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Che-1 attenuates hypoxia/reoxygenation-induced cardiomyocyte apoptosis by upregulation of Nrf2 signaling

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Abstract. – OBJECTIVE: Hypoxia/reoxygenation (H/R)-induced cardiomyocyte apoptosis plays a critical role in the development of myocardial infarction. Che-1 has been reported as an anti-apoptotic gene in response to various cellular stresses. However, whether Che-1 regulates cardiomyocyte apoptosis in myocardial infarction remains unclear. In this study, we aimed to investigate the role of Che-1 in regulating H/R-induced cardiomyocyte apoptosis and the underlying molecular mechanism.

MATERIALS AND METHODS: The expression of mRNA and protein was detected by Real-time quantitative polymerase chain reaction and Western blot. Cell viability was detected by cell counting kit-8 assay. Cell cytotoxicity was measured by lactate dehydrogenase assay. Cell apoptosis was assessed by caspase-3 activity assay. Intracellular ROS generation was determined using a Reactive Oxygen Species Assay Kit. The activity of antioxidant response elements was detected by luciferase reporter assay.

RESULTS: We found that Che-1 expression was significantly upregulated in cardiomyocytes in response to H/R treatment. Functional experiments showed that silencing of Che-1 promoted H/R-induced cell apoptosis and oxidative stress. By contrast, overexpression of Che-1 significantly alleviated H/R-induced cell apoptosis and oxidative stress. Interestingly, we found that Che-1 promoted the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and upregulated the activity of antioxidant response elements. Moreover, Che-1 significantly upregulated the expression of Nrf2 downstream target genes, including heme oxygenase-1 and NADPH-quinone oxidoreductase 1.

CONCLUSIONS: Our results showed that Che-1 alleviates H/R-induced cardiomyocyte apoptosis by upregulation of Nrf2 signaling. Our study suggests that Che-1 may serve as a potential and promising therapeutic target for the treatment of myocardial infarction.

Key Words:
Che-1, Hypoxia/reoxygenation, Myocardial infarction, Nrf2.

Introduction

Myocardial ischemia/reperfusion injury is one of the leading causes of death and disability after cardiac surgery and myocardial infarctions[1]. Cell apoptosis induced by ischemia/reperfusion is one of the major mechanisms of myocardial injury[2]. The sudden loss of oxygen and nutrient supply to the cardiomyocytes induces the overproduction of reactive oxygen species (ROS) and activates the apoptotic pathway[3]. The cardiomyocytes are terminally differentiated cells that lack a regeneration ability[4]. Therefore, preventing the loss of cardiomyocytes is essential for maintaining cardiac function during myocardial ischemia/reperfusion injury.

Che-1 (also known as apoptosis antagonizing transcription factor, AATF) is a human RNA polymerase II binding protein, which regulates the transcription of various genes[5-7]. The Che-1 gene is located on chromosome 17, which is very rich in segmental duplications and protein-coding genes and is home to genes implicated in various human genetic diseases[8]. Che-1 is highly conserved and ubiquitously expressed in eukaryotes playing important roles in regulating cell cycle, cell proliferation, and cell apoptosis[9,10]. Moreover, Che-1 is suggested as a strong inhibitor of apoptosis and is involved in regulating neurotoxicity, tumorigenesis and chemoresistance[9,11-13]. Previous reports have also shown that Che-1 is responsive to various cellular stresses, such as hypoxia[14,15] and endoplasmic reticulum stress[16]. However, whether Che-1 regulates cardiomyocytes during myocardial ischemia/reperfusion injury remains unclear.

Nuclear factor-E2-related factor 2 (Nrf2) is a highly conserved transcription factor that regulates the antioxidant process[17-19]. Under oxidative stress, Nrf2 transfers into the nucleus and binds

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to the antioxidant responsive elements (ARE), which activates antioxidant enzymes, such as heme oxygenase-1 (HO-1) or NADPH-quinone oxidoreductase 1 (NQO1)[20]. The activation of Nrf2/ARE prevents oxidative cardiac cell injury *in vitro* and *in vivo*[21,22]. Therefore, Nrf2 has been proposed as a promising target for the treatment of myocardial ischemia/reperfusion injury.

In this study, we aimed to investigate the potential role of Che-1 in regulating cardiomyocyte apoptosis during myocardial ischemia/reperfusion injury using an *in vitro* cellular model induced by hypoxia/reoxygenation (H/R). We found that Che-1 expression was significantly upregulated in cardiomyocytes in response to H/R treatment. Functional experiments showed that silencing of Che-1 promoted H/R-induced cell apoptosis and oxidative stress, while overexpression of Che-1 showed protective effects. Interestingly, we found that Che-1 promoted the expression of Nrf2 and upregulated the activity of ARE. Moreover, Che-1 significantly upregulated the expression of Nrf2 downstream target genes, including HO-1 and NQO1. Taken together, our results showed that Che-1 alleviates H/R-induced cardiomyocyte apoptosis by upregulation of Nrf2 signaling, providing a novel and promising therapeutic target for the treatment of myocardial ischemia/reperfusion injury.

Materials and Methods

Cell Culture

The primary neonatal cardiomyocytes were isolated from 1-day-old neonatal C57BL/6 mouse using enzymatic disassociation method as previously described[23]. In brief, the mice were euthanized, and the ventricular myocardial tissues were dissected under aseptic conditions. After washing with phosphate-buffered saline (PBS), the tissues were cut into small pieces and digested using 1.2 mg/ml pancreatin and 0.625 mg/ml type II collagenase in a 37°C water bath for 40 min. The cardiomyocytes were then re-suspended in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The isolated cells were then filtered with a 70 μm cell strainer (Millipore, Billerica, MA, USA). After centrifugation at 500 × g for 5 min, the cells were re-suspended in DMEM/F12 and were pre-plated for 90 min to remove non-cardiomyocytes and enrich the culture with cardiomyocytes at 37°C for 1 h. Finally, the non-adherent cardiomyocytes were collected and plated on 1% gelatin-coated well plates and cultured at 37°C in a humidified incubator containing 5% CO_2/95% air. To establish the H/R model, cardiomyocytes were cultured in a tri-gas incubator containing 94% N_2/5% O_2/1% CO_2 for 6 h followed by reoxygenation for 24 h in an atmosphere containing 5% CO_2/95% air at 37°C. Cells cultured under normal conditions served as control. This study was reviewed and approved by the Institutional Animal Care and Use Committee of Ankang City Central Hospital. The experiments were performed in accordance with the National Institute of Health Guide (National Institute of Health Publications No. 80-23, Revised 1978) for the care and use of Laboratory Animals for experimental procedure.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted for cultured cardiomyocytes using TRIzol (Invitrogen, Carlsbad, CA, USA) and RNA was then reverse transcribed into cDNA using M-MLV Reverse Transcriptase (TaKaRa, Dalian, China) according to the manufacturer's instructions. The PCR reactions were performed with SYBR Premix Ex Taq II assay (TaKaRa) on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) following the thermal cycle conditions: 30 s at 95°C, followed by 40 cycles of 95°C for 40 s, 60°C for 35 s and 72°C for 40 s. β-actin was used as internal control for normalization of mRNA expression. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

Equal amounts of proteins were separated using a 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Non-specific binding sites on the membranes were blocked by 5% fat-free milk at 37°C for 1 h. The membranes were incubated with primary antibodies including anti-Che-1, anti-Nrf2 and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. The membranes were then probed with secondary antibodies for 1 h at room temperature. The protein bands were visualized by enhanced chemiluminescence using a Western blot detec-

tion kit (Millipore, Billerica, MA, USA). Band intensity was measured by Image-Pro Plus 6.0 software for relative protein quantitation.

Cell Transfection

The siRNAs for targeting Che-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and transfected into cells according to the manufacturer's instructions. The Che-1 expression vector was generated by inserting the Che-1 open reading frame into a pcDNA3.1 vector and it was transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Cell Counting Kit-8 (CCK-8) Assay

Cell viability was evaluated using a CCK-8 assay. Briefly, cells were seeded into 96-well plates at 3×10^5 cells/well and cultured overnight. After the indicated treatments, 10 μL of CCK-8 solution were added to each well and incubated at 37°C for 2 h. The optical density (OD) value at 450 nm was measured using a microplate spectrophotometer (Bio-Tek, Winooski, VT, USA).

Lactate Dehydrogenase (LDH) Assay

Cell cytotoxicity was measured using an LDH Cytotoxicity Assay Kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's protocol. In brief, cells were lysed after the indicated treatments. Lysates were treated with NADH and pyruvate and incubated at 37°C for 15 min. The OD value at 530 nm was measured by a microplate spectrophotometer (Bio-Tek, Winooski, VT, USA).

Cell Apoptosis Assay

Cell apoptosis was detected using a Caspase-3 Activity Assay Kit (Beyotime Biotechnology, Haimen, China) according to the manufacturer's instructions. In brief, cells were lysed and then treated with 2 mM Ac-DEVD-pNA at 37°C for 1-4 h. The OD value at 405 nm was assessed using a microplate spectrophotometer (Bio-Tek, Winooski, VT, USA).

Measurement of Intracellular ROS Generation

Intracellular ROS generation was determined using a Reactive Oxygen Species Assay Kit (Beyotime Biotechnology) according to the manufacturer's instructions. In brief, cells were incubated with 2',7'-dichlorofluorescin diacetate (DCFH-DA) at 37°C for 30 min. The fluorescence intensity reflecting ROS levels was measured using a fluorescence spectrophotometer (Bio-Tek, Winooski, VT, USA).

Luciferase Reporter Assay

For detection of ARE activity, cells were transfected with Che-1 siRNA or a Che-1 expression vector in the presence of an ARE reporter vector and a phRL-TK Renilla luciferase vector (Promega, Madison, WI, USA). Luciferase activity was determined using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Statistical analyses were carried out using SPSS 18.0 statistics software (SPSS Inc., Chicago, IL, USA). For experiments with two groups, data were analyzed using a Student's *t*-test. For experiments with multiple groups ($\geq$ 3), data were analyzed using one-way analysis of variance followed by post hoc least significant difference tests. Differences with $p < 0.05$ were accepted as statistically significant.

Results

Expression of Che-1 in Response to H/R Treatment in Cultured Cardiomyocytes

To investigate the potential role of Che-1 in regulating cardiomyocyte apoptosis, we first examined the expression of Che-1 in cardiomyocytes in response to H/R treatment by RT-qPCR and Western blot analysis. The results showed that both the mRNA (Figure 1A) and protein (Figure 1B) expression of Che-1 was significantly increased in response to H/R treatment. The data indicate that Che-1 is a hypoxia-responsive gene, which may play an important role in regulating H/R-induced cardiomyocyte apoptosis.

Knockdown of Che-1 Aggravates H/R-Induced Cell Injury in Cultured Cardiomyocytes

To investigate the precise biological function of Che-1 in regulating H/R-treated cardiomyocytes, we performed loss-of-function experiments by using siRNA targeting Che-1. We found that transfection of Che-1 siRNA significantly decreased the expression of Che-1 in cardiomyocytes (Figure 2A and B). We then detected the effect of Che-1 knockdown on H/R-induced cell injury. The CCK-8 assay

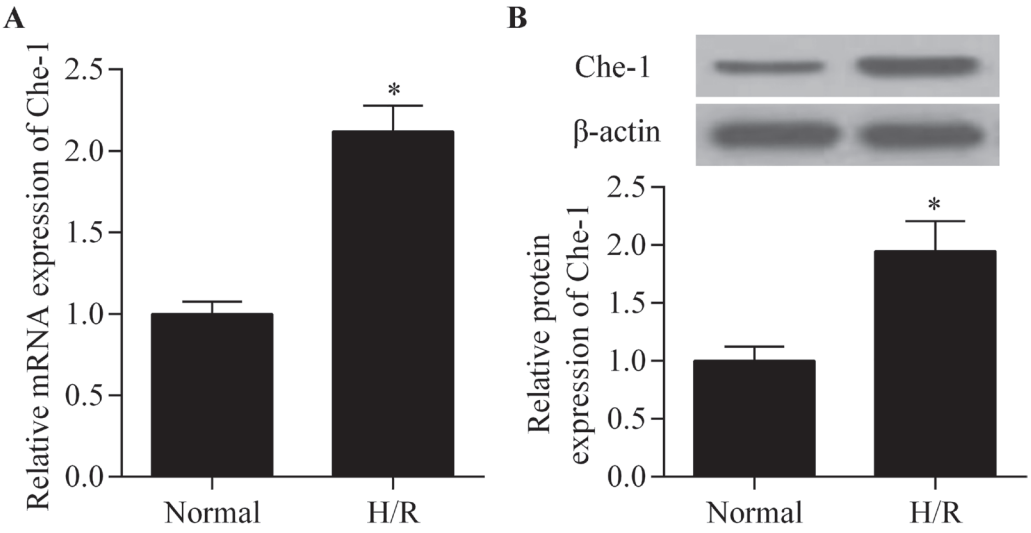

Figure 1. Che-1 is reduced in cardiomyocytes with H/R treatment. The mRNA ***(A)*** and protein ***(B)*** expression were detected by RT-qPCR and Western blot analysis, respectively, in primary mouse cardiomyocytes with H/R treatment. Cells under normal conditions served as control. *$p < 0.05$ *vs.* normal.

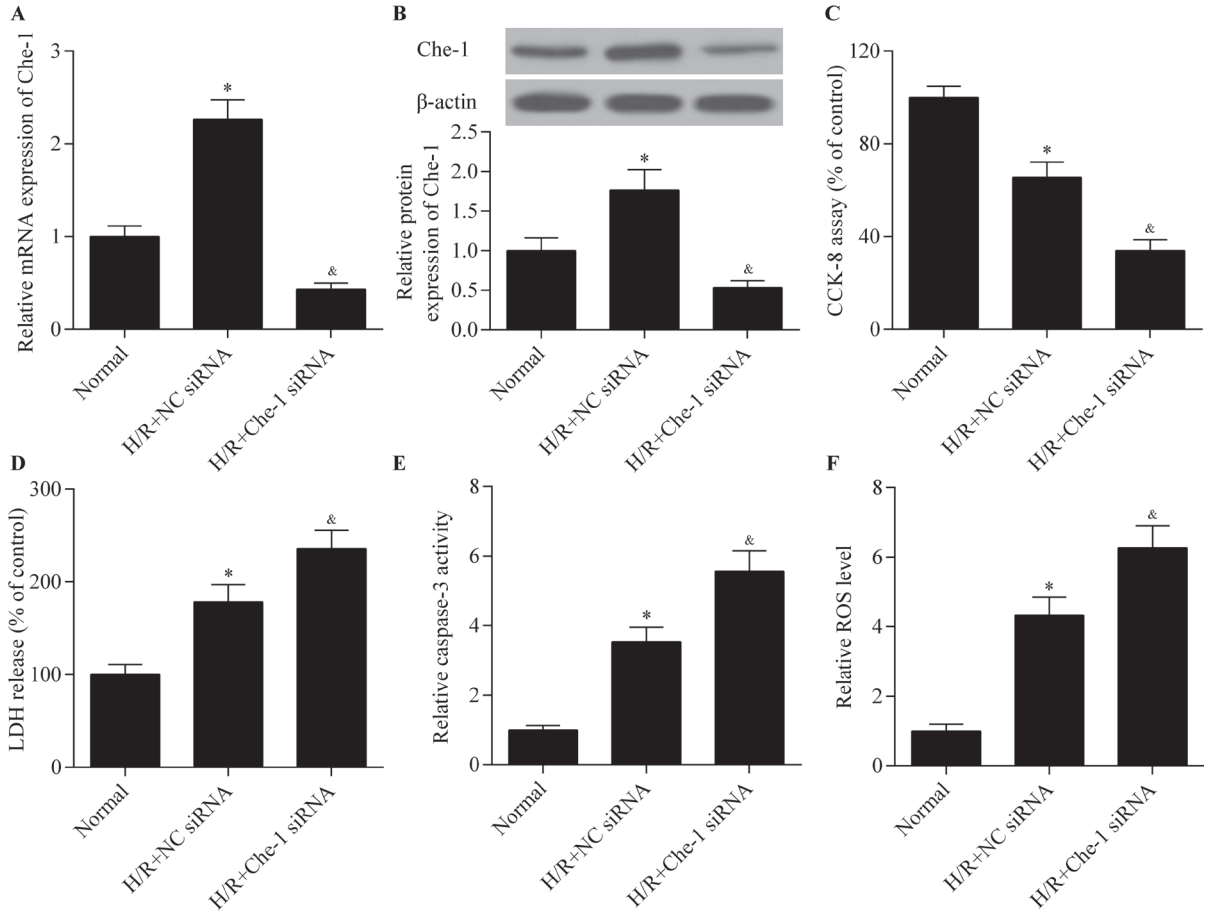

Figure 2. The effect of Che-1 knockdown on H/R-induced cell injury. Cardiomyocytes were transfected with Che-1 siRNA or negative control (NC) siRNA for 24 h followed by H/R treatment. The mRNA ***(A)*** and protein ***(B)*** expression of Che-1 was detected by RT-qPCR and Western blot analysis. ***(C)*** Cell viability was detected by CCK-8 assay. ***(D)*** Cell cytotoxicity was assessed by LDH assay. ***(E)*** Cell apoptosis was detected by caspase-3 activity. ***(F)*** Intracellular ROS levels were measured by DCFH-DA assay. *$p < 0.05$ *vs.* normal and &$p < 0.05$ *vs.* H/R+NC siRNA.

showed that H/R treatment significantly reduced cell viability, which was further decreased by Che-1 knockdown (Figure 2C). The LDH assay showed that H/R-induced cytotoxicity was further increased by Che-1 knockdown (Figure 2D). The caspase-3 activity assay showed that cell apoptosis induced by H/R treatment was further promoted by Che-1 knockdown (Figure 2E). Moreover, the H/R-induced generation of ROS was further increased by Che-1 knockdown (Figure 2F). These results suggest that knockdown of Che-1 aggravates H/R-induced cell injury in cultured cardiomyocytes.

Overexpression of Che-1 Alleviates H/R-Induced Cell Injury in Cultured Cardiomyocytes

Considering that silencing of Che-1 aggravates H/R-induced cell injury, we suggested that overexpression of Che-1 may protect cardiomyocytes against H/R-induced injury. To verify the hypothesis, we performed gain-of-function experiments using the Che-1 expression vector. We found that transfection of the pcDNA3.1/Che-1 expression vector significantly increased the expression of Che-1 in cultured cardiomyocytes (Figure 3A and B). As expected, the CCK-8 assay showed that overexpression of Che-1 significantly reversed the inhibitory effect of H/R on cell viability (Figure 3C). The LDH assay showed that H/R-induced cytotoxicity was markedly decreased by Che-1 overexpression (Figure 3D). Cell apoptosis induced by H/R treatment was significantly attenuated by Che-1 overexpression (Figure 3E). Moreover, the H/R-induced ROS generation was also inhibited by Che-1 overexpression (Figure 3F). These results suggest that overexpression of Che-1 protects cardiomyocytes against H/R-induced injury.

Che-1 Regulates the Expression of Nrf2

To investigate the underlying mechanism by which Che-1 regulates H/R-induced injury, we detected the regulatory effect of Che-1 on Nrf2,

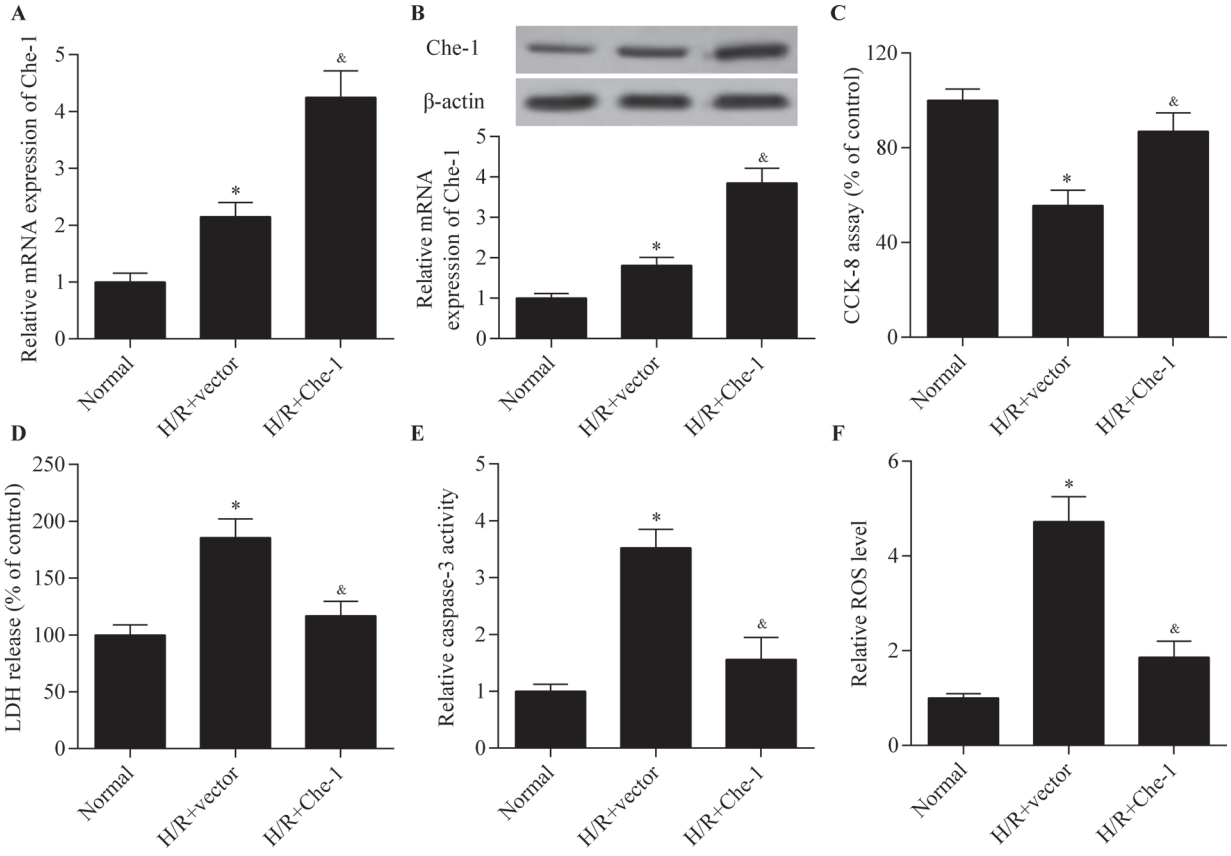

Figure 3. Effect of Che-1 overexpression on H/R-induced cell injury. Cardiomyocytes were transfected with a pcDNA3.1/Che-1 expression vector or a pcDNA3.1 empty vector for 24 h followed by H/R treatment. The mRNA ***(A)*** and protein ***(B)*** expression of Che-1 was detected by RT-qPCR and Western blot analysis. ***(C)*** Cell viability was detected by CCK-8 assay. ***(D)*** Cell cytotoxicity was assessed by LDH assay. ***(E)*** Cell apoptosis was detected by caspase-3 activity. ***(F)*** Intracellular ROS levels were measured by DCFH-DA assay. *$p < 0.05$ *vs*. normal and &$p < 0.05$ *vs*. H/R+vector.

a transcription factor regulating antioxidant in cardiovascular homeostasis[24]. Notably, we found that knockdown of Che-1 significantly decreased the expression of Nrf2 (Figure 4A and B) and decreased ARE activity (Figure 4C). In contrast, overexpression of Che-1 promoted Nrf2 expression (Figure 5A and B) and promoted ARE activity (Figure 5C). Overall, these results indicate that Che-1 regulates the expression of Nrf2.

Che-1 Regulates the Expression of Antioxidant Genes Downstream of Nrf2

To further verify the regulatory effect of Che-1 on Nrf2, we detected the effect of Che-1 on Nrf2 downstream signaling. We found that the expression of HO-1 (Figure 6A) and NQO1 (Figure 6B), the target genes of Nrf2, were significantly decreased by Che-1 knockdown. In contrast, overexpression of Che-1 significantly promoted the expression of HO-1 (Figure 6C) and NQO1 (Figure 6D). These results mean that Che-1 regulates Nrf2 signaling.

Discussion

We showed for the first time an important role of Che-1 in regulating cardiomyocyte injury; Che-1 is a gene responsive to H/R treatment in cultured cardiomyocytes. Loss of Che-1 aggravated H/R-induced injury, while overexpression of Che-1 showed a promising protective effect in H/R-treated cardiomyocytes. Importantly, we found that the underlying mechanism was associated with the promoting effect of Che-1 on Nrf2 signaling.

A growing body of evidence has documented that Che-1 has strong anti-apoptotic activity[9]. Che-1 was originally identified as a strong apoptotic inhibitor, by antagonizing the activity of Dlk/ZIP[5]. Che-1 counteracts NRAGE-induced neuronal apoptosis during neuronal development[25]. Studies[11,26,27] show that Che-1 also inhibits neuronal apoptosis through interaction with prostate apoptosis response-4, Tau protein or cyclin-dependent kinase 5. Bruno et al[28] reported that Che-1 activated the expression of an X-linked inhibitor of an apoptosis protein in response to DNA damage induced by anticancer drugs. Of note, Che-1 inhibits DNA damage-induced apoptosis through maintaining the G2/M checkpoint and inhibiting the expression of pro-apoptotic genes through interacting with p53 [29-31]. These findings suggest an anti-apoptotic role of Che-1. However, a pro-apoptotic role of Che-1 is also reported. Ferraris et al[32] reported that overexpression of Che-1 promoted UV-induced apoptosis via the interacting and activating pro-apoptotic factor c-Jun. Otherwise, we found that the loss of Che-1 enhanced H/R-induced cardiomyocyte apoptosis, while overexpression of Che-1 attenuated H/R-induced cardiomyocyte apoptosis, supporting an anti-apoptotic role of Che-1 in cardiomyocytes.

An increasing number of researches have shown that Che-1 is a responsive gene to various cellular stresses. Hyperosmotic stress has been

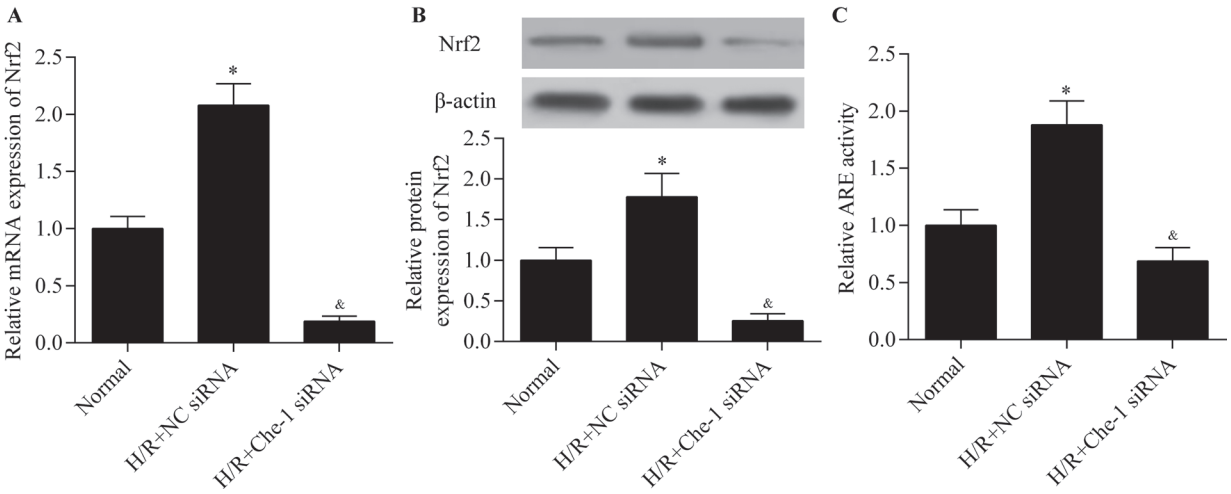

Figure 4. Silencing of Che-1 downregulates the expression of Nrf2. Cardiomyocytes were transfected with Che-1 siRNA or NC siRNA for 24 h followed by H/R treatment. The mRNA ***(A)*** and protein ***(B)*** expression of Nrf2 were detected by RT-qPCR and Western blot analysis. ***(C)*** ARE activity was detected by luciferase reporter assay. *$p < 0.05$ *vs*. normal and &$p < 0.05$ *vs*. H/R+NC siRNA.

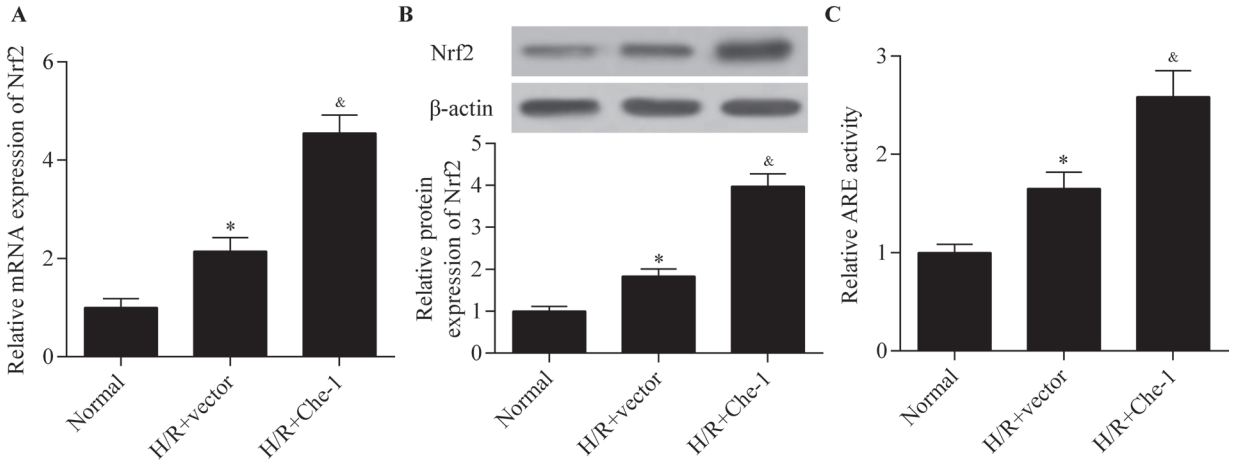

Figure 5. Overexpression of Che-1 upregulates the expression of Nrf2. Cardiomyocytes were transfected with a pcDNA3.1/Che-1 expression vector or a pcDNA3.1 empty vector for 24 h followed by H/R treatment. The mRNA ***(A)*** and protein ***(B)*** expression of Nrf2 were detected by RT-qPCR and Western blot analysis. ***(C)*** ARE activity was detected by luciferase reporter assay. *$p < 0.05$ *vs.* normal and &$p < 0.05$ *vs.* H/R+vector.

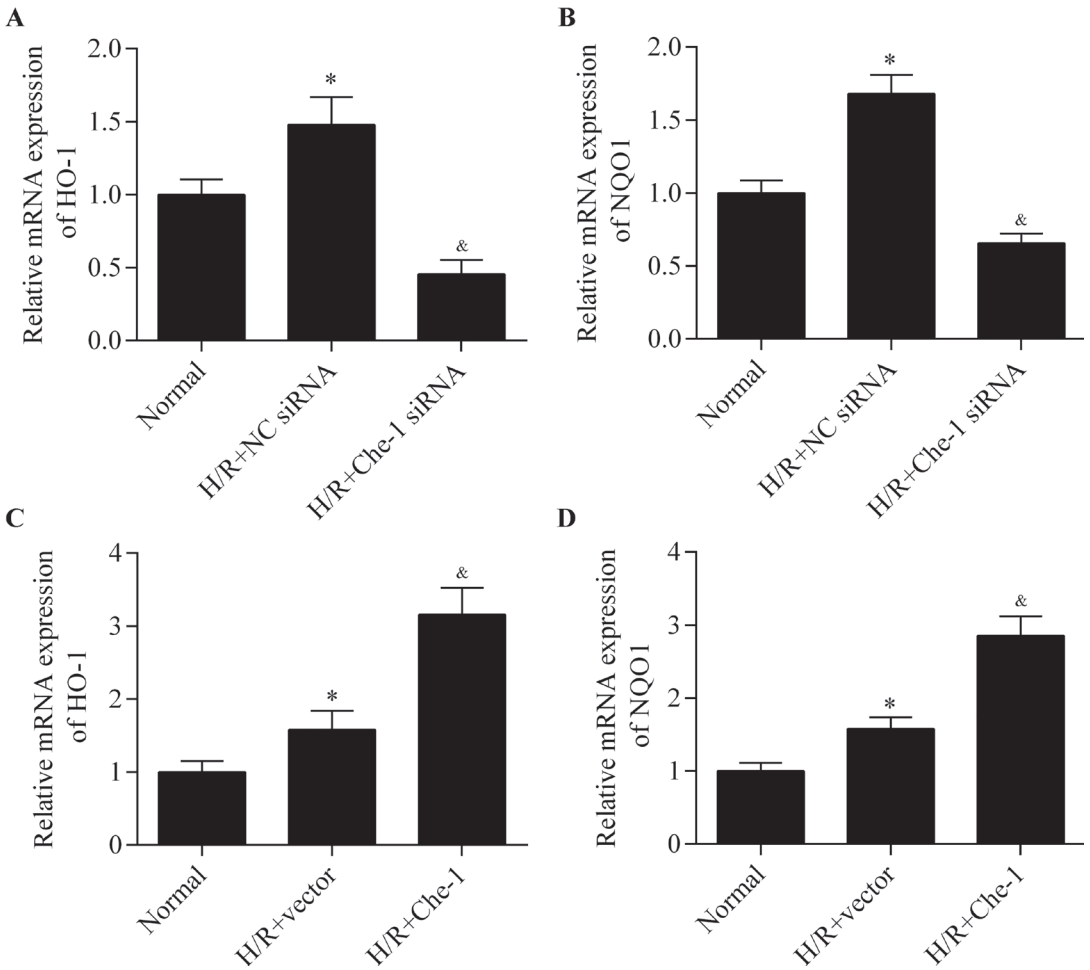

Figure 6. Che-1 regulates the expression of HO-1and NQO1. The mRNA expression of HO-1 ***(A)*** and NQO1 ***(B)*** were detected by RT-qPCR in cardiomyocytes transfected with Che-1 siRNA or NC siRNA for 24 h followed by H/R treatment. *$p < 0.05$ *vs.* normal and &$p < 0.05$ *vs.* H/R+NC siRNA. The mRNA expression of HO-1 ***(C)*** and NQO1 ***(D)*** were detected by RT-qPCR in cardiomyocytes transfected with a pcDNA3.1/Che-1 expression vector or a pcDNA3.1 empty vector for 24 h followed by H/R treatment. *$p < 0.05$ *vs.* normal and &$p < 0.05$ *vs.* H/R+vector.

shown to activate Che-1 by inducing MK2-mediated phosphorylation[30]. Upon endoplasmic reticulum stress, Che-1 is induced and promotes cell survival by activating the STAT3/Akt1 signaling pathway[16]. A recent study[33] revealed that Che-1 is induced by ionizing radiation, glucose deprivation or hypoxia, and it protects the cells against these insults by inducing mTOR-mediated autophagy. Moreover, hypoxia-induced Che-1 expression contributes to maintaining the adaptation of cancer cells to hypoxia by affecting hypoxia-inducible factor-1α stabilization[15]. Che-1 has been reported to exhibit a protective role against experimental renal ischemia/reperfusion injury[34]. However, whether Che-1 is involved in regulating cardiomyocytes in response to H/R treatment remains unclear. In this work, we showed that Che-1 was significantly increased in cultured cardiomyocytes in response to H/R treatment. We further showed that overexpression of Che-1 protected cardiomyocytes against H/R-induced injury. These results suggest that Che-1 may be also involved in regulating myocardial ischemia/reperfusion injury.

Nrf2 is a redox transcription factor that plays an important role in cardio-protection[21,24,35]. Che-1 has been suggested as a transcriptional cofactor involved in regulating gene expression through connecting specific transcription factors to the general transcriptional machinery. A previous study[36] has shown that Che-1 can enhance transactivation of several steroid hormone receptors by interacting with nuclear hormone receptors. Che-1 interacts with TSG101 to enhance the transcription of androgen receptor[37]. In addition, several transcription factors, including the retinoblastoma protein, STAT3, and p53 were also found as the targets of Che-1[16,31,38]. However, whether Che-1 regulates Nrf2 remains unknown. In this study, we showed that Che-1 increased the expression of Nrf2 and promoted the activation of Nrf2/ARE signaling. We found that the transcription activity of Nrf2 was enhanced by Che-1 overexpression, suggesting a regulatory effect of Che-1 on Nrf2 signaling. Therefore, the protective effect of Che-1 against H/R-induced oxidative stress and apoptosis may be associated with the promotion of Nrf2 signaling. An increasing number of reports[39-42] have shown that activating Nrf2 signaling by various pharmaceuticals shows promising protective effects against myocardial ischemia/reperfusion injury or H/R injury *in vivo* and *in vitro*. We indicate that Che-1 may serve as a potential target for modulating Nrf2 signaling.

Conclusions

We showed for the first time a critical role of Che-1 in regulating cardiomyocyte apoptosis in response to H/R injury. We further elucidated that the underlying mechanism may involve the regulatory effect of Che-1on Nrf2 signaling. We suggest that Che-1 may serve as a potential and promising therapeutic target for inhibiting cardiomyocyte apoptosis in the treatment of myocardial ischemia/reperfusion injury.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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