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ORIGINAL ARTICLE

Dexmedetomidine alleviates H_2O_2-induced oxidative stress and cell necroptosis through activating of α2-adrenoceptor in H9C2 cells

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Abstract

Oxidative stress induced necroptosis is important in myocardial ischemia/reperfusion injury. Dexmedetomidine (Dex), an α2-adrenoceptor (α2-AR) agonist, has protective effect on oxidative stress induced cell apoptosis, but effects of Dex and Dex-mediated α2-AR activation on oxidant induced necroptosis was unclear. H9C2 cardiomyocytes were pre-treated with or without Dex and α2-AR antagonist yohimbine hydrochloride (YOH) before being exposed to H_2O_2 to induce oxidative cellular damage. Cell viability and lactate dehydrogenase (LDH) were detected by ELISA kits, protein expressions of Heme Oxygenase 1(HO-1), receptor interacting protein kinase 1 (RIPK1) and receptor interacting protein kinase 3 (RIPK3) were observed by WB, and TUNEL was used to detected cell apoptosis. H_2O_2 significantly decreased cell viability and increased LDH release and necroptotic and apoptotic cell deaths (all $p < 0.05$, H_2O_2 vs. Control). Dex preconditioning alleviated these injuries induced by H_2O_2. Dex preconditioning significantly increased expression of protein HO-1 and decreased expressions of proteins RIPK1 and RIPK3 induced by H_2O_2, while all these protective effects of Dex were reversed by YOH (all $p < 0.05$, Dex + H_2O_2 vs. H_2O_2; and YOH + Dex + H_2O_2 vs. Dex + H_2O_2). However, YOH did not prevent this protective effect of Dex against H_2O_2 induced apoptosis (YOH + Dex + H_2O_2 vs. Dex + H_2O_2, $p > 0.05$). These findings indicated that Dex attenuates H_2O_2 induced cardiomyocyte necroptotic and apoptotic cell death respectively dependently and independently of α2-AR activation.

Keywords Dexmedetomidine · Necroptosis · Oxidative stress

Introduction

The death of terminally differentiated cardiomyocytes is a major cause of heart remodeling and dysfunction [1–3]. Cardiomyocyte death occurs in myocardial ischemia/reperfusion (I/R) injury [4, 5]. Many factors (such as oxidative stress, intracellular Ca^{2+} overload, and inflammation etc.) have been validated to contribute to myocardial I/R injury [3]. Among these factors, an increasing number of studies have demonstrated that oxidative stress plays a key and initiating role in the pathology of I/R injury [6]. Oxidative stress triggers numerous detrimental reactions in cells such as the aforementioned intracellular calcium overload and leads to cell apoptosis and necrosis [7]. Cellular apoptosis is a form of programmed cell death which is tightly controlled, and it has obtained much attention over the past three decades [8]. However, treatments that aimed to reducing or inhibiting apoptosis have not yield optimal effects in attenuating post-ischemic myocardial I/R injury.

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Necrosis has always been considered to be an accidental or passive form of cell death which cannot be mediated. However, more recent studies show the existence of a form of necrosis, termed necroptosis, which can be precisely programmed and regulated [9]. Previously, it was reported that necroptosis plays an essential role in myocardia I/R injury, while decreasing necroptosis can remarkably protect against I/R injury [9–11]. Therefore, strategies to prevent oxidative stress induced necroptosis are attractive approaches to ameliorate myocardial I/R injury.

Cardiac surgery may result in complicated cardiovascular and other complications, such as stroke, cardiac arrest and myocardial infarction. The imbalance of oxygen supply and demand, ischemia–reperfusion and the companied oxidative stress were major detrimental factors leading to these complications. We reported that perioperative dexmedetomidine (Dex) use was associated with a decrease in postoperative mortality up to 1 year and decreased incidence of postoperative complications in patients undergoing cardiac surgery with cardiopulmonary bypass [12], a procedure that is accompanied with myocardial I/R injury and oxidative stress. However, the underlying mechanism attributable to the beneficial effects of Dex has yet to be determined.

Dex is a potent, highly selective agonist of α2-adrenoceptor (α2-AR), which has been widely used in perioperative patients for sedation and analgesia [13]. Additionally, it has been validated that Dex has sufficient potential to alleviate I/R injury in many organs, such as heart, brain, intestinal and lung [14]. In vivo and ex vivo rat heart I/R models, Dex preconditioning significantly improves post-ischemia myocardial function and decreases myocardial infarct size [14–16]. These protective effects are associated with the activation of α2-AR [16]. These findings suggest that the cardioprotection of Dex might depend on the activation of α2-AR.

H_2O_2 exposure has been always used to mimic oxidative stress in cardiomyocytes following I/R injury [17–19]. H_2O_2 induces both cardiomyocyte apoptosis and necroptosis, and the extent (or degree) of apoptosis or necroptosis was largely dependent on the time and concentration of H_2O_2 exposure [9]. Dex preconditioning has been demonstrated to protect cardiomyocytes against H_2O_2-induced cell injury by inhibiting apoptosis [20, 21]. However, whether Dex preconditioning has protective effect on H_2O_2-induced necroptosis is still unknown, and in particular, the relative role of α2-AR receptor in Dex mediated attenuation of H_2O_2 induced cardiomyocyte apoptosis and necroptosis has not been explored. In this study, we exposed H9C2 cells to H_2O_2 to induce cell death, and investigated if α2-AR is involved in the effects of Dex preconditioning on H_2O_2-induced apoptosis and necroptosis.

Materials and methods

Reagents and antibodies

Dex and H_2O_2 solution were purchased from Sigma-Aldrich. Dex was dissolved into PBS and then diluted with PBS to obtain the desired concentration. H_2O_2 solution was diluted with sterile water. Yohimbine hydrochloride (YOH) was purchased from MedChem Express, and was dissolved into dimethylsulfoxide (DMSO), YOH (1 μM) was administrated 10 min before the administration of Dex, while Dex (10 μM) was given 120 min before the H9C2 cells were subjected to H_2O_2 exposure (Dex preconditioning) according to our preliminary experiments and other studies [16, 20, 22]. Antibodies that were purchased from Cell Signaling Technology included anti-cleaved caspase3 (#9661), anti-receptor-interacting serine/threonine-protein kinase 1(RIPK) (#3493), anti-receptor interacting protein kinase 3 (RIPK3) (#95702) and anti-GAPDH (#5174) antibodies. Anti-heme oxygenase-1 (HO-1) antibodies (ab13248) were obtained from Abcam. HRP-labeled Goat Anti-Rabbit IgG (H + L) and HRP-labeled Goat Anti-Mouse IgG (H + L) were obtained from beyotime (Shanghai, China).

Cell culture

H9C2 (embryonic rat heart-derived myoblast) cells used in this study was from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle medium (DMEM; GIBCO Laboratories, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/mL) and penicillin (100 units/mL) in a humidified incubator under the condition of 37 °C, 95% air and 5% CO_2.

Experimental protocols

The first set of the study was designed to determine the optimal concentration of H_2O_2 and Dex. H9C2 cells were exposed to various concentrations (100 μM, 500 μM, and 1000 μM) of H_2O_2 for 12 h. After confirmed the optimal concentration of H_2O_2, cells pretreated with different concentrations (0.001 μM, 0.01 μM, 0.1 μM, 1 μM and 10 μM) of Dex for 120 min and then exposed to H_2O_2 (500 μM) for 12 h. Then H9C2 cells were divided into four groups: (1) Control group, (2) H_2O_2 group, (3) Dex + H_2O_2 group, (4) YOH + Dex + H_2O_2 group.

Cell viability assay

Cell viability was measured by using cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan). 8×10^3 cells were seeded in the 96 well, after the completion of the experimental protocol, CCK-8 agent was added into each well (ratio between CCK-8 agent and cell culture media was 1:10) and incubated at 37 °C for 2 h. The absorbance which indirectly reflected the viabilities of cells was examined on a microplate reader at a wavelength in the 450 nm. All measurements were performed in triplicate by an expert examiner in a blinded manner and results of the treated groups were expressed in comparison to the control group.

Detection of lactate dehydrogenase (LDH)

LDH is expressed extensively in tissues or cytoplasm in the body. When cell injury occurred, LDH is rapidly released out of cell through the damaged cell membrane. Hence, we determined the degree of cell injury by LDH release assay. 2×10^6 cells were seeded in the 6 well plates. Cell culture medium was collected and assayed, LDH release was detected using LDH kit (Roche, Mannheim, Germany) following the manufacturer' instructions. The absorbance at a wavelength in the 492 nm reflected the level of LDH in cell culture medium. More LDH release led to higher absorbance. All measurements were performed in triplicate by an independent investigator in a blinded manner and results of the treated groups were expressed in comparison to the control group.

Assessment of reactive oxygen species

Intracellular ROS was assessed by 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma) staining. 1×10^6 H9C2 cells were plated into 12-well plates. After the completion of the experimental protocol, cells were washed twice with PBS, and then incubated in free FBS medium with 10 μM DCFH-DA (Beyotime, Shanghai, China) for 10 min. Fluorescence was observed under a fluorescence microscope (Leica, Dmi8 + DFC 7000T) at an absorption wave length of 488 nm and an emission wave length of 530 nm. The average fluorescence intensities of DCFH-DA in the cells which reflected the level of intracellular ROS were analyzed using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA). Fluorescence was observed under a fluorescence microscope (Leica, Dmi8 + DFC 7000T) at an absorption wave length of 488 nm and an emission wave length of 530 nm. The number of positive H9C2 cells which were stained red, and was determined by counting them with in defined areas (35 × 35 grids) of each slide and the average of 10 histological sections. Referred to previous study [23], we also detected the SOD activity and the expression of protein HO-1 to verify the result of ROS detection.

Detection of superoxide dismutase

Intracellular content of superoxide dismutase (SOD) was detected by Enzyme-linked immunosorbent assay (ELISA). Briefly, 2×10^6 cells were seeded in the 6 well plate, after the experiments were completed, cells were harvested by centrifugation and were resuspended with 200 μL PBS. Three freeze (− 80 °C, 10minutes)-thaw (room temperature, 10 min) cycles were performed to lyse cells. Following lysis, samples were centrifuged at 2000×*g*, 4 °C for 20 min, and collected the supernatant cautiously. The protein concentration in each sample was determined via bicinchoninic acid (BCA) protein assay kit (Novagen, MA, USA). At last, SOD content was detected by using rat SOD ELISA kit (Shanghai Jianglai Industrial Limited By Share Ltd, Shanghai, China) following the manufacture's instruction and the content of SOD was expressed as ng/mg protein.

Terminal deoxynucleotidySl transferase dUTP nick end labeling (TUNEL) assay

TUNEL analysis was used to measure the degree of cell apoptosis via the In Situ Cell Death Detection kit (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's instructions. H9C2 cells were cultured in suitable glass slides, after H_2O_2 exposure with treatment of dexmedetomidine (Dex) in the presence or absence of YOH, cells were washed in sterilized PBS three times (5 min each) and fixed in triformol for 30 min, followed by washing with PBS three times. The cells were blocked with BSA for 60 min before analyzed using a TdT-mediated DUTP-X nick end labeling (TUNEL stain) according to the manufacturer's instruction (Roche Applied Science, Indianapolis, IN, USA) to quantify apoptosis, and 4′, 6-diamidino-2-phenylindole (DAPI) staining solution (Beyotime, Shanghai, China) was used to visualize nuclei. Fluorescence was observed under a fluorescence microscope (Leica, Dmi8 + DFC 7000T). The number of positive H9C2 cells (which were stained bright green was determined by counting them with in defined areas (35 × 35 grids) of each slide and the average of 10 histological sections was reported as means ± SEM, all morphometric measurements were performed by 3 independent expert examiners in a blinded manner and results of the treated groups were expressed in comparison to the control group. The apoptotic index was expressed as the total number of TUNEL-positive nuclei (green)/the total number of DAPI-stained nuclei (blue) × 100%. 2 × 106 cells were seeded in the 6 well plate, considering that the limitations of

TUNEL [24], we also perform WB experiments to observe the expression changes of apoptosis protein cleaved-caspase3 to testify the effects of Dex.

Propidium iodide staining

Propidium Iodide (PI), an intercalating red fluorescent agent, cannot cross the intact membrane of cells. When necrosis happened, PI can enter into cell through the damaged plasma membrane and bind to DNA. Upon binding to DNA, the fluorescence of PI is enhanced dramatically, so it can used to detect cell necrosis. The detailed procedures for PI staining are as follows: H9C2 cells were washed with PBS after the H_2O_2 treatment, and then incubated in free FBS medium with 1.25 μg/mL PI (ImmunoChemistry Technologies, Bloomington, USA) for 10 min at room temperature. After washing with PBS twice, cells were fixed with 4% PFA for 30 min. Following a wash in PBS, the H9C2 cells were mounted with DAPI Staining Solution and examined with a fluorescence microscope. The necrosis index was expressed as the total number of PI-positive nuclei (red)/the total number of DAPI-stained nuclei (blue) × 100%.

Western blotting assay for proteins

The cells were collected and lysed for 30 min on ice in RIPA buffer containing Phenylmethanesulfonyl fluoride (Beyotime, Shanghai, China), then centrifuged for 15 min at 13,500 rpm, the supernatant was collected as total cell protein and assessed by Western Blotting (WB). The RIPA buffer we used mainly contained 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, leupeptin and cocktail. Equal protein samples were respectively added to 12% SDS–polyacrylamide gel for electrophoresis and transferred to PVDF (0.22 μm) membrane, in each well, 30 μg protein was loaded to detect the expression changes of protein. After blocking with 5% non-fat milk for 2 h at room temperature, the PVDF membranes were incubated together with antibody against Heme Oxygenase 1(HO-1), cleaved caspase-3, receptor interacting protein kinase 1 (RIPK1), and receptor interacting protein kinase 3 (RIPK3) at 4 °C overnight. Then, the membranes were washed with TBST (Tris-buffered saline with 0.1% Tween-20) and exposed to the corresponding secondary HRP-labeled antibodies for 2 h at room temperature. The blots were visualized by using enhanced chemiluminescence reagents (Millipore, Billerica, MA, USA), ChemiScope Series (Model No. 6000Touch) was used as our chemiluminescence reader. The densities of the immunoreactive bands were analyzed using ImageJ software.

Statistical analysis

All data are presented as the means ± standard errors of the means (SEM). Statistical differences were assessed by one-way analysis of variance (GraphPad Software, Inc., LaJolla, CA) followed by the Tukey test for multiple comparisons. P values less than 0.05 were considered statistically significant.

Results

Dex dose-dependently attenuated H_2O_2-induced cardiomyocytes injury

As shown in Fig. 1, H_2O_2 caused significant reductions in cell viability and increment in LDH release in a concentration dependent manner (Fig. 1a, b), with H_2O_2 at 500 μM caused about 50% cell viability reduction and 20% increase of LDH release increment. Therefore, H_2O_2 at 500 μM was used in the ensuring studies. Dex attenuated H_2O_2-induced cardiomyocytes injury evidenced by increasing cell viability (approximate 10%) and decreasing LDH release (approximate 15%) at the concentration of 10 μM (all $p < 0.05$, Dex 10 uM vs. H_2O_2).

Inhibition of α2-AR cancelled the protective effects of Dex on H_2O_2-induced cardiomyocytes injury

YOH, an α2-AR antagonist, has been widely used to inhibit the effect of Dex on α2-AR [16, 25]. As shown in Fig. 2a, cell viability was significantly increased with Dex (10 μM) pretreatment for 2 h (H_2O_2 *vs.* control, Dex + H_2O_2 *vs.* H_2O_2, all $p < 0.01$) and this effect was blunted by the addition of YOH (1 μM) (YOH + Dex + H_2O_2 vs. Dex + H_2O_2, $p < 0.01$). Figure 2b showed that H_2O_2 significantly increased LDH release and Dex reduced the increment of LDH release induced by H_2O_2 (YOH + Dex + H_2O_2 vs. Dex + H_2O_2, $p < 0.05$), this cardioprotection effect of Dex was abolished by YOH (YOH + Dex + H_2O_2 vs. Dex + H_2O_2, $p < 0.05$).

Inhibition of α2-AR weakened Dex-mediated reduction of H_2O_2-induced oxidative stress in cardiomyocytes

Oxidative stress arisen because of the accumulation of ROS and/or reduction of antioxidant enzymes. As Fig. 3a and b showed, Dex preconditioning significantly decreased the accumulation of intracellular ROS induced by H_2O_2 (H_2O_2 vs. control, Dex + H_2O_2 vs. H_2O_2, all $p < 0.01$), after treatment with YOH, the effects of Dex on ROS was weakened (YOH + Dex + H_2O_2 vs. Dex + H_2O_2, $p < 0.01$). Figure 3c showed that SOD level was significantly

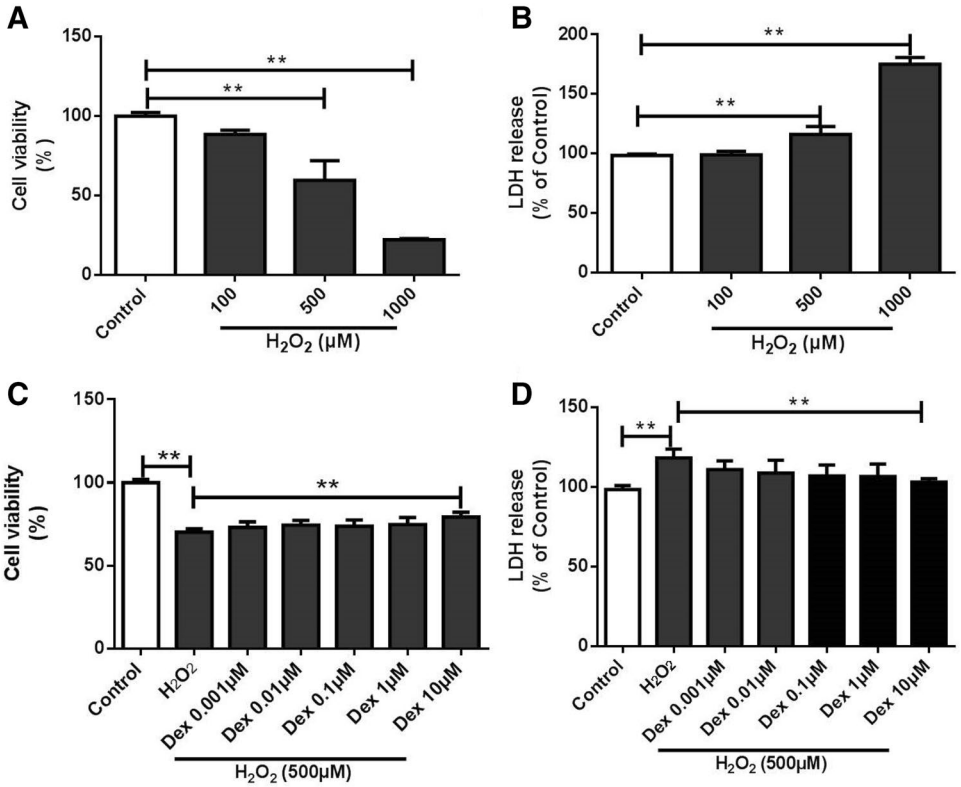

Fig. 1 Effects of Dex on H_2O_2-induced cell injury in H9C2 cells. **a** Concentration -dependent effects of H_2O_2 on cell viability. **b** Concentration-dependent effects of H_2O_2 on LDH release. **c** Effect of Dex pretreatment with different concentrations on cell viability in H_2O_2 exposure-injured H9C2 cells. **d** Effect of Dex pretreatment with different concentrations on LDH release in H_2O_2 exposure-injured H9C2 cells for 12 h. Data are mean ± SEM, n = 6 per group. $^{**}p < 0.01$

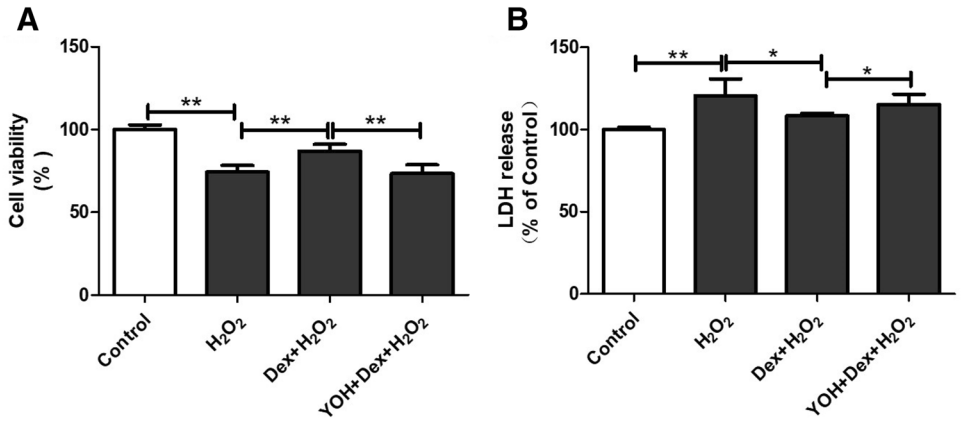

Fig. 2 Inhibition of α2-AR reversed the protective effects of dexmedetomidine (10 μM) on H_2O_2-induced cardiomyocytes injury. **a** The effects of Dex and YOH on cell viability. **b** The effects of Dex and YOH on LDH release. H9C2 cells were subjected to H_2O_2 for 12 h. Data are mean ± SEM, n = 6 per group. $^{*}p < 0.05$; $^{**}p < 0.01$

decreased after H9C2 suffered to H_2O_2 (H_2O_2 vs. control), treatment with Dex significantly increased the SOD level (Dex + H_2O_2 vs. H_2O_2, $p < 0.01$), YOH cancelled Dex-mediated enhancement in the content of SOD (YOH + Dex + H_2O_2 vs. Dex + H_2O_2, $p < 0.01$). The Protein HO-1 could defense against oxidative damage to confer cardioprotective effect. As Fig. 3d showed, the expression of protein HO-1 was significantly enhanced after treatment with Dex (Dex + H_2O_2 vs. H_2O_2, $p < 0.01$), however, YOH pretreatment abolished the protective effect of Dex on HO-1(YOH + Dex + H_2O_2 vs. Dex + H_2O_2, $p < 0.01$). All of the above suggested that the benefit effects of Dex on H_2O_2-induced oxidative stress were associated with or mediated by the activation of α2-AR in cardiomyocytes.

Dex ameliorated H_2O_2-induced apoptosis in cardiomyocytes independent of the activation of α2-AR

H_2O_2 is widely experimentally used as a study to induce cell apoptosis. As shown in Fig. 4a and b, Dex preconditioning significantly ameliorated H_2O_2-induced apoptosis evidenced by decreases in TUNEL-positive cells ratio (H_2O_2 vs. control, Dex + H_2O_2 vs. H_2O_2, all $p < 0.05$, Fig. 4a, b), and the

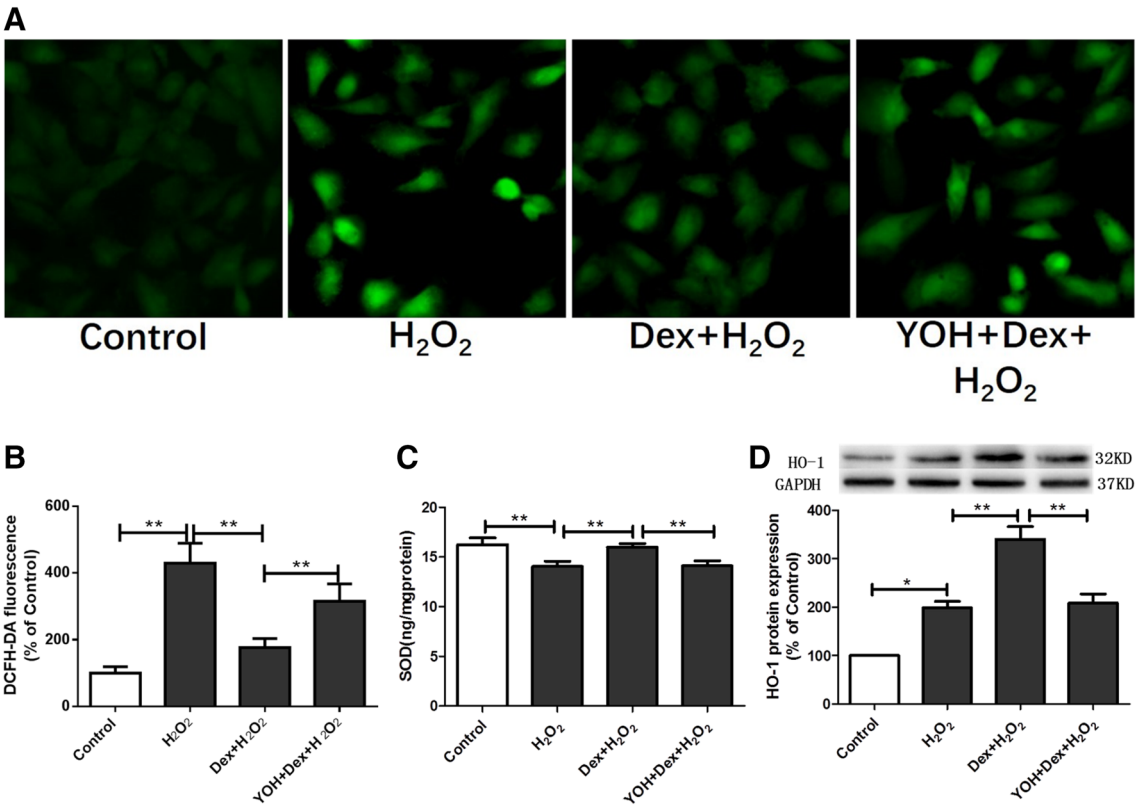

Fig. 3 Effects of Dex preconditioning on H_2O_2-induced oxidative stress were attenuated after α2-AR inhibited. **a** Representative fluorescence microscopy images of the cells stained with DCFH-DA. **b** Quantification of fluorescence density of the cells stained with DCFH-DA. **c** Quantitative analysis of SOD by ELISA. **d** Western blot analysis of HO-1 expression in cells. H9C2 cells were subjected to H_2O_2 for 12 h. Data are mean ± SEM, n = 6 per group. $*p < 0.05$; $**p < 0.01$

expression of the pro-apoptosis protein cleaved caspase-3 was also significantly decreased when cells were treated with Dex (H_2O_2 vs. control, Dex + H_2O_2 vs. H_2O_2, all $p < 0.05$, Fig. 4c). Interestingly, application of YOH did not influence the protective effects of Dex on H_2O_2-induced apoptosis (YOH + Dex + H_2O_2 vs. Dex + H_2O_2, $p > 0.05$).

Dex prevented H_2O_2-induced necroptosis by activating α2-AR in cardiomyocytes

H_2O_2 treatment triggered not only apoptosis, but also necrosis. It has been validated that exposure of H9C2 cardiomyocytes H_2O_2 (500 μM) for 12 h predominantly caused necrosis [9]. As shown in Fig. 5a and b, necrosis was assessed by PI staining, Dex preconditioning significantly reduced H_2O_2-induced augment in number/percentage of necrotic cells (H_2O_2 vs. control, $p < 0.05$), YOH cancelled the protective effect of Dex in reducing number/percentage of necrotic cells. RIPK1 and RIPK3 are well known to be the crucial factors of necroptosis. During the process of necroptosis, the expression of RIPK1 and RIPK3 were increased [9]. Dex preconditioning significantly attenuated the increase of RIPK1 and RIPK3 induced by H_2O_2 (H_2O_2 vs. control, Dex + H_2O_2 vs. H_2O_2, all $p < 0.01$, Fig. 5d, e). After pretreatment with YOH, the expressions of protein RIPK1 and RIPK3 were all significantly increased compared to Dex + H_2O_2 group (YOH + Dex + H_2O_2 vs. Dex + H_2O_2, $p < 0.05$), which suggest that the effects of Dex on decreasing the necrosis was abolished. According to these results, Dex precongditioning prevented H_2O_2-induced cardiomyocytes necroptosis, but this effect was eliminated after the inhibition of α2-AR.

Discussion

In our study, Dex preconditioning conferred protective effects on H_2O_2-induced cell injury, as confirmed by improving cell viability, decreasing LDH release intracellular oxidative stress, cell apoptosis and necroptosis. However, these protective effects were weakened by administration of the selective α2-AR inhibitor YOH, but it had no influence on the protective effects of Dex on H_2O_2-induced apoptosis, these findings demonstrated that Dex preconditioning protected cardiomyocytes against oxidative stress induced necroptosis by activating α2-AR and partly

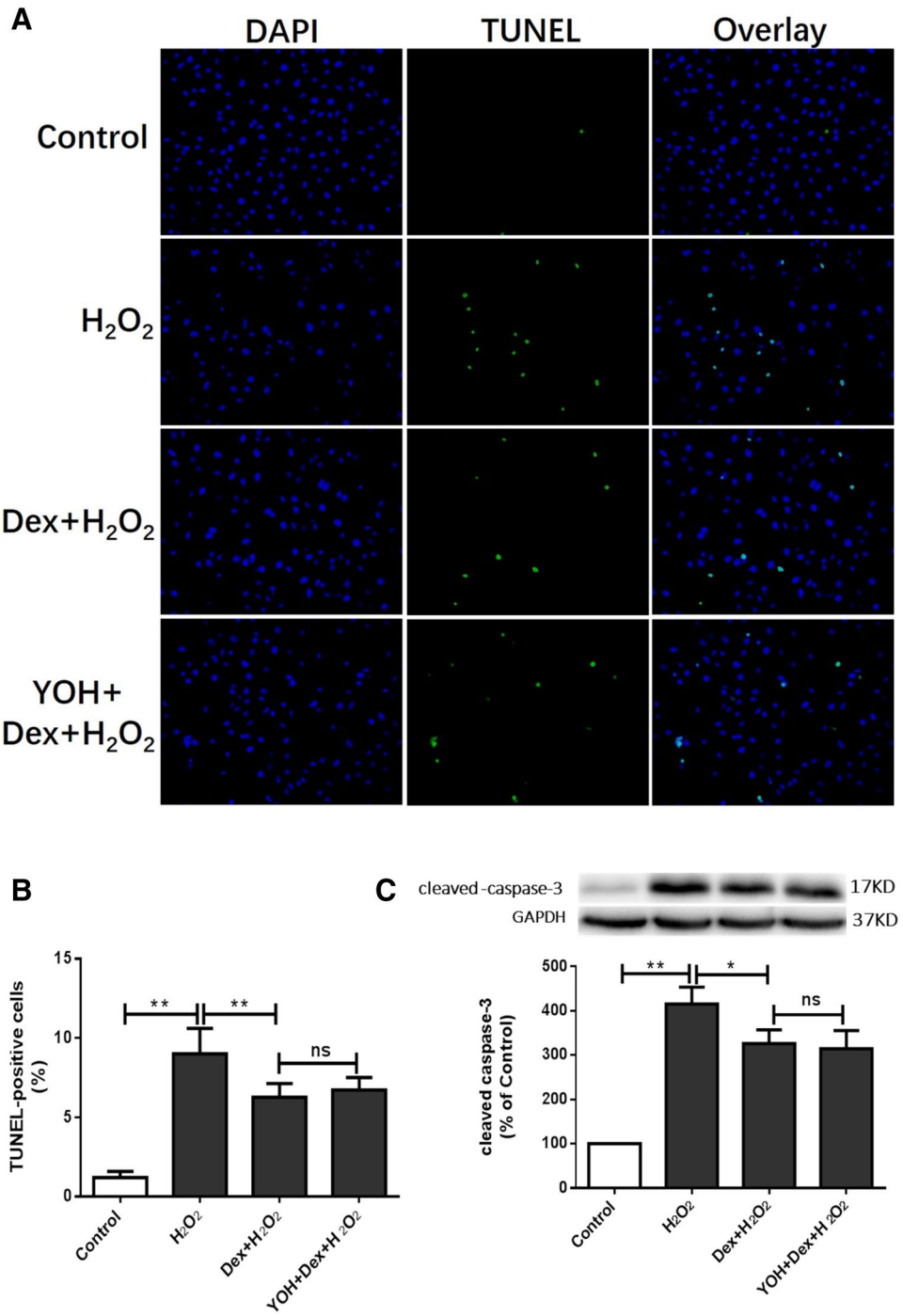

Fig. 4 Inhibition of α2-AR had no effect on the protective effects of Dex on H_2O_2-induced apoptosis. **a** Apoptosis was assessed by TUNEL staining. **b** Statistical analysis of the proportion of the TUNEL positive cells. **c** Western blot analysis of cleaved caspase3 expression. H9C2 cells were subjected to H_2O_2 for 12 h. Data are mean ± SEM, n = 6 per group. $^*p < 0.05$; $^{**}p < 0.01$

through reducing apoptosis. Dexmedetomidine (Dex) is a highly selective α2-adrenoceptor agonist, whose superiority is sedative, anxiolytic, analgesic, and with sympatholytic properties [21]. Clinical studies showed that Dex was associated with improved outcomes and reduced mortality in patients undergoing cardiac surgery [12, 26]. In in vivo study, Dex preconditioning reduced the myocardial infarct size by inhibiting inflammation via the α2-adrenergic receptor activation in rat myocardial I/R injury [12, 16], but the definite mechanism was not fully illuminated.

As a precursor of ROS, H_2O_2 was often used to generate oxidative stress in cells. The results of this study showed that H_2O_2 exposure elevated ROS level in H9C2 cells. When cells were exposed to exogenous H_2O_2, H_2O_2 not only directly damage plasma membrane [27], but also entered into cell through the water channels on plasma membrane

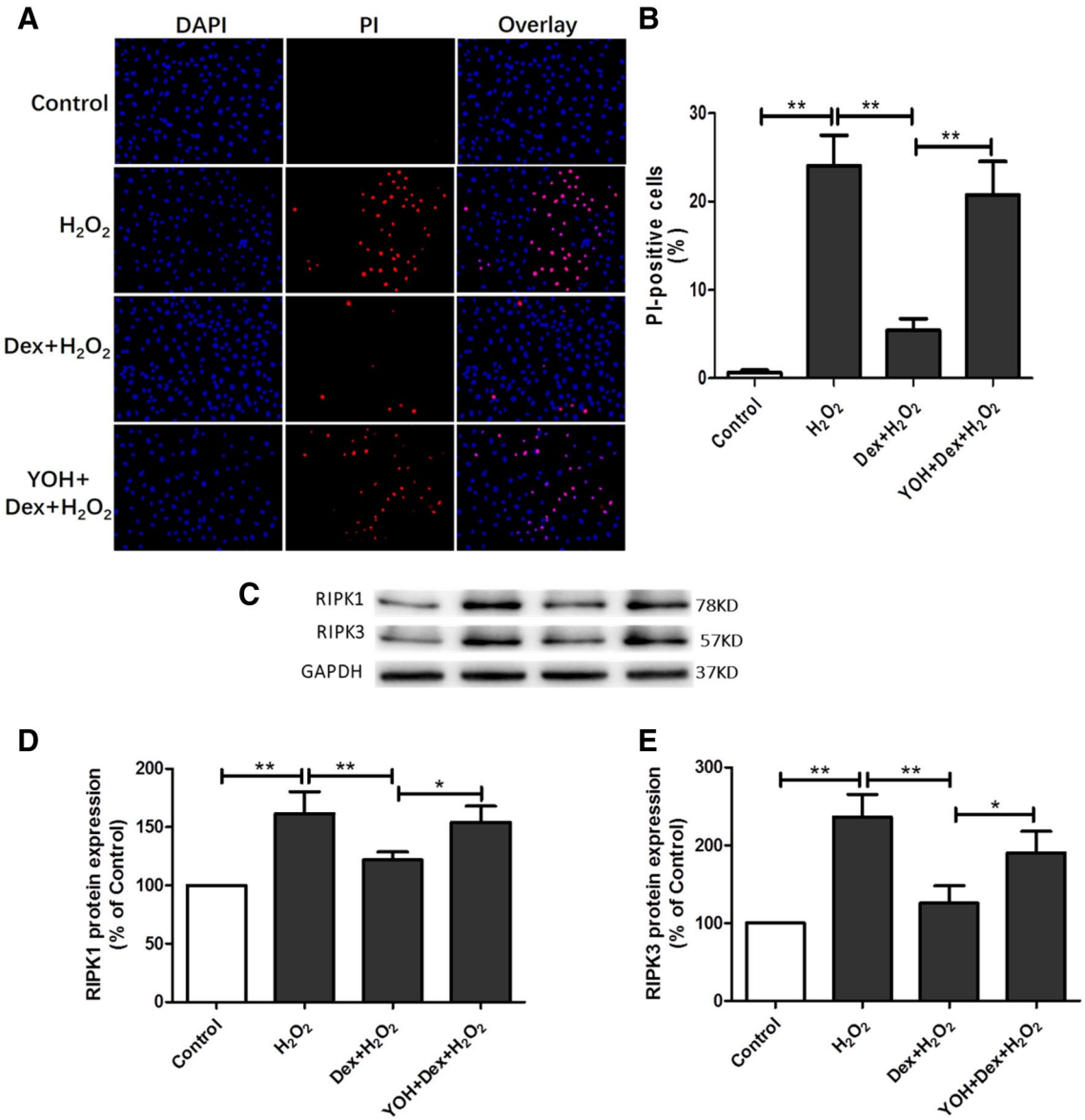

Fig. 5 Inhibition of α2-AR cancelled or weakened the protective effect of Dex on H_2O_2-induced necroptosis. **a** Necroptosis was assessed by PI staining. **b** Statistical analysis of the proportion of the PI positive cells. **c** The WB bands of RIPK1, RIPK3 and GAPDH **d** Western blot analysis of expression of protein RIPK1. **e** Western blot analysis of expression of protein RIPK1. H9C2 cells were subjected to H_2O_2 for 12 h. Data are mean ± SEM, n = 6 per group. $^{*}p < 0.05$; $^{**}p < 0.01$

to elevate the level of intracellular ROS [28]. ROS level elevated by exogenous or endogenous factors could stimulate mitochondria to produce much more ROS, this phenomenon is known as ROS-induced ROS release (RIRR) [29]. The accumulation of intracellular ROS can modify proteins, glucose, lipids, and nucleic acids in cells and tissues to cause dysfunction and cell death [30]. It has been reported that Dex pretreatment attenuates the accumulation of intracellular ROS induced by H_2O_2 through improving the function of mitochondria and endoplasmic reticulum [20, 21]. Similarly, in our study, Dex preconditioning attenuated the accumulation of intracellular ROS in cardiomyocytes, however, YOH administration cancelled this protective effect of Dex, suggesting that the activation of α2-AR is required for Dex to confer protective effect on the excessive ROS production. H9C2(2-1) is a subclone of the original clonal cell line derived from rat heart and exhibits almost whole properties of cardiomyocyte. It has been demonstrated that alpha-2 adenoreceptor is expressed in neonatal rat cardiomyocytes [21] and that Dex is a highly selective α-2 adrenoceptor agonist. Therefore, in the current study, YOH, an alpha 2-adrenoreceptor antagonist, was employed to address the role of alpha-2 adenoreceptor activation in Dex preconditioning mediated protective effects in H9C2 cells. Additionally, we

also found that Dex enhanced the expression of SOD and HO-1 which are potent antioxidant enzyme, but this beneficial effect of Dex was reversed by YOH administration. These findings demonstrated an essential role of α2-AR in Dex mediated protection of cardiomyocytes against oxidative stress.

Apoptosis, a form of programmed cell death, has been demonstrated to play an important role in myocardial I/R injury [31]. Apoptosis is aroused by the activation of caspases, especially caspase-3 [32]. The excessive ROS can lead to mitochondria dysfunction and caspase activation, and considered as a trigger factor of cell apoptosis [33]. Previous studies have reported that apoptosis takes place in various cell types after H_2O_2 treatment [34]. In our study, we found that Dex preconditioning significant reduced H9C2 apoptosis induced by H_2O_2 and these findings are consistent with previous reports which showed that Dex preconditioning protected cardiomyocytes against apoptosis induced by H_2O_2 [20, 21]. Further, we also found that administration of YOH did not alter the effects of Dex preconditioning on H_2O_2-induced apoptosis, thus, we speculated that Dex may have inhibited H_2O_2-induced cell apoptosis primarily via activation other receptors in cardiomyocytes at least in the current experimental settings, and Zhang et al. demonstrated that Dex conferred protection effect against oxygen–glucose deprivation-induced injury through the I2 imidazoline receptor-PI3K/AKT pathway in rat C6 glioma cells [13], this potentially intriguing hypothesis may need to be tested in future studies.

Both apoptosis and necrosis can occur in cardiomyocyte during the process of myocardial I/R [35], under some circumstances of more severe insult, necroptosis may be the major form of cell death during myocardial I/R injury [4, 17]. Apoptosis has always been recognized as a gene-directed and regulated process, and it is mainly rely on caspase activation. Necrosis was defined as an uncontrolled form cell death, and recently it has been shown that necrosis occurred also in a highly regulated way and both apoptotic and necrosis are considered to be regulated by, in part, overlapping molecular machineries [36]. Caspase-independent programmed necrosis has been termed necroptosis, and receptor-interacting erine/threonine protein kinase (RIPK)1 or the phosphorylation of the pseudo kinase mixed-lineage kinase domain-like protein (MLKL) (necrosulfonamide), are central proteins of the necroptosis signalling cascade. The differences between apoptosis and necrosis was that in apoptosis, the plasma membrane remains intact until the dying cell undergoes phagocytosis, whereas in necrosis cell membrane become leaky and pro-inflammatory molecules was released [37]. Previous studies showed that treatment with H_2O_2 (500 μM) for 12 h predominantly triggered necroptosis in cardiomyocytes [9, 17], and reducing necroptosis significantly attenuated myocardial I/R injury [9]. In the current study, we successfully induced necroptosis by exposing cardiomyocytes to 500 μM H_2O_2 for 12 h, and we found that Dex preconditioning remarkably prevented cell necroptosis manifested by decreasing the expressions of protein RIPK1 and RIPK3, which are major representative proteins in the process of necroptosis [9, 38, 39]. However, the protective effect of Dex preconditioning on necroptosis was cancelled after the administration of YOH which demonstrated that the effect of Dex in attenuating oxidative stress-induced necroptosis in cardiomyocytes is mediated.

It should be noted that our current study has its limitations. Although we showed Dex preconditioning suppressed oxidative stress, reduced cell death by inhibiting apoptosis and necroptosis, we did not identify whether the reduction of the cell death was only in response to the oxidative stress suppression. Our study also showed the protective effects of Dex were not absolutely depend on the activation of α2-AR, but we did not further investigate the role of other receptor, such as imidazoline receptor. Actually, as reported, in part, overlapping molecular machineries were found in apoptosis and necrosis [36]. Tumor necrosis factor (TNF) family may play different roles in promoting cell survival, apoptosis and necrosis depending on other signals and conditions in the cell during apoptosis and necrosis process [37]. Necroptosis is a caspase-independent cell death which is mainly controlled by RIP1 and RIP3, and thus necroptosis is a category of necrosis that is highly regulated. Until now, the recognized pathway of necroptosis activation is mediated by death receptors most often by tumour necrosis factor receptor1(TNFR1), although studies also reported that the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas receptors can also induce necroptosis. Briefly, during the necroptosis, when a ligand binds to TNFR1, prosurvival complex I which included TNFR-associated death domain (TRADD), RIP1 and several ubiquin E3 was recruited. In complex I, deubiquitination of RIP1 induced either complex IIa or IIb formating. Complex IIa activates caspase 8 leading to apoptosis, in another condition, when caspase 8 is inhibited, complex IIb is formed and activates necroptosis. To initiate necroptosis via complex IIb, RIP1 recruits RIP3 and phosphorylated RIP3 and necrosome (a multiprotein complex resembling amyloid) appeared. Mixed lineage kinase domain-like pseudokinase (MLKL) which is phosphorylated by RIP3 is also involved in necroptosis. Following phosphorylation, MLKL oligomerizes and then translocate from the cell cytoplasm to the membrane and this results in necrosis and cell death [11, 40]. In our study, we perform the experiments about RIP1 and RIP3, in future study, we should study the signaling pathway: TNFR-RIP1-RIP3-MLKL to clarify the mechanism of cardioprotection effects of Dex. Future studies will also be performed on the effects of Dex related to other

receptors to further consummate the mechanisms of Dex beneficial effects.

Conclusion

H_2O_2 induced H9C2 cells injury through increasing oxidative stress, apoptosis and necroptosis. Dex preconditioning significant increased cell viability, SOD level and the expression of protein HO-1, decreased apoptosis cells, the expressions of protein cleaved-caspase3, RIPK1 and RIPK3, and consequently, suppressed H_2O_2 exposure induced oxidative stress, apoptosis and necroptosis in cardiomyocytes. YOH, which is selective α-2 adrenoceptor agonist cancelled the protection effects of Dex on attenuating oxidative stress and necroptosis, but had no influence on apoptosis, according to this, we conclude that Dex conferred it protective effects respectively dependently and independently of α2-AR activation.

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Compliance with ethical standards

Conflict of interest All the authors declared that they have no conflict of interest.

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