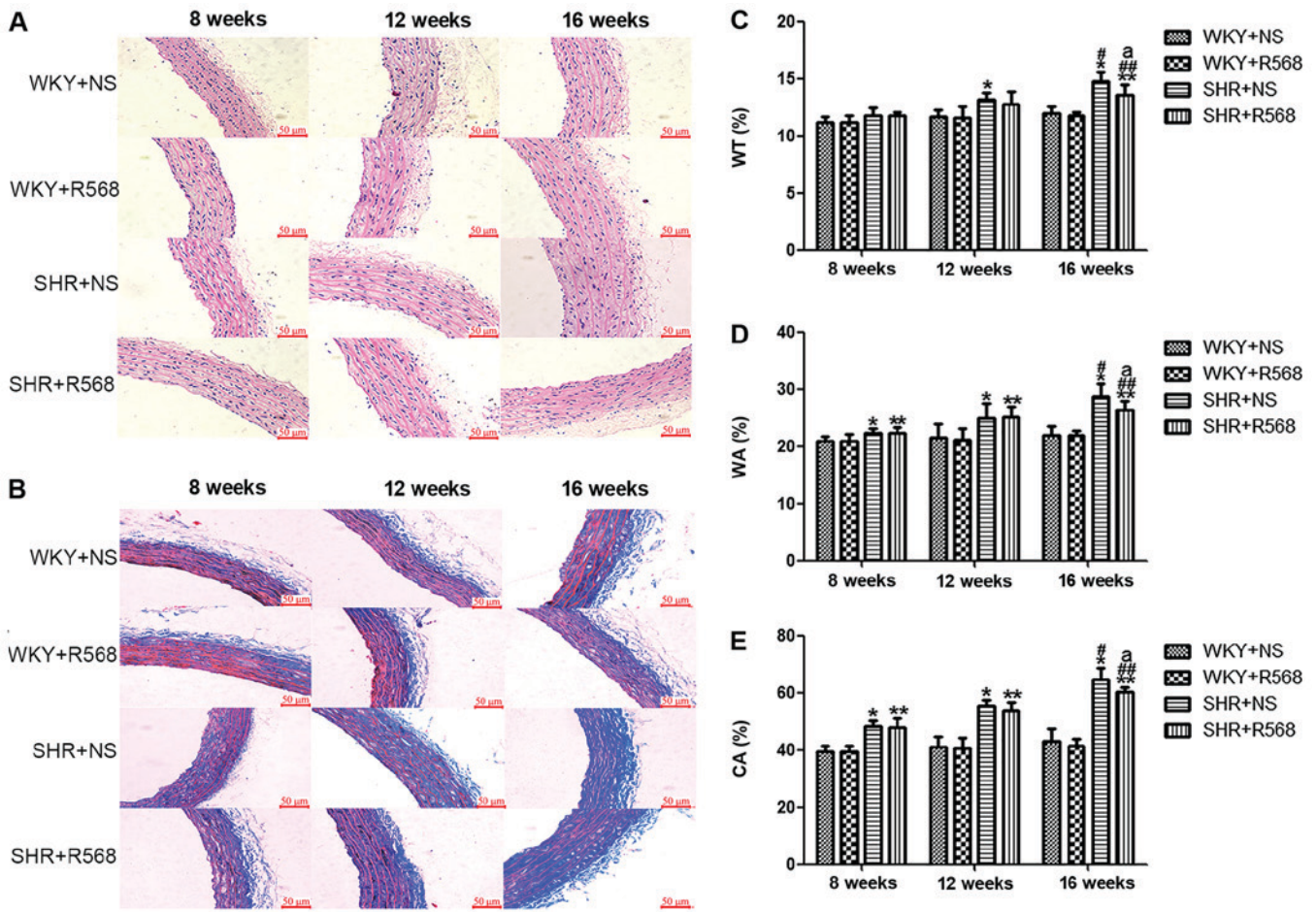
Figure 2. Changes of proliferation and remodeling indices in thoracic aortic smooth muscle of rats. (A) HE staining. (B) Masson staining. (**C**) WT%, the wall thickness percentage of the external diameter. (D) WA%, total vessel wall area as a percentage of the total area (WA%). (E) CA%, total area occupied by collagen as a percentage of the total vessel wall area. Data are means ± standard deviation (n=7). SHR+NS groups vs. the age-matched WKY+NS groups, [*]P<0.05; SHR16w+NS groups vs. SHR8w+NS groups, [#]P<0.05; SHR+R568 groups vs. the age-matched WKY+R568 groups, [**]P<0.05; SHR16w+R568 groups vs. SHR8w+R568 groups, [##]P<0.05; SHR+R568 vs. SHR+NS groups, [a]P<0.05. WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; NS, normal saline; R568, NPSR568; WT%, the wall thickness percentage of the external diameter; WA%, total vessel wall area as a percentage of the total area (WA%); CA%, total area occupied by collagen as a percentage of the total vessel wall area. Scale bar, 50 μM.

thoracic aortas in SHRs (P<0.05), and the expression of OPN, a synthetic/dedifferentiated phenotype marker protein, and PCNA was significantly increased (P<0.05; Figs. 3 and 4). The same trend was observed when comparing the SHR16w group with the SHR8w group (P<0.05); however, activation of the CaSR by R568 increased the expression of calponin and α-SMA, and reduced the expression of OPN and PCNA protein in the thoracic aortas of SHR16w group (P<0.05; Figs. 3 and 4). R568 had no significant effect on the expression of proliferative and remodeling proteins in WKY groups.

R568 reverses the low expression of the CaSR in rat thoracic aortas. Western blotting and immunohistochemistry showed that expression of the CaSR in SHRs was lower than age-matched WKY rats (P<0.05). Compared with SHR16w group and SHR8w group, expression of the CaSR protein in the thoracic aortas of rats increased as the BP increased in SHRs (P<0.05; Fig. 5A-D); however, R568 reversed expression of the CaSR in SHRs at 16 weeks (P<0.05; Fig. 5A-D). R568 had no significant effect on CaSR protein expression in thoracic aortas of WKY rats.

R568 reverses the low expression of the CaSR in plasma of SHRs and has no significant effect on aortic homogenates. The concentration of the CaSR in plasma and thoracic aortas of rats was determined with an enzyme-linked immunosorbent assay (ELISA). The CaSR concentration in SHRs plasma was significantly lower than age-matched WKY rats (P<0.05; Fig. 5E). The same trend was observed in comparing SHR16w group with SHR8w group (P<0.05; Fig. 5E). Interestingly, the content of the CaSR in SHR-16 group thoracic aortas was significantly higher than in WKY-16 group (P<0.05; Fig. 5F). After treatment with R568, the CaSR concentration was significantly elevated in the plasma of SHR16w group (P<0.05; Fig. 5E). However, R568 had no significant effect on the CaSR content in the thoracic aortas (Fig. 5F).

R568 inhibits renin and AT1R protein increases while decreasing renin and Ang II levels in the SHR thoracic aorta. Western blotting and ELISA results showed that compared with age-matched WKY rats, renin, AT1R protein expression, and renin and Ang II concentration in thoracic aortas were increased in SHRs; the above indicators increased more significantly in SHR16w compared with those in SHR8w
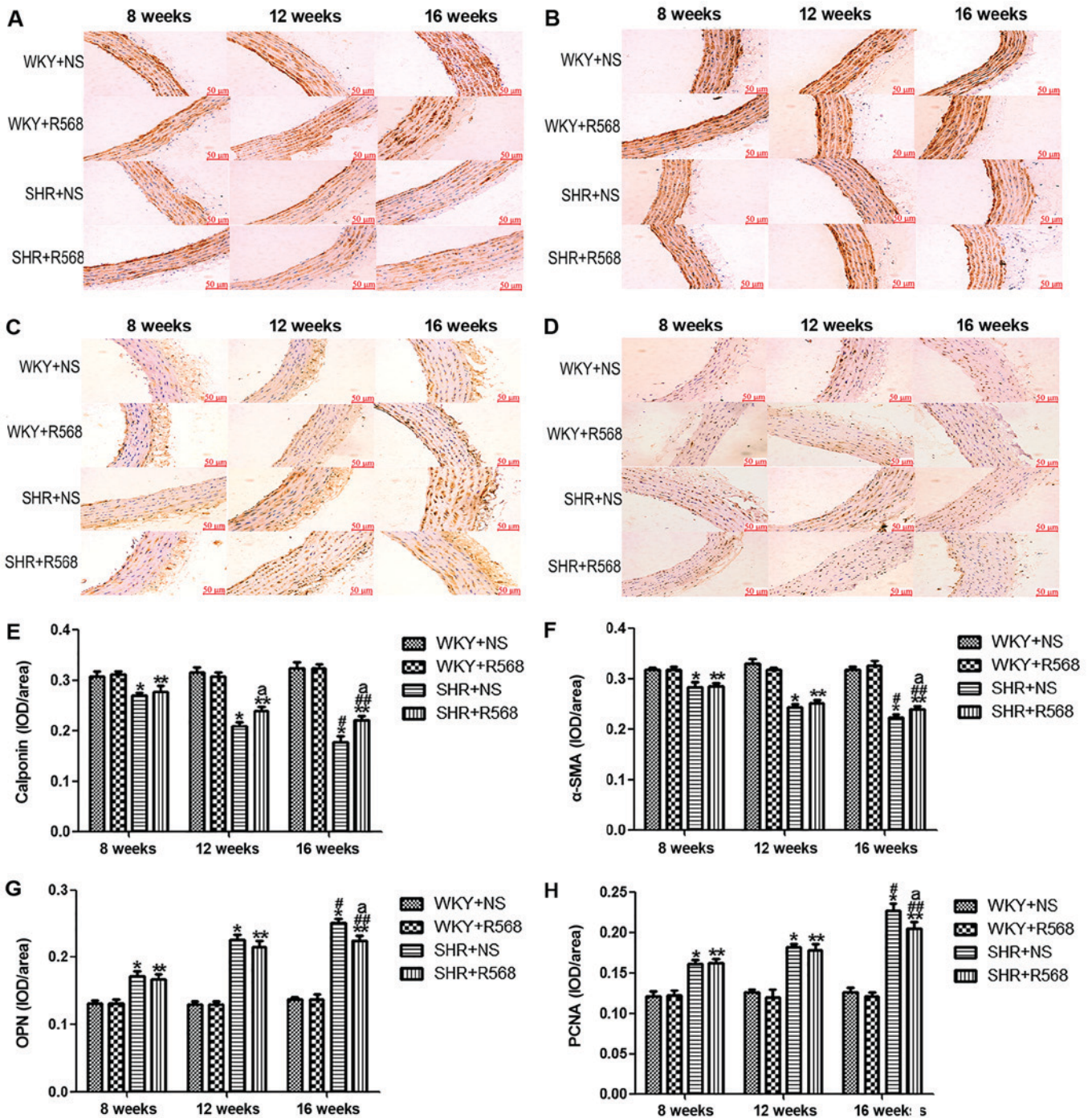
Figure 3. Immunohistochemical analysis of proliferating remodeling protein expression in the thoracic aorta of rats. Representative images of (A) Calponin; (B) α-SMA, smooth muscle α-actin; (C) OPN, osteopontin; (D) PCNA, proliferating cell nuclear antigen. Quantification of (E) Calponin; (F) α-SMA, smooth muscle α-actin; (G) OPN, osteopontin; (H) PCNA, proliferating cell nuclear antigen. Data are means ± standard deviation (n=7). SHR+NS groups vs. the age-matched WKY+NS groups, *$P<0.05$; SHR16w+NS groups vs. SHR8w+NS groups, #$P<0.05$; SHR+R568 groups vs. the age-matched WKY+R568 groups, **$P<0.05$; SHR16w+R568 groups vs. SHR8w+R568 groups, ##$P<0.05$; SHR+R568 groups vs. SHR+NS groups, a$P<0.05$. WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; NS, normal saline; R568, NPSR568; α-SMA, smooth muscle α-actin; OPN, osteopontin; PCNA, proliferating cell nuclear antigen. Scale bar, 50 μM.

group ($P<0.05$; Fig. 6). R568 treatment reduced renin and AT1R protein expression, and renin and Ang II levels in the thoracic aortas of SHR16w group ($P<0.05$; Fig. 6). R568 had no significant effect on RAS-related protein expression in the thoracic aorta of WKY rats (Fig. 6).

R568 has no significant effect on cAMP, renin, and Ang II levels in the plasma of rats. Our ELISA results showed that plasma levels of cAMP and Ang II increased and the renin levels decreased with increasing BP in SHRs compared with age-matched WKY rats ($P<0.05$; Fig. 7); the same trend can be observed compared with SHR16w and SHR8w groups ($P<0.05$; Fig. 7). There were no significant changes in plasma cAMP, renin, and Ang II levels in SHRs and WKY rats after treatment with R568 for 8 weeks (Fig. 7).
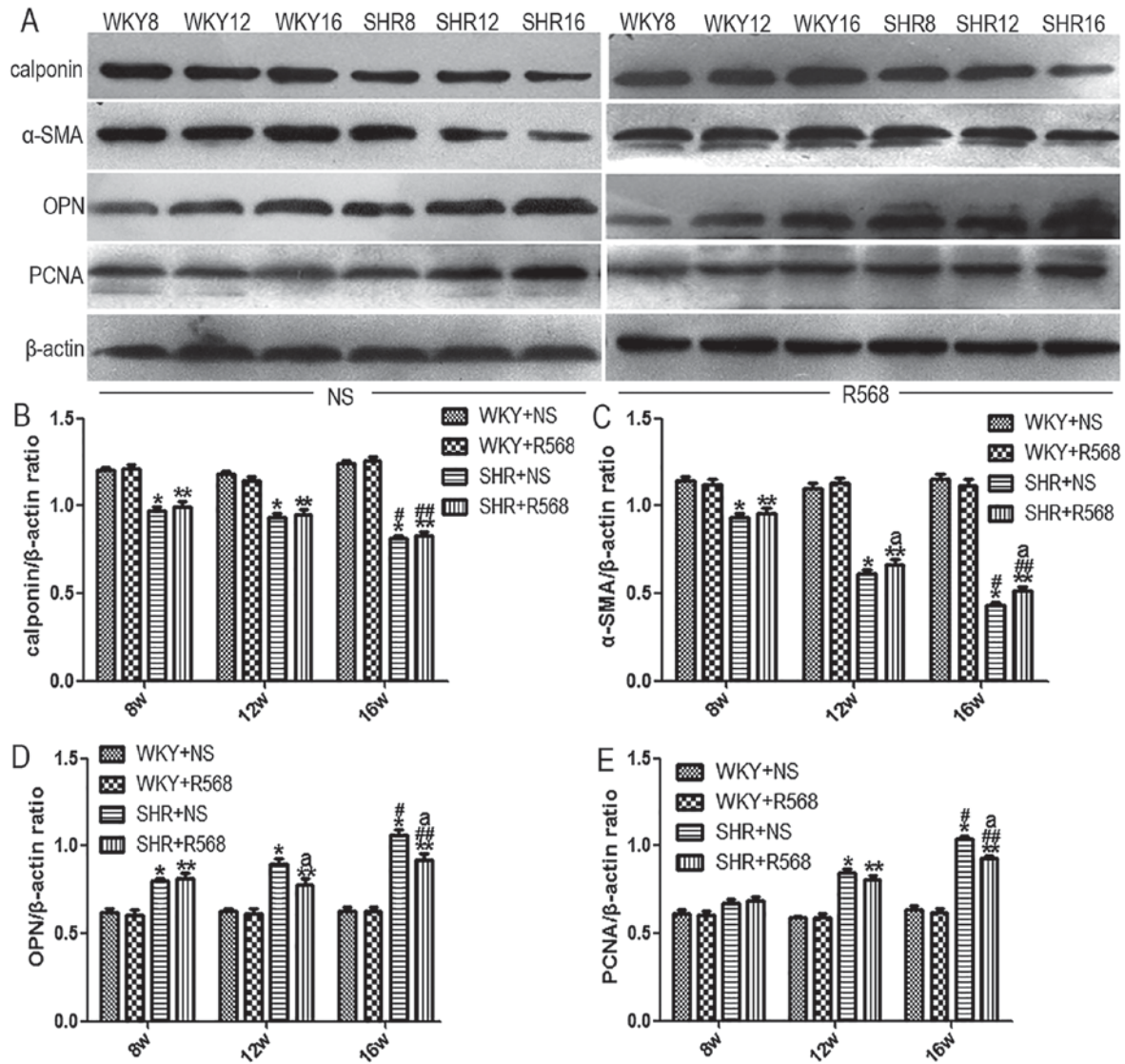
Figure 4. Western blot analysis of proliferating remodeling protein expression in the thoracic aorta of rats. (A) Western blot analysis of the protein expression. (B) Calponin. (C) α-SMA, smooth muscle α-actin. (D) OPN, osteopontin. (E) PCNA, proliferating cell nuclear antigen. Data are means ± standard deviation (n = 7). SHR+NS groups vs. the age-matched WKY+NS groups, *P<0.05; SHR16w+NS groups vs. SHR8w+NS groups, #P<0.05; SHR+R568 groups vs. the age-matched WKY+R568 groups, **P<0.05; SHR16w+R568 groups vs. SHR8w+R568 groups, ##P<0.05; SHR+R568 groups vs. SHR+NS groups, aP<0.05. WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; NS, normal saline; R568, NPSR568; α-SMA, smooth muscle α-actin; OPN, osteopontin; PCNA, proliferating cell nuclear antigen.

Discussion

CaSR activation has a protective effect on EH and hypertension-induced aortic smooth muscle proliferation and remodeling. By analyzing the effects of a R568-activated CaSR on rat BP, aortic remodeling, and RAS, we demonstrated the following: i) decreased CaSR expression is involved in hypertension development; ii) low CaSR expression can activate circulating and vascular local RAS through the cAMP pathway and promote the development of hypertension; and iii) the CaSR may regulate BP by regulating local RAS activity. These data provide new insight into the regulatory mechanism of the CaSR on EH from the perspective of the RAS. To date, this is the first study to explore CaSR-regulated EH mechanisms through the RAS pathway.

The CaSR, a member of the GPCR superfamily, exerts biological effects by regulating the PLC-IP$_3$ signaling pathway to induce an increase in [Ca^{2+}]$_i$ (7). Recent studies have suggested that the CaSR agonist, R568, can reduce BP, but the underlying mechanism is not clear. Maillard *et al* (18) suggested that the calcimimetic R568 can regulate renin release via CaSR, and renin plays an important role in the occurrence of EH. Ogata *et al* (14) compared the effects of NPSR568 and parathyroidectomy on the progression of renal failure and showed that R568 decreased BP in uremic rats and SHRs, but had no effect on normotensive rats. Rybczyńska *et al* (20) also observed the effect of intravenous administration of R568 on the MAP of SHRs and WKY rats in the presence or absence of thyroparathyroidectomy and found that in the presence of parathyroid, calcimimetic R568 lowered the BP of SHRs, but has no effect on BP in WKY rats. In the current study, we first used SHRs and WKY rats of different ages to dynamically observe the anti-hypertensive effect of the CaSR agonist, R568. The results showed that R568 activation of CaSR reduced BP in
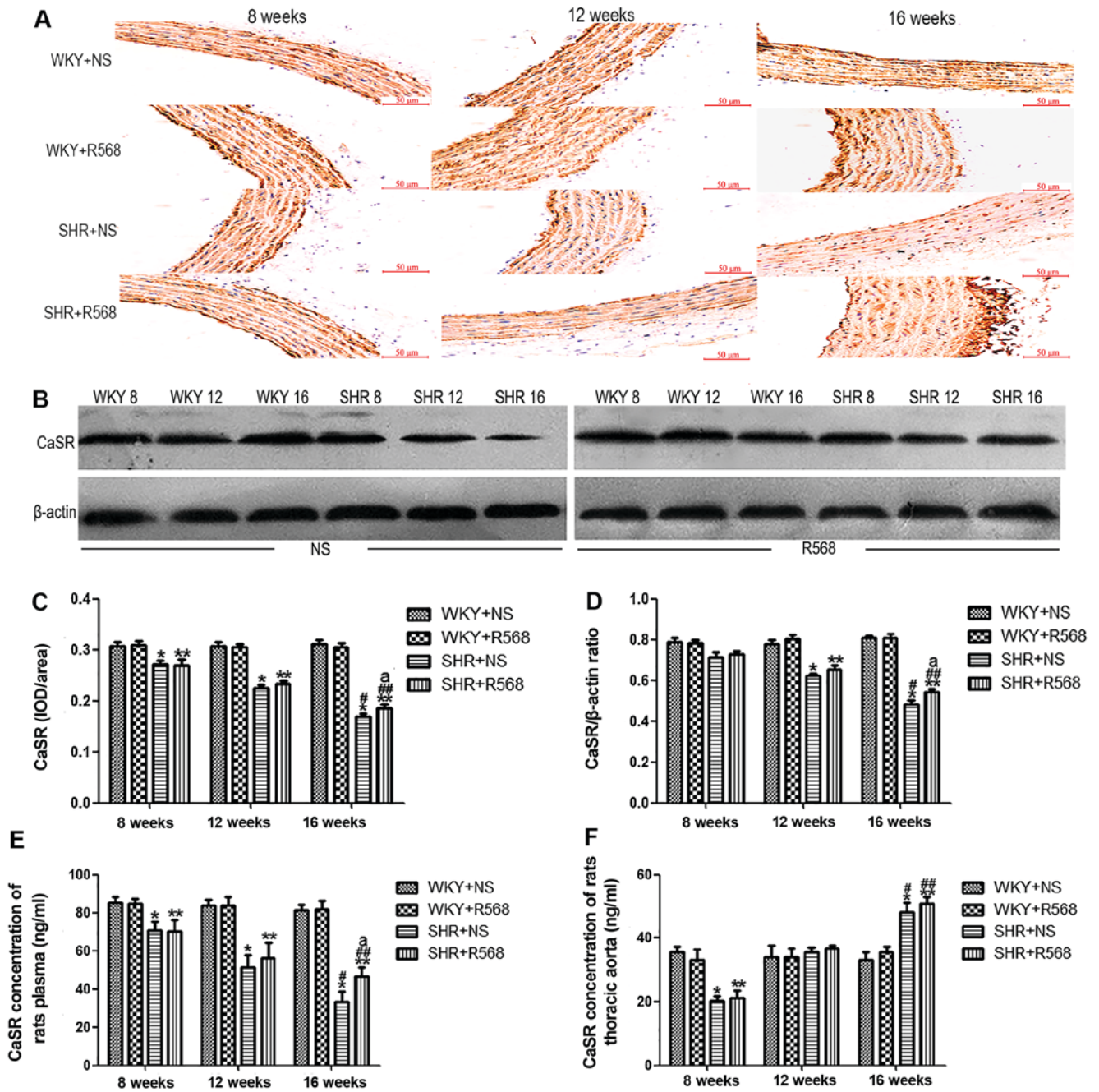
Figure 5. Detection of CaSR protein expression and content in plasma and thoracic aorta of rats. (A) Immunohistochemical analysis. (B) Western blotting analysis. (C) Densitometric analysis of A. (D) Densitometric analysis of B. (E) CaSR level in rat plasma (as detected by ELISA). (F) CaSR content in thoracic aorta (as detected by ELISA). Data are means ± standard deviation (n=7). SHR+NS groups vs. the age-matched WKY+NS groups, *P<0.05; SHR16w+NS groups vs. SHR8w+NS groups, #P<0.05; SHR+R568 groups vs. the age-matched WKY+R568 groups, **P<0.05; SHR16w+R568 groups vs. SHR8w+R568 groups, ##P<0.05; SHR+R568 groups vs. SHR+NS groups, aP<0.05. WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; NS, normal saline; R568, NPSR568; CaSR; calcium-sensing receptor. Scale bar, 50 μM.

SHRs but had no significant effect on BP in normotensive rats. Therefore, we believe that low CaSR expression is involved in the development of hypertension. Different views have also been proposed by other researchers. Nakagawa et al (21) observed the changes in MAP after acute injection of femoral vein R568 and the enantiomer, S568, in Sprague-Dawley (SD) rats. The results showed that R568 exerts an acute, CaSR-independent antihypertensive effect through vasodilation and negative degeneration at concentrations exceeding those required to modulate PTH secretion. Because S568 has

little effect on the CaSR, Nakagawa et al (21) suggested that the effect of R568 on lowering BP may not be CaSR-mediated. From this point of view, the researchers believe that R568 is a phenylalkylamine compound, its effect on CaSR is basically stereotactic, and both isomers R568 and S568 are equivalent in blocking voltage or ligand-gated Ca^{2+} influx (21). The lack of stereoselectivity and the hypotensive effect of R568 suggest that exclusion of voltage-gated Ca^{2+} channels, except for CaSR-mediated activity, is still required; however, this remains to be demonstrated (21,22).
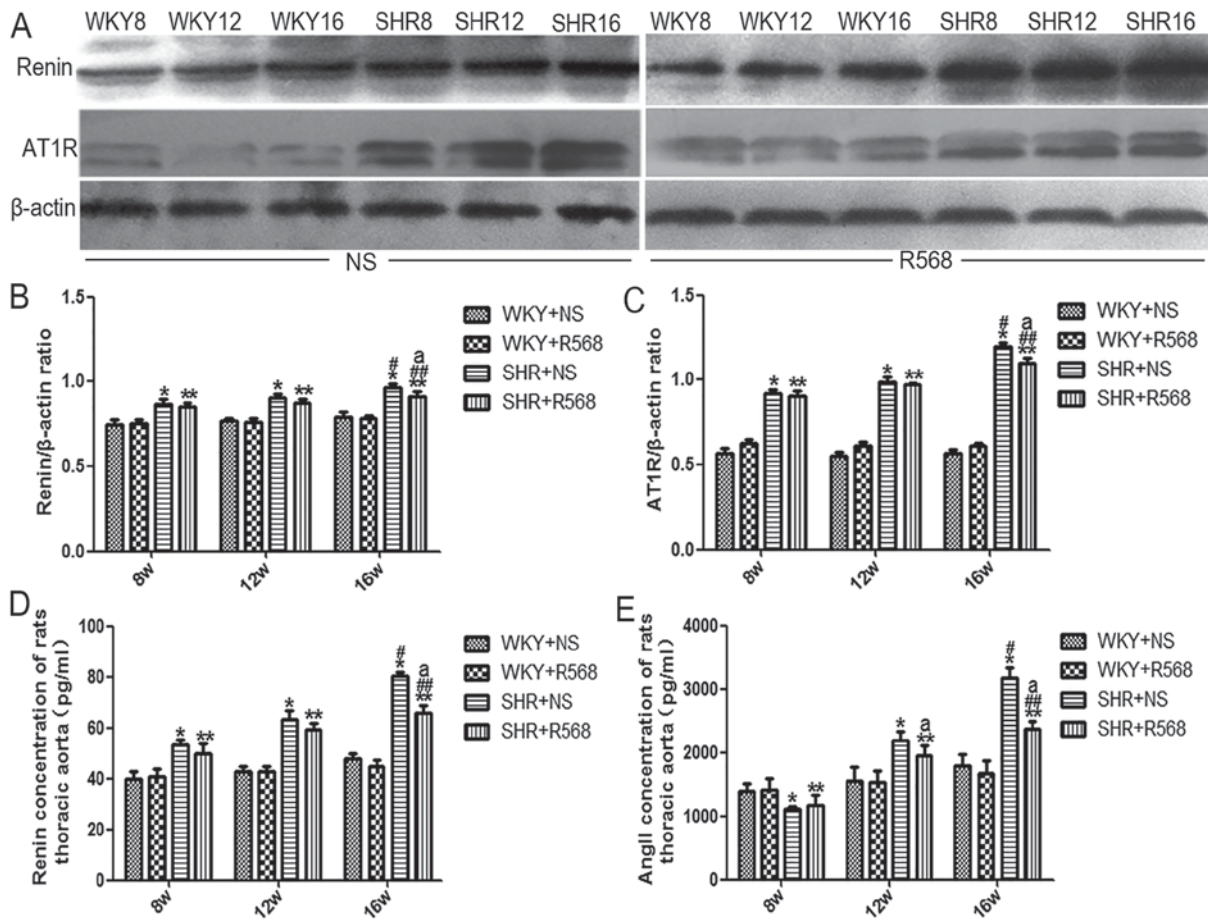
Figure 6. Determination of renin, AT1R protein expression and renin, Ang II levels in thoracic aorta of rats. (A) Western blotting analysis of Renin and AT1R expression. (B) Densitometric analysis of Renin expression. (C) Densitometric analysis of AT1R expression. (D) ELISA detection of renin concentration. (E) ELISA detection of Ang II concentration. Data are means ± standard deviation (n=7). SHR+NS groups vs. the age-matched WKY+NS groups, *P<0.05; SHR16w+NS groups vs. SHR8w+NS groups, #P<0.05; SHR+R568 groups vs. the age-matched WKY+R568 groups, **P<0.05; SHR16w+R568 groups vs. SHR8w+R568 groups, ##P<0.05; SHR+R568 groups vs. SHR+NS groups, aP<0.05. WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; NS, normal saline; R568, NPSR568; Ang II, Angiotensin II; AT1R, Ang II type 1 receptor.

As an adaptive response, the progression of hypertension is often accompanied by changes in the structure and function of blood vessels [i.e., vascular remodeling (VR)], which is an independent risk factor for the increased incidence of cardiovascular events in hypertension (23). The central part of VR is the proliferation of VSMCs, migration to the sub-endocardium, and secretion of the extracellular matrix (24). VSMCs play an important role in maintaining BP, but progression of hypertension leads to phenotypic changes in VSMCs. The conversion of VSMCs from a contractile phenotype to a synthetic phenotype leads to thickening of the vessel wall. In this experiment, we determined the expression of SMAα and calponin (two contractile/differentiated phenotypic markers) (25), OPN (a synthetic/dedifferentiated phenotypic marker) (26), and PCNA (an important marker of cell proliferation) (27) in the thoracic aortas of rats. Protein expression in thoracic aortas in WKY rats and SHRs was detected with immunohistochemistry and western blotting, respectively. We observed that the expression of α-SMA and calponin decreased, while OPN and PCNA increased significantly with increasing age in SHRs; however, these changes were inhibited by the CaSR agonist, R568. HE and Masson staining showed that the WA%, WT%, and CA% of SHR thoracic aortas were significantly increased

compared with WKY rats, which was reduced by R568. The above results indicate that R568 improved the proliferation and remodeling of thoracic aortas in SHRs.

Ziegelstein *et al* (28) suggested that the CaSR is expressed in aortic endothelial cells; whereas Smajilovic *et al* (29) demonstrated that the CaSR also exists in aortic VSMCs and that the CaSR can affect the proliferation of aortic VSMCs. Our previous study also put forward a similar view; expression of the CaSR protein in rat thoracic aorta VSMCs and the CaSR content were decreased with the increase in BP (9). In our experiments, similar results were obtained. We found that reduction in the CaSR could be reversed after treatment with R568 for 8 weeks in the plasma and thoracic aortas of SHRs rather than in thoracic aorta homogenates; however, the opposite result was observed in thoracic aorta homogenates when compared with SHR16w and WKY16w. Moreover, the same reverse trend was detected in CaSR protein expression in the thoracic aortas when SHR16w were compared with SHR8w. Unfortunately, no strong evidence has been found to explain this phenomenon. We presume this finding may be a compensatory manifestation for the increase of BP in SHRs, which provides a direction for further research in the future. Taken together, our results partially demonstrate that
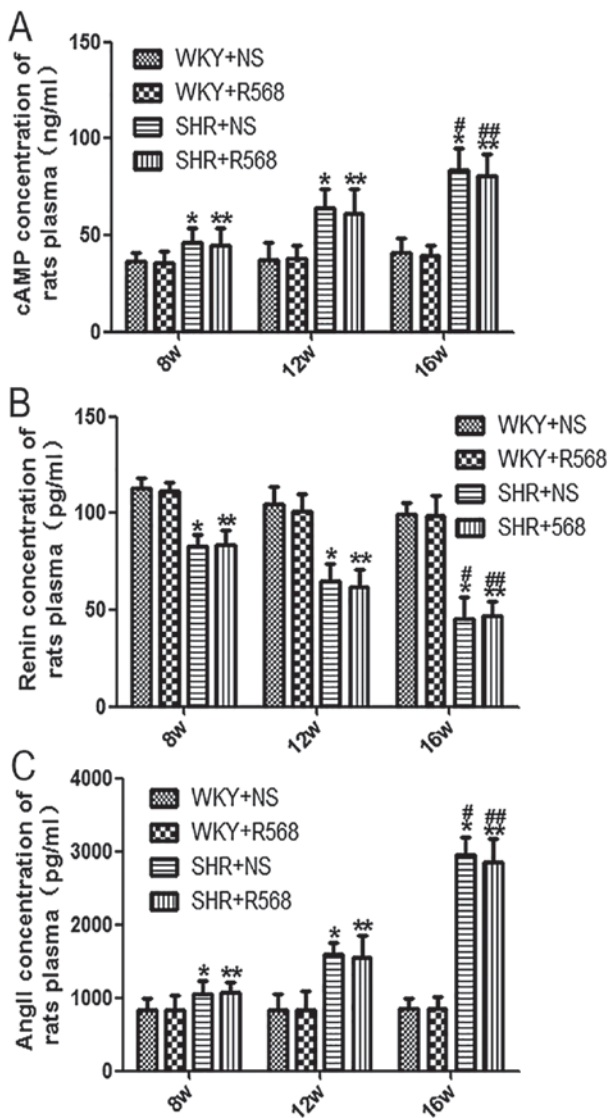
Figure 7. Determination of renin, Ang II and cAMP levels in plasma of rats. ELISA was used to detect the levels of (A) renin, (B) Ang II and (C) cAMP. Data are means ± standard deviation (n=7). SHR+NS groups vs. the age-matched WKY+NS groups, $^*P<0.05$; SHR16w+NS groups vs. SHR8w+NS groups, $^#P<0.05$; SHR+R568 groups vs. the age-matched WKY+R568 groups, $^{**}P<0.05$; SHR16w+R568 groups vs. SHR8w+R568 groups, $^{##}P<0.05$. WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; NS, normal saline; R568, NPSR568; Ang II, Angiotensin II; cAMP, cyclic adenosine monophosphate.

the CaSR agonist (R568) may play a protective role in EH, and EH evokes proliferation and remodeling of thoracic aortic cells in SHRs.

There is a complex network of contacts between the RAS, VR, and hypertension. Studies suggest that CaSR regulation of BP may involve six related pathways, but the molecular mechanism underlying this association has not been fully elucidated (30). To investigate the mechanism underlying the CaSR in hypertensive vascular remodeling, we selected the RAS pathway for further study, which is closely related to the development of clinical hypertension. Renin, which is closely linked to hypertension, is the first rate-limiting enzyme in the RAS, and cAMP plays a key role in this system (4). Alderman et al (31) divided hypertension into three types (high, low, and intermediate renin). At present, low renin

hypertension (LRH), a subtype of EH, has been unanimously approved by researchers (32). The majority of EH patients in China are LRH (33). Angiotensinogen produces angiotensin I (Ang I) under the action of renin and angiotensin converting enzyme (ACE) acts on Ang I to produce angiotensin II (Ang II) (34). Ang II is the main active substance of the RAS, and binds to Ang II type 1 receptor (AT1R), thus causing vasoconstriction and cell proliferation (35). AT1R mediates the biological effects of Ang II and plays an important role in the regulation of BP (36). RAS is an important body fluid regulating system in the body. Circulating RAS mainly exists in plasma, and local RAS exists in the heart, aorta, brain, kidneys, and many other tissues (37). Recent studies have shown that intracellular RAS plays a more important role in the function and regulation of local tissues under certain cell types or pathologic conditions (38,39). Local RAS operates independently of circulating RAS and many of the adverse effects after AT1R activation are caused by locally-generated Ang II (40). Blaine et al (41) believes that the vascular local RAS, through the independent regulation of vascular function, promotes the occurrence of hypertension. Unger et al (42) also suggests that Angiotensin converting enzyme inhibitors (ACEIs), such as captopril, reduce BP in SHRs by inhibiting local RAS activity. In our experiments, it was observed that renin, Ang II, and AT1R in thoracic aortas increased with the increase in BP, whereas R568 inhibited the increase in these factors. In circulating blood, renin was lower in SHRs, whereas Ang II and cAMP exhibited the opposite trend, which is consistent with our previous study (9). Unfortunately, R568 treatment failed to significantly alter renin and Ang II levels in plasma. Li et al (43) believe that in the early stage of hypertension, circulatory RAS is activated and involved in the initial rise of BP, but after 8 weeks, circulating blood RAS is decreased, while BP continues to rise; at which time RAS in the heart, brain, kidneys, and other local tissues actively participate in EH and target organ damage maintenance, which is consistent with our point of view. Meanwhile, Ferrario et al (44) also noted that the effects of RAS blockers on Ang II concentrations in circulating plasma and local tissues were different, suggesting that local RAS intervention may be viable in the prevention and treatment of related diseases. As mentioned above, our results suggested that the CaSR agonist (R568) is viable to suppress local RAS activity to lower BP and improve VSMC proliferation and remodeling.

The limitations of our experiments were a lack of inhibition of the CaSR activity with NPS2314 or Calhex231, and further observations regarding the relationship between BP, thoracic aortic proliferation, and remodeling and RAS activity are needed. Despite these limitations, our study partially explains the basic mechanism by which the CaSR mediates BP through the RAS pathway.

In summary, our results indicate that low expression of the CaSR is involved in the development of hypertension by cAMP pathway activation of circulatory and vascular local RAS. Regulation of the CaSR on BP in SHRs, but not WKY rats, may be through regulation of local RAS activity. The clinically used ACEIs and Ang II receptor blockers lower BP by inhibiting RAS activity, but have significant side effects. We found that R568 can reduce BP by activating the CaSR to regulate RAS activity, thus preventing the side effects of these

drugs. Continued studies are being conducted in our laboratory to further determine the mechanism by which the CaSR regulates BP at the molecular level. Our findings provide new insight into the pathogenesis of this complex disease and suggest that the CaSR may be a valuable pharmacologic target in patients with hypertension.

Acknowledgements

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

RS and FH contributed to the conception and design of the study. RS drafted the manuscript. WZ and NT performed the data analyses. HZ, LW and YL performed Masson's staining and immunohistochemical analysis. YZ and TZ performed and analyzed western blotting and ELISA. FH reviewed the manuscript and gave final approval to the submitted and final versions. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Shihezi Medical University (Shihezi, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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