Suppression of calcium-sensing receptor ameliorates cardiac hypertrophy through inhibition of autophagy

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Abstract. The calcium-sensing receptor (CaSR) releases intracellular calcium ($[Ca^{2+}]_i$) by accumulating inositol phosphate. Changes in $[Ca^{2+}]_i$ initiate myocardial hypertrophy. Furthermore, autophagy associated with $[Ca^{2+}]_i$. Autophagy has previously been demonstrated to participate in the hypertrophic process. The current study investigated whether suppression of CaSR affects the hypertrophic response via modulating autophagy. Isoproterenol (ISO) was used to induce cardiac hypertrophy in Wistar rats. Hypertrophic status was determined by echocardiographic assessment, hematoxylin and eosin, and Masson's staining. The protein expression levels of CaSR and autophagy level were observed. Changes of hypertrophy and autophagy indicators were observed following intravenous injection of a CaSR inhibitor. An ISO-induced cardiomyocyte hypertrophy model was established and used determine the involvement of $GdCl_3$. $[Ca^{2+}]_i$ was determined using Fluo-4/AM dye followed by confocal microscopy. The expression levels of various active proteins were analyzed by western blotting. The size of the heart, expression levels of CaSR and autophagy level were markedly increased in hypertrophic myocardium. In addition, the present study demonstrated that the indicators of hypertrophy and autophagy were effectively suppressed by CaSR inhibitor. Furthermore, similar effects were demonstrated in neonatal rat hypertrophic cardiomyocytes treated with ISO. It was also observed that CaSR regulates the Ca^{2+}/calmodulin-dependent protein kinase kinase β ($CaMKK_β$)-AMP-activated protein kinase (AMPK)-mammalian target of rapamycin (mTOR) signaling pathway induced by ISO in cardiomyocytes. Furthermore, the AMPK inhibition significantly reduced the autophagy level following CaSR stimulation ($P<0.05$). The results of the present demonstrated that inhibition of CaSR may ameliorate cardiac hypertrophy induced by ISO and the effect may be associated with the inhibition of autophagy and suppression of the $CaMKK_β$-AMPK-mTOR signaling pathway.

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Introduction

Cardiac hypertrophy is a general adaptive process in response to almost all types of cardiac disease (including pressure overload, cardiac arrhythmias, exercise training and endocrine disorders) (1-3). Various extrinsic physiological or pathological factors stimulate the development of cardiac hypertrophy (1,4). Although various treatments are available, advances in therapeutic strategies that are suitable for preventing the progression of cardiac hypertrophy have been limited, as the pathophysiology of cardiac hypertrophy remains to be elucidated.

The calcium-sensing receptor (CaSR) stimulates the phospholipase C system to release intracellular calcium ($[Ca^{2+}]_i$) (5). The expression of CaSR has previously been observed in the kidney, bone, intestine and other tissues (6,7). The functional expression of CaSR was first described in rat cardiac tissue in 2003 (8). One of the crucial functions of CaSR is regulating systemic Ca^{2+}. A change in the $[Ca^{2+}]_i$ is an initiating factor in cardiac hypertrophy (9,10). However, the regulation of CaSR during the hypertrophic process remains poorly characterized. It was previously reported that the expression of CaSR is increased in hypertrophic cardiomyocytes (8). Cardiac hypertrophy is a complex pathophysiological process regulated by various signal pathways and gene networks. Thus, identifying the underlying molecular mechanism of cardiac hypertrophy is crucial for developing improved therapeutic strategies.

Pathologically, autophagy, necrosis and apoptosis are the predominant modes of cell death (11). Autophagy is a catabolic process mediated by lysosomes leading to degradation of damaged organelles and macromolecules to achieve effective cell recycling (12). This pathophysiological process is involved in immune defense, cell differentiation and cell death (12). Previous studies have demonstrated that autophagy participates in pathological cardiac hypertrophy (2,13,14). It is well known that the level of physiological autophagy is important for cellular homeostasis, whereas autophagic cell death is a result of excessive autophagy (14). Numerous studies have indicated that the autophagy may be upregulated in response to pathological stress, including endoplasmic reticulum stress, cardiac hypertrophy and heart failure (11,13). Upregulated autophagy may induce ventricular hypertrophy by facilitating protein degradation during the development from cardiac hypertrophy to heart failure (13).

However, the effect of CaSR modulation on autophagy in cardiac hypertrophy remains to be elucidated. The present study investigated whether the expression of CaSR was changed during isoproterenol (ISO)-induced hypertrophy and whether CaSR was involved in cardiac hypertrophy via the modulation of autophagy.

Materials and methods

Materials. ISO, $Calhex_{231}$, $GdCl_3$, compound C and 3-methyladenine (3-MA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following primary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA): Rabbit polyclonal anti-calcium/calmodulin-dependent protein kinase II (CaMKII; cat. no. CST-3362), rabbit polyclonal anti-phosphorylated (p)-CaMKII (cat. no. CST-3361); rabbit polyclonal anti-sequestosome 1 (p62; cat. no. CST-5114); rabbit monoclonal anti-Beclin-1 (cat. no. CST-3495); rabbit monoclonal anti-microtubule-associated protein light chain 3 (LC3; cat. no. CST-3868); rabbit monoclonal anti-caspase-3 (cat. no. CST-9664); rabbit monoclonal anti-AMP-activated protein kinase (AMPK; cat. no. CST-2535); rabbit monoclonal anti-p-AMPK (cat. no. CST-5832); rabbit monoclonal anti-p-$CaMKK_{\beta}$ (cat. no. CST-12818); rabbit monoclonal anti-mammalian target of rapamycin (mTOR; cat. no. CST-2972); rabbit monoclonal anti-p-mTOR (cat. no. CST-2971); and rabbit monoclonal anti-GAPDH (cat. no. CST-5174). Mouse monoclonal anti-$CaMKK_{\beta}$ (cat. no. SC-100364) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal anti-CaSR (cat. no. ACR-004) was purchased from Alpha Diagnostic International Inc. (San Antonio, TX, USA). Secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG; cat. no. S3731) was obtained from Promega Corporation (Madison, WI, USA). Polyvinylidene difluoride membranes were purchased from Whatman, GE Healthcare Life Sciences (Little Chalfont, UK) and alkaline phosphatase-conjugated horse anti-mouse IgG (cat. no. ZB-2310; Zhongshan Golden bridge Biotechnology, Co., Ltd., Beijing, China).

ISO-induced cardiac hypertrophy in vivo. Male Wistar rats (age, 10-12 weeks; n=70) were kept at 21±2˚C, 60±5% humidity and a 12-h light-dark cycle. The rats were housed together and had free access to food and water. They were injected subcutaneously with ISO once a day to activate β-adrenergic receptors, according to the previously described method (15). Wistar rats (weight, 200-250 g) were randomly assigned to seven treatment groups, as follows: i) Control group (Control, n=10), rats were subcutaneously injected with saline; ii) ISO-1d group (ISO-1d; n=10), rats were subcutaneously injected with ISO (5 mg/kg in saline) for 1 day to induce cardiac hypertrophy; iii) ISO-3d group (ISO-3d; n=10), rats were subcutaneously injected with ISO for 3 days; iv) ISO-5d group (ISO-5d; n=10), rats were subcutaneously injected with ISO for 5 days; v) ISO-7d group (ISO-7d; n=10), the rats were subcutaneously injected with ISO for 7 days; vi) ISO group (ISO, n=10), the rats were subcutaneously injected with normal saline for 2 weeks following the administration of ISO for 7 days; and vii) ISO + $Calhex_{231}$ group (ISO + $Calhex_{231}$; n=10), rats were intravenously injected with the specific CaSR inhibitor $Calhex_{231}$ (10 μmol/kg/day in saline) for 2 weeks following the administration of ISO for 7 days. Subsequently, three rats were sacrificed in each group by overdose of 10% chloral hydrate (0.75-1 ml/100 g). All animals were obtained from the Experimental Animal Center of Harbin Medical University (Harbin, China) and the present study was approved by the Institutional Animal Research Committee of Harbin Medical University.

Echocardiographic assessment. Cardiac function was noninvasively monitored by transthoracic echocardiography with a Vivid 7 Dimension echocardiographic system (GE Healthcare Life Sciences). Briefly, the rats were anesthetized with 10% chloral hydrate (0.25-0.35 ml/100 g; Shanghai Fanke Biotechnology Co. Ltd., Shanghai, China) as described previously (1), and echocardiograms were obtained and analyzed as reported previously.

Histological analysis. Following anesthesia with 10% chloral hydrate (0.25-0.35 ml/100 g), the hearts were excised and immediately placed in 4% paraformaldehyde (Shanghai Fanke Biotechnology Co. Ltd.) at room temperature for 24 h. The myocardial specimens were embedded in paraffin (Shanghai Fanke Biotechnology Co. Ltd.), cut into 4-μm sections and stained with hematoxylin and eosin (H&E; Zhuhai Baso Biotechnology Co., Ltd., Zhuhai, China) and Masson's trichrome reagent (Zhuhai Baso Biotechnology Co., Ltd.). The fibrotic areas stained blue, and the normal tissues stained red. Tissues were analyzed using an E2000 Nikon microscope (Tokyo, Japan).

Electron microscopy. Hearts were removed from three mice in the control, ISO and ISO+$Calhex_{231}$ groups. Cardiac tissue was cut into 1-mm cubes and fixed with 2.5% glutaraldehyde (Shanghai Fanke Biotechnology Co. Ltd.) in 0.1 M phosphate buffer (Shanghai Fanke Biotechnology Co. Ltd.) (pH 7.4) overnight at 4˚C. Following fixation, the sections were immersed in 1% osmium tetroxide (Shanghai Fanke Biotechnology Co. Ltd.) for 2 h, dehydrated in graded ethanol solutions graded ethanol (Shanghai North Connaught Biotechnology Co, Ltd., Shanghai, China), embedded in epoxy resin (Shanghai Fanke Biotechnology Co. Ltd.) and then cut into ultrathin sections (60-70 nm) with an ultramicrotome (Leica Microsystems, Shanghai, China). Sections were then post-stained with

uranyl acetate and lead citrate (Yuanye Technology Co., Ltd., Shanghai, China) prior to examination under a JEM-1010 transmission electron microscope (JEOL, Ltd., Tokyo, Japan).

Establishing an in vitro model of ISO-induced cardiomyocyte hypertrophy. As previously described (8), neonatal rat cardiomyocytes were prepared from 60 2-to-3 day-old neonatal Wistar rats (obtained from the Experimental Animal Center of Harbin Medical University, Harbin, China; wieght, 20-30 g). The rats were immersed in 70% (v/v) alcohol (Shanghai North Connaught Biotechnology Co., Ltd.), and placed on a flat board. Then the mice were sacrificed by decapitation with scissors. The ventricles were removed and washed three times in D-Hank's balanced salt solution (BosterBio, Beijing, China) (0.4 g/l KCl; 0.06 g/l KH_2PO_4; 8.0 g/l NaCl; 0.35 g/l $NaHCO_3$; and 0.06 g/l $Na_2HPO_4{\cdot}7H_2O$, pH 7.2) at 4˚C. They were then homogenized and incubated with 0.25% (w/v) trypsinase for 10 min at 37˚C. Next, an equal volume of cold Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT, USA) containing 10% (v/v) newborn calf serum was added to terminate the digestion. The supernatant was discarded and cells were incubated with fresh 0.25% trypsinase (Beyotime Institute of Biotechnology, Jiangsu, China) for 15 min at 37˚C, and the supernatant was then collected. The latter digestion step was repeated four times. Cells in the supernatant were isolated by centrifugation at 286 x g and room temperature for 10 min, then resuspended in DMEM containing 20% (v/v) newborn calf serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Yuanye Technology Co., Ltd.). The cells were cultured in a monolayer at a density of $5x10^4$ cells/cm^2 at 37˚C in a humidified atmosphere containing 5% (v/v) CO_2. The medium contained 2 μM fluorodeoxyuridine (Shanghai Fanke Biotechnology Co. Ltd.) to prevent proliferation of nonmyocytes.

Three days after seeding, the neonatal rat cardiomyocytes were starved in serum-free DMEM for 24 h then divided randomly into six groups as follows: i) Normal control group; ii) ISO group, cardiomyocytes treated with 10 μM ISO for 48 h; iii) $GdCl_3$ + ISO group, cardiomyocytes preincubated with 30 μM $GdCl_3$ (specific CaSR agonist) for 1 h and then treated with 10 μM ISO for 48 h; iv) $GdCl_3$ + $Calhex_{231}$+ISO group, the cardiomyocytes were preincubated with 3 μM $Calhex_{231}$ (specific CaSR inhibitor) for 30 min prior to the addition of ISO; v) $GdCl_3$ + 3-MA + ISO group, the cardiomyocytes were preincubated with 5 mM 3-MA (specific autophagy inhibitor) for 30 min prior to the addition of ISO; and vi) $GdCl_3$ + compound C + ISO group, the cardiomyocytes were preincubated with 5 μM compound C (AMPK inhibitor) for 30 min prior to the addition of ISO.

Measurement of $[Ca^{2+}]_i$ in cardiomyocytes. Following the described treatments, cardiomyocytes were loaded with 1 μM Fluo-4/AM (Sigma-Aldrich) at 37˚C for 30 min. The cells were washed twice with Ca^{2+}-free phosphate-buffered saline to remove the remaining dye and then further incubated in DMEM. Changes in $[Ca^{2+}]_i$ were measured by the fluorescence intensity induced by Fluo-4 in the cardiomyocytes recorded for 5 min using an IX-70 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan; x600 magnification) with excitation and emission at 488 and 530 nm, respectively. Image Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) was used for analysis.

Western blotting. Protein was isolated from rat cardiac tissues and neonatal rat cardiomyocytes, which were homogenized in 0.5 ml of RIPA buffer prior to being transferred into small tubes and rotated 1 h at 4˚C. Protein concentrations were determined by the Coomassie method (Beyotime Institute of Biotechnology) using bovine serum albumin as the standard. All samples (containing 80 μg protein) were mixed with loading buffer (Beyotime Institute of Biotechnology) and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins in the samples from different experimental groups were separated and transferred onto polyvinylidene difluoride membranes (GE Healthcare Life Sciences) by electroblotting (300 mA for 2 h). Membranes were blocked in Tris-buffered saline with 0.1% (v/v) Tween 20 [TBS-T; 137 mM NaCl, 20 mM Tris (pH 7.6)] (Applygen Technologies Inc., Beijing, China) containing 5% (w/v) skimmed milk at 37˚C for 1 h. Membranes were then incubated overnight at 4˚C with antibodies against CaSR (1:800), and CaMKII, p-CaMKII, p62, Beclin-1, LC3, caspase-3, AMPK, p-AMPK, $CaMKK_\beta$, p-$CaMKK_\beta$, mTOR, p-mTOR and GAPDH (all 1:1,000). Membranes were then washed with TBST three times for 5 min and incubated with an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (alkaline phosphatase-conjugated IgG; 1:5,000) and alkaline phosphatase-conjugated anti-mouse IgG (Zhongshan Golden bridge Biotechnology, Co., Ltd.) in TBS-T for 1 h at room temperature. The densities of the protein bands were quantified using a Chemi Doc EQ densitometer and Quantity One 4.6.2 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and GAPDH served as an internal control for the semi-quantitative assay.

Statistical analysis. All data were obtained from at least three independent experiments that were replicated two to four times under each condition. All values are expressed as the mean ± standard error of the mean. Comparisons between the groups were performed using Kruskal-Wallis two-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ISO induced cardiac hypertrophy in rats. An increase in cardiomyocyte volume and extracellular matrix deposition is characteristic of myocardial hypertrophy. To investigate the *in vivo* effects of CaSR in hypertrophic hearts, a model of cardiac hypertrophy model was established by administering ISO to rats for 7 days. Myocardial function was assessed using echocardiography. At 1, 3 and 5 days after ISO injection, animals injected with ISO exhibited an increase in interventricular septum (IVS) thickness, diastolic left ventricle posterior wall (LVPWd) thickness and a decrease in left ventricle ejection fraction (LVEF) compared with the control group (Table I). However, there was no statistical difference between the control and ISO-1d, -3d and -5d animals with the exception of LVIDd. At 7 days after ISO injection, the myocardial dysfunction was

Table I. Echocardiographic analysis of left ventricular wall and chamber dimension in control, ISO-1d, ISO-3d, ISO-5d, ISO-7d, and ISO + $Calhex_{231}$-treated rats.

Parameter	Control	ISO-1d	ISO-3d	ISO-5d	ISO-7d	ISO + $Calhex_{231}$
IVSd (cm)	0.17±0.02	0.17±0.01	0.19±0.01	0.19±0.02	0.22±0.02[a]	0.19±0.01[b]
IVSs (cm)	0.22±0.03	0.23±0.02	0.26±0.02	0.27±0.02	0.32±0.06[a]	0.25±0.02[b]
LVPWd (cm)	0.16±0.02	0.17±0.01	0.19±0.02	0.21±0.01	0.23±0.03[a]	0.18±0.02[b]
LVIDd (cm)	0.51±0.07	0.56±0.07	0.52±0.05	0.68±0.04[a]	0.75±0.06[a]	0.65±0.04[b]
LVEF (%)	86.20±4.68	83.37±5.58	77.13±12.78	73.93±10.96	57.55±6.46[a]	70.76±5.70[b]

Values are expressed as the mean ± standard error of the mean. [a]P<0.05 vs. control group, [b]P<0.05 vs. ISO-7d group. ISO, isoproterenol; IVSd, diastolic interventricular septum thickness; IVSs, systolic interventricular septum thickness; LVPWd, diastolic left ventriclular posterior wall thickness; LVIDd, diastolic left ventricular internal dimension; LVEF, left ventricular ejection fraction.

further exacerbated, with a significant increase in diastolic and systolic IVS (IVSd and s), LVPWd and diastolic left ventricular internal dimension (LVIDd), and a significant decrease in LVEF, compared with the control group (all P<0.05). The results indicated that cardiac hypertrophy was occurring at 7 days post-ISO injection (Table I).

H&E staining demonstrated that ISO markedly increased the cell cross-sectional area of the myocardial tissue compared with the control group (Fig. 1A). Morphological analysis of the control group demonstrated that the cardiomyocytes exhibited a clear arrangement into defined rows, intercalated discs and transverse stripes with loose nuclear chromatin. However, following ISO injection, the rat cardiac tissues were expanded, and the muscle fibers became thickened and disorganized. These changes were the most marked in the ISO group (Fig. 1A).

Masson's staining demonstrated that the hearts of ISO treated rats exhibited extensive interstitial fibrosis in the ventricular wall compared with control hearts. Morphological analysis revealed that the cytoplasm, collagen fibers and red blood cells were stained blue, and the nuclei stained blue-brown. The control group exhibited normal myocardial fibers, whereas, the ISO group exhibited a large number of blue-stained collagen fibers (Fig. 1A).

As demonstrated by western blot analysis, the protein expression level of CaSR in the rat heart was significantly increased in the ISO-7d group compared with the control group (P<0.01; Fig. 1B).

$Calhex_{231}$ ameliorates cardiac hypertrophy induced by ISO in rats. The CaSR inhibitor, $Calhex_{231}$, binds to the transmembrane domains of CaSR to compete with Ca^{2+} molecules (16,17). Echocardiography demonstrated that cardiac systolic and diastolic functions were improved following $Calhex_{231}$ administration compared with the ISO group. $Calhex_{231}$ treatment ameliorated cardiac functions, with IVSd, IVSs, LVPWd and LVIDd significantly decreased, and LVEF increased compared with the ISO group (all P<0.05; Table I). $Calhex_{231}$ markedly attenuated the levels of ventricular hypertrophy and fibrosis, as well as cardiomyocyte apoptosis induced by ISO (Fig. 1A). In addition, the protein expression level of CaSR was significantly decreased following $Calhex_{231}$ treatment compared with the ISO group (P<0.05; Fig. 1B), suggesting that $Calhex_{231}$ treatment ameliorated cardiac hypertrophy induced by ISO.

Levels of autophagy increased during ISO-induced cardiac hypertrophy in rats. Transmission electron microscopy indicated that the administration of $Calhex_{231}$ attenuated the disorganized sarcomere structure and mitochondrial disarray observed in ISO-induced hypertrophic hearts, which was consistent with the observation of increased autophagosomes. The electron microscopy results prompted the present study to further investigate the effect of $Calhex_{231}$ on autophagy (Fig. 2A).

Autophagy is a bulk degradation mechanism for damaged cytosolic organelles and proteins with long half lives. The protein expression levels of Beclin-1 and LC3II were measured as markers of autophagy (18) and autophagosome formation (19), respectively. Beclin-1 and LC3II levels were increased by ISO treatment compared with control (P<0.05), whereas the expression of P62, which transports a number of ubiquitinated substrates to autophagosomes (20), was reduced in the ISO group (P<0.05). Furthermore, the administration of $Calhex_{231}$ decreased the protein expression levels of Beclin-1 and LC3II, and increased P62 expression levels compared with that of ISO group (all P<0.05) (Fig. 2B).

Administration of CaSR inhibitor ameliorated hypertrophy in neonatal cardiomyocytes. CaMKII is an essential signaling molecule involved in cardiac hypertrophy. The present study demonstrated that the protein content and expression of p-CaMKII were increased in cardiomyocytes of the ISO group compared with the control group (P<0.05; Table II; Fig. 3A). In addition, the protein content and p-CAKKII levels were significantly increased in the $GdCl_3$ + ISO group compared with the ISO group (P<0.05). $Calhex_{231}$ treatment also attenuated these increases, as the phosphorylation of p-CAMKII level were significantly reduced compared with the $GdCl_3$ + ISO group (P<0.05). Notably, treatment with 3-MA, an autophagy inhibitor, also significantly decreased the level of p-CaMKII compared with that of the $GdCl_3$ + ISO group (P<0.05).

The protein expression level of CaSR in cardiomyocytes was significantly increased in the ISO and $GdCl_3$ + ISO groups compared with the control group (P<0.05). $Calhex_{231}$ treatment attenuated the increase of CaSR expression induced by ISO

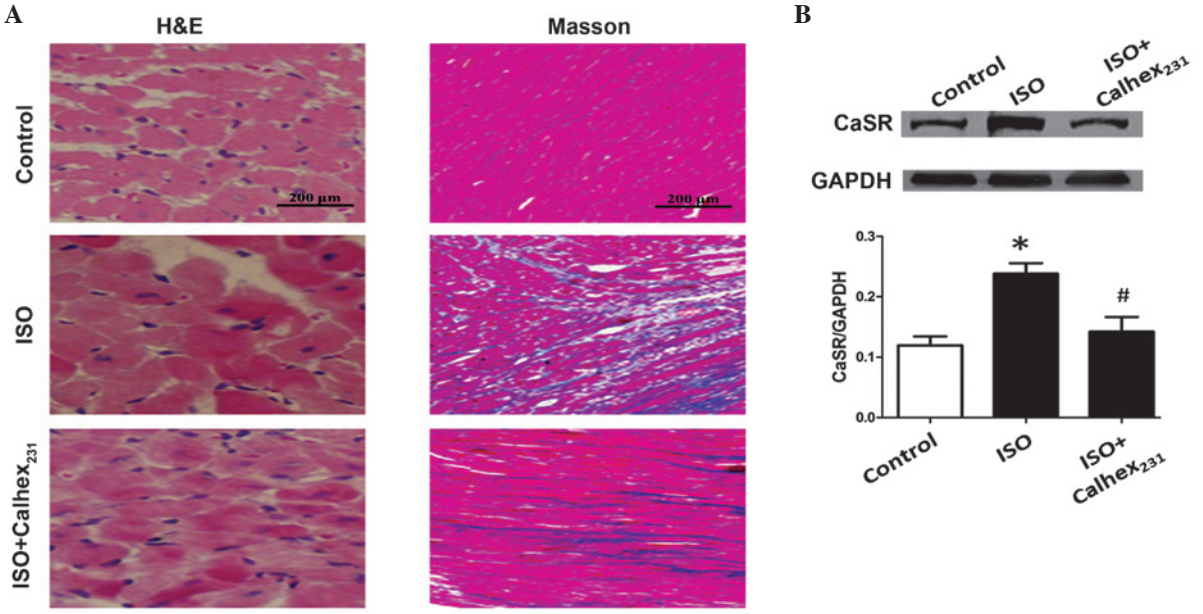

Figure 1. $Calhex_{231}$ ameliorated cardiac hypertrophy induced by ISO in rats. (A) Morphological changes of rat cardiac tissue with H&E and Masson's staining in different groups (magnification, x200). (B) Protein expression of CaSR in rat heart tissues as determined by western blot analysis. The intensity of each band was quantified by densitometry and the data were normalized to the GAPDH protein band intensity. The fold change values are represented as the mean ± standard error of the mean from three independent determinations. *P<0.05 vs. the control group, #P<0.05 vs. the ISO group. H&E, hematoxylin and eosin; ISO, isoproterenol; CaSR, calcium-sensing receptor.

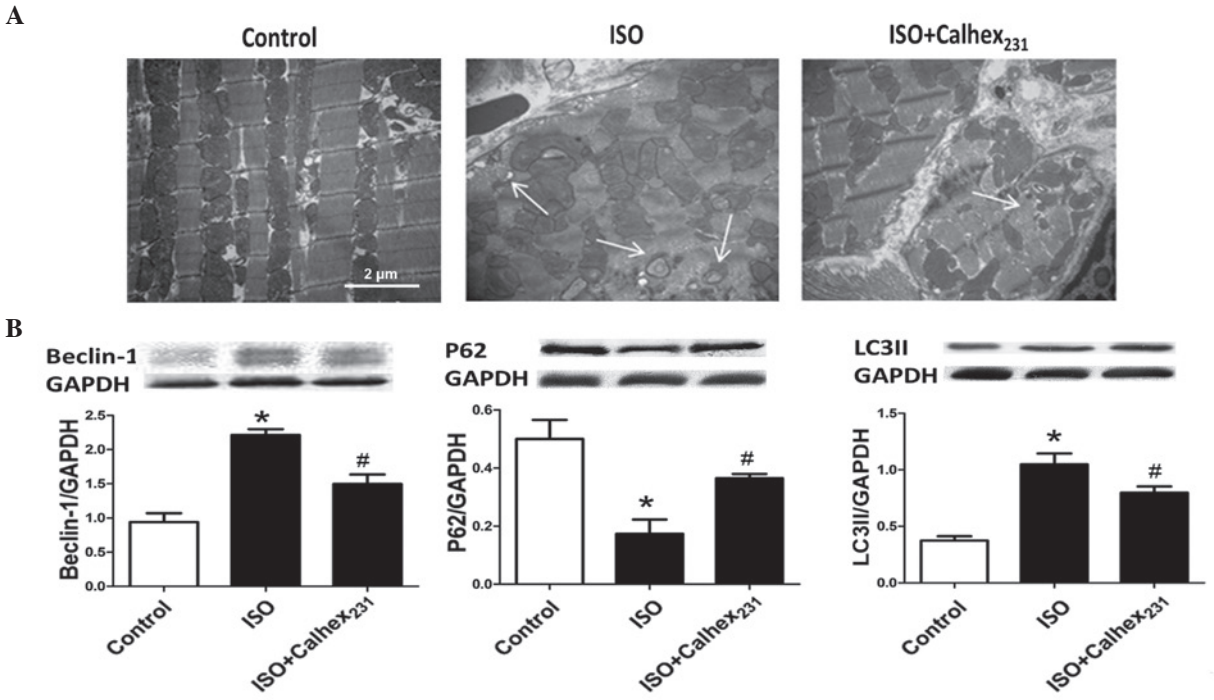

Figure 2. Effect of CaSR on autophagy in rats. (A) Representative autophagic ultrastructure of heart tissue following ISO injection under transmission electron microscopy (magnification, x15,000). (B) Protein expression of Beclin-1, p62, and LC3II, as analyzed by western blotting. The intensity of each band was quantified using densitometry and the data were normalized to the GAPDH protein band intensity. Values are represented as the mean ± standard error of the mean. *P<0.05 vs. the control group, #P<0.05 vs. the ISO group. ISO, isoproterenol; p62, sequestosome 1; LC3II, microtubule-associated protein light chain 3 II.

and $GdCl_3$; the levels were significantly decreased compared with the $GdCl_3$ + ISO group (P<0.05; Fig. 3B).

The current study demonstrated that ISO markedly increased $[Ca^{2+}]_i$ compared with the control group, and that $GdCl_3$ exerted a synergistic effect to induce a further increase of $[Ca^{2+}]_i$ in the $GdCl_3$ + ISO group compared with the ISO group (P<0.05). Furthermore, this effect was blocked by $Calhex_{231}$ treatment compared with the $GdCl_3$ + ISO group (Fig. 3C).

CaSR inhibition ameliorated hypertrophic cardiomyocytes via suppression of autophagy. In agreement with the results

Table II. Changes of protein content in the control, ISO, $GdCl_3$ + ISO, $GdCl_3$ + $Calhex_{231}$ + ISO and $GdCl_3$ + 3-MA + ISO group rats.

Treatment group	Rats	Protein content
Control	6	3.06±0.96
ISO	6	5.61±1.83[a]
$GdCl_3$ + ISO	6	8.45±1.97[b]
$GdCl_3$ + $Calhex_{231}$ + ISO	6	5.07±2.11[c]
$GdCl_3$ + 3-MA + ISO	6	4.82±2.01[c]

Values are expressed as the mean ± standard error of the mean. [a]P<0.05 vs. control group, [b]P<0.05 vs. ISO group and [c]P<0.05 vs. $GdCl_3$ + ISO group. ISO, isoproterenol; 3-MA, 3-methyladenine.

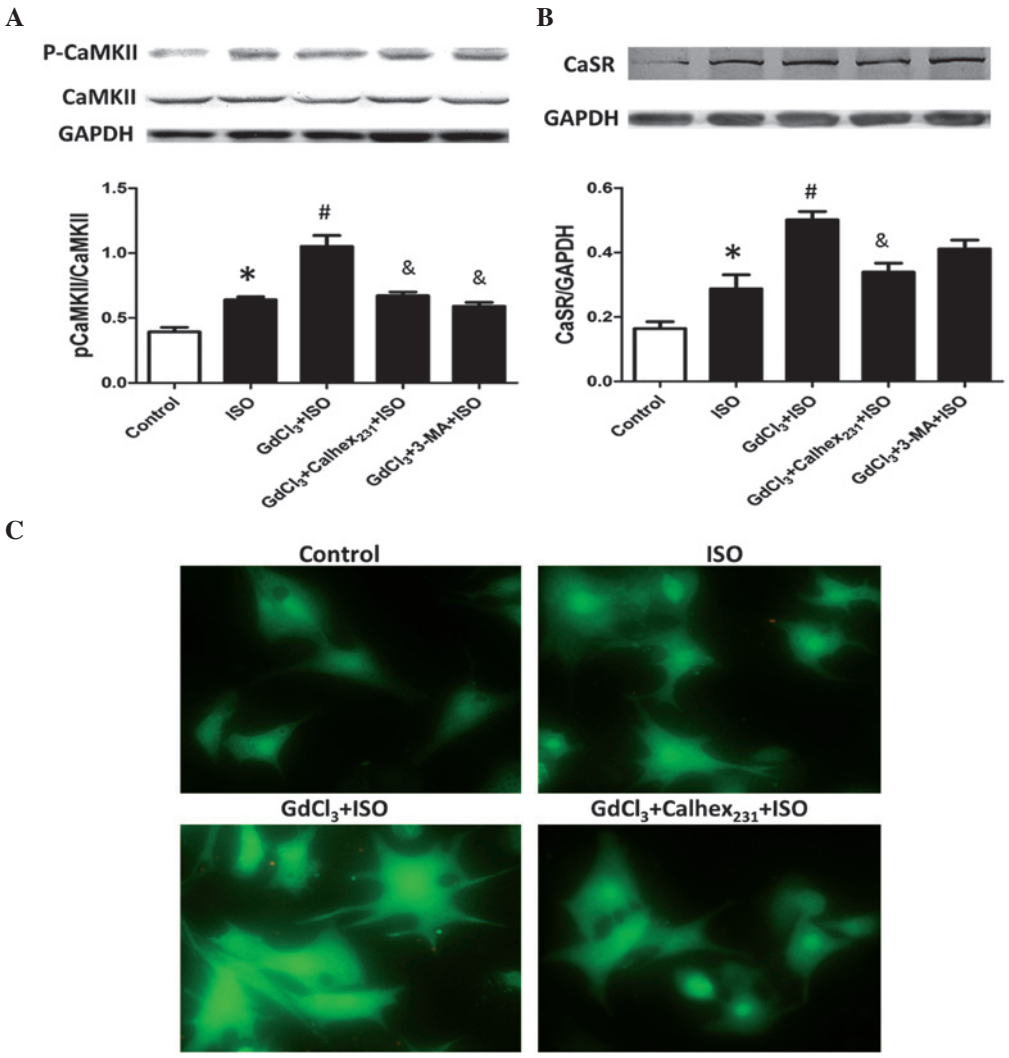

Figure 3. CaSR inhibitor administration inhibited ISO-induced neonatal cardiomyocytes hypertrophy. Expression of (A) the hypertrophic marker CaMKII and (B) CaSR in neonatal rat cardiomyocytes was determined by western blot analysis. The intensity of each band was quantified by densitometry, and the data were normalized to the GAPDH protein band intensity. The fold change values are represented as the mean ± standard error of the mean from three independent determinations. *P<0.05 vs. the control group, #P<0.05 vs. the ISO group and &P<0.05 vs. the $GdCl_3$ + ISO group. (C) Fluorescent images of cardiomyocytes in the control, ISO, $GdCl_3$ + ISO group and $GdCl_3$ + $Calhex_{231}$ + ISO groups. p-CAMKII, calcium/calmodulin-dependent protein kinase II; CaSR, calcium-sensing receptor; ISO, isoproterenol; 3-MA, 3-methyladenine.

from the *in vivo* ISO-induced hypertrophy model, the protein expression levels of Beclin-1 and p62 in the *in vitro* cardiomyocyte model were significantly increased and decreased, respectively, in the ISO group compared with the control (P<0.05). In addition, the expression levels of Beclin-1 and p62 in the $GdCl_3$ + ISO group were significantly increased and decreased, respectively, compared with the ISO group (P<0.05). $Calhex_{231}$ inhibited these effects; the levels of

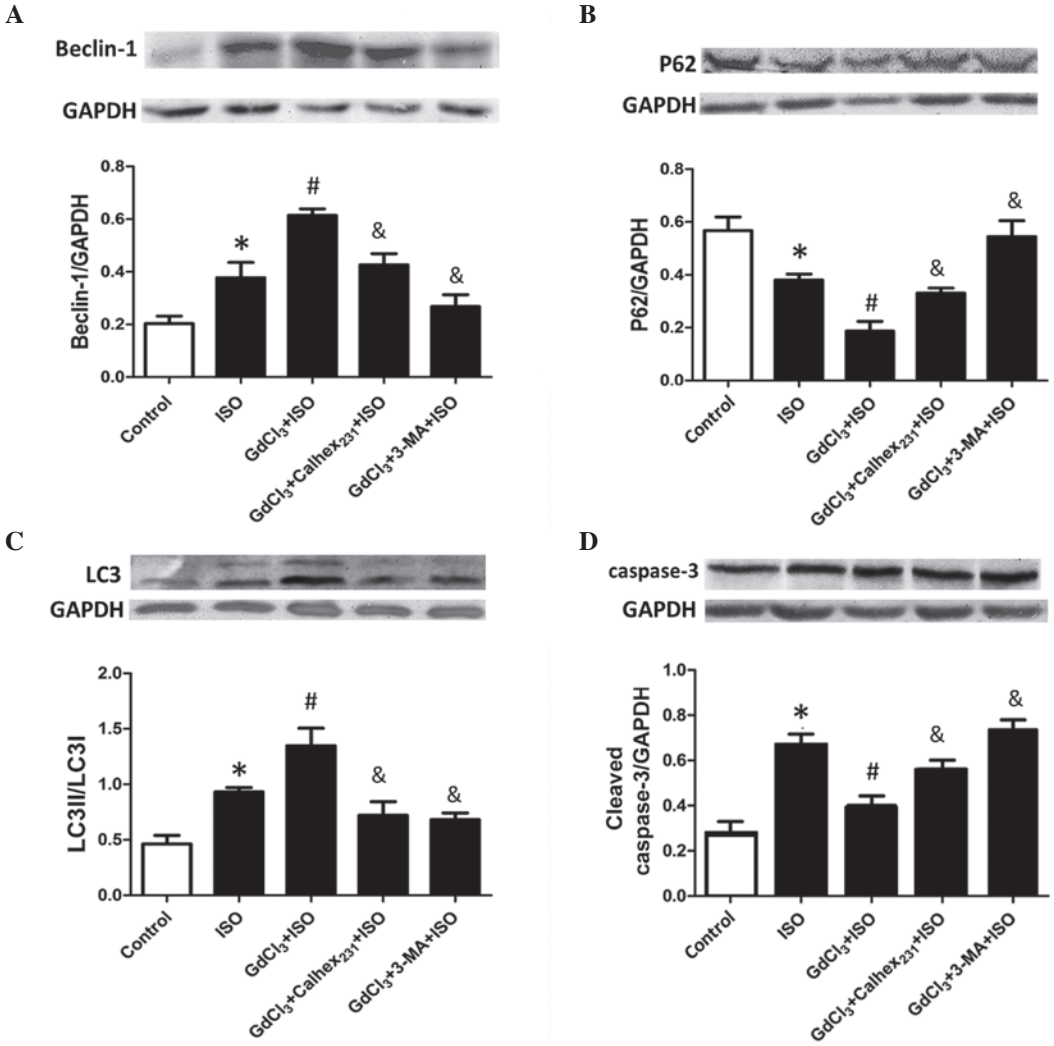

Figure 4. Protein expression of (A) Beclin-1, (B) P62, (C) LC3 and (D) cleaved caspase-3 was determined by western blot analysis in neonatal rat cardiomyocytes. The intensity of each band was quantified using densitometry, and the data were normalized to the GAPDH protein band intensity. Values are represented as the mean ± standard error of the mean. *P<0.05 vs. the control group, #P<0.05 vs. the ISO group and &P<0.05 vs. the $GdCl_3$ + ISO group. P62, sequestosome 1; ISO, isoproterenol; 3-MA, 3-methyladenine; LC3, microtubule-associated protein light chain 3.

Beclin-1 and p62 were significantly decreased and increased, respectively, in the $GdCl_3$ + $Calhex_{231}$ + ISO group compared with the $GdCl_3$ + ISO group (P<0.05). Furthermore, autophagy was significantly inhibited by 3-MA treatment (P<0.05) (Fig. 4A and B).

To investigate the functional association between apoptosis and autophagy, the current study analyzed the protein expression level of cleaved caspase-3 in the *in vitro* cardiomyocyte model. The LC3 isoform, LC3I, is soluble and exists in the cytosol, whereas LC3II is membrane-bound. The level of LC3II increases when autophagy is induced, reflecting the enhanced lipidation reaction. Thus, the level of LC3II provides a measure of autophagy induction, however the rate of the LC3II increase depends on the cell type (21). ISO treatment increased the cleaved caspase-3 and LC3 protein expression levels in the ISO group compared with the control group (P<0.05; Fig. 4C and D). In addition, the current study demonstrated that $GdCl_3$ significantly increased the LC3II/LC3I ratio (P<0.05), whereas it decreased the expression of caspase-3 (P<0.05) in the $GdCl_3$ + ISO group compared with the ISO group. Notably, although 3-MA inhibited CaSR-augmented autophagy, the apoptotic index was markedly increased. Compared with the $GdCl_3$ + ISO group, pretreatment with 3-MA significantly decreased the LC3II/LC3I ratio (P<0.05) and increased the expression of cleaved caspase-3 (P<0.05) in the $GdCl_3$ + 3-MA + ISO group. These results indicated that $Calhex_{231}$ suppresses autophagy against hypertrophic stimuli to ameliorate cardiomyocyte survival.

CaSR inhibition reduced autophagy via suppressing the $CaMKK_{\beta}$-AMPK-mTOR signaling pathway. To elucidate the underlying molecular mechanisms involved in CaSR-induced autophagic responses, the expression levels of $CaMKK_{\beta}$, p-$CaMKK_{\beta}$, AMPK, p-AMPK, mTOR and p-mTOR were measured. In the *in vitro* cardiomyocyte model, increased p-$CaMKK_{\beta}$ and p-AMPK levels, and decreased p-mTOR levels were demonstrated in the ISO group compared with the control group (P<0.05). Furthermore, compared with the ISO group, these effects were significantly enhanced in the $GdCl_3$ + ISO group (P<0.05). However, $Calhex_{231}$ blocked these effects; compared with the $GdCl_3$ + ISO group, p-$CaMKK_{\beta}$ and p-AMPK levels were significantly decreased, and p-mTOR

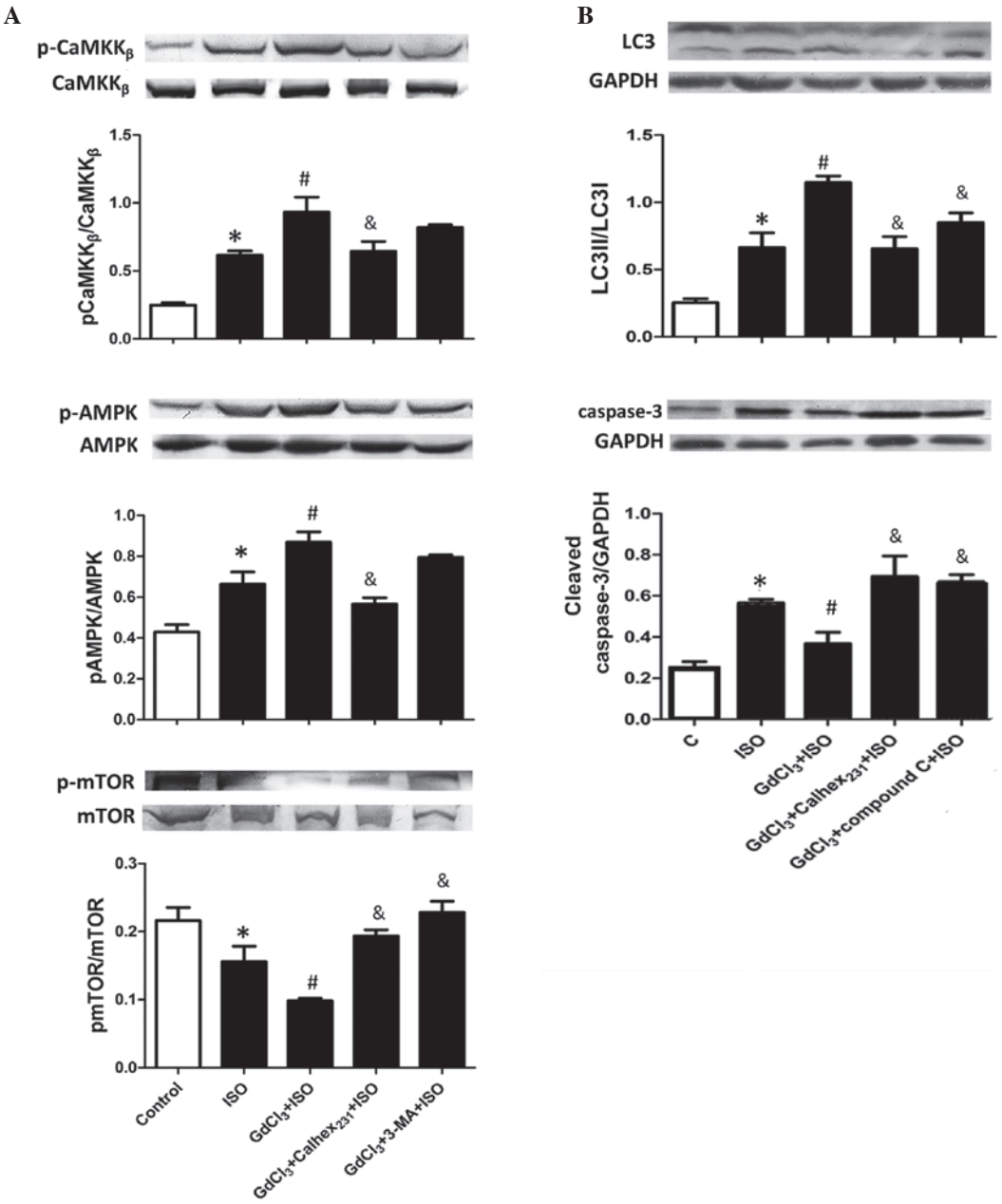

Figure 5. Effect of calcium-sensing receptor on autophagy initiation signaling during ISO-induced cardiac hypertrophy. Protein expression levels of (A) $CaMKK_{\beta}$, AMPK and mTOR, and (B) LC3 and cleaved caspase-3 were determined by western blot analysis of neonatal rat cardiomyocytes. The intensity of each band was quantified by densitometry and the data were normalized to the GAPDH protein band intensity. The fold change values are represented as the mean ± standard error of the mean from three independent determinations. $^{*}P<0.05$ vs. the control group, $^{\#}P<0.05$ vs. the ISO group and $^{\&}P<0.05$ vs. the $GdCl_3$ + ISO group. p-, phosphorylated-; $CaMKK_{\beta}$, $Ca2^{+}$/calmodulin-dependent-protein kinase kinase 2 β, LC3, microtubule-associated protein light chain 3; AMPK, AMP-activated protein kinase; ISO, isoproterenol; 3-MA, 3-methyladenine; mTOR, mechanistic target of rapamycin.

levels increased by $Calhex_{231}$ ($P<0.05$; Fig. 5A). The results of the current study indicated that CaSR stimulates autophagy and the effect is mediated by $CaMKK_{\beta}$-AMPK-mTOR signaling.

The effect of compound C (a specific AMPK inhibitor) on the $Calhex_{231}$-induced suppression of autophagy was investigated. Fig. 5B demonstrates the cellular molecular signaling pathways induced by CaSR to increase autophagy and decrease apoptosis. Compared with the control group, ISO treatment significantly increased the LC3II/LC3I ratio and the expression level of cleaved caspase-3 ($P<0.05$). In addition, $GdCl_3$ treatment increased the LC3II/LC3I ratio compared with the ISO group, however, the cleaved caspase-3 level was decreased. A negative association between autophagy and apoptosis induced by CaSR was observed. However, compared with the $GdCl_3$ + ISO group, the LC3II/LC3I ratio was significantly reduced and the caspase-3 level was increased in the $GdCl_3$ + compound C+ ISO group, suggesting that CaSR regulates the autophagy level, which is mediated by the $CaMKK_{\beta}$-AMPK-mTOR signaling pathway ($P<0.05$). $Calhex_{231}$ reduced the CaSR-augmented autophagy via suppressing the $CaMKK_{\beta}$-AMPK-mTOR signaling pathway (Fig. 5B).

Discussion

In the present study, a rat cardiac hypertrophy model was established using ISO. The levels of CaSR expression and

autophagy were markedly increased in hypertrophic hearts. Furthermore, CaSR inhibition significantly reduced autophagy signaling and ameliorated cardiac hypertrophy (P<0.05). In addition, the experimental results of the neonatal rat hypertrophic cardiomyocytes induced by ISO *in vitro* were consistent with the results obtained from the animal model, supporting the findings of the present study.

Previous studies have demonstrated that CaSR is expressed throughout the cardiovascular system and is important in cardiac physiology and pathophysiology (22,23). CaSR releases $[Ca^{2+}]_i$ by accumulation of inositol phosphate. Increased $[Ca2+]_i$ activates certain Ca^{2+}-dependent signaling pathways, which result in myocardial hypertrophy (24). CaSR is activated by Gd^{3+} and Mg^{2+} (type I activators), which are present in extracellular fluids, whereas, calcilytics, including $Calhex_{231}$ inhibit the effect of Ca^{2+} on CaSR (25).

Myocardial hypertrophy is characterized by an increase in cardiomyocyte size and protein content (26,27). The manifestation of cardiac hypertrophy involves increases in the heart size and interstitial fibrosis (28). Numerous reports have indicated that ISO is important for mediating load-induced myocardial hypertrophy (4,9). In the present study, when rats were administered ISO for 7 days, the heart size, quantity of interstitial collagen and the cardiomyocyte size were markedly increased, indicating dysfunction of the heart. The current study demonstrated that ISO induced cardiac hypertrophy. Furthermore, the protein expression level of CaSR was markedly increased in hypertrophic myocardium. A similar effect was observed in cardiomyocytes treated with ISO *in vitro*, with a notable increase in the cardiomyocyte size, $[Ca^{2+}]_i$, p-CaMKII expression level, protein content and expression of CaSR observed. CaSR inhibitor markedly protected cardiomyocytes against cardiac hypertrophy induced by ISO injection *in vivo* and *in vitro*. The present study demonstrated that treatment with CaSR inhibitor decreased cardiomyocyte size, CaMKII expression and protein content in hypertrophic hearts and cardiomyocytes, and markedly improved the cardiac functions. These results indicate that CaSR inhibition effectively reduced myocardial hypertrophic remodeling.

Autophagy maintains cellular homeostasis and degrades proteins with long half-lives or damaged organelles to avoid apoptosis initiation (29,30). Consistent with the results of the present study, autophagy is markedly increased during cardiac dysfunction resulting from hypertensive heart disease, ischemic heart disease and dilated cardiomyopathy (31,32). The current study demonstrated that autophagy was markedly increased in ISO-induced hypertrophic rat hearts and cardiomyocytes. CaSR inhibition significantly suppressed autophagy to aid the survival of cardiomyocytes under hypertrophic conditions (P<0.05). In addition, ISO treatment markedly increased apoptosis *in vivo* and *in vitro*. However, ISO treatment following pre-incubation with a CaSR activator significantly increased the level of autophagy and decreased apoptosis *in vitro*, compared with the ISO treatment alone (P<0.05). By contrast, treatment with a CaSR inhibitor significantly increased ISO-induced apoptosis and decreased autophagy in cardiomyocytes. As the balance between autophagy and apoptosis maintains homeostasis, inactivation of autophagy may result in the accumulation abnormal proteins and organelles, thus, promoting apoptosis (33). CaSR-induced autophagy was investigated further by treatment of cardiomyocytes with 3-MA. Compared with the $GdCl_3$ + ISO group, the autophagy level was decreased and cardiomyocyte apoptosis was significantly increased following 3-MA treatment (P<0.05), consistent with a previous report (34).

To investigate the potential underlying mechanisms of CaSR-induced autophagy, the current study examined intracellular signaling pathways regulated by CaSR that upregulate autophagy. $CaMKK_\beta$ has previously been demonstrated to be activated by increased $[Ca^{2+}]_i$ and stimulates AMPK (35). AMPK is responsible for sensing energy and nutrients, and is involved in promoting autophagy by directly activating the mammalian autophagy-initiating kinase unc-51 like autophagy activating kinase 1 via phosphorylation of Ser317 and Ser777 (36). Thus, $CaMKK_\beta$ may induce autophagy by activating AMPK and inhibiting the mTOR signaling pathway. ISO treatment increased the phosphorylation of $CaMKK_\beta$ and AMPK, and decreased mTOR. Notably, compared with the ISO only, treatment with CaSR activator and ISO increased $CaMKK_\beta$ and AMPK phosphorylation, whereas mTOR phosphorylation was decreased. However, the CaSR inhibitor blocked these effects. The results of the current study indicate that inhibition of CaSR in the $CaMKK_\beta$-AMPK-mTOR signaling pathway may contribute to cardiomyocyte protection. Furthermore, the present study demonstrated that compound C inhibits AMPK and significantly decreases CaSR-induced autophagy in cardiomyocytes treated with ISO (P<0.05). These data demonstrate that CaSR stimulates autophagy in hypertrophic cardiomyocytes via activation of the $CaMKK_\beta$-AMPK-mTOR signaling pathways.

In conclusion, the result of the present study indicate that the expression of CaSR is upregulated in ISO-induced cardiac hypertrophy. Furthermore, inhibition of CaSR may ameliorate ISO-induced cardiac hypertrophy. This effect may be associated with inhibition of autophagy and suppression of the $CaMKK_\beta$-AMPK-mTOR signaling pathway. Cardiac hypertrophy is induced by multiple factors, including pressure overload, ISO, swimming and exercise. The present study investigated cardiac hypertrophy in ISO-induced models, thus, the results require further validation in other models of cardiac hypertrophy. The results of the current study may support the potential use of a CaSR inhibitor as a novel therapeutic agent for the treatment of cardiac hypertrophy.

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