Original Research

Calcimimetic R568 improved cardiac remodeling by classic and novel renin-angiotensin system in spontaneously hypertensive rats

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Impact statement

Our study reveals that low calcium-sensitive receptor (CaSR) expression is associated with the occurrence and development of essential hypertension-mediated myocardial remodeling. The activation of CaSR can reverse adverse myocardia remodeling by inhibiting local classical renin-angiotensin system (RAS) and activating novel RAS in cardiac tissues. CaSR is closely related to many cardiovascular diseases, but its specific mechanism remains not to be elucidated. To date, CaSR has not been investigated with regard to cardiovascular treatment; however, given the important relationship between CaSR and cardiovascular disease, CaSR regulators can be potential drugs for the treatment of cardiovascular disease.

Abstract

One major cause of cardiac mortality is heart disease caused by hypertension. The formation of cyclic adenosine monophosphate (cAMP) is inhibited by calcium-sensitive receptor (CaSR) activation which increases intracellular Ca^{2+} concentrations and suppresses renin release. As we know, renin-angiotensin system (RAS) is closely related to development of essential hypertension (EH). Therefore, we focused on exploring the roles of NPSR568 (R568)-activated CaSR in cardiac remodeling of spontaneously hypertensive rats (SHRs), as well as the activity of classic and novel RAS. Wistar-Kyoto rats (WKYs) and SHRs were treated by R568 for four and eight weeks, respectively, and their blood pressure (BP), echocardiographic values, heart-to-body weight ratio (HW/BW%), and left ventricle-to-body weight ratio (LVW/BW%) were evaluated. Then Masson's trichrome staining and hematoxylin and eosin staining as well as RT-qPCR analysis of β-isoform of myosin heavy chain and brain natriuretic peptide mRNA expression were performed. A Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay and analysis of apoptosis marker proteins were used to assess the extent of myocardial apoptosis. The CaSR expression and the activity of classic and novel RAS were examined by immunohistochemistry, western blotting, and enzyme-linked immunosorbent assay. The present study revealed that the development of hypertension was accompanied by increased BP, apoptosis, hypertrophy, and fibrosis, along with decreased expression of CaSR, decreased novel RAS, and increased classic RAS in myocardial tissues. R568 administration for four and eight weeks reduced BP and myocardial remodeling and reversed the low expression of CaSR; moreover, classic RAS was suppressed and novel RAS was activated in the myocardium. Taken together, these data indicate that R568 may effectively inhibit EH myocardial remodeling by inhibiting classic RAS and activating novel RAS in SHRs.

Keywords: Essential hypertension, cardiac remodeling, calcium-sensing receptor, NPSR568, renin-angiotensin system

Introduction

Hypertension is one of the most common types of cardiovascular disease; it seriously endangers human health, and 90–95% of cases are diagnosed as essential hypertension (EH).[1] Further development of hypertension may involve damage to target organs such as the heart, brain, and kidneys. Myocardial remodeling is one of the most important manifestations of hypertensive heart disease, which is defined as a change in the gene expression leading to molecular, cellular, and interstitial changes; clinical manifestations of cardiac hypertrophy; fibrosis; and decompensation of the heart. Ventricular hypertrophy is an independent risky factor for death in hypertensive patients.[2] Reversing cardiac hypertrophy can decrease the risk of cardiovascular complications in patients.

The renin-angiotensin system (RAS) is widely distributed throughout the human body. However, the pathological activation of RAS can lead to excessive vasoconstriction, vascular smooth muscle and myocardial hypertrophy, fibrosis, and extracellular matrix hyperplasia and remodeling. This system is first limited by renin, the secretion of which occurs through complex interactions between four different regulatory pathways, including intrarenal baroreceptors, sympathetic nerves, dense plaques, and several hormones and endocrine factors; the common factor in all these pathways is cAMP.[3] As research has progressed, new factors were identified in addition to those in the classic RAS family, which have been classified as novel RAS, including new members, pathways, and receptors such as ACE2 (angiotensin converting enzyme 2)/ angiotensin (1–7) (Ang (1–7))/ MasR (Mas receptor), ACE2/ angiotensin (1-9) (Ang(1-9))/ AT2R (angiotensin II type 2 receptor) pathways.[4] It has been proven that the physiological effect of novel RAS is antagonistic to that of classic RAS.

The relationship between Ca^{2+} and hypertension has long been known in a clinical setting. A low-calcium diet is a significant risk factor for hypertension, and blood pressure (BP) is effectively lowered by calcium intake.[5] This conclusion has also been confirmed in animal models.[6] However, the mechanism for this has remained unknown for a long time. Calcium-sensitive receptor (CaSR), an extracellular Ca^{2+} ($[Ca^{2+}]_o$) receptor, is a member of the C subfamily of the G-protein-coupled receptor (GPCR) superfamily, which is mainly distributed in parathyroid glands, bone, kidneys, and intestinal tissues, and is related to calcium metastasis and homeostasis.[7,8] Its main function is to maintain the homeostasis of systemic Ca^{2+} and other metal ions.[8] In 2003, the expression of CaSR was first confirmed in rat myocardia. Since then, many studies have confirmed the important role of CaSR in the process of myocardial hypertrophy, ischemia reperfusion injury, atherosclerosis, pulmonary hypertension, and other cardiovascular diseases.[9,10] Using neonatal cardiomyocyte models, it was found that reduced DNA synthesis was elicited by CaSR activation, indicating that CaSR may potentially inhibit hypertrophy.[11] In a model of angiotensin-II-induced hypertrophy, CaSR was found to be involved in cardiac hypertrophy through a Ca^{2+}-dependent phosphatase calcineurin pathway in cultured neonatal rat cardiomyocytes.[12] By employing isoproterenol-induced hypertrophy models, it was found cardiac hypertrophy was ameliorated by suppressed CaSR via inhibiting autophagy.[13] However, the methodologies used in the above cases showed limited success owing to the complexity of *in vivo* environment and that the hypertrophic signaling mechanism at this cell stage was only applicable to adult cardiomyocytes to a limited extent.[14] In addition, other studies have shown that CaSR may regulate BP. CaSR binding and activation of PLC (G-protein-phospholipase C)-IP3 (inositol 1,4,5-trisphosphate) receptor pathway are caused by increased $[Ca^{2+}]_o$, leading to raised intracellular Ca^{2+} ($[Ca^{2+}]_i$), which is closely related to hypertension development.[7] Atchison *et al.* have shown that $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ are inversely proportional to the secretion of renin from juxtaglomerular cells;[15] this mechanism is related to the finding that increased $[Ca^{2+}]_o$ inhibits adenylyl cyclase isoform V (AC-v), the generation of cAMP, and the release of renin by increasing $[Ca^{2+}]_i$ and calmodulin (CaM) activity.[16,17] Nevertheless, it remains unclear whether CaSR functions in cardiac hypertrophy related to RAS, classic RAS and/or novel RAS.

In this study, CaSR activity was disrupted by using NPSR568 (R568), an allosteric agonist of CaSR, to explore CaSR's effect on BP and myocardial remodeling in spontaneously hypertensive rats (SHRs), as well as its relationship with AC (cAMP content), classic RAS activity, and novel RAS activity.

Materials and methods

Animals and ethics statement

Male SHRs and Wistar-Kyoto rats (WKYs) (Vital River Laboratory Animal Science and Technology Co., Ltd, Beijing, P.R.C.) which were age-matched, aged 16 weeks and weighing approximately 300 g, were enrolled in this study. The SHR and WKY rats were classified into three groups randomly, namely WKY ($n = 15$), SHR + NS ($n = 15$), and SHR + R568 groups ($n = 15$). Because our previous study found that R568 had no effect on the BP of WKYs, WKY + R568 group was not used in this study.[18] Rats in each group were treated with the CaSR agonist R568 (Tocris Bioscience R&D Systems, Minneapolis, MN, USA) or NS (normal saline) (0.12 mg/100 g body weight/ day)[18] through intraperitoneal injection for 0 week (16-week-old), four weeks (20-week-old), and eight weeks (24-week-old). Rats were housed at the room temperature and humidity with a 12-h light/dark cycle and allowed access to tap water and standard chow *ad libitum*. Approval of the protocol was obtained from the Institutional Animal Research Committee of Shihezi Medical University, and Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health was followed.

Measurement of BP and the heart-to-body weight ratio

Mean arterial pressure (MAP), diastolic blood pressure (DBP), and systolic blood pressure (SBP) were examined by the tail-cuff method (BP-98A-L; Softron, Tokyo, Japan) at the identical time of one day once weekly, and the

average value of three measurements was recorded. Chloral hydrate (10%) was used for anesthesia, and blood specimens were taken from the rats' abdominal aorta. After centrifugation, the blood specimens were kept at –80°C after 16 weeks, 20 weeks, and 24 weeks. Then, the heart was removed and the left ventricle (LV) was resected to calculate the heart-to-body weight ratio (HW/BW%) and the left ventricle-to-body weight ratio (LVW/BW%).

Hematoxylin and eosin staining

The heart tissues were fixed in neutral buffered formalin (10%), and then embedded in paraffin. They were sliced into sections (5-μm thick) and stained by hematoxylin and eosin (H&E). Finally, the cross-sectional area of myocytes and extent of cardiac hypertrophy were measured.

Masson's trichrome staining

Masson's trichrome staining was performed on the cardiac sections for collagen fibers. Then, three fields were selected randomly from each slice, and we calculated the ratio of collagen area to the total cardiac area. The cardiac tissues were photographed using a microscope (Olympus BX40), and Image-Pro Plus 6.0 software (provided by Media Cybernetics Inc., Buckinghamshire, United Kingdom) was used for analysis.

Real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was extracted from tissues in each group and 3 μg total RNA was reversely transcribed to cDNA (Tiangen Biotech, Shanghai, China) based on the manufacturer's manuals. RT-qPCR was performed in a reaction system (20-μL) which contained SYBR Green/Fluorescein qPCR Master Mix, forward primers, reverse primers, and cDNA. The relative gene expression was normalized to *GAPDH* (the internal reference gene). The ABI7500 real-time PCR system (provided by Applied Biosystems, CA, America) was employed for PCR. The primers for the skeletal isoform of *β-isoform of myosin heavy chain* (*β-MHC*), brain natriuretic peptide (*BNP*), and *GAPDH* were as follows:

β-MHC: forward, 5′-AAGGCCAAGATCGAGACGG-3′; reverse, 5′-CCACTTATAGGGGGTTGACGGTG-3′.
BNP: forward, 5′-TCCAGGAGAGACTTCGAAATTC-3′; reverse, 5′-GCAAGTTTGTGCTGGAAGATAA-3′.
GAPDH: forward, 5′-TGGCCTTCCGTGTTCCTAC-3′; reverse, 5′-GAGTTGCTGTTGAAGTCGCA-3′.

Alterations in the mRNA expression in cardiac tissues were assessed by the $2^{-\Delta\Delta Ct}$ method.

Assessment of heart function and cardiac structures

After weighing, live rats were intraperitoneally injected with 10% chloral hydrate at a dose of 350 mg/kg. The limbs of the rats were fixed on the operation table, chest fur was shaved on the left side, a small amount of coupling agents was applied, and a probe was placed on the left side of the chest for M-mode imaging. An animal-specific instrument (VisualSonics Vevo3100, VisualSonics Inc., Toronto, Canada) was employed to record left ventricular M-mode images; at least five consecutive cardiac cycles were sampled, and sampling was repeated three times. Left ventricular end-systolic diameter (LVIDs), left ventricular end-diastolic posterior wall thickness (LVPWd), left ventricular end-diastolic diameter (LVIDd), percent fractional shortening (FS%), and percent ejection fraction (EF%) were measured to evaluate the extent of hypertrophy and function of the heart at 24 weeks.

TUNEL assay

To determine cardiac apoptosis, a TUNEL assay was carried out with *In Situ* Cell Death Detection Kit (POD Protocol; Roche Diagnostics, Basel, Switzerland), and the manufacturer's manual was followed. Three to four slides were selected from each group for TUNEL assays, and negative controls (no nucleotide or enzyme) were set up. High-power fields (×200) were chosen randomly under the microscope, and the mean number of TUNEL-positive cells was determined by normalization to that of WKY samples.

Immunohistochemistry analysis

The expression of CaSR and renin in cardiac tissues was detected by immunohistochemistry. After deparaffinization, cardiac sections were subjected to incubation with primary mouse monoclonal antibodies anti-CaSR (provided by Abcam, Cambridge, UK; 1:50) and primary rabbit polyclonal antibodies anti-renin (provided by Biosynthesis, Beijing, China; 1:50) at four centigrade degree overnight. Then the sections were subjected to incubation with the corresponding horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) at 37°C for half an hour. Diaminobenzidine/peroxidase substrate was utilized for color development. Integral optical density/myocardial area was employed to analyze the positive staining of tissue sections. Negative control sections were incubated with the secondary antibody alone.

Enzyme-linked immunosorbent assay

Plasma samples were extracted as described above. Enzyme immunoassay (commercially available) was employed to detect angiotensin II (Ang II), renin, cAMP (Elabscience Biotechnology, Wuhan, China), and Ang (1–7), Ang (1–9) (USCN, Wuhan, China) levels in plasma and heart tissues. A microplate reader (purchased from Bio-Rad Laboratories, Hercules, America; Bio-Rad Model 3550-UV) was used to measure the absorbance at a wavelength of 450 nm.

Western blotting

From the frozen LV tissues, proteins were extracted, and the concentrations of which were measured with a Bicinchoninc acid (BCA) protein assay. Samples were subjected to incubation with primary antibodies anti-

CaSR (provided by Abcam; 1:800), renin (provided by Bioss, Beijing, China; 1:250), AT2R (provided by Abcam; 1:800), angiotensin II type 1 receptor (AT1R) (provided by Abcam; 1:800), MasR (Abcam; 1:800), B cell leukemia-2 (Bcl-2) (provided by Cell Signaling Technology, Danvers, America; 1:1000), Bcl-2-associated X protein (Bax) (provided by Cell Signaling Technology; 1:800), and caspase-3 (provided by Abcam; 1:500), and normalized to GAPDH (provided by Sugisuke Bridge, China; 1:1000). Bio-Rad Quantity One software (provided by Bio-Rad) was used to quantify the intensity of protein bands.

Statistical analysis

Quantitative data were described as the mean ± SEM and one-way analysis of variance (ANOVA) was used for the comparison among multiple experimental groups, followed by Bonferroni *post hoc* tests. $P < 0.05$ indicated significant difference. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was employed for statistical analysis.

Results

R568 reduces BP in SHRs

The average SBP, DBP, and MAP in SHR+NS group were higher than that in WKYs group ($P < 0.05$). Additionally, treatment with R568 reduced the SBP, DBP, and MAP at 20 weeks and 24 weeks ($P < 0.05$). There were no significant differences between SHR+NS groups ($P > 0.05$; Figure 1).

R568 reverses myocardial hypertrophy and fibrosis

The HW/BW% and LVW/BW% were used as parameters to reflect the size of the entire heart and to identify hypertrophy in the LV. In SHR+NS group, there was an increase in the average HW/BW% and LVW/BW% at various time points ($P < 0.05$). The ratios in SHR24w+NS group were significantly higher compared to SHR16w+NS group ($P < 0.05$). The HW/BW% and LVW/BW% were reduced in SHR+R568 at both 20 weeks and 24 weeks ($P < 0.05$; see Figure 2(a) and (b)). As shown in Figure 2(c) and (d), H&E staining revealed increased cross-sectional area of cardiocytes in SHR+NS groups compared to age-matched WKYs ($P < 0.05$). These changes were attenuated following R568 injection after four and eight weeks ($P < 0.05$), as is seen in

Figure 2(c) and (d). Besides morphological alterations, RT-qPCR was employed for determination of the mRNA expression related to cardiac hypertrophy. The expression of *β-MHC* and *BNP* mRNA in SHR+NS group was significantly higher compared to WKYs group ($P < 0.05$). Moreover, their expression was decreased in SHR+R568 group at 20 and 24 weeks ($P < 0.05$; Figure 2(e) and (f)). Masson's trichrome staining revealed an increase in interstitial fibrin and collagen in the SHR+NS group compared to age-matched WKYs group ($P < 0.05$). These changes in the extracellular matrix were significantly reversed in SHR+R568 group at 20 and 24 weeks ($P < 0.05$; Figure 2 (g) and (h)).

R568 reduces LVPWd but exhibits no effect on cardiac function in SHRs

The degree of myocardial hypertrophy and malfunction was detected by *in vivo* echocardiographic measurements at 24 weeks (Figure 3(a)). The results showed an increase in LVPWd, a significant reduction in LVIDs and LVIDd in SHR+NS group compared to WKYs group ($P < 0.05$; Figure 3(b) to (d)). The change in LVPWd was reversed after fight weeks of treatment with R568 ($P < 0.05$; Figure 3(b)); nevertheless, there were no significant differences in LVIDs and LVIDd after R568 treatment for eight weeks ($P > 0.05$; Figure 3(c) and (d)). Other parameters, including EF% and FS%, were not significantly different in any groups ($P > 0.05$; Figure 3(e) and (f)), indicating that cardiac function was not significantly affected in SHR+NS group at 24 weeks, possibly due to the powerful compensatory mechanisms of the heart.

R568 reduces myocardial apoptosis

Several TUNEL-positive myocytes were found in SHR+NS group; in contrast, there were less TUNEL-positive myocytes in WKYs group ($P < 0.05$; Figure 4(a) and (b)). SHR+R568 group showed a reduction in the TUNEL-positive cell count at 20 and 24 weeks ($P < 0.05$), as shown in Figure 4(a) and (b). To further investigate this phenomenon, expressions of apoptosis marker proteins were analyzed. The results demonstrated a reduced expression of Bcl-2 in the SHR+NS group compared to age-matched WKYs group; conversely, the expression of Bcl-2
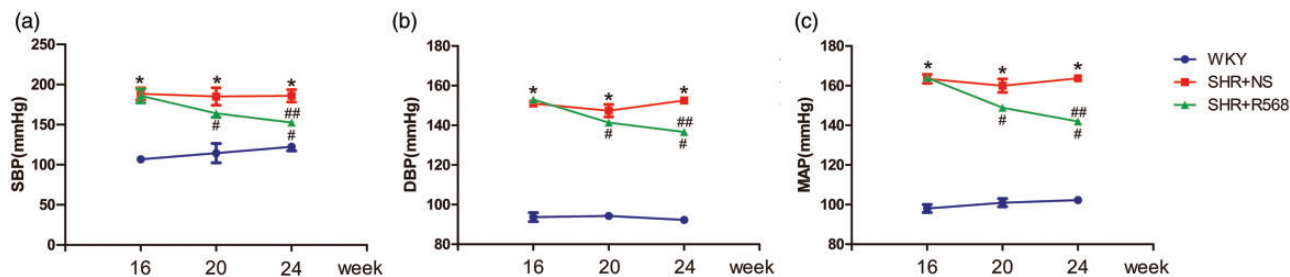

Figure 1. Comparison of SBP, DBP, and MAP of rats. (a) SBP; (b) DBP; (c) MAP. Values, mean ± SEM; $n = 5$; *$P < 0.05$, SHR+NS groups vs. the age-matched WKY groups; #$P < 0.05$, SHR+R568 groups vs. the age-matched SHR+NS groups; ##$P < 0.05$, SHR24w+R568 groups vs. SHR16w+R568 groups. (A color version of this figure is available in the online journal.)
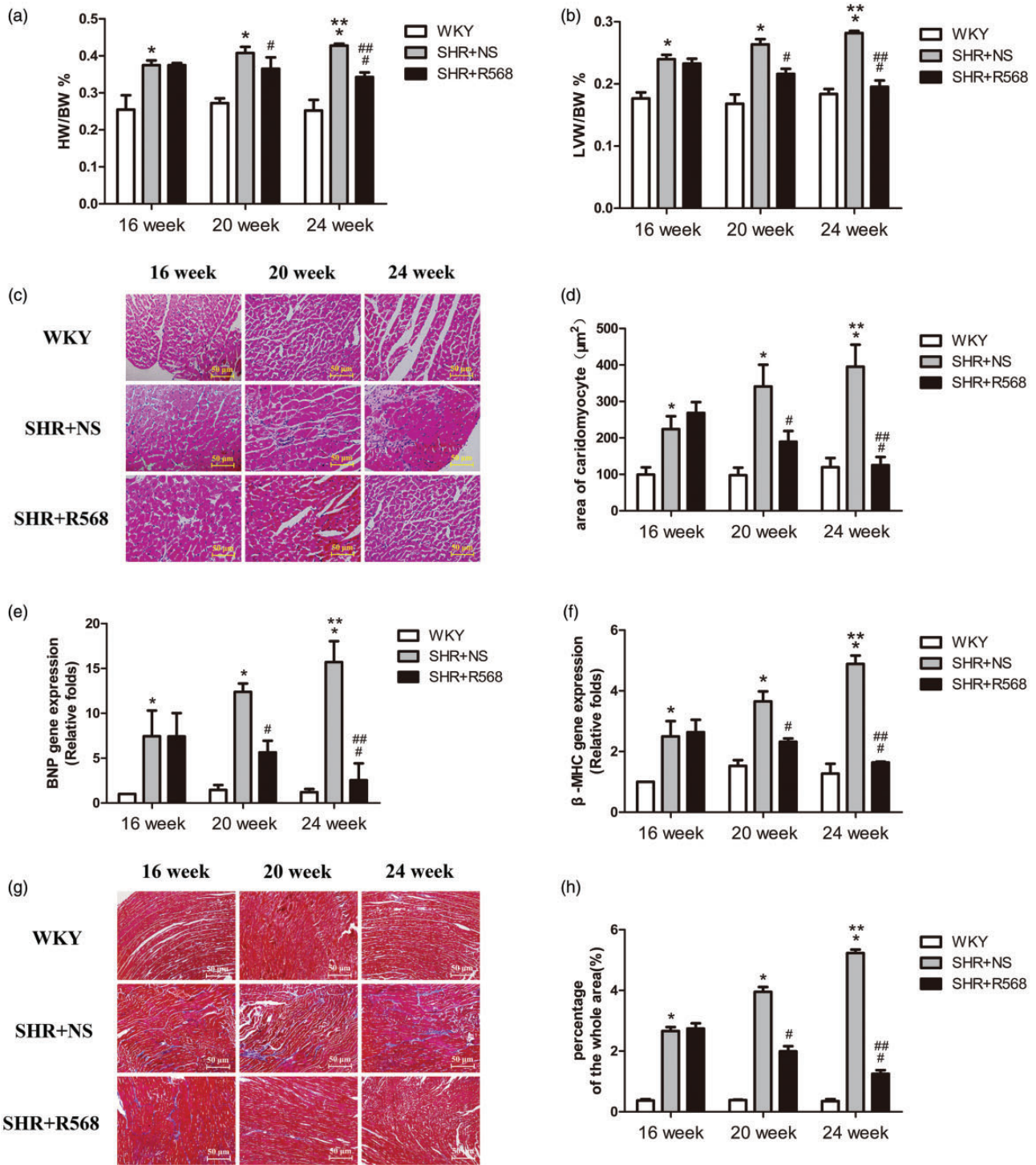
Figure 2. Assessment of myocardial hypertrophy and fibrosis between different groups of rats. (a) HW/BW%; (b) LVW/BW%; (c) representative images of H&E staining; (d) quantitative analysis of the cell size (μm^2) of cardiac myocytes in all groups; (e,f) RT-qPCR analysis of cardiac-specific fetal genes BNP and β-MHC; (g) representative images of Masson's trichrome stain; (h) quantitative analysis of fibrosis in all groups. Values, mean $\pm$ SEM; $n = 5$; *$P < 0.05$, SHR+NS groups vs. the age-matched WKY groups; #$P < 0.05$, SHR+R568 groups vs. the age-matched SHR+NS groups; **$P < 0.05$, SHR24w+NS groups vs. SHR16w+NS groups; ##$P < 0.05$, SHR24w+R568 groups vs. SHR16w+R568 groups. HW/BW%: heart-to-body weight ratio; LVW/BW%: left ventricle-to-body weight ratio; Images $\times 200$ power, scale bars $= 50$ μm. (A color version of this figure is available in the online journal.)

was increased significantly in SHR+R568 group at 20 and 24 weeks ($P < 0.05$; Figure 4(c) and (d)). Furthermore, Bax was significantly upregulated in SHR+NS group compared to age-matched WKYs group; this increase in the

expression was reversed in SHR+R568 group at 20 and 24 weeks ($P < 0.05$), as shown in Figure 4(c) and (e). Moreover, the changes in the caspase-3 expression were consistent with those of Bax ($P < 0.05$), as revealed by Figure 4(c)
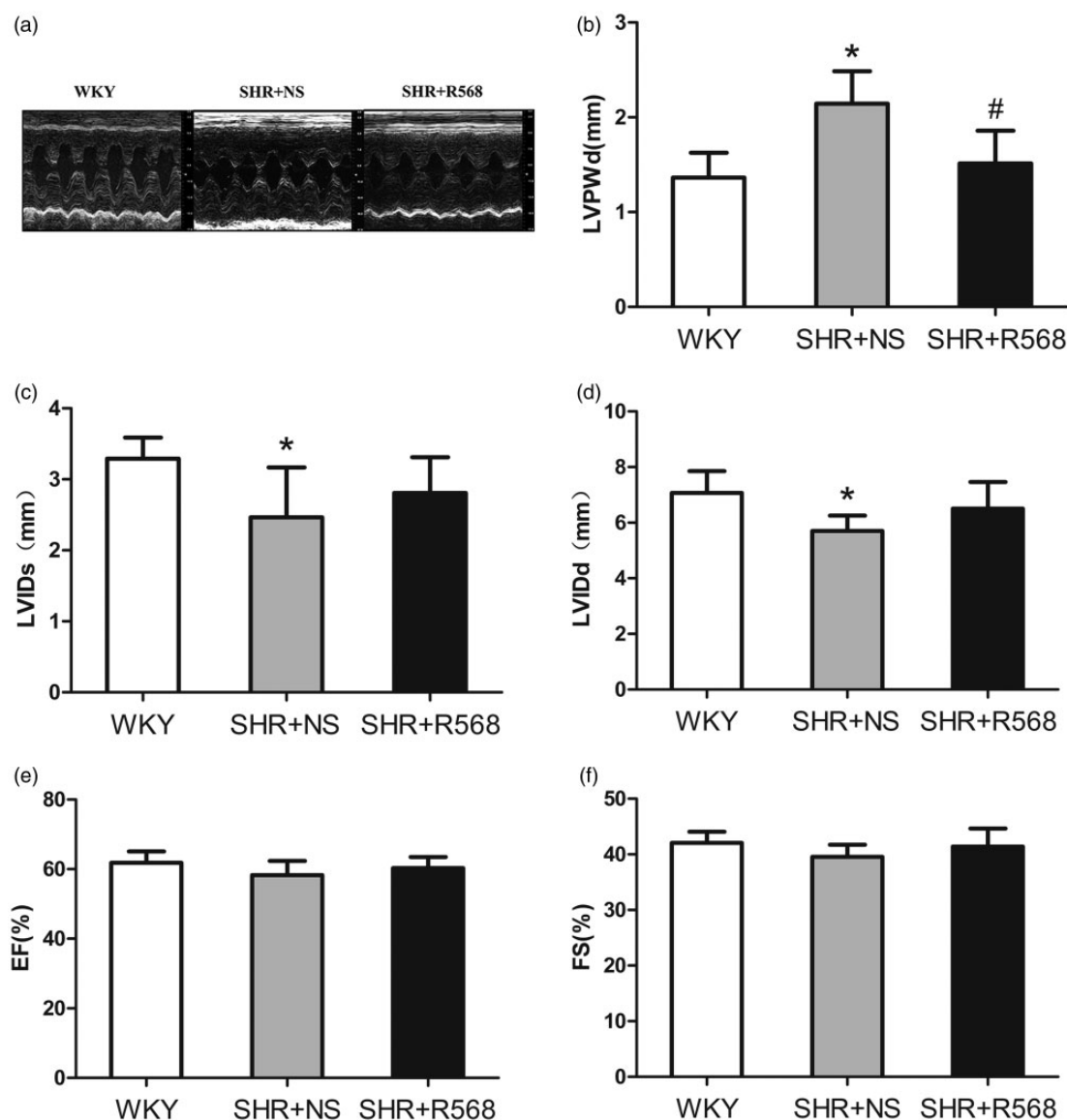
Figure 3. Echocardiographic data of LVPWd, LVIDs, LVIDd, EF%, and FS% of rats. (a) Images of M-mode of LV; (b–f) LVPWd, LVIDs, LVIDd, EF%, FS%. Values, mean ± SEM; $n = 5$; *$P < 0.05$, SHR+NS groups vs. the age-matched WKY groups; #$P < 0.05$, SHR+R568 groups vs. the age-matched SHR+NS groups. LVPWd: left ventricular end-diastolic posterior wall dimension; LVIDs: left ventricular end-systolic diameter; LVIDd: left ventricular end-diastolic diameter; EF%: ejection fraction; FS%: fractional shortening.)

and (f). The data above suggested R568 might reduce myocardial apoptosis in SHRs.

The low expression of CaSR is reversed by R568 in cardiac tissues of SHRs

Immunohistochemistry (Figure 5(a) and (b)) and western blotting analysis (Figure 5(c) and (d)) of rat cardiac tissues showed that the CaSR expression in SHR+NS group was lower compared to WKYs group, with significant difference ($P < 0.05$). Moreover, the CaSR expression in SHR24w+NS group was also lower compared to SHR16w+NS group, with significant difference ($P < 0.05$). In SHR+R568 group, the expression of CaSR was significantly higher at 20 and 24 weeks ($P < 0.05$).

R568 down-regulates AT1R and renin expressions while levels of cAMP, Ang II, and renin in SHR myocardia are decreased

Immunohistochemistry of rat cardiac tissues indicated that the renin expression in SHR+NS group was increased compared to WKYs group, with significant difference ($P < 0.05$); furthermore, the renin expression in SHR24w+NS group was higher compared to SHR16w+NS group, with significant difference ($P < 0.05$). The expression of renin in SHR+R568 group was significantly lower compared to SHR+NS group at 20 and 24 weeks ($P < 0.05$), as shown in Figure 6(a) and (c). The findings above were consistent with western blot analysis results of renin and AT1R protein expressions ($P < 0.05$; Figure 6(b), (d), and (e)). Furthermore, enzyme-linked immunosorbent assay (ELISA) results indicated that Ang II and cAMP levels of
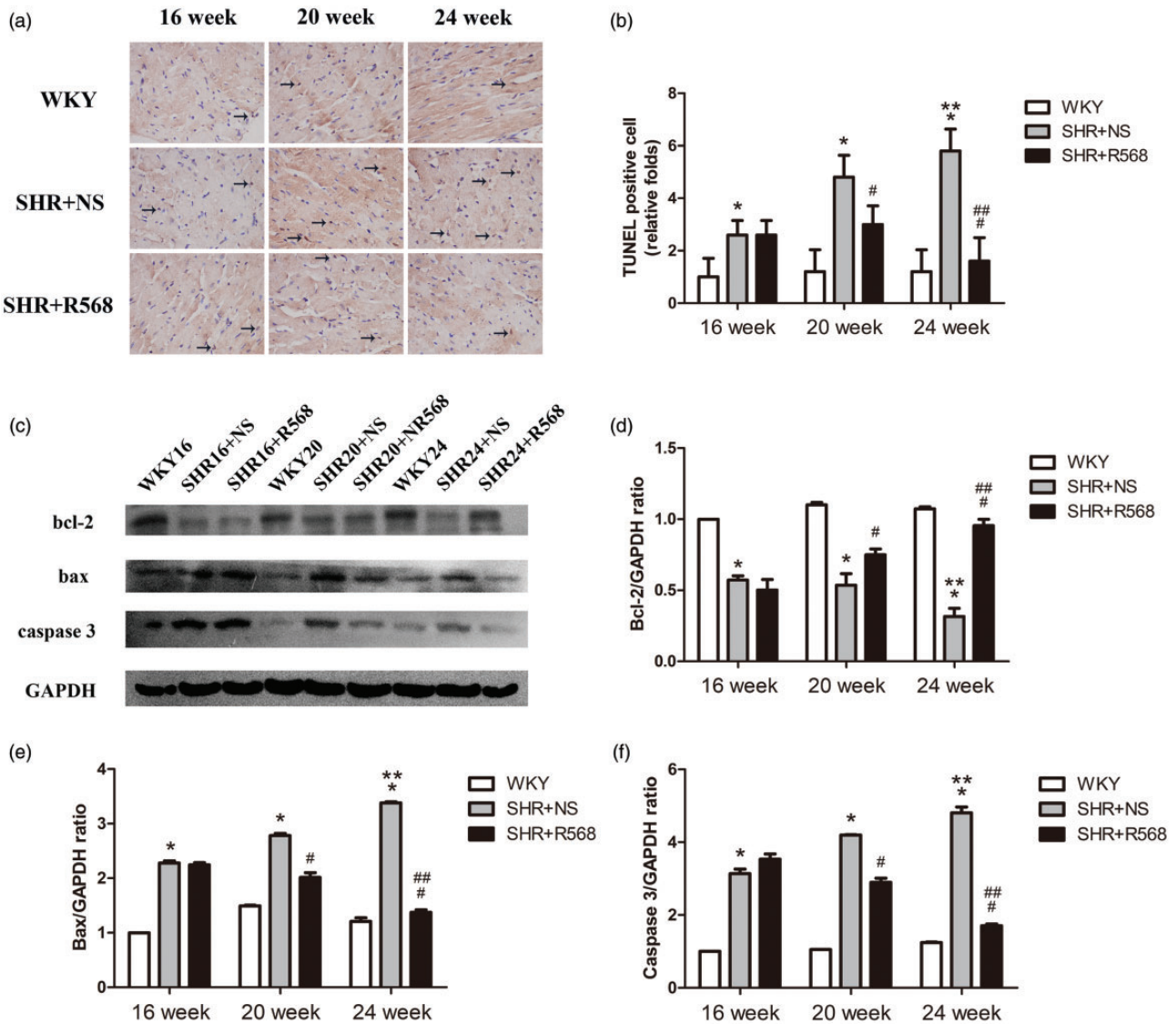
Figure 4. Detection of myocardial cell apoptosis in all groups. (a) Representative images of TUNEL staining, arrow indicating a TUNEL-positive cardiomyocyte in brown color; (b) quantitative analysis of TUNEL-positive cardiac myocytes in all groups and normalized to the WKY controls; (c) apoptotic protein expression of left ventricle by western blot in all groups; (d–f) densitometric analysis of (c). Values, mean ± SEM; $n = 5$; *$P < 0.05$, SHR+NS groups vs. the age-matched WKY groups; #$P < 0.05$, SHR+R568 groups vs. the age-matched SHR+NS groups; **$P < 0.05$, SHR24w+NS groups vs. SHR16w+NS groups; ##$P < 0.05$, SHR24w+R568 groups vs. SHR16w+R568 groups. Images ×200 power. (A color version of this figure is available in the online journal.)

rat plasma in SHR+NS group were higher compared to WKYs group, with significant difference ($P < 0.05$; see Figure 6(f) and (h)), while renin levels of rat plasma were lower in SHR+NS group compared to WKYs group, with significant difference ($P < 0.05$; see Figure 6(g)); the levels of Ang II, renin, and cAMP were not significantly different between SHR+NS and SHR+R568 groups ($P > 0.05$; Figure 6(f) to (h)). However, the opposite was observed in myocardia, where the levels of the above factors in SHR+NS group were higher compared to WKYs group, with significant difference ($P < 0.05$; Figure 6(i) to (k)); their expression in SHR24w+NS group was higher than those in SHR16w+NS group. Meanwhile, these changes were significantly reversed in SHR+R568 group at 20 and 24 weeks ($P < 0.05$; Figure 6(i) to (k)).

R568 increases the level of Ang (1–9) and Ang (1–7) in myocardial tissues of SHRs

To further explore the potential role of novel RAS, the expression of MasR and AT2R, two novel RAS-related proteins, was evaluated. Unfortunately, neither of them showed any significant differences in any groups ($P > 0.05$; Figure 7(a) to (c)). Next, Ang (1–9) and Ang (1–7) levels in plasma and myocardia were analyzed by ELISA. The levels of Ang (1–9) and Ang (1–7) were all reduced in the plasma and myocardia of SHR+NS group compared to WKYs group ($P < 0.05$; see Figure 7(d) to (g)). Treatment with R568 resulted in increased Ang (1–7) and Ang (1–9) expression levels in myocardia ($P < 0.05$; see Figure 7(e) and (g)), but showed no significant effects in plasma ($P > 0.05$), as shown in Figure 7(d) and (f). This
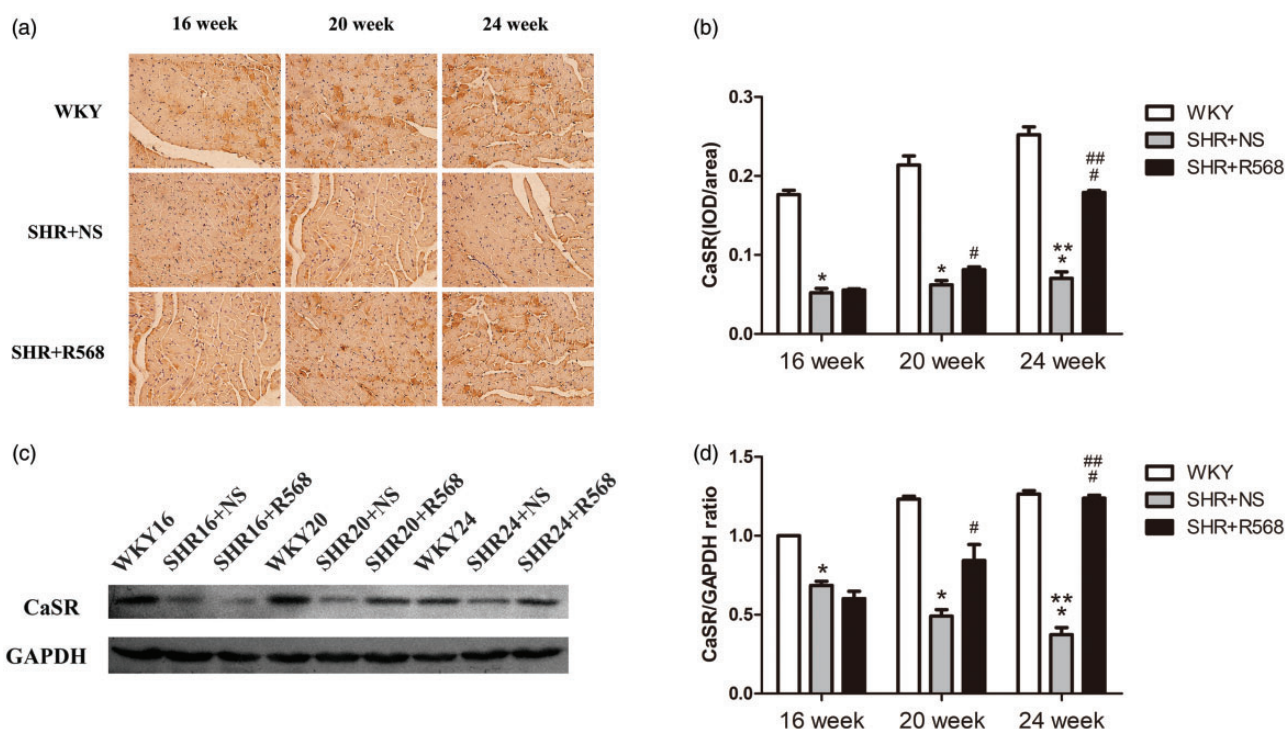
Figure 5. Detection of CaSR protein expression in heart tissue of rats. (a) Immunohistochemical analysis; (b) densitometric analysis of (a); (c) western blotting analysis; (d) densitometric analysis of (c). Values, mean ± SEM; $n = 5$; *$P < 0.05$, SHR+NS groups vs. the age-matched WKY groups; #$P < 0.05$, SHR+R568 groups vs. the age-matched SHR+NS groups; **$P < 0.05$, SHR24w+NS groups vs. SHR16w+NS groups; ##$P < 0.05$, SHR24w+R568 groups vs. SHR16w+R568 groups. Images ×200 power. (A color version of this figure is available in the online journal.)

indicated that R568 might play a role in activating novel RAS in cardiac tissues.

Discussion

For the first time, our study provides a characterization of hypertensive myocardial remodeling through molecular mechanisms caused by downregulated CaSR. We demonstrated that BP, apoptosis, cardiac hypertrophy, and fibrosis significantly increased with the age of SHRs, as well as with decreased CaSR expression; the selective activation of CaSR by NPSR568 in the myocardium is a crucial trigger to reverse adverse cardiac remodeling in the heart. Furthermore, we found that the role of CaSR in hypertensive myocardial remodeling is mediated by the cAMP-RAS pathway, including both classic and novel RAS. Moreover, we determined a critical target for RAS signaling pathways to block EH-mediated cardiac remodeling and cardiac malfunction in the absence of RAS signaling pathway blockers.

Previous research has shown that CaSR may regulate the remodeling of the thoracic aorta in hypertensive rats by regulating local RAS activity.[18] It is still unknown whether it would be associated with hypertensive cardiac remodeling; the specific mechanism controlling this also remains unknown. In this study, we provided structural, functional, and molecular evidence for mechanisms controlling cardiac remodeling. We clearly demonstrated that BP, myocardial apoptosis, hypertrophy, fibrosis, and cardiac function were aggravated with increased age in SHRs. In addition, the results of echocardiography showed that LVPWd was significantly thickened, LVIDs and LVIDd were decreased,

and EF% and FS% were not significantly changed in SHRs; this indicates that the rats selected in our study were in the early stages of compensatory hypertrophy, which is characterized by hypertension. Heart function was not majorly affected during this period, making it ideal for drug interventions to reverse adverse myocardial remodeling. The Bcl-2 protein family is one of the primary regulatory factors for apoptosis.[19,20] Bcl-2 inhibits apoptosis and Bax promotes apoptosis;[21] together, these factors contribute to the regulation of caspase-3, which determines whether cells will initiate apoptosis. Indeed, we identified higher percentages of TUNEL-positive cells and increased caspase-3 protein expression in the myocardia of SHRs. Gonzalez et al.[22] performed a biopsy on the myocardial tissues of hypertensive patients with heart failure and hypertensive patients with left heart hypertrophy and found that apoptosis may play a significant role in the development of heart failure and myocardial remodeling. Studies have reported that the myocardial apoptosis index increased in SHRs with heart failure compared to WKY control rats with normal BP; this is considered to be one of the main mechanisms for the transition from cardiac compensatory hypertrophy to heart failure. The pathways of apoptosis are closely related and complex; further study is required to uncover interventions that can reduce apoptosis in myocardial cells.

In addition to the enhancement of BP, apoptosis, cardiac hypertrophy, and myocardial fibrosis in SHRs, we found that decreased levels of CaSR occurred in conjunction with the above changes, indicating that the low expression of CaSR may be associated with the progression of
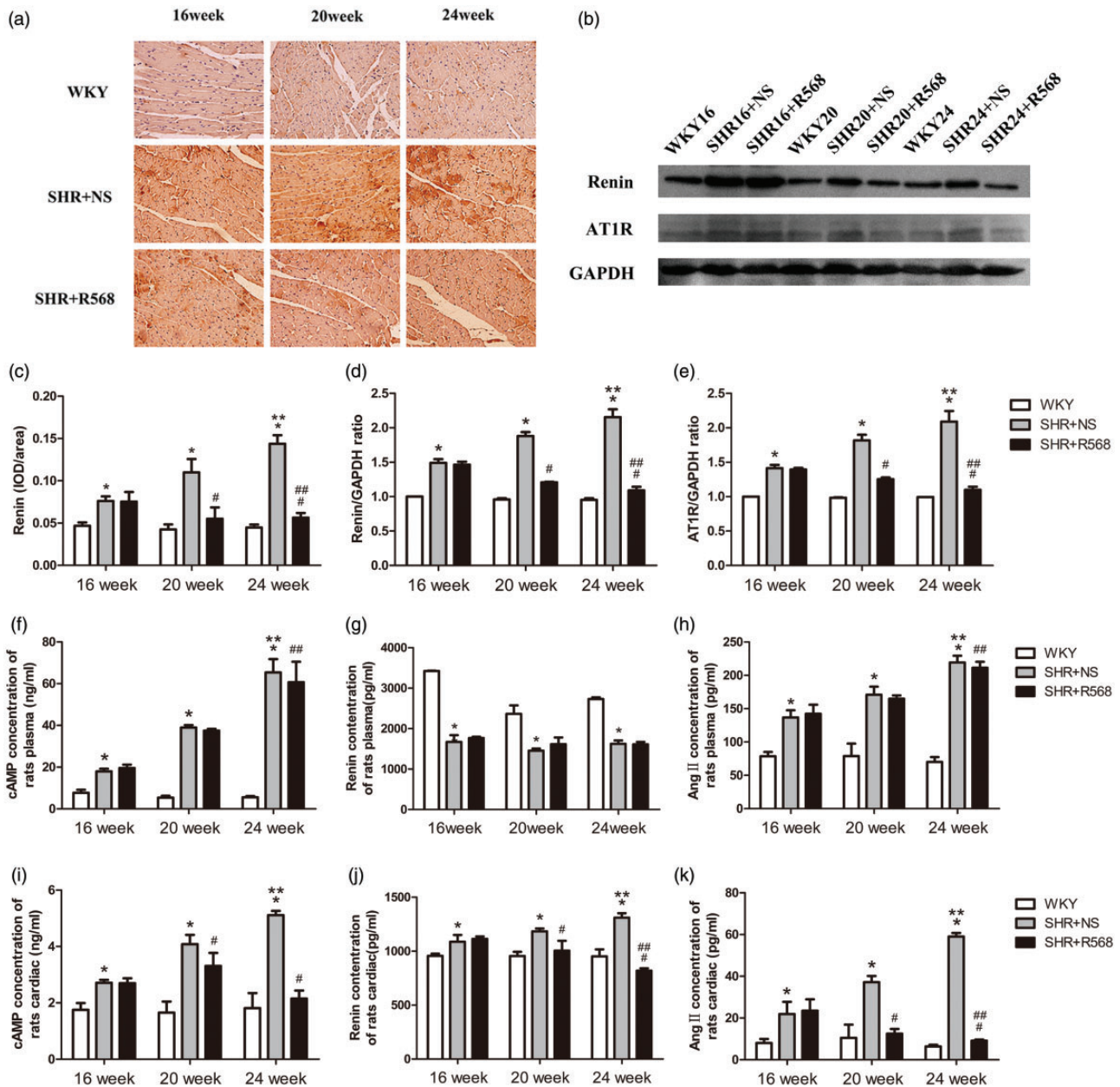
Figure 6. Determination of renin, AT1R protein expression and cAMP, renin, Ang II levels in plasma and cardiac of rats. (a) Immunohistochemical analysis of renin; (b) western blotting analysis of renin and AT1R; (c) densitometric analysis of (a); (d,e) densitometric analysis of (b); (f–h) ELISA detection of cAMP, renin, Ang II concentration in plasma; (i–k) ELISA detection of cAMP, renin, Ang II concentration in cardiac. Values, mean ± SEM; $n = 5$; $*P < 0.05$, SHR+NS groups vs. the age-matched WKY groups; $#P < 0.05$, SHR+R568 groups vs. the age-matched SHR+NS groups; $**P < 0.05$, SHR24w+NS groups vs. SHR16w+NS groups; $##P < 0.05$, SHR24w+R568 groups vs. SHR16w+R568 groups. Images ×200 power. (A color version of this figure is available in the online journal.)

hypertension and may promote cardiac remodeling. CaSR, which belongs to the GPCR superfamily, activates the PLC-IP3 signaling pathway and elicits increased $[Ca^{2+}]_i$ concentrations, hence exerting biological effects.[8] It has been reported that CaSR participates in development of myocardial ischemia-reperfusion injury, apoptosis, and myocardial hypertrophy.[12,23,24] For example, CaSR expression levels have been found to be increased in Ang II-induced heart hypertrophy and ischemia reperfusion-triggered myocardial apoptosis.[25] Sun and Murphy[26] found that CaSR plays a protective role in myocardial ischemic preconditioning, which shows that CaSR plays different roles in

different heart diseases. Subsequently, Ogata *et al.*[27,28] found that the CaSR isomorphic agonist R568 successfully reduced the BP of uremia rats and SHRs; however, it showed no antihypertensive effect in rats with normal BP, and its mechanism of action remains unknown. According to our results, CaSR activation using R568 treatment declined BP and reversed adverse cardiac remodeling in SHRs. The relationship between EH and CaSR demonstrates CaSR may play an important role in myocardial remodeling in the SHR model.

It is well known that RAS is closely related to the progression of cardiovascular disease. RAS system is first
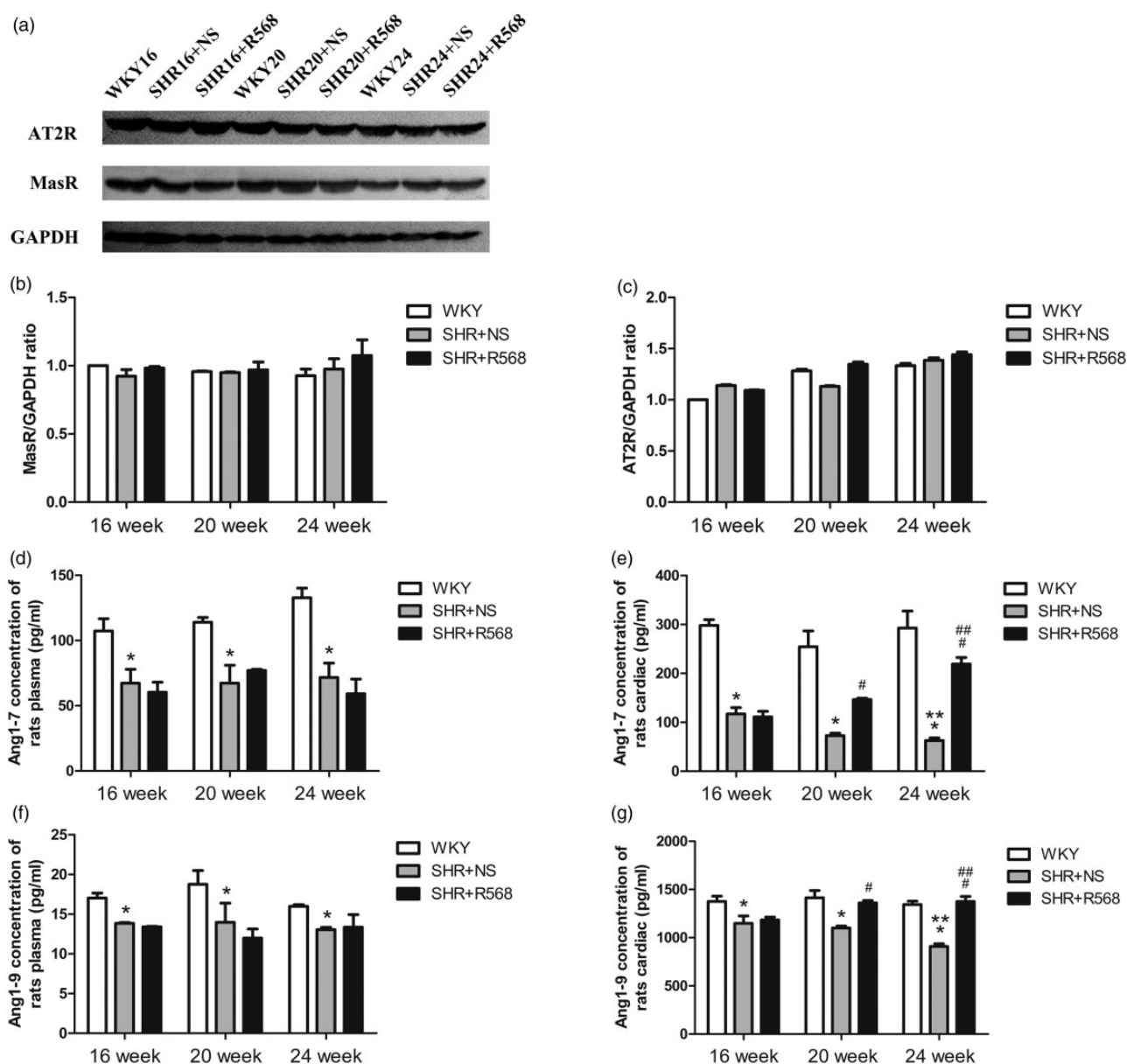
Figure 7. Determination of novel RAS related protein expression of MasR, AT2R, and level of Ang (1–7), Ang (1–9) of rats. (a) Western blotting analysis of MasR and AT2R; (b,c) densitometric analysis of (a); (d,e) ELISA detection of Ang (1–7) concentration in plasma and cardiac tissues; (f,g) ELISA detection of Ang (1–9) concentration in plasma and cardiac tissues. Values, mean ± SEM; $n = 5$; $^*P < 0.05$, SHR+NS groups vs. the age-matched WKY groups; $^\#P < 0.05$, SHR+R568 groups vs. the age-matched SHR+NS groups; $^{**}P < 0.05$, SHR24w+NS groups vs. SHR16w+NS groups; $^{\#\#}P < 0.05$, SHR24w+R568 groups vs. SHR16w+R568 groups.

limited by renin, and cAMP is significant for this system. Ang II is the primary factor in RAS, as the binding of Ang II to AT1R elicits Gq activation, hence resulting in PLC stimulation, $[Ca^{2+}]_i$ mobilization, production of diacylglycerol (DAG) and IP3, and downstream cellular effectors activation.[29] AT1R activation explains most classical effects of Ang II, including water and sodium reabsorption, vasoconstriction, cell proliferation, cell growth, and extracellular matrix deposition.[30] RAS channel blockers, although highly effective for the clinical treatment of hypertension and target organ damage, should be administered in combination with other treatments for the majority of hypertension patients in order to achieve effective antihypertensive effects. Therefore, finding new targets to disrupt the RAS pathway would contribute to novel preventive and

therapeutic strategies of hypertension and target organ damage. Maillard *et al.* previously reported that the release of renin can be regulated by calcimimetic R568 through CaSR;[31] based on this, the mechanisms of CaSR acting in hypertensive cardiac remodeling were investigated from perspectives of the RAS pathway. RAS can be divided into circulatory RAS and local RAS. The former mainly affects the plasma, while local RAS affects aorta, the heart, kidneys, brain, and numerous other tissues.[32] Local RAS is independent of circulatory RAS, and many harmful AT1R-activated effects are caused by the local activation of Ang II.[33] It is worth noting that the components of RAS may differ depending on the pathological conditions or cell type;[34] therefore, it can be inferred that there are differences in the effects of drugs on local RAS and circulatory RAS.

In our experiments, renin, cAMP, AT1R, and Ang II expressions increased in cardiac tissues as the rise of BP, while their expressions were suppressed by R568. In the circulatory blood, the renin expression was significantly lower in SHRs, while cAMP and Ang II showed the reverse trend. Unfortunately, R568 treatment failed to significantly alter the expression of these indexes in plasma. Animal studies have shown that RAS blockers have different effects on circulatory and local Ang II concentrations; Ferrario et al.[35] indicated that treatment with the angiotensin-converting enzyme inhibitors losartan and lisinopril for 12 days significantly reduced rat plasma Angiotensin II concentrations and increased plasma Ang (1–7) concentrations by 1.8-fold, while Ang (1–7) and Angiotensin II concentrations in the LV did not change significantly. It has been shown that circulatory RAS activation is induced in the early stage of hypertension, which is involved in the early increase of BP. However, after eight weeks, circulatory RAS is downregulated, while BP continues to increase. At this point, RAS in the local tissues, such as brain, heart, kidneys, is activated, leading to the progression of EH and target organ damage maintenance.[36] These findings are consistent with our results, suggesting that disrupting the molecular mechanisms of local RAS may be feasible for disease management.

In addition, it is generally acknowledged that novel RAS activation plays an essential positive role in cardiovascular protective action. Kostenis et al.[37] found that MasR can form a heterodimer with AT1R to antagonize the function of AT1R. Adding Ang (1–9) to cardiomyocyte culture medium can reverse cardiomyocyte hypertrophy caused by Ang II or pituitrin. In a rat model of renal hypertension,[38] the long-term application of Ang (1–9) significantly improved cardiovascular damage caused by hypertension. The most important finding of this study was our investigation of the novel RAS signal transduction pathway, under which CaSR was activated in hypertensive myocardial hypertrophy; through this, we found an unexpected protective role of CaSR. We found that Ang (1–9) and Ang (1–7) concentrations were reduced in the plasma and myocardial tissues of SHRs, and that R568 intervention increased Ang (1–9) and Ang (1–7) levels in the myocardium without affecting their plasma concentrations. These observations demonstrate that the local tissue-specific effects of novel RAS might differ from its systemic effects, and that Ang (1–7) and Ang (1–9) may regulate cardiac repair/remodeling in an autocrine/paracrine manner. In MI (myocardial infarction) in transgenic mice, tissue-specific effects were also reported for Ang (1–7);[39] the lentiviral delivery of Ang (1–7) improved their cardiac function in rats with MI. Other studies have also shown that Ang (1–9) has antihypertrophic effects independent of Ang (1–7),[40] supporting the hypothesis that local cardiac Ang (1–7) and Ang (1–9) exert beneficial effects. Further studies have demonstrated that injecting normal rats with Ang (1–7) downregulated cardiac MasR expression;[41] in contrast, MasR was found to be increased in dysfunctional hearts at four weeks post-MI in rats.[42] In another study, Fattah et al.[43] found that MasR expression was downregulated in adeno-associated virus-treated Ang (1–9)-injected mice. However, in contrast to the above-mentioned findings, in our experiments, no significant changes in MasR and AT2R were observed in SHRs, and R568 had no obvious effect on their expression. Possibly, R568-induced Ang (1–7) mediates at least part of its cardioprotective effects by acting as an endogenous β-arrestin-biased agonist targeting AT1R.[44] The inconsistency of our findings may result from differences in animal models and experimental protocols, as well as the complexity of the in vivo environment. More evidence is needed to further clarify the role of novel RAS in myocardial remodeling protective action mediated by CaSR. Nevertheless, our study has provided strong evidence that R568 can reverse cardiac remodeling by mediating local novel RAS.

Further research is needed on inhibition of CaSR activity and regarding the relationship between BP, cardiac remodeling, and RAS activity. In the case of treatment with the CaSR agonist R568, its cardioprotective effects are mainly exerted by local Ang (1–9) or Ang (1–7), and its specific mechanism of action needs further investigation. Furthermore, cytological-level model experiments will further support our findings. However, we have identified the basic mechanisms by which CaSR partially mediates BP and cardiac remodeling.

Taken together, our study reveals that low expression of CaSR is involved in the occurrence and development of EH-mediated myocardial remodeling. The activation of CaSR can reverse adverse myocardia remodeling by inhibiting local classical RAS and activating novel RAS in cardiac tissues. CaSR is closely related to many cardiovascular diseases, but its specific mechanism still not to be elucidated. Until now, CaSR has not been investigated in terms of cardiovascular treatment, but given the important relationship between CaSR and cardiovascular disease, CaSR regulators are expected to become important drugs for the treatment of cardiovascular disease.

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