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Involvement of the calcium-sensing receptor in cyclosporin A-induced cardiomyocyte apoptosis in rats

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In this study, we sought to determine whether the calcium-sensing receptor (CaSR) is involved in Cyclosporin A (CsA)-induced cardiomyocyte apoptosis and identify its signal transduction pathway. Forty Wistar rats were randomly divided into four groups: the control group, the CsA group (CsA 15 mg/kg/day intraperitoneally, i.p.), the $GdCl_3$ group ($GdCl_3$ 10 mg/kg, every other day, i.p.), and the CsA + $GdCl_3$ group (CsA 15 mg/kg/day, i.p. and $GdCl_3$ 10 mg/kg, every other day, i.p.). The groups were treated for two weeks. Cardiomyocyte apoptosis and injury were observed by light microscopy, electron microscopy and TUNEL staining. CaSR mRNA expression was determined by RT-PCR, and CaSR protein expression was detected by western blot and immunohistochemistry. The protein expression levels of cytochrome c, cleaved caspase-9, cleaved caspase-3, Bax, and Bcl-2 were detected by western blot and immunohistochemistry. CsA increased the expression of CaSR mRNA and protein and enhanced cardiomyocyte apoptosis. $GdCl_3$, a specific activator of CaSR, further enhanced CaSR expression and cardiomyocyte apoptosis and led to the upregulation of cytochrome c, cleaved caspase-9, cleaved caspase-3, and Bax, as well as the down-regulation of Bcl-2. The present *in vivo* study provides further information on CsA-induced cardiomyocyte apoptosis. We determined for the first time that CaSR is involved in CsA-induced cardiomyocyte apoptosis in the rat through the activation of downstream cytochrome c-caspase-3 pathways. Furthermore, we offer evidence that the Bcl-2 family is involved in this process. These findings could provide novel strategies for the prevention and cure of CsA-induced cardiotoxicity.

1. Introduction

Cyclosporin A (CsA) is an effective immunosuppressant drug that is widely used to both prevent immunological organ rejection and to treat autoimmune diseases (Rezzani 2006). However, the use of CsA is limited by its side effects like hepatotoxicity (Rezzani et al. 2005), nephrotoxicity (Wongmekiat and Thamprasert 2005) and cardiotoxicity (Florio et al. 2003). At present, there is no effective solution to these negative effects. Although studies have shown that CsA can inhibit cardiac hypertrophy (Molkentin et al. 1998), protect myocardial cells from heart failure (Sharov et al. 2007), and inhibit cardiac ischemia-reperfusion injury (Bès et al. 2005), some studies have found that the use of CsA can lead to myocardial structural damage and increased myocardial fibrosis in rats (Rezzani 2004). In a previous study, we found that CsA could result in cardiomyocyte injury in a time-dependent manner (Tang et al. 2011). Therefore, CsA cardiotoxicity cannot be ignored. To date, although the mechanism of CsA-induced cardiotoxicity has not been clarified, some studies have shown that CsA can induce cardiomyocyte apoptosis (Tang et al. 2011; Rezzani et al. 2009), which could be a major cause of CsA-induced cardiotoxicity. Therefore, analysis of the pathway of cardiomyocyte apoptosis induced by CsA would contribute to a better understanding of the mechanism of CsA-induced cardiotoxicity.

The calcium-sensing receptor (CaSR) is a G protein-coupled receptor that belongs to the C family of the superfamily of seven-transmembrane receptors. It was first discovered and cloned in bovine parathyroid cells in 1993. It has three structural domains: the NH_2-terminal extracellular portion, a seven-transmembrane region, and an intracellular COOH-terminal tail (Brown et al. 1993). Subsequently, its expression was localized mainly to the parathyroid, kidney, thyroid, bone, and gastrointestinal tract tissues. In the cardiovascular system, Wang et al. (2003) first observed CaSR expression in cardiac tissue. Later, Tfelt-Hansen et al. (2006) showed that CaSR also exists in neonatal ventricular cardiomyocytes. CaSR plays an important role in the homeostasis of systemic calcium and other metal ions (Fudge and Kovacs 2004) and is involved in apoptosis (Zhang et al. 2006; Mizobuchi et al. 2007), hormone secretion (Squires et al. 2000), cell differentiation and proliferation (Yano et al. 2000). The trivalent cation Gd^{3+} is a strong agonist of CaSR (Sun et al. 2006).

However, the role of CaSR in CsA-induced cardiomyocyte apoptosis in rats is unknown. Therefore, in this study, our aim was to identify (i) whether CsA induces apoptosis in the rat heart; (ii) whether CaSR is involved in CsA-induced apoptosis;

and (iii) the CaSR downstream intracellular apoptotic signaling pathways caused by CsA.

2. Investigations and results

2.1. H&E staining

Light microscopic examination revealed the effect of CsA and $GdCl_3$ on myocardial cells in rats (Fig. 1a). No significant changes were observed in the control group. In the CsA group, myocardial cells shrank and were arranged in a disordered manner, with increased amounts of collagen fibers between the cells. In the CsA + $GdCl_3$ group, myocardial cells shrank and were arranged in a disordered manner. Fibrosis emerged in the myocardial interstitial space. In the $GdCl_3$ group, some cells shrank, and the number of collagen fibers increased slightly.

2.2. Ultrastructural changes of cardiomyocyte apoptosis

The ultrastructural changes of cardiomyocyte apoptosis were observed by transmission electron microscopy (Fig. 1b). There were no significant ultrastructural changes in the control groups. In the CsA group, many apoptotic bodies formed surrounding the myocardial cells, the density of the mitochondrial matrix increased, and some of the myofilaments were destroyed. In the CsA + $GdCl_3$ groups, multiple apoptotic bodies formed surrounding the myocardial cells, mitochondrial swelling was observed, and the structure of the sarcomere was diminished. There were dark particles in the mitochondria, which could be calcium. Mitochondrial swelling was observed in the $GdCl_3$ group. In this group, the structure of the sarcomere was unclear, and some of the myofilaments had dissolved.

2.3. Apoptotic cell death by TUNEL staining

We used the TUNEL assay to investigate CsA-induced cardiomyocyte apoptosis. We detected a low level of TUNEL-positive cells in the control group (8.19 ± 1.0 %). The number of apoptotic cells in the CsA group was 27.5 ± 1.3 % ($p < 0.05$ vs. control), 38.4 ± 2.1 % in the CsA + $GdCl_3$ group ($p < 0.05$ vs. control, $p < 0.05$ vs. CsA), and 16.5 ± 0.7 % in the $GdCl_3$ group ($p < 0.05$ vs. control) (Fig. 1c, d). These results demonstrated that CsA could induce cardiomyocyte apoptosis in rats and that $GdCl_3$ further enhanced cyclosporine A-induced cardiomyocyte apoptosis.

2.4. Expression of CaSR mRNA

RT-PCR was used to measure the expression of CaSR mRNA in the various groups (Fig. 2a, b). The 234-bp PCR fragment was observed, as reported by Wang et al. (2003). We found that CsA and $GdCl_3$ up-regulated mRNA expression of CaSR ($p < 0.05$ vs. control). Moreover, the increase was more significant in the CsA + $GdCl_3$ group than in the CsA group ($p < 0.05$).

2.5. Expression and distribution of CaSR protein

We also used Western blot to analyze CaSR protein expression for the various groups. Three bands of CaSR proteins with relative molecular masses of 110–130 kDa, 130–170 kDa and 170–180 kDa were detected in the rat ventricle, as reported by Sun et al. (2006). CaSR protein expression was increased in the CsA group, $GdCl_3$ group and CsA + $GdCl_3$ group, compared to the control group ($p < 0.05$). Moreover, the increase was significant in the CsA + $GdCl_3$ group ($p < 0.05$ vs. CsA) (Fig. 2c, d).

The distribution of CaSR protein was detected by immunohistochemistry. In rat cardiac tissue, brown immunohistostaining was observed throughout the cardiomyocytes. Increased expression of CaSR protein was observed in the CsA group, $GdCl_3$ group and CsA + $GdCl_3$ group; the most significant increase was observed in the CsA + $GdCl_3$ group (Fig. 2e). Semiquantitative analysis of CaSR expression is indicated in Table.

2.6. Western blot analysis of cytochrome c and Bax

We performed Western blot to assay the expression levels of cytochrome c and Bax for the various groups. Cytochrome c expression was increased in the CsA group, $GdCl_3$ group and CsA + $GdCl_3$ group compared to the control group ($p < 0.05$); the most significant increase was observed in the CsA + $GdCl_3$ group ($p < 0.05$ vs. CsA) (Fig. 3a, b). Bax expression was increased in both the CsA group and the CsA + $GdCl_3$ group ($p < 0.05$ vs. control) (Fig. 3c, d).

2.7. Immunohistochemical analysis of cytochrome c, cleaved caspase-9, cleaved caspase-3, Bax, and Bcl-2

The expression levels of cytochrome c, cleaved caspase-9, cleaved caspase-3, Bax, and Bcl-2 proteins were determined by immunohistochemistry. The expression levels of cytochrome c, cleaved caspase-9, cleaved caspase-3, and Bax were increased in the CsA and CsA + $GdCl_3$ groups, with the most significant increase observed in the CsA + $GdCl_3$ group. By contrast, Bcl-2 expression was decreased in the CsA and CsA + $GdCl_3$ groups (Fig. 4). Semiquantitative analysis of the protein expression levels is indicated in the Table.

3. Discussion

Accumulating evidence suggests that CsA can induce apoptosis in multiple cell types. Thomas et al. (1998) showed that CsA causes a marked increase in apoptosis of tubular and interstitial cells, which is associated with CsA nephropathy and interstitial fibrosis. Tu et al. (2009) demonstrated that CsA enhances apoptosis in gingival keratinocytes of rats and in OECM1 cells *via* the mitochondrial pathway. Recently, Rezzani et al. (2009) showed that CsA could induce an enhancement of cardiomyocyte apoptotic processes in rats. Our previous study (Tang et al. 2011) indicated that CsA induces cardiomyocyte apoptosis in neonatal rats and that increasing the CsA treatment period led to a gradual aggravation of the cardiomyocyte damage. Although previous studies demonstrated that CsA has protective effects on the myocardium, the role of CsA-induced myocardial injury should not be ignored. In this study, cardiomyocyte apoptosis was detected by TUNEL assay and observed by H&E staining and transmission electron microscopy in the CsA and CsA + $GdCl_3$ groups. Our study further confirmed that a conventional dose of CsA was able to induce cardiomyocyte apoptosis in rats. We used $GdCl_3$, which is a strong CaSR agonist, to study the role of CaSR in CsA-induced cardiomyocyte apoptosis and its downstream intracellular signaling pathways in apoptosis. The TUNEL assay indicated that cardiomyocyte apoptosis increased with the addition of $GdCl_3$.

To test whether CsA induces cardiomyocyte apoptosis in rats *via* a CaSR-mediated calcium overload, we assayed the expression and distribution of CaSR using RT-PCR, Western blot, and immunohistochemistry. The expression of CaSR mRNA and CaSR protein significantly increased in the CsA group compared to the control, and $GdCl_3$ further enhanced the expression of CaSR. Wang et al. (2003) demonstrated that CaSR activation can lead to increased myocardial intracellular calcium

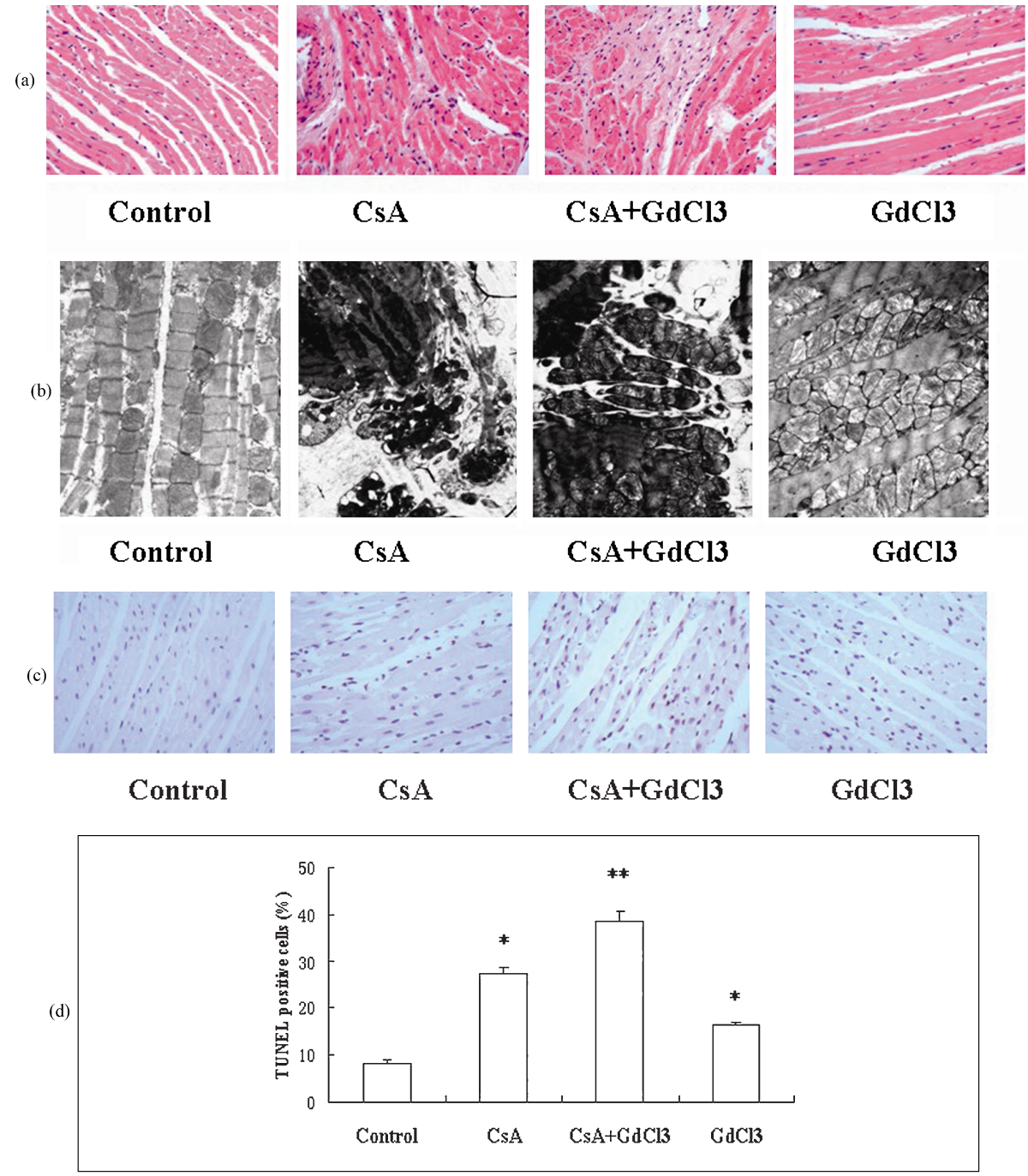

Fig. 1: Morphological characterization of cardiomyocyte apoptosis in rat tissues. (a) H&E staining in rat tissues (400 ×). (b) Ultrastructural changes of cardiomyocyte apoptosis. (A) Control, (B) CsA, (C) CsA + $GdCl_3$, and (D) $GdCl_3$. Magnification is 10,000 × (A,D), 5,000 × (B), and 8,000 × (C). (c) Representative illustration of TUNEL staining. Nuclei with brown staining indicate TUNEL-positive cells (400 ×). (d) Quantitative results of the TUNEL staining for each group. Values represent the group means ± SEM. $^{*}p < 0.05$ vs. control, $^{**}p < 0.05$ vs. CsA

through the PLC-inositol, 4,5-triphosphate (IP3) pathway. Sun et al. (2006) suggested that $GdCl_3$ could induce cardiomyocyte apoptosis and speculated that CaSR activation might initiate a cascade of increased intracellular calcium, destroyed mitochondria, and the release of apoptosis promoters. Our previous *in vitro* study demonstrated that CsA induces cardiomyocyte injury through a CaSR-mediated calcium overload (Tang et al. 2011). Florio et al. (2003) also demonstrated that CsA increases

Table: Semiquantitative analysis of CaSR, cytochrome c, cleaved caspase-9, cleaved caspase-3, Bax, and Bcl-2 proteins in rat cardiac tissue

Group	CaSR	Cytochrome c	Cleaved Caspase-9	Cleaved caspase-3	Bax	Bcl-2
Control	+	+	−	−	+	+++
CsA	++	++	+	++	++	+
CsA+$GdCl_3$	+++	+++	++	+++	+++	+
$GdCl_3$	++	++	+/−	+/−	+	++

The data are expressed as negative (−), very weak (+/−), weak (+), moderate (++) and strong (+++) positivity.

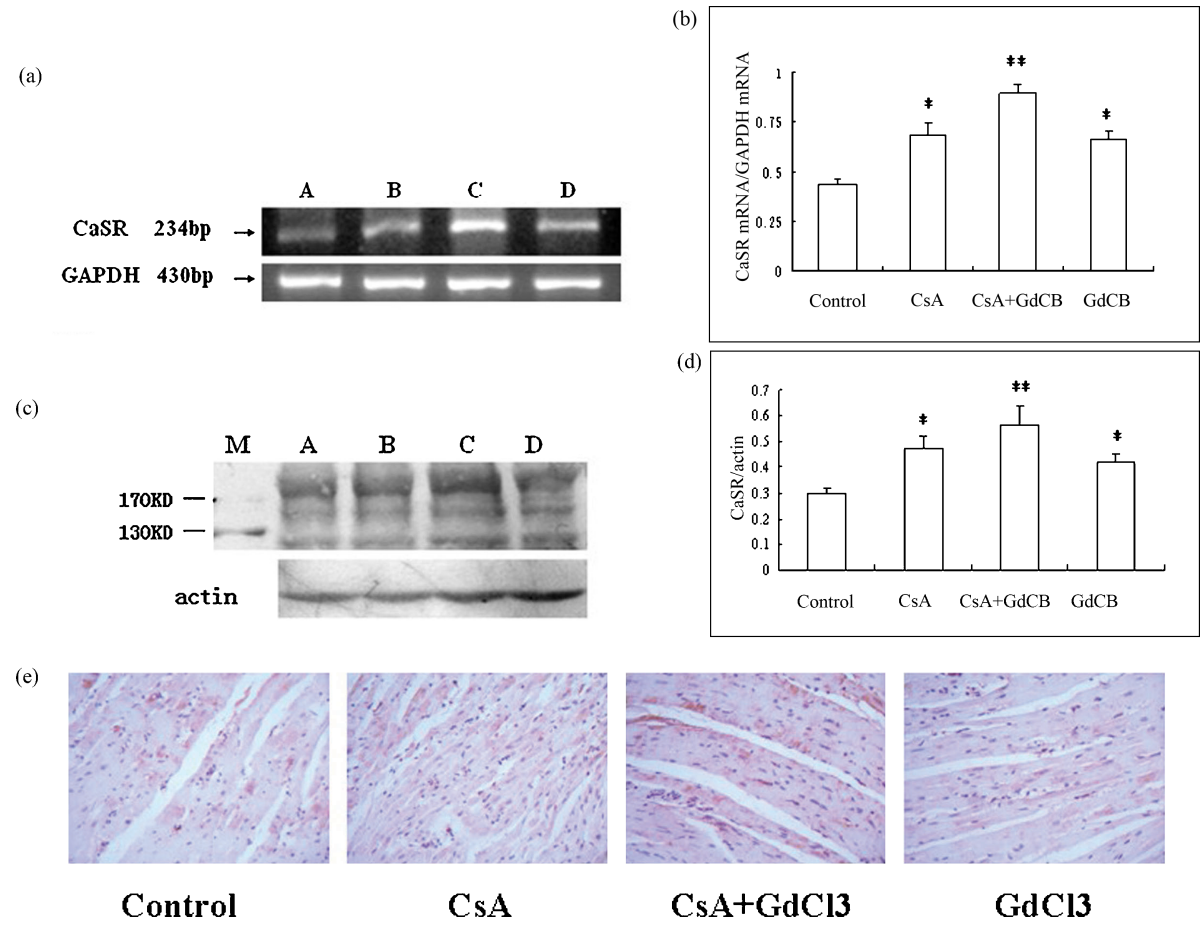

Fig. 2: The expression and distribution of CaSR in rat cardiac tissues for each group. (a) RT-PCR analysis for CaSR mRNA in rat cardiac tissues for each group. (A) Control, (B) CsA, (C) CsA + $GdCl_3$, and (D) $GdCl_3$. (b) Quantitative analysis of CaSR mRNA in rat cardiac tissues for each group. (c) Western blot analysis for CaSR protein in rat cardiac tissues. M: marker, (A) Control, (B) CsA, (C) CsA + $GdCl_3$, and (D) $GdCl_3$. (d) Quantitative analysis of CaSR protein in rat cardiac tissue for each group. (e) Immunohistochemical analysis of CaSR in rat cardiac tissues (400×). Semiquantitative analysis of the CaSR levels in rat cardiac tissue is indicated in Table. Values represent group means ± SEM. $^{*}p < 0.05$ vs. control, $^{**}p < 0.05$ vs. CsA

intracellular calcium in a dose-dependent manner. They speculated that the CsA toxicity observed was due to calcium overload. In summary, our results demonstrated that CsA can increase or upregulate CaSR expression, increase intracellular calcium levels, and induce cardiomyocyte apoptosis.

Two major apoptotic signaling pathways have been defined: an extrinsic pathway and an intrinsic pathway. Mitochondria play a critical role in intrinsic apoptosis (Hallak et al. 2008). The permeability change in the mitochondrial outer membrane induces the release of cytochrome c to the cytosol (Kroemer and Reed 2000), and cytochrome c promotes the formation of the so-called "apoptosome," which consists of the caspase-9 activating complex, apoptosis protease activating factor 1 (APAF-1) and ATP/dATP (Riedl and Salvesen 2007). Subsequently, activated caspase-9 can activate the effectors caspase-3, -6 and -7, which ultimately contribute to apoptosis (Thornberry and Lazebnik

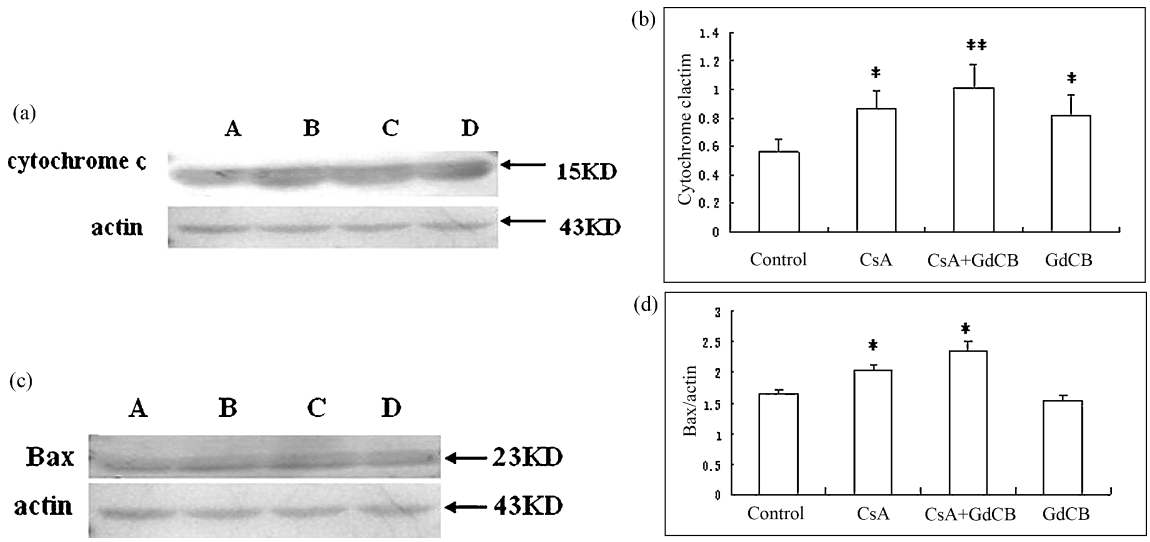

Fig. 3: Western blot analysis of cytochrome c and Bax proteins in rat cardiac tissue (a, c). (A) Control, (B) CsA, (C) CsA + $GdCl_3$, and (D) $GdCl_3$. Quantitative analysis of cytochrome c and Bax proteins in rat cardiac tissue for each group (b, d). Values represent group means ± SEM. $^{*}p < 0.05$ vs. control, $^{**}p < 0.05$ vs. CsA

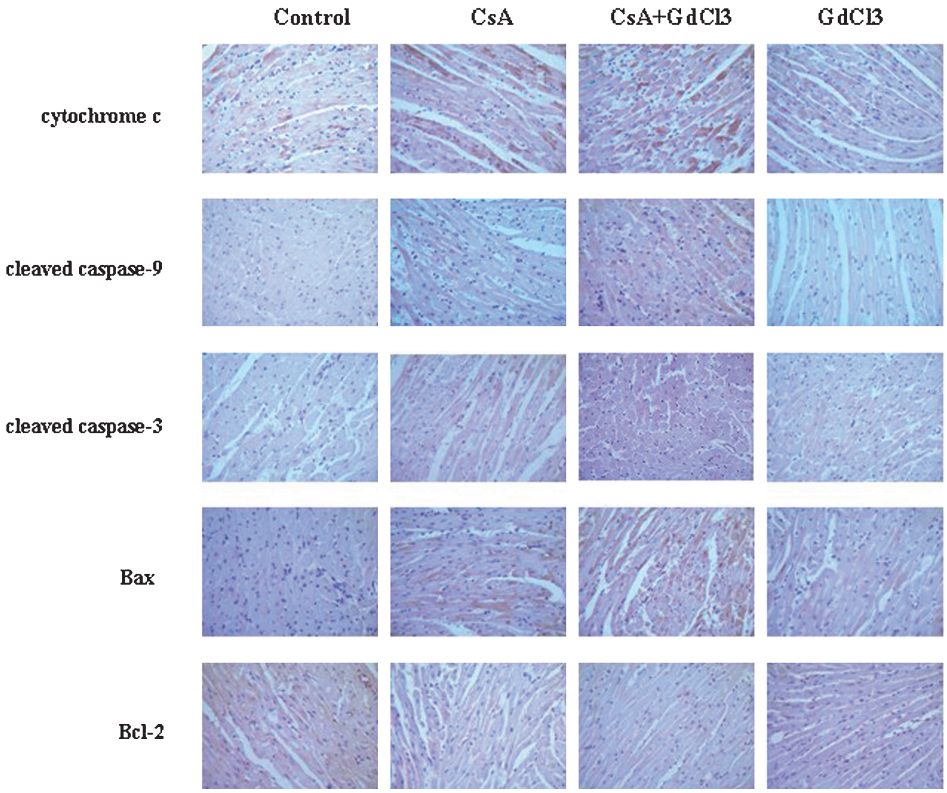

Fig. 4: Immunohistochemical analysis of cytochrome c, cleaved caspase-9, cleaved caspase-3, Bax, and Bcl-2 (400×). Semiquantitative analysis of the protein levels in rat cardiac tissue is indicated in the Table

1998). The Bcl-2 protein family is made up of significant regulators of the mitochondrial pathway of apoptosis. The proapoptotic proteins of this family, such as the Bax proteins, induce mitochondrial outer membrane permeabilization (MOMP), which leads to the release of cytochrome c from the intermembrane space of the mitochondria into the cytosol (Kluck et al. 1997). Anti-apoptotic proteins, such as the Bcl-2 proteins, antagonize the actions of proapoptotic proteins (Kim et al. 2006). Apoptosis is controlled by many factors, of which mitochondrial injury appears to be the most important. Calcium overload can damage mitochondria, activating apoptosis promoters and the caspase cascade (Jin et al. 2005).

To further detect the signaling pathways of CsA-induced cardiomyocyte apoptosis with respect to CaSR in rats, we assayed the expression levels of cytochrome c, cleaved caspase-9, cleaved caspase-3, Bax, and Bcl-2 by Western blot and immunohistochemistry. The expression levels of cytochrome c, cleaved caspase-9, cleaved caspase-3, and Bax proteins were significantly increased in the CsA group relative to the control group. The addition of $GdCl_3$ further increased the expression levels of cytochrome c, cleaved caspase-9, cleaved caspase-3, and Bax. Conversely, the expression levels of Bcl-2 proteins were significantly decreased in the CsA and CsA + $GdCl_3$ groups compared to the control and $GdCl_3$ groups. Studies have shown that intracellular calcium can accumulate in the mitochondria (Schanne et al. 1979; Ott et al. 2002), inducing a change in the mitochondrial permeability transition pore and promoting the release of pro-apoptotic proteins, such as cytochrome c and apoptosis-inducing factor. Our data strongly suggest that CaSR is involved in CsA-induced apoptosis in rats. We explain this process in the following way.

On one hand, CsA induces an increase in the expression level of CaSR, which leads to a CaSR-mediated increase in the level of intracellular calcium (Tang et al. 2011), an induction of MOMP, destruction of the mitochondria, and downstream activation of the mitochondrial apoptotic pathways. One the other hand, increased intracellular calcium can be taken up by the mitochondria, in which case CsA, which is a potent inhibitor of the mitochondrial permeability transition pore, can inhibit calcium release from the mitochondria, cause mitochondrial calcium overload and induce mitochondrial injury. Finally, cytochrome c released from the mitochondria can activate caspase-9 and then caspase-3, resulting in apoptosis. Millane et al. (1994) observed mitochondrial calcium deposition in the myocardial biopsies of patients taking CsA, supporting the theory of mitochondrial calcium overload. Bax and Bcl-2 proteins may also be involved in this apoptotic process.

The present *in vivo* study provides further information on CsA-induced cardiomyocyte apoptosis. For the first time, we identified that CaSR is involved in CsA-induced cardiomyocyte apoptosis in rats through the activation of downstream cytochrome c and caspase-3 pathways. Furthermore, the Bcl-2 family is also involved in this process. Our study could provide novel strategies for the prevention and cure of CsA-induced cardiotoxicity.

4. Experimental

4.1. Materials

Cyclosporin A was purchased from Novartis Pharma AG (Basel, Switzerland). $GdCl_3$ was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The terminal deoxynucleotidyl transferase (TdT)-mediated-UTP nick end labeling (TUNEL) kit was obtained from Roche (Mannheim, Germany). Anti-CaSR antibody was obtained from Alpha Diagnostic International Inc. (San Antonio, TX, USA), and anti-cleaved caspase-9 and anti-cleaved caspase-3 antibodies were purchased from Cell Signaling Technologies. Anti-cytochrome c, anti-Bcl-2, and anti-Bax antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

4.2. Animal model

Forty male Wistar rats (220 ± 10 g) (Animal Research Institute of Harbin Medical University, China) were used for this study. All animals were handled in accordance with the Guide for the Care and Use of Laboratory

Animals, published by the National Institutes of Health. Rats were randomly divided into four groups. CsA was used at a dose of 15 mg/kg/day and dissolved in olive oil. $GdCl_3$ was used at a dose of 10 mg/kg, every other day, and dissolved in saline. The groups were as follows: the control group was injected intraperitoneally (i.p.) with olive oil; the CsA group was injected i.p. daily with CsA; the $GdCl_3$ group was injected i.p. with $GdCl_3$ every other day; and the CsA + $GdCl_3$ group was injected daily with CsA and every other day with $GdCl_3$. Two weeks later, the rats were anesthetized, and the hearts were removed.

4.3. Hematoxylin & eosin (H&E) staining

Rat ventricular tissues were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin, and the embedded hearts were cut into 5-μm thick slices, dewaxed and stained with H&E. The morphology of the myocardial tissue was observed by light microscopy.

4.4. Transmission electron microscopy

Ventricular tissues were first fixed with 2.5% glutaraldehyde and then postfixed with 1% osmium tetroxide. After dehydration, the tissues were embedded in epoxy resin, stained with uranyl ethanoate and lead citrate, and visualized using a transmission electron microscope.

4.5. TUNEL assay

Apoptotic cells in paraffin sections were identified using an in situ cell death detection kit (Roche, Mannheim, Germany). Cardiac ventricular tissues were fixed in 4% paraformaldehyde for 24 h at room temperature, dehydrated and embedded in paraffin. The sections were incubated with protease K for 15 min at room temperature, immersed in 3% H_2O_2 for 10 min, rinsed, transferred into 50 μL TUNEL reaction mixture for 60 min at 37 °C, and incubated with 50 μL POD solution for 30 min at 37 °C. DAB solution was added to the slides for 10–30 min, then the slides were counterstained with hematoxylin, dehydrated and mounted. Labeled myocytes were analyzed using light microscopy.

4.6. RNA extraction and RT-PCR

Total RNAs of rat ventricular myocardium tissues were extracted according to the Trizol reagent (Invitrogen) protocol and quantified by spectrophotometry at 260 nm. RT reactions were performed using the RT kit protocol (Promega). The PCR reactions involved an initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 20 s, exposure to an appropriate annealing temperature (50 °C) for 40 s, and then incubation at 72 °C for 40 s. A final cycle of 72 °C for 2 min was included after the completion of 35 cycles. The nucleotide sequences of the primers (TaKaRa Co. Ltd.) used were as follows: (1) CaSR (forward), 5'-ttcggcatcagctttgtg-3'; CaSR (reverse), 5'-tgaagatgatttcgtcttcc-3'. (2) GAPDH (forward), 5'-ctcaactacatggtctacatg-3'; GAPDH (reverse), 5'-tggcatggactgtggtcatgag-3'. Amplified RT-PCR products were analyzed on 1.2% agarose gels and visualized with ethidium bromide. The optical density (OD) for each band was measured using a Bio-Rad Chemi Doc™ EQ densitometer and Bio-Rad Quantity One software (Bio-Rad laboratories, Hercules, USA). GAPDH was used as an internal control for the semi-quantitative assay.

4.7. Western blot analysis of CaSR, cytochrome c and Bax

Frozen ventricular tissue was homogenized in cold lysis buffer with PMSF (Solarbio, Beijing), and the homogenate was centrifuged at 15,000 g for 40 min at 4 °C. The protein concentrations were determined using a BCA protein assay kit (Beyotime, Jiangsu). Equal amounts of protein were loaded onto 10% or 15% gels and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). All samples were transferred onto nitrocellulose (CaSR) or PVDF membranes and blocked with 5% nonfat milk in TBST for 1 h at 37 °C. The membranes were incubated overnight at 4 °C with the primary antibodies anti-CaSR (1:500, rabbit polyclonal), anti-actin (1:500, rabbit polyclonal), anti-cytochrome c (1:200, mouse monoclonal) or anti-Bax (1:200, rabbit polyclonal) diluted in blocking buffer. Secondary antibodies conjugated to alkaline phosphatase (CaSR) or conjugated to horseradish peroxidase for 1 h at 37 °C. The reaction was visualized by Western Blue Stabilized Substrate (CaSR) or DAB solution. Protein–antibody complexes conjugated to secondary antibodies were scanned on a GIS Imaging System and analyzed by a Bio-Rad Chemi Doc ™ EQ densitometer and Bio-Rad Quantity One software. Actin was used as an internal control for the semi-quantitative assay.

4.8. Immunohistochemical analysis of CaSR, cytochrome c, cleaved caspase-9, cleaved caspase-3, Bax, and Bcl-2

All of the sections were dewaxed, rehydrated, and immersed in 3% H_2O_2 for 15 min. They were then incubated overnight at 4 °C with the primary antibodies anti-CaSR (1:100, rabbit polyclonal), anti-cytochrome c (1:200, mouse monoclonal), anti-Bax (1:200, rabbit polyclonal), anti-Bcl-2 (1:100, rabbit polyclonal), cleaved caspase-3 (1:50, rabbit monoclonal) or cleaved caspase-9 (1:100, rabbit polyclonal). The sections were then treated with biotinylated goat anti-rabbit or goat anti-mouse horseradish peroxidase (HRP). The sections were stained by immersing the slides in DAB solution. They were then counterstained with hematoxylin, dehydrated and mounted. Control reactions were performed in the absence of primary antibodies. The immunohistochemical data were expressed as: negative (−), very weak (+/−), weak (+), moderate (++), and strong (+++) positivity. All samples were analyzed and semiquantitatively scored blindly.

4.9. Statistical analysis

All values are expressed as mean ± SEM. Multiple comparisons among groups were tested using one-way ANOVA. Differences between two groups were detected using unpaired two-tailed Student's *t* tests. Values of $P < 0.05$ were considered statistically significant.

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