

## Casr-Inhibitor Treatment Prevents Hypertension Induced Cardiac Fibrosis and Remodeling

Xinhua Yin\*, Siting Hong, Xin Zhang, Lei Gao, Jinyu Chi

Department of Cardiology, the First Affiliated Hospital of Harbin Medical University, Harbin150001, and Heilongjiang Province, China

### Abstract:

**Introduction:** Myocardial fibrosis induced by hypertension results in cardiac remodeling and dysfunction. It has been demonstrated that calcium sensing receptor (CaSR) activation is involved in calcium overload during cardiac fibrosis. This study investigated the role of Calhex<sub>231</sub>, a CaSR inhibitor in preventing cardiac fibrosis in a model of hypertension in spontaneously hypertensive rat (SHR).

**Methods and results:** Wistar Kyoto (WKY) rats were used in this study as normotensive controls. Cardiac function, hypertrophy index and blood pressure were determined at the end of the study. The results showed that Calhex<sub>231</sub> treatment reduced the blood pressure, cardiac fibrosis and extracellular matrix (ECM) secretion in SHRs. Moreover, western blot results showed that the elevated expression levels of CaSR, matrix metalloproteinase-2 and -9 (MMP-2 and -9) induced by hypertension were suppressed by Calhex<sub>231</sub> administration.

**Conclusions:** We found inhibition of CaSR through decreasing the deposition of ECM can treat cardiac remodeling.

**Keywords:** Calcium sensing receptor (CaSR); Hypertension; Cardiac fibrosis; Spontaneously hypertensive rats (SHRs); Cardiac remodeling

### Introduction

Myocardial fibrosis, which is characterized by accumulation of extracellular matrix (ECM) and deposition of excessive collagen, develops to left ventricular (LV) diastolic dysfunction, myocardial remodeling and heart failure<sup>[1-3]</sup>. Studies have shown that the cardiomyocytes replaced by cardiac fibroblasts and pressure overload leading to ECM deposition are the two reasons of fibrosis<sup>[4]</sup>. Since the mechanism of fibrosis is complex, seeking protective effect of novel treatment is urgent.

Calcium sensing receptor (CaSR), is a Class C G protein-coupled receptor (GPCR) involved in Ca<sup>2+</sup> homeostasis<sup>[6,7]</sup>. Brown et al<sup>[8]</sup> have first discovered its expression in the parathyroid, and then its existence was found in different tissues<sup>[6]</sup>. Increased extracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>e</sub>) binds to CaSR, activating phospholipase C (PLC) signalling pathway, leading to accumulation of inositol 1,4,5-trisphosphate (IP<sub>3</sub>)<sup>[6]</sup>. A previous work demonstrated that CaSR promoted cardiac fibroblast proliferation via Ca<sup>2+</sup> signaling involved in cardiac fibrosis through the PLC-IP<sub>3</sub> pathway<sup>[9]</sup>. However, no evidence had shown that CaSR was involved in hypertensive cardiac remodeling at present. In this study, we aimed to explore the effects of Calhex<sub>231</sub>, the inhibitor of CaSR, on myocardial fibrosis by modulating ECM secretion in vivo.

### Methods

**Materials and reagents:** 20 weeks old male Spontaneously hypertensive rats (SHRs) and Wistar Kyoto (WKY) rats were obtained from Vital River Laboratories (Beijing, China). All animal experimental procedures were complied with the Guide for the Care and Use of Laboratory Animals of National Institutes of Health. Calhex<sub>231</sub> was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibody against CaSR was purchased from Alpha Diagnostic International Inc. (San Antonio, Texas, USA). Antibodies against MMP-2, MMP-9,  $\alpha$ -SMA, TIMP-2 and  $\beta$ -actin were from Abcam Inc. (Cambridge, MA, USA). All secondary antibodies G were purchased from Rockland (PA, USA). Poly vinylidene difluoride (PVDF) membranes were acquired from what man (now part of GE Healthcare Life Sciences, Buckinghamshire, (UK)).

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**\*Corresponding author:** Department of Cardiology, the First Affiliated Hospital of Harbin Medical University, No.23, You Zheng Street, Nan Gang District, Harbin 150001, Heilongjiang Province, China, Tel: 86 451 85555063; E-mail: [yinxinhua5063@163.com](mailto:yinxinhua5063@163.com)

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**Animal study:** Twenty male spontaneously hypertensive rats (SHRs) and ten age-matched male Wistar Kyoto (WKY) rats were randomly divided into three groups: (1) WKY(n=10),the 20-week-old Wistar-Kyoto rat group were intraperitoneal (i.p) injected with saline (2 mL/kg/day) for 4 weeks; (2) SHR (n=10), the 20-week-old SHRs group were i.p. Injected with saline (2 mL/kg/day) for 4 weeks, and (3) SHR plus Calhex<sub>231</sub> (n=10,SHR+Calhex<sub>231</sub>),the 20-week-old SHRs were received a daily i.p injection of Calhex<sub>231</sub> (10 μmol/kg) which was administered in saline for 4 weeks.

All animals were housed in separate cages under standard conditions with free access to food and water. The blood pressure of the rats was carried out by the caudal artery using the RBP-1 method in each rat once a week. Four weeks later, the rats were anesthetized before echocardiograms, and then the whole hearts were quickly removed and stored at -80 °C until western blot analyses.

**Echocardiograph evaluation:** Cardiac functions were obtained using a Vivid 7 Dimension echocardiography machine (GE Healthcare, Waukesha, WI, USA). The rats were anaesthetized with 10% chloral hydrate at 0.3 ml/100 g body weight i.p and adjusted to the left lateral decubitus position self-breathing. The images and data were recorded for analysis.

**Histological analysis:** The hearts were fixed in 4% para formaldehyde at room temperature for 24 h and then embedded in paraffin, sliced into 4-μm thick sections, and stained with Masson's trichrome reagent. Fibrosis tissue which was stained blue, quantified by Image-Pro Plus v4.0software.

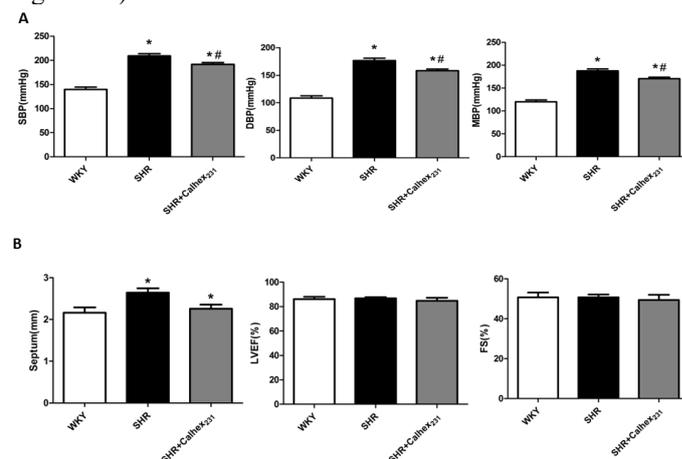
**Western-blot analysis of proteins in left ventricular tissues**  
Protein concentrations were determined using an enhanced BCA protein assay kit (Beyotime, Nantong, China). The equal amounts of protein were subjected to sodium do-decyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) .Then the samples were transferred to PVDF membranes (Millipore) and blocked with 5% non-fat dry milk dissolved in TBST for 1 h at room temperature. The membranes were incubated at 4 °C overnight with the primary antibodies anti- CaSR (1:500), anti-MMP-2 (1:1000), anti-MMP-5 (1:1000), anti-α-SMA (1:500), anti-TIMP-2(1:500), and anti-β-actin (1:1000). After washing with TBST, the membranes were incubated with secondary antibodies fluorescence-conjugated goat anti-rabbit oranti-mouse IgGs (1:5,000; Rockland) in TBST for 1 h at room temperature. Bands were scanned by an Odyssey infrared imaging system (LI-COR) and Odyssey v3.0 software. The results were analyzed with Image J software, and β-actin was used as an internal control for the normalized assay.

**Statistical analysis:** The data are expressed as the mean ± standard deviation (SD). Graph Pad Prism 5.0 software was used for the data analyses. We use done-way ANOVA to compare more than two groups. For all analyses, values of *P* < 0.05 were considered statistically significant. Each experiment was repeated a minimum of three times.

## Results

### Effects of CaSR on myocardial function and cardiac fibrosis in spontaneously hypertensive rats

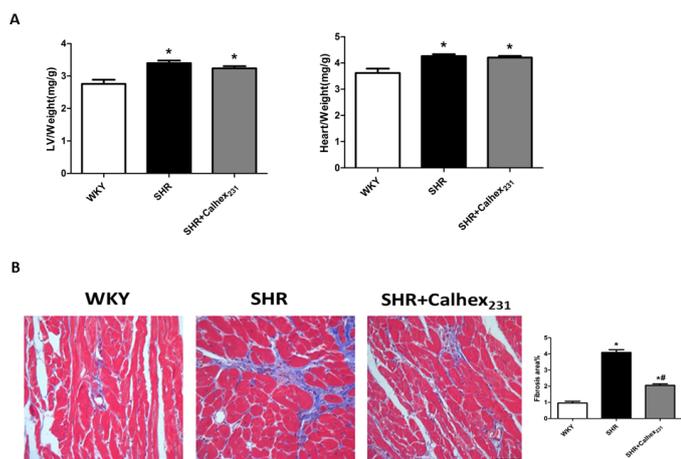
To investigate the effect of CaSR on myocardial fibrosis in the hypertensive heart, we used a model of SHRs to study the development of cardiac hypertrophy and cardiac remodelling. In the SHR and SHR + Calhex<sub>231</sub> groups, systolic blood pressure , diastolic blood pressure and mean arterial pressure were significantly higher compared to the WKY group (*P* < 0.05); however, the elevated blood pressure was reduced by the treatment of Calhex<sub>231</sub> compared with that in the SHRs group (*P* < 0.05, Figure.1A).



**Figure 1:** Effect of Calhex<sub>231</sub> on the blood pressure and cardiac function in SHRs. WKY: normo tensive age-matched control rats, SHR: 24 week-old SHRs, SHR + Calhex<sub>231</sub>: 24 week-old SHR treated with Calhex<sub>231</sub> for 4 weeks. (A)Calhex<sub>231</sub> treatment influences the blood pressure in SHRs.SBP: systolic blood pressure, DBP: diastolic and blood pressure, MBP: mean arterial blood pressure. Values are presented as the mean ± SD. \**P*<0.05 versus the WKY group. #*P*< 0.05 versus the SHR group. n = 7 per group. (B) Effect of Calhex<sub>231</sub> on cardiac function in SHRs heart. The data are detected by echocardiography. Septum: thickness of septum, EF: ejection fraction, FS: fractional shortening. Values are presented as the mean ± SD. \**P* < 0.05 versus the WKY group. n = 7 per group.

Echocardiography results showed myocardial function. Compared with the WKY group, the thickness of the septum was increased in the SHR group (*P* < 0.05, Figure.1B). As shown in Figure.1, no differences were observed in LV systolic functions (EF and FS) between the three groups at 24 weeks of age. The degree of hypertrophy were determined by HW/BW (heart weight to body weight ratio) and LVW/BW (left ventricular weight to body weight ratio).Our results showed that SHR shad an increased HW/BW ratio compared to the control WKY rats (all *P* < 0.05, Figure.2A). Similar result was observed in LVW/BW ratio (*P* < 0.05, Figure.2A) .These results show the presence of hypertrophy but not congestive heart failure in the SHR group.

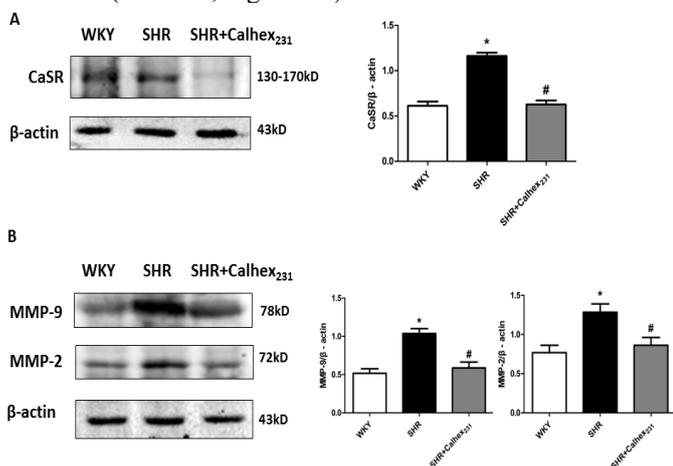
Masson's staining was to detect the collagen deposition in the cardiac tissue. SHRs presented a remarkable increase in the muscle fibres compared with the WKY group (*P* < 0.05). Moreover, Calhex<sub>231</sub> treatment ameliorated cardiac fibrosis induced by hypertension compared with the SHR group (*P* < 0.05, Figure.2B).



**Figure 2:** Effect of Calhex<sub>231</sub> on gravimetric parameters and cardiac fibrosis of SHRs. (A) LVW: weight of left ventricle, HW: heart weight, BW: body weight. Data are presented as the mean  $\pm$  SD. \* $P < 0.05$  versus the WKY group.  $n = 7$  per group. (B) Calhex<sub>231</sub> treatment decreased the deposition of interstitial collagen in SHRs heart. Sections of cardiac tissue were observed by Masson staining, magnification: 400, Scale bar: 25  $\mu$ m. The bar graph presents statistical analysis of myocardial fibrotic area. Values are represented as the mean  $\pm$  SD from three independent experiments. \* $P < 0.05$  versus the WKY group; # $P < 0.05$  versus the SHR group.  $n=6$  per group.

### Expression of CaSR protein

Western blot analysis showed that the expression of CaSR was increased in the SHR group, compared to the WKY group (Figure.3A). Moreover, the increase was reduced after Calhex<sub>231</sub> treatment ( $P < 0.05$ , Figure.3A).



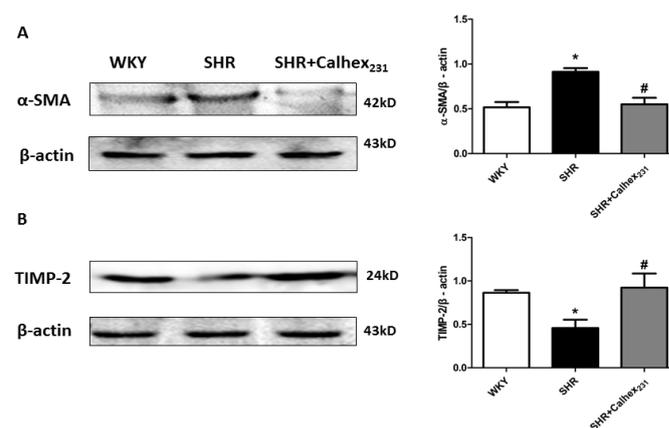
**Figure 3:** Protein expression of CaSR and MMPs in rat heart tissues was determined by western blot analysis. (A) Western blots analysis and summarized data of CaSR. Protein levels were normalized to  $\beta$ -actin. Values represent mean  $\pm$  SD. \* $P < 0.05$  versus the WKY group. # $P < 0.05$  versus the SHR group.  $n=6$  per group. (B) Western blots analysis and summarized data of MMP-9 and MMP-2. Protein levels were normalized to  $\beta$ -actin. Values represent mean  $\pm$  SD. \* $P < 0.05$  versus the WKY group. # $P < 0.05$  versus the SHR group.  $n=6$  per group.

### Western blot analysis of indicator of cardiac fibrosis

We performed Western blot to explore the protein levels of indicators of myocardial fibrosis for the three groups. The expression of MMP-9 and MMP-2 were increased in the SHR group compared to the WKY group ( $P < 0.05$ , Figure.3B). But Calhex<sub>231</sub> treatment decreased the protein levels of these proteins ( $P < 0.05$ , Figure.3B).

< 0.05, Figure.3B).

The expression of  $\alpha$ -SMA (alpha-smooth muscle actin) was increased in the SHR group compared to the WKY group and Calhex<sub>231</sub> treatment decreased the protein level of  $\alpha$ -SMA ( $P < 0.05$ , Figure.4A). Our result showed that TIMP-2 expression was significantly decreased in the SHR group compared to the WKY group, but was elevated in SHR + Calhex<sub>231</sub>-treated group ( $P < 0.05$ , Figure. 4B).



**Figure 4:** The effect of Calhex<sub>231</sub> on extracellular matrix production in SHRs. (A) Western blot analysis and summarized data of  $\alpha$ -SMA. Protein levels were normalized to  $\beta$ -actin. Values represent mean  $\pm$  SD. \* $P < 0.05$  versus the WKY group. # $P < 0.05$  versus the SHR group.  $n=6$  per group. (B) Western blots analysis and summarized data of TIMP-2. Protein levels were normalized to  $\beta$ -actin. Values represent mean  $\pm$  SD. \* $P < 0.05$  versus the WKY group. # $P < 0.05$  versus the SHR group.  $n=6$  per group.

### Discussion

Calcium sensing receptor (CaSR) distributes primarily on the parathyroid glands, regulating the release of the calcium-retaining hormone, parathyroid hormone (PTH)<sup>[6]</sup>. The significant role of CaSR is regulating intracellular calcium homeostasis through  $Ca^{2+}$  signaling pathways<sup>[10]</sup>. Studies have observed the existence of CaSR in cardiovascular system, like neonatal rat ventricular myocytes (NRVMs)<sup>[11]</sup>, rat cardiac fibroblasts<sup>[9]</sup> and vascular smooth muscle<sup>[12]</sup>. Moreover, CaSR plays an important role in many diseases, such as hypoxia/reoxygenation<sup>[13]</sup>, cardiomyocyte apoptosis<sup>[11]</sup>, myocardial infarction<sup>[14]</sup>, cardiac hypertrophy and heart failure<sup>[15]</sup>.

Previous studies have also reported that the activation of CaSR induces the release of PTH which lowers blood pressure<sup>[7,8,16]</sup>. However, Schluter et al noted that inhibition of CaSR maybe not reduce the release of PTH<sup>[17]</sup>. And Sutherland found that low level of  $[Ca^{2+}]_o$  could stimulate the secretion of parathyroid hypertensive factor (PHF) in parathyroid gland organ cultures<sup>[18]</sup>. There are many factors involving the regulating the blood pressure, such as effect of the  $Ca^{2+}$  or CaSR on vascular tone, secretion of hormonal (like renin, PTH and so on) and the drug's pharmacological effects<sup>[19]</sup>. So, the mechanism of CaSR in the regulating blood pressure needs further investigation in the following study.

In human cardiac fibroblasts, people found that calcium homeostasis is regulated by IP3 receptor (IP3R)<sup>[20]</sup>. A study revealed that extracellular calcium activated of CaSR increasing

intracellular  $\text{Ca}^{2+}$  [ $\text{Ca}^{2+}$ ]<sub>i</sub> levels through the PLC-IP3 pathway and promoted proliferation and migration of neonatal cardiac fibroblast and ECM remodeling<sup>[9]</sup>. Our study showed that Calhex<sub>231</sub> was able to suppress myocardial fibrosis in SHR. This result demonstrates that CaSR is involved in the hypertension induced cardiac remodeling. So we hypothesize that activation of the CaSR promotes cardiac fibrosis through the PLC-IP3 pathway.

Because of the longtime increased workload, the heart progresses to hypertrophy, fibroblast proliferation and ECM secretion<sup>[21]</sup>. And the fibroblasts cooperate with the collagen network influencing myocardial relaxation and contractility<sup>[22]</sup>. In our present study, Masson staining results showed an obvious increase in collagen deposition in the SHR group, whereas Calhex<sub>231</sub> diminished collagen area of myocardial fibrosis. The expression of MMP-2, MMP-9 and  $\alpha$ -SMA were elevated in SHR group indicating of fibrotic tissue remodeling, and treatment with Calhex<sub>231</sub> decreased the expression levels of these proteins. And Calhex<sub>231</sub> unregulated the decreased expression of TIMP-2 in SHR. Moreover, MMPs have a significant role in the ECM remodeling. During repairing of the damage, MMPs destroy the network of the collagen and promote inflammatory cell gathering, leading to releasing of cytokines. Then it results in fibroblast proliferation and excessive deposition of ECM<sup>[23]</sup>. Additionally, studies showed that many factors, including inflammatory cytokines, hormones and mechanical stretch can affect proliferation, migration and gene expression of fibroblasts<sup>[24,25]</sup>. Our data prove that CaSR can be involved in the mechanism of cardiac remodeling mentioned above.

## Conclusion

The study demonstrated that CaSR is involved in cardiac fibrosis induced by hypertension. For the first time, we provide experimental evidence for treating cardiac remodeling by inhibition of CaSR through decreasing the deposition of ECM. The present study could provide novel approaches for the preventing cardiac fibrosis.

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