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The role of Hsp90α in heat-induced apoptosis and cell damage in primary myocardial cell cultures of neonatal rats

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ABSTRACT. To understand the mechanism underlying the sudden animal death caused by acute heart failure during heat stress, the relationships among the heat-induced pathological changes and apoptosis and the variations in the levels of protective Hsp90α and its mRNA in the heat-stressed primary myocardial cells of neonatal rats *in vitro* were studied by cytopathological observation, immunoblotting, RT-PCR, and analysis of the related enzymes. After a period of adaptive cell culture, the myocardial cells were immediately exposed to heat stress at 42°C for 10, 20, 40, 60, 120, 240, 360, and 480 min. Levels of creatine kinase increased from the beginning of heat stress, and the cells exposed to heat stress showed acute cellular

lesions characterized by vacuolar degeneration and necrosis after 40 min of heat stress, suggesting that the myocardial cells *in vitro* were obviously stressed and damaged by higher temperature. The levels of cleaved caspase-3 and cytochrome C, which were related to apoptosis, increased significantly after 40 min of heat stress while the Hsp90α protein level significantly decreased. In contrast, after 6 h of exposure to heat stress, the levels of cleaved caspase-3 and cytochrome C decreased while those of Hsp90α significantly increased, suggesting that early depletion of Hsp90α coincides with a high rate of necrosis and apoptosis in heat-stressed myocardial cells, while the Hsp90α level in surviving cells increases again with significantly less apoptosis after 6 h of heat stress. These findings also indicate that apoptosis of myocardial cells occurs through the activation of the cytochrome C and caspase-3 pathway. The cell repair capacity of Hsp90α is overstrained in the early phase of heat treatment and needs some hours to stabilize. As a result, in the primary myocardial cells *in vitro*, Hsp90α shows protective activity against damage at the end period of the heat exposure.

Key words: Heat stress; Hsp90α; Myocardial cells; Rat; Apoptosis

INTRODUCTION

High stress levels can have negative effects on living organisms and its organs, as demonstrated in various studies with transport-stressed pigs and heat-stressed chicken, which eventually show heat shock and sudden death (Lee et al., 1996; Yu et al., 2008). The mechanisms underlying the cellular damage and death of animals exposed to these stresses are not yet well understood, although a number of factors and physiological reactions are already known (Kamarck and Jennings, 1991). For instance, heat stress has been reported to cause myocardial cell damage in chicken and other animals (Heads et al., 1995; Yu et al., 2008; Yan et al., 2009). Apoptosis is one of the biochemical events that can occur in all tissues; in heart cells, for example, apoptosis causes a loss of adult cardiomyocytes under conditions like heat stress and leads to conditions like myocardial ischemia, which is a potent inducer of the stress protein response of cells (Knowlton et al., 1991; Morris et al., 1996) and finally to death.

The role of heat shock proteins (Hsp) in the regulation of cell survival, proliferation, apoptosis, and cell death resulting from stress has been described (Parsell and Lindquist, 1993). Heat shock proteins are highly conserved proteins whose expression in cells of warm-blooded animals (Latchman, 2001) is induced in response to a wide variety of physiological and environmental influences, including heat exposure (McCormick et al., 2003; Ganter et al., 2006; Staib et al., 2007). Among the numerous types of HSP families, members of the HSP90 family are the most expressed stress proteins synthesized by normal unstressed cells and increased in response to stressful stimuli. The functions of HSP90 include assisting in protein folding, cell signaling, and tumor repression (Prodromou et al., 2000). Hsp90α and Hsp90β are major isoforms of HSP90 (Csermely et al., 1998; Sreedhar et al., 2004). Hsp90α, one of the main types of HSP90, is a copious, well-preserved cytosolic protein that accounts for 1-2%

of all cellular proteins in nearly all cells under basal, non-stress conditions and shows an increase in response to heat stress and other proteotoxic insults (Bagatell et al., 2000). Because of its highly conserved and inducible nature, Hsp90α acts as a good mediator of cellular stress (Prohaszka and Fust, 2004). However, the mechanisms responsible for the protective function of Hsp90α are not fully understood. Therefore, the purpose of this study was to understand the relationships among the heat stress-induced pathological changes and apoptosis of heat-stressed myocardial cells, and the variations in the levels of the protective Hsp90α and its corresponding mRNA *in vitro*.

MATERIAL AND METHODS

Cell culture and experimental treatment

Primary myocardial cells of a neonatal rat (Fu Meng Biological Technology Ltd., Shanghai, China) were incubated at 37°C in humidified atmosphere of 5% CO_2 and 95% air for 72 h to allow the cells to adapt to the temperature. When a minimum of 85% of the cells in a culture were alive and viable after this period, the cells were suddenly exposed to heat stress *in vitro* for 10, 20, 40 min, 1, 2, 4, 6, and 8 h in another incubator with 95% air and 5% CO_2 at 42°C. The control group was maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Meanwhile, the primary myocardial cells were grown on polylysine-coated coverslips (size of the plates, 35 cm^2) at a density of 2-8 x 10^4 cells/plate for detection of pathological changes.

Determination of creatine kinase (CK) activity

From each plate, 1.5 mL of the supernatant liquids of the myocardial cell cultures was collected, transferred to centrifuge tubes, and stored at -80°C. The activity of CK was measured according to the instructions provided in the commercial kits (A032; Nanjing Jiancheng Biochemical Reagent Co., Nanjing, China).

Cytopathological examination

After collecting the supernatants, the myocardial cells grown on polylysine-coated coverslips were fixed with 4% paraformaldehyde for 30 min at room temperature (RT). After fixation, the cells were dehydrated with graded series of alcohol, stained by hematoxylin and eosin staining, and examined by light microscopy.

Detection of *hsp90α mRNA* by fluorescence quantitative real-time PCR (FQ-RT-PCR)

Isolation of total RNA and RT-PCR

After exposing the experimental culture plates to 42°C for the respective periods, the plates were taken out of the incubator and washed with phosphate-buffered saline (PBS). Total RNA was isolated by using Trizol reagent (Invitrogen, USA) according to manufacturer instructions. The concentration of RNA was determined by a spectrophotometer (Mx3000P;

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 the Transcript M-MLV kit (AM1710; Invitrogen) according to the manufacturer protocol, and finally stored at -80°C.

Design of primers for *hsp90α mRNA*

Primer sets were specifically designed to anneal to each target *mRNA*. The sequences of *hsp90α mRNA* and *β-actin mRNA* were obtained from the National Center for Biotechnology Information's (NCBI) Genbank (accession NC_005105.2 and NC_005111.2, respectively). Using this sequence, primers were designed for *hsp90α* and *β-actin* by the Primer Premier 5.0 software for RT-PCR amplification. Primer sequences for these genes were as follows: *hsp90α* gene (forward primer: 5'-CCCGGTGCGGTTAGTCACGT-3'; reverse primer: 5'-TCCAGAGCGTCTGAGGAGTTGGA-3'). The expect width of PCR products was 214 bp. *β-actin* gene (forward primer: 5'-CCCATCTATGAGGGTTCA-3'; reverse primer: 5'-TCACGCACGATTTCC-3'). The expect width of PCR products was 143 bp.

FQ-RT-PCR

Each DNA sample (2 μL, 25X dilution) was suspended in 2X SYBR Premix Ex Taq™ (15218-019, Invitrogen) with primer (25 pmol of both sense and anti-sense primer), and double-distilled water to a total volume of 25 μL. Quantitative PCR was performed using an ABI 7300 RT-PCR thermocycler (Applied Biosystems, USA). The thermal profile was established according to the manufacturer protocol. Briefly, this protocol involved enzyme activation at 95°C for 3 min, followed by denaturation at 95°C for 5 s, and annealing and elongation at 52°C for 30 s, for a total of 45 cycles. 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 FQ-PCR. The *hsp90α mRNA* of all samples was normalized using the following formula: Relative quantity of *hsp90α mRNA* = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{hsp90\alpha\ mRNA} - Ct_{\beta\text{-}actin\ mRNA})$ control group - $(Ct_{hsp90\alpha\ mRNA} - Ct_{\beta\text{-}actin\ mRNA})$ test group.

Western blot analysis

After exposure to heat stress at 42°C, myocardial cells 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 transferred to a nitrocellulose membrane by electrotransfer. The membrane was blocked with 5% non-fat milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 0.1% Tween-20 (TBST) for 1 h at RT, and incubated with anti-rat Hsp90α monoclonal antibody (ab79849; Abcam, USA), anti-caspase-3 (ab7980; Abcam), and anti-cytochrome C (ab13575; Abcam) for 16 h at 4°C. After washing with TBST, the membrane was further incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (ab6789; Abcam) at RT for 1 h. Then, the antibody-antigen complexes were detected using Western blotting luminal reagent. The bands on the developed film were quantified with the Quantity one-4.6.2 software (Bio-rad, USA). The density of each band was normalized to that of β-actin protein.

Statistical analysis

All analyses were performed using one-way analysis of variance (ANOVA) with the Statistical Package for Social Sciences (SPSS version 16.0). The Duncan test for multiple comparisons was carried out to compare the mean value of the control group with that of each experimental group. Differences were regarded as significant at $P < 0.05$. Three repetitions were performed for all the experiments above.

RESULTS

CK levels

The levels of CK increased gradually from the beginning of exposure to heat stress and reached the highest level at 2 h of heat stress ($P < 0.05$). The CK levels remained obviously higher than that in control myocardial cells, although they started to decrease from 4 h of heat stress (Figure 1).

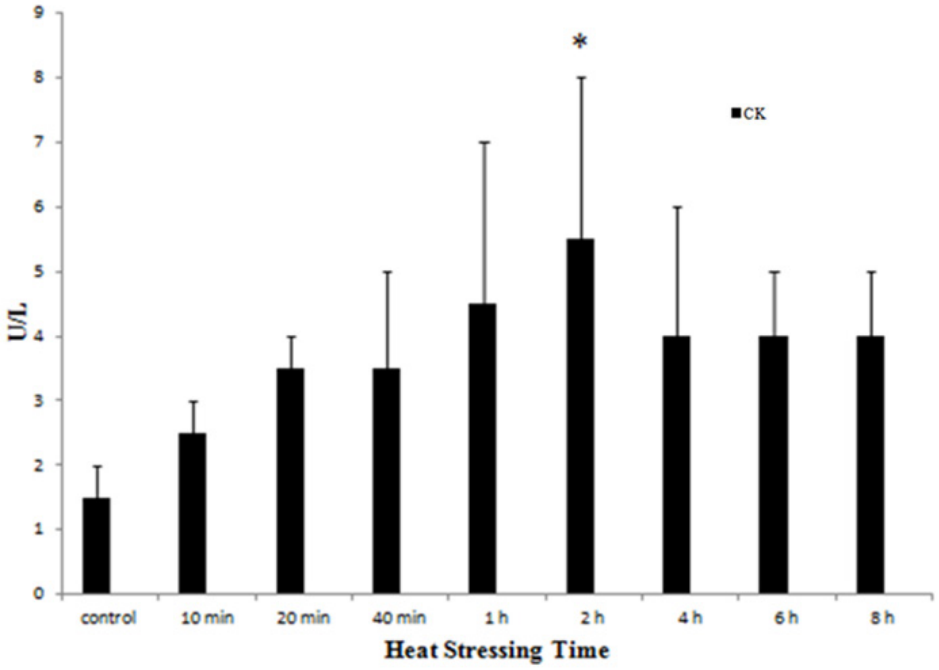

Figure 1. Levels of enzyme creatine kinase (CK) in the heat-stressed myocardial cells of rat *in vitro* (U/L). *P < 0.05 when comparing the heat stressed groups with the control group. Values are reported as means ± SD; N = 3.

Cytopathological observation

Cytopathological changes of the primary myocardial cells *in vitro* are illustrated in Figure 2. The myocardial cells showed acute degeneration characterized by enlarged cell size

and small vacuoles in the cytoplasm as soon as they were exposed to high temperature. After 40 min of heat stress, the myocardial cells showed obvious vacuolar degeneration characterized by numerous small vacuoles, fine granular particles in the cytoplasm, and enlarged cell size. Necrotic cells characterized by pyknosis of the nucleus and cytoplasmic vacuolar degeneration were occasionally observed in the cytoplasm of myocardial cells after about 2 h of heat stress. Admixture of degenerated cells characterized by small vacuoles and deep red staining granules in the cytoplasm and dead cells characterized by pyknosis was still observed in the heat-stressed myocardial cells after 8 h of heat stress. No obvious pathological changes were found in the myocardial cells of the control group.

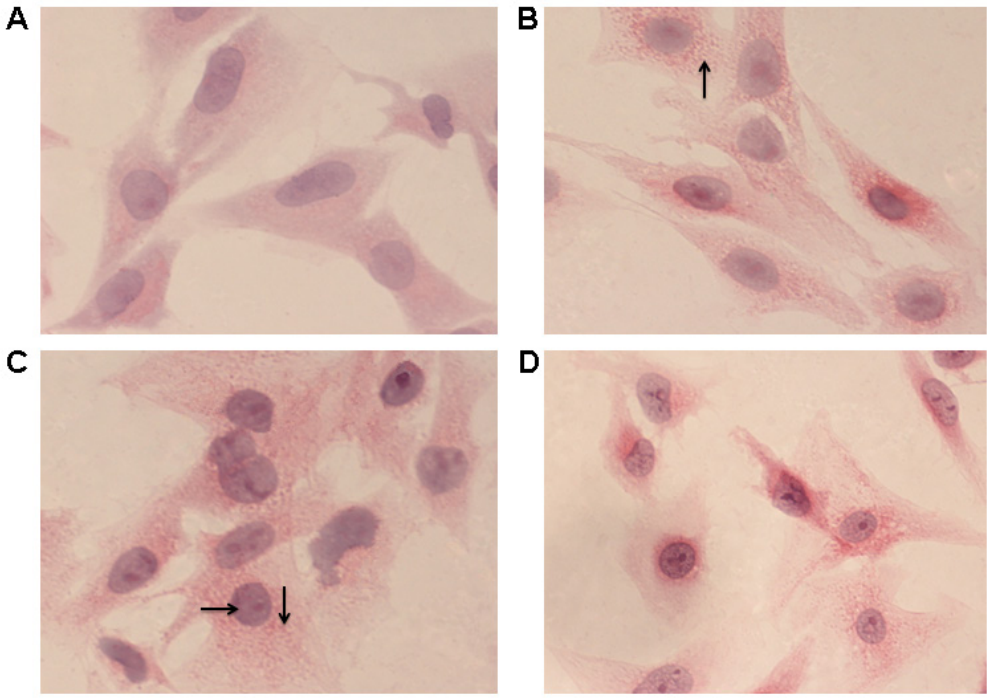

Figure 2. Representative photomicrographs of primary myocardial cells *in vitro* H&E stainning. Scale bar = 20 μm. **A.** Primary myocardial cells of a control group. **B.** Acute degeneration characterized by small vacuoles, lightly red staining granules in the cytoplasm and enlarged cell size of myocardial cells (arrow) after 40 min of heat stress. **C.** Acute degeneration characterized by small vacuoles (vertical arrow) in the cytoplasm and necrosis characterized by pyknosis (horizontal arrow) after 2 h of heat stress. **D.** Degeneration and necrosis characterized by deep red staining granules and pyknosis in the cytoplasm of myocardial cells after 8 h of heat stress.

Caspase-3 expression

The expression levels of caspase-3 at various stages of apoptosis is shown in Figure 3. The levels of caspase-3 increased significantly ($P < 0.01$) after 20 min of heat stress and remained high from 40 min to 4 h of heat stress. Except for a reduction at 6 h of heat stress.

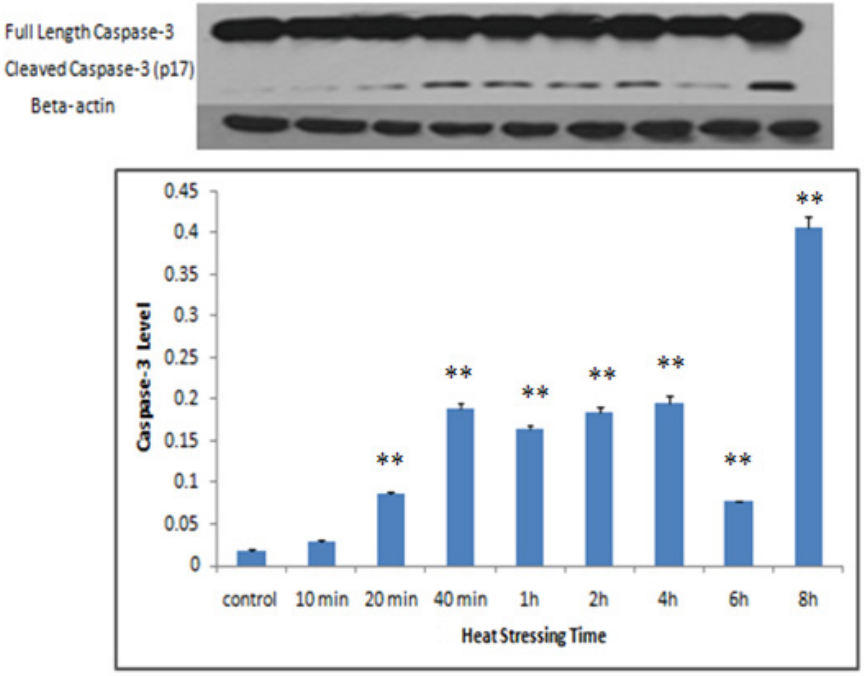

Figure 3. Levels of caspase-3 in the heat-stressed myocardial cells *in vitro*. **P < 0.01 when comparing the heat-stressed groups with the control group. Values are reported as means ± SD; N = 3.

Cytochrome C levels

The levels of cytochrome C increased significantly at 20 min of heat stress and reached the highest level at 40 min of heat stress (P < 0.01), in comparison with the control levels. Although the levels of cytochrome C decreased gradually from 1 to 4 h of heat stress, they remained obviously higher (P < 0.01) than those in the control myocardial cells (Figure 4). At 8 h of heat stress, the levels of cytochrome C continued to increase (P < 0.01), except for a reduction at 6 h of heat stress.

Transcription levels of *hsp90α* mRNA in the heat-stressed myocardial cells of rat

The transcription levels of *hsp90α* mRNA increased immediately from the beginning (10 min) of heat stress (P < 0.05) and, in comparison with control myocardial cells, showed significant inductions over the period during which the cells were exposed to heat stress (P < 0.01). From 20 min to 1 h of heat stress, the transcription levels of *hsp90α* mRNA in the heat-stressed myocardial cells increased from 28 to 50% (28- to 50-fold) higher than that of the control group. From 2 to 8 h of heat stress, the levels of *hsp90α* mRNA increased from 200 to 280% (200- to 280-fold) higher than those in the control group, and *hsp90α* mRNA transcription reached its peak level (P < 0.01) at 4 h of heat stress. After 4 h of heat stress, the transcription level of *hsp90α* mRNA still remained at obviously higher levels than that in control myocardial cells (P < 0.01) although its transcription levels decreased gradually after 6 and 8 h of heat stress (Figure 5).

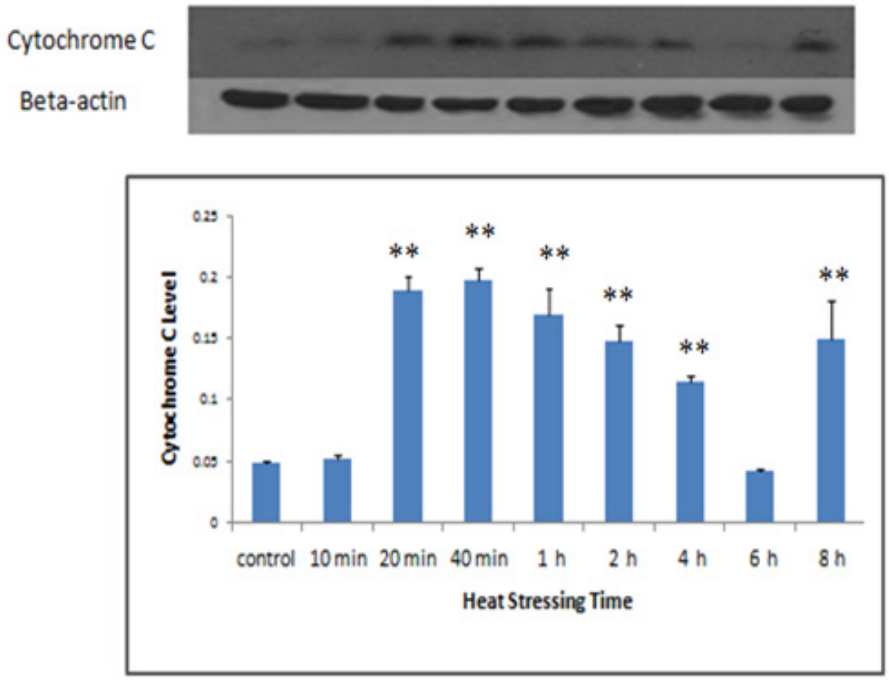

Figure 4. Levels of cytochrome C in the heat-stressed myocardial cells *in vitro*. **P < 0.01 when comparing the heat stressed groups with the control group. Values are reported as means ± SD; N = 3.

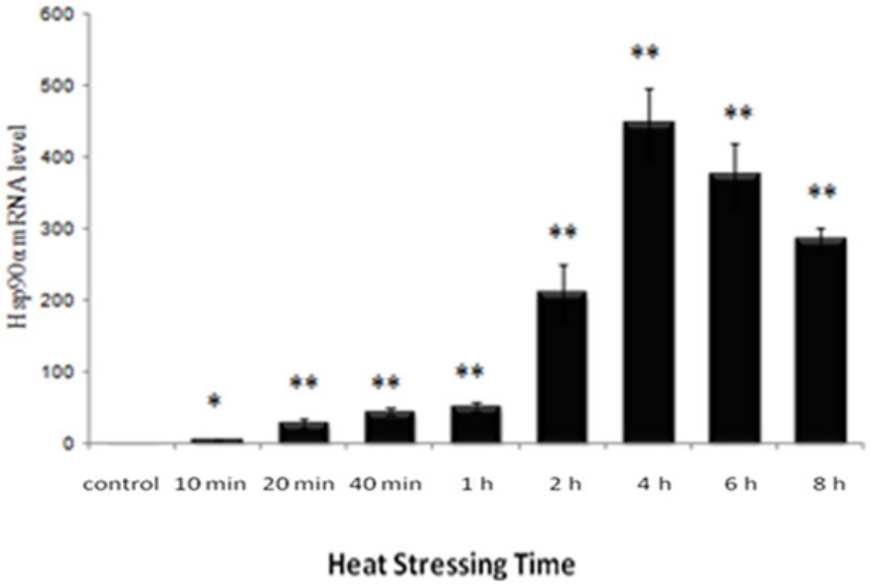

Figure 5. Transcription levels of *hsp90α* mRNA in the heat-stressed myocardial cells of rat *in vitro*. *P < 0.05; **P < 0.01 when comparing the heat-stressed groups with the control group. Values are reported as means ± SD; N = 3.

Western blot analysis of Hsp90α in the heat-stressed myocardial cells of rats

Hsp90α was consistently detected in the rat myocardial cells of both the heat-stressed and control groups (Figure 6). However, during exposure to heat stress, the Hsp90α protein was not expressed to a similar extent. After heat stress, Hsp90α expression in the myocardial cells *in vitro* decreased immediately and significantly at 10 min ($P < 0.01$), 20 min ($P < 0.01$), 40 min ($P < 0.01$), and 1 h ($P < 0.05$) of heat stress, in comparison with the corresponding levels in the control. Hsp90α levels in the rat myocardial cells started to increase gradually after 2 h ($P > 0.05$) and 4 h ($P > 0.05$) of heat stress, although there were no significant differences compared to the control. The levels of Hsp90α expression increased significantly at 6 h ($P < 0.01$) and 8 h ($P < 0.01$) of heat stress and were 1-fold higher than those in the control group.

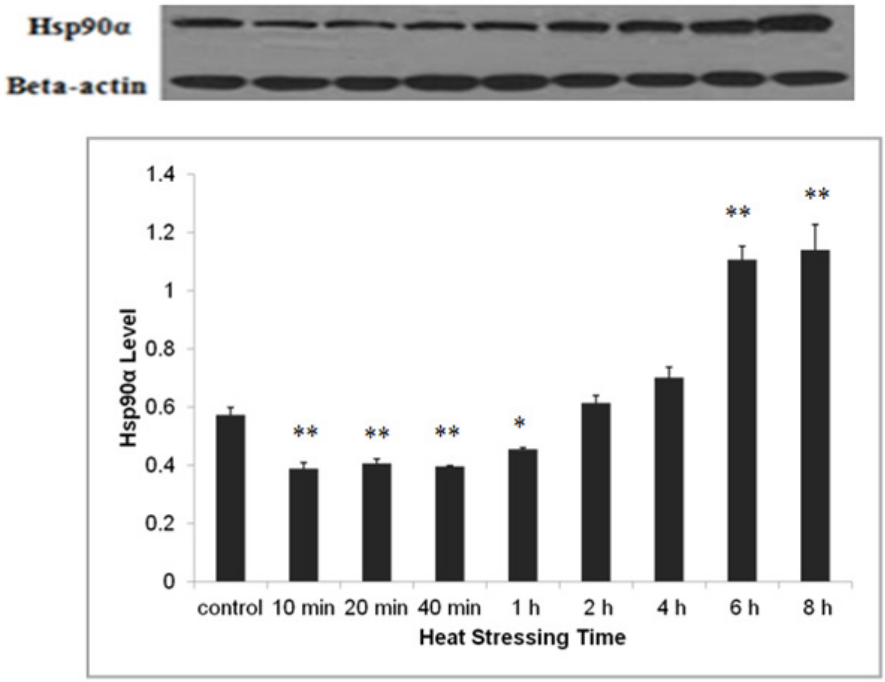

Figure 6. Levels of Hsp90α expression in the heat-stressed myocardial cells of rat *in vitro*. *P < 0.05; **P < 0.01 when comparing the heat-stressed groups with the control group. Values are reported as means ± SD; N = 3.

DISCUSSION

Heat shock proteins are believed to control a regulatory mechanism for cell survival, proliferation, apoptosis, and cell death resulting from stress (Parsell and Lindquist, 1993). Our present results showed that CK levels increased significantly in the primary myocardial cells *in vitro* from the start of exposure to heat stress and reached the peak level at about 2 h of heat stress. These results are in line with the cytopathological changes in the heat-stressed myocar-

dial cells, which are characterized by vacuolar and granular degeneration and even necrosis after 40 min and 2 h of heat stress. It indicates that heat stress induces myocardial cell damage as early as heat treatment does. A similar result demonstrated that heat stress causes myocardial cell damage in broilers *in vivo*, which is accompanied by an elevation in the levels of CK and an increase in apoptotic cells, consistent with our results (Sun et al., 2007). However, the serum activity levels of CK increased after rats were exposed to 6 and 24 h of heat stress (Manjoo et al., 1985). This implies that heat stress induces cellular damage and apoptosis of myocardial cells during the first stage of heat treatment and needs some hours to stabilize. In the primary myocardial cells, Hsp90α shows protective function against damage at the end of the heat exposure.

Interestingly, our results also showed that the levels of caspase-3 and cytochrome C, which are related to apoptosis, increased significantly at 40 min of heat stress in comparison with the controls, indicating that the heat-induced myocardial cell apoptosis is increased from early period of heat stress exposure. The pathway to apoptosis is mediated by a family of cysteine-dependent aspartate-specific proteases known as caspases, which have specificity for aspartate residues (Wolf and Green, 1999). Caspases are always found in higher concentrations in the cell when birds/animals are exposed to heat stress, although little is known about the exact pathway leading to cell death in heat-stressed myocardial cells. Cytochrome C interacts with the apoptotic protease activation factor-1, and this complex then activates caspase-9, which in turn activates caspase-3, leading to the cytosolic activation of a caspase cascade that leads to the initiation of apoptosis (Li et al., 1997). In view of the above findings, it can be concluded that apoptosis of the myocardial cells occurs through the activation of the cytochrome C and caspase-3 pathway.

The lower levels of Hsp90α expression in the early phase of heat stress could be used as a risk indicator of pathological damage and massive apoptosis, which may be followed by heart failure. The present results showed that Hsp90α expression levels in the myocardial cells significantly decreased after 40 min of heat stress with the inductions of enzyme CK, which was related to cell damage, and caspase-3 and cytochrome C, which were related to apoptosis. However, the results also showed that Hsp90α levels were elevated at 6 and 8 h of heat stress, while the levels of caspase-3 and cytochrome C (especially cytochrome C) decreased at 6 h of heat stress; meanwhile, the levels of CK decreased, indicating that the damage sustained by heat-stressed myocardial cells begin to reduce after 6 and 8 h of heat stress. This indicates that the elevation of Hsp90α in the myocardial cells *in vitro* has a protective role in such adverse conditions and can be an important marker of the stage of heat shock. This also implies that the apoptosis of the myocardial cells occurred through the activation of the cytochrome C and caspase-3 pathways. However, the cell repair capacity of Hsp90α is overstrained in the early phase of heat treatment and needs some hours to stabilize. Our present results cannot be sustained by some earlier reports that showed that the overexpression of Hsp90 could increase the rate of apoptosis in the monoblastoid cell line U937 following induction with TNFα and cycloheximide (Galea-Lauri et al., 1996). Furthermore, treatment of human embryonic fibroblasts with the Hsp90α inhibitor geldanamycin increases the resistance of these cells to nicotine, which may induce apoptosis via Hsp90α expression in human cells tested (Wu et al., 2002). Heat shock proteins can inhibit or aid the apoptotic mechanism through their chaperone functions by affecting protein folding, ubiquitin degradation pathways, and protein translocation (Takayama et al., 2003). Their most prominent abilities are to protect and repair cells and

tissues (Luh et al., 2007), including the myocardial cells, by suppressing apoptosis from the damaging effects of ischemia and reperfusion (Currie et al., 1988; Samali and Orrenius, 1998; Wu and Tanguay, 2006; Seok et al., 2007).

Variations in Hsp90α protein levels did not correspond to changes in *hsp90α* mRNA levels in the myocardial cells of neonatal rats after heat stress. The present study showed that the level of *hsp90α* mRNA increased significantly during the course of heat stress, while Hsp90α levels decreased during the first hours and increased at the end of the heat treatment. It is possible that Hsp90α may be affected in the heat-stressed myocardial cells during its expression. It was demonstrated that the low correlation between both mRNA and protein levels could be due to the energy spent on regulating mRNA levels (Ostlund and Sonnhammer, 2012).

In conclusion, this study indicates that heat stress induces cellular damage and apoptosis of myocardial cells during the first stage of heat treatment and the apoptosis occurs through the activation of the caspase-3 and cytochrome C pathway. Low Hsp90α levels indicate that the repair capacity of this protein is overstrained in the first phase of sudden heat stress in myocardial cells and needs some hours to stabilize. Hsp90α shows protective function against damage at the end period of the heat exposure in the primary myocardial cells, and the consumption of Hsp90α exceeded its production at the early stage of heat stress, but sufficient quantities of Hsp90α were produced in myocardial cells after 6 h of heat stress.

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