Expression and location of HSP60 and HSP10 in the heart tissue of heat-stressed rats

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Abstract. The present study aimed to analyze the expression levels and localizations of heat shock protein (HSP) 60 and HSP10 in the heart tissue of rats subjected to heat stress (42˚C) for 0, 20, 80 and 100 min. Histopathological injuries and increased serum activities of serum lactate dehydrogenase and creatine kinase isoenzyme MB were detected in the heated rat myocardial cells. These results suggested that heat stress-induced acute degeneration may be sufficient to cause sudden death in animals by disrupting the function and permeability of the myocardial cell membrane. In addition, the expression levels of HSP60 were significantly increased following 20 min heat stress, whereas the expression levels of its cofactor HSP10 were not. Furthermore, the location of HSP60, but not of HSP10, was significantly altered during periods of heat stress. These results suggested that HSP60 in myocardial tissue may be more susceptive to the effects of heat stress as compared with HSP10, and that HSP10 is constitutively expressed in the heart of rats. The expression levels and localizations of HSP60 and HSP10 at the different time points of heat stress were not similar, which suggested that HSP60 and HSP10 may not form a complex in the heart tissue of heat-stressed rats.

Introduction

The exposure of animals to heat stress has previously been associated with increased morbidity and mortality, which in turn have led to substantial economic losses to animal product industries (1). Typically, the increased mortality rate associated with heat stress is a result of organ failure, particularly heart failure preceded by cardiovascular damage (2).

Heat shock proteins (HSPs), which are ubiquitously expressed and highly conserved in prokaryotes and eukaryotes (3) are classified into families according to their molecular size, including small HSPs, HSP27, HSP40, HSP60, HSP70, HSP90 and HSP110 (4). HSPs are molecular chaperones that have been shown to perform important functions in the folding, unfolding (5) and translocation (6,7) of proteins, in addition to the assembly and disassembly of protein complexes (8,9).

HSP60 and HSP10 are two important chaperones that interact in a two-step folding mechanism in the mitochondria of prokaryotic and eukaryotic cells (10). HSP10 has been reported to be a cofactor involved in HSP60-mediated protein folding and sorting (11). Previous studies investigating HSP60 and HSP10 have focused on their roles in prokaryotic (12) and tumor cells (13,14). In addition, in eukaryotic cells, there have been reports that HSP60 may be involved in angiocardiopathy (15,16). Furthermore, a previous study reported significantly increased levels of HSP60 in the heart tissue, but not in the liver and kidney tissues, of heat-stressed chickens and pigs that have been transported for a long period of time (17). In a previous study, the abnormal trafficking of HSP60 to the cell surface was suggested to be an early trigger for myocyte loss and the progression of heart failure (18). Previous studies have associated HSP10 with numerous cellular processes, including cellular differentiation (19,20), cell proliferation (21,22), cell apoptosis (23,24) and cytoprotection (25,26). Although there is an increasing awareness regarding the protective functions of HSPs, and their importance in numerous regulatory pathways, little is known regarding the expression levels of HSP60 and HSP10 in the heart tissue of mammals under conditions of heat stress. Therefore, the present study aimed to investigate the dynamic expression levels of HSP60 and HSP10, and their associations, in the heart tissue of heat-stressed rats *in vivo*.

Materials and methods

Animals and experimental design. A total of 40 adult Sprague-Dawley (SD) rats (20 female and 20 male rats; weight, ~220 g) were purchased from Qinglong Mountain

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Animal Breeding Ground of the Nanjing Jiangning District (Nanjing, China) for use in the present study. The rats were randomly divided into four groups (n=10 per group). The rats were maintained under standard conditions for 7 days to allow them to acclimatize to their new surroundings and to recover from environmental stress. During this period, the humidity of the chamber was maintained at 60±10% and the room temperature was 25±1˚C. On day 8, all rats were transferred to a controlled climate chamber (RX8-500D; New Jiangnan, Co., Ltd., Ningbo, China) and exposed to 42±1˚C for 0 (control), 20, 80 and 100 min. During the heat stress period, the rats received *ad libitum* access to feed-stuff and water. The rats were sacrificed by decapitation, after which blood was collected to prepare serum, manually eviscerated and the hearts were rapidly dissected. Half of the heart tissue samples were fixed in 10% neutral-buffered formalin (G fan Biological Technology Co., Ltd., M003, Shanghai, China) for histopathological analyses and the other half was stored in liquid nitrogen (-196˚C) for biological analyses.

The present study was conducted in accordance with the recommendations outlined in the Guide for the Care and Use of Laboratory Animals of the Nanjing Agricultural University (Nanjing, China), and the guidelines of the Animal Ethics Committee of Jiangsu Province (Nanjing, China). The protocol was approved by the Committee on the Ethics of Animal Experiments of Nanjing Agricultural University (permit no. SYXK (su) 2011-0036).

Determination of enzyme activity. The activities of serum lactate dehydrogenase (LDH; A020-2) and creatine kinase isoenzyme MB (CKMB; H197) were determined using commercial kits (Nanjing Jiancheng Biochemical Reagent Co., Nanjing, China) and a clinical autoanalyzer (Vital Scientific NV, Dieren, Netherlands), according to the manufacturer's protocols.

Histopathological analysis. Paraffin-embedded heart tissues that had been fixed in 10% neutral-buffered formalin were serially sliced into 4-μm sections. One of the sections was stained with hematoxylin and eosin (HE; Liansuo Biological Technology Co., Ltd., Shanghai, China), and examined under a light microscope (Axioskop 2 plus; Zeiss GmbH, Jena, Germany).

Immunohistochemical analysis. The heart tissue sections from the heat-stressed and control groups were examined using the streptavidin-biotin-peroxidase complex procedure (85-6643; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The sections were dewaxed and rehydrated, then incubated with 3% hydrogen peroxide in methanol for 10 min in order to inhibit endogenous peroxidases. Subsequently, the tissue sections were placed in 10 mM citric acid buffer (pH 6.0), then heated in a microwave oven at 800 W for 3 min and 400 W for 10 min. The sections were incubated with antibodies against HSP60 and HSP10 [1:200 in phosphate-buffered saline (PBS; Wuhan Boster Biological Technology, Ltd., Wuhan, China); polyclonal rabbit HSP10 (ADI-SPA-110; Enzo Life Sciences, Inc., Farmingdale, NY, USA); mouse monoclonal HSP60 (ab5478; Abcam, Cambridge, UK)] overnight at 4˚C. For the negative control, PBS was run instead of the primary antibody. The slices were incubated with a biotinylated secondary mouse antibody from a Histostain-Plus IHC Kit (85-6643; Thermo Fisher Scientific, Inc.) for 20 min at 37˚C in a humidified chamber. Subsequently, the tissue sections were washed three times with PBS, incubated for 20 min in horseradish peroxidase-streptavidin (85-6643; Thermo Fisher Scientific, Inc.), then washed three times with PBS for 5 min each. Antibody complexes were visualized by incubating the tissue sections with 3-3'-diaminobenzidine (00-2014; Thermo Fisher Scientific, Inc.), after which the tissue sections were incubated with hematoxylin for 30 sec for nuclear counter-staining, followed by mounting. The corresponding negative control sections were prepared by omitting the antibodies.

Western blot analysis. Total protein was extracted from the heart tissues of the rats in the control and heat-stressed groups using ultrasonication (JY99-IIDN; Ningbo New Cheese Instrument Co., Ltd., Ningbo, China) and Radio Immunoprecipitation Assay lysis buffer (Beyotime Institute of Biotechnology, Nanjing, China), and protein concentrations were determined using the bicinchoninic acid assay (232235; Micro BCA™ Protein Assay kit; Thermo Fisher Scientific, Inc.). Heart protein extract (80 μg) was electrophoresed using a 5% sodium dodecyl sulfate (SDS) polyacrylamide spacer gel (60 V; 30 min) and a 12% SDS separation gel (100 V; 1.5 h; both from Tiandz, Inc., Beijing, China), then transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) by electrotransfer (200 mA; 1 h). The membranes were washed four times in washing buffer [20 mM Tris base, pH 7.6; 12.5 mM NaCl; and 0.5% Tween-20 (TBST buffer); Beijing Donglinchangsheng Biotechnology Co., Ltd., Beijing, China] then 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 room temperature. Subsequently, the membranes were incubated with anti-rat monoclonal antibodies against HSP10 (1:2,000), HSP60 (1:20,000; ab13532; Abcam), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1,000; ab8224; Abcam) for 1 h at 37˚C. After washing with TBST, the membranes were incubated with peroxidase-conjugated secondary antibody (1:1,000; BA1038; Boster Systems, Inc., Pleasanton, CA, USA) at room temperature for 1 h. Western blotting luminal reagent (Thermo Fisher Scientific, Inc.) was used to detect the antibody-antigen complexes. Bands on the developed film were quantified using Quantity One software, version 4.6.2 (Bio-Rad Laboratories, Inc.). The densities of the HSP60 and HSP10 protein bands were normalized against GAPDH.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the heart tissues of the rats using the RNAiso Plus reagent (D9108A; Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. The optical density (OD) at 260 nm (OD260)/OD280 value of all the RNA samples was between 1.8 and 2.0, and the concentration of each RNA sample exceeded 1,000 ng/μl. RNA samples were reverse transcribed into cDNA using the PrimeScript RT Master Mix (DRR036A; Takara Biotechnology, Co., Ltd.), according to the manufacturer's protocol, and the reaction products were stored at -80˚C until further experimentation. cDNA samples (2 μl)

were suspended in a qPCR reaction system containing 10 μl 2X SYBR Premix Ex Taq (DRR041S; Takara Biotechnology, Co., Ltd.), 0.6 μl each of the forward and reverse primers, and double-distilled water to a total volume of 20 μl. PCR primers were designed according to target mRNAs using Primer Premier software, version 5.0 (Premier Biosoft International, Palo Alto, CA, USA). The accession numbers of the mRNA sequences obtained from the GenBank database (www.ncbi.nlm.nih.gov/genbank/) were NM_012966.1, NM_022229.2 and NM_031144.3 for *HSP10, HSP60* and *β-actin*, respectively. The primer sequences were as follows: *HSP10* (147 bp) forward, 5'-GAGTATTGGTTGAAAGGAGTG-3' and reverse, 5'-TGACAGGCTGAATCTCTCC-3'; *HSP60* (128 bp) forward, 5'-CCGCCCCGCAGAAATGCTTCGA-3' and reverse, 5'-AGGCTCGAGCATCCGCACCAA-3'; and *β-actin* (110 bp) forward, 5'-TGCGCAAGTTAGGTTTTGTCA-3' and reverse, 5'-GCAGGAGTACGATGAGTCCG-3'. PCR was conducted using the Bio-Rad iQ5 Real-Time PCR Thermocycler (Bio-Rad Laboratories, Inc.), according to the manufacturer's protocol. Briefly, enzyme activation was performed at 95˚C for 3 min, followed by 40 cycles of denaturation at 95˚C for 20 sec, annealing at 60˚C for 30 sec, and elongation at 72˚C for 30 sec. For each run, a negative control without cDNA was analyzed along with the experimental samples to ensure that there was no contaminating genomic DNA. A fourfold multiproportion dilution series of the cDNA was used in the qPCR reactions to obtain standard curves as follows: *HSP10* mRNA slope=-3.39 and r^2=0.995; *HSP60* mRNA slope=-3.43 and r^2=0.998; and *β-actin* mRNA slope=-3.49 and r^2=0.998. The amplification efficiencies of the target and reference genes were approximately equal. Therefore, the *HSP60* and *HSP10* mRNA levels were normalized against *β-actin* mRNA levels using the $2^{-\Delta\Delta Cq}$ method (27).

Statistical analysis. Differences between two groups were compared using one-way analysis of variance, followed by Fisher's Least Significant Difference and Duncan's new multiple range test, conducted using SPSS software, version 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation of at least three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Mortality of the rats during the heat stress period. After 20 min heat stress the rats began to exhibit signs of polypnea and nervousness (identified by signs of agitation). After 40 min heat stress, the rats were sweating and exhibited signs of thirst (identified by a high frequency of drinking water). After 60 min heat stress, a few of the rats were pronated, while after 100 min heat stress all the rats were pronated and appeared comatose, such that the experiment was terminated.

Enzyme activities and clinical symptoms of heat-stressed rats. The serum activities of CKMB and LDH exhibited similar patterns in the heat-stressed rats exposed to heat for various time periods (Figs. 1 and 2). The activities of LDH and CKMB were significantly decreased following 20 min heat stress, as compared with the control group (P<0.05); however,

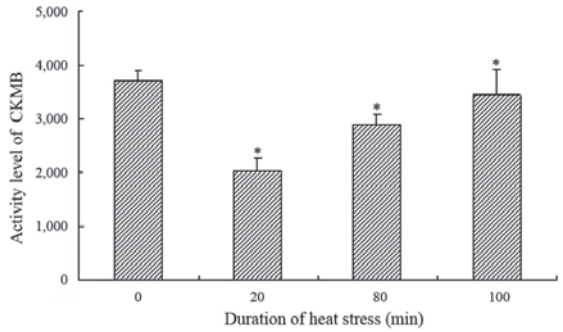

Figure 1. Alterations in the serum activity of CKMB in heat-stressed rats. Activity levels of CKMB showed an increasing trend with duration of heat stress exposure; however, enzyme levels were significantly lower at 20, 80 and 100 min heat stress, as compared with the control group (0 min). *P<0.05 vs. the control group. Data are presented as the mean ± standard deviation. CKMB, creatine kinase isoenzyme MB.

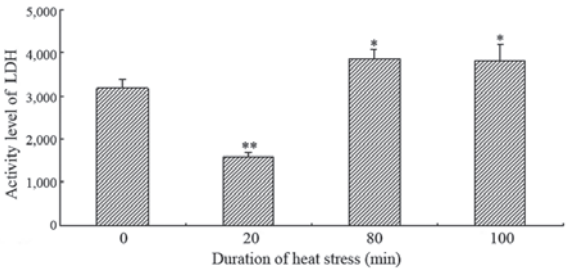

Figure 2. Alterations in serum activity of LDH in heat-stressed rats. Activity levels of LDH were decreased at 20 min heat exposure and then increased with time of exposure. *P<0.05 and **P<0.01 vs. the control group (0 min). Data are presented as the mean ± standard deviation. LDH, lactate dehydrogenase

they showed an overall increasing trend with exposure time. The serum LDH activity was significantly increased after 80 min heat stress in the heat-stressed rats, as compared with the control group (P<0.05). Conversely, although the serum activity of CKMB showed the same increasing trend, the CKMB activity was significantly lower at 80 and 100 min heat stress, as compared with the control group.

Histopathological analyses. Heat stress-induced acute degeneration in the heart tissue of the heat-stressed rats was detected by histopathological analyses (Fig. 3). After 20 min heat stress, edema, which was characterized by increased interstitial spaces between the muscle fibers, a cloudy cytoplasm in swollen myocardial fibers and light hyperemia, was observed. After 80 min heat stress, granular degeneration, which was characterized by an enlarged cell size, a cloudy cytoplasm in myocardial fibers and obvious hyperemia in blood capillaries, was observed. Furthermore, after 100 min heat stress, necrosis, which was identified by karyolysis in the myocardial fibers, and obvious edema, were observed. Throughout the heat stress period, necrosis was occasionally observed.

Immunohistochemical analyses. Immunohistochemical analyses demonstrated that HSP60- and HSP10-positive signals were predominantly located in the cytoplasm of myocardial

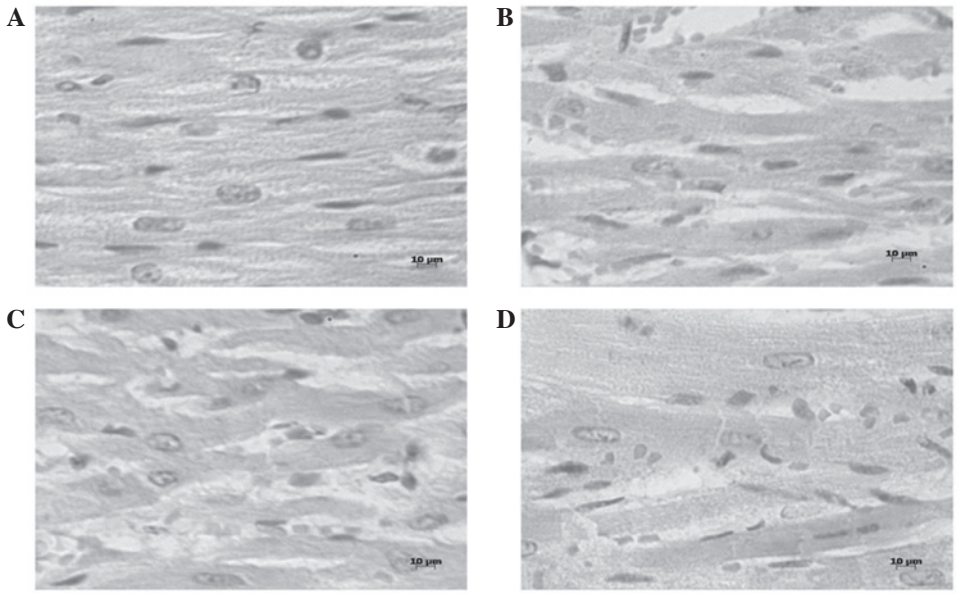

Figure 3. Heat-stressed myocardial cells underwent hematoxylin and eosin staining and were visualized using light microscopy (scale bar, 10 μm). (A) No obvious histopathological changes were observed in the myocardial cells of the control rats. (B) After 20 min heat stress, edema characterized by increased interstitial spaces between muscle fibers, a cloudy cytoplasm in swollen myocardial fibers and light hyperemia, was observed. (C) After 80 min heat stress, the cytoplasm of myocardial fibers appeared cloudy and eosinophilic and light hyperemia was observed. (D) After 100 min heat stress, necrosis of the enlarged myocardial fibers, characterized by karyolysis, was observed.

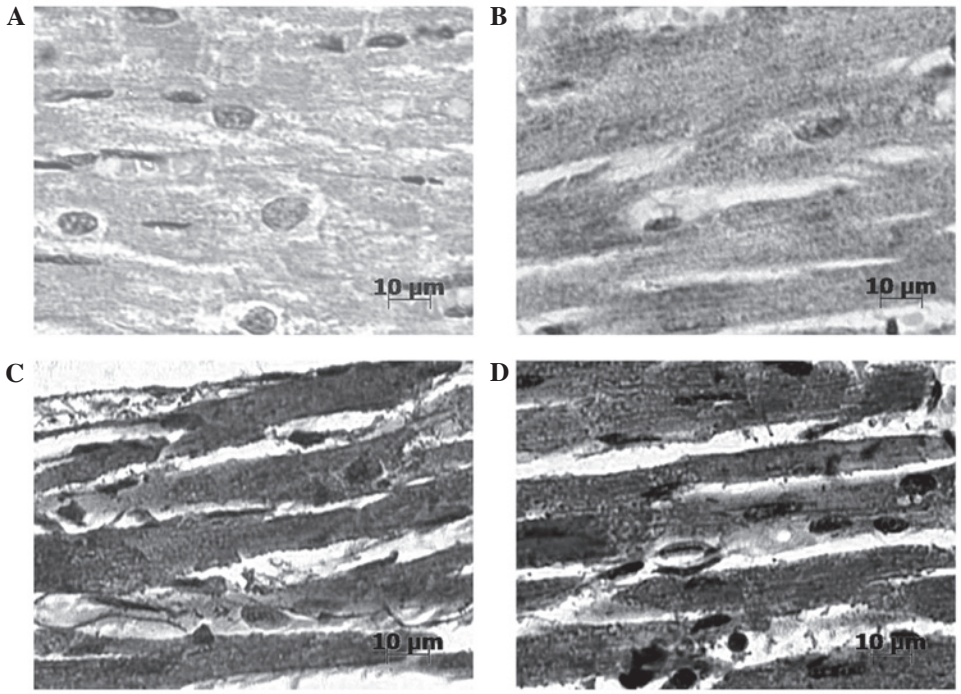

Figure 4. Localization of HSP60 and HSP10 in the heart tissue of heat-stressed rats was determined by immunostaining of rat myocardial cells (scale bar, 10 μm). (A) Prior to heat stress, HSP60 was not clearly detectable in the heart tissue of rats. (B) After 100 min heat stress, relatively strong positive signals of HSP60, exhibiting a punctiform distribution, were detected in the cytoplasm of the myocardial cells. (C) Prior to heat stress, HSP10 is strongly stained and was found to be predominantly located in the cytoplasm of myocardial cells. (D) After 100 min heat stress, immunoreactivity for HSP10 was present at significantly higher levels in the cytoplasm of the myocardial cells. HSP, heat shock protein.

cells (Fig. 4). Prior to heat stress, HSP60 was not clearly detectable in the heart tissue of rats. However, following 100 min heat stress, markedly stronger positive signals of HSP60, showing a punctiform distribution, were detected in the cytoplasm of the myocardial cells. Prior to heat stress, HSP10 staining was strongly positive and was predominantly located in the cytoplasm of myocardial cells. After 100 min heat stress, immunoreactive HSP10 was present at markedly higher levels in the cytoplasm of the myocardial cells.

HSP60 and HSP10 protein expression levels. The protein expression levels of HSP60 and HSP10 were detected in rat

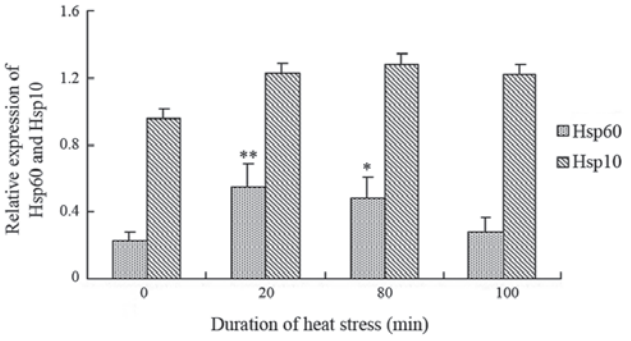

Figure 5. Relative protein expression of HSP60 and HSP10 in the heart tissues of heat-stressed rats were determined using western blotting. *P<0.05 and **P<0.01 vs. the control group (0 min). Data are presented as the mean ± standard deviation. HSP, heat shock protein.

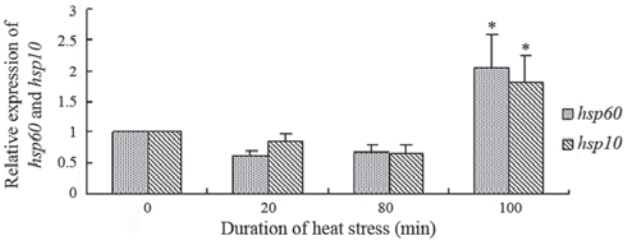

Figure 6. Relative mRNA expression levels of *HSP60* and *HSP10* in the heart tissues of heat-stressed rats were determined using reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. the control group (0 min). Data are presented as the mean ± standard deviation. HSP, heat shock protein.

heart tissues and were normalized against GAPDH (Fig. 5). After 20 min heat stress, the protein expression levels of HSP60 were significantly increased (P<0.01), and remained constant until 80 min heat stress (P<0.05), as compared with the control group. However, the protein expression levels of HSP60 returned to normal at 100 min heat stress. The protein expression levels of HSP10 exhibited a similar trend, although HSP10 was constitutively expressed under normal conditions and heat stress. The protein expression levels of HSP10 did not significantly alter in the heat-stressed rats; however, there was a slight increasing trend (P>0.05) from the beginning of heat stress.

HSP60 and HSP10 mRNA expression levels in the rat heart tissues. Fig. 6 presents the mRNA expression levels of *HSP60* and *HSP10*, normalized against *β-actin* mRNA levels, in the heat-stressed heart tissues. Following 100 min heat stress, there were significant increases in the mRNA expression levels of *HSP60* and *HSP10*, as compared with the control group (P<0.05). The mRNA expression levels of *HSP10* in the heart tissue of the heat-stressed rats showed a similar trend to *HSP60*.

Discussion

The results of serum enzyme (CKMB and LDH) assays are typically used as indexes of myocardial injury (28). A previous study demonstrated that the diversity of aspartate aminotransferase, LDH and CK often foreshadow heat shock-induced changes in cellular structure (29), and the activity of these enzymes in intercellular fluid has been associated with heart disease (30). Furthermore, these enzymes serve as molecular predictors of damage to cardiac muscle cells during heat stress (31). In the present study, the serum activities of LDH and CKMB were detected as indicators of heart damage under heat stress conditions, and an increasing trend with exposure to heat stress was observed. An elevation of plasma CKMB levels, which has been shown to be indicative of heart muscle damage, results from the disruption of the function and permeability of the muscle cell membrane (sarcolemma) (32). The activity of LDH has previously been evaluated as an indicator of stress during transportation, and a previous study reported acute cellular lesions in the hearts of transported pigs (33). This is consistent with the present study in which obvious lesions, characterized by granular and vascular degeneration and even necrosis, were observed in the heart tissue of heat-stressed rats using histopathological analyses. Furthermore, the activities of enzymes associated with myocardial cell damage were gradually increased with the duration of heat stress and reached statistical significance at 100 min. Histopathological analyses revealed acute degeneration, including granular and vascular degeneration of myocardial cells; however, there was no obvious and extensive myocardial cell necrosis. These results suggested that acute degeneration may be sufficient to cause sudden death in animals during heat stress by disrupting the function and permeability of the myocardial cell membrane.

The distribution of HSPs may be associated with their protective function (34). In the present study, a higher density of HSP60-positive signals were detected in the heat-stressed groups, as compared with the control group, and strong positive signals of HSP10 that exhibited a punctiform distribution were detected in the cytoplasm of the heat-stressed myocardial cells. These results suggested that HSP60 and HSP10 were synthesized in response to heat stress. In addition, HSP60 and HSP10 exhibited a punctiform distribution in the mitochondria of heat-stressed cells. This was consistent with the results of a previous study, in which HSP60 was reported to be predominantly located in the cytoplasm and mitochondria of muscle fibers in humans (35). Furthermore, another study demonstrated that mammalian HSP60 was rapidly transported into the mitochondria following dehydration (36). Therefore, these molecules may be transported into the mitochondria under conditions of heat stress; however, further studies are required in order to confirm this.

In the present study, western blotting demonstrated that the protein expression levels of HSP60 were significantly increased following 20 and 80 min heat stress; thus suggesting that the elevation of HSPs in the heart may confer protection against stress-induced myocardial injury (37,38). The protein expression levels of HSP60 were decreased following 100 min heat stress; however, the serum levels of HSP60 were high and histopathological analysis of the heat-stressed tissue revealed obvious lesions. In a previous study, HSP60 expression in the cytoplasm of myocardial cells was more prominent in intact areas than in degenerated areas (39). Concordantly, in the present study, HSP60 staining was markedly reduced in the cytoplasm of granular degenerated areas in myocardial cells. These results suggested that HSP60 may be considered a potential biomarker of heat stress-induced injuries of the

heart. In the present study, the presence and localization of HSP60 and HSP10 in rat heart tissue in response to heat stress were evaluated. Although HSP60 and HSP10 should be functionally correlated, HSP10 was present in a higher number of specimens and had a higher expression level, as compared with HSP60; thus suggesting that HSP10 may have a different role in the heart tissue of rats. Similar results were reported in a recent study in which the HSP60 and HSP10 expression levels were investigated in a series of normal human bone marrows and within the cytoplasm of tumor cells (40). HSP10 was more obviously and constitutively expressed in unstressed myocardial cells under normal conditions, as compared with HSP60 in heat-stressed rats.

A previous study demonstrated that the expression levels of *HSP60* and *HSP10* were regulated simultaneously during carcinogenesis, since these genes are localized head-to-head on the chromosome (41). However, the quantitative relationship between the mRNA and protein expression levels of a gene has yet to be completely recognized. In the present study, the mRNA expression levels of *HSP60* and *HSP10* were gradually and significantly increased when the duration of heat stress was increased to 100 min ($P<0.05$). The HSP60 protein expression levels were significantly increased following 20 min heat stress, but returned to normal at 100 min, whereas HSP10 was constitutively expressed. These results suggested that HSP60 in myocardial tissue may be more susceptive to the effects of heat stress, as compared with HSP10 (42). HSP60 and HSP10 have previously been shown to form mitochondrial chaperone complexes that are believed to have a role in maintaining normal mitochondrial function (43). However, the detailed functions of these two HSPs in myocardial cells are yet to be fully elucidated.

Acknowledgements

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