Expression of heat shock protein 90 alpha (Hsp90α) in primary neonatal rat myocardial cells exposed to various periods of heat stress in vitro

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ABSTRACT. The objective of this study was to investigate the mechanism of heat shock protein 90 alpha (Hsp90α) protection against heart damage resulting from heat stress by detecting Hsp90α mRNA, Hsp90α protein, protein localization, and cell damage in primary myocardial cells of neonatal rats in response to heat stress in vitro. The cells were heat-stressed at 42°C in an incubator with 95% air and 5% CO₂ for different periods. Levels of Hsp90α, protein localization, enzymes, and cytopathological lesions were detected using Western blot, immunocytochemistry enzymatic assays, and cytopathological techniques. Aspartate aminotransferase, lactate
dehydrogenase, and creatine kinase enzyme levels were elevated during heat stress, and acute cellular lesions that were characterized by vacuolar degeneration and necrosis were observed. Hsp90α levels decreased between 10 and 60 min of heat stress and increased after 360 and 480 min, while Hsp90α mRNA decreased after 360 min. These results indicate that heat stress might induce irreversible damage in certain myocardial cells. The elevated Hsp90α level at the end of heat stress and its positive signal in the cytoplasm of myocardial cells after heat stress could be associated with its protective role. Additionally, the consumption of Hsp90α exceeded its production in the first period of treatment.

**Key words:** Heat stress; Heat shock protein 90 alpha (Hsp90α); Creatine kinase; Primary myocardial cells

**INTRODUCTION**

Heat shock proteins (HSPs) are normally present in mammalian cells and are activated by physical, chemical, and biological stress (Staib et al., 2007). Heat stress is a major aspect that affects mammals, and its effect on HSPs needs to be investigated. HSPs are classified by their molecular weight, which ranges from 10 to 150 kDa (Benjamin and McMillan, 1998). HSPs are known to act as molecular chaperones in protein assembly and disassembly (Haslbeck et al., 2005), protein folding and unfolding (Zietkiewicz et al., 2004), protein translocation (Ryan and Pfanner, 2001), and the refolding of damaged proteins (Marques et al., 2006). HSPs support the folding of proteins into biologically active conformations (Schwarz et al., 1996) and can bind proteins that are in a non-native conformation because of environmental stress-induced protein denaturation. These molecules minimize the aggregation of non-native proteins and target damaged proteins for hydrolysis and removal from the cell (Hofmann et al., 1999), suggesting that HSPs can protect and repair cells and tissues. The over-expression of one or more HSPs confers protection against stress (Luh et al., 2007) because they regulate apoptosis and cell death resulting from stress. Furthermore, HSPs can inhibit or aid the apoptotic mechanism through their chaperone functions by affecting protein folding, ubiquitin degradation, and protein translocation pathways (Takayama et al., 2003).

Hsp90 (83-99 kDa) is the most highly expressed stress protein in the cytoplasm, nucleus, and endoplasmic reticulum of eukaryotic cells (Lindquist and Craig, 1988; Welch, 1990). The two major isoforms of Hsp90, namely, Hsp90α and Hsp90β, are highly expressed at basal levels, and various stresses increase the expression of both forms to different degrees (Romanucci et al., 2006). Hsp90α (86 kDa) is a highly conserved cytosolic protein (Cserrmely et al., 1998) whose levels increase in response to heat stress and other environmental conditions (Bagatell et al., 2000). It maintains cell structures and supports maturation (Maloney and Workman, 2002; Neckers, 2002), and, because it is highly conserved and inducible, it is a good mediator of cellular stress (Prohaszka and Fust, 2004). As a molecular chaperone, Hsp90α is involved in regulating productive interactions of steroids with their cellular receptors, prevents protein denaturation, and refolds proteins into their proper structures with assistance from the prolyl isomerase family. The induction of Hsp90α in response to environmental, physical, and chemical stresses is therefore essential for the maintenance of cellular integrity and survival of the organisms (Bose et al., 1996; Ekambaram et al., 2008). Hsp90 also crosslinks with actin filaments in a Ca²⁺- and ATP-dependent manner (Nishida et al., 1986), which, in turn, might...
help modulate the cytoskeletal dynamics in stressed cells and for myocardial protection.

Heat stress can cause damage to different organs, including the heart (Yu et al., 2008; Yan et al., 2009), while HSPs can protect the myocardium from the damaging effects of ischemia and reperfusion. A shortage of HSPs associated with lesions in the myocardium may cause electromechanical dissociation, which can result in acute heart failure and death, as observed under poor environmental and management conditions, such as stressful transport (Luh et al., 2007). Although Hsp90 was able to protect cultured primary cardiac cells (Cumming et al., 1996), little information is available to support a specific role for Hsp90α and its expression in response to heat stress in primary cultures of cardiac myocytes in vitro. Although it has been demonstrated that a lack of HSP in cardiac cells results in acute heart failure (Anrani et al., 1993), the mechanisms by which Hsp90α offers protection against this remain unclear. The aim of this study was to detect the expression and localization of Hsp90α in primary myocardial cells from neonatal rats and to correlate this with heart tissue damage resulting from exposure to heat stress.

MATERIAL AND METHODS

Cell culture and experimental treatment

Primary myocardial cells from neonatal rats were obtained from Fu Meng Biological Technology Ltd., Shanghai, China. The experiments were also conducted in the same laboratory. Cells were grown on 35 cm² polylysine-coated coverslips at a density of 2-8 x 10⁴ cells/plate, and the cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 72 h to allow the cells to adapt. When a minimum of 85% of the cells in a culture were alive, viable, and beating after this period, the culture was divided into 9 groups: the control group and 8 groups exposed to heat stress for 10, 20, 40, 60, 120, 240, 360, and 480 min by incubation in a 42°C incubator with a humidified atmosphere of 5% CO₂ and 95% air. The ambient temperature of the control group was maintained at 37°C.

Determination of lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and creatine kinase (CK) activities

The supernatants of the cell cultures were collected from each plate, transferred into centrifuge tubes, and stored at -80°C. The activities of AST, CK, and LDH were measured according to the instructions given in the commercial kits (A020-1, C010, and A032, respectively, Nanjing Jiancheng Biochemical Reagent Co., China).

Immunocytochemical detection of Hsp90α

After collecting the supernatants, the cultures were fixed with 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, the cells were overlaid with anti-rat Hsp90α monoclonal antibody (ab79849, Abcam, USA) at a dilution of 1:200 in a moist chamber at 37°C for 1 h. After washing 3 times with PBS, the cells were incubated with rhodamine red-conjugated goat anti-mouse IgG antibody (BA1031, Boster, China) at a 1:100 dilution at 37°C for 1 h and then were washed again with PBS before counterstaining with 4’,6-diamidino-2-phenylindole (DAPI) solution and being observed with an immunofluo-
Rescence microscope (Beckman, USA).

Detection of hsp90α mRNA by fluorescence quantitative real-time polymerase chain reaction (qRT-PCR)

Isolation of total RNA and reverse transcription

After the heat exposure at 42°C, the cultures were washed with PBS, and total RNA was isolated using TRIZOL (Invitrogen, USA) according to manufacturer instructions. The concentration of RNA was determined using a spectrophotometer (Mx3000P, USA) at 260 nm. Serial dilutions of RNA were prepared with ribonuclease-free water, and 2 µg each sample was synthesized into DNA using the Transcript M-MLV kit (AM1710, Invitrogen, USA) following manufacturer protocol and stored at -80°C.

Primer design 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 (NCBI) GenBank database (accessions NC_005105.2 and NC_005111.2, respectively). Using these sequences, primers were designed for hsp90α and β-actin (Table 1) by the Primer Premier 5.0 software for real-time reverse transcription-PCR (RT-PCR) amplification.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amplicon size (bp)</th>
<th>Sense primer (5’-3’)</th>
<th>Antisense primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp90α mRNA</td>
<td>214</td>
<td>CCCCGTTGCGGTAGTCACGT</td>
<td>TCCAGAGCGTCTCAGGAGTTGGA</td>
</tr>
<tr>
<td>β-actin mRNA</td>
<td>143</td>
<td>CCCATCTATGAGGGTTCAA</td>
<td>TCACGCACGATTCCC</td>
</tr>
</tbody>
</table>

qRT-PCR

Each DNA sample (2 µL, 25 times dilution) was suspended in 2X SYBR Premix Ex Taq™ (15218-019, Invitrogen, USA) with primers (25 pmol each of sense and anti-sense primer), and double-distilled water to a total volume of 25 µL. Quantitative PCR was performed using an ABI 7300 real-time PCR thermocycler (Applied Biosystems, USA). The thermal profile was 95°C for 3 min for enzyme activation, followed by denaturing 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 without DNA was performed. A 2-fold dilution series of the template was used in the qRT-PCR reactions. The hsp90α mRNA in all samples was normalized using the following formula: Relative quantity of hsp90α mRNA = 2^(-ΔΔCt) = (Ct_hsp90α mRNA - Ct_β-actin) control group - (Ct_hsp90α mRNA - Ct_β-actin) test group.

Western blot analysis

After treatment at 42°C, cardiac muscle 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 10% SDS-polyacrylamide gel electrophoresis and transferred onto 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 (TTBS) for 1 h at RT and incubated with anti-rat Hsp90α monoclonal antibody (ab79849, Abcam, USA) for 16 h at 4°C. After washing with TTBS, the membrane was further incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (ab6789, Abcam, USA) for 1 h at RT. The antibody-antigen complexes were detected using Western blotting luminal reagent. The bands on the developed film were quantified with the Quantity One v.4.6.2 software (Bio-Rad, USA). The density of each band was normalized to that of β-actin.

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS). Where significant differences existed, Duncan’s multiple range test was used to compare the means. Differences are reported as significant at P < 0.05. All experiments were repeated in triplicate.

RESULTS

Enzyme levels of LDH, AST, and CK

As illustrated in Figure 1, AST activity levels increased significantly during the course of heat exposure (P < 0.01) compared to the control. The levels of LDH increased at 20 min (P < 0.05) and then decreased quickly after 40 min of heat stress. The LDH activity remained higher than that of the control cells, but the difference was not statistically significant. After 480 min, the levels of LDH increased again to a significant level (P < 0.05). The levels of CK also increased, but the change was only significant after 120 min of heat stress (P < 0.05).

Figure 1. Activity levels of LDH, AST, and CK in primary rat neonatal myocardial cells (U/L). *P < 0.05; **P < 0.01 compared with the control group; values are indicated as means ± SD; N = 3.

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

The hsp90α mRNA data for the rat myocardial cells after different periods of heat stress
are displayed in Figure 2. The level of *hsp90α* mRNA increased after 10 min of heat stress (*P* < 0.05) and reached the highest level after 240 min (*P* < 0.01). All other periods of heat stress resulted in significantly (*P* < 0.01) higher levels of *hsp90α* mRNA transcription than the control.

Expression levels of Hsp90α in heat-stressed rat myocardial cells

The Hsp90α expression levels that were detected in the rat myocardial cells after different periods of heat stress are shown in Figure 3. Hsp90α expression decreased significantly (*P* < 0.01) within the first 10 min of heat stress. The expression level stayed almost constant until 60 min of treatment. After 120 min, the level returned to the control level. During treatments longer than 2 h, Hsp90α expression increased and reached the highest level after 360 min.
Immunocytochemical observations

Immunocytochemical staining for Hsp90α in primary rat myocardial cells is shown in Figure 4. Positive staining for Hsp90α was detected in both the nucleus and cytoplasm of the myocardial cells. However, the positive staining in the cytoplasm was stronger than that in the nucleus.

![Figure 4](image)

DISCUSSION

Heat stress is known to damage the integrity of various tissues, including myocardial cells, and it induces the expression of HSPs (Yu et al., 2008; Yan et al., 2009). Among the key indicators of tissue damage caused by heat stress, the activity of AST, CK, and LDH are well recognized (Li et al., 2008). In pigs, a relationship between environmental stressors including transport, the expression of Hsp90, and myocardial cell damage has been postulated (Yue et al., 2010) with heat stress being applied as a defined stressor. A gradual change in LDH, AST, and CK takes place when the ambient temperature is increased from 37 to 42°C, and the presence of CK and AST in the blood serum is often associated with muscle damage (Fabrega et al., 2002).

The serum activity levels of the AST, LDH, and CK increased after rats were exposed to 6 and 24 h heat stress (Manjoo et al., 1985). This was also evident in our study: the levels of LDH, AST, and CK increased over the course of heat stress. CK is found in cardiac muscle...
fibers, increases rapidly after intense physical exertion (Britton et al., 1980), and is the most sensitive parameter of tissue damage (Manjoo et al., 1985). We saw a gradual increase in CK levels during the course of heat stress, and CK levels were the highest at 120 min of heat stress. These results agree with those of earlier studies demonstrating that plasma CK concentrations increased in heat stressed broilers in vivo during the period of heat exposure time and reached a peak level after 10 h of heat stress (Sun et al., 2007; Yu et al., 2008). In contrast, another group reported that CK was significantly reduced in the hearts of rats exposed to 15 min of heat shock after 5 minutes of reperfusion (Currie et al., 1988). In this study, the levels of all 3 tested enzymes were elevated with the application of heat stress, confirming that heat stress induces damage in myocardial cells in vitro.

Temperatures from 3 to 5°C cause cells from all organisms to initiate the expression of HSPs (Locke and Noble, 1995). However, our results demonstrated that Hsp90α protein levels decrease significantly during the first hour of heat stress treatment, while the enzyme activities and cell damage indicators were elevated, as exemplified by the CK enzyme. This indicates that heat stress induces the irreversible damage and death of certain myocardial cells because of the decrease in Hsp90α levels in the myocardial cells, and it suggests that the consumption of Hsp90α by the cells exceeded its production at the beginning of heat stress in myocardial cells. HSPs can act as protective responses to external stressors, thereby maintaining the cell structure and preventing protein aggregation and denaturation (Marruchella et al., 2004). Several studies have demonstrated the cytoprotective activity of HSPs against ischemia and reperfusion-associated damage in the heart (Seok et al., 2007). Therefore, the reduced HSP levels in the myocardium may lead to acute heart failure, while accumulating HSPs in cardiac cells enhances myocardial resistance to ischemia/reperfusion injury and improves posts ischemic recovery of cardiac function (Amrani et al., 1993). Hsp90 has the ability to regulate the activity of specific proteins, such as enzyme and hormone receptors, but it regulates the transcription of other HSPs to an even greater extent by its association with transcription factors that modulate the transcriptional activity and expression of other HSPs (Nadeau et al., 1993). A significant increase in Hsp90α in primary cardiac muscle cells after 360 min of heat stress was recorded, which contrasted with observations made during the first hour. Over the same time period, the CK enzyme associated with heart disease also demonstrated decreased activity levels. Although the stress damage of myocardial cells still existed in the sustaining hyperthermia situation, these results showed that Hsp90α started to increase after 120 min and reached significantly high levels after 360 min of heat stress, while CK started to decrease. The over-expression of Hsp90 can protect either cultured primary cardiac cells (Cumming et al., 1996) or H9c2 cells against heat stress (Heads et al., 1995). However, the cytoprotective activity of Hsp90α was delayed in its action against heat stress at the beginning of the heat treatment, which could be attributed to the consumption of Hsp90α exceeding its production. This was reversed after 120 min, when Hsp90α levels started to increase and reached a peak after 360 min of heat stress.

The expression of Hsp90α in both the cytoplasm and nucleus of the myocardial cells may have a functional significance for the refolding of denatured proteins caused by heat stress. As revealed by immunocytochemical results, Hsp90α expressed positive granules in the primary muscle cells that were exposed to 37°C, while the expression of Hsp90α was increased after 60 and 240 min of heat stress, which was consistent with previous findings that Hsp90α is a constitutively expressed cytosolic protein under physiological conditions and that its levels increase in response to heat stress (Csermely et al., 1998). The role of Hsp90 is to protect the cytoskeleton. Hsp90 associated with actin microfilaments and tubulin microtubules, which
are major constituents of the cell cytoskeleton, and influenced microfilament organization. Hsp90 possesses an ATP-binding site and low ATPase activity and can bind unoccupied steroid hormone receptors (Kellermayer and Csermely, 1995). Hsp90 has been characterized as a molecular chaperone that is able to keep proteins in a folding-competent state. It has enhanced chaperone activity in an oligomeric form at high temperature. Taken together, Hsp90α may have a significant role in the survival and structural integrity of the cell after stress and protection of cardiac muscle cells. Cellular damage and ATP depletion in stressed cells that are induced by hyperthermia leads to protein aggregation and affects cytoskeletal structures (Kabakov and Gabai, 1997), but they may be overcome by inducting Hsp90α. Therefore, Hsp90α seems to contribute to the stabilization of the intracellular protein structure by refolding denatured proteins and steroid activation (Ekambaram et al., 2008). However, Hsp90α expression in response to high temperature and its association with myocardial cell protection against heat stress still needs further investigation. The heat stress model that was used in this experiment may be a helpful tool to further elucidate the relationship between HSP expression and tissue damage in the heart and other organs.

The levels of \textit{hsp90} mRNA increased immediately and steadily from the beginning of heat stress treatment, while the level of Hsp90α protein expression decreased continuously until 60 min of heat stress. \textit{hsp90α} mRNA showed a significant and continuous induction from the start of heat stress and reached the highest level at 240 min of heat stress \textit{in vitro}; however, the obvious elevation of Hsp90α protein occurred in the primary myocardial cells after 360 min of heat stress. The possible explanation for this may be that heat stress overwhelmed the repair mechanism of the myocardial cells. The results imply that the consumption of Hsp90α exceeded its production at the beginning of treatment, and the irreversible damages were induced by the decrease of protective Hsp90α protein in the myocardial cells. The expression of Hsp90α and its corresponding mRNA therefore depends on a number of unknown factors related to the trigger that is applied to elicit Hsp90α synthesis. Several studies have documented the protective role of HSPs against stress-induced myocardial damage. Myocytes express high levels of Hsp90β, which reflects significant preservation against heat stress (Heads et al., 1995). In addition, the low correlation between the level of \textit{hsp90α} mRNA and the level of Hsp90α protein may refer to the complicated post-transcriptional mechanisms that are involved in turning mRNA into protein. These results are supported by earlier reports that demonstrate the reasons for the poor correlation between the mRNA and protein levels. For example, many complicated post-transcriptional mechanisms that are involved in turning mRNA into protein are not yet sufficiently well-defined to allow the computation of protein concentrations from mRNA, and proteins may differ substantially in their \textit{in vivo} half-lives (Baldi and Long, 2001; Ostlund and Sonnhammer, 2012).

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