Original Paper

Beta-1-Adrenergic Receptors Mediate Nrf2-HO-1-HMGB1 Axis Regulation to Attenuate Hypoxia/Reoxygenation-Induced Cardiomyocytes Injury *in Vitro*

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Key Words

Nuclear factor erythroid 2-related factor 2 • Heme oxygenase-1 • High mobility group box 1 protein

Abstract

Background/Aims: The aim of the study was to evaluate the effects of beta1-adrenergic receptors (β 1-ARs) -mediated nuclear factor erythroid 2-related factor 2 (Nrf2)-heme oxygenase-1 (HO-1)-high mobility group box 1 protein (HMGB1) axis regulation in hypoxia/reoxygenation (H/R)-induced neonatal rat cardiomyocytes. **Methods:** The neonatal cultured cardiomyocytes were concentration-dependently pretreated by dobutamine (DOB), a selective β 1-adrenergic receptor agonist, in the absence and/or presence of LY294002 (a phosphatidylinositol 3-kinase (PI3K) inhibitor), SB203580 (a p38mitogen-activated-protein kinase (p38MAPK) inhibitor), Nrf2siRNA and HO-1siRNA, respectively, and then treated by H/R. The effects and mechanisms by which H/R-induced cardiomyocytes injury were evaluated. **Results:** Significant increase of HO-1 was found in neonatal cultured cardiomyocytes treated with DOB, when compared to the control group. Significant change for Nrf2 translocation was also revealed in neonatal cultured cardiomyocytes treated with DOB. Insignificant decreases of NF-kappaB p65 activation and HMGB1 release were observed in H/R-induced neonatal cultured cardiomyocytes treated with DOB, when compared to the control group. Importantly, DOB treatment significantly increased the cell viability and decreased the levels of LDH and MDA in H/R-induced cardiomyocytes injury. However, DOB failed to increase HO-1, inhibit NF-kappaB p65 activation, prevent HMGB1 release and attenuate H/R-induced cardiomyocytes injury when the cultured cardiomyocytes were pretreated by Nrf2siRNA, HO-1siRNA, PI3K inhibitor (LY294002) and p38MAPK inhibitor (SB203580), respectively. **Conclusions:** β 1-ARs-mediated Nrf2-HO-1-HMGB1 axis regulation plays a critical protective role in H/R-induced neonatal rat cardiomyocytes injury *in vitro* via PI3K/p38MAPK signaling pathway.

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Introduction

Myocardial reperfusion therapy is the optimal therapeutic strategy for ischemic heart disease which reverses myocardial ischemia and limits the infarct size [1]. However, the subsequent ischemia/reperfusion (I/R) injury may reduce the therapeutic advantage [2]. Myocardial I/R is a complex pathophysiological process that involves various factors and pathways, in which the inflammatory response has been proved to be a major cause of I/R-induced tissue injury [1-3]. High mobility group box 1 protein (HMGB1), a highly conserved DNA binding protein that plays an important role in upregulating pro-inflammatory cytokines, has been reported to contribute to the pathophysiological progression of myocardial I/R injury [4, 5]. Hence, reducing HMGB1 release may become a novel therapeutic approach for myocardial I/R injury [6].

Heme oxygenase (HO)-1 has been reported to be expressed in endothelial, epithelial, and mononuclear and smooth muscle cells and plays an important role in anti-inflammatory, anti-apoptotic and anti-proliferating processes [7, 8]. Meanwhile, it has been further confirmed that β 1-adrenergic receptors (ARs)-mediated HO-1 induction via nuclear factor erythroid 2-related factor 2 (Nrf2) translocating from cytosol to nucleus in RAW 264.7 cells, could inhibit HMGB1 release in LPS-activated RAW 264.7 cells [9]. Otherwise, Salie et al. [10] revealed that PI3K activation may be associated with the cardioprotective effects of pre-ischemic β 1-ARs stimulation during myocardial I/R injury. Furthermore, Wang et al. [11] demonstrated that DOB mediated the induction of HO-1 by stimulating β 1-adrenergic receptors via PI3K and p38 MAPK pathway and inhibited HMGB1 release for attenuating rat myocardial I/R injury *in vivo*.

Taken together, the aim of the study was to evaluate the effects of β 1-ARs-mediated Nrf2-HO-HMGB1 axis regulation in hypoxia/reoxygenation (H/R)-induced neonatal rat cardiomyocytes.

Materials and Methods

Materials

DOB hydrochloride, SB203580 and LY294002 were purchased from Sigma-Aldrich, St. Louis., MO, USA. The MTT was purchased from Sigma-Aldrich, USA. The antibodies used to recognize Nrf2, HO-1, HMGB1 and NF- κ B p65 were purchased from Sigma-Aldrich, St. Louis., MO, USA. The control siRNA, Nrf2siRNA, HO-1 siRNA and transfection reagents were purchased from Santa Cruz Biotechnology, inc. CA, USA.

Ethics Statement and Study Protocol

The study protocol was approved by the Ethical Committee of Renmin Hospital of Wuhan University, and all animal handling was performed in accordance with the Wuhan Directive for Animal Research and the current Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85-23, revised 1996). The 1-to-3 day old SD rats were supplied by the experimental animal center of medical school of Wuhan University and kept in an environmentally controlled breeding room (temperature: $23 \pm 2^\circ\text{C}$, humidity: $60 \pm 5\%$, 12 h dark/light cycle). Neonatal rat ventricular myocytes were prepared from the hearts of 1-to-3 day old SD rats by enzymatic dissociation, as described previously [12]. Briefly, the rats were anesthetized with sodium pentobarbital (45 mg/kg, i.p.) to ameliorate suffering. Then the hearts were excised for ventricular myocytes preparation. In this study, the details of animal welfare have been seriously taken into consideration.

Cell culture

After scalpel homogenization, the heart tissue was incubated with 0.25% (w/v) trypsin overnight at 4°C , following a 0.1% (w/v) collagenase treatment for 20 min at 37°C . Cardiomyocytes were enriched by percoll gradient centrifugation (Amersham, USA) and plated at a density of $5 \times 10^5/\text{ml}$ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum at 37°C and 5% (v/v) CO_2 for 24 hours as described previously [13]. Then the cardiomyocytes were washed by balanced salt solution and the medium was replaced. The cardiomyocytes were then underwent 72-hour further incubation.

MTT assay for determination of cell viability

The cell viability was determined colorimetrically using the MTT reagent as described previously [13]. Briefly, the cultured cells were seeded at a density of 1×10^5 /ml per well. After 72-hour further incubation, MTT was added (final concentration, 0.5 mg/ml) to each well. After 3 h of additional incubation, 100 μ l of a solution of 10% SDS and 0.01 N HCl was added to dissolve the crystals for 16 h. Absorbance values at the test wavelength of 570 nm was determined with an automatic Microplate Reader. The relative cell viability was expressed as a percentage of the control group.

Establishment of hypoxia/reoxygenation (H/R)-induced cardiomyocytes injury

The cultured cardiomyocytes from neonatal rats were exposed to 6 h of hypoxia followed by 3 h of H/R as described previously [12]. Briefly, injury of cultured cardiomyocytes induced by H/R was conducted *in vitro* to simulate the myocardial I/R injury *in vivo*. To induce hypoxia, the myocytes were incubated in glucose-free anoxic Hank's solution and transferred to a gas chamber in an atmosphere of 95% N₂/5% CO₂. After 6-hour hypoxia, the culture medium was replaced with fresh oxygenated DMEM supplemented with 15% new bovine serum, and the dishes were transferred to a normoxic incubator full of 95% air / 5% CO₂ for 3-hour reoxygenation.

Nrf2 and HO-1 small interfering RNA transfection study

Cultured cardiomyocytes were transfected with 300 nM control siRNA and Nrf2 or HO-1 siRNA using transfection reagents according to the manufacturer's instructions. The cells were incubated for 24 h in serum-free media. The transfected cells were washed with 4ml of PBS and pretreated with or without DOB, following H/R treatment.

Drugs treatment and H/R-induced cardiomyocytes injury

Nrf2siRNA, HO-1siRNA, PI3K inhibitor (LY294002), p38MAPK inhibitor (SB203580), and dimethyl sulfoxide (DMSO) were administered 30 min before DOB, respectively. Then the cultured cardiomyocytes were treated with DOB (50, 100, 200 μ M) or without DOB for 8 h. After 8 hours, the cultured cardiomyocytes were exposed to 6 h of hypoxia followed by 3 h of H/R as described above. The effects of drugs treatment in H/R-induced cardiomyocytes injury were evaluated.

Assessment of lactate dehydrogenase (LDH) and malondialdehyde (MDA) in H/R-induced cardiomyocytes injury

Standard techniques using commercialized assay kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China) were performed for assessing the levels of LDH and MDA in H/R-induced cardiomyocytes injury.

Western blot analysis

Cytoplasmic and nuclear protein extracts were prepared from cultured cardiomyocytes as reported previously [13]. Western blotting was performed according to the manufacturer's procedures and n values for western blotting in each group were 6. Briefly, 50 μ g of cytoplasmic or nuclear proteins was separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. Non-specific binding sites were blocked with 5% non-fat dry milk in Tris-buffer saline (TBS)-0.05% Tween. The membrane was subsequently probed with primary antibody (anti-HO-1 antibody, anti-Nrf2 antibody, anti-HMGB1 antibody and anti-NF- κ B p65 antibody) and incubated in horseradish peroxidase-conjugated secondary antibody. The protein bands were visualized by an enhanced chemiluminescence system, and β -actin was used as an internal control to correct the variations of different samples. The expression levels of HO-1, Nrf2, HMGB1 and NF- κ B p65 were indicated as a ratio of HO-1, Nrf2, HMGB1 and NF- κ B p65 to β -actin.

Statistical analysis

All continuous values were expressed as mean $\pm$ S.D. Student t-test was used for between-group comparisons. One-way ANOVA or Welch was used for comparisons among groups and the Student-Neuman-Keuls or Dunnett T3 was used for post-hoc multiple comparisons. P values less than 0.05 was considered statistically significant.

Fig. 1. Effects of DOB on cell viability in cultured cardiomyocytes of neonatal rat. Data represented as means $\pm$ SD. **P < 0.01 compared to control group.

Results

Cell viability

To select optimal concentration of dobutamine, we tested cellular toxicity by MTT assay. As shown in Figure 1, the cell viability of myocardial cell cultured under normoxic conditions with DOB (50, 100, 200, 300 μ M) was decreased in a concentration-dependent manner. When the concentration of DOB was more than 200 μ M, the cell viability of myocardial cell was significantly decreased compared to that in the control group ($P < 0.01$). Therefore, the concentration of DOB was limited to 200 μ M throughout the experiment.

β 1-ARs-mediated HO-1 induction in a time- and concentration-dependent manner in neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes were incubated under normoxic conditions with DOB for 8 h at different concentrations (Fig. 2A). Cells were incubated for the indicated period of time with a fixed concentration of DOB (200 μ M) (Fig. 2B). As shown in Figure 2A and 2B, we confirmed that DOB increased HO-1 expression in a time- and concentration-dependent manner through the β 1-ARs in neonatal rat cardiomyocytes.

β 1-ARs mediated HO-1 induction via Nrf2 translocation in neonatal rat cardiomyocytes

To confirm whether Nrf2 translocation is involved in HO-1 induction by DOB, cytosol and nuclear fraction were separated after 3 h of incubation under normoxic conditions with the indicated concentrations of DOB [9] (Fig. 3A, 3B). As shown in Figure 3A and 3B, DOB significantly and concentration-dependently translocated Nrf2 from cytosol to nucleus as required initiating HO-1 induction. Furthermore, DOB (200 μ M) failed to induce HO-1 induction in Nrf2 siRNA-transfected cells (Fig. 3C), suggesting that Nrf2 is an important regulator involved in β 1-ARs-mediated HO-1 induction by DOB.

β 1-ARs mediated HO-1 induction via a PI3K- and p38 MAPK-dependent pathway in neonatal rat cardiomyocytes

The neonatal rat cardiomyocytes were incubated under normoxic conditions and pretreated 30 min with different concentrations of LY294002, a PI3K inhibitor (Fig. 4A) or SB203580, a p38 MAPK inhibitor (Fig. 4B) prior to addition of DOB (200 μ M) to identify the signaling pathway involved in HO-1 induction. HO-1 protein was detected after 8 h of incubation under normoxic conditions and treated with DOB. The results indicated that LY294002 and SB203580 significantly and concentration-dependently inhibit HO-1 protein expression induced by DOB in neonatal rat cardiomyocytes. Furthermore, DMSO did not involve in β 1-ARs mediated HO-1 induction via a PI3K- and p38 MAPK-dependent pathway in neonatal rat cardiomyocytes (Fig. 4C).

β 1-ARs mediated HMGB1 inhibition via a PI3K- and p38 MAPK-dependent pathway in H/R-induced neonatal rat cardiomyocytes

To address whether PI3K/p38 MAPK pathway is also involved in β 1-ARs-mediated HMGB1 inhibition, cardiomyocytes were pretreated with the indicated concentrations of

Fig. 2. β 1-ARs mediated HO-1 induction in a time- and concentration-dependent manner in neonatal rat cardiomyocytes. Western blot analysis was performed. Data represented as means $\pm$ SD. * P < 0.05, compared to the control group.

Fig. 3. β 1-ARs mediated HO-1 induction via Nrf2 translocation in neonatal rat cardiomyocytes. Western blot analysis was performed. Data represented as means $\pm$ SD. * P < 0.05, compared to corresponding control group; # P < 0.05, compared to corresponding control group; ▲ P < 0.05, compared to DOB (200 μ M) group.

DOB 1 h before induction of H/R. LY294002 (10 μ M) or SB203580 (10 μ M) was administered 30 min prior to treatment with DOB [9]. Cardiomyocytes were incubated for 16 h after H/R-Induced for detecting HMGB1. As shown in Figure 5A and 5B, DOB significantly and concentration-dependently inhibited HMGB1 release, which was reversed by LY294002 and SB203580 in H/R-induced neonatal rat cardiomyocytes, respectively. Furthermore, DMSO did not involve in β 1-ARs mediated HMGB1 inhibition via a PI3K- and p38 MAPK-dependent pathway in neonatal rat cardiomyocytes (Fig. 5C).

β 1-ARs mediated NF- κ B p65 inhibition via HO-1 induction in the H/R-induced neonatal rat cardiomyocytes

The present study addressed whether β 1-ARs mediated NF- κ B p65 inhibition via HO-1 induction in H/R-induced neonatal rat cardiomyocytes. As shown in Figure 6, DOB concentration-dependently inhibited NF- κ B p65 activation in H/R-induced neonatal rat cardiomyocytes. However, the effect was significantly reversed by the presence of HO-1siRNA.

β 1-ARs mediated HO-1 induction to reduce HMGB1 release in H/R-induced neonatal rat cardiomyocytes

To further confirm whether β 1-ARs-mediated HMGB1 inhibition is due to the induction of HO-1 by the treatment of DOB, cardiomyocytes were pretreated with HO-1siRNA which was co-administered with DOB (100, 200 μ M) 1 h prior to H/R-Induced. As indicated in Figure 7, H/R-induced injury increased HMGB1 release in the media after 16 h in neonatal rat cardiomyocytes, while pretreatment with DOB(100, 200 μ M) significantly and concentration-dependently reduced the release of HMGB1. However, these effects were significantly reversed by the presence of HO-1siRNA.

β 1-ARs-mediated Nrf2-HO-1-HMGB1 axis attenuated H/R-induced cardiomyocytes injury

To further evaluate whether DOB could attenuate H/R-induced neonatal rat cardiomyocytes injury via β 1-ARs-mediated Nrf2-HO-1-HMGB1 axis regulation, the cell viability and the levels of LDH and MDA in H/R-induced cardiomyocytes injury were assessed. As shown in Figure 8 (A, B, C), DOB treatment could significantly increase the cell viability (Fig. 8A) and decrease the levels of LDH (Fig. 8B) and MDA (Fig. 8C) in H/R-induced

cardiomyocytes injury. However, DOB failed to attenuate H/R-induced cardiomyocytes injury when the cultured cardiomyocytes pretreated by Nrf2siRNA, HO-1siRNA, LY294002, and SB203580, respectively.

Discussion

Nuclear factor erythroid 2-related factor 2 (Nrf2), a nuclear transcriptional factor, has been reported to play an important role in anti-inflammatory and anti-oxidative stress in

Fig. 7. β 1-ARs mediated HMGB1 release via HO-1 induction in the H/R-induced neonatal rat cardiomyocytes. After 16 h, the detection of HMGB1 in the nucleus was performed by Western blot analysis. Data represented as means $\pm$ SD. * P < 0.05, compared to corresponding control; # P < 0.05, compared to corresponding control group; ΔP < 0.05, compared to DOB (200 μ M) group.

Fig. 8. β 1-ARs-mediated Nrf2-HO-1-HMGB1 axis regulation attenuates H/R-induced cardiomyocytes injury. Data represented as means $\pm$ SD. * P < 0.05 versus Sham group; # P < 0.05 versus H/R group; ΔP < 0.05 versus DOB-H/R group.

liver, kidney, lung, vascular endothelial [14-16]. As known, Nrf2 is usually present within the cytosol as a complex with Keap-1 protein and released from this complex and transported into nucleus. Then the transported Nrf2 forms a new complex with Maf protein, and induces the transcription of various antioxidant by binding to antioxidant response element (ARE) on

DNA and the specific enzymes that are activated, include HO-1, which may elicit antioxidant and anti-inflammatory effects in many cells [9, 17-22]. Meanwhile, HO-1 has been reported to depend on PI3K/p38MAPK signaling pathway in many cells [9, 23-25]. Otherwise, HO-1 has been further demonstrated to be induced by dobutamine and inhibit HMGB1 release during myocardial I/R injury [11]. Importantly, cultured neonatal cardiomyocytes may present with a very stable phenotype and the contractile profile of cultured neonatal cardiomyocytes during H/R has been proved to be comparable with that of in situ adult hearts during I/R [26-29].

In the present study, before cells exposure to H/R, DOB concentration-dependently induced HO-1 protein expression and caused Nrf2 translocated from cytosol to nucleus in cardiomyocytes of neonatal rat via β 1-adrenergic receptors stimulation. However, these effects were significantly abolished by LY294002, a specific PI3K inhibitor; SB203580, a p38 MAPK inhibitor and Nrf2siRNA, respectively. Hence, β 1-ARs-mediated Nrf2 translocation may initiate HO-1 induction via PI3K/p38MAPK signaling pathway in cardiomyocytes.

HMGB1, as a novel pro-inflammatory cytokine, could be first detectable 8 hours after the onset of various stimuli, then increases to plateau levels from 16 to 32 hours, and maintains at elevated level for at least 72 hours [30]. Importantly, HMGB1 has been further proved to contribute to the pathophysiological progression of myocardial I/R injury by promoting the apoptosis of myocardium and inflammatory response in rats [4-6]. Hence, reducing HMGB1 release may become a novel therapeutic approach for myocardial I/R injury [6]. Furthermore, Takamiya et al [31] had demonstrated that the expression levels of HMGB1 release were higher in HO-1^{-/-} mice than in HO-1^{+/+} mice. Likewise, the HO-1 induction has been further proved to inhibit the release of HMGB1 in endotoxin-activated macrophages *in vitro* and septic animals *in vivo* [32], which was further demonstrated in H/R-induced cardiomyocytes in the present study. Hence, β 1-ARs-mediated Nrf2-HO-1 may inhibit HMGB1 release in H/R-induced cardiomyocytes.

In addition, the NF- κ B activation has been proved to be critical for induction of inflammatory cytokines such as TNF- α , IL-1 β , NO, and release of HMGB1 in macrophages [33]. Meanwhile, Nrf2 has been further demonstrated to suppress NF- κ B-activation, but the precise mechanisms require future elucidation [34]. In the current study, neonatal cultured cardiomyocytes were subjected to 6 h of hypoxia followed by 3 h of reoxygenation as a cellular model to mimic adult I/R. The results demonstrated that DOB inhibited the NF- κ B p65 activation which was paralleled with the decrease of HMGB1 release. Thus, it is reasonable to speculate that the β 1-ARs-mediated Nrf2-HO-1 may play an important role in anti-NF- κ B p65 activation and anti-HMGB1 release in H/R-induced neonatal rat cardiomyocytes.

In conclusion, the present study demonstrated an important axis regulation in H/R-induced neonatal rat cardiomyocytes, i.e., stimulation of the β 1-ARs by DOB make it possible for Nrf2 to move to the nucleus to bind to the ARE promoter site, leading to the upregulation of HO-1 gene expression. Significant increase of HO-1 expression inhibits NF- κ B p65 activation and HMGB1 release via PI3K/p38MAPK signaling pathway to attenuate cardiomyocytes H/R-induced injury *in vitro*.

Abbreviations

AR (Adrenergic receptor); ARE (Antioxidant response element); CLP (Cecal ligation and puncture); CRP (C-reactive protein); DOB (Dobutamine); DMSO (Dimethyl sulfoxide); HMGB1 (High mobility group box 1 protein); HO-1 (Heme oxygenase-1); H/R (Hypoxia/Reoxygenation); I/R (Ischemia and reperfusion); LPS (Lipopolysaccharide); LDH (Lactate dehydrogenase); MDA (Malondialdehyde); MTT (3-(4, 5-dimethylthiazolyl)-2, 5 diphenyltetrazolium bromide); NF- κ B (Nuclear factor-kappa B); Nrf2 (Nuclear factor erythroid 2-related factor 2); PI3K (Phosphatidylinositol 3-kinase); P38 MAPK (P38 mitogen-activated-protein kinase); 2-MCA (2-Methoxycinnamaldehyde).

Acknowledgements

The present study only demonstrated that DOB inhibited the NF- κ B p65 activation which was paralleled with the anti-HMGB1 release by inducing HO-1 expression. However, the regulation relationship between NF- κ B p65 activation and HMGB1 and how HO-1 regulates NF- κ B p65 activation and HMGB1 in the cardiomyocytes should be investigated in future studies.

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Disclosure Statement

None declared.

Reference

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