Calhex\textsubscript{231} Ameliorates Cardiac Hypertrophy by Inhibiting Cellular Autophagy \textit{in Vivo} and \textit{in Vitro}

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Key Words
Calcium-sensing receptor (CaSR) • Calhex\textsubscript{231} • Autophagy • Cardiac hypertrophy

Abstract

\textbf{Background/Aims:} Intracellular calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) homeostasis, an initial factor of cardiac hypertrophy, is regulated by the calcium-sensing receptor (CaSR) and is associated with the formation of autolysosomes. The aim of this study was to investigate the role of Calhex\textsubscript{231}, a CaSR inhibitor, on the hypertrophic response via autophagy modulation.

\textbf{Methods:} Cardiac hypertrophy was induced by transverse aortic constriction (TAC) in 40 male Wistar rats, while 10 rats underwent a sham operation and served as controls. Cardiac function was monitored by transthoracic echocardiography, and the hypertrophy index was calculated. Cardiac tissue was stained with hematoxylin and eosin (H&E) or Masson’s trichrome reagent and examined by transmission electron microscopy. An angiotensin II (Ang II)-induced cardiomyocyte hypertrophy model was established and used to test the involvement of active molecules. Intracellular calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) was determined by the introduction of Fluo-4/AM dye followed by confocal microscopy. The expression of various active proteins was analyzed by western blot.

\textbf{Results:} The rats with TAC-induced hypertrophy had an increased heart size, ratio of heart weight to body weight, myocardial fibrosis, and CaSR and autophagy levels, which were suppressed by Calhex\textsubscript{231}. Experimental results using Ang II-induced hypertrophic cardiomyocytes confirmed that Calhex\textsubscript{231} suppressed CaSR expression and downregulated autophagy by inhibiting the Ca\textsuperscript{2+}/calmodulin-dependent-protein kinase-kinase-β (CaM KK\textsubscript{β})–AMP-activated protein kinase (AMPK)–mammalian target of rapamycin (mTOR) pathway to ameliorate cardiomyocyte hypertrophy.

\textbf{Conclusions:} Calhex\textsubscript{231} ameliorates myocardial hypertrophy induced by pressure-overload or Ang II via inhibiting CaSR expression and autophagy. Our results may support the notion that Calhex\textsubscript{231} can become a new therapeutic agent for the treatment of cardiac hypertrophy.

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Introduction

Cardiac hypertrophy, which results from a compensatory mechanism in response to mechanical stimuli (i.e., pressure-overload and hemodynamic changes) and humoral stimuli (i.e., norepinephrine and Ang II), leads to heart failure or sudden death [1, 2]. Although therapies are available, advances in preventing the progression of cardiac hypertrophy have been limited due to the lack of knowledge regarding the involved pathophysiological mechanisms.

Our previous study has reported that activation of calcium-sensing receptor (CaSR) can promote myocardial hypertrophy via an increase of intracellular calcium concentration ([Ca^{2+}]_{i}) [3]. CaSR, a member of the C subfamily of membrane G-protein coupled receptors, releases intracellular Ca^{2+} by accumulating inositol phosphate [4]. The functional expression of CaSR was initially discovered in rat cardiac tissue in 2003 [5]. Calhex_{231}, a negative allosteric modulator, inhibits the parathyroid receptor and blocks the effects of Ca^{2+}; therefore, it is used as a calcilytic agent to inhibit CaSR [6]. It binds to the transmembrane domains of CaSR and competes with the cellular flux of Ca^{2+} [7]. However, the role of Calhex_{231} in cardiac hypertrophy is still unknown. Cardiac hypertrophy is controlled by a complex signal transduction and gene regulatory network [8, 9]. Recently, autophagy has been shown to be involved in the pathogenesis of cardiac hypertrophy [10].

Autophagy is a self-digestive process that targets internal or damaged organelles and misfolded proteins for lysosomal degradation [11]. Under physiological conditions, autophagy plays an important role in the maintenance of cardiac geometry and function [12]. However, various stresses may induce an extensive (or persistent) autophagy, causing cell death via different molecular mechanisms [13]. Numerous studies have demonstrated that autophagy may be upregulated in response to pathological stresses such as endoplasmic reticulum stress, ischemia/reperfusion injury, and heart failure [14]. Moreover, [Ca^{2+}]_{i} plays a role in autophagy [15]. Consequently, a thorough understanding of the relationship between autophagy and [Ca^{2+}]_{i} may provide a better rationale for the therapeutic application of Calhex_{231} to treat cardiac hypertrophy. In the present study, we investigated the effect of Calhex_{231} on autophagy modulation upon hypertrophic response.

Materials and Methods

Materials

Ang II, Calhex_{231}, R568, GdCl_{3}, compound C, and 3-methyladenine (3-MA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for calcium/calmodulin-dependent protein kinase II (CaMKII), p-CaMKII, p62, Beclin1, microtubule-associated protein light chain 3 (LC3), caspase-3, AMP-activated protein kinase (AMPK), p-AMPK, Ca^{2+}/calmodulin-dependent-protein kinase-kinase-β (CaMKKβ), p-CaMKKβ, mammalian target of rapamycin (mTOR), p-mTOR, and GAPDH were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-CaSR was from Alpha Diagnostic International Inc. (San Antonio, TX, USA). The secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG) was from Promega Corporation (Madison, WI, USA). Polyvinylidene difluoride membranes were from Whatman (Buckinghamshire, UK).

Pressure overload-induced cardiac hypertrophy in vivo

Pressure overload and cardiac hypertrophy were induced by transverse aortic constriction (TAC) in male Wistar rats, according to the method reported previously [16]. Wistar rats, weighing 200–250 g, were anesthetized with 10% chloral hydrate (0.25 mL/100 g of body weight (BW), intraperitoneal injection). Animals were placed in the supine position. The abdomen was opened, and the abdominal aorta was identified. A 7-0 silk suture was placed around the transverse aorta and tied with a 6-0 silk suture against a blunt needle (26 gauge), which was subsequently removed [17]. Rats were randomly assigned into five experimental groups: Sham group (Sham, n = 10), sham-operated animals serving as controls; TAC-4W group (TAC-4W, n = 10), cardiac hypertrophy was induced by TAC without further treatment, and the rats...
were analyzed at 4 weeks after surgery; TAC-8W group (TAC-8W, n = 10), cardiac hypertrophy was induced by TAC without further treatment, and the rats were analyzed at 8 weeks after surgery; TAC-12W group (TAC-12W, n = 10), cardiac hypertrophy was induced by TAC without further treatment, and the rats were analyzed at 12 weeks after surgery; and TAC + Calhex$_{231}$ group (TAC + Calhex$_{231}$, n = 10), TAC was performed and the rats were treated with Calhex$_{231}$ (10 μmol/kg/d in saline) for 4 weeks after 4 weeks post-TAC, and the rats were analyzed at 12 weeks after surgery. The rats were sacrificed, and the hearts were quickly excised and weighed in cold (4 °C) buffer. The left and right ventricles were then separated and weighed, with the left ventricular tissue being rapidly frozen in liquid nitrogen and stored at -80 °C for subsequent western blot analyses. All animals 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 United States National Institutes of Health. The 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, Waukesha, WI, USA). Briefly, the rats were anesthetized as described previously, and echocardiograms were obtained and analyzed as reported previously.

Hypertrophy index analysis
Rats were weighed and received 10% chloral hydrate (0.25 mL/100 g of BW, intraperitoneal injection), and then the heart was cut and washed with cold saline. The heart heavy weight (HW) and left ventricular weight (LVW) were recorded to calculate the heart weight index (HW/BW) and left ventricular weight index (LVW/BW), which were used to assess cardiac hypertrophy.

Histological analysis
Following anesthesia, the hearts were excised and immediately placed in 4% paraformaldehyde at room temperature for 24 h. The myocardial specimens were embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin (H&E) and Masson’s trichrome reagent. The fibrotic areas were stained blue, and the normal tissues were stained red.

Electron microscopy
Cardiac tissue was quickly cut into 1-mm cubes and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4 °C. After fixation, the sections were immersed in 1% osmium tetroxide for 2 h, dehydrated in graded ethanol solutions, embedded in epoxy resin, and then cut into ultrathin sections (60–70 nm) with an ultramicrotome, which were then post-stained with uranyl acetate and lead citrate, prior to examination under a JEM-1010 transmission electron microscope.

Immunohistochemistry
Paraffin-embedded tissue sections (0.2 μm) were deparaffinized with xylenes and rehydrated with graded ethanol solutions followed by phosphate-buffered saline (PBS). Then, each slice was treated with 30 μL of 3% H$_2$O$_2$ (reagent A), incubated at room temperature for 20 min, and washed twice with PBS. Next, 30 μL of goat serum (reagent B) was added, followed by incubation at room temperature for 20 min and washing twice with PBS. Each slice was incubated with primary antibody (anti- Beclin1 and anti-LC3, 1:50 dilution) and placed in a wet box at 4 °C overnight. After washing with PBS, the slices were incubated in 30 μL of biotinylated polyclonal secondary antibody (reagent C) at room temperature for 30 min, followed by washing with tap water. Slices were then counterstained with hematoxylin, incubated in ammonia, dehydrated with graded ethanol solutions, transparentized with xylenes, and finally sealed with neutral gum. Cells with brown-stained particles in their cytoplasm and nucleus were denoted as positive under a light microscope.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay
The TUNEL assay was used to detect the apoptosis of rat cardiomyocytes. The heart tissues were fixed in 4% paraformaldehyde overnight, dehydrated, and then embedded in paraffin. The tissue was analyzed by
a TdT-DNA Fragmentation Detection kit, obtained from Roche (Basel, Switzerland), and the procedure was performed according to the kit’s protocol.

Establishment of an in vitro model of Ang II-induced cardiomyocyte hypertrophy

Neonatal rat cardiomyocytes 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 in 70% (v/v) alcohol. The ventricles were removed and washed three times in D-Hank’s balanced salt solution (0.4 g/L KCl, 0.06 g/L KH₂PO₄, 8.0 g/L NaCl, 0.35 g/L NaHCO₃, and 0.06 g/L Na₂HPO₄·7H₂O, pH 7.2) at 4 °C. They were then minced 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) containing 10% (v/v) newborn calf serum was added to terminate the digestion. The supernatant was discarded. Then, cells were incubated with fresh 0.25% trypsinase for 15 min at 37 °C, and the supernatant was collected. The latter digestion step was repeated four times. Cells in the supernatant were isolated by centrifugation at 2000 rev/min and room temperature for 10 min before they were resuspended in DMEM containing 20% (v/v) newborn calf serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were cultured in a monolayer at a density of 5 × 10⁴ cells/cm² at 37 °C in a humidified atmosphere containing 5% (v/v) CO₂. The medium contained 2 µM fluoro deoxyuridine to prevent proliferation of nonmyocytes.

Three days after being seeded, the neonatal rat cardiomyocytes were starved in serum-free DMEM for 24 h. Then, they were divided randomly into six groups: (1) normal control group; (2) Ang II group: cardiomyocytes were treated with 100 nM Ang II for 48 h; (3) GdCl₃/R-568+Ang II group: cardiomyocytes were preincubated with 30 µM GdCl₃/5 µM R-568 (a specific agonist of CaSR) for 1 h and then treated with 100 nM Ang II for 48 h; (4) GdCl₃/R-568+Calhex ²⁺₁/R-568+Ang II group: the procedure was similar to that for group (3), except the cardiomyocytes were preincubated with 3 µM Calhex ²⁺₁ (a specific inhibitor of CaSR) for 30 min, before the addition of Ang II; (5) GdCl₃/R-568+3-MA+Ang II group: the procedure was similar to that of group (3), except the cardiomyocytes were preincubated with 5 mM 3-MA (a specific inhibitor of autophagy) for 30 min, before the addition of Ang II; (6) GdCl₃/R-568+compound C+Ang II group: the procedure was similar to that of group (3), except the cardiomyocytes were preincubated with 5 µM compound C (an inhibitor of AMPK) for 30 min, before the addition of Ang II.

Measurement of intracellular calcium in cardiomyocytes

After the treatments described above, cardiomyocytes were loaded with 1 µM Fluo-4/AM at 37 °C for 30 min. The cells were rinsed twice with Ca²⁺-free PBS to remove the remaining dye, and further incubated in DMEM. Changes in [Ca²⁺], were analyzed by the fluorescence intensity induced by Fluo-4 in the cardiomyocytes, which was recorded for 5 min using a confocal laser scanning microscope (Olympus, Tokyo, Japan) with excitation at 488 nm and emission at 530 nm.

Western blotting

Protein concentrations were determined by the Coomassie method using bovine serum albumin as the standard. All samples were mixed with loading buffer and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins in the samples from different experimental groups were separated and transferred onto nitrocellulose membranes by electroblotting (300 mA for 2 h). Membranes were blocked in TBS-T [137 mM NaCl, 20 mM Tris (pH 7.6), and 0.1% (v/v) Tween 20] containing 5% (w/v) skim milk at 37 °C for 1 h. Membranes were then incubated overnight at 4 °C with antibodies against CaSR (1:800), CaMKI (1:1000), p-CaMKII (1:1000), p62 (1:1000), Beclin1 (1:1000), LC3 (1:1000), caspase-3 (1:1000), AMPK (1:1000), p-AMPK (1:1000), CaMKK, (1:1000), p-CaMKK (1:1000), mTOR (1:1000), p-mTOR (1:1000), and GAPDH (1:1000). Membranes were incubated with secondary antibody (alkaline phosphatase-conjugated IgG) (Promega) (1:5000 dilution) in TBS-T for 1 h at room temperature. The densities of the protein bands were quantified using a Bio-Rad Chemi DocTM EQ densitometer and Bio-Rad Quantity One software (Bio-Rad, Hercules, CA, USA), and GAPDH was used 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 means ± standard error of the mean (SEM).
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Comparisons among the groups were performed using Kruskal–Wallis two-way analysis of variance. Values of P < 0.05 were considered statistically significant.

Results

**TAC induces cardiac hypertrophy in rats**

Cardiac hypertrophy was histologically characterized by an increase in myocyte mass and extracellular matrix deposition. To investigate the *in vivo* effects of Calhex
231 in hypertrophic hearts, we first established a cardiac hypertrophy model by TAC surgery. Myocardial function was assessed using echocardiography. At 4 and 8 weeks after the TAC surgery, we observed that the animals showed an increase in interventricular septum (IVS) thickness, left ventricular posterior wall (LVPW) thickness, left ventricular internal dimension (LVID), left ventricular diastolic volume (LVDV), and left ventricular systolic volume (LVSV) together with a decrease in the left ventricular fractional shortening (LVFS) and left ventricular ejection fraction (LVEF), respectively, compared with the sham group. Nevertheless, there was no statistical disparity among the sham group and TAC-surgery animals after 4 and 8 weeks. At 12 weeks after the TAC surgery, the myocardial dysfunction was further exacerbated, with an increase in diastolic and systolic IVS, LVDV, LVSV, LVID, and diastolic and systolic LVPW, and a decrease in LVEF and LVFS (all P < 0.05), compared with those of the sham group. These results indicated that cardiac hypertrophy occurs between 8 and 12 weeks after TAC surgery (Table 1).

The rats gained weight during the period of study. We calculated the HW/BW and LVW/BW ratios and observed that TAC significantly increased these ratios by 12 weeks after the TAC surgery, compared with the sham group (all P < 0.05) (Fig. 1A).

TAC remarkably increased the cell cross-sectional area of the myocardial tissue, which was stained with H&E, compared with that of the sham group. Morphological analysis of the sham group revealed that the cardiomyocytes exhibited a clear arrangement into neat rows, intercalated discs, and transverse stripes with loose nuclear chromatin. However, after TAC surgery, the rat cardiac tissues were expanded, and the muscle fibers became thickened and disorganized. These changes were the most significance in the TAC-12W group (Fig. 1B).

Table 1. Echocardiographic analysis of rat left ventricular wall and chamber dimensions in the sham, TAC alone at various stages, and TAC with Calhex
231, treatment groups. IVSd, diastolic interventricular septum thickness; IVSs, systolic interventricular septum thickness; LVPWd, diastolic left ventricular posterior wall thickness; LVPWs, systolic left ventricular posterior wall thickness; LVIDd, diastolic left ventricular internal dimension; LVIDs, systolic left ventricular internal dimension; LVDV, left ventricular diastolic volume; LVSV, left ventricular systolic volume; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening. *P < 0.05 versus the sham group, #P < 0.05 versus the TAC-12W group.
With Masson's staining, the hearts after TAC surgery displayed extensive interstitial fibrosis in the ventricular wall compared with that in the sham hearts. Morphological analysis revealed that the cytoplasm, collagen fibers, and red blood cells were stained blue and the nuclei were stained blue-brown; while the sham group displayed normal myocardial fibers. The TAC-12W group exhibited a large number of blue-stained collagen fibers (Fig. 1B).

As shown by the western blot results, the expression of CaSR in the rat heart was increased in the TAC-12W group (P < 0.05), compared with that in the sham group (Fig. 2).

**Calhex$_{231}$ ameliorates rat cardiac hypertrophy induced by TAC**

Echocardiography showed that the cardiac systolic and diastolic functions were improved after Calhex$_{231}$ administration, compared with those of the TAC-12W group. Calhex$_{231}$ treatment significantly ameliorated the cardiac functions, with decreases of diastolic and systolic IVS, LVDV, LVSV, LVIDs, and diastolic and systolic LVPW as well as increases of LVEF and LVFS in the TAC+Calhex$_{231}$ group, compared with those of the TAC-12W group (all P < 0.05) (Fig. 3A and Table 1). Calhex$_{231}$ remarkably reduced the TAC-induced increases of the HW/BW and LVW/BW levels, compared with those of the TAC-12W alone group (all P < 0.05) (Fig. 3B). Calhex$_{231}$ markedly attenuated the levels of ventricular hypertrophy and fibrosis as well as increased cardiomyocyte apoptosis induced by TAC (Fig. 3C); and the expression of CaSR was significantly decreased after Calhex$_{231}$ treatment (P < 0.05) (Fig. 3D), suggesting that Calhex$_{231}$ ameliorated cardiac hypertrophy induced by TAC. Treatment with Calhex$_{231}$ alone for 1W and 2W did not affect hypertrophy index, tissue and cellular morphology and CaSR expression in the experimental rats (Fig. 5A, 5B and 5C).
Autophagy during TAC-induced rat cardiac hypertrophy

Transmission electron microscopy revealed that the administration of Calhex$_{231}$ attenuated the disorganized sarcomere structure and mitochondrial disarray in TAC-induced hypertrophic hearts, which was in parallel with the observation of increased autophagosomes, supporting the notion that Calhex$_{231}$ has an effect on autophagy (Fig. 4A).

Autophagy is a bulk degradation mechanism for cytosolic damaged organelles and long-lived proteins. We observed that the expression levels of Beclin-1, an accepted target gene
responsible for autophagy [18], and LC3II, an indicator of autophagosome formation [19], were both increased (all \( P < 0.05 \)) and that the expression of p62, a deliverer of several ubiquitinated substrates to autophagosomes [20], was reduced in the TAC-12W group (\( P < 0.05 \)), compared with the expression levels of these proteins in the sham group. After the administration of Calhex\(_{231}\), the expression levels of Beclin-1 and LC3II were decreased and the expression of p62 was increased, compared with the expression levels of these proteins after TAC alone (all \( P < 0.05 \)) (Fig. 4B). Treatment with Calhex\(_{231}\) alone for 1W and 2W did not affect autophagy level in the experimental rats (Fig. 5C).

In comparison with the sham samples, tissues from the TAC-12W group showed a moderate increase in Beclin-1-positive and LC3-positive fibers by immunohistochemical analysis, which was attenuated by Calhex\(_{231}\) treatment (Fig. 4C), consistent with the western blot results.

**Calhex\(_{231}\) ameliorates hypertrophy in neonatal cardiomyocytes**

We demonstrated that Ang II significantly increased [Ca\(^{2+}\)], and GdCl\(_3\)/R-568 added a synergetic effect with a further increase of [Ca\(^{2+}\)], in the GdCl\(_3\)/R-568+Ang II group (\( P < 0.05 \)), compared with that in the Ang II group. However, Calhex\(_{231}\) abolished these effects (Fig. 6A).

CaMKII is an essential signaling molecule that governs cardiac hypertrophy. We found that the expression of p-CaMKII in cardiomyocytes was increased in the Ang II group (Fig. 6B–C) (all \( P < 0.05 \)), compared with that in the control group, and were remarkably increased in the GdCl\(_3\)/R568+Ang II group (all \( P < 0.05 \)), compared with that in the Ang II group. Calhex\(_{231}\) treatment also attenuated these increases. Interestingly, treatment with 3-MA, an autophagy inhibitor, significantly decreased the expression level of p-CaMKII, compared with that of the GdCl\(_3\)/R568+Ang II group (all \( P < 0.05 \)).
The expression of CaSR in cardiomyocytes was increased progressively in the Ang II and GdCl$_3$/R568+Ang II groups, compared with that in the control group (both $P < 0.05$). Calhex$_{231}$ treatment also attenuated the increase of CaSR expression (Fig. 6D).

**Calhex$_{231}$ ameliorates hypertrophic cardiomyocytes via suppression of autophagy**

In agreement with the results from the TAC model, the expression levels of Beclin-1 and p62 of the *in vitro* cardiomyocyte model were increased and decreased, respectively, in the Ang II group (both $P < 0.05$) and were more remarkable in the GdCl$_3$/R568+Ang II group (all $P < 0.05$), compared with those in the Ang II alone group. Calhex$_{231}$ abolished these effects. Moreover, autophagy was significantly inhibited by 3-MA treatment (Fig. 7).

To discover the functional relationship between apoptosis and autophagy, we analyzed the cleaved caspase-3 level using the *in vitro* cardiomyocyte model. Ang II increased the cleaved caspase-3 and LC3 levels (both $P < 0.05$) in the Ang II group, compared with those in the control group (Fig. 7). In addition, we observed that GdCl$_3$/R568 significantly increased the LC3II/LC3I ratio ($P < 0.05$), whereas it decreased the expression of caspase-3 ($P < 0.05$) in the GdCl$_3$/R568+Ang II group, compared with that in the Ang II group. Interestingly, although 3-MA inhibited the CaSR-augmented autophagy, the apoptotic index was remarkably increased. Compared with the GdCl$_3$/R568+Ang II group, pretreatment with 3-MA decreased the LC3II/LC3I ratio ($P < 0.05$), whereas it increased the expression of cleaved caspase-3 ($P < 0.05$) in the GdCl$_3$/R568+3-MA+Ang II group. These results indicated that Calhex$_{231}$ suppressed autophagy against hypertrophic stimuli to ameliorate cardiomyocyte survival.
Calhex$_{231}$ suppresses autophagy via suppressing the CaMKK$_{\beta}$–AMPK–mTOR signaling pathway

To reveal the molecular mechanisms involved in CaSR-induced autophagic responses, the levels of CaMKK$_{\beta}$, p-CaMKK$_{\beta}$, AMPK, p-AMPK, mTOR, and p-mTOR were measured. In the cardiomyocyte model, increased p-CaMKK$_{\beta}$ and p-AMPK levels as well as decreased p-mTOR levels were observed in the Ang II group (all $P < 0.05$), compared to those in the control group. Furthermore, compared with the Ang II group, these results were significantly more remarkable in the GdCl$_3$/R568+Ang II group (all $P < 0.05$). However, Calhex$_{231}$ abolished these
Fig. 7. Protein expression levels of Beclin-1, p62, LC3, and cleaved caspase-3 in neonatal rat cardiomyocytes as analyzed by western blot. The intensity of each band was quantified using densitometry, and the data were normalized to the GAPDH protein band intensity. *P < 0.05 versus the control group; †P < 0.05 versus the Ang II group; ‡P < 0.05 versus the GdCl₃/R568+Ang II group.

Fig. 8. Effect of Calhex₂₃₁ on autophagy initiation signaling during Ang II-induced cardiac hypertrophy. Protein expression levels of CaMKKβ, AMPK, and mTOR as 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 ± SEM from three independent determinations. *P < 0.05 versus the control group, †P < 0.05 versus the Ang II group; ‡P < 0.05 versus the GdCl₃/R568+Ang II group.
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Effects (Fig. 8). Our results supported that CaSR stimulated autophagy, which is mediated by CaMKKβ–AMPK–mTOR signaling.

We further investigated the role of compound C (a specific AMPK inhibitor) in the Calhex 231-induced suppression of autophagy. Figure 9 shows the cellular molecular pathways struck by CaSR to increase autophagy and decrease apoptosis. Compared with the control group, Ang II treatment significantly increased the LC3II/LC3I ratio and the cleaved caspase-3 level (all P < 0.05), and GdCl₃/R568 cotreatment further augmented the LC3II/LC3I ratio but decreased the cleaved caspase-3 level in the Ang II group. We observed an intense negative relationship between autophagy and apoptosis induced by CaSR. However, compared with the GdCl₃/R568+Ang II group, the LC3II/LC3I ratio was significantly reduced and the caspase-3 level was remarkably increased in the GdCl₃/R568+compound C+Ang II group, suggesting that CaSR activated the autophagy level, which is mediated by the CaMKKβ–AMPK–mTOR signaling pathway. Calhex 231 reduced the CaSR-augmented autophagy via suppressing the CaMKKβ–AMPK–mTOR signaling pathway.

Discussion

In the present study, the rat cardiac hypertrophy model was established with TAC, and the expression of CaSR and autophagy level were remarkably increased in the hypertrophic hearts. Moreover, Calhex 231 significantly inhibited the upstream signaling of autophagy and ameliorated cardiac hypertrophy. Additionally, the experimental results of the in vitro neonatal rat hypertrophic cardiomyocytes induced by Ang II were fully aligned with the results obtained with the in vivo TAC model, confidently confirming our discoveries.

Cardiac hypertrophy involves an imbalance in cardiovascular homeostasis, which is characterized by excessively activated local catecholamine and the renin–angiotensin–aldosterone systems...
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Fig. 10. Schematic diagram summarizes the molecular mechanisms by which Calhex 231 ameliorates cardiac hypertrophy by inhibiting cellular autophagy. Cardiac hypertrophy can be ameliorated by Calhex 231, which is involved in inhibiting autophagy via suppression of the CaMKKβ–AMPK–mTOR pathway. CaSR, calcium-sensing receptor; PLC, phospholipase C; PIP2, phosphatidylinositol bisphosphate; DAG, diacylglycerol; IP3, inositol triphosphate; CaMKKβ, Ca2+/calmodulin-dependent-protein kinase-kinase-β; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin.

to stimulate cardiac hypertrophy development in an autocrine/paracrine manner [21, 22]. Prolonged pressure overload induces heart hypertrophy, which advances to heart failure. In addition to the blood pressure stimulation on cardiomyocytes, abnormal intracellular Ca2+ homeostasis in the myocardium has been suggested to be a cause of cardiac hypertrophy [23, 24]. An elevated [Ca2+]i activates several Ca2+-dependent signaling pathways, which eventually results in cardiac hypertrophy [25-27]. The activation of CaSR induces the release of Ca2+ from the sarcoplasmic reticulum [28] of the cardiovascular system and is involved in cardiac ischemia/reperfusion injury [29, 30].

CaSR is activated by Gd3+ and Mg2+ (type I activators), which are present in extracellular fluids. Recently, the activation of CaSR with positive allosteric modulators (type II activators), including NPS R-568, NPS R-467, and cinacalcet, has been discovered [31]. They interact allosterically within the seven-transmembrane domain to potentiate the effect of Ca2+. On the other hand, negative allosteric modulators such as Calhex 231 inhibit the effect of Ca2+ on CaSR [32] through binding to the seven-transmembrane domain of CaSR distanced from the Ca2+ orthosteric binding site [28].

We demonstrated that TAC was indeed able to induce cardiac hypertrophy and resulted in an increased heart size, HW/BW, cross-sectional cardiomyocyte area, and interstitial collagen in association with cardiac dysfunction and that the in vitro cardiomyocyte hypertrophy model produced results that align with remarkable increases in the cell size, [Ca2+], and p-CaMKII expression. Despite the different incentives, the cardiomyocytes had a common response to hypertrophic stimuli. Calhex 231 strongly protected cardiomyocytes against hypertrophy induced by hypertrophic stimuli in vivo and in vitro. We demonstrated that Calhex 231 supplementation significantly decreased heart size, HW/BW, cardiomyocyte size, [Ca2+], and CaMKII expression in hypertrophic hearts and cardiomyocytes as well as markedly improved the cardiac functions. These results indicated that Calhex 231 could effectively inhibit myocardial hypertrophic remodeling.

Autophagy, a highly conserved cytoprotective pathway, serves as a cell survival mechanism [33] and is involved in the pathogenesis of cardiac hypertrophy caused by pressure overload [10]. Cardiac-specific autophagy-related gene Atg5 or Atg7 deficiency leads to cardiac hypertrophy [34]. Under baseline conditions, constitutive autophagy plays a homeostatic role in maintaining cardiomyocyte size, and global cardiac structure and function, while upregulation of autophagy in failing hearts can be an adaptive response for protecting cells from hemodynamic stress [35]. In contrast, the degree of autophagic activity correlates with the magnitude of hypertrophic growth and the rate of transition to heart failure demonstrated in a pressure overload model [36]. It has been proposed that
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excessive activation of autophagy leads to type II programmed cell death, aggravates cardiac hypertrophy by pressure overload, and stimulates the process of heart failure [37]. Therefore, autophagy can be maladaptive under conditions of severe pressure overload. Regardless of the controversial on the effect of autophagy in the progress of cardiac hypertrophy induced by pressure overload, our present results demonstrated that autophagy was remarkably increased in the TAC-induced rat hearts as well as in the hypertrophic cardiomyocytes induced by Ang II and that Calhex_231 suppressed autophagy to help the cardiomyocytes survive under hypertrophic conditions. Our results also suggested that enhanced autophagic activity can have pathological consequences, potentially leading to cardiac hypertrophy through autophagic cell death.

Autophagy is a highly dynamic and multistep process. The accumulation of autophagosomes could only indicate either autophagic activation or a blockage of downstream steps in autophagy, such as inefficient fusion or decreased lysosomal degradation [38]. Therefore, the mere detection of the number of autophagosomes or the presence of LC3 processing may be insufficient for an overall evaluation of the entire autophagic system or the dynamic process of autophagy termed “autophagic flux”. As P62 serves as a link between LC3 and ubiquitinated substrates, and is efficiently degraded by autophagy [39], it can be used to monitor autophagic flux. The present results demonstrated that autophagic suppression correlates with an increased p62 level, while, autophagic activation correlates with a decreased p62 level, supporting its role in the functional autophagic flux process.

Concurrently, Calhex_231 also increased apoptosis in both the TAC and Ang II models. As the balance between autophagy and apoptosis maintains homeostasis, inactivation of autophagy may cause abnormal proteins and organelles to be accumulated, thereby promoting apoptosis. Excessive autophagic activity induced by severe stimuli can destroy a large fraction of the cytosol and organelles, leading to the complete loss of all cellular functions and abnormal cell morphology. Thus, the dead and dying cells can simultaneously show characteristics of autophagy, apoptosis, and necrosis [37].

Autophagy in cardiomyocytes is a complex process that involves numerous signaling molecules. To provide further evidence that reveals the underlying mechanisms of CaSR-induced autophagy, we investigated the intracellular signaling pathways leading to autophagy activation. AMPK is responsible for sensing energy and nutrients and is involved in promoting autophagy by directly activating the mammalian autophagy-initiating kinase Ulk1 via phosphorylation of Ser317 and Ser777 [40]. CaMKK_β is stimulated by an increase in the [Ca^{2+}]_i and activates AMPK [41] to induce autophagy, thus inhibiting the mTOR signaling pathway. In this study, we observed that AMPK inhibition by compound C effectively reduced the CaSR-augmented autophagy level in Ang II-induced cardiomyocytes. These results revealed that the inhibition of CaSR in the CaMKK_β–AMPK–mTOR signaling pathway might contribute to its cardiac cytoprotection, in agreement with previous studies [41, 42].

In summary, our results demonstrated that cardiac hypertrophy induced by pressure overload or Ang II upregulates the expression of CaSR and can be ameliorated by Calhex_231, which is involved in inhibiting autophagy via the suppression of the CaMKK_β–AMPK–mTOR pathway (Fig. 10).

Cardiac hypertrophy can be induced by multiple factors, such as pressure overload, isoproterenol, swimming exercise, etc. The present study only investigated the effects of pressure overload and Ang II-induced cardiac hypertrophic models. Our conclusion needs to be further validated in other cardiac hypertrophic models. As myocardial fibroblasts also participate in the development of hypertrophy, further investigations on the effect of Calhex_231 on cardiac fibroblasts may supply useful information on the potential therapeutic efficacy of this agent on cardiac hypertrophy.

Disclosure Statement

The authors declare no conflict of interest.
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