Original Paper

Calcium Sensing Receptor Promotes Cardiac Fibroblast Proliferation and Extracellular Matrix Secretion

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Key Words

Calcium-sensing receptor • Cardiac fibroblast • Intracellular calcium • Cardiac fibrosis

Abstract

Aims: Calcium-sensing receptor (CaR) acts as a G protein coupled receptor that mediates the increase of the intracellular Ca^{2+} concentration. The expression of CaR has been confirmed in various cell types, including cardiomyocytes, smooth muscle cells, neurons and vascular endothelial cells. However, whether CaR is expressed and functions in cardiac fibroblasts has remained unknown. The present study investigated whether CaR played a role in cardiac fibroblast proliferation and extracellular matrix (ECM) secretion, both in cultured rat neonatal cardiac fibroblasts and in a model of cardiac hypertrophy induced by isoproterenol (ISO).

Methods and Results: Immunofluorescence, immunohistochemistry and Western blot analysis revealed the presence of CaR in cardiac fibroblasts. Calcium and calindol, a specific activator of CaR, elevated the intracellular calcium concentration in cardiac fibroblasts. Pretreatment of cardiac fibroblasts with calhex231, a specific inhibitor of CaR, U73122 and 2-APB attenuated the calindol- and extracellular calcium-induced increase in intracellular calcium ($[\text{Ca}^{2+}]_i$). Cardiac fibroblast proliferation and migration were assessed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), cell count and the cell scratch assay. ECM production was detected by expression of matrix metalloproteinase-3 and -9 (MMP-3 and -9). Activation of CaR promoted cardiac fibroblast proliferation and migration and ECM secretion. More importantly, calhex231, suppressed cardiac fibroblast proliferation and migration and MMP-3 and -9 expression. To further investigate the effect of CaR on cardiac fibrosis, a model of ISO-induced cardiac hypertrophy was established. Pretreatment with calhex231 prevented cardiac fibrosis and decreased the expression of MMP-3 and -9 expression. **Conclusions:** Our results are the first report that CaR plays an important role in Ca^{2+} signaling involved in cardiac fibrosis through the phospholipase C- inositol 3,4,5 phosphate (PLC-IP₃) pathway.

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Introduction

Cardiac fibroblasts are the main non-myocyte cell type in the heart. Under physiological circumstances, cardiac fibroblasts play an important role in maintaining homeostasis of the extracellular matrix, in addition to producing growth factors, cytokines and matrix metalloproteinases (MMPs) [1]. However, cardiac fibroblasts promote myocardial scarring and remodeling in injured hearts in ischemic, hypertensive and hypertrophic heart diseases. Excessive proliferation and migration of cardiac fibroblasts result in fibrosis [2, 3]. Myocardial fibrosis can alter the mechanical properties of the hearts, impairing cardiac function and promoting heart failure [4]. Some evidence has shown that the cytosolic calcium ion ($[Ca^{2+}]_i$) acts as a second messenger to regulate versatile cell functions including excitation-condition coupling, gene transcription, cell growth, differentiation and apoptosis. However, little information implies that the Ca^{2+} signaling pathway is involved in cardiac fibroblast proliferation and extracellular matrix (ECM) production [5].

Calcium sensing receptor (CaR), a G protein coupled receptor, activates the phospholipase C (PLC) signaling pathway, resulting in an increase in inositol phosphate (IP) accumulation and Ca^{2+} mobilization [6]. Our previous work demonstrated that CaR induced cardiomyocyte apoptosis through the mitochondrial and endo(sarco)plasmic reticulum apoptotic pathway in the failing heart. Moreover, our results showed obvious myocardial fibrosis following administration of a CaR activator [7]. However, at present, no evidence had shown that CaR was expressed in cardiac fibroblasts.

In this study, our results demonstrate that CaR is present in rat cardiac fibroblasts. We identified both the protein levels and the functional regulation of CaR in cardiac fibroblasts.

Materials and Methods

Materials

Isoproterenol (ISO), calindol, calhex231, sodium tauroursodeoxy cholate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for CaR, MMP-2, MMP-3, MMP-9, TIMP-3, TIMP-4, α -SMA and GAPDH were from Santa Cruz Biotechnology. A secondary antibody was AP-IgG (Promega). Polyvinylidene difluoride (PVDF) membranes were from Whatman (now part of GE Healthcare Life Sciences, Buckinghamshire, UK).

Neonatal rat cardiac fibroblasts incubation

Neonatal rat cardiac fibroblasts were prepared from 2- to 3-day-old neonatal Wistar rats (Animal Research Institute of Harbin Medical University, China). The rats were anesthetized and sacrificed by immersion into 75% (v/v) alcohol. The ventricles were removed and washed three times in D-Hanks balanced salt solution (mmol/L: 0.4 KCl, 0.06 KH_2PO_4 , 8.0 NaCl, 0.35 $NaHCO_3$, and 0.06 $Na_2HPO_4 \cdot 7H_2O$, pH 7.2) at 4 °C, then minced and incubated with 0.25 % (w/v) trypsin for 10 min at 37°C. To terminate the digestion, an equal volume of cold Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) newborn calf serum was added. The supernatant was discarded. Cells were incubated with fresh 0.25 % trypsin for 15 min at 37 °C, and the supernatant was collected. The latter digestion step was repeated four times. Cells in the supernatant were then isolated by centrifugation for 13 min at 2400 g/min at room temperature. Cells were re-suspended in DMEM containing 20 % (v/v) newborn calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin, and then were cultured as monolayers at a density of 5×10^4 cells/cm² at 37 °C in a humidified atmosphere containing 5 % (v/v) CO₂. After 1 h incubation in flasks at 37 °C, in a humidified atmosphere with 5 % CO₂, the unattached cells were discarded and the attached cells were continuously cultured. The medium was changed two times per week.

Experimental protocol

Three days after being seeded, the cardiac fibroblasts were divided randomly into four groups: (1) control group: no treatment; (2) angiotensin (Ang) II group: pre-treated with 100 nmol/L AngII followed by treatment with 2.5 mM $CaCl_2$ for 24 h; (3) Calindol+ angiotensin (Ang) II group: pre-incubated with 100

nmol/L AngII followed by treatment with 3 μ M calindol for 24 h; (4) Calhex231+ angiotensin (Ang) II group: pre-incubated with 100 nmol/L AngII followed by treatment with 2 μ M calhex231 for 24 h.

Immunofluorescent and immunohistochemical staining

Paraffin-embedded tissue sections (0.2 μ m) were deparaffinized with xylenes and rehydrated with graded washes with ethanol followed by phosphate-buffered saline (PBS). Then, each slice was treated with 30 μ l 3 % H₂O₂ (reagent A), incubated at room temperature for 20 min and washed twice with PBS. Then, 30 μ l goat serum (reagent B) was added, followed by incubation at room temperature for 20 min and two washes with PBS. Each slice was incubated in 30 μ l primary antibody (mouse anti-rat CaR monoclonal antibody, 1:200 dilution) and placed in a wet box at 4°C overnight. After washing with PBS, the slices were incubated in 30 μ l biotinylated polyclonal secondary antibody (reagent C) at room temperature for 30 min, followed by washing with PBS. The diaminobenzidine (DAB) method was used for color development, followed by washing with tap water. Slices were then counterstained with hematoxylin, incubated in ammonia, dehydrated with gradient ethanol, transparentized with xylenes and finally sealed with neutral gum. Cells with brown staining particles in their cytoplasm and nucleus were denoted positive under a light microscope.

Cytoplasmic protein isolation from cardiac fibroblasts

Protein isolation and analysis were performed as described previously [8]. Briefly, whole-cell lysates were collected in lysis buffer (62.5 mmol/L of Tris-HCl, 2 mmol/L of EDTA, 2.3% SDS and 10% glycerol [pH 6.8]), with a protease inhibitor mixture, and total protein content was determined by the bicinchoninic acid method (Pierce). Equal amounts of protein samples were separated by SDS-PAGE and transferred to nitrocellulose using standard techniques.

Measurement of intracellular calcium in cardiac fibroblasts

Cardiac fibroblasts were loaded with 1 μ mol/L Fluo-4/AM at 37°C for 30 minutes in HEPES buffered saline (in mmol/L: 130 NaCl, 5 KCl, 10 glucose, 1 MgCl₂, 1.0 CaCl₂, and 25 HEPES [pH 7.4]), and groups of 5 to 8 cells were monitored using an inverted Olympus IX-70 microscope. Spectrofluorometric measurements were collected using the Delta Scan System spectrofluorometer (Photon Technology), where the field was excited at 488 nm, with emission at 530 nm [9].

MTT Proliferation Assay

Cardiac fibroblasts (1x10⁵ cells per well) were seeded in 6-well plates and treated with or without different reagents for 48 hours. Thereafter, MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/mL) was added to each well, and plates were incubated at 37°C for 4–6 hours. The medium was then replaced with 150 μ l dimethyl sulfoxide (DMSO) and agitated for 10 minutes. Absorbance was measured at 490 nm using a microplate reader.

Cell Counting

Cardiac fibroblasts (1 x10⁵ per well) were seeded in 6-well plates and treated with or without different reagents for a designated period. The cells were then digested by 0.25% trypsin for 6–8 minutes at 37°C. The cell number was counted using a hemocytometer.

Scratch wound repair assay

Rat cardiac fibroblasts were treated with calcium, calindol and calhex231, and then subjected to the *in vitro* scratch assay as described previously [10, 11]. Images were captured at 0, 24 and 48 hours after treatment using phase-contrast microscopy.

Animals

Male Wistar rats (200–250 g) were obtained from the Experimental Animal Center of Harbin Medical University (Harbin, People's Republic of China). All animal experimental protocols complied with the "Guide for the Care and Use of Laboratory Animals" published by the Chinese National Institutes of Health. The study was approved by the Institutional Animal Research Committee of Harbin Medical University. All animals were housed at the animal care facility of Harbin Medical University at 25°C with 12/12 h light/

dark cycles and were allowed free access to normal rat chow and water throughout the study period. Rats were randomly assigned to different treatment groups.

Experimental groups

β -Adrenergic stimulation can be induced by a high dose of isoproterenol (ISO), injected subcutaneously as reported before [12]. Sixty rats were randomly divided into four groups. In group 1 (n= 10), the rats were subcutaneously injected with saline for 4 days as a control. In group 2 (ISO, n= 15), the rats were subcutaneously injected with ISO (170 mg/kg/d in saline) for 4 days to induce cardiac hypertrophy. In group 3 (ISO-A, n= 18), the rats were subcutaneously injected with calindol (10 μ mol/kg/d in saline) for 4 weeks after administration of ISO for 4 days. Calindol is a specific activator of CaR. In group 4 (ISO-I, n= 17), the rats were subcutaneously injected with calhex231 (10 μ mol/kg/d in saline) for 4 weeks after administration of ISO for 4 days. Calhex231 is a specific inhibitor of CaR.

Hemodynamic measurement

Cardiac functions were assessed using an echocardiography system (GEVIVID7 10S, USA). Echocardiograms were performed on self-breathing rats under anesthesia (intraperitoneal injection of 10 % chloral hydrate at 0.3 ml/100 g body weight). Left ventricular (LV) parameters measured include inlet ventricular septal defects (IVSd) and left ventricular ejection fraction (LVEF). All parameters represent the means of five consecutive cardiac cycles [13].

Masson's staining

Hearts from different groups, fixed in 10% formalin, were processed and paraffin embedded. Heart sections (4 mm) were then stained with Masson's trichrome reagent [14]. The percent of fibrosis (blue staining) to total tissue was analyzed and calculated by the NIS-ELEMENTS quantitative automatic program (Nikon, Tokyo, Japan), with the average value representing at least 8 images per heart, analyzed in double-blind fashion. Serial, transverse cryosections (7 mm thick) of hearts were cut with the CM1950 Frigocut (Leica, Wetzlar, Germany) at -20°C and were stored at -80°C.

Statistical analysis

Data are expressed as the mean $\pm$ SEM. One-way analysis of variance (ANOVA) was used to determine statistical significance between groups and also the two-tailed Student's t-test. A probability value of $p < 0.05$ was taken to indicate statistical significance.

Results

Protein expression of CaR in rat neonatal cardiac fibroblasts

To determine whether CaR is expressed in cardiac fibroblasts, CaR expression was detected by immunofluorescence and immunohistochemical staining. In neonatal cardiac fibroblasts, deep yellow immunostaining or green fluorescence was present throughout all cardiac fibroblasts indicating the expression of CaR at the protein level in these cells. A lack of specific staining was demonstrated in the control group, in the absence of anti-CaR antibody (Fig. 1a and b).

The expression of CaR protein was also examined in neonatal cardiac fibroblasts by western blotting. CaR proteins with a relative molecular mass between 120-140 kDa were detected in neonatal cardiac fibroblasts (Fig. 1c). Furthermore, preadsorption of anti-CaR antibody with standard CaR antigen eliminated the 130 kDa band. Together, these results indicate that CaR is expressed in neonatal cardiac fibroblasts.

Activation of CaR elevated the intracellular calcium concentration through the PLC-IP3 pathway

To confirm that CaR functionally regulates intracellular calcium concentration in neonatal cardiac fibroblasts, cells were treated with 2.5 mM CaCl_2 , NiCl_2 (1 mM), an inhibitor of the Na^+ - Ca^{2+} exchanger, and CdCl_2 (200 μ M), an inhibitor of the L-type calcium channel, in the

Fig. 1. CaR is expressed in neonatal cardiac fibroblasts. (a) Immunofluorescence staining for CaR (green) and DAPI (blue). (b) Immunohistochemistry for CaR (yellow) protein expression in neonatal cardiac fibroblasts. The bar graph shows the quantification of the areas of positive cells (right panel; $n = 8$). Data are presented as the mean $\pm$ SEM. Magnification: $\times 400$. (c) Western blotting analysis of CaR expression in neonatal cardiac fibroblasts.

Fig. 2. CaR activation induces Ca^{2+} release from the ER in neonatal cardiac fibroblasts. (a) CaR activation induced Ca^{2+} release from the ER with the treatment of 2.5 mM CaCl_2 . (b) Calindol induced Ca^{2+} release from the ER. The change in fluorescence intensity, corresponding to intracellular calcium concentration, was measured by Fluo-4 AM staining, ($n=4-5$). * $p < 0.05$ vs. control.

bath solution. These treatments induced a time-dependent increase in intracellular calcium. Similarly, administration of 10 μM calindol evoked an increase in intracellular calcium concentration in more than 95 % of neonatal cardiac fibroblasts in a given observation field. After pretreatment with calhex231, a specific inhibitor of CaR, intracellular calcium concentration was not significantly increased compared to administration of calindol alone.

Neonatal cardiac fibroblasts were pretreated for 10 min with 10 μM TG (thapsigargin), which inhibits refilling of the IP_3 -sensitive calcium release pools. Subsequently, cells

Fig. 3. CaR induced cardiac fibroblast proliferation and migration. Cell proliferation induced by activation of CaR, as evidenced by (a) cell viability and (b) cell number. (c) Scratch wound repair assay for observation of neonatal cardiac fibroblast migration.

were treated with 2.5 mM CaCl_2 and 10 μM calindol, which failed to elicit any increase of intracellular calcium concentration. Preincubation of neonatal cardiac fibroblasts for 10 min with U73122, a phosphatidylinositol-specific PLC blocker, abolished the increase in intracellular calcium induced by 10 μM calindol or 2.5 mM CaCl_2 . Pretreatment with 2-APB, an IP_3 receptor-specific inhibitor, for 10 min eliminated the effect of 10 μM calindol and 2.5 mM CaCl_2 on the induction of intracellular calcium release (Fig. 2). These results demonstrate that the activation of CaR resulted in intracellular calcium increase in neonatal cardiac fibroblasts through stimulation of the PLC- IP_3 pathway.

Effects of CaR on cell proliferation and migration in neonatal cardiac fibroblasts

To investigate whether neonatal cardiac fibroblast proliferation is regulated by CaR, the cells were starved overnight and pretreated with CaR activators, either CaCl_2 (2.5 mM) or calindol (10 μM), for 1 hour and then incubated with AngII for 48 hours. The results indicated that both CaCl_2 and calindol enhanced cell density, whereas the CaR blocker calhex231 abolished these effects. As shown in Figures 3a and b, CaCl_2 and calindol induced cardiac fibroblast proliferation, as evidenced by cell viability (Fig. 3a) and increased cell number (Fig. 3b).

To address whether the migration of neonatal cardiac fibroblast is induced by CaR, cell migration was studied in a wound-healing assay. Cells in culture were scraped off with a pipette tip, producing a wide acellular area. Cardiac fibroblasts migrating into this acellular area were counted and expressed as the number of migrated cells. Treatment with 2.5 mM CaCl_2 and 10 μM calindol significantly increased the migration of neonatal cardiac fibroblasts after 24 h incubation, while administration of calhex231 reduced this effect (Fig. 3c).

Fig. 4. The effect of CaR on extracellular matrix production. Expression of (a) CaR, (b) MMP-2, (c) MMP-9, (d) TIMP-4 and (e) α-SMA proteins, as induced by the activators calindol and 2.5 mM CaCl₂. Protein levels were normalized to GAPDH (n=4-5). *p<0.05 vs. control group, # p<0.05 vs. 2.5 mM CaCl₂ group.

Effects of CaR on extracellular matrix (ECM) production in neonatal cardiac fibroblasts

To investigate CaR regulating ECM production, CaR expression was detected. In the calindol and CaCl₂ groups, CaR expression was upregulated compared to control and calhex231-treated group (Fig. 4 a).

Fig. 5. Involvement of CaR in the functional changes of ISO-induced cardiac hypertrophy. (a) and (b) CaR induced changes in myocardial function in cardiac hypertrophy, as shown by analysis of the left ventricular ejection fraction (LVEF) and the interventricular septal diastolic thickness (IVSd). (c) Representative Masson trichrome staining of heart sections from ISO-induced cardiac hypertrophy. The bar graph shows the quantification of the fibrotic areas in histological sections (right panel; $n = 8$). Magnification: 40×200 .

MMPs not only play a role in matrix degradation, but are also involved in collagen synthesis regulation. To reveal whether CaR effecting on ECM secretion, MMP-2 and MMP-9 were detected by Western blotting. As expected, $10\text{ }\mu\text{M}$ calindol and 2.5 mM CaCl_2 significantly enhanced the expression of MMP-2 and MMP-9 compared to control and calhex231-treated groups (Fig. 4b, c). TIMP-4 expression was significantly decreased in calindol and CaCl_2 groups, but was increased in calhex231-treated group (Fig. 4d).

α -SMA (alpha-smooth muscle actin) positive fibroblasts were identified as primary cell type responsible for interstitial matrix accumulation in fibrotic diseases. Our results showed that α -SMA expression was increased in the calindol and CaCl_2 groups (Fig. 4e).

Effects of CaR activation on cardiac fibrosis and myocardial function in ISO-induced cardiac hypertrophy

To further investigate the *in vivo* effect of CaR on cardiac fibrosis in cardiac hypertrophy, we established a model of cardiac hypertrophy by injecting rats with a high dose of ISO (170 mg/Kg) for 4 days. Masson's staining analyses showed that collagen deposition was increased in the ISO-4w and ISO-4w-calindol groups, compared with the ISO-4w-calhex231 groups (Fig. 5c).

Fig. 6. The effect of CaR on extracellular matrix production in cardiac hypertrophy. (a) Western blotting analysis showing CaR expression in cardiac hypertrophy model. Expression of (b) MMP-2, (c) MMP-3 and (d) MMP-9 proteins induced by the activators calindol. Protein levels were normalized to GAPDH (n=4-5). *p<0.05 vs. control group, # p<0.05 vs. ISO 4w+calindol group.

Hemodynamic parameters were monitored by echocardiography. Compared with the control group, the interventricular septal diastolic thickness (IVSd) was increased in the ISO-4w, ISO-4w-calindol and ISO-4w-calhex231 groups. The left ventricular ejection fraction was decreased in the ISO-4w and ISO-4w-calindol groups after ISO administration for 4 weeks, compared with the control group (Fig. 5a,b).

We also found that the expression of CaR in heart tissues was significantly increased in the ISO-4w and ISO-4w-calindol groups (Fig. 6a). To explore the *in vivo* effect of CaR on

cardiac fibrosis in cardiac hypertrophy, we detected the expression of several molecular indicators of cardiac fibrosis. The expression of both MMP-2, 3 and -9 in the ISO-4w and ISO-4w-calindol groups was increased compared to the ISO-4w-calhex231 and control groups. These results further demonstrate that activation of CaR is involved in the formation of cardiac fibrosis in ISO-induced cardiac hypertrophy (Fig. 6 b,c,d).

Discussion

The expression of CaR in various cells is related to multiple cellular functions, such as cell proliferation, apoptosis and the regulation of systemic calcium homeostasis [15]. For instance, Brown et al. have confirmed that CaR is activated by extracellular calcium in parathyroid cells and is responsible for the release of PTH [16-18]. Our previous studies demonstrated that the activation of CaR induced cardiomyocyte apoptosis during ischemia/reperfusion injury and in failing hearts [7, 8]. Our present study demonstrates, for the first time, the existence of CaR in rat cardiac fibroblasts. This conclusion is based on several lines of evidence: (a) CaR proteins were identified in neonatal cardiac fibroblasts by Western blot analysis; (b) CaR proteins were shown to be located in neonatal cardiac fibroblasts by immunohistological and immunofluorescence staining; (c) extracellular calcium and calindol, a specific activator of CaR, increased intracellular calcium levels, thereby activating the PLC-IP₃ pathway and inducing the release of calcium from ER; (d) extracellular calcium and calindol promoted neonatal cardiac fibroblast proliferation, migration and ECM secretion through the activation of CaR; and (e) CaR played a role in ISO-induced cardiac fibrosis in rats.

Ca²⁺ signals are well studied in cardiac myocytes [19, 20]. However, Ca²⁺ signaling pathways are not well understood in neonatal cardiac fibroblasts. Li et al. have revealed that Ca²⁺ activity in human cardiac fibroblasts is mediated by multiple Ca²⁺ signaling pathways, including IP₃Rs, SERCAs, NCX, PMCAs and SOCs [21]. CaR, as a G protein coupled receptor, senses changes in extracellular calcium concentration, triggering an increase in intracellular calcium [18]. CaR, in both rat cardiac myocytes and human antral gastrin cells, is sensitive to changes in extracellular calcium concentration [15, 22]. Our results in neonatal cardiac fibroblasts are similar to the results described above.

The sarcoplasmic/endoplasmic reticulum, a specialized calcium-storing organelle, is well known to be intimately involved in regulating Ca²⁺ movement within cells. IP₃Rs and RyRs participate in the release of Ca²⁺ from the sarcoplasmic/endoplasmic reticulum [23, 24]. Interestingly, Li et al. found that RyRs may not be involved in Ca²⁺ activity in human cardiac fibroblasts. These results demonstrated that intracellular calcium in cardiac fibroblasts are mediated by IP₃Rs [21]. Our results reveal that increased intracellular calcium concentrations were inhibited by U73122 and 2-APB. In addition, the CaR activator calindol induced significantly increased intracellular calcium concentrations. These results demonstrate that CaR is involved in increasing the intracellular Ca²⁺ concentration through the PLC-IP₃ pathway.

Cardiac fibrosis is vital for ventricular remodeling [25]. Cardiac fibroblasts, highly active cells that exhibit increased proliferative, migratory and secretory properties [26, 27], represent the largest class of cells residing in the heart. Proliferation of cardiac fibroblasts is the main characteristic of myocardial fibrosis. Although the importance of fibrosis in various cardiac diseases has been well established, the Ca²⁺ signaling mechanisms involved in cardiac fibrosis are not known. Some studies revealed that TRPM7 is located in the membrane of cardiac fibroblasts and mediates higher [Ca²⁺]_i oscillation frequencies indicative of higher ECM stiffness, which would result in more efficient ECM remodeling [28-30]. Ca²⁺ may play an essential role in cardiac fibroblasts proliferation and differentiation [19]. Our results confirm that activation of CaR could promote migration and elevate the expression levels of MMP-2 and 9 in neonatal cardiac fibroblasts. To further demonstrate the involvement of

CaR in cardiac fibrosis, a rat model of ISO-induced cardiac hypertrophy and fibrosis was established, followed by administration of calindol and calhex231. Masson's staining showed that calindol enhanced cardiac fibrosis, whereas calhex231 diminished cardiac fibrosis. The expression of both MMP-2 and -9 was elevated by treatment with calindol.

The CaR-mediated triggering of cardiac fibrosis via Ca^{2+} signaling may serve as a common pathway in the fibrosis cascade. If so, inhibition of Ca^{2+} release from the ER through CaR may attenuate cardiac fibrosis. The contribution of CaR-mediated Ca^{2+} signal in cardiac fibroblasts shown in this study is the first demonstration of an important role of this receptor in heart disease.

In conclusion, our results are the first to report an important role for CaR in Ca^{2+} signaling involved in cardiac fibrosis and that this role is mediated by the PLC-IP3 pathway. Our study opens up a new avenue to explore the potential roles of Ca^{2+} signaling in cardiac fibrosis and could lead to the development of more effective approaches for treating cardiac fibrosis.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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