Original Article
Relationship between CaSRs and LPS-injured cardiomyocytes

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Abstract: Objective: Calcium-sensing receptors (CaSRs) regulate systemic calcium homeostasis. Intracellular calcium concentration changes are initiating factors of endoplasmic reticulum stress and cell autophagy. Recent research has revealed that CaSRs play an important role in myocardial ischemia/reperfusion injury and other cardiovascular diseases. However, it remains unclear whether CaSRs are involved in lipopolysaccharide (LPS)-induced cardiomyocyte injury. Methods: Cultured neonatal rat cardiomyocytes were treated with LPS, with or without pretreatment by a CaSR specific agonist SC-211006 or CaSR specific antagonist SC-207394. The ultrastructure of cardiomyocytes was observed using a transmission electron microscope, and the expression of CaSR, GRP78, LC3B, CytC and Bcl-2 proteins were detected by western blot. Results: Compared with the control group, LPS increased cardiomyocyte injury and the expression of CaSR, GRP78, LC3B and CytC proteins, but decreased the expression of Bcl-2. Compared with the LPS group, pretreatment with SC-211006 further enhanced cardiomyocyte damage and the expression of CaSR, GRP78, LC3B and CytC, but reduced the expression of Bcl-2. Conversely, pretreatment with SC-207394 decreased cardiomyocyte injury and the protein expression of CaSR, GRP78, LC3B and CytC, but increased the expression of Bcl-2. Conclusion: Our results suggest that CaSRs are involved in LPS-induced rat cardiomyocyte injury via the activation of endoplasmic reticulum stress and autophagy.

Keywords: Calcium-sensing receptor, cardiomyocyte, lipopolysaccharide, endoplasmic reticulum stress, autophagy

Introduction

Sepsis is defined as the excessive and irregular response of a host against an existing infection [1]. It is a complex disorder that arises from an uncontrolled systemic inflammatory response to an infection, and is a major cause of mortality in critically ill patients, especially in neonates [2]. Despite the increase of our knowledge on the pathophysiology of sepsis, sepsis remains a serious clinical problem [3]. The heart is an important target organ in sepsis induced by exposure to lipopolysaccharide (LPS). In a study, it was found that LPS can inhibit rat cardiomyocyte contractility *in vitro* [4].

Furthermore, approximately 60% of patients admitted to intensive care units present a clinical picture of cardiac dysfunction that has been recognized as a serious manifestation of severe sepsis, with mortality ranging from 70% to 90%, in contrast with septic patients without cardio-

vascular involvement, with a mortality of 20% [5]. Hence, there is an urgent need to investigate the mechanism of myocardial injury induced by LPS.

Calcium handling is essential for the homeostatic control of cardiovascular function. The alteration of intracellular calcium ($[Ca^{2+}]_i$) is a fundamental pathway to decrease cardiomyocyte and ventricular contractility. Calcium-sensing receptors (CaSRs) are a member of the family of G protein-coupled receptors, which were first cloned in 1993 from a bovine parathyroid gland by Brown *et al.* The role of CaSRs in general calcium homeostasis has now been well-established [6, 7]. Wang *et al.* and Hansen *et al.* reported that CaSRs are functionally expressed in rat cardiac tissues and rat neonatal ventricular cardiomyocytes, respectively. The activation of CaSRs resulted in intracellular calcium increase through the PLC-inositol, 4, 5-trisphosphate (IP_3) pathway [8, 9]. We have demonstrated that CaSRs were involved in

apoptosis in isolated adult rat hearts and rat neonatal cardiomyocytes during ischemia/reperfusion [10, 11]. CaSRs are also involved in cardiac hypertrophy through the inhibition of autophagy. Liu et al. used Calhex 231 to inhibit CaSRs to regulate autophagy and reduce myocardial hypertrophy [12, 13].

Recent studies have found that endoplasmic reticulum (ER) stress and autophagy are involved in LPS-induced acute lung injury [14, 15]. Other studies have demonstrated that LPS-induced cardiac damage is involved in autophagy, and is associated with age [16, 17]. Furthermore, it was found that aged mice were more prone to cardiac damage.

Our previous studies have found that the expression of CaSRs increased in endotoxemia rat myocardial tissues, and CaSR was involved in LPS-induced myocardial injury [18]. However, its mechanism remains unclear. In the study, we investigated whether CaSR was involved in LPS-induced cardiomyocyte injury through the modulation of ER stress and autophagy.

Materials and methods

Animals

Sprague-Dawley rats (1-3 days old) were obtained from the Laboratory Animal Center of the Second Affiliated Hospital of Harbin Medical University (license: SCXK [black], 2013-001). All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the China National Institutes of Health, and approved by the Animal Care Committee of Harbin Medical University, China.

Reagents

LPS was obtained from Sigma (St. Louis, USA), SC-211006 and SC-207394 were obtained from Santa Cruz Biotechnology, anti-CaSR antibody (product number: ACR-004) was purchased from Alomone Labs (Jerusalem, Israel), anti-GRP78 antibody (product number: 3158-1) was purchased from Epitomics (Burlingame, USA), and anti-LC3B antibody (product number: TDY357), anti-CytC antibody (product number: TDY378) and anti-Bcl-2 antibody (product number: TDY061) were obtained from Tian De Yue (Beijing, China).

Cell culture and treatment

The primary cultures of neonatal rat ventricular cardiomyocytes were prepared based on a previously described method [10]. Three days after the cells were seeded,the cultured cardiomyocytes were randomly divided into four groups (*n*=8): (1) control group, cardiomyocytes were continuously cultured for four hours in DMEM medium; (2) LPS group, cardiomyocytes were incubated for four hours with LPS (4 µg/mL) [19] alone; (3) LPS+SC-211006 group, cardiomyocytes were cultured with DMEM medium containing 4 µg/mL of LPS and 1 µM of SC-211006 for four hours (activator of CaSR) [20]; (4) LPS+SC-207394 group, cardiomyocytes were cultured with DMEM medium containing 4 µg/mL of LPS and 1 µM of SC-207394 (antagonist of CaSR) [20] for four hours.

Transmission electron microscopy

Cardiomyocytes were fixed with 2.5% glutaraldehyde overnight at 4°C. After fixation, the sections were immersed in 1% osmium tetroxide for two hours, dehydrated in graded ethanol solutions, embedded in epoxy resin, and cut into ultrathin sections (60-70 nm) with an ultramicrotome. Then, the sections were post-stained with uranyl acetate and lead citrate prior to examination under a transmission electron microscope (H-7650, Japan).

Western blot analysis

The total proteins of the neonatal rat myocytes were prepared according to manufacturer's instructions. Protein concentrations were determined by BCA protein assay using BSA as the standard. All samples were mixed with the loading buffer and subjected to 8% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins in samples obtained from different experimental groups were separated and transferred onto nitrocellulose membranes by electroblotting (300 mA for 0.5-2.0 hours). The membranes were blocked with TBS-T (137 mM of NaCl, 20 mM of Tris [pH 7.6], and 0.1% [v/v] Tween 20) containing 5% (w/v) skim milk at 37°C for one hour. Then, the membranes were incubated overnight at 4°C with antibodies against CaSR (1:2,500), GRP78 (1:2,000), LC3B (1:1,000), Bcl-2 (1:1,000) and GAPDH (1:2,000), respectively. Next, these membranes were incubated with secondary anti-
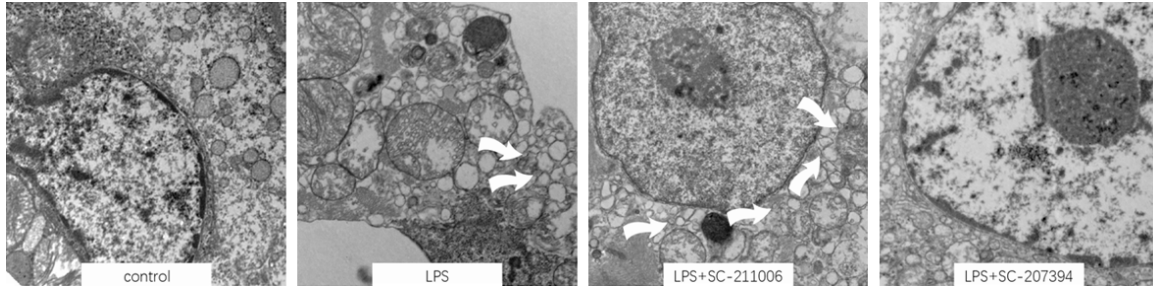
Figure 1. Morphologic changes in cardiomyocytes by transmission electron microscopy (10,000×). Nuclear chromatin underwent no significant changes in the control group. In the LPS group, mitochondrial vacuoles and lysosomes were apparent. Lysosomes and autophagosomes were increased in the LPS+SC-211006 group (labeled by the arrow). In the LPS+SC-207394 group, the injury of mitochondria and sarcoplasmic reticulum were clearly reduced.

body (alkaline phosphatase-conjugated IgG, diluted at 1:2,000; Tian De Yue) in TBS-T for 40 minutes at room temperature. Antibody antigen complexes were detected using Western Blue Stabilized Substrate for alkaline phosphatase. The densities of the protein bands were quantified using a Bio-Rad Chemi Doc TM EQ densitometer and the Bio-Rad Quantity One software (Bio-Rad, Hercules, CA, USA). GAPDH was used as an internal control for the semi-quantitative assay.

Detection of Cyt c release from cytosolic fraction

The cytosolic fraction of cyt *c* was analyzed by western blot, as previously described. Briefly, cells were harvested, washed twice with ice-cold PBS and incubated in ice-cold Tris-sucrose buffer (0.35 mM of sucrose, 10 mM of Tris-HCl at pH 7.5, 1 mM of EDTA, 0.5 mM of dithiothreitol, and 0.1 mM of phenylmethylsulfonyl fluoride). After 40 minutes of incubation, cells were centrifuged at 1,000×g for five minutes at 4°C, and the supernatant was further centrifuged at 40,000×g for 30 minutes at 4°C. The supernatant was retained as the cytosolic fraction, and analyzed by western blot with a primary rat anti-Cyt *c* monoclonal antibody (1:2,000) and a secondary goat anti-rat immunoglobulin G (Promega). GAPDH expression was used as the control.

Statistical analysis

All data were obtained from at least three independent experiments that were replicated two to four times under each condition. These results were expressed as mean ± standard deviation (SD), and statistical differences were

evaluated by *t*-test. A *P*-value <0.05 was considered statistically significant.

Results

Observation of cardiomyocyte ultrastructure under transmission electron microscope

In the control group, there were no evident histopathologic changes. After treatment with LPS, mitochondrial vacuoles and lysosomes were detected. In the CaSR agonists group, lysosomes and autophagosomes were significantly increased. Mitochondrial swelling and sarcoplasmic reticulum expansion were noted. However, in the CaSR inhibitor group, histopathologic injury was reduced (**Figure 1**).

LPS increased CaSR expression

As revealed by western blot, the expression of CaSR in rat cardiomyocytes increased in the LPS group (*P*<0.05 *vs.* the control group). When incubated with LPS and SC-211006 for four hours, CaSR expression was further upregulated (*P*<0.05 *vs.* the LPS group), while SC-207394 reduced the expression of CaSRs (*P*<0.05 *vs.* the LPS group) (**Figure 2**).

Influence of CaSR on autophagy in LPS-induced cardiomyocyte injury

Autophagy is a bulk degradation mechanism for cytosolic damaged organelles and long-lived proteins. The activation of autophagy was examined through the immunoblotting of Light Chain 3 (LC3). The results of the present study revealed that the LC3II/LC3I ratio was increased in the LPS group, compared with the control group (*P*<0.05). Compared with the LPS
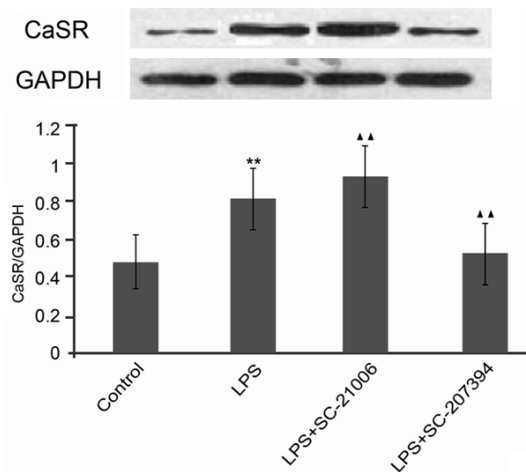
Figure 2. Expression of CaSR in cardiomyocytes as indicated by western blot (*n*=8). A basal level of CaSR was detected in the control group. This served as a baseline for the comparison of fold increases in other groups. The expression of CaSR in the LPS group was higher than that in the control group. Pretreatment with SC-211006 further increased CaSR expression. However, SC-207394 was found to inhibit the expression of CaSR. **$P<0.05$ *vs.* the control group, ▲▲$P<0.05$ *vs.* the LPS group.
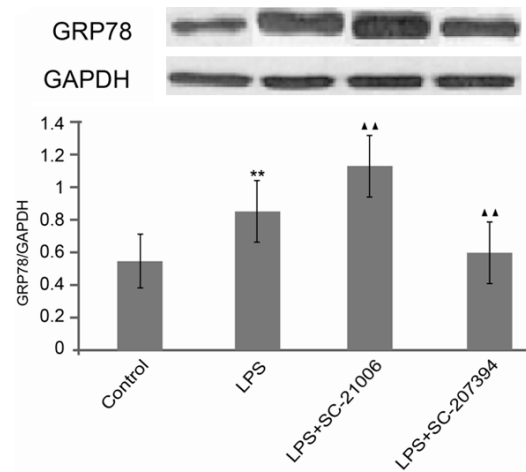

Figure 4. The expression of GRP78 in cardiomyocytes indicated by western blot (*n*=8). The basal level of GRP78 was detected in the control group. This served as a baseline for comparing the fold increases in other groups. The expression of GRP78 in the LPS group was higher than that in the control group. SC-211006 alone was showed to further increase the expression of GRP78, while SC-207394 was showed to inhibit the expression of GRP78. **$P<0.05$ *vs.* the control group, ▲▲$P<0.05$ *vs.* the LPS group.
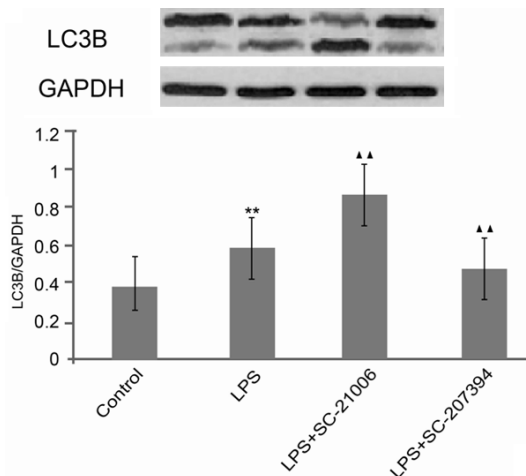

Figure 3. Analysis of LC3 protein expression in cardiomyocytes by western blot (*n*=8). The intensity of each band was quantified by densitometry, and the data were normalized to the GAPDH protein band intensity. The fold change values were presented as mean ± standard deviation (SD). In the LPS group, the LC3II/LC3I ratio increased. SC-211006 could further increase the LC3II/LC3I ratio, while SC-207394 could reduce this ratio. **$P<0.05$ *vs.* the control group, ▲▲$P<0.05$ *vs.* the LPS group.

group, the LC3II/LC3I ratio became further elevated in the LPS+SC-211006 group ($P<0.05$), while the LC3II/LC3I ratio was reduced in the LPS+SC-207394 group ($P<0.05$) (**Figure 3**).

Effect of CaSRs on ES stress in LPS-induced cardiomyocyte injury

In order to investigate whether CaSR can affect ER stress in LPS-stimulated cardiomyocyte injury, the expression of GRP78 was evaluated. Results revealed that the expression of GRP78 was upregulated in the LPS group ($P<0.01$ *vs.* the control group). Furthermore, CaSR inhibitor SC-207394 attenuated GRP78 upregulation ($P<0.05$ *vs.* the LPS group). However, CaSR agonist SC-211006 increased LPS-stimulated GRP78 upregulation ($P<0.05$ *vs.* the LPS group) (**Figure 4**).

Effects of CaSRs on the expression of Cyt c and Bcl-2 in cardiomyocytes

In order to further understand the functional relationship between CaSRs and apoptosis, Cyt c and Bcl-2 levels were analyzed. LPS increased the levels of Cyt c and reduced the levels of Bcl-2, when compared with the control group ($P<0.05$). In addition, it was observed that CaSR agonist SC-211006 significantly increased the expression of Cyt c and reduced the expression of Bcl-2 ($P<0.05$ *vs.* the LPS group), while CaSR inhibitor SC-207394 decreased the expression of Cyt c and upregulated the expres-
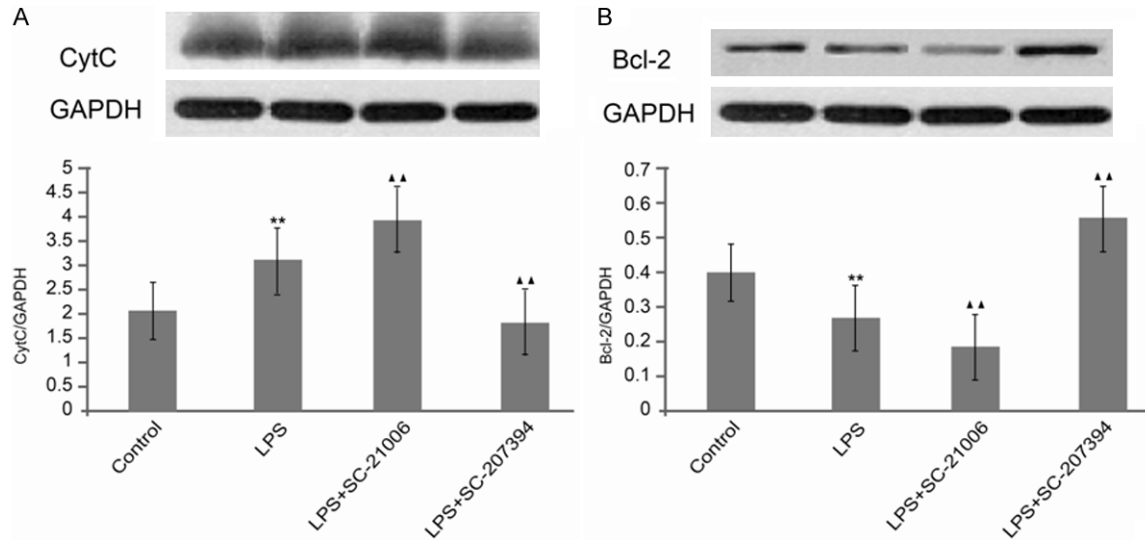
Figure 5. Detection of Bcl-2 and CytC expression in cardiomyocytes by western blot (*n*=8). A. Western blot assay for CytC expression in cardiomyocytes; B. Western blot assay for Bcl-2 expression in cardiomyocytes. LPS increased the levels of CytC and reduced Bcl-2. SC-211006 increased CytC and reduced Bcl-2 significantly, whereas SC-207394 decreased the expression of CytC and upregulated Bcl-2. **$P<0.05$ *vs.* the control group, ▲▲$P<0.05$ *vs.* the LPS group.

sion of Bcl-2 (*P*<0.05 vs. the LPS group) (**Figure 5**).

Discussion

CaSRs plays a significant pathophysiologic role in cardiac diseases [21]. However, the role of CaSRs in LPS-induced myocardial dysfunction remains unclear. Our study showed that LPS increased cardiomyocyte injury and the expression of CaSR, GRP78, LC3B and CytC proteins. Activator of CaSR further enhanced cardiomyocyte damage and the expression of CaSR, GRP78, LC3B and CytC, but reduced the expression of Bcl-2. Conversely, antagonist of CaSR decreased cardiomyocyte injury and expression of CaSR and protein related to autophagy.

Cyt c, a water-soluble protein located in the mitochondrial outer membrane lacuna, plays a vital role in the process of cell apoptosis. Cyt c is released from injured mitochondria and triggers cytosolic caspase-3 activation through the formation of the cytochrome c/Apaf-1/caspase-9-containing complex apoptosome, leading to apoptosis. Bcl-2 belongs to a potent inhibitor of apoptosis, and inhibits mitochondrial disruption and subsequent Cyt c release, as well as the activation of caspase [22].

In the present study, results revealed that LPS increased the severity of cardiomyocyte injury and the expression of Cyt c, compared with the control group. This indicates that the endotoxemia cardiomyocyte injury model was established with LPS.

Although Neomycin, Mg^{2+} and Gd^{3+} can potentiate CaSR, in this study, we selected SC-211006 i.e. Calindol hydrochloride, a new calcimimetic acting at CaSR, as CaSR activator. It can activate an extracellular ligand-binding domain-deleted Rhodopsin-like seven-transmembrane structure in the absence of Ca^{2+}. Our results revealed that SC-211006 further increased cardiomyocyte injury and Cyt c expression, and decreased Bcl-2 expression, when compared with the LPS group. However, CaSR inhibitor SC-207394 (an inhibitor of the CaSR *via* negative allosteric modulation, blocks increases in [^{3}H]inositol phosphate levels) decreased cardiomyocyte injury and Cyt c expression, and increased Bcl-2 expression, when compared with the LPS group. These results indicate that CaSR activation promotes LPS-induced cardiomyocyte injury and apoptosis.

The exact mechanism of CaSR-induced cardiomyocyte injury remains unelucidated. An increase in [Ca^{2+}]$_i$ activates some Ca^{2+}-dependent signaling pathways, which finally results in cardiomyocyte injury.

ER plays pivotal roles in regulating Ca^{2+} homeostasis. When ER homeostasis is disordered, ER

stress follows; and an adaptive process called unfolded protein response (UPR) is initiated [23]. Recent evidence has shown that UPR can also be activated by plasma membrane signaling in the absence of ER stress [24]. If various UPR-induced mechanisms fail to alleviate ER stress, both the intrinsic and extrinsic pathways for apoptosis can be activated. ER stress response is involved in different neurodegenerative disorders, and it is also involved in endocrine disorders [12].

The 78 kDa glucose-regulated protein (GRP-78) is a marker protein for ER stress. GRP-78 is mainly located in the ER, where it binds denatured or incorrectly folded polypeptides and initiates a cascade of protective reactions, helping the cell survive under conditions of stress induced by protein overload, viral infection, and so on [25]. GRP-78 is a multifunctional regulator of ER homeostasis and stress response. It was initially identified as a key component of the cellular stress pathway induced by the accumulation of unfolded proteins.

Antiapoptotic protein Bcl-2 plays a major role in preventing apoptosis. Bcl-2 could affect cellular metabolism including calcium homeostasis. In addition to its role in the mitochondrial pathway of apoptosis, Bcl-2 also localizes at the ER membrane and protects against ER stress. It is believed that the cytoprotective function of Bcl-2 is mainly due to its ability to lower the steady-state levels of ER Ca^{2+} *via* IP_3R. In the present study, CaSR agonist SC-211006 upregulated the expression of CaSR and GRP78, and downregulated the expression of Bcl-2, compared to the LPS group. However, this effect was reversed by adding CaSR inhibitor SC-207394. These results demonstrate that CaSR activation causes cardiomyocyte apoptosis by increasing ER stress and decreasing Bcl-2 protein levels in LPS-induced cardiomyocyte damage.

Autophagy maintains cellular homeostasis and removes old proteins or damaged organelles, and it may also trigger apoptosis [26]. Autophagy is remarkably enhanced during cardiac dysfunction due to hypertensive heart disease, ischemic heart disease, and dilated cardiomyopathy,consistent with the results of the present study. The autophagy protein microtubule associated protein LC3 plays an essential role in autophagosome formation, and is commonly used as a marker protein for autophagosomes. LC3 is predominantly present in cells in its nonlipidated form, that is, LC3-I. When autophagy is induced, a series of conjugation reactions mediated by a specific set of autophagy-related protein leads to the covalent linkage of LC3 to the phosphatidylethanolamine present in autophagosomal membranes; and this is called LC3-II. Therefore, the LC3-II/LC3-I ratio is usually used to represent the activation level of autophagy. Recent studies have shown that LPS-induced cardiomyocyte contractile dysfunction was prevented by antioxidant N-acetylcysteine and autophagy inhibitor 3-methyladenine (3-MA) [27]. Our studies revealed that autophagy was remarkably increased in endotoxin rat myocardial cells induced by LPS. CaSR agonists SC-211006 further promoted LPS-induced cardiomyocyte autophagy. CaSR inhibitor SC-207394 significantly suppressed autophagy to help cardiomyocytes survive under LPS conditions. This suggests that CaSR activation induces cardiomyocyte apoptosis by enhancing autophagy.

In summary, the present study demonstrates that CaSR activation is involved in endotoxin rat myocardial cell injury and apoptosis induced by LPS through the upregulation of endoplasmic reticulum stress and autophagy pathways.

Acknowledgements

Disclosure of conflict of interest

None.

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