

## Heat Shock Protein 70 Gene Transfection Protects Rat Myocardium Cell Against Anoxia-Reoxygeneration Injury

## ABSTRACT

The cultured primary neonatal rat myocardiocytes with an acute myocardial A/R injury model and the HS-treated rat myocardiocyte model were used. Three-day cultured myocardiocytes were randomly divided into four groups (n=8): control group, A/R group, HS+A/R group and pCDNA HSP<sub>70</sub> +A/R group. A liposome-coated HSP<sub>70</sub> pCDNA plasmid was transfected into the primary neonatal rat myocardiocytes; HSP<sub>70</sub> mRNA and its protein were confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting. The cell viability was assayed by monotetrazolium (MTT) and the lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) activity of cells during incubation and the changes in cells ultrastructure were examined. NF- $\kappa$ B activity in the primary neonatal rat myocardiocytes was measured with flow cytometry.

Keywords: gene transfection, HSP<sub>70</sub> gene, NF-KB, cardiac myocyte, anoxia-reoxygeneration injury

## 1. Introduction

Heat shock proteins (HSPs) are a family of inducible and constitutively expressed self-preservation proteins which maintain cell homeostasis under environmental stress. In a protocol involving prolonged cardioplegic arrest and reperfusion, Amrani et al<sup>1</sup> has shown improved recovery of both ventricular and coronary endothelial function of rat hearts after heat stress. Heat shock protein 70 (HSP<sub>70</sub>) is one of the members which is strongly induced in the myocardium under various forms of stress. It appears that the induction of HSP70 confers a protective effect on cardiac function against exposure to an ischemia-reperfusion injury. Currie et al<sup>2</sup> has shown that a rise in levels of a particular HSP<sub>70</sub>, induced by heat stress, is associated with protection against ischemia-reperfusion injury. However, the protective effects may be contaminated by other factors resulting from stress, such as expression of catalase, superoxide dismutase (SOD) or other members of the HSP family<sup>3</sup>. To study the role of individual HSP<sub>70</sub> separated from the many complex pathways, the technique of gene transfection was adopted in this study with cultured primary cardiomyocytes.

## 2. Methods

## 2.1. Preparation of Neonatal Rat Cardiac Myocyte Cultures

Ventricular myocytes were isolated from the hearts of neonatal rats (Sprague-Dawley) which were less than 3 days old and were cultured prepared according to Simpson and Savion<sup>4</sup> with the following modifications: neonatal rat pups were killed by swift decapitation within the first 3 days after birth. The heart was immediately removed under aseptic conditions and placed in ice-cold sterile Hank's balanced salt solution (HBSS; no Ca<sup>++</sup> or Mg<sup>++</sup>). Enzymatic and mechanical dissociation of cardiomyocytes were then performed using the Neonatal Cardiomyocyte Isolation System. The hearts were minced on ice and digested overnight at 4°C with purified trypsin (10 µg/ml) in HBSS. In the following morning, the digested tissue was transferred to a 50-ml conical tube and purified soybean trypsin inhibitor (40 µg/ml) was added to terminate trypsinization. The tissue was oxygenated and warmed to 37°C. Purified collagenase (10 U/ml) was added and digestion proceeded for 45 minutes at 37°C with intermittent gentle swirling. Mild titration was then used to mechanically dissociate the

digested tissue and single-cell suspensions were obtained by filtering this digested material through 70  $\mu$ m sterile mesh filters. The cells were collected by low-speed centrifugation. The supernatant was discarded and the cell pellet resuspended in DMEM (GIBCO, USA) culture medium containing 10% defined iron-supplemented bovine calf serum, 2 mmol/L glutamine, and gentamicin sulfate (50  $\mu$ g/ml). The cells were then "preplated" to remove fibroblasts and the remaining cells counted and seeded onto multi-well culture plates at a density of 10<sup>5</sup> cells/well. The media was changed every other day beginning the day after seeding. Bromodeoxyuridine (0.1 mmol/L) was added to the culture media to further minimize contamination from fibroblasts. At the third day the myocytes were grouped at random.

#### 2.2. Anoxia-reoxygeneration Model

Cells were plated in 14-mm-diameter glass bottom microwell dishes and ischemia was introduced by a buffer exchange to ischemia-mimetic solution (NaH<sub>2</sub>PO<sub>4</sub> 0.9 mmol/L, NaHCO<sub>3</sub> 6.0 mmol/L, CaCl<sub>2</sub> 1.8 mmol/L, MgSO<sub>4</sub> 1.2 mmol/L, HEPES 20 mmol/L, NaCl 98.5 mmol/L, KCl 10.0 mmol/L, pH 6.8, 37°C) and placing of the dishes in hypoxic pouches equilibrated with 95% N2-5% CO<sub>2</sub>. After 3 hours of ischemia, reoxygeneration was initiated by a buffer exchange to normoxic solution (NaCl 129.5 mmol/L, KCl 5.0 mmol/L, NaH2PO4 0.9 mmol/L, NaHCO<sub>3</sub> 20 mmol/L, CaCl<sub>2</sub> 1.8 mmol/L, MgSO<sub>4</sub> 1.2 mmol/L, glucose 55 mmol/L, HEPES 20 mmol/L, pH 7.4, 37°C) and incubation at 95% room air-5% CO<sub>2</sub><sup>5,6</sup>. Controls incubated in normoxic solution were run in parallel for each condition for periods of time that corresponded with those of the experimental groups. Under control conditions, cell viability was not compromised.

## 2.3. Heat Shock Model

In heat shock group, the cells were subjected to hyper-

thermia of 42°C for 1 hour in a water bath. Forty-eight hours after treatment, cells were used for the following experiments.

#### 2.4. Construction of Expression Vector and Lipofection

Full-length human HSP70 DNA (donated by Prof. WANG Shan-ming, Chicago University, USA) was cloned at the EcoRI/BamHI site of pCDNA, which has a cytomegalovirus promoter. pCDNA HSP70 was mixed with liposomes according to the protocol provided by the manufacturer. Briefly, for each transfection, 2 µl of liposomes (1 mg/ml) were diluted with 100 µl serum-free Opti-MEM and kept at room temperature for 35 minutes. They were then mixed with 100 µl serum-free Opti-MEM containing 2 µg plasmid DNA and the mixture (0.2 ml DNA-liposome complex) was left at room temperature for 10 minutes before being diluted with 0.8 ml serum-free Opti-MEM. The diluted DNA-liposome complex was added to the cultures. Prior to transfection cultures were rinsed twice with a serum-free medium. After incubation at 37°C for 12 hours the transfection medium was replaced with a fresh culture medium containing 10% fetal bovine serum  $(FBS)^7$ .

#### 2.5. Experiment Protocols and Grouping

The cultures were grouped at random, including control group, anoxia-reoxygeneration group (A/R group), heat shock (HS)+ A/R group and pCDNA HSP<sub>70</sub> + A/R group. The protocols of each group were showed in Figure 1.

#### 2.6. MTT Assay for Cell Viability

Cardiomyocytes were seeded in 96-well plates at a density of  $10^5$  cells/well, 20 µl monotetrazolium (MTT) were added to each well under sterile conditions (with a final concentration of 0.5 mg/ml) and the plates were incubated for 4 hours at 37°C. Untransformed MTT was removed by aspiration and formazan crystals were



Figure 1. Experimental protocols and grouping

dissolved in dimethyl sulfoxide (150  $\mu$ l/well). Formazan was quantified spectroscopically at 490 nm using an automated enzyme immunoassay analyzer<sup>8</sup>.

#### 2.7. Biochemistry Detection

After the experiment, 200  $\mu$ l of the culture supernatant were assayed and the activity of lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) were detected by a biochemical autoanalyser (Beckman, USA).

# 2.8. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)<sup>9</sup>

Cultures harvested for total RNA immediately after treatment were washed in phosphate buffered saline solution (PBS) and RNA from five wells pooled after Trizol harvesting (Promega, China). Total RNA, 1000 ng was reverse transcribed for polymerase chain reaction (PCR) using avian myeloblastosis virus (AMV) reverse transcriptase (Promega). Primer sequences for HSP<sub>70</sub> were 5-AAC-GTG-CTG-CGG-ATC-ATC-AA-3(sense), 5-CT G-GAT-GGA-CGT-GTA-GAA-GT-3 (antisense) (347 bp)<sup>10</sup> for  $\beta$ -actin 5-TCA-TCA-CCA-TTG-GCA-ATC-AG-3 (sense), 5-GTC-TTG-GCG-TAC-AGG-3 (antisense) (154 bp); PCR was performed at 55°C (annealing temperature) for 30 cycles. Products were analyzed on ethidium bromide-stained 1.5% agarose gels.  $\beta$ -Actin was used as RNA input control.

#### 2.9. Western Blotting Analysis

After incubation in serum-free DMEM cells were washed with ice-cold PBS and homogenized in buffer containing 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 10 µg/ml leupeptin, and 5 µg/ml pepstatin, centrifuged at 12 000 r/min for 10 minutes at 4°C and the supernatants were collected. The protein concentration in the supernatant was determined by using a protein assay reagent (Bio-Rad Laboratories, Hercules, USA). The same amounts (10 µg each lane) of proteins from cell homogenates were electrophoresed on 8% polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes by electro blotting. The membranes were blocked for 1 hour at room temperature with 5% nonfat dried milk and 0.1% bovine serum albumin in tris-buffered saline (TBS) containing 0.1% (vol/vol) Tween 20 (TBS-T) and incubated for 1 hour with sheep anti-rat antibody diluted in TBS-T containing 5% FBS. After washing with TBS-T the blots were developed by enhanced chemiluminescence and exposed to X-ray film<sup>11</sup>.

#### 2.10. Transmission Electron Microscope (TEM) for Ultramicrostructure<sup>12</sup>

Cells were washed twice with PBS at 4°C and immediately fixed in 2.5% glutaraldehyde solution and then kept in the refrigerator at 4°C for two hours. Samples were later post-fixed in 1% osmium tetroxide, dehydrated in ascending concentration series of ethyl alcohol and embedded in Spur's resin. Ultrathin sections were prepared using diamond knives, stained with uranyl acetate and lead acetate and then examined at 80 kV under the transmission electron microscope (Hitachi, H-600 Japan).

## 2.11. Detection of NFκB by Flow Cytometric Assay<sup>13</sup>

After reoxygeneration cardiac myocytes were washed with PBS containing 10% endotoxin-free FCS. Nuclei were prepared by incubating the cells with 200 µl Pipes-Triton buffer (10 mmol/L Pipes, 0.1 mol/L NaCl, 2 mmol/L MgCl<sub>2</sub>; Sigma-Aldrich, Steiheim, Germany and 0.1% Triton X 100) in PBS for 30 minutes at 4°C. After two washes in PBS-FCS nuclei were stained with anti-NF- $\kappa$ B antibody at 5  $\mu$ g/ml for 45 minutes at 4°C. Mouse anti-NF-kB p65 monoclonal antibody (Santa Cruz, USA) recognizes epitopes mapping to the C amino-acid terminus of mouse NF-kB p65. After two washes in PBS-FCS nuclei were incubated for 45 minutes at 4°C with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse anti-Ig antibody fragments (1/50; Santa Cruz, CA, USA). After washings in PBS-FCS nuclei were analyzed by flow cytometry on a FACSC Caliber (BD Biosciences, San Jose, CA, USA). Fluorescence intensity of single nuclei was detected at random. A total of  $10^4$  events were recorded for each sample.

#### 2.12. Statistical Analysis

Data analysis was performed using the Statictical Package for Social Science (SPSS 11.5). All data are presented as mean  $\pm$  standard deviation (SD). One way analysis of variance (ANOVA) was used for multiple group comparison. If a significant *F* value was obtained, further comparisons were determined with least significant difference (LSD) test. Significance was accepted at the level of *P*<0.05.

## 3. Results

#### 3.1. MTT Assay for Cell Viability

The results from the MTT assay indicated that in the control group the viability of cardiac myocytes were  $(87.3 \pm 11.4)\%$ . In the A/R group the cell viability dropped to  $(35.4 \pm 6.9)\%$ . However, the cell viability was improved in the HS+A/R group and the pCDNA

 $HSP_{70} + A/R$  group. These data showed that HS stress and  $HSP_{70}$  gene transfection could lessen the injury from the A/R procedure and improve the cell viability (Figure 2).

#### 3.2. Analysis for Biochemistry Detection

As shown in the table the activity of LDH and CPK was significantly elevated in the A/R group. However, in the HS+A/R group and the pCDNA HSP<sub>70</sub>+A/R group clear decreases in activity were observed. There were statistically significant differences between these groups. It showed that HS stress and HSP<sub>70</sub> gene transfection could inhibit the elevation of activity of CPK and LDH induced by A/R injury.

#### **3.3. TEM for Ultrastructural Analysis**

The cells subjected to TEM examination were analyzed for ultrastructural differences. The irregular pattern of muscular fibril was observed in cardiac myocytes of the A/R group. The sarcoplasmic reticulum was extended to the vacuole and the mitochondria were swollen and misshapen. However, in the HS+A/R group and the pCDNA HSP<sub>70</sub>+A/R group the muscular fibril was arranged regularly (Figure 3A) and the structure of the sarcoplasmic reticulum and mitochondria were normal. No apparent differences in the microscopic appearance were seen between the HS+A/R (Figure 3B) and pCDNA HSP<sub>70</sub> + A/R groups (Figure 3C).

#### 3.4. Expression of HSP<sub>70</sub> Gene in Cardiac Myocytes

To determine the expression of the  $HSP_{70}$  gene in cardiac myocytes, RT-PCR and Western analysis were performed. In the control group, there was no expression of the  $HSP_{70}$  gene. There was a slight upregulation for expression of  $HSP_{70}$  in the A/R group and a clear increase in the expression of the  $HSP_{70}$  gene was observed by RT-PCR in the HS+A/R group and pCDNA  $HSP_{70}+A/R$  group compared with the A/R group (Figure 4). Increased expression of  $HSP_{70}$  protein was confirmed by Western blot analysis. Compared with the A/R group, an obvious increase in the  $HSP_{70}$  protein was found in the



Figure 2. Effect of various treatments on viable cell in rat's cultured ventricular myocytes (n=8, mean±SD) (<sup>\*</sup>P>0.05, vs control group; <sup>#</sup>P<0.01, vs A/R group)



Figure 3. Representative TEM photographs showing for ultrastructure of cardiac myocytes. A: A/R group. B: HS + A/R group. C: pcDNA HSP<sub>70</sub> + A/R group

Groups	п	СРК	LDH
Control	8	10.5±1.2	2.9±0.5
A/R	8	38.6±4.2	22.0±4.0
HS+A/R	8	28.2±3.1*	14.3±2.6*
pcDNAHSP <sub>70</sub> + A/R	8	24.6±2.8*	14.6±2.9*

Table 1. Activity of CPK and LDH (IU/L)

\*P<0.01, compared with A/R group

HS+A/R group and in the pCDNA HSP<sub>70</sub>+A/R group. In addition, there was a significant difference in expression of the HSP<sub>70</sub> gene between the HS+A/R group and the pCDNA HSP<sub>70</sub>+A/R group (Figure 5). These data show that the HSP<sub>70</sub> gene could be induced by heat shock stress and gene transfection by liposome. And more gene expression could be induced by gene transfection.

#### 3.5. Analysis for activity of NF-κB

High activity of NF-κB (5.76±0.64) was detected in the A/R group. Compared with the control group, there was a significant difference (1.72±0.31; *P*<0.01). It showed that A/R injury could lead to an obvious increase in activity of NF-κB. But in the HS+A/R group, there was a statistically significant decrease in the activity of NF-κB compared with the A/R group (3.11±0.52 vs 5.76±0.64, *P* <0.01). The same statistically significant difference was also observed in the pCDNA HSP<sub>70</sub> + A/R and A/R groups (2.83±0.49 vs 5.76±0.64, *P*<0.01). These data indicate that HS stress and HSP<sub>70</sub> gene transfection could inhibit the increased of activity of NF-κB induced by A/R injury.

#### 4. Discussion

The present study provided evidence that upregulation of HSP<sub>70</sub> by a mild heat shock attenuates anoxia- reoxygeneration. Furthermore, transfection experiments directly showed that elevated expression of HSP<sub>70</sub> alone was sufficient for this protection. Using neonatal rat cardiac myocytes, we provided evidence that upregulation of HSP<sub>70</sub> by gene transfection attenuates A/R injury.

The important role of  $HSP_{70}$  in protecting the heart against ischemia-reperfusion has been clearly shown by many experiments. Using a protocol that mimics clinical donor heart preservation, Jayakumar et al<sup>3</sup> demonstrated that overexpression of  $HSP_{70}$  in whole hearts led to the protection of mitochondrial respiratory function and ventricular function after prolonged cold cardioplegic arrest. Wischmeyer et al<sup>14</sup> showed that administration of glutamine could significantly reduce the deleterious changes in myocardial metabolism and preserve cardiac output after reoxygeneration, which was owed to the enhanced expression of HSP<sub>70</sub> induced by glutamine. However, the protective effects may involve many pathways<sup>15,16</sup>. To confirm the direct effect of HSP<sub>70</sub> on cardiac protection gene transfection was adopted to modify neonatal rat cardiac myoctyes in our study and the results were excited.

The development of techniques to introduce exogenous DNA into mammalian somatic cells has opened up the possibility of treating inherited and acquired diseases at the genetic level. Gene transfer strategies involve viral and nonviral techniques. The concerns with viral techniques are that some viruses might disturb the host DNA transcription and synthesis, regain their pathogenic activity or generate immune responses<sup>17,18</sup>. Nonviral gene transfer techniques are attractive alternatives because they produce fewer side effects<sup>19</sup>. Lipofection, which is liposome-mediated transfection, is one such technique. In the present study the HSP<sub>70</sub> gene was transfected into cardiac cells successfully by lipofection. This result was confirmed by RT-PCR analysis and Western blot analysis.

In the present study the pCDNA HSP<sub>70</sub> was transfected into neonatal rat cardiac cells by lipofection and the overexpression of HSP<sub>70</sub> mRNA and protein were achieved. An A/R injury model was used to test HSP<sub>70</sub> protection: cell viability was improved, the activity of LDH and CPK were attenuated and the cell ultrastructure was nearly normal. There was no difference in cardiac cell protection between heat shock stress and HSP<sub>70</sub> gene transfection. It showed that the effect of cardiac protection could be achieved by HSP<sub>70</sub> gene transfection. And it also indicated that the effect of heat shock stress on cardiac protection is mediated by overexpression of HSP<sub>70</sub> gene.

Ischemia and reperfusion injury is a complex phenomenon directly associated with inflammatory changes. The traditional studies on the mechanisms of cardiac injury induced by A/R focused on the direct effect of toxic substances including proinflammatory cytokines,



Figure 4. Effect of various treatments on expression of HSP<sub>70</sub> mRNA in rat's cultured ventricular myocytes (*n*=8, mean±SD). A: representative electrophoretic diagram of agarose gels for HSP<sub>70</sub> mRNA. B: densitometric fold of A/R. (\**P* <0.01, vs A/R group; #*P* <0.01, vs HS+A/R group)

chemokines and adhesion molecules. However, a recent study shows a role of these factors in gene regulation of NF- $\kappa$ B in cardiac injuries. NF- $\kappa$ B is an inducible eukaryotic transcription factor that normally exists in an inert cytoplasmic complex bound to inhibitory proteins of the I $\kappa$ B family and is induced by a variety of pathogenic stimuli. Zhang et al<sup>20</sup> showed in their study that NF- $\kappa$ B was markedly increased in the ischemia- reperfusion injury heart. And they demonstrated that the cardioprotective effects of pentoxifylline against I/R injury were due to reduction in the activation of NF- $\kappa$ B. It has been demonstrated that exposure to a number of chemical inducers not only leads to HSP induction but also to inhibition of NF- $\kappa$ B<sup>21</sup>. In the present study, the activity of NF- $\kappa$ B were detected in each group and an obvious reduction in the activation of NF- $\kappa$ B was confirmed in HS+A/R group and in the pCDNA HSP<sub>70</sub> + A/R group. The results indicated that cardioprotective effect of HSP<sub>70</sub> may be due to inactivation of NF- $\kappa$ B. In summary, we demonstrated that overexpression of HSP<sub>70</sub> alone by gene transfection leads to the protection for cardiac myocytes against anoxia-reoxygeneration. These cardioprotective effects were related to the reduction in activation of NF- $\kappa$ B.



Figure 5. Effect of various treatments on expression of HSP<sub>70</sub> protein in rat's cultured ventricular myocytes (*n*=8, mean±SD). A: representative Western blots for HSP<sub>70</sub> protein. B: densitometric fold of A/R ( $^*P < 0.01$ , vs A/R group;  $^#P < 0.01$ , vs HS+A/R group)

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