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Original Article

Effect of heat shock pretreatment on apoptosis and metallothionein expression in rat cardiomyocytes

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Abstract: To investigate the effect of heat shock pretreatment on apoptosis and mitochondrial metallothionein (MT) expression in rat cardiomyocytes. *In vitro* cultured H9C2 cells were randomly divided into three groups: control, hydrogen peroxide (H_2O_2) injury, and H_2O_2 injury after heat shock pretreatment (n = 6 per group). Cardiomyocyte apoptosis and caspase-3 activity were assayed after treatment. Mitochondrial cytochrome (cyt) c and MT expression was assayed by Western blotting. Compared with the control group, the H_2O_2 injury group had a growing number of apoptotic cardiomyocytes ($P < 0.01$) and significantly elevated caspase-3 activity ($P < 0.01$) with markedly increased mitochondrial cyt c and MT expression ($P < 0.01$). After heat shock pretreatment, the numbers of apoptotic and necrotic cardiomyocytes ($P < 0.01$) and the caspase-3 activity significantly declined ($P < 0.01$), while mitochondrial cyt c and MT expression continued to increase ($P < 0.01$) compared with the H_2O_2 injury group. Heat shock pretreatment inhibits cardiomyocyte apoptosis, which may have a protective effect on cardiomyocytes by increasing the expression of myocardial protective MT and reducing the release of mitochondrial cyt c.

Keywords: Heat shock pretreatment, cardiomyocyte, metallothionein, apoptosis, cytochromec

Introduction

In recent years, the incidence and mortality of cardiovascular disease have significantly grown due to the continuous improvement of people's living standards and the impact of population aging. Because of the great harmfulness of cardiovascular disease, cardiovascular protection is particularly important. There have been an increasing number of treatment methods for cardiovascular disease, such as medications, interventions, and surgeries, especially the latter two treatments. Because surgical anaesthesia and stress can increase the probability of cardiovascular accidents, higher requirements are put forward for cardiovascular protection in clinics [1-3].

The heat shock response is a defensive adaptive response characterised by gene expression changes in living organisms under heat stress (or other stresses). The resultant proteins, called heat shock proteins, have been demonstrated to have an excellent myocardial protective effect [4]. Additionally, recent studies have found that expression of a molecular weight protein, called metallothionein (MT), can be induced in organisms under stress conditions such as heat stress. MT is considered an endogenous cell protective agent that has a protective role in a variety of cardiovascular diseases [4-6]. However, little is known about mitochondrial MT expression and apoptosis in cardiomyocytes in the context of heat shock injury. Therefore, the present study applied heat shock pretreatment to H_2O_2-injured rat cardiomyocytes and further investigated the myocardial protective mechanism of heat shock pretreatment.

Materials and methods

Cell culture and grouping

Rat cardiomyocytes (H9C2) were aseptically cultured and passaged *in vitro*. The cells were harvested and centrifuged to remove waste liquid. A small amount of dimethylsulphoxide (DMSO) cryopreservation solution was added to adjust the cell density to $(1\text{-}3)^3 \times 10^3$ cells/mL.

The cell suspension was mixed well, dispensed into cryotubes, and stored in liquid nitrogen until use.

In vitro cultured cells were randomly divided into three groups (n = 6 samples per group). In the control group, cardiomyocytes were cultured in serum-containing Dulbecco's modified Eagle's medium (DMEM) and placed in a 37°C, 5% CO_2 incubator for 3 h. In the hydrogen peroxide (H_2O_2) injury group, cardiomyocytes were cultured in serum-free DMEM containing 0.5 mmol/L H_2O_2 and placed in a 37°C, 5% CO_2 incubator. In the H_2O_2 injury after heat shock pretreatment (HSP) group, cardiomyocytes were cultured in serum-containing DMEM, and the culture flask was aseptically placed in a 42°C water bath for 60 min. After 6 h of recovery in a 37°C, 5% CO_2 incubator, the serum-containing DMEM was aspirated and the cells were rinsed. Then, the cardiomyocytes were placed in a 37°C, 5% CO_2 incubator and cultured in serum-free DMEM medium containing 0.5 mmol/L H_2O_2.

Flow cytometry assay of cardiomyocyte apoptosis

The cultured cells were harvested into centrifuge tubes, (1-10) × 10^5 cells per tube. After centrifugation (4°C, 500 *g*, 3 min), the waste liquid was removed, and the cell pellet was washed with incubation buffer. After centrifugation (4°C, 500 *g*, 3 min), the waste liquid was removed, and the cells were resuspended in approximately 100 μL of marking solution and then centrifuged, washed, and resuspended to a volume of 100 μL. The cell suspension contained 5 μL of Alexa Fluor 488 Annexin V and 1 μL of 100 μg/L propidium iodide (PI) working solution, followed by incubation at room temperature for 15-30 min. Then, 400 μL of 1 × Annexin V buffer was added, and the cell suspension was loaded onto a BD flow cytometer to assay the level of apoptosis.

Caspase-3 activity assay in rat cardiomyocytes

The cultured cells were harvested into centrifuge tubes and centrifuged (4°C, 500 *g*, 3 min) to remove waste liquid. The cell pellet was washed with incubation buffer and then centrifuged (4°C, 500 *g*, 3 min) to remove waste liquid. Then, the cells were resuspended in lysis buffer (50 μL of lysis buffer per 1 × 10^6 cells) and incubated in an ice bath for 30 min after mixing. After 10 min of centrifugation at 12000 rpm, the supernatant (i.e., total cellular protein) was collected. A small amount of the supernatant (1-2 μL) was taken for protein quantification by Bradford or bicinchoninic acid (BCA) assay. The protein was diluted to 2 μg/μL with cell lysis buffer. After addition of the sample, the release of 7-amino-4-methylcoumarin was immediately measured using a Polarstar fluorescence spectrophotometer. Fluorescence intensity was analysed with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The measurement was performed at 37°C every 10 min for 120 min of continuous monitoring at 37°C. The "fold increase" in fluorescence intensity was taken as the caspase-3 activity. Each sample assay was repeated in at least triplicate wells.

Western blotting assay of cyt c and MT expression

The cultured cells were subject to washing, digestion, dispersion, homogenisation, and denucleation by centrifugation, high-speed centrifugation, and protein extraction. Protein quantification was performed by BCA assay. The protein samples (100 μg each) were loaded for sodium dodecyl sulphide-polyacrylamide gel electrophoresis (SDS-PAGE) and then blocked for 20 min after membrane transfer. The membrane was incubated with rabbit anti-rat MT or cyt c antibody (1:500 dilution) and incubated at 4°C overnight. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:5000 dilution) and incubated at room temperature for 1 h. Thereafter, enhanced chemiluminescence (ECL) and X-ray radiography were performed. The banding pattern of the X-ray films was analysed using Quantity One software (Bio-Rad). Protein expression was quantified by the ratio of the protein density to the internal control.

Statistical analysis

Statistical analysis and was performed using Statistical Package for Social Science (SPSS) 17.0. Graphs were plotted using GraphPad PRISM 5.0. Multiple group comparisons were performed using single-factor analysis of variance (ANOVA). Measurement data were expressed as the mean ± standard deviation. A

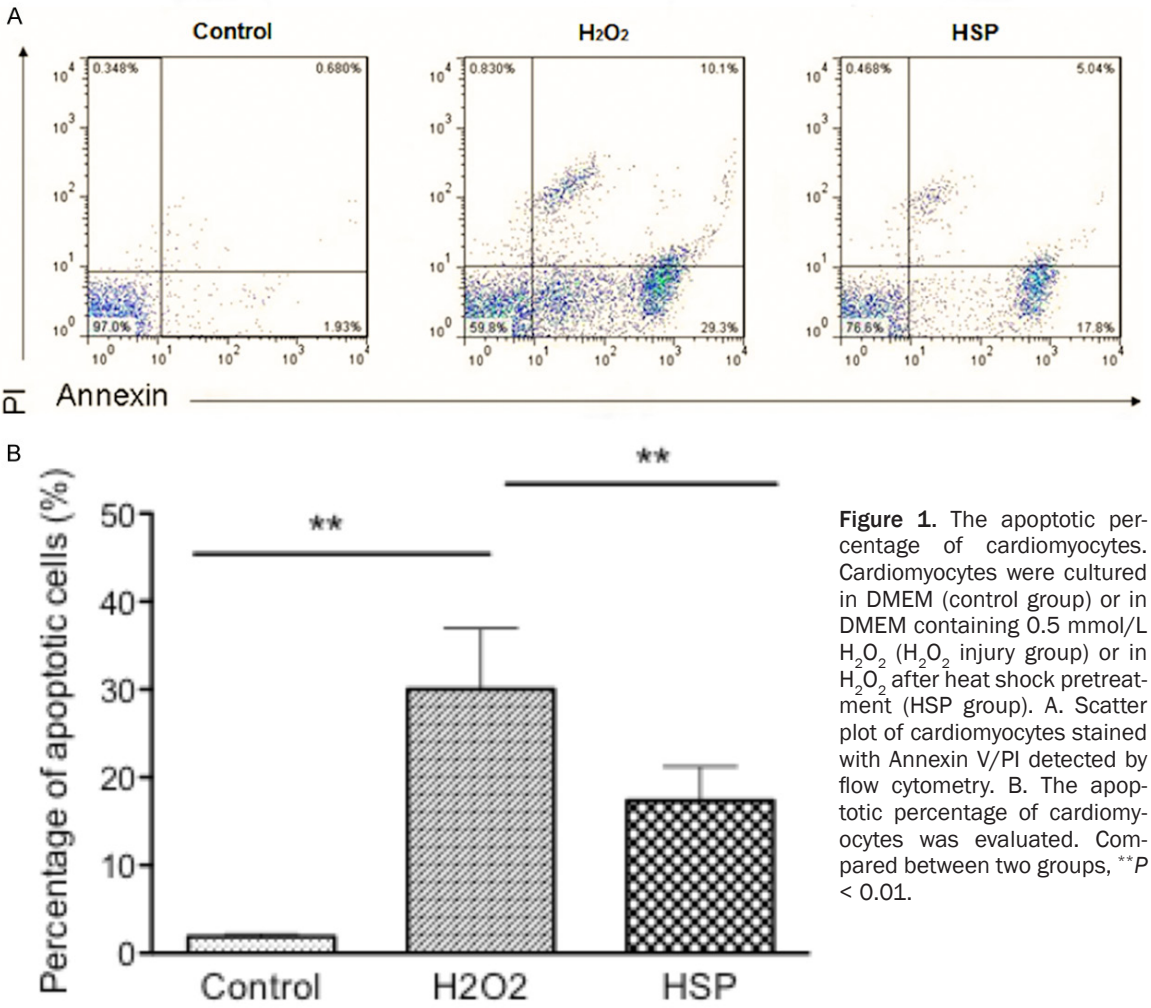

Figure 1. The apoptotic percentage of cardiomyocytes. Cardiomyocytes were cultured in DMEM (control group) or in DMEM containing 0.5 mmol/L H_2O_2 (H_2O_2 injury group) or in H_2O_2 after heat shock pretreatment (HSP group). A. Scatter plot of cardiomyocytes stained with Annexin V/PI detected by flow cytometry. B. The apoptotic percentage of cardiomyocytes was evaluated. Compared between two groups, $^{**}P < 0.01$.

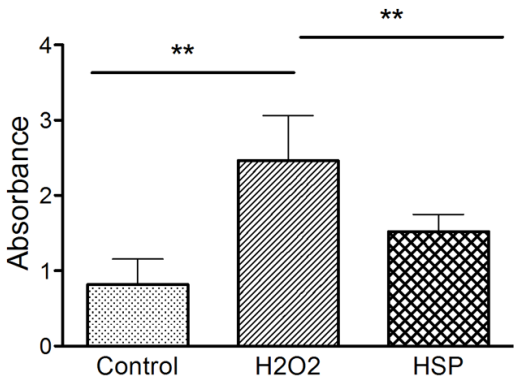

Figure 2. Caspase-3 activity in the three groups (the control group, H_2O_2 injury group and HSP group) of rat cardiomyocytes. Fluorescence intensity was taken as the caspase-3 activity. $^{**}P < 0.01$.

P value < 0.05 was considered statistically significant, and *P* < 0.01 was considered extremely statistically significant.

Results

Cardiomyocyte apoptosis in different groups as detected by flow cytometry

Levels of apoptosis and necrosis in rat cardiomyocytes were detected by flow cytometry. The cell scatter plot shows normal cells in the lower left quadrant, injured cells in the upper left quadrant, necrotic cells in the upper right quadrant, and apoptotic cells in the lower right quadrant. **Figure 1A** and **1B** showed that only a few apoptotic cells were observed in the control group, which accounted for 1.91 ± 0.27% of the total cells and was significantly less than in the H_2O_2 group (29.4 ± 5.4%, *P* < 0.01). In the HSP group, the percentage of apoptotic cells was 17.4 ± 3.1%, which was also significantly less than in the H_2O_2 group (*P* < 0.01). Additionally, the number of necrotic cells was markedly increased in the H_2O_2 group but substantially decreased in the HSP group.

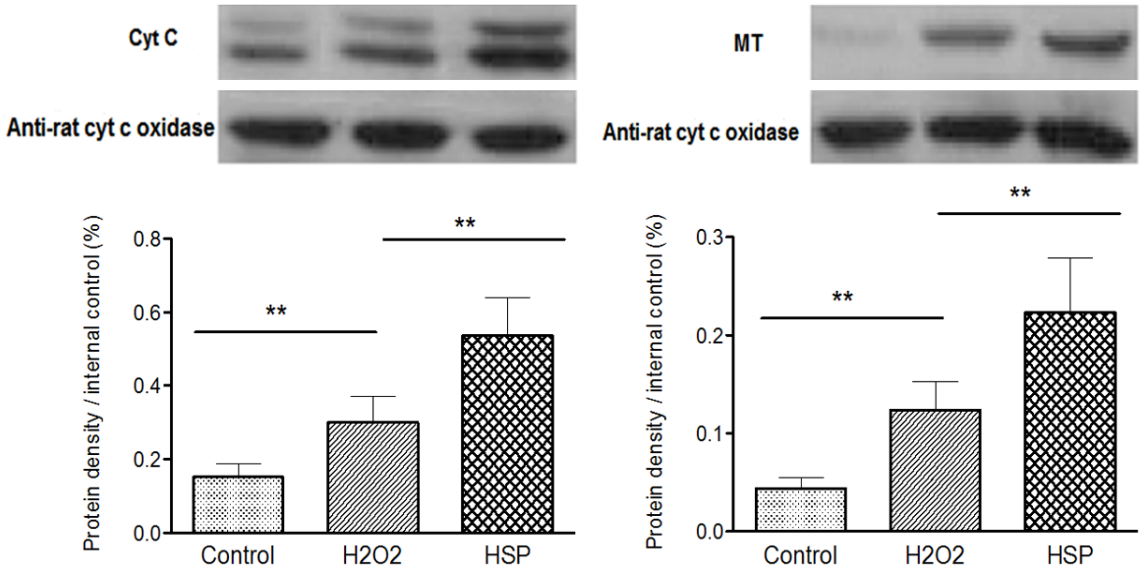

Figure 3. Expression of mitochondrial cyt c and MT. Western blotting was used to determine cyt c and MT expression in each group (the control group, H_2O_2 injury group and HSP group). Relative band intensities of each protein were quantified by densitometry. $^{**}P < 0.01$.

Caspase-3 activity in rat cardiomyocytes

The absorbance of the H_2O_2 group (2.5 ± 0.6) was the highest among the three groups, showing a statistically significant increase compared with the control group (0.9 ± 0.41, $P < 0.01$). There was a significant decrease in the absorbance in the HSP group (1.4 ± 0.45) compared with the H_2O_2 group ($P < 0.01$). The absorbance of the HSP group was slightly higher than that of the control group, but the difference was not statistically significant ($P = 0.07$) (**Figure 2**).

Mitochondrial cyt c and MT expression

Mitochondrial cyt c and MT expression levels were significantly higher in the H_2O_2 group than in the control group ($P < 0.01$). In the HSP group, cyt c and MT expression further increased and reached 0.5531 ± 0.14 and 0.2204 ± 0.05, respectively, significantly higher than that in the H_2O_2 and control groups ($P < 0.01$) (**Figure 3**).

Discussion

In recent years, the endogenous anti-injury mechanisms of the body have become an intense area of research for the prevention and treatment of cardiovascular disease. An increasing number of studies show that HSP has an endogenous protective effect on cardiomyocytes in the body. The protective role of HSP may be implemented through inhibiting the activity of cysteine protease, suppressing stress-activated protein kinase, and preventing initiation of the pro-apoptotic program in the cell. Stimulation of the body's cells by apoptotic signals can induce mitochondrial swelling, increased permeability, and outer membrane potential decline, resulting in destruction of the mitochondrial outer membrane. Then, mitochondrial factors such as cyt c and Ca^{2+} are released into the cytoplasm, which initiate the apoptotic program by activating caspase and directly damaging chromatin within the nucleus or other Ca^{2+}-dependent proteins, leading to apoptosis [5-9]. Additionally, HSP can induce the synthesis of heat shock proteins to inhibit p53 activity or Bax translocation. This mechanism significantly reduces the release of apoptotic proteins (the second mitochondria-derived activator of caspase, Smac, and cyt c) from mitochondria, exerting an important influence on the apoptotic pathway [10]. As a water-soluble protein, cyt c is localised between the inner and outer membranes of mitochondria and is loosely connected to the mitochondrial inner membrane. When oxidative stress injury occurs, the mitochondrial membrane potential changes, and mitochondrial permeability tran-

sition pores open. After membrane shedding, cyt c is released into the cytoplasm to initiate programmed cell death pathways, further accelerating apoptosis. A series of studies has recently found that mitochondrial cyt escapes from the cytoplasm during the early stages of apoptosis, and this process is believed to be closely associated with apoptosis progression. However, Bcl-2 in the cytoplasm and mitochondria can prevent the opening of mitochondrial permeability transition pores and the release of cyt c to inhibit the apoptotic program [10, 11].

Research in an ischemia/reperfusion (IR) model has found that MT in cardiomyocytes reduces lipid peroxidation injury induced by oxidative stress from oxygen free radicals, thus playing a protective role in cardiomyocytes. The use of exogenous MT can also reduce injuries caused by the myocardial IR process and the accumulation of Ca^{2+}. Additionally, other studies have found that MT has a significant inhibitory effect on the abnormal automaticity of cardiomyocytes generated during IR, mainly by inhibiting calcium influx in the plateau phase and suppressing free radicals [12, 13]. Moreover, in one study, morphological examination following isoproterenol treatment of injured cardiomyocytes after pretreatment with zinc chloride showed reduced cardiomyocyte injury, with significantly increased MT at the mRNA and protein levels. The above study suggests that exogenous injury can induce cardiomyocytes to synthesise MT and increase protein expression. This mechanism is protective against cardiomyocyte injury at the transcriptional level [10, 14].

Compared with the control group, the H_2O_2 group had a growing number of apoptotic cardiomyocytes and significantly elevated caspase-3 activity with substantially increased mitochondrial cyt c and MT expression. After HSP, the numbers of apoptotic and necrotic cardiomyocytes both significantly decreased, and the caspase-3 activity, which is involved in apoptosis progression, also significantly decreased. In contrast, mitochondrial cyt c and MT expression continued to increase compared with the H_2O_2 group. These results reveal that HSP can inhibit apoptosis in cardiomyocytes, increase the expression of myocardial protective MT, and reduce the release of mitochondrial cyt c, thus playing a protective role in cardiomyocytes.

Disclosure of conflict of interest

None.

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