Original Research

H_2O_2 Evokes Injury of Cardiomyocytes Through Upregulating HMGB1

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Introduction: Reactive oxygen species (ROS) have been shown to induce cell apoptosis in cardiomyocytes. However, the underlying mechanism remains unclear. This study aimed to investigate the role of high-mobility group box 1 protein (HMGB1) in cardiomyocytes undergoing H_2O_2 treatment.

Methods: Neonatal rat cardiomyocytes were treated with H_2O_2 (100, 200, 500 μM) or pre-treated with antioxidant N-acetylcysteine (NAC 200 μM) or HMGB1 neutralizing antibody (20 μg/ml) in an appropriate concentration of H_2O_2 (200 μM). The cell viability, apoptosis rate, lactate dehydrogenase (LDH), and the activity of superoxide dismutase were measured. HMGB1 expression was assessed by immunoblotting.

Results: H_2O_2-induced ROS significantly decreased cell viability, promoted the apoptosis of neonatal myocytes, and upregulated the expression of HMGB1 in a dose-dependent manner. However, NAC or HMGB1 neutralizing antibody suppressed the loss of cell viability and the rate of cell apoptosis induced by H_2O_2. NAC or HMGB1 neutralizing antibody also significantly suppressed the release of LDH and the expression of HMGB1.

Conclusion: The present study suggests that H_2O_2-induced ROS evoke injury to cardiomyocytes that may be associated with upregulating HMGB1.

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It has been recognized that an excess of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (H_2O_2) can induce cell death, while apoptosis is involved in the pathological process of many cardiovascular diseases, including heart failure, myocardial infarction, cardiac ischemia and reperfusion injury, and atrial fibrillation.[1-3] Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase are the two major enzymatic sources of ROS in the myocardium.[2] N-acetylcysteine (NAC), the N-acetyl derivative of the amino acid cysteine, has been demonstrated to reduce NADPH oxidase activity.[1] There is evidence to indicate that elevated ROS can regulate the activity of toll-like receptor-4-nuclear factor-κB (TLR-4-NF-κB) or mitogen-activated protein kinase (MAPK) pathways, promote the release of inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), that are detrimental to cardiomyocytes, and finally cause cell death and apoptosis.[4,5] A recent study showed that mitochondrial apoptosis also participated in the redundant death pathway.[6] However, the underlying mechanisms have not been fully investigated.

High-mobility group box 1 protein (HMGB1), a nuclear protein that is actively secreted by innate immune cells (such as macrophages and monocytes) or passively released by necrotic and apoptotic cells, can act as a novel proinflammatory cytokine, which in turn functions to mediate cytokine release, inflamma-

tion and tissue injury by activating innate immunity through signal transduction in the receptor for advanced glycation end products (RAGE) and TLRs in the extracellular space.[7,8] Our previous studies of cardiac ischemia and reperfusion models indicated that inhibition of HMGB1 expression by protective drugs could suppress the release of inflammatory cytokines and attenuate the oxidant stress injury.[9-11] Tsung et al showed that ROS upregulated the expression of HMGB1 in cultured hepatocytes during H_2O_2-induced oxidant stress injury,[12] whereas HMGB1-neutralizing antibody could provide protective effects against the process of liver ischemia–reperfusion or acute lung injury.[13-15] However, whether HMGB1 was involved in the process of ROS-induced cardiomyocyte injury remains unclear. That hypothesis was tested in this study.

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 3-day-old Sprague-Dawley (SD) rats were purchased from the Experimental Animal Center in Wuhan University. Primary cultures of neonatal rat cardiomyocytes were prepared from the ventricles of these SD rats, as described previously.[4] Briefly, the hearts were harvested and minced into pieces, the heart tissue was dissociated with 0.125% (w/v) trypsin and 0.08% collagenase I for 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) fetal bovine serum (FBS), 1% penicillin (100 U/ml), 1% streptomycin (100 μg/ml) at 37°C, and 5% (v/v) CO_2 incubated for 4 days before the experiment.

Experimental designs

The cultured cells were divided into 4 groups that received the following treatments:

- Group 1 (control group): cardiomyocytes were incubated in DMEM F12 with 15% FBS.
- Group 2 (H_2O_2 group): cardiomyocytes were treated with H_2O_2 (final concentrations: 100, 200, 500 μM)
- Group 3 (H_2O_2 and NAC group): cardiomyocytes were pretreated with NAC (final concentrations: 200 μM) for 30 min before H_2O_2 (final concentrations: 200 μM) co-incubation for 24 hours
- Group 4 (H_2O_2 and HMGB1 Ab group): cardiomyocytes were pretreated with HMGB1-neutralizing antibody (IBL, Germany, final concentrations: 20 μg/ml)16 for 30 min before H_2O_2 (final concentrations: 200 μM) co-incubation for 24 hours.

Assay of cell viability

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

Assay of lactate dehydrogenase (LDH) and creatine kinase (CK)

The extent of cell injury was assessed by the concentrations of LDH and CK contained in the culture medium. The protocols were followed according to the manufacturer's instructions (JianCheng Bioengineering Institute, Nanjing, China).

Assay of superoxide dismutase (SOD) activity and malondialdehyde (MDA)

The oxidant stress injury was measured by the indexes of oxygen free radical and lipid superoxide level in the cardiomyocytes. MDA concentration and SOD activity in cardiomyocytes were measured following the protocol according to the manufacturer's instructions (JianCheng Bioengineering Institute, Nanjing, China).

Western blotting analysis

The cell extracts were prepared for quantitative immunoblotting using HMGB1 antibody as described

previously.[9,10] GAPDH antibody (1:5000 dilution; cell signaling) was used as the loading control.

Flow cytometric analysis of apoptosis

Apoptosis was assessed by flow cytometric analysis of propidium iodide (PI) and Annexin V double staining. The cardiomyocytes were harvested after treatment, then rinsed in PBS and suspended in 500 μl binding buffer, after which 5 μl Annexin V and 5 μl PI were added. The stained cells were analyzed using a BD flow cytometer in the FL1-H, FL2-H or FL3-H channels.

Statistical analysis

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

Results

Cell viability

To assess the cytotoxicity of H_2O_2 and the protective effect of NAC or HMGB1-neutralizing antibody on cardiomyocytes, the cells were treated with different concentrations of H_2O_2 (100, 200, 500 μM) or pretreated with NAC (200 μM) or HMGB1 neutralizing antibody (20 μg/ml). Higher concentrations of H_2O_2 caused a greater loss of cardiomyocyte viability. NAC or HMGB1-neutralizing antibody significantly prevented the loss of cardiomyocyte viability resulting from H_2O_2 (200 μM) treatment ($p<0.05$; Figure 1).

LDH and CK levels

Cardiomyocytes treated with H_2O_2 (100, 200, 500 μM) showed a release of LDH and CK according to the concentration. However, NAC or HMGB1-neutralizing antibody suppressed the release of LDH and increased the level of CK in cardiomyocytes undergoing H_2O_2 treatment ($p<0.05$; Figure 2).

MDA level and SOD activity

Cardiomyocytes treated with H_2O_2 (100, 200, 500 μM) suppressed the activity of SOD and increased the level of MDA ($p<0.05$). However, NAC or HMGB1-neutralizing antibody countered the loss of SOD activity and the increasing MDA level ($p<0.05$; Figure 3).

Effect of NAC on the expression of HMGB1 in cardiomyocytes undergoing H_2O_2 treatment

The expression of HMGB1 was significantly increased in cardiomyocytes treated with H_2O_2 (100, 200, 500 μM). In contrast, HMGB1 expression was significantly decreased in the NAC + H_2O_2 and HMGB1 Ab + H_2O_2 groups (both $p<0.05$; Figure 4)

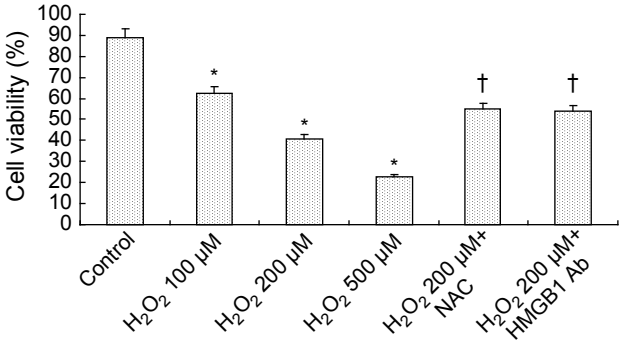

Figure 1. Effect of different concentrations of H_2O_2, NAC and HMGB1-neutralizing antibody on the viability of myocytes. *$p<0.05$ vs. control group; †$p<0.05$ NAC + H_2O_2 (200 μM) group or HMGB1 Ab + H_2O_2 (200 μM) group vs. H_2O_2 (200 μM) group. NAC – N-acetylcysteine; HMGB1 – high-mobility group box 1 protein.

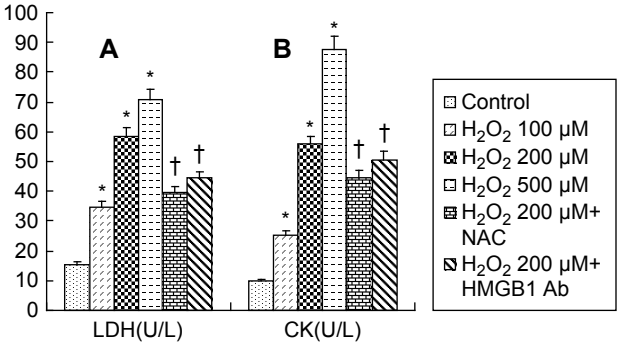

Figure 2. Effect of different concentrations of H_2O_2, NAC and HMGB1-neutralizing antibody on the release of LDH (A) and the activity of CK (B). *$p<0.05$ vs. control group; †$p<0.05$ NAC + H_2O_2 (200 μM) group or HMGB1 Ab + H_2O_2 (200 μM) group vs. H_2O_2 (200 μM) group. LDH – lactate dehydrogenase; CK – creatine kinase. Other abbreviations as in Figure 1.

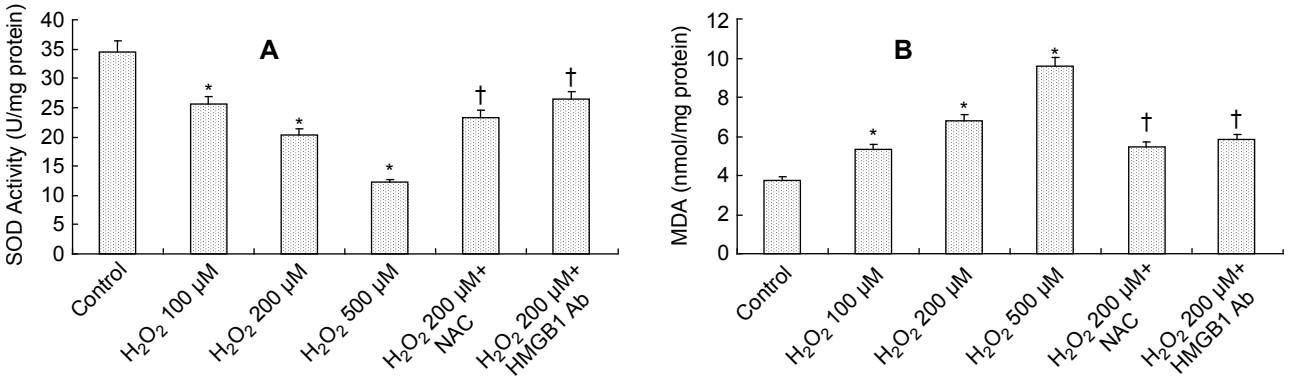

Figure 3. Effect of different concentrations of H_2O_2, NAC and HMGB1-neutralizing antibody on the activity of SOD (A) and the level of MDA (B). *p<0.05 vs. control group; †p<0.05 NAC + H_2O_2 (200 μM) group or HMGB1 Ab + H_2O_2 (200 μM) group vs. H_2O_2 (200 μM) group. MDA – malondialdehyde; SOD – superoxide dismutase. Other abbreviations as in Figure 1.

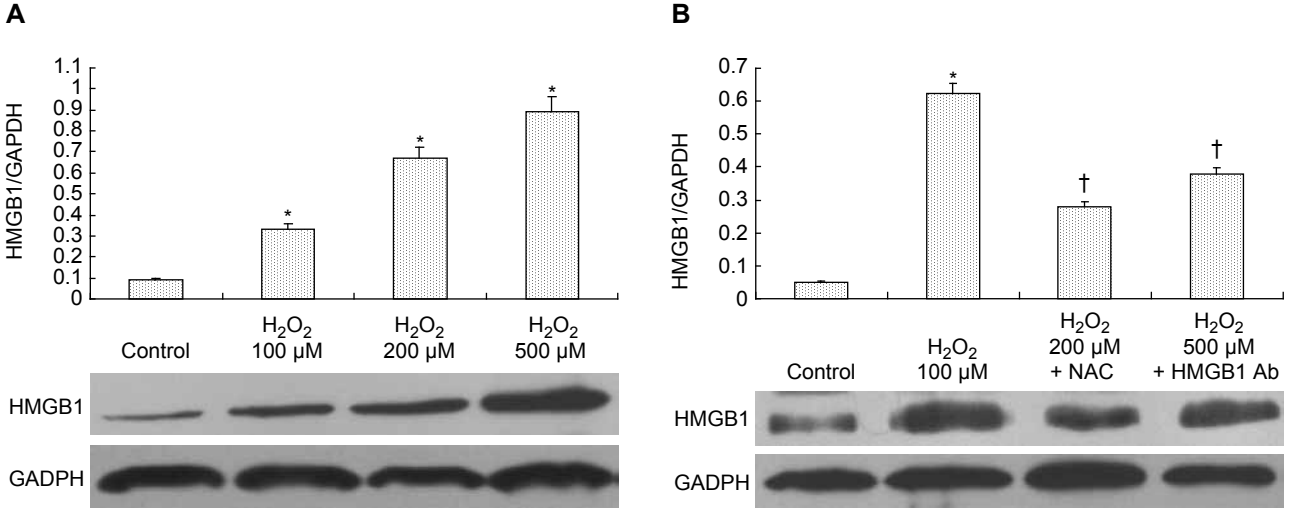

Figure 4. Effect of different concentrations of H_2O_2, NAC and HMGB1-neutralizing antibody on the expression of HMGB1. *p<0.05 vs. control group; †p<0.05 NAC + H_2O_2 (200 μM) group or HMGB1 Ab + H_2O_2 (200 μM) group vs. H_2O_2 (200 μM) group. Abbreviations as in Figure 1.

Cardiomyocyte apoptosis

The rate of cell apoptosis was significantly increased in cardiomyocytes treated with H_2O_2 (100, 200, 500 μM). Moreover, the rate of cardiomyocyte apoptosis was increased by treatment with NAC + H_2O_2 or HMGB1 Ab + H_2O_2 (both p<0.05; Figure 5).

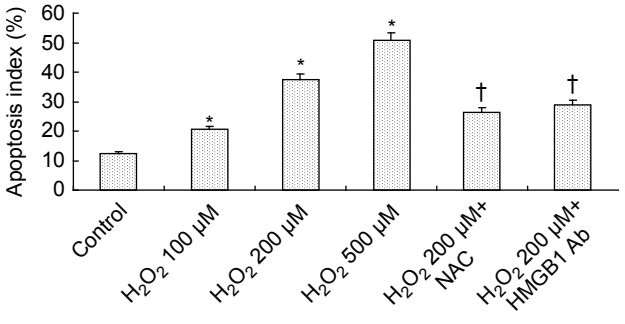

Figure 5. Effect of different concentrations of H_2O_2, NAC and HMGB1-neutralizing antibody on the apoptosis rate of myocytes. *p<0.05 vs. control group; †p<0.05 NAC + H_2O_2 (200 μM) group or HMGB1 Ab + H_2O_2 (200 μM) group vs. H_2O_2 (200 μM) group. Abbreviations as in Figure 1.

Discussion

The novel findings of this study may be summarized as follows. First, H_2O_2, a type of ROS, caused a loss of cell viability and the release of LDH and CK in a dose-dependent manner. Meanwhile, the activity of SOD was suppressed and the level of MDA was elevated following treatment with H_2O_2. Second, we found that H_2O_2 promoted the expression of HMGB1 and the

apoptosis of cardiomyocytes in a dose-dependent manner. Third, we found that NAC, which has been demonstrated to reduce NADPH oxidase activity, was able to prevent the loss of cell viability and suppress the expression of HMGB1, while reducing the apoptosis rate of cardiomyocytes undergoing H_2O_2 treatment, implying that antioxidant therapy may protect cardiomyocytes from ROS injury by downregulating HMGB1. Finally, HMGB1-neutralizing antibody, which assists in the cytoprotective process of acute organ injury by inhibiting HMGB1 expression,[13-15] could also attenuate the injury and apoptosis of cardiomyocytes undergoing H_2O_2 treatment.

This study showed that the level of HMGB1 elevation was associated with the severity of oxidative stress and cell injury. Our previous work has indicated that exogenous HMGB1 could significantly decrease the cell viability and promote the apoptosis of neonatal myocytes in a dose-dependent manner. However, HMGB1 can be passively released from necrotic and apoptotic cells undergoing damage signal stimulation.[7,8] Moreover, necrotic cells lacking HMGB1 are not able to induce the secretion of proinflammatory cytokines.[8] These observations suggest that ROS-induced cardiomyocyte death causes the release of endogenous HMGB1, which promotes cell apoptosis, eliciting a further inflammatory response.

HMGB1 could be actively released by macrophages, monocytes or cultured hepatocytes undergoing ROS treatment.[12,17] Our previous work also indicated that downregulation of HMGB1 by several pharmaceuticals, such as ethyl pyruvate or minocycline, may attenuate ROS-induced ischemia and reperfusion injury in rats.[9,10] However, HMGB1-neutralizing antibody, acting as a specific function-blocking antibody, has been demonstrated to improve cell survival in sepsis and acute lung injury by inhibiting HMGB1 expression.[13-14,18] Tsung et al also found that inhibition of HMGB1 by neutralizing antibody significantly reduced the liver damage induced by ischemia–reperfusion injury.[15] In the present study, inhibition of the elevated level of HMGB1 by neutralizing antibody also protected against ROS-induced cardiomyocyte injury. These observations, together with our results in the present study, indicate that HMGB1 is associated with the ROS-induced injury of cardiomyocytes. Moreover, downregulation of HMGB1 may offer a potential therapeutic approach to diseases caused by ROS-induced cell injury. Therefore, the underlying mechanism of HMGB1-induced cardiomyocyte injury during ROS treatment will require further investigation.

Conclusions

The present study demonstrated that H_2O_2 evokes the injury and apoptosis of cardiomyocytes, which may be associated with the upregulation of HMGB1.

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