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Cyclosporin A induces cardiomyocyte injury through calcium-sensing receptor-mediated calcium overload

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The aim of this study was to investigate whether Cyclosporin-A (CsA)-induced myocardial injury is mediated by elevating the intracellular calcium concentration ($[Ca^{2+}]_i$) through the Calcium sensing receptor (CaSR). Cultured neonatal rat cardiomyocytes were treated with CsA, with or without pretreatment with the CaSR-specific antagonist NPS2390 or the CaSR-specific agonist gadolinium chloride ($GdCl_3$). At 2 h, 4 h, 6 h and 8 h after CsA treatment, the ultrastructural changes of the cardiomyocytes were observed. In addition, the lactate dehydrogenase (LDH) and creatine kinase (CK) release from the cardiomyocytes, the $[Ca^{2+}]_i$ and the level of CaSR expression were determined. With increasing time of CsA treatment, ultrastructural damage of cardimyocytes gradually aggrevated, LDH and CK release and $[Ca^{2+}]_i$ also gradually increased. CaSR mRNA and protein expression increased at 4 h after CsA treatment. Compared with CsA treatment alone, pretreatment with NPS2390 lessened the ultrastructural damage of the cardiomyocytes as well as decreased the LDH and CK release, $[Ca^{2+}]_i$ and the expression of the CaSR mRNA and protein. Conversely, pretreatment with $GdCl_3$ aggravated the ultrastructural damage of the cardiomyocytes as well as increased LDH and CK release, $[Ca^{2+}]_i$ and the expression of the CaSR mRNA and protein. These results demonstrate that CsA induced cardiomyocyte injury in a time-dependent manner. Moreover, CsA-induced cardiomyocyte injury was related to CaSR-mediated intracellular calcium overload. These findings provide new insight into the mechanisms involved in CsA-induced myocardial injury.

1. Introduction

Cyclosporin A (CsA) is an effective immunosuppressive agent that is used in preventing organ rejection and treating autoimmune disease (Borel et al. 1996). Some clinical investigations have shown that several patients who underwent orthotopic heart transplantation with CsA treatment developed severe diastolic dysfunction, and their endocardial biopsy showed myocardial fibrosis (Laczkovics et al. 1987; Siostrzonek et al. 1993). Several experimental studies have also shown that CsA can lead to myofibril disorganization and myocardial fibrosis in the rat heart (Rezzani et al. 2003; Selcoki et al. 2007). Thus, treatment with CsA is limited by its cardiotoxicity. Although the mechanism of CsA-induced cardiotoxicity remains poorly understood, some studies have shown that intracellular calcium overload could be a major cause (Florio et al. 2003). Therefore, analysis of the pathway of CsA-induced calcium overload would aid in understanding the mechanism of CsA-induced cardiotoxicity. The calcium-sensing receptor (CaSR) is a member of the G protein-coupled receptor (GPCR) superfamily. The CaSR was initially discovered in parathyroid chief cells (Brown et al. 1993), and expression was subsequently found in various tissues and organs (Brown and MacLeod 2001). Moreover, the CaSR was shown to play an important role in the regulation of systemic calcium homeostasis (Fudge and Kovacs 2004; Kifor et al. 2001). The CaSR is also involved in cell differentiation and proliferation (Quarles 1997), gene expression (Tfelt-Hansen et al. 2003), ion channel opening (van den Hurk et al. 2005) and hormone secretion (Squires et al. 2000). Wang et al. (2003) reported that the level of the CaSR that is present in cardiac tissue and the activation of the CaSR increased $[Ca^{2+}]_i$ through the PLC-inositol, 4, 5-triphosphate (IP_3) pathway. During cardiac ischemia/reperfusion (I/R), CaSR expression increased, and the CaSR was found to be involved in calcium overload-induced cardiomyocyte apoptosis (Zhang et al. 2006). However, the relationship between the CaSR and CsA-induced myocardial injury remains unknown. Our previous study showed that CaSR mRNA level increased in rat ventricular muscle after one week of CsA administration. Therefore, we speculated that the CaSR could be directly or indirectly involved in CsA-induced myocardial injury. With this hypothesis in mind, we investigated the injury induced by CsA and the effect of CsA on CaSR expression in neonatal rat cardiomyocytes. Furthermore, we studied the role of the CaSR in CsA-induced cardiomyocyte injury using the CaSR-specific antagonist NPS2390 and the CaSR-specific agonist $GdCl_3$.

2. Investigations and results

2.1. Effect of CsA on the ultrastructure of cardiomyocytes

Transmission electron microscopy revealed a normal ultrastructure for the cardiomyocytes in the control group characterized by evenly distributed chromatin, clear mitochondrial cristae and

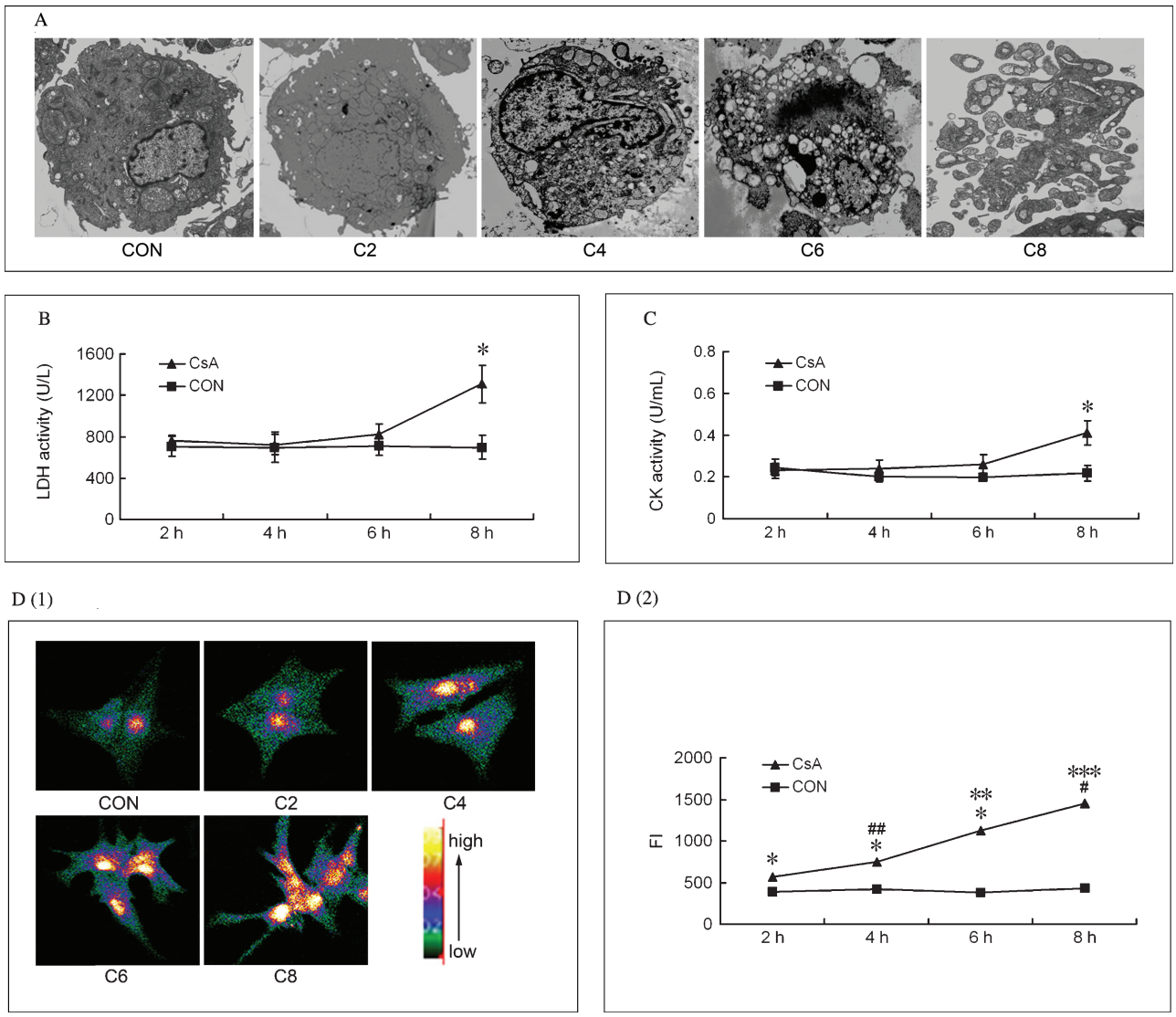

Fig. 1: Effect of CsA on neonatal rat cardiomyocytes. Neonatal rat cardiomyocytes were treated with 10 μg/ml CsA. At 2 h, 4 h, 6 h and 8 h after CsA treatment, the ultrastructural changes of the cardiomyocytes were observed, and the LDH and CK release from the cardiomyocytes and their $[Ca^{2+}]_i$ were determined. CON: control; C2: CsA 2 h; C4: CsA 4 h; C6: CsA 6 h; C8: CsA 8 h. A. Ultrastructral changes of cardiomyocytes. The ultrastructural damage of the cardiomyocytes aggravated with treatment time, progressing from slight mitochondrial swelling to cellular apoptosis and secondary necrosis. (Original magnification: CON, C2, C6 × 6000; C4, C8 × 8000) B. and C. LDH and CK release from cardiomyocytes. D. $[Ca^{2+}]_i$ changes in neonatal rat cardiomyocytes. D(1). Fluorescent images of cardiomyocytes. D(2). Fluorescent intensity analysis for $[Ca^{2+}]_i$ in cardiomyocytes. $^{*}p < 0.05$ vs. control; $^{\#}p < 0.01$ vs. control; $^{\#\#}p < 0.05$ vs. CsA 2 h; $^{**}p < 0.05$ vs. CsA 4 h; $^{***}p < 0.05$ vs. CsA 6 h

plentiful muscle fibers. After 2 h of CsA treatment, slight mitochondrial swelling was observed; after 4 h, the ultrastructural changes of the cardiomyocytes included cell contraction, cytoplasmic condensation and slight nuclear pyknosis, which are characteristics of early apoptosis; after 6 h, the ultrastructural changes of the cardiomyocytes included nuclear pyknosis, mitochondrial distension, chromatin margination and the formation of apoptotic bodies, characteristics of late apoptosis; after 8 h, apoptotic bodies and secondary necrosis were seen in most cardiomyocytes (Fig. 1).

2.2. *Effect of CsA on LDH and CK release from cardiomyocytes*

The control group showed no differences over time in LDH and CK release from cardiomyocytes treated with DMEM for 2 h, 4 h, 6 h and 8 h. Compared with the control group, LDH and CK release did not change significantly at 2 h after CsA treatment. At 4 h and 6 h, the release was slightly but not significantly elevated ($p > 0.05$), and at 8 h, release was significantly higher than the control ($p < 0.05$) (Fig. 1).

2.3. *Effect of CsA on the $[Ca^{2+}]_i$ of cardiomyocytes*

Measurement of $[Ca^{2+}]_i$ using laser confocal scanning microscopy showed no differences among the $[Ca^{2+}]_i$ of cardiomyocytes treated with DMEM for 2 h, 4 h, 6 h and 8 h. The $[Ca^{2+}]_i$ of the cardiomyocytes increased to various degrees at 2 h, 4 h, 6 h and 8 h after CsA treatment compared to that in the control group ($p < 0.05$ and $p < 0.01$). Moreover, CsA increased $[Ca^{2+}]_i$ in a time-dependent manner (Fig. 1).

2.4. *CaSR expression in neonatal rat cardiomyocytes*

2.4.1. *CaSR mRNA expression*

After CsA treatment for 4 h, the level of CaSR mRNA was higher than that of the control group ($p < 0.05$). NPS pretreatment attenuated the increase in the CaSR mRNA level ($p < 0.05$), and $GdCl_3$ further increased the CaSR mRNA level ($p < 0.05$) (Fig. 2).

2.4.2. *CaSR protein expression*

In the western blot analysis, two bands corresponding to the CaSR with relative molecular masses of 150 kD and 170–180 kD

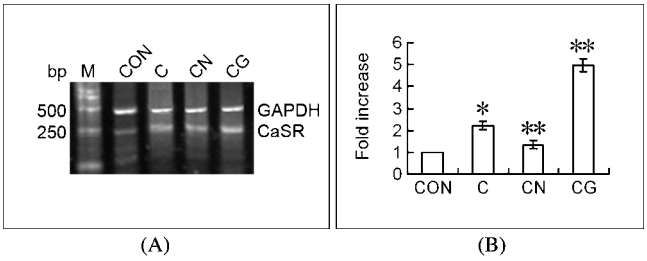

Fig. 2: CaSR mRNA expression in neonatal rat cardiomyocytes as determined by RT-PCR. M: Marker; CON: control; C: CsA; CN:CsA + NPS2390; CG: CsA + $GdCl_3$. A. RT-PCR result. B. Analysis of CaSR mRNA expression. The intensity of each band was quantified using densitometry, and the data were normalized to the actin signal. $^{*}p < 0.05$ vs. control; $^{**}p < 0.05$ vs. CsA

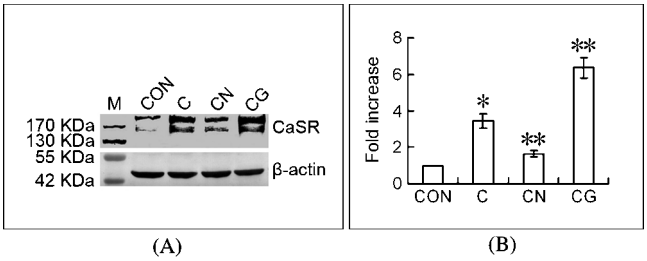

Fig. 3: CaSR protein expression in neonatal rat cardiomyocytes as determined by western blot analysis. M: Marker; CON: control; C: CsA; CN: CsA + NPS2390; CG: CsA + $GdCl_3$. A. Western blot result. B. Analysis of CaSR protein expression. The intensity of each band was quantified using densitometry, and the data were normalized to the actin signal. $^{*}p < 0.05$ vs. control; $^{**}p < 0.05$ vs. CsA

were detected. After CsA treatment for 4 h, the CaSR protein level was higher than that of the control group ($p < 0.05$). NPS pretreatment attenuated the increased level of the CaSR protein ($p < 0.05$), and $GdCl_3$ further increased the CaSR protein level ($p < 0.05$) (Fig. 3).

2.5. Effect of inhibition or activation of the CaSR on CsA-induced cardiomyocyte ultrastructure damage

Compared with CsA treatment alone for 4 h, NPS pretreatment alleviated the cardiomyocyte ultrastructural damage, including mitochondrial swelling and mild nuclear swelling, and $GdCl_3$ pretreatment aggravated the cardiomyocyte ultrastructural damage, including the formation of apoptotic bodies and secondary necrosis (Fig. 4).

2.6. Effect of inhibition or activation of the CaSR on CsA-induced LDH and CK release

Compared with CsA treatment alone for 8 h, NPS pretreatment attenuated the increase in LDH and CK release ($p < 0.05$), and $GdCl_3$ pretreatment further increased LDH and CK release ($p < 0.05$) (Fig. 4).

2.7. Effect of inhibition or activation of the CaSR on CsA-induced $[Ca^{2+}]_i$ increase

Compared with CsA treatment alone for 4 h, NPS pretreatment attenuated the increase in $[Ca^{2+}]_i$ ($p < 0.05$), and $GdCl_3$ pretreatment further increased $[Ca^{2+}]_i$ ($p < 0.05$) (Fig. 4).

3. Discussion

CsA has been reported to have dual effects on the myocardium. Some studies have shown that CsA leads to degenerative changes in normal rat cardiac tissues, including a greate disorganization of the myofibrils and myocardial fibrosis in both the atria and ventricles (Bianchi et al. 2003; Jurado et al. 1998). Conversely, other studies have shown that CsA inhibits cardiac hypertrophy (Molkentin et al. 1998) and protects the myocardium against ischemia/reperfusion injury (Bes et al. 2005; Griffiths and Halestrap 1993) and failure (Sharov et al. 2007). Although these studies have demonstrated that CsA has opposing effects on the myocardium, CsA-induced myocardial injury cannot be neglected. In our previous studies, CsA was administered to normal rats and this treatment resulted in widespread edema, muscle fiber fracture and cardiac fibrosis in cardiac tissues. In the present study, we found that the ultrastructural damage of cardiomyocytes aggravated with increasing CsA treatment time, progressing from slight mitochondrial swelling to cellular apoptosis and secondary necrosis. Meanwhile, LDH and CK release, indicators of cardiomyocyte injury, gradually increased and were significantly higher at 8 h after CsA treatment compared with the control. This result demonstrated that CsA induced cardiomyocyte injury in a time-dependent manner.

The maintenance of intracellular calcium homeostasis plays an important role in cell growth, development and survival. Exposure to a variety of stimuli can lead to an imbalance of intracellular calcium homeostasis, including calcium overload and calcium depletion, which can lead to reversible or irreversible cell injury. Commonly, this imbalance in intracellular calcium homeostasis is manifested as calcium overload (Dong et al. 2006). Recent studies showed that intracellular calcium overload-induced oxidative stress could be important in CsA-induced myocardial injury (Florio et al. 2003). Therefore, in the present study we investigated the effect of CsA on $[Ca^{2+}]_i$. The result showed that CsA treatment increased $[Ca^{2+}]_i$ in a time-dependent manner, with $[Ca^{2+}]_i$ gradually increasing with treatment time. Meanwhile, CsA-induced cardiomyocyte injury aggravated over time. This indicated that the degree of intracellular calcium overload seemed to determine the extent of cardiomyocyte injury. Our data suggested a key role for intracellular calcium overload in CsA-induced cardiomyocyte injury. Numerous studies have shown that the CaSR is expressed throughout the cardiovascular system, such as in the myocardium (Wang et al. 2003), vascular smooth muscle (Smajilovic et al. 2006) and endothelial cells (Ziegelstein et al. 2006). Furthermore, the CaSR plays an important role in a variety of physiological and pathological conditions, such as myocardial ischemia/reperfusion (Zhang et al. 2006, 2007), cardiomyocyte apoptosis (Sun et al. 2006) and cardiac hypertrophy (Wang et al. 2008). However, there is a lack of information concerning the relationship between the CaSR and CsA-induced myocardial injury. In our study, we used the CaSR-specific antagonist NPS2390 and the CaSR-specific agonist $GdCl_3$ to reveal the role of the CaSR in CsA-induced myocardial injury. First, we determined the level of CaSR expression by RT-PCR and western blot analysis. During CsA-induced cardiomyocyte injury, CaSR mRNA and protein expression increased, which indicated the involvement of the CaSR in CsA-induced cardiomyocyte injury. In order to further elucidate the role of the CaSR in CsA-induced cardiomyocyte injury, neonatal rat cardiomyocytes were pretreated with NPS2390 or $GdCl_3$ before CsA treatment. Compared with CsA treatment alone, pretreatment with NPS2390 ameliorated the ultrastructural damage of the cardiomyocytes, decreased the release of LDH and CK and decreased the expression of the CaSR mRNA and protein. Conversely, pretreatment with $GdCl_3$ aggravated the ultrastructural damage of the cardiomyocytes, increased the release of LDH and CK and increased the expression of the CaSR mRNA and protein. These results suggested that the CaSR could be involved in CsA-induced cardiomyocyte injury. Recently, an

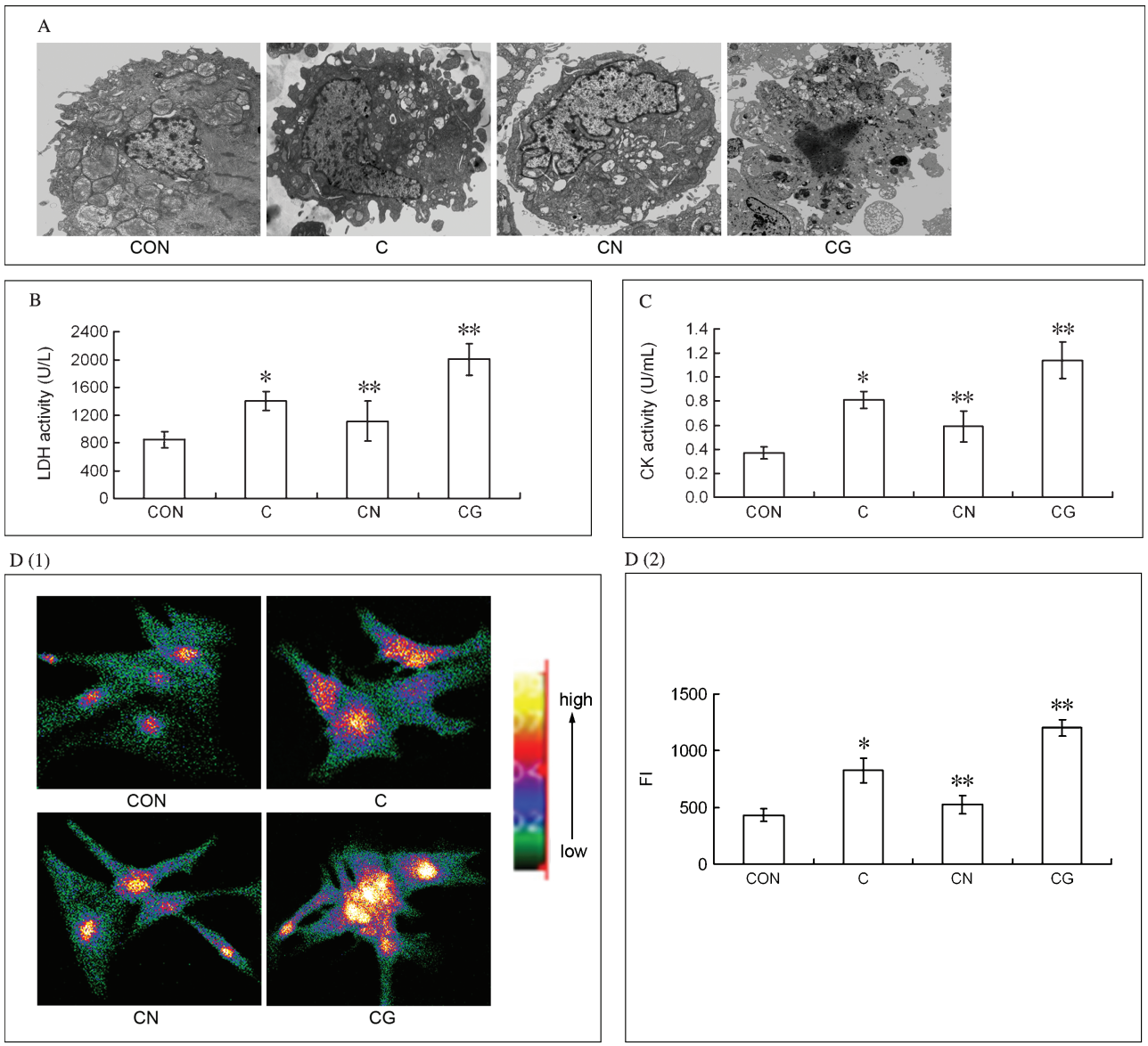

Fig. 4: Effect of the inhibition or activation of the CaSR on CsA-treated cardiomyocytes. CON: control; C: CsA; CN: CsA + NPS2390; CG: CsA + $GdCl_3$. A. Ultrastructral changes of cardiomyocytes. Compared with the CsA treatment alone for 4 h, pretreatment with NPS alleviated the cardiomyocyte ultrastructural damage, including mitochondrial swelling and mild nuclear swelling. Conversely, $GdCl_3$ pretreatment aggravated the cardiomyocyte ultrastructure damage, including the formation of apoptotic bodies and secondary necrosis. (Original magnification: CON × 10000; C, CN, CG × 6000) B. and C. LDH and CK release from cardiomyocytes. D. $[Ca^{2+}]_i$ changes in neonatal rat cardiomyocytes. D(1). Fluorescent images of cardiomyocytes. D(2). Fluorescent intensity analysis for $[Ca^{2+}]_i$ in cardiomyocytes. $^{*}p < 0.05$ vs. control; $^{**}p < 0.05$ vs. CsA

accumulating body of evidence has shown that the CaSR was an important regulator of calcium homeostasis in cardiomyocytes (Sun et al. 2006; Wang et al. 2003; Zhang et al. 2006). Therefore, we examined the involvement of the CaSR in calcium overload during CsA-induced cardiomyocyte injury. Our findings that CsA-induced calcium overload was attenuated by NPS2390 and exacerbated by $GdCl_3$ suggested that the CaSR could be involved in CsA-induced calcium overload. These results demonstrated that the CaSR could mediate intracellular calcium overload, thus inducing cardiomyocyte injury.

Previous studies have shown that the CsA-induced increase in the frequency of Ca^{2+} oscillation and in the level of free calcium in the cytosol is likely related to a higher probability of endoplasmic reticulum (ER) Ca^{2+}-release channel openings (Bandyopadhyay et al. 2000). The binding of extracellular calcium to the CaSR could induce calcium release from the ER through the G protein-PLC-IP_3 pathway (Wang et al. 2003). One attractive hypothesis is that CsA activates the CaSR and its G protein-PLC-IP_3 pathway, followed by ER calcium release. Calcium is an important messenger for intracellular signal transduction (Berridge et al. 2003). Cellular calcium is remarkable in its ability to bind and regulate various proteases such as phospholipase, calpain and endonuclease. An increase in $[Ca^{2+}]_i$ induces the activation of these Ca^{2+}-dependent proteases, which leads to membrane phospholipid hydrolysis, cytoskeletal disruption and DNA damage, resulting in cell injury (Dong et al. 2006). Therefore, CsA-induced cardiomyocyte injury might be related to proteases activated by calcium overload. In addition, mitochondria are important organelles in the maintenance of intracellular calcium homeostasis. Upon an increase in $[Ca^{2+}]_i$, calcium uptake by the mitochondria increases and mitochondrial calcium overload ensues, resulting in cell apoptosis and cell injury (Ott et al. 2002; Schanne et al. 1979). Millane et al. (1994) found that mitochondrial calcium overload could cause CsA-induced myocardial injury. Further study is required to determine whether CaSR-mediated intracellular calcium overload results in mitochondrial calcium accumulation during CsA-induced cardiomyocyte injury.

4. Experimental

4.1. Materials

Cyclosporin A, fluo-3 acetoxymethyl ester (Fluo-3/AM), the CaSR-specific agonist $GdCl_3$ and the CaSR-specific antagonist NPS2390 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The lactate dehydrogenase (LDH) and creatine kinase (CK) kits were obtained from Nanjing Jiancheng Biotechnology Co., Ltd. (Nanjing, Jiangsu, China). The primary anti-CaSR antibody was obtained from Alpha Diagnostic International Inc. (San Antonio, TX, USA).

4.2. Neonatal rat cardiomyocyte culture

Neonatal rat cardiomyocytes were prepared according to a previously described method (Xu et al. 2004) with minor modifications. Briefly, hearts from 1- to 3-day-old neonatal Wistar rats (Animal Research Institute of Harbin Medical University, China) were dissected, minced and placed in a Petri dish. The tissue was trypsinized at 37 °C in D-Hanks' balanced salt solution (8.00 g/L NaCl, 0.4 g/L KCl, 0.06 g/L KH_2PO_4, 0.35 g/L $NaHCO_3$, 0.09 g/L $Na_2HPO_4 \cdot 7H2O$ and 0.25% trypsin). After centrifugation (1000 rpm, 10 min), cells were collected and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) newborn calf serum. The cells were diluted to 5×10^6 cells/ml and then cultured at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2.

4.3. CsA incubation

After 72 h of culture and subsequent starving in serum-free medium for 24 h, the cardiomyocytes were incubated with 10 μg/ml CsA for 2 h, 4 h, 6 h or 8 h, in 5% CO_2 at 37 °C. A control group was incubated in DMEM for the same time periods.

4.4. Preincubation with NPS2390 or $GdCl_3$

After 72 h of culture and subsequent starving in serum-free medium for 24 h, the cardiomyocytes were preincubated with or without NPS2390 (10 μM, 1 h) or $GdCl_3$ (30 μM, 2 h) and then incubated with 10 μg/ml CsA for 4 h or 8 h in 5% CO_2 at 37 °C.

4.5. Transmission electron microscopy

The ultrastructural changes of the cardiomyocytes were examined by transmission electron microscopy. Electron microscopy analysis was performed as published previously (Ho et al. 1996). After the treatment described above, the cells were fixed with 2.5% glutaraldehyde in Hank's modified salt solution and then postfixed in 1% OsO_4 in 0.1 M cacodylate buffer. The OsO_4-fixed samples were dehydrated in an ethanol series and embedded in Araldite. Ultrathin sections were stained with saturated uranyl acetate and lead citrate and then observed under a JEM-1220 electron microscope.

4.6. LDH and CK assays

The cardiomyocytes were assayed for LDH and CK release into the culture media, which are indicators of cardiomyocyte injury. After the treatment described above, the culture media samples were collected and measured spectrophotometrically using an LDH or CK kit. With this kit, LDH catalyzes lactate to yield pyruvate, which reacts with 2,4-dinitrophenylhydrazine to produce pyruvate dinitrophenylhydrazone. The change in absorbance at 440 nm was monitored with a spectrophotometer and LDH activity was expressed as U/L. CK phosphorylates creatine to yield creatine phosphate, which dissociates into inosine and inorganic phosphate. Inorganic phosphate reacts with ammonium molybdate to produce phosphomolybdic acid, which is reduced to molybdenum blue. The change in absorbance at 660 nm was monitored with a spectrophotometer and CK activity was expressed as U/ml.

4.7. Reverse transcription-polymerase chain reaction (RT-PCR) of CaSR

Total RNA was isolated from cultured cardiomyocytes using TRIzol Reagent (Invitrogen) 4 h after CsA treatment. Total RNA (2 μg) was reverse transcribed with a RT kit (Promega). The RT reaction was performed with random primers according to the manufacturer's protocol. For each RT product, aliquots of the final reaction volume were amplified in a PCR reaction with CaSR-specific primers (sense 5'-TTCGGCATCAGCTTTGTG-3', antisense 5'-TGAAGATGATTTCGTCTTCC-3') or GAPDH-specific primers (sense 5'-CTCAACTACATGGTCTACATG-3', antisense 5'-TGGCATGGACTGTGGTCATGAG-3'). Amplification was performed over 35 cycles, each consisting of 2 min at 94 °C, 40 s at 50 °C and 40 s at 72 °C. A final extension period of 2 min at 72 °C was included after the 35 cycles. The PCR products were electrophoresed on a 1.2% agarose gel and visualized with ethidium bromide. The optical density (OD) of each band was measured using a Bio-Rad Chemi Doc™ EQ densitometer and Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, USA).

4.8. Western blot analysis for CaSR

Western blot analysis was performed according to a previously described method (El-Hajj et al. 2007). Briefly, the cells were washed with PBS and resuspended in cold lysis buffer with PMSF. The cell lysate was incubated on ice for 30 min and centrifuged at 12,000 rpm for 15 min at 4 °C. The protein concentration of the lysate was determined with a BCA-200 protein assay kit (Beyotime). Equal amounts of protein were denatured by boiling and then electrophoresed in an 8% SDS-polyacrylamide gel. The protein was transferred to nitrocellulose. After blocking with 5% nonfat milk in TBS-T [137 mM NaCl, 20 mM Tris (pH 7.6), and 0.1% (v/v) Tween 20] for 1 h at 37 °C, the blots were incubated overnight at 4 °C with a primary antibody against CaSR (1:2000, rabbit monoclonal). The membrane was incubated with an AP-conjugated secondary antibody for 1 h at 37 °C. The volumes of the protein bands were quantified using a Bio-Rad Chemi Doc™ EQ densitometer and Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, USA). β-actin was used as an internal control for the semiquantitative assay.

4.9. Measurement of $[Ca^{2+}]_i$

The cardiomyocytes were loaded with 5 μmol/L of the Ca^{2+}-selective fluorescent probe Fluo-3/AM for 30 min at 37 °C. Excess extracellular dye was removed by washing three times with Ca^{2+}-free PBS. The fluorescence intensity for $[Ca^{2+}]_i$ was measured using a laser scanning confocal microscope (Olympus, Japan) with excitation at 488 nm and emission at 530 nm.

4.10. Statistical analysis

All results are expressed as the mean ±SEM. A one-way ANOVA was used for multiple comparisons. Differences between two groups were tested with unpaired two-tailed Student's t tests. A value of $p < 0.05$ was considered statistically significant.

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