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Temporal variations of Hsp60 and HSF-1 in primary rat myocardial cells *in vitro* under heat stress

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ABSTRACT. The mechanisms involved in sudden animal death due to acute heart failure during heat stress are not well understood. We examined the relationship between heat stress-induced variations of protective Hsp60 and expression of its regulatory factor, HSF-1, in heat-stressed primary myocardial cells of neonatal rats *in vitro* through cardiac enzyme detection, immunoblotting, immunocytochemistry, and qPCR. Increases in cardiac damage-related enzyme levels demonstrated injury to myocardial cells after heat exposure at 42°C. Hsp60 expression levels fluctuated during heat stress; they decreased significantly after 20 min, then increased at 120 min and decreased again at 360 min after initiation of heat stress. The highest levels of Hsp60 were observed at 240 min, while the lowest were at 60 min. Damage to myocardial cells was characterized by increases in cardiac

enzyme levels and low levels of Hsp60 due to functional disorder of myocardial cells at early stages of heat stress. However, the significant induction of *hsp60* mRNA levels from the beginning up to 240 min of heat stress was not consistent with the classic regulatory mechanisms that link transcription and translation, suggesting that Hsp60 expression is delayed due to loss of Hsp60 during the early stages of heat stress. *hsf-1* mRNA levels were significantly increased from 10 min of heat stress; however, HSF-1 protein levels did not simultaneously increase, indicating that HSF-1 is not the sole regulator of Hsp60 expression.

Key words: Heat shock protein 60; Heat shock protein messenger RNA; Heat stress; Myocardial cells; Heat shock factor-1

INTRODUCTION

Stress produces deleterious effects in living organisms. It can suppress growth and reduce production in animals. Animals respond to environmental stress with a series of reactions depicted as the "fight-or-flight" response (Banerjee et al., 2009). Studies on chronic heat stress have demonstrated altered physiological, metabolic, biochemical, and cellular response in animal models and poultry (Hu et al., 2007; Lu et al., 2007). Stressors are also responsible for eventual shock and sudden death in transported pigs and heated poultry (Lee et al., 1996). Myocardial ischemia is a known potent inducer of the stress protein response. Several stimuli trigger the upregulation of myocardial stress proteins in response to ischemia (Benjamin et al., 1992; Iwaki et al., 1993).

Hsps are the most broadly distributed class of proteins and are also among the most highly conserved proteins in nature. Hsps are named according to their molecular weight, for example, HSP60, HSP70, and HSP90 (Li and Srivastava, 2004). Hsp60 is a predominantly intracellularly located protein (Martin et al., 1992), and as a mitochondrial chaperone, it controls the transportation of proteins from the cytoplasm into the mitochondrial matrix where refolding occurs. In addition to its role as a heat shock protein, Hsp60 functions as a chaperonin to facilitate the folding of linear amino acid chains into their respective 3-dimensional structures. Hsps are primarily responsible for maintaining the integrity of cellular proteins particularly in response to environmental changes and stresses, such as temperature, concentration imbalance, pH changes, and toxins. Hsp60 constitutes approximately 15-30% of all cellular proteins (Ranford et al., 2000). Under normal growth conditions, Hsp60 expression changes following thermal challenge or stimulation from a variety of environmental stressors (Sunita et al., 2006; Sharma and Settleman, 2006). Cell death, particularly necrosis, may lead to the release of considerable amounts of Hsp60 into the circulation, rendering it accessible to the immune system where it can decisively influence the anti-Hsp immune reaction (Schett et al., 1999). Many studies have revealed the importance of Hsps for the survival of cells under stress conditions (McCormick et al., 2003; Romano et al., 2004).

Although stress increases the synthesis of Hsps, some Hsps such as Hsp60 are also constitutively expressed, and they play an essential role in protecting cells against stress. They are upregulated when cells are exposed to elevated temperatures or other stress (De Maio, 1999). This increase in Hsp expression is transcriptionally regulated. The dramatic upregula-

tion of Hsps is a key component of the heat shock response, and this upregulation is induced primarily by heat shock factor (HSF) (Wu, 1995). Previous studies reported that the effects of different types of stressors, e.g., transport in pigs and heat in poultry, induce animal mortality at certain stages, especially at an early stage of stress, and may be due to a variety of events that occur in response to transportation or heat stress (Yu et al., 2008; Zhang et al., 2011). However, there is still uncertainty in the research and control of the type and intensity of stressors that induce damages *in vivo* during stress conditions. Therefore, this problem may be better approached through *in vitro* studies to determine whether or not myocardial cell damages occur due to stress, as conditions can be more easily controlled.

In addition, little is known about the expression of Hsp60 in the dynamic state response to heat stress as well as the potential role of Hsp60 in protecting against hyperthermia-induced cellular damage. The aims of this study were to monitor the localization of Hsp60, the expression of Hsp60 and its corresponding mRNA levels, and the expression of HSF-1 and its corresponding mRNA levels in *in vitro* neonatal rat primary myocardial cells, and to correlate Hsp60 expression with cellular damage resulting from heat exposure for various times and the mechanism of HSF-1 on regulating Hsp60 expression.

MATERIAL AND METHODS

Cell culture and experimental treatment

The myocardial cells used in the present experiment were provided by Fu Meng Biological Technology Limited, Shanghai, China. The cells were incubated in DMEM medium in a 37°C incubator for 3 days for adaptation to the culture temperature. After 3 days, more than 85% of the cells were found viable, and the cultured cells were divided into different experimental groups. Experimental groups consisting of 9-cell culture plates were placed in a humidified atmosphere with 5% CO_2 and 95% air at 42°C, and control group plates were placed in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. A plate from each group was removed from the incubator after 0, 10, 20, 40, 60, 120, 240, 360, and 480 min.

Tests for LDH, AST, CK, and CK-MB activities

The supernatant of myocardial cells incubated on each plate was collected from both experimental and control groups, transferred to 1.5-mL centrifuge tubes, and labeled, after which it was stored at -80°C. The activities of LDH (A-020-1), AST (C010), CK (A032), and CK-MB (H197) were measured according to the instructions given in the commercial kits (Nanjing Jiancheng Biochemical Reagent Co., Nanjing, China).

Immunocytochemistry

Myocardial cells (2-8 x 10^4 cells per 35-cm^2 plate) grown on glass coverslips coated with polylysine solution were fixed in 4% paraformaldehyde for 30 min at room temperature (RT) and permeabilized with 0.4% Triton X-100 in phosphate-buffered saline (PBS). After blocking with 5% skim milk in PBS for 1 h, anti-rat Hsp60 monoclonal antibody (ab59457, Abcam, USA) at a 1:200 dilution was added to the coverslip, and the coverslips were incubated

in a moist chamber for 1 h at 37°C. The coverslips were then washed with PBS 3 times, and rhodamine red-conjugated goat anti-mouse IgG antibody at a 1:100 dilution (Boster, China) was added to the coverslips, which were further incubated at 37°C for 1 h. After washing with PBS, the coverslips were stained with DAPI solution. After again washing with PBS, the coverslips were dry-mounted. Finally, cardiac muscle cells were examined using an immunofluorescence microscope (Beckman, USA).

Western blotting

Myocardial cells treated at 42°C were washed with PBS and lysed with sodium dodecyl sulfate (SDS)-polyacrylamide gel Laemmli sample buffer. Cell lysates were collected and boiled for 5 min. Equal amounts of protein (10 μg) were subjected to SDS-10% polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 for 1 h at RT. The membranes were blocked with 5% skim milk in TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 0.1% Tween-20 and incubated with anti-rat Hsp60 monoclonal antibody (ab59457) for 16 h at 4°C. After washing with TBS, the membrane was further incubated with peroxidase-conjugated goat anti-mouse IgG antibody at RT for 1 h. The antibody-antigen complexes were then detected using Western blotting luminal reagent. The bands on the developed film were quantified using the Quantity One software version 4.6.2 (Bio-Rad, USA). The density of each band was normalized to that of β-actin protein.

Detection of *hsp60* mRNA by fluorescence quantitative real-time PCR (qPCR)

Isolation of total RNA for RT-PCR

After treatment of myocardial cells at 42°C, cells were removed from the incubator and washed in PBS. Total RNA was then isolated using TRIzol reagent according to manufacturer instructions. The concentration of RNA was detected by spectrophotometer (Mx3000P, Stratagene, USA) at 260 nm. Serial dilutions of RNA were prepared with ribonuclease-free water, and 2 μg of each sample was synthesized into DNA using a Transcript Moloney murine leukemia virus (M-MLV) kit (28025013, Gibco) following the manufacturer protocol and finally stored at -80°C. The M-MLV Reverse Transcriptase was used in the first step to extend a primer hybridized to an RNA sample. M-MLV has higher stability and lower intrinsic RNase H activity than AMV Reverse Transcriptase. Both random decamers and oligo dT are provided in the RETROscript® kit (Catalogue No. AM1710).

Primers designed for hsp60 mRNA

Primer sets were specifically designed to anneal each target mRNA, and the sequences of *hsp60*, *hsf-1*, and *β-actin* mRNA were obtained from the National Center for Biotechnology Information (NCBI) GenBank database (accession Nos. NC_005108.2, NP_077369.1, and NC_005111.2, respectively). The primers were designed using the Primer Premier 5.0 software for conventional and RT-PCR amplification. The primer sequences for these genes were as follows:

hsp60 sense: 5'-CCGCCCCGCAGAAATGCTTCGA-3'
hsp60 antisense: 5'-AGGCTCGAGCATCCGCACCAA-3'
hsf-1 sense: 5'-ACCCCAGCCTCTGCCTGCT-3'
hsf-1 antisense: 5'-TTCCCACTCGGGCTCCAGCA-3'
β-actin sense: 5'-CCCATCTATGAGGGTTCA-3'
β-actin antisense: 5'-TCACGCACGATTTCC-3'

The expected lengths of the PCR products for *hsp60*, *hsf-1*, and *β-actin* were 128, 153, and 143 bp, respectively.

qPCR

Each DNA sample (2 µL, 25X dilution) was suspended in 2X SYBR Premix Ex Taq™ (DRR041S, Takara, China) with primers (25 pmol of each sense and antisense primer), and double-distilled water was added to a total volume of 25 µL. qPCR was performed using an ABI 7300 qPCR thermocycler (7300, Applied Biosystems, USA). The thermal profile was established according to the manufacturer protocol. Briefly, this protocol consisted of enzyme activation at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 5 s and annealing and elongation at 52°C for 30 s. For each run, a negative control tube without DNA was run along with the experimental samples. A 2-fold dilution series of the template was used in the fluorescence quantitative-PCRs. The *hsp60* mRNA expression of all samples could be normalized using the following formula:

$$\text{Relative quantity of } hsp60 \text{ mRNA} = 2^{-\Delta\Delta Ct}$$

$$\Delta\Delta Ct = (Ct_{hsp60\ \text{mRNA}} - Ct_{\beta\text{-}actin\ \text{mRNA}})_{\text{control group}} - (Ct_{hsp60\ \text{mRNA}} - Ct_{\beta\text{-}actin\ \text{mRNA}})_{\text{test group}}$$

Statistical analysis

Statistical analysis of the differences between the experimental group and control group values was performed with one-way analysis of variance followed by the least significant difference multiple test comparison test provided by SPSS version 17.0 for Windows. The results are reported as means ± standard deviation of at least 3 independent experiments. $P < 0.05$ was considered to be statistically significant.

RESULTS

Levels of AST, LDH, CK, and CK-MB in cell supernatant

The AST, LDH, and CK levels in the supernatant from the incubated primary myocardial cells are shown in Figure 1. AST activity was obviously induced in the heat-stressed groups ($P < 0.01$) in comparison with the control levels throughout the heat stress period. LDH activity displayed the same increase as AST levels, although statistically significant induction was only observed after 20 and 480 min of heat stress ($P < 0.05$). CK activity levels increased immediately in response to heat stress; however, significant increases in CK activity were

not observed until 120 min ($P < 0.05$). The levels of CK-MB were immediately increased in response to heat stress, and high levels of CK-MB activity were observed after 60 ($P < 0.05$) and 120 min ($P < 0.05$) of heat stress.

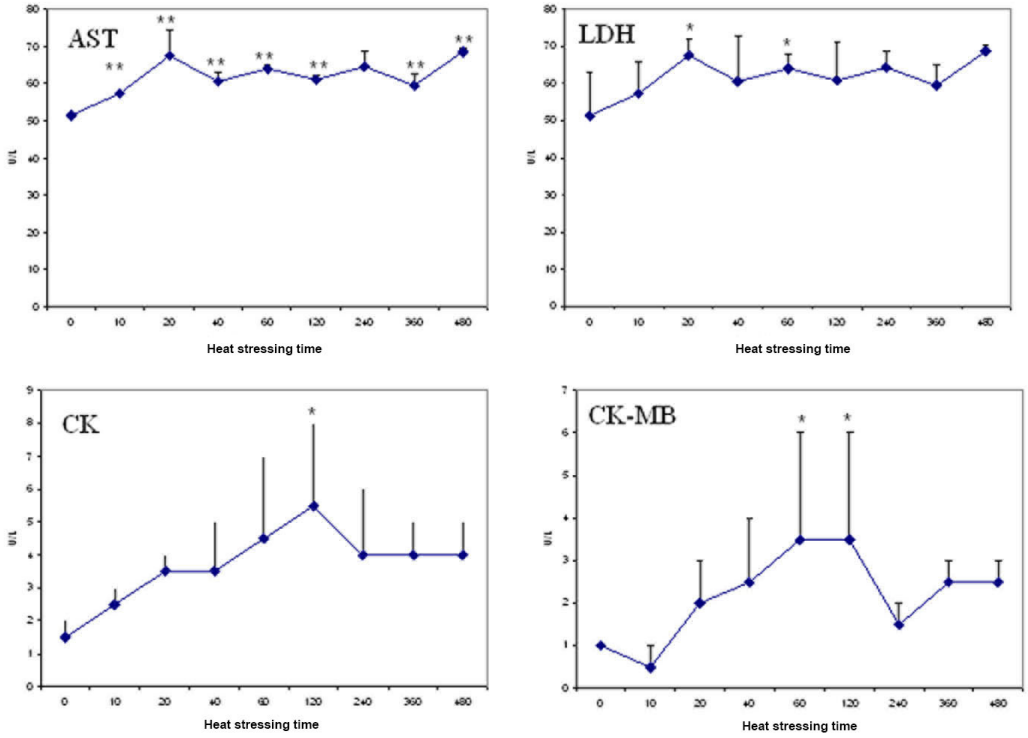

Figure 1. Activity levels of serum enzymes in the supernatant of myocardial cells (U/L). *$P < 0.05$; **$P < 0.01$; values are reported as means ± SD.

Localization and distribution of Hsp60 and HSF-1 in myocardial cells *in vitro*

Immunocytochemical staining of Hsp60 and HSF-1 in the neonatal rat primary cardiac muscle cells is shown in Figures 2 and 3, respectively.

Hsp60 was consistently present in the cytoplasm of myocardial cells both in the experimental and control groups. The density of Hsp60 was higher in control group than in the experimental group after 60 min of heat stress. However, the highest density of Hsp60 signals was observed in the cytoplasm of the myocardial cells after 240 min of heat stress. The most conspicuous positive signals of Hsp60 were observed after 240 min of heat stress at 42°C. The granular staining pattern of Hsp60 was scattered with low density in the cytoplasm of control myocardial cells; however, the density of Hsp60-positive staining was increased in the cytoplasm of cells after 240 min of heat stress. The staining density of Hsp60 appeared to increase gradually with heat stress time after 60 min at 42°C.

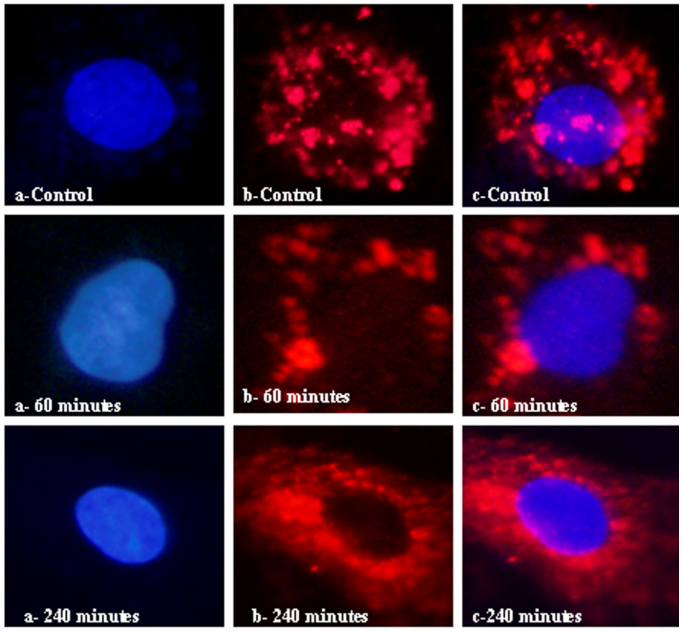

Figure 2. Localization and distribution of Hsp60 in myocardial cells *in vitro*. **a.** DAPI; **b.** Hsp60; **c.** Merge.

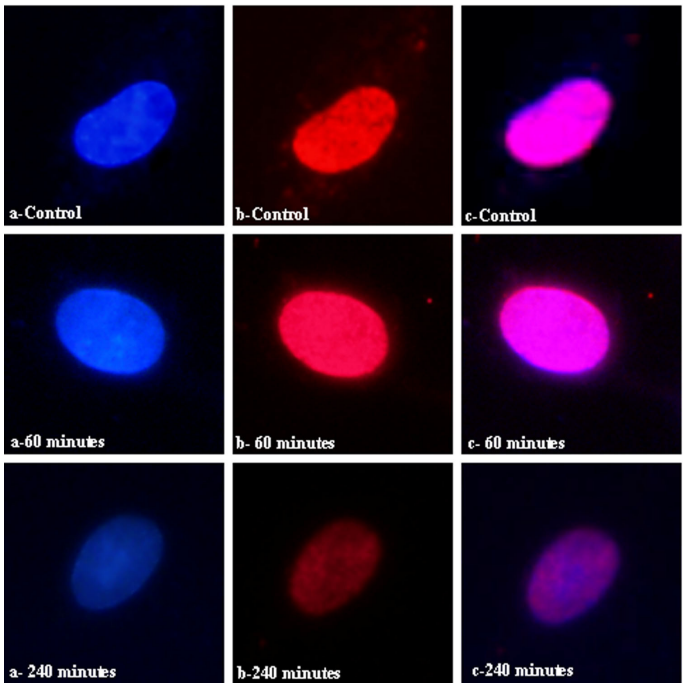

Figure 3. Localization of HSF-1 in the primary cultured myocardial cells of neonatal rats after heat stress. **a.** DAPI; **b.** HSF-1; **c.** Merge.

Immunocytochemical staining of HSF-1 in the primary myocardial cells of neonate rats is shown in Figure 3. The positive staining of HSF-1 revealed that the HSF-1 protein was present in the cytoplasm as well as in the nucleus.

Transcription levels of *hsp60* and *hsf-1* in heat-stressed myocardial cells

The levels of *hsp60* and *hsf-1* mRNA normalized to heart tissue *β-actin* mRNA are displayed in Figures 4 and 5, respectively.

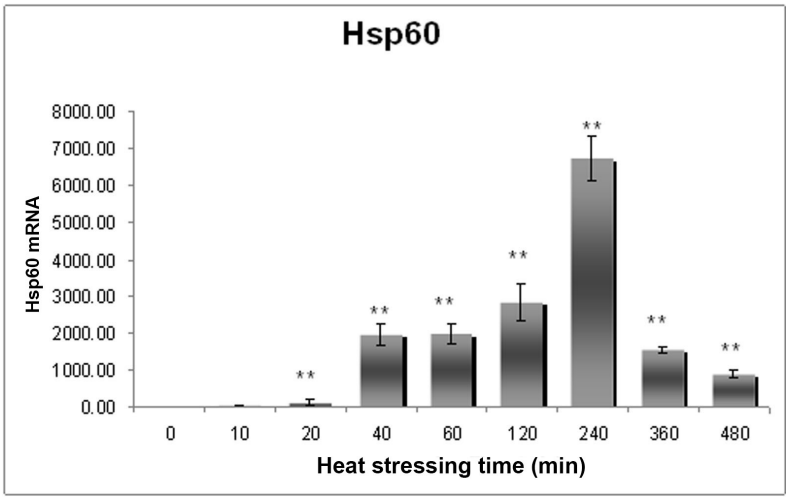

Figure 4. Transcription levels of *hsp60* mRNA in neonatal rat primary cultured myocardial cells after heat stress. **P < 0.01; values are reported as means ± SD.

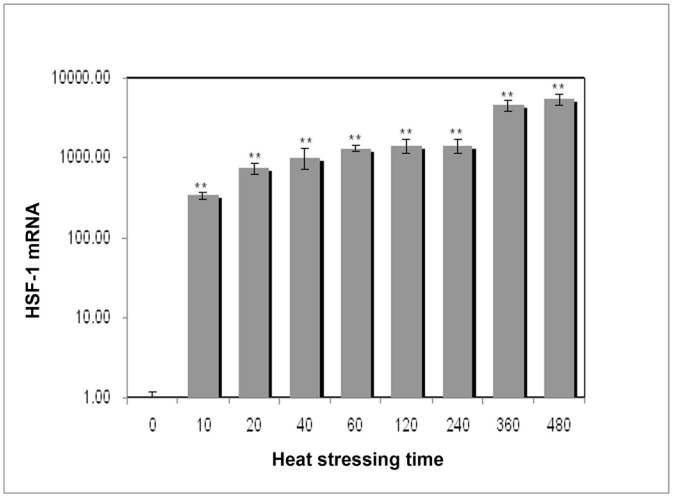

Figure 5. Transcription levels of *hsf-1* mRNA in neonatal rat primary cultured myocardial cells after heat stress. *P < 0.05; **P < 0.01; values are reported as means ± SD.

The levels of *hsp60* mRNA in the rat myocardial cells *in vitro* were increased by heat exposure, with significant increases observed after 20 min of heat stress ($P < 0.01$). *hsp60* mRNA transcription peaked after 240 min of heat stress ($P < 0.01$) but was significantly lower after 360 min of heat exposure ($P < 0.01$), albeit obviously higher than that in control myocardial cells ($P < 0.01$).

The transcription levels of *hsf-1* in the primary myocardial cells of neonatal rats are shown in Figure 5. The graph shows that the levels of *hsf-1* mRNA were significantly increased after 10 min of heat stress ($P < 0.01$) and sustained for 480 min of heat stress ($P < 0.01$).

Quantitative detection of Hsp60 and HSF-1 expression in heat-stressed myocardial cells

The results of Western blot analysis are shown in Figures 6 and 7. Hsp60 expression was significantly decreased ($P < 0.01$) in response to heat stress compared with that in the control group, particularly at 20 and 40 min, after which it increased gradually. After 120 min of heat exposure, Hsp60 expression was significantly increased ($P < 0.01$) and sustained after 240 min of heat exposure ($P < 0.05$). Beyond this point, Hsp60 expression started to gradually decrease, and its expression was significantly lower than that in the control cells after 360 and 480 min of heat stress ($P < 0.01$).

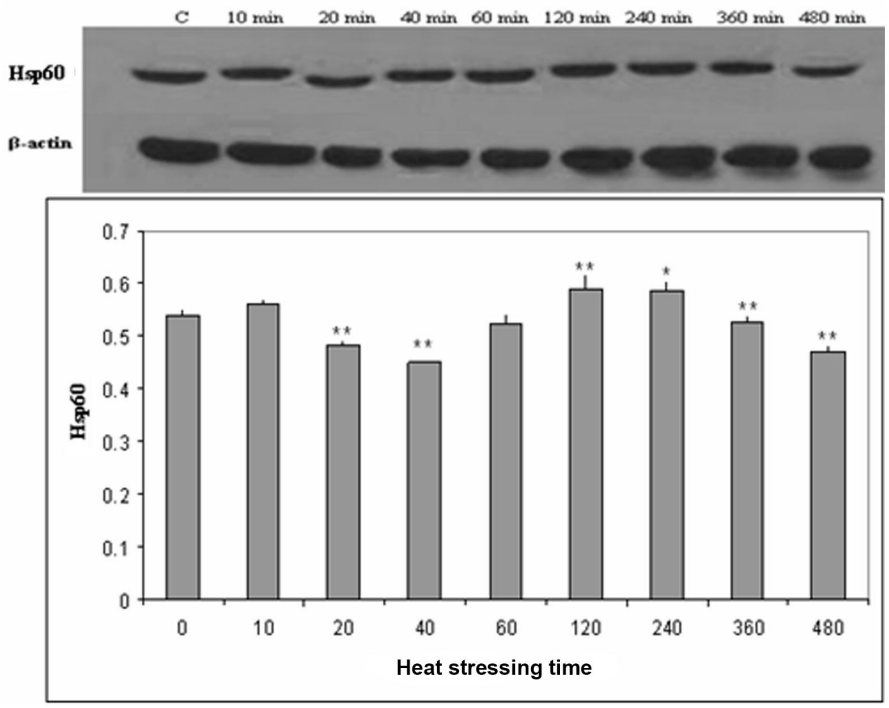

Figure 6. Expression levels of Hsp60 in neonatal rat primary cultured myocardial cells after heat stress. *$P < 0.05$; **$P < 0.01$; values are reported as means ± SD.

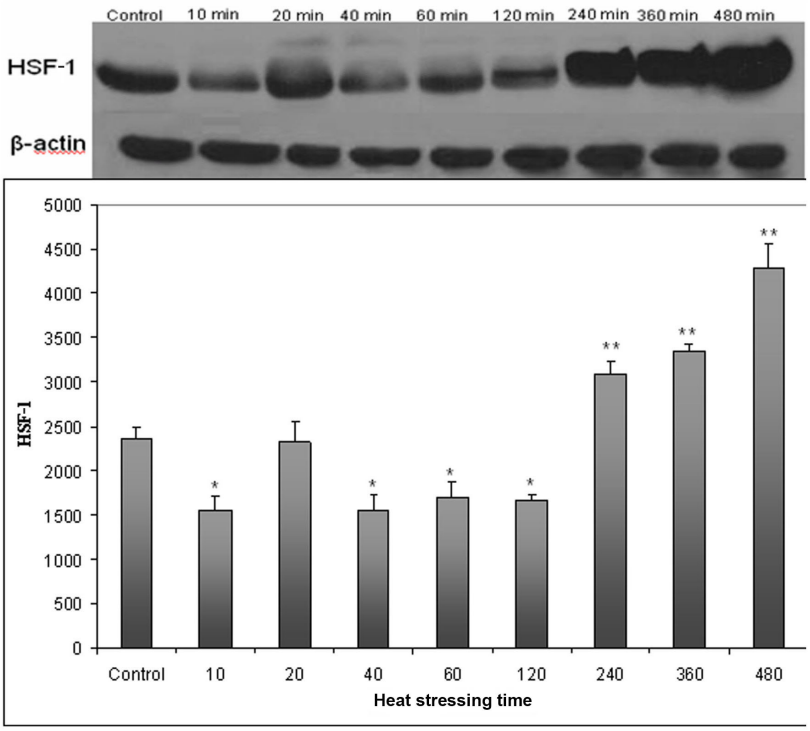

Figure 7. Expression levels of HSF-1 in neonatal rat primary cultured myocardial cells after heat stress. *P < 0.05; **P < 0.01; values are reported as means ± SD.

Compared to the control group levels, the levels of HSF-1 were obviously decreased (P < 0.01) in response to heat stress for up to 120 min. Beginning at 240 min of heat exposure, the levels of HSF-1 gradually and significantly increased and remained elevated after 480 min of heat stress.

DISCUSSION

In this study, we were successful in developing a heat stress model of primary myocardial cells characterized by increased levels of cardiac function markers. Many studies have reported that increased levels of AST, ALT, LDH, GGT, and CPK are consistently correlated with cardiac infarcts (Yogeeta et al., 2006; Rajadurai and Stanely, 2007). Myocardial cell damage induced by heat stress was observed through the gradual elevation of LDH, AST, and CK levels in the present study. CK is primarily distributed in myocardial cells, but its distribution changes in response to myocardial injury. When an external stress causes membrane damage in myocardial cells, CK is released from myocardial cells, resulting in elevated blood levels of CK *in vivo* and elevated levels of CK in culture medium *in vitro* (Britton et al., 1980). AST is mainly distributed in the heart, brain, liver, muscle, kidney, and other organs and can be utilized for the diagnosis of myocardial injury (Oswald et al., 1986). During myocardial infarction, CK and CK-MB levels are elevated, and this elevation plays a role in diagnosis,

especially in myocarditis (Oswald et al., 1986). CK is usually found in muscles and also in cardiac muscle fibers after intensive physical exertion, which can also be associated with the suffering of animals. Elevated serum levels of ALT and AST are often connected with heart and liver disease or damage (Wada and Kamiike, 1990). In this study, the levels of LDH, AST, CK-MB, and CK were higher in the experimental groups than in the control group during the period of heat stress, indicating that they are sensitive parameters of myocardial damage.

Hsps are a family of highly conserved cytosolic proteins that are transiently expressed after exposure of the cell to sublethal environmental stimuli such as hyperthermic and oxidative stress. Several studies have demonstrated that controlled *in vivo* hyperthermic preconditioning resulting in elevated Hsp levels can improve myocardial functional recovery in isolated ischemic heart models (Jayakumar et al., 1998). The localization of Hsps may be related to the protection function of molecular chaperones (Georgopoulos and Welch, 1993). The immunohistochemical results revealed the higher density of Hsp60-positive signals in the control group and after 240 min of heat stress than after 60 min of heat stress. This finding indicates that Hsp60 consumption exceeded its production after 60 min of heat stress; however, after 240 min, the cells produced sufficient Hsp60 protein to protect them against heat stress. As revealed by immunocytochemistry, Hsp60 was primarily expressed in the cytoplasm of myocardial cells, consistent with previous findings of Hsp60 in muscle fibers in humans. Hsp60 expression in the cytoplasm of myocardial cells was more prominent in intact areas than in degenerated areas (Kervinen et al., 2003). In myocardial cells, Hsp60 staining was clearly lower in the cytoplasm of granular degenerated areas (Yu et al., 2008). ATP depletion is known to have detrimental effects that range from protein aggregation and collapse of the cytoskeleton to the loss of ionic balance. Heat stress and subsequent Hsp60 accumulation give rise to an ATP-sparing effect (Kabakov and Gabai, 1997). Our Western blot results also revealed that the level of Hsp60 was significantly decreased after 20 and 40 min of heat stress but significantly increased after 120 min of heat stress. Hsp60 expression remained elevated after 240 min of heat stress but gradually decreased after this point. These results are in accordance with the results of immunocytochemistry and enzyme concentrations, which revealed the reduction of Hsp60 signals in the cytoplasm at 60 min and the subsequent increase of Hsp60 levels after 240 min of heat exposure. The damage of myocardial cells characterized by enzyme elevations and over consumption of Hsp60 concerned to the functional disorder of myocardial cells at early stage of heat stress. The concentrations of various myocardial enzymes such as AST, LDH, CK, and CK-MB are indicative of damage in myocardial cells after heat exposure. The levels of Hsp60 remain elevated above control levels after 3, 5, and 10 h of heat exposure (Yu et al., 2008). A 5-fold increase in Hsp60 levels has been shown in the hearts of patients with dilated cardiomyopathy relative to those in normal subjects (Latif et al., 1999). Therefore, mitochondrial HSP60 plays an important role in maintaining mitochondrial integrity, function, and capacity for ATP generation, which are crucial factors for determining the survival of myocardial cells after heat stress-induced injury. Molecular chaperones influence several cellular components including the cytoskeleton, an assortment of filamentous and tubular polymers composed of microtubules, microfilaments, and intermediate filaments (Liang and MacRae, 1997).

Variations in Hsp60 protein levels did not correspond to changes in *hsp60* mRNA levels in the myocardial cells of neonatal rats after heat stress. These observations are not consistent with the classic regulatory mechanisms that link transcription and translation. In the present study, the transcription levels of *hsp60* mRNA in the myocardial cells of neonatal

rats were increased significantly ($P < 0.01$) after 10 min of heat stress and remained increased after 240 min of heat stress. Although the transcription of *hsp60* mRNA gradually and significantly decreased ($P < 0.01$) after 360 min of heat treatment, it remained much higher than that observed in the control group. Conversely, the transcription levels of *hsf-1* mRNA increased significantly after 10 min of heat stress and remained elevated throughout the stress treatment. Several studies have demonstrated that elevation of Hsps in the heart provides protection against stress-induced myocardial injury. For example, overexpression of Hsp72 reduced infarct size in an *in vivo* transgenic mouse model of myocardial ischemia and reperfusion (Hutter et al., 1996). Elevations of *hsp60*, *hsp70*, and *hsp90* mRNA (especially *hsp60* mRNA) occur in the heart after 2 h of heat stress, and these factors may act as important markers and protective proteins in response to adverse environmental conditions (Yu et al., 2008). In eukaryotic cells, the heat shock response involves transcriptional activation mediated by a transcription factor known as HSF (Morimoto, 1993). Our results are in line with those revealing that the levels of *hsp60* mRNA transcripts in the heart decrease sharply after 3 h of heat stress (Yu et al., 2008). When the Hsp protein concentration increases to a high level, it can bind HSF, thereby inhibiting HSF activation and reducing the HSF- and heat shock element (HSE)-specific binding that controls heat shock gene transcription. Consequently, *hsp* transcription can be sustained at a certain level (Morimoto, 1993). The overexpression of Hsp60 may be the reason for the increased requirement for ATP under heat stress in the heart due to the increasing rate of heart contraction (Koelkebeck and Odom, 1995). Thus, heat stress response results in the accumulation of heat stress proteins.

HSF-1 is a mammalian cell-mediated response to heat shock, the major heat shock transcription factor (Rabindran et al., 1991). In nonstressed states, HSF-1 is usually distributed in the cytoplasm in an inactive monomer form. In the present study, the immunofluorescence assay indicated that HSF-1 was stably distributed in the nucleus of cardiac cells before and after heat stress, which is different from previous reports (Vujanac et al., 2005). However, our results also indicated that *hsf-1* mRNA levels were significantly increased after 10 min of heat stress. HSF-1 protein expression was not simultaneously increased. The level of *hsf-1* mRNA remained low up to 120 min of heat exposure and then increased significantly after longer exposure times. HSFs assemble into trimers and accumulate within the nucleus. Activation and binding of HSFs to HSEs, the specific DNA recognition sequences located in the 5'-flanking sequences of heat shock-responsive genes, are detected within minutes of temperature elevation (Perisic et al., 1989; Sarge et al., 1993). In response to stress, a single HSF-1 phosphorylation event results in the rapid aggregation of HSF-1 into a trimer that can bind to HSE and initiate the transcription process (Sorger and Pelham, 1988; Larson et al., 1988; Cotto et al., 1996; Winegarden et al., 1996).

In summary, a model of myocardial cells was developed and analyzed by release of myocardial enzymes. The immunohistochemical results indicated the consumption of Hsp60 after 60 min of heat stress and reproduction after 240 min of heat stress to protect the myocardial cells against heat stress. The Western blot results also revealed that the level of Hsp60 were decreased after 20 and 40 min of heat stress but increased after 120 min of heat stress so that it may be able to protect the cells against heat stress. Hsp60 expression remained elevated after 240 min of heat stress but decreased gradually after this point. *hsf-1* transcription was stimulated and mRNA levels significantly increased after 10 min of heat stress. HSF-1 protein expression was not simultaneously increased. However, the detailed mechanism is still unclear and requires further study.

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