**Original Article**

Reduction of apoptosis as a target of dexmedetomidine effects on ischemia/reperfusion injury in diabetic myocardium

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**Abstract:** Dexmedetomidine (DEX) is a α₂-adrenergic receptor agonist commonly used in clinical anesthesia that has established cardioprotective effects against myocardial apoptosis injury. We investigated whether DEX has anti-apoptotic effects in type 2 diabetic rats subjected to ischemia/reperfusion (I/R) injury. The type 2 diabetes model was established by providing a high-fat diet, followed by low dose streptozotocin (STZ) injection. The myocardial I/R model consisted of left anterior descending coronary artery occlusion for 30 minutes followed by reperfusion for two hours. DEX and its antagonist yohimbine were administered intravenously together or alone before ischemia. At the end of reperfusion, the rats were sacrificed, and plasma samples were collected to measure the levels of nitric oxide (NO) and inducible nitric oxide synthase (iNOS). The hearts were isolated for immunohistochemistry and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end label (TUNEL) assay. Western blot analysis was used to measure the expression of B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and caspase 3. DEX preconditioning significantly increased the levels of NO (75.36 ± 2.7) compared with the Control group (63.14 ± 2.65) (P<0.05). DEX preconditioning also significantly increased the levels of iNOS (43.76 ± 3.09) compared with the Control groups (32.66 ± 2.62) (P<0.05). DEX preconditioning significantly reduced the apoptosis index (AI) in TUNEL assay and the expression of caspase 3. DEX preconditioning also significantly reduced the ratio of Bax/Bcl-2 (2.29 ± 0.03) compared with the Control group (8.87 ± 0.93) (P<0.05). All of these protective effects of DEX were reversed by co-administration of yohimbine. These results suggest that dexmedetomidine preconditioning exerted a cardioprotective effect against myocardial apoptosis injury in a rat model of type 2 diabetic myocardial ischemia/reperfusion.

**Keywords:** Myocardial ischemia/reperfusion, type 2 diabetes, apoptosis, dexmedetomidine

**Introduction**

Ischemic heart disease (IHD) is the leading cause of death in modern society [1]. Reperfusion is recognized as an effective therapy for IHD. However, complications caused by reperfusion have attracted increasing attention. Myocardial reperfusion injury consists of four basic forms: lethal cardiac myocyte injury, vascular injury, stunned myocardium, and reperfusion arrhythmia [2]. The risk of heart disease increases two- to three-fold in diabetic patients, and the diabetic patients who suffering from acute myocardial infarction are more likely going to death than nondiabetic patients [3, 4]. Therefore, myocardial protection for diabetic patients with I/R injury during the perioperative period warrants further investigation.

Dexmedetomidine (DEX) is a highly selective α₂-adrenergic receptor agonist [5]. Previous experiments have shown the benefit of DEX preconditioning in the I/R-injured heart, which is mediated by the activation of pro-survival kinases after cardiac α₂-adrenergic receptor stimulation [5]. DEX has also been reported to elicit cardioprotection against myocardial apoptosis injury in a grave scalding rat model [6]. Currently, the anti-apoptotic effect of DEX on regional I/R injury in type 2 diabetes is unknown.
We hypothesized that DEX may exert protective effects against myocardial apoptosis injury in diabetic rats.

Materials and methods

Animals

This study was approved by the Institutional Animal Care and Use Committee of Harbin Medical University in Harbin, China. Sprague-Dawley rats aged 8-9 weeks were purchased from the Animal Center of the Second Affiliated Hospital of Harbin Medical University (SYXK, 2013-002).

The type 2 diabetes model was established by a high-fat diet for two weeks, followed by a low dose injection of streptozotocin (STZ) (Sigma, St.Louis, MO, USA), as described previously [7]. After 4 weeks of dietary manipulation, diabetes was confirmed based on a randomly sampled blood glucose level greater than 16.7 mmol/L. After successful induction of diabetes, the animals were prepared for the regional heart I/R model. The rats were anesthetized with pentobarbital 60 mg/kg administered intraperitoneally. The trachea was intubated with a specific cannula. The right femoral vein and artery were cannulated for drug administration and continuous heart rate monitoring, respectively. After intravenously injection of rocuronium, positive-pressure artificial respiration was started with pure O₂, using a volume of 8-10 ml/kg body weight, at a rate of 45-55 beats/min. Then, the chest was opened via a left thoracotomy through the fourth or fifth intercostal space. A 5-0 ophthalmic suture was placed below the left anterior descending coronary artery (LADCA). Before tying the suture, a polyethylene tube was placed on the surface of the artery to form a reversible occlusion. Then, the heart was subjected to 30 minutes of regional ischemia followed by 120 minutes of reperfusion. Ischemia was confirmed by visually detectable cyanosis on the surface of the myocardium. By loosening the ligature, reperfusion was successful as epicardial hyperemia resulted.

Experimental protocols

All animals were allowed to stabilize for 10 min before being randomly assigned to one of the following groups: a) a Control group, which was not subjected to preconditioning but was administered physiological solution for 30 min; b) a DEX group, which was preconditioned with a 10 min bolus of 1 μg/kg DEX, followed by 15 min of 0.7 μg/kg/h DEX administration; c) a Yohimbine (YOH) group, which was preconditioned with a 5 min bolus of 1 mg/kg YOH, followed by 20 min of 0.5 mg/kg/h YOH administration; d) a YOH/DEX group, which was preconditioned with 5 min bolus of 1 mg/kg YOH, followed by 25 min of 0.5 mg/kg/h YOH administration. In the YOH/DEX group, 5 min into YOH administration, a 10 min bolus of 1 μg/kg DEX was performed, followed by 15 min of 0.7 μg/kg/h DEX administration. All the drugs were administered intravenously. The doses of DEX and YOH were selected according to the manufacturer’s recommendation and the report of Ibacache et al [5].

Determination of No and iNOS levels

At the end of reperfusion, blood samples were obtained from the femoral artery using a heparinized syringe, immediately centrifuged and stored at -80°C until use. Nitric oxide (NO), inducible nitric oxide synthase (iNOS) were measured using a diagnostic assay kit (Jiancheng Institute of Biotechnology, Nanjing, China). Absorbance was determined using a UV-2100 spectrophotometer (UNIC Medical Inc., Shanghai, China) at 530 nm according to the manufacturer’s instructions [8].

TUNEL assay

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end label (TUNEL) assay was used to detect cardiomyocyte apoptosis induced by DEX. Myocardial tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), washed, dehydrated, and embedded in paraffin. Paraffin-embedded tissue sections were sectioned at 5 μm according to standard procedures. TUNEL staining was then performed according to the manufacturer’s instructions with the TUNEL detection kit (Roche, Indianapolis, IN, USA). We randomly chose five fields in each slide (400× magnification) to calculate the TUNEL-positive percentage. The apoptosis index (AI) was calculated by expressing the number of TUNEL-positive cells per 100 cells [9].

Immunohistochemistry

Paraffin-embedded specimens were sliced into 5-μm tissue sections, deparaffinized and
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hydrated in PBS (pH 7.4). Then, the sections were incubated in 3 % H$_2$O$_2$ for 10 min and placed in sodium-citrate buffer for antigen retrieval in a steaming water bath.

A primary antibody against caspase 3 (Jiancheng Bio-Technology, Nanjing, China) was applied, followed by washing and incubation in a biotinylated secondary antibody (Vector Labs, Burlingame, CA, USA) for 30 min. Photographs were taken using a digital camera. Five scopes of each section were chosen for measuring the integral optical density (IOD) of caspase 3 expression [10].

Western blot analysis

Western blot analysis was performed using a previously described method [11]. Primary antibodies against Bax (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-2 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), caspase 3 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) and appropriate secondary antibodies (Vector Labs, Burlingame, CA, USA) were used. The band density was quantified using Image J software (v1.33, NIH, Bethesda, MD, USA). The same membranes were subjected to Western blot for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2000, ABeclonal-Bio, Wuhan, China) as a loading control.
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Figure 3. The proteinic expression of caspase 3. Western blot showed the caspase 3 expression in rat myocardium. Mean value rates of caspase 3/GAPDH in the Control, DEX, YOH and YOH/DEX groups are shown as mean ± SD, n=5 per group, *P<0.01 DEX vs. Control.

Figure 4. The caspase 3 was localized by immunohistochemical staining. The expression of caspase 3 was visualized as yellow particles, in the cytosol and the nucleus. The expression of caspase 3 in the Control, YOH and YOH/DEX groups were more positive than the DEX group, which was confirmed by the integral optical density (IOD).

Results

Dexmedetomidine preconditioning increased the levels of NO and iNOS

The plasma NO and iNOS levels are shown in Figure 1. Preconditioning with DEX resulted in significantly higher NO levels (75.36 ± 2.7) compared with the Control groups (63.14 ± 2.65) (P<0.05). A YOH co-administration with DEX reversed the above effect. The data in YOH group and YOH/DEX group are 62.67 ± 2.03 and 62.84 ± 2.45. Preconditioning with DEX resulted in significantly higher iNOS levels (43.76 ± 3.09) compared with the Control groups (32.66 ± 2.62) (P<0.05). A YOH co-administration with DEX reversed the above effect. The data in YOH group and YOH/DEX group are 31.03 ± 2.02 and 31.51 ± 1.88.

TUNEL assay results

The myocardial tissues used for the TUNEL assay were the same as those used for immunohistochemistry. The nucleolus of the control myocardial cells was stained blue, whereas in the I/R injured myocardium, the nucleolus stain appeared buffy suggesting that these cells were apoptotic. The apoptosis index (AI) was lower in the DEX group than in the Control group, which indicated that DEX exerted an anti-apoptotic effect
Dex inhibited the apoptosis of diabetic I/R injury rat models. This effect was reversed by YOH co-administration (Figure 2).

Analysis of caspase 3 by western blot assay and immunohistochemistry

The expression of caspase 3 is shown in Figures 3 and 4. The expression of caspase 3 in the Control group was much higher than in the DEX group. DEX substantially reduced the expression of caspase 3. YOH co-administration could reverse this effect.

Bax and Bcl-2 expression by western blot assay

The protein Bcl-2 and the protein Bax expression of different groups in the myocardium is shown in Figure 5. I/R injury of the myocardium in type 2 diabetes increased the expression of Bax, but decreased the expression of Bcl-2. Compared with the Control group, DEX preconditioning decreased the expression of Bax and increased the expression of Bcl-2. As a result, the ratio of Bax/Bcl-2 was decreased by DEX preconditioning (2.29 ± 0.03). There was no difference among the Control group (8.87 ± 0.93), the YOH group (9.11 ± 0.89) and the YOH/DEX group (8.65 ± 0.45) with respect to the Bax/Bcl-2 ratio.

Discussion

The main findings of this study on diabetic I/R myocardium are as follows: a) DEX pretreatment increased the level of NO, as well as the expression of iNOS. b) The AI was reduced by DEX preconditioning; and c) DEX preconditioning inhibited myocardial apoptosis, causing a decrease in caspase 3 expression and the Bax/Bcl-2 ratio. DEX clearly exerted an anti-apoptotic effect against myocardial I/R injury, and we are the first to demonstrate that this protective effect is also present in myocardial I/R injury in type 2 diabetes.

DEX is an α₂-adrenergic receptor agonist that shares physiological similarities with clonidine [6]. In previous studies, DEX improved myocardial function and reduced myocardial infarction size after I/R injury [5], exerted a cardioprotective effect on global ischemia in an isolated rat heart model [12]. DEX also activated pro-survival kinases in rat models of myocardial I/R injury to protect the I/R-injured heart and exerted a cardioprotective effect against myocardial apoptosis injury [6].

Apoptosis is an important component of the pathogenesis of myocardial I/R injury, and inhibition of apoptosis in cardiomyocytes can attenuate cardiac injury induced by myocardial I/R. The signaling pathways of apoptosis mainly include the extrinsic (or death receptor) pathway and the intrinsic (or mitochondrial) pathway. Extrinsic signaling is initiated by the binding of a death ligand trimer to its cognate cell surface receptor; tumor necrosis factor-α may be the ligand [13]. The intrinsic pathway integrates a broad spectrum of extracellular and intracellular stressors. DEX, an extracellular stimulus, may exert an anti-apoptotic effect through the intrinsic pathway. The signal pathways converge on the mitochondria leading to the dysfunction of organelles and the release of apoptogenic proteins [14]. The mitochondrial pathway is activated by upstream apoptotic signals to the mitochondria. Then, the Bcl-2 protein family is activated, and the mitochondrial...
permeability transition pore opens. Translocation of Bax to the mitochondria is triggered, which then stimulates the release of cytochrome c into the cytosol. Bcl-2 is an anti-apoptotic protein and exerts effects through interactions with many factors and pathways [15]. All of these events induce the activation of the downstream caspase family and the initiation of apoptosis. The Bax/Bcl-2 ratio reflects the extent of apoptosis [16]. When Bax expression is high, a Bax/Bcl homodimer is formed to stimulate apoptosis and when Bcl-2 expression is high, a Bcl-2/Bax heterodimer is formed to inhibit apoptosis [17, 18]. We further investigated the effect of DEX on Bax, Bcl-2, and caspase 3 expression in a type 2 diabetic rat models after I/R injury. Western blotting and immunohistochemistry showed that the Bax/Bcl-2 ratio and the expression of caspase 3 were markedly increased by I/R in the hearts of type 2 diabetic rats. DEX preconditioning significantly reduced the Bax/Bcl-2 ratio and the expression of caspase 3; these effects were blocked by YOH. These results indicate that DEX inhibited myocardial apoptosis partly by inhibiting Bax expression and by upregulating Bcl-2 expression.

NO, which is generated from L-arginine, has been demonstrated to exert protective effects against cardiac injury [19, 20]. Previous studies have demonstrated that NO regulates cytoprotection during I/R injury [21]. Furthermore, both NOS and NO have been reported to have anti-apoptotic properties [22]. In the present study, the expression of NO and iNOS were significantly increased by DEX preconditioning compared with the Control group.

Myocardial apoptosis is also directly related to the pathogenesis of diabetes [23]. The development of apoptosis is one of the major events associated with the development cardiovascular complications and myocardial I/R injury in diabetes. And STZ-induced chronic diabetes significantly increased cardiac apoptosis in myocardial I/R injury compared with the control group [24]. Apoptosis can also reduce cardiac function in diabetic rat hearts and increase reperfusion-induced injury. Taken together, our results suggest that DEX may exert an anti-apoptotic effect through the intrinsic apoptosis pathway by reducing the Bax/Bcl-2 ratio and activating the expression of caspase 3. In addition, DEX exerts anti-apoptotic effects by increasing the levels of NO and iNOS. As a result, apoptosis in type 2 diabetes I/R myocardium is inhibited following DEX treatment.

There were several limitations to this study. First, the number of apoptosis-associated proteins is enormous, and we detected only some of them. Although our results partly demonstrate that the anti-apoptosis effect of DEX occurs via the intrinsic pathway, more factors and proteins need to be investigated to consolidate our hypothesis. Second, we discussed the mechanism of apoptosis at the protein level, further investigation at the ribonucleic acid level is required in the future.

Conclusions

The present study showed that dexmedetomidine exerts a cardioprotective effect against myocardial apoptosis injury in a type 2 diabetic myocardial I/R model. The results indicate that high risk cardiovascular patients with type 2 diabetes undergoing surgery will get benefit from dexmedetomidine.

Disclosure of conflict of interest

None.

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