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Original Article

Effects of high-mobility group box 1 on the expression of Beclin-1 and LC3 proteins following hypoxia and reoxygenation injury in rat cardiomyocytes

Weipan Xu, Hong Jiang, Xiaorong Hu, Wenwen Fu

Department of Cardiology, Renmin Hospital of Wuhan University, Cardiovascular Research Institute of Wuhan University, Wuhan, China

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Abstract: The mechanisms underlying autophagy during myocardial ischemia and reperfusion remain unclear. The present study investigated the relationship between high-mobility group box 1 protein (HMGB1) and autophagy in hypoxia/reoxygenation (H/R)-induced neonatal rat cardiomyocytes. Neonatal rat cardiomyocytes were treated with recombinant HMGB1 (200 ng/L) or ammonium glycyrrhizinate (100 μM) at appropriate concentrations. Cell viabilities and lactate dehydrogenase (LDH) and creatine kinase (CK) activity levels were measured. HMGB1, LC3 and Beclin-1 expression were assessed by Western blot. The results demonstrated that HMGB1-induced myocardial cells have increased levels of Beclin-1 protein and even higher levels of LC3 protein, while HMGB1-inhibited myocardial cells have decreased levels of Beclin-1 and LC3 proteins. In addition, HMGB1 induction significantly increased LDH and CK levels in the cell culture medium; the inhibition of HMGB1 significantly reduced LDH and CK expression in cardiomyocyte culture medium. In conclusion, the results of the present study suggest that HMGB1 is able to regulate Beclin-1 and LC3 levels following hypoxia and reoxygenation injury in rat cardiomyocytes.

Keywords: High-mobility group box 1 protein, autophagy, myocardial hypoxia and reoxygenation

Introduction

Autophagy is a regulated cellular process responsible for the degradation and disposal of damaged and dysfunctional organelles and protein aggregates [1]. It has been reported that autophagy is up-regulated during myocardial ischemia/reperfusion (I/R) due to ATP depletion as well as intracellular calcium overload, the generation of reactive oxygen species (ROS) and mitochondrial permeability transition pore opening [2-4]. Some studies have reported that enhanced autophagy contributes to cell death during I/R [5, 6]. However, another study has suggested that autophagy is protective during ischemia but that it plays a detrimental role during reperfusion [7]. However, the causative role of autophagy in mediating either the survival or the death of cardiac myocytes during stress conditions remains to be elucidated.

High-mobility group box 1 protein (HMGB1), a highly conserved nuclear protein that is released from necrotic cells and secreted by activated macrophages, natural killer cells, and mature dendritic cells, can function as an extracellular signalling molecule during inflammation, cell differentiation, cell migration, and tumour metastasis [8]. Tang et al. have shown that HMGB1 promotes autophagy in response to metabolic stress and oxidant stress, promoting pro-autophagic activities by, for example, controlling Beclin1-Bcl-2 complex formation [9]. Kang et al. have found that the cytoplasmic translocation of HMGB1 is necessary to promote and sustain autophagy in stressed cardiac myocytes, while the inhibition of autophagy can limit HMGB1 translocation [10]. However, whether HMGB1-regulated autophagy is involved in the process of myocardial ischemia/reperfusion (similar to myocardial hypoxia and reoxygenation) injury remains unclear; thus, we evaluated this hypothesis in this study.

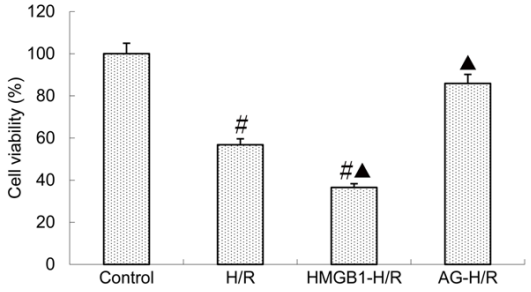

Figure 1. Effects of recombinant HMGB1 and AG on viability of cardiomyocytes. #P<0.05 vs. control group; ▲P<0.05 HMGB1 + H/R group or AG (100 μM) + H/R group vs. H/R group. HMGB1, high-mobility group box 1 protein; AG, ammonium glycyrrhizinate.

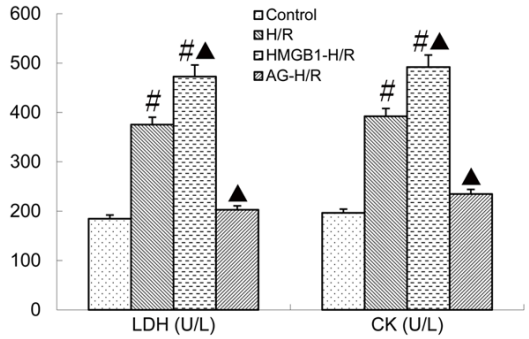

Figure 2. Effects of recombinant HMGB1 and AG on the release of LDH and the activity of CK. #P<0.05 vs. control group; ▲P<0.05 HMGB1 + H/R group or AG (100 μM) + H/R group vs. H/R group. LDH, lactate dehydrogenase; CK, creatine kinase; other abbreviations are identical with those of **Figure 1**.

Materials and methods

Cell culture and treatment

The experimental protocol conformed to the Guideline for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, revised 1996) and was approved by the Institutional Animal Care and Use Committee. One- to three-day-old Sprague-Dawley rats were purchased from the Centre of Experimental Animals at Wuhan University. Primary cultures of neonatal rat cardiomyocytes were prepared from the ventricles of 1-3-day-old SD rats. Briefly, the hearts were harvested and minced into pieces, and the heart tissue was dissociated with 0.125% (w/v) trypsin and 0.08% collagenase I 5 times at 37°C. Cardiomyocytes were enriched and plated at a density of 1×10^6/ml in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal bovine serum (FBS), 1% penicillin (100 U/ml), and 1% streptomycin (100 μg/ml) at 37°C and 5% (v/v) CO_2. They were incubated for 4 days before conducting the experiment.

Experimental design

The cultured cells were divided into 4 groups. In group 1, the control group (Control), cardiomyocytes were incubated in DMEM F12 with 15% FBS. In group 2, the hypoxia and reoxygenation (H/R) group, cardiomyocytes were incubated in DMEM after 24 hours of synchronisation and were then subjected to hypoxia for 2 hours at 37°C and 95% N_2 at 5% (v/v) CO_2. Next, they were incubated in DMEM F12 for 4 hours. In group 3, the HMGB1 + H/R group (HMGB1-H/R), cardiomyocytes were pre-treated with recombinant HMGB1 (200 ng/L) at 24 hours before incubation in DMEM and were then were subjected to hypoxia for 2 hours at 37°C and 95% N_2 at 5% (v/v) CO_2. Next, they were incubated in DMEM F12 for 4 hours at 37°C and 5% (v/v) CO_2. In group 4, the ammonium glycyrrhizinate + H/R group (AG-H/R), cardiomyocytes were pre-treated with ammonium glycyrrhizinate (100 μM, an inhibitor of HMGB1) at 24 hours before incubation in DMEM and then subjected to hypoxia for 2 hours at 37°C and 95% N_2 at 5% (v/v) CO_2. Next, they were incubated in DMEM F12 for 4 hours at 37°C and 5% (v/v) CO_2.

Assay of cell viability

Cell viability was determined using the Cell Counting Kit (CCK)-8 assay (Dojindo, Tokyo, Japan), and the experimental procedure was based on the manufacturer's recommendations. Cardiomyocytes were seeded in 96-well plates at 1×10^5 cells/well and incubated for 4 days before treatments as described above. The absorbance of each well at 490 nm was measured with a microplate reader (Bio-Rad Laboratories, Hercules, CA). The percent cell viability was calculated using the following formula: % cell viability = (mean absorbance in test wells)/(mean absorbance in control wells) ×100.

LDH and CK activities

The extent of cell injury was assessed by the concentrations of LDH and CK in the culture

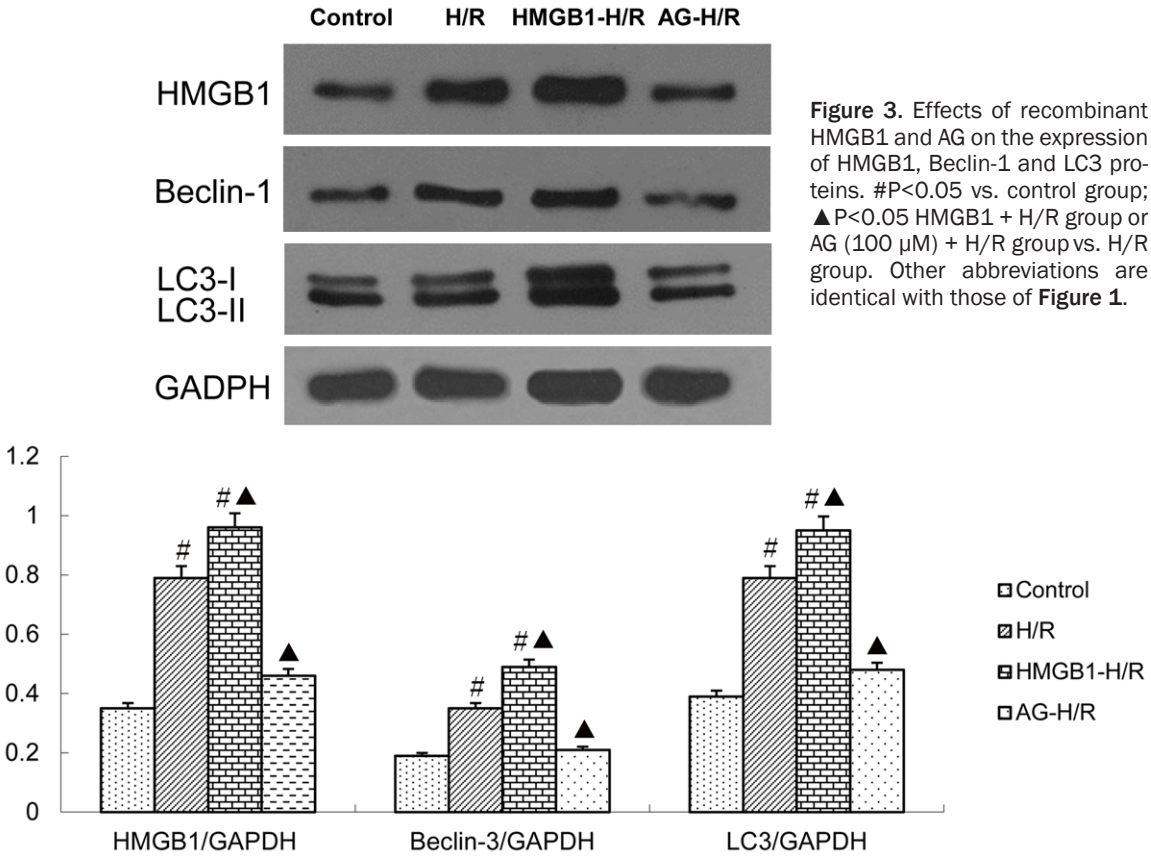

Figure 3. Effects of recombinant HMGB1 and AG on the expression of HMGB1, Beclin-1 and LC3 proteins. #P<0.05 vs. control group; ▲P<0.05 HMGB1 + H/R group or AG (100 μM) + H/R group vs. H/R group. Other abbreviations are identical with those of **Figure 1**.

medium. The protocols were followed according to the manufacturer's instructions (JianCheng Bioengineering Institute, Nanjing, China).

Western blot analysis

The cell extracts were prepared for quantitative immunoblotting using HMGB1, LC3 and Beclin-1 antibodies as described previously [9]. GAPDH antibody (1:5000 dilution; Cell Signaling) was used as the loading control.

Statistical analysis

Statistical analysis was conducted with SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). All data are expressed as the mean ± SD. One-way ANOVA or the Welch test was used for comparisons among groups, and the Student-Newman-Keuls test or Dunnett's T3 test was used for post-hoc multiple comparisons. P<0.05 was considered statistically significant.

Results

Cell viability

As shown in **Figure 1**, after 2 hours of hypoxia exposure, cardiomyocyte viability in the H/R group and HMGB1 + H/R group was significantly decreased compared to that in the control group (P<0.05). Moreover, cardiomyocyte viability was greatly decreased in the HMGB1 + H/R group compared with the H/R group but significantly increased in the AG + H/R group.

Levels of LDH and CK

As shown in **Figure 2**, Cardiomyocytes subjected to hypoxia and reoxygenation were able to cause the release of LDH and CK (P<0.05). Furthermore, recombinant HMGB1 increased the levels of LDH and CK (P<0.05), while HMGB1 suppression was able to suppress their release (P<0.05).

Expression of HMGB1, Beclin-1 and LC3

The Western blot result is as shown in **Figure 3**, the expression of HMGB1 significantly increased after hypoxia and reoxygenation (P<0.05). In addition, the expression of HMGB1 significantly increased in the HMGB1 + H/R group but decreased in the AG + H/R group (P<0.05). The expression of the Beclin-1 and LC3 proteins significantly increased after hypoxia and reoxygenation (P<0.05). Furthermore,

the expression of the Beclin-1 and LC3 proteins significantly increased in the HMGB1 + H/R group but decreased in the AG + H/R group ($P<0.05$).

Discussion

The present study showed that elevated levels of autophagy were associated with the expression of HMGB1 during hypoxia and reoxygenation in cardiomyocytes.

HMGB1, which is a novel pro-inflammatory cytokine that is actively released by macrophages and monocytes, has been proven to contribute to myocardial ischemia/reperfusion injury [11]. Our previous study also found that the downregulation of HMGB1 by several drugs, such as minocycline and ethyl pyruvate, can reduce myocardial ischemia/reperfusion injury in rats [12, 13]. Recently, some studies demonstrated that HMGB1 plays a critical role in promoting autophagy, contributing to the removal of protein aggregates and damaged or excess organelles [9, 14].

Importantly, it has been reported that autophagy is increased in the heart, which may be caused by ATP depletion, intracellular calcium overload, hypoxia, reactive oxygen species (ROS), reactive nitrogen species, mitochondrial permeability transition pore opening, endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) during both ischemia/reperfusion [3, 15-18]. Interestingly, there are two types of views regarding autophagy in the heart. The first is that the upregulation of autophagy is a beneficial response that is able to promote cardiomyocyte survival [19, 20], and the second is that enhanced autophagy contributes to cell death during I/R [5, 21]. Therefore, autophagy may play a complex, dual role in ischemia/reperfusion, and excess autophagy can cause cell death due to the excessive degradation of essential proteins and organelles [5, 22].

In addition, the present study demonstrated that the inhibition of the level of HMGB1 by ammonium glycyrrhizinate could significantly reduce the expression of the LC3 and Beclin-1 proteins, which are effective biomarkers of autophagy, suggesting that the downregulation of HMGB1 can attenuate myocardial hypoxia and reoxygenation injury by reducing autophagy. In accordance with these findings, some reports have also shown that the induction of autophagy, such as that due to starvation and rapamycin treatment, can promote HMGB1 translocation from the nucleus to the cytosol in participation with ROS, further enhancing autophagy [14]. Moreover, Kang et al. have found that HMGB1 regulates autophagy through the dissociation of the Beclin 1-Bcl-2 complex by binding to Beclin 1 [23]. These observations, together with the results obtained in the present study, indicate that HMGB1-regulated autophagy is associated with myocardial hypoxia and reoxygenation injury. Therefore, the mechanisms underlying the regulation of autophagy by HMGB1 during myocardial hypoxia and reoxygenation injury will require further investigation.

In conclusion, the results of the present study suggest that HMGB1 is able to regulate Beclin-1 and LC3 levels following hypoxia and reoxygenation injury in rat cardiomyocytes. HMGB1 may promote hypoxia and reoxygenation injury, which may be associated with the mediation of autophagy.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hong Jiang, Department of Cardiology, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuchang, Wuhan City 430060, PR. of China. Tel: +86-027-88041911; Fax: +86-027-88040334; E-mail: jianghwurm@163.com

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