Cyclosporin A Protects H9c2 Cells Against Chemical Hypoxia-Induced Injury via Inhibition of MAPK Signaling Pathway

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SUMMARY

This study aimed to investigate the effects and molecular mechanism of cyclosporin A (CsA) on cobalt chloride (CoCl2)-induced injury in H9c2 embryonic rat cardiac cells. The results showed that CsA could protect H9c2 cells against CoCl2-induced hypoxic injury. CsA effectively improved cell viability, and decreased LDH leakage, cell apoptosis, MDA concentration, and ROS generation, and increased SOD activity, GSH production, and CAT activity in a dose-dependent manner. In addition, CsA treatment blocked the CoCl2-induced increases in ROS production and mitochondrial dysfunction, including a decrease in membrane potential, cytochrome c (cyto-c) release, Bax/Bcl-2 imbalance, as well as the ratios of cl-casp-9/casp-9 and cl-casp-3/casp-3 ratios, via the inhibition of p38 and ERK MAPK signaling pathways. The results also suggested that CsA protected H9c2 cells against CoCl2-induced hypoxic injury, possibly by suppressing the MAPK signaling pathway. Thus, CsA is a potential therapeutic agent for cardiac hypoxic injury. (Int Heart J 2016; 57: 000-000)

Key words: CoCl2

Cyclosporin A (CsA) is a powerful immunosuppressive drug. Recent evidence indicates that CsA may serve as an important biological cytoprotective agent. CsA has been reported to be a multifactorial neuroprotective agent that putatively exerts neuroprotective and neurotrophic effects on traumatic brain injury and sciatic nerve injuries, as well as focal and global ischemia.1 However, the cardioprotective effects of CsA are still unknown.

Such cytoprotection is associated with the suppression of myocardial oxidative stress and preservation of mitochondrial structure and function.2 The oxidative stress injury caused by cardiac ischemia/reperfusion injury has been attributed to the activation of mitogen activated protein kinase (MAPK) pathways, including the p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) pathways. ERK promotes cell survival, whereas JNK and p38 lead to cell death. MAPK plays a pivotal role in cardiovascular diseases and is involved in cell survival and the recovery of damaged myocardial apoptosis.3

Cobalt chloride (CoCl2) is a chemical hypoxia mimetic agent. CoCl2 can mimic hypoxic/ischemic conditions, including ROS generation, in various cultured cells.4,5 H9c2 embryonic rat cardiac cells, a subclone of an original clonal cell line, are derived from rat embryonic hearts and have been used as in vitro models for exploring the mechanisms underlying hypoxia-induced apoptosis of cardiomyocytes.6 Therefore, in the present study, we used CoCl2-treated H9c2 cells as a model with which to investigate whether CsA protects H9c2 cells from chemical hypoxia-induced injury and reduces oxidative stress and mitochondrial damage by the inhibition of MAPK signaling pathways.

METHODS

Materials: CsA and CoCl2 were purchased from Sigma. Bax, Bcl-2, cleaved caspase-9, caspase-9, cleaved caspase-3, caspase-3, cyto-c, p-p38, p38, p-ERK1/2, ERK1/2, p-JNK1/2, JNK1/2, and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture and treatment: H9c2 embryonic rat cardiac cells were purchased from the Chinese Academy of Sciences (Shanghai, China). Briefly, the H9c2 cells were cultured in DMEM-F12 medium supplemented with 20% FBS at 37°C under an atmosphere of 5% CO2 and 95% air.

Chemical hypoxia was achieved by adding CoCl2 at the concentrations indicated into the medium and incubating cells in the presence of CoCl2 for 24 hours. In order to explore the...
effects of CsA on CoCl2-induced cell injury, after CoCl2-induced cell injury, H9c2 cells were maintained in complete medium with 10 ng/mL, 50 ng/mL, and 50 ng/mL CsA or MAPK inhibitors, 15 μM SB203580 (Abcam, Cambridge, UK), 15 μM U0126 (Abcam), and 15 μM SP600125 (Abcam). The control cells were incubated without CsA and CoCl2.

Cell viability assay: A Cell Counter Kit-8 (CCK-8) assay was used to investigate the cell viability of H9c2 cells cultured in 96-well plates at a density of 5000 cells/well. When the cells had grown to 70-80% confluence, CoCl2-induced cell injury was performed. Therefore, 10 μL CCK-8 solution was added to each well and the cells were incubated for a further 4 hours at 37°C. Absorbance was measured at 450 nm with a microplate reader. The mean optical density (OD) of 4 wells in each group was used to calculate cell viability as follows:

Cell viability (%) = (OD_treatment group/OD_control group) × 100

Experiments were performed in triplicate.

Measurement of intracellular ROS: The DCF-DA method was used to detect the level of intracellular ROS. H9c2 cells were seeded on culture slides (5 × 10^4 cells/well) for 24 hours and then the CoCl2-induced cell injury protocol was performed. CsA (10, 30, and 50 ng/mL) was added to the plates. The cells were incubated for an additional 24 hours at 37°C. After the addition of 25 μM of DCF-DA solution for 10 minutes, the fluorescence of DCF was detected using a fluorescence-activated cell sorting (FACS) caliber.

LDH, MDA, SOD, GSH, and CAT assays: LDH, MDA, SOD, GSH, and CAT levels were measured using commercial kits, according to the manufacturer’s protocol.

Hoechst 33342/PI assay: Cell apoptosis was analyzed by staining with Hoechst 33342/PI staining. H9c2 cells were collected, washed in PBS, resuspended in 0.8-1 mL of cell staining buffer containing 5 μL of Hoechst 33342 and 5 μL of PI incubated for 20-30 minutes at 4°C in the dark, washed in PBS, and then smeared on slides. The red fluorescence and the blue fluorescence were observed under a fluorescence microscope.

Measurement of mitochondrial membrane potential: A JC-1 fluorescent, lipophilic, and cationic probe was used to measure the mitochondrial membrane potential (Δψm) of H9c2 cells according to the manufacturer’s protocol. H9c2 cells were plated in 96-well plates at a density of 5000 cells/well. When the cells had grown to 70-80% confluence, CoCl2 (700 μM) was added. The cells were then incubated with JC-1 staining solution for 20 minutes at 37°C. The fluorescence was detected with a FACS caliber. The wavelengths of excitation and emission were 490 and 535 nm for detection of the monomeric state, and 585 and 633 nm for detection of the aggregated state. These results suggest that CsA protects H9c2 cells against CoCl2-induced cytotoxicity.

Effects of CsA on CoCl2-induced ROS generation: The involvement of ROS in the CoCl2-induced apoptosis of H9c2 cells was evaluated by measuring the level of ROS production. After H9c2 cells had been treated with 700 μM CoCl2 for 24 hours, intracellular ROS levels increased compared with the control level. However, treatment with CsA decreased intracellular ROS levels, further demonstrating the antioxidant effects of CsA (Figures 2A and 2A’).

Effects of CsA on CoCl2-induced changes in MDA concentration, SOD activity, GSH production, and CAT activity in H9c2 cells: Treatment of H9c2 cells with CoCl2 significantly increased the MDA concentration and reduced SOD activity, GSH production, and CAT activity. The oxidative abnormalities were clearly ameliorated by CsA treatment, as manifested by the significant reduction in MDA concentration and the increases in SOD activity, GSH production, and CAT activity (Figures 2B, 2C, 2D, and 2E).

Effects of CsA on CoCl2-induced mitochondrial abnormalities: The Δψm disruption is believed to be a common early event of apoptosis prompted by a variety of stimuli, including hypoxia. As shown in Figure 3A, exposure of cells to CoCl2 for 24 hours induced a significant loss of Δψm in H9c2 cells. Interestingly, treatment with CsA markedly attenuated the loss of Δψm. These results suggest that CsA protects H9c2 cells against the loss of Δψm by inhibiting ROS overproduction. CoCl2 treatment resulted in a significantly in-

**Results**

Effects of CsA on CoCl2-induced cytotoxicity: As shown in Figure 1A, the treatment of H9c2 cells with CoCl2 (400-900 μM) for 24 hours reduced cell viability in a concentration-dependent manner. The choice of 700 μM for the concentration of CoCl2 applied to all the experiments was based on the CCK-8 assay of survival in the H9c2 cells. At this concentration, nearly 50% of the CoCl2-treated cells died within 24 hours. Other H9c2 cells were treated with various concentrations of CsA alone for 24 hours. Cell viability was not significantly changed when cells were treated with CsA at concentrations ranging from 10 ng/mL to 50 ng/mL, whereas CsA at concentrations of 60-70 μg/mL induced cell viability reduction (Figure 1B). H9c2 cells treated with CsA (10, 30, and 50 μg/mL) for 24 hours had significantly inhibited CoCl2-induced cytotoxicity and enhanced cell viability in a dose-dependent manner (Figure 1C). Cell apoptosis percentage in H9c2 cells treated with CoCl2 was determined using the Hoechst 33342/PI method. Exposure of cells to 700 μM CoCl2 for 24 hours increased the percentage of apoptotic H9c2 cells. The CoCl2-induced increase in the percentage of apoptotic H9c2 cells was reversed by treatment with CsA, revealing the cytoprotective effect of CsA against chemical hypoxia-induced apoptosis (Figures 1D and 1D’). The results also suggested that CsA effectively reduced LDH leakage induced by CoCl2 (Figure 1E). These results suggest that CsA could protect H9c2 cells against CoCl2-induced cytotoxicity.

Statistical analyses: All data are presented as the mean ± SEM. For multiple comparisons, the statistical analysis was performed using one-way ANOVA followed by Tukey’s multiple comparison test. A P < 0.05 was considered significant.
creased cytosolic cyto-c protein expression. Treatment with CsA reduced the level of cytosolic cyto-c in a dose-dependent manner. Both the p38 inhibitor SB203580 and the ERK inhibitor U0126 further reduced the level of cytosolic cyto-c. The JNK inhibitor SP600125 did not have a significant effect (Figure 3B).

Effects of CsA on CoCl$_2$-induced upregulation of Bax/Bcl-2, cl-casp-9/casp-9, and cl-casp-3/casp-3 ratios: The results of the Western blot analysis showed that treatment with 700 μM CoCl$_2$ for 24 hours markedly up-regulated the Bax/Bcl-2, cl-casp-9/casp-9, and cl-casp-3/casp-3 ratios in H9c2 cells. Importantly, treatment with CsA significantly inhibited the increases in the Bax/Bcl-2, cl-casp-9/casp-9, and cl-casp-3/casp-3 ratios. SB203580 and U0126 further reduced the ratios of Bax/Bcl-2, cl-casp-9/casp-9, and cl-casp-3/casp-3. SP600125 did not attenuate these effects (Figure 3C).

CsA protects H9c2 cells against CoCl$_2$-induced hypoxic injury by suppressing MAPK signaling pathways: The phosphorylation of JNK1/2 after CoCl$_2$-induced injury (Figure 4A). These results suggested that CsA protects H9c2 cells against CoCl$_2$-induced injury by suppressing MAPK signaling pathways.

**Discussion**

Recently, CsA has been reported to be capable of modulating many pathological processes in the body. CsA is one of the highly effective drugs in preventing brain ischemic damage.$^{7}$ CsA is also believed to be an important pathological mediator capable of regulating myocardial ischemic damage. This study investigated whether CsA can protect H9c2 cells from hypoxic/ischemic injury in vitro. The results showed that CsA inhibited CoCl$_2$-induced apoptosis in H9c2 cells by regulating ROS-initiated MAPK signaling pathways.

CoCl$_2$-treated H9c2 cells may serve as a simple and useful in vitro model for exploring the mechanisms underlying the hypoxia-linked damage in cardiac cells because H9c2 cells can exhibit electrophysiological and biochemical properties of cardiac tissues.$^{8}$ The present study evaluated the potential mecha-
nisms of CsA-mediated cardioprotection with the use of an *in vitro* chemical hypoxia-induced injury model. This study also demonstrated that CoCl$_2$, a chemical hypoxia-mimetic agent, significantly suppressed cell viability and induced apoptosis in H9c2 cells.

According to the results of this study, CsA reduced oxygen demand, thereby protecting H9c2 cells from CoCl$_2$-induced injury. CsA also protected H9c2 cells against CoCl$_2$-induced injury, enhancing cell viability and reducing the percentage of apoptotic cells. The increased LDH activity in a culture medium in generally known to correspond to the degree of cell death. We assessed CoCl$_2$-induced H9c2 cell death and cardiotoxicity through LDH assays and confirmed that CoCl$_2$ significantly increased LDH release. However, treatment with different concentrations of CsA markedly attenuated the increase in LDH release. Hypoxia/ischemia induced oxidative stress injuries by scavenging ROS and lipid peroxidation production of MDA. SOD is one of the principal enzymes that scavenge free radicals. GSH, which is a major cellular antioxidant, is not only the immediate donor of electrons to neutralize H$_2$O$_2$ and lipoperoxide, but also a scavenger of oxygen and nitrogen-based free radicals. CAT is a defense molecule against toxic oxygen metabolites. CoCl$_2$ significantly enhanced MDA concentration and inhibited SOD activity, GSH production, and CAT activity. By contrast, CsA prevented CoCl$_2$-induced cell injuries, decreased MDA levels, and increased SOD, GSH, and CAT levels. The present study provides novel evidence to demonstrate that CoCl$_2$, a chemical hypoxia mimetic agent, damages H9c2 cells by increasing ROS production and MDA concentration and suppressing SOD activity, GSH production, and CAT activity. Our previous studies showed that CsA protected H9c2 cells from oxidative damage by reversing these effects.

The results of a recent study indicated that CoCl$_2$-induced apoptosis of H9c2 cells might be associated with the mitochondrial apoptotic pathway. The mitochondria-dependent apoptotic pathway may be activated under hypoxic/ischemic conditions by increasing ROS production, disrupting mitochondrial membrane potential, releasing cyto-c from the damaged mitochondria to the cytosol, and activating caspase-9 and caspase-3, which subsequently contribute to apoptotic cell death. Caspase-9 is an initiator of apoptosis and caspase-3 is an effector of apoptosis, both of which regulate cell apoptosis. Pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 on the outer mitochondrial membrane could regulate membrane permeability and mitochondrial integrity, thereby affecting the release of cyto-c from the mitochondrion and catalyzing mitochondria-dependent caspase-9 and caspase-3 activation. To explore whether CsA prevented the CoCl$_2$-induced apoptosis of H9c2 cells by inhibiting the factors involved in the mitochondrial apoptotic pathway, we evaluated the effect of CoCl$_2$ on ROS production. Intracellular ROS levels increased in CoCl$_2$-treated H9c2 cells, and this increase was suppressed by CsA. These results indicate that ROS production...
may be involved in CoCl₂-induced apoptosis of H9c2 cells and CsA is capable of scavenging ROS, which could be one of the mechanisms underlying the protective effects of CsA against CoCl₂-induced injury in H9c2 cells. These results suggest that CoCl₂ induces the apoptosis of H9c2 cells via the mitochondrial apoptotic pathway. The results also suggest that CsA treatment reversed the Bax/Bcl-2, cl-casp-9/casp-9, and cl-casp-3/casp-3 ratios promoted by CoCl₂ exposure. This finding correlates well with the blockage of cyto-c release and inhibition of Δψm by CsA treatment. We demonstrated that CsA protects H9c2 cells against CoCl₂-induced injury by suppressing oxidative stress and preserving mitochondrial function. In this study, CsA was involved in myocardial injury initiated by CoCl₂-induced injury. Furthermore, CsA significantly decreased the ratios of Bax/Bcl-2, cl-casp-9/casp-9, and cl-casp-3/casp-3, as well as blocked cyto-c release from the mitochondrion; such a release may be related to MAPK signaling pathways.

MAPK signaling pathways are considered mediators of cardiac ischemia/reperfusion injuries. Oxidative stress can result in the activation of MAPK cascade in cardiac ischemia/reperfusion injury. The activation of p38 and ERK during cardiac ischemia/reperfusion injury is associated with mitochondrial dysfunction. The mitochondria are important and central mediators of death. Bax and Bcl-2 in the outer mitochondrial membrane could result in cell apoptosis and the activation of caspase-9 and caspase-3. In our study, CsA inhibited p38 and ERK MAPK activation but did not affect the increased JNK expression. These results suggest that the p38 and ERK MAPK pathways could be involved in the reduced H9c2 CoCl₂-induced injury effects of CsA. CsA protects H9c2 cell death by inhibiting p38 and ERK MAPK pathways.

ROS can activate MAPK signaling pathways, including p38, ERK, and JNK, which have pivotal roles in cardiovascular diseases. The activation of MAPK signaling pathways is linked to ROS generation and the progression of cell apoptosis. Our study showed that CoCl₂-induced hypoxic injury significantly enhanced the expression levels of p-p38, p-ERK, and p-JNK MAPK, indicating the activation of MAPK by ROS. CsA protects H9c2 cells by suppressing the hypoxic injury-induced activities of p38 and ERK phosphorylation but does not affect the increased JNK phosphorylation expression. Indeed, in this study, CsA inhibited p38 and ERK MAPK but did not affect the increased JNK expression. H9c2 cells were pretreated with 15 μM SB203580 (a selective inhibitor of p38 MAPK), 15 μM U0126 (a selective inhibitor of ERK MAPK), and 15 μM SP600125 (a selective inhibitor of JNK MAPK) before exposure to CoCl₂-induced hypoxic injury. The activation of p38 and ERK MAPK significantly attenuated the myocardial protective effects of CsA on hypoxic injury-induced death in H9c2 cells because the factors (p38 and ERK) inhibited by CsA were inhibited in the presence of SB203580 or U0126 pharmacological inhibitors. However, SP600125 did not affect the growth of H9c2 cells. CsA can have a myocardial protection effect on CoCl₂-induced hypoxic injury through inhibition of the ROS/MAPK pathway.

The endogenous defense system, primarily the antioxidant enzyme system SOD, is critical to attenuate the injury induced by hypoxia. CsA enhanced the level of the antioxidants...
activity of SOD with respect to the CoCl2-induced hypoxic injury group. Thus, we suppose that treatment with CsA can increase SOD activities to reduce hypoxic injury. Extensive research has shown that ROS generation may result in apoptosis during hypoxic injury and reduced ROS generation can suppress hypoxic injury via antioxidants and antioxidative enzymes, such as SOD. SOD is a powerful antioxidant that catalyzes redox reactions by converting superoxide radicals into hydrogen peroxide and oxygen. We provided clear evidence that CsA treatment can protect from apoptosis by inhibiting the p38 and ERK MAPK pathways. Furthermore, we found a positive interaction between ROS, p38, and ERK MAPK activation and SOD activity reduction: CsA treatment decreased ROS generation and suppressed the p38 and ERK MAPK pathways and increased SOD activities, indicating that CsA has partial antioxidative activity by modulating the ROS-induced p38 and ERK MAPK pathways.

Two major limitations of the present study need to be acknowledged and addressed. First, we attempted to demonstrate the cardioprotection effects of CsA by performing the study on a cell line but not on a system that is more relevant to an in vivo situation. Second, in this study, we used the H9c2 cell line (rat cardiomyocyte cell line) and not real myocardial cells, although H9c2 cells do display some myocardial functions. More experiments must be conducted using an animal model or real myocardial cells, particularly primary myocardial cells, in future studies to verify the cardioprotection effect of CsA.

In conclusion, our results indicate that CsA may protect H9c2 cells against chemical hypoxia-induced injury by suppressing the p38 and ERK MAPK signaling pathways (summarized in Figure 4B). Thus, CsA is potentially a novel therapeutic target for the treatment of cardiac ischemia/reperfusion injury.

**Disclosures**

**Conflict of interest:** There is no conflict of interest to report for any author.

**Ethical standards:** The study complies with current ethical standards.

**REFERENCES**


