GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Evaluation of Hsp47 expression in heat-stressed rat myocardial cells *in vitro* and *in vivo*

A. Abdelnasir[1,2], J.R. Sun[1], Y.F. Cheng[1], H.B. Chen[1], S. Tang[1], N. Kemper[3], J. Hartung[3] and E.D. Bao[1]

[1]Department of Basic Veterinary Science, College of Veterinary Medicine, Nanjing Agricultural University, Weigang 1, Nanjing, China
[2]Department of Pathology, Faculty of Veterinary Science, University of Nyala, Nyala, Sudan
[3]Institute for Animal Hygiene, Animal Welfare and Farm Animal Behaviour, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany

Corresponding author: E.D. Bao
E-mail: b_endong@njau.edu.cn

Genet. Mol. Res. 13 (4): 10787-10802 (2014)
Received November 26, 2013
Accepted February 10, 2014
Published December 18, 2014
DOI http://dx.doi.org/10.4238/2014.December.18.20

ABSTRACT. The aim of the present study was to identify the correlation between expression of heat shock protein 47 (Hsp47) and stress injury in heat-stressed myocardial cells and to compare variations in Hsp47 expression in rat myocardial cells exposed to different heat stress for varying periods *in vitro* and *in vivo*. Exposure to heat stress at 42°C resulted in similar induction patterns of the heart damage-related enzyme aspartate aminotransferase in the supernatants of H9c2 cells and in the serum of rats. Histological analysis revealed that both H9c2 cells and heart tissues displayed cellular degeneration in response to different periods of heat stress. Hsp47 was constitutively expressed in the cytoplasm of H9c2 cells at all time points during heat stress, which was consistent with observations in heart fibers *in vivo*. Immunoblotting analysis revealed no significant difference between the expression of Hsp47 in H9c2 cells and heart tissue. However, the expression of *hsp47* mRNA in response to heat stress was significantly increased in

H9c2 cells at 60 min ($P < 0.01$) and 100 min ($P < 0.01$), which was comparable to that at 100 min ($P < 0.01$) in the rat heart. Thus, Hsp47 was elevated significantly after hyperthermia at the mRNA level but not at the protein level both *in vitro* and *in vivo*. The results suggest that Hsp47 turnover may increase during heat stress or that Hsp47 consumption exceeds its production.

Key words: H9c2 cells; Heat shock protein 47; Heat stress; *In vivo*; *In vitro*; Rat heart

INTRODUCTION

Heat shock proteins (HSPs) are a group of highly conserved molecules that play a vital role in protein integrity during transcription in cells under stress conditions (Vasques et al., 2013). HSP expression is induced by several environmental factors, including physical factors (e.g., exposure to high temperature, noise, ultraviolet light, and radiation), chemical factors (e.g., thousands of industrial xenobiotics such as carbon monoxide, heavy metals, and dust) and biological factors (e.g., infection by viruses, bacteria, parasites, and fungi) (Wu and Tanguay, 2006). The response to these environmental agents is transient, providing cellular protection and preventing organisms from being severely damaged (Schöffl et al., 1998). Stress factors that cause protein unfolding, misfolding, or aggregation trigger the stress response, leading to the induction of gene transcription of proteins that stabilize and refold proteins; these proteins restore the balance between protein synthesis, assembly, and degradation (Pathan et al., 2010). Prior induction of HSPs by mild stress confers a protective effect against more severe stress in heart tissue. Moreover, overexpression of an individual HSP has direct protective effects in cardiac cells and tissues, which has been confirmed *in vitro* and *in vivo* (Latchman, 2001). Undifferentiated neonatal rat cardiomyoblast (H9c2) cells subjected to sub-lethal heat stress at 43°C for 30 min were shown to express Hsp70, Hsp90, and Hsp60 at different levels and confer varying patterns of protection against subsequent exposure to lethal heat stress at 47°C for 2 h (Heads et al., 1995). However, the role of Hsp47 in the protection against myocardial cells and heart tissue exposed to heat shock for different periods has not been thoroughly studied.

Mammalian HSPs are classified into different families based on their molecular weights, including HSP100, HSP90, HSP70, HSP60, and small HSPs, and play vital roles in various aspects of protein folding. Most HSP families are strictly stress-inducible, although some proteins are constitutively expressed. Molecules in each family are targeted to different subcellular compartments (Schmitt et al., 2007). Stress-inducible Hsp47 (Lele et al., 1997) is a collagen-binding glycoprotein present in the endoplasmic reticulum (ER) of collagen-secreting cells (Nagata, 1996; Taguchi and Razzaque, 2007). Some ER-localized stress proteins are typically induced increased concentrations of unfolded proteins in the ER. However, Hsp47 is the only ER resident protein that is induced by cytosolic and not ER stresses (Yasuda et al., 2002). Hsp47 belongs to the serine protease inhibitor (serpin) superfamily (Whisstock et al., 1998). Under stress conditions, Hsp47 expression is upregulated as part of the heat shock/stress response that mitigates cell damage from harmful stimuli, such as elevated temperature, heavy metals, and oxidative stress (Morimoto et al., 1997; Hart et al., 2000). Hsp47 was found to be elevated at 42°C or higher (Nagata et al., 1986). Both *in vitro* and *in vivo* studies have

demonstrated that diverse environmental stress factors can induce overexpression of HSPs to protect cells or eliminate them if they become irreversibly damaged. Additionally, highly significant upregulation of Hsp47 has been detected following hyperthermia and photodynamic therapy with a photosensitizing agent such as riboflavin, which can lead to collagen damage (Verrico et al., 2001). Hsp47 was reported to be localized in collagen-producing cells, such as fibroblasts, smooth muscle cells, chondrocytes, and endothelial cells, in chicks (Miyaishi et al., 1992). Expression of Hsp47 has also been correlated with collagen type I and type II expression in different mouse tissues during development, indicating that it acts as a collagen-specific chaperone in the mouse developmental program (Masuda et al., 1998). Upregulation of Hsp47 by heat-shock treatment has been detected in different cell types. For example, porcine fibroblasts were reported to upregulate Hsp47 expression at both the transcriptional and translational levels in response to heat stress (Wang et al., 2002). Exposure of oral ulcers to a defocused high-energy diode laser inducing a transient mild increase in local temperature of the superficial layers led to significant upregulation of Hsp47, which is thought to enhance wound repair by improving collagen synthesis and release (Vasques et al., 2013).

Cardiomyocytes (either neonatal or adult myocardial cells) were reported to stimulate the upregulation of collagen types I and III at the transcriptional level when co-cultured with adult rat fibroblasts in the presence of supplemented angiotensin II. This suggests that factors produced by cardiomyocytes are necessary to increase the expression of collagen genes *in vitro* and that fibroblast-myocyte crosstalk is required in this process (Pathak et al., 2001). Hsp47 is thought to be important for fibroblast-myocyte crosstalk, because induction of *hsp47* mRNA expression was reported to parallel that of type I and type III collagen mRNA in response to lung tissue damage (Hagiwara et al., 2007). The role of sub-lethal heat stress on Hsp47 expression in H9c2 cells and heart tissue has not been reported. Therefore, we investigated variations in Hsp47 expression to determine the correlation between Hsp47 expression and stress injury in heat-stressed myocardial cells *in vitro* and *in vivo*.

MATERIAL AND METHODS

Experimental design and heat-stress treatment of rats

Sixty-day-old Sprague-Dawley male and female rats (Qing Long Shan Company, Nanjing, China) were given free access to food and water and housed in cages in the animal facility at room temperature (RT) for 3 days for adaptation. After this period, 60 rats were divided randomly into 6 groups and exposed to heat stress for 0 (control), 20, 40, 60, 80, or 100 min by rapidly increasing the temperature from 25° to 42° ± 1°C in a controlled climate chamber (RX8-500D, New Jiangnan Co., Ltd., China). At the end of the heat-stress periods, all experimental rats were humanely sacrificed by decapitation. The abdomen was opened by midline incision and blood samples were taken from the heart and stored in anticoagulant-free test tubes for serum separation. Heart tissue samples were acquired and divided into 2 parts: one was fixed in 10% neutral buffered formalin for histopathological analysis, while the other was stored in liquid nitrogen for biological analysis.

The study protocol was reviewed and approved by the Animal Care and Use Committee of Nanjing Agricultural University, and the experiment was conducted in accordance with the guidelines of the regional Animal Ethics Committee.

Cell culture and heat-stress treatment of myocardial cells *in vitro*

Cardiac H9c2 cells from the American Type Culture Collection (ATCC, Manassas, VA, USA), which are undifferentiated neonatal rat cardiomyoblasts, were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% antibiotics (streptomycin and penicillin). Cells were incubated in a 5% CO_2 incubator in 25-cm^2 cell culture flasks until 80-90% confluency. The medium was changed at least 1 day before exposure to heat stress.

Heat shock was carried out according to a previously described method (Chen et al., 2011), with minor modifications. Briefly, cells were heat stressed by incubation in a water bath for 0 (control), 20, 40, 60, 80, or 100 min at 42°C by rapidly increasing the culture temperature from 37° to 42° ± 1°C. After heat shock, cells were lysed immediately in lysis buffer containing protease inhibitors, and supernatants were stored at -20°C for subsequent analysis. Each set of experimental conditions for the entire course of heat-stress treatment was repeated 3 times in duplicate.

Detection of myocardial cell damage-related enzymes

Approximately 2 mL cell culture supernatants of H9c2 cells and 1.5 mL serum from heat-stressed rats were collected at different time points as described above and stored at -80°C for further analysis. Levels of specific myocardial cell damage-related enzymes, such as aspartate aminotransferase (AST), creatine kinase (CK), creatine kinase MB (CK-MB), and lactate dehydrogenase (LDH), were assessed according to manufacturer instructions (Nanjing Jiancheng Biochemical Reagent Co., Nanjing, China).

Cytopathological and histopathological observations

For the *in vitro* assay, cardiac H9c2 cells were seeded on 35-mm culture dishes, each containing a poly-L-lysine-coated coverslip. These cells were divided into 6 groups for heat treatment 0, 20, 40, 60, 80, or 100 min in a water bath at 42°C or kept at 37°C as a control. After heat stress, the medium was discarded, and the cells were washed 2 times with phosphate-buffered saline (PBS) and fixed in 95% ethanol for 20 min at RT. After 2 additional washes with PBS, the cells were stained with hematoxylin, rinsed with tap water, submersed in acid alcohol, and washed again with tap water before staining with eosin. After subsequent dehydration with ascending concentrations of alcohol and clearing, the cells were mounted with a coverslip for observation and photographing under a microscope.

Heart samples from rats were obtained after different time periods of heat stress and fixed in 10% neutral buffered formalin. Samples were embedded in paraffin and cut into 5-μm thick sections. Sections were stained with hematoxylin and eosin (H&E), and images were acquired to examine histopathological changes among groups.

Immunofluorescent and immunohistochemical staining

After exposure to heat stress for various periods of time in a water bath at 42°C, cardiac H9c2 cells subcultured in poly-L-lysine-coated coverslips were washed in PBS and

fixed in 95 and 100% alcohol for 15 min each. After washing with pre-cooled PBS for 15 min, cells were permeabilized with 0.5% (v/v) Triton X-100 in PBS for 15 min. Nonspecific protein binding in permeabilized cells was blocked by incubation with 5% bovine serum albumin (BSA)/PBS for 30 min at RT. Cells were then incubated with a specific mouse monoclonal antibody against rat Hsp47 (ADI-SPA-470, Enzo Life Science, Farmingdale, NY, USA) diluted at 1:50 with 1% BSA/PBS, followed by incubation with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (1:50 dilution) (101274, Jackson Immuno Research Labs, West Grove, PA, USA) in 1% BSA/PBS. Images were acquired using a fluorescent microscope.

A series of 5-µm paraffin-embedded heart sections similar to those used for routine H&E staining were mounted on polylysine-coated slides and dried for 1 h at 65°C. Sections were then deparaffinized with xylene and hydrated with descending concentrations of alcohol. For antigen unmasking, sections were incubated with hydrochloric acid in distilled water for 20 min at RT and then washed in PBS containing 0.05% Tween-20. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min at RT. Nonspecific binding sites were blocked by incubating with 5% BSA for 30 min at 37°C. Sections (except controls) were then incubated for 2 h at 37°C with a mouse monoclonal antibody against rat Hsp47 (ADI-SPA-470, Enzo Life Science) at 1:50 dilution. Sections were rinsed in PBS containing 0.05% Tween-20 and subsequently incubated with a horseradish peroxidase goat anti-mouse IgG-horseradish peroxidase secondary antibody (SN133, Sun Shine Bio, Nanjing, China) at a 1:500 dilution for 1 h at 37°C. Samples were rinsed in PBS containing 0.05% Tween-20 and the reaction was developed with 2 drops of prepared diaminobenzidine tetrahydrochloride (DAB) substrate chromogen solution (AR1022, Boster Immunoleader, Auhan, China) for 10 min at RT until the desired color was obtained. Sections were counterstained with hematoxylin solution, mounted with aqueous mounting medium, and photographed under a microscope.

Detection of *hsp47* mRNA by quantitative real-time PCR (qRT-PCR)

Primer design for qRT-PCR

Primer pairs were designed to anneal specifically to the target *hsp47* or *β-actin* mRNA using corresponding sequences obtained from the NCBI (accession Nos. NM_017173.1 and NM_031144.3, respectively). Primer specificity was confirmed using the NCBI BLAST database. The ensemble website was used to determine exon regions of the *hsp47* mRNA for the final design of primer sequences using the Primer Premier 5.0 software (Table 1). The expected sizes of PCR products were 110 and 184 bp for *β-actin* and *hsp47*, respectively. Primers were synthesized by Invitrogen (Carlsbad, CA, USA).

Table 1. Primers for qRT-PCR analysis.

Gene name	Type	Sequence
hsp47 mRNA	Forward	5'-TCTCCTTCTGGGCACCTTA-3'
	Reverse	5'-CTCCACCGCCTGATCTTT-3'
β-actin mRNA	Forward	5'-TGCGCAAGTTAGGTTTTGTCA-3'
	Reverse	5'-GCAGGAGTACGATGAGTCCG-3'

RNA extraction for qRT-PCR

Total RNA was extracted from cultured H9c2 cells using Trizol-RNAiso Plus reagent according to manufacturer instructions (D9108A, Takara, Dalian, China). Briefly, after H9c2 myocytes were heat-stressed at 42°C for different time periods, 1 mL RNAiso Plus reagent was added to each 10-mm^2 cell culture plates. For each rat sample, 100 μg heart tissue was homogenized, and then 1 mL RNA extraction buffer was added according to manufacturer instructions. RNA concentration was determined by spectrophotometry (M200PRO, Tecan Infinite 200 Pro, Grödig, Austria). RNA samples were used to synthesize cDNA with PrimeScript RT Master Mix (Perfect Real Time, DRR036A, Takara) following manufacturer instructions and stored at -80°C for further analysis.

Quantitative RT-PCR

Quantitative RT-PCR was carried out in an iQ5 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Each 2 μL cDNA sample diluted 10X was suspended in 2X iQ™ SUPER® Green Supermix (Bio-Rad Laboratories) with 0.6 μL of each primer (Table 1), and double-distilled water was added such that the total reaction volume was 20 μL. The PCR conditions were as follows: enzyme activation was carried out at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s.

For each run, a negative control sample without cDNA was analyzed along with the experimental groups. A 4-fold dilution series of template was used in the PCR amplification. Using the Bio-Rad iQ5 software, the data were analyzed and normalized using the following formula: relative *hsp47* mRNA levels = $2^{-\Delta\Delta Ct}$, where

$$\Delta\Delta Ct = [(Ct_{hsp47\ mRNA} - Ct_{\beta\text{-}actin\ mRNA}) \text{ experimental group}] - [(Ct_{hsp47\ mRNA} - Ct_{\beta\text{-}actin\ mRNA}) \text{ control group}].$$

Western blotting

After exposure to *in vivo* heat stress for different periods of time, all experimental rats were humanely sacrificed by decapitation. Approximately 100 μg heart tissue was taken from each specimen and placed in 1 mL PBS for homogenization using a Fluko® Super Fine Homogenizer (623003, Fluko Equipment Co. Ltd., Shanghai, China). After centrifugation at 1500 *g*, the cell pellets were resuspended in 200 μL ice-cold RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and l mL PMSF) (WB-0071, Dingguo Changsheng Biotechnology Co. Ltd., Beijing, China). The homogenates were then centrifuged at 14,000 *g* for 5 min at 4°C, and the supernatants were collected and used as total protein extracts.

After 0, 20, 40, 60, 80, or 100 min of exposure to heat stress in a water bath at 42°C, H9c2 cells were washed 2 times in PBS and lysed in M-PER® Mammalian Protein Extraction Reagent (28501, Thermo Scientific, Rockford, IL, USA) supplemented with Halt Protease Inhibitor Cocktail according to manufacturer instructions. Cell homogenates were then centrifuged at 14,000 *g* for 5 min at 4°C, and the supernatants were collected and used as total protein extracts.

All protein concentrations were measured using the Micro-BCA™ protein assay kit (Thermo Scientific). After addition of protein loading buffer and boiling of the protein samples, they were electrophoresed on 10% SDS-polyacrylamide gels. Proteins on the gels were transferred to polyvinylidene difluoride membranes (Bio-Rad), blocked in Tris-buffered saline (TBS) containing 0.05% Tween-20 (v/v) (TBST) and 5% (w/v) nonfat dry milk and then reacted with the mouse monoclonal antibody against rat Hsp47 (1:1000) dilution (ADI-SPA-470, Enzo Life Science) diluted in TBST containing 5% nonfat dry milk overnight at 4°C. After washing 3 times for 10 min with TBST, the membranes were incubated with goat anti-mouse IgG secondary antibody at 1:10,000 dilution (SN133, Sun Shine Bio). After washing with TBST, proteins in the membranes were visualized using SuperSignal® West Pico (Thermo Scientific). The intensity of the Hsp47 signal was normalized against that of glyceraldehyde-3-phosphate dehydrogenase detected using a mouse monoclonal antibody at 1:1000 dilution (KC-5G4, Kangchen, Shanghai, China), and goat anti-rabbit IgG secondary antibody at 1:10,000 dilution (SN134, Sun Shine Bio). Bands generated from the Western blot were further quantified by densitometry.

Statistical analysis

All data are reported as means ± standard deviation (SD). Data were analyzed by one-way analysis of variance using the Statistical Package for Social Sciences (SPSS 16.0; SPSS, Inc., Chicago, IL, USA). For multiple comparisons, the least significant difference test was used to compare the mean values of the control groups with other experimental groups. All experiments were performed in triplicate (N = 3). Statistically significant results were noted for $P < 0.05$ and $P < 0.01$.

RESULTS

Clinical symptoms of heat-stressed animals

All heat-stressed rats exhibited clinical signs such as rapid breathing, sweating, restlessness, and thirst at the beginning of the treatment period. Prolonging the heat-stress period caused the rats to become dull or comatose, and some rats died. Forty percent of rats died in the group exposed to 100 min of heat treatment at 42°C, while no deaths occurred in the normal control and other groups.

Detection of enzyme levels in H9c2 cell supernatant and rat serum after heat treatment

Levels of heart cell injury-related enzymes such as AST, CK, CK-MB, and LDH in the supernatant of H9c2 cells and in the serum of rats after exposure to different periods of heat stress are shown in Figure 1. AST levels gradually increased in the supernatant of heat-stressed H9c2 cells, with a significant induction occurring after 60 min ($P < 0.01$) and reaching a maximum at 100 min ($P < 0.01$) of heat stress. AST levels in the serum of rats were initially reduced at 20 min ($P < 0.01$) of heat stress, but then started to increase to reach a maximum at 100 min ($P < 0.01$). CK levels in the serum of heat-stressed rats and in the supernatants of the heat-stressed H9c2 cells showed similar patterns, with no significant difference among

the various time periods of heat stress. During the entire course of heat-stress treatment, no significant change in CK-MB was observed in the supernatant of H9c2 cells, while CK-MB levels were decreased in the serum of heat-stressed rats, particularly at 20 min ($P < 0.01$) and 80 min ($P < 0.01$) of exposure to high temperatures. Aside from the reduction at 20 min of heat stress, LDH activity in the serum of rats showed clear induction at 60 min ($P < 0.01$) and 100 min ($P < 0.05$) of heat stress. In the supernatant of H9c2 cells, a significant reduction in LDH enzyme occurred after 40 min ($P < 0.01$) of heat stress.

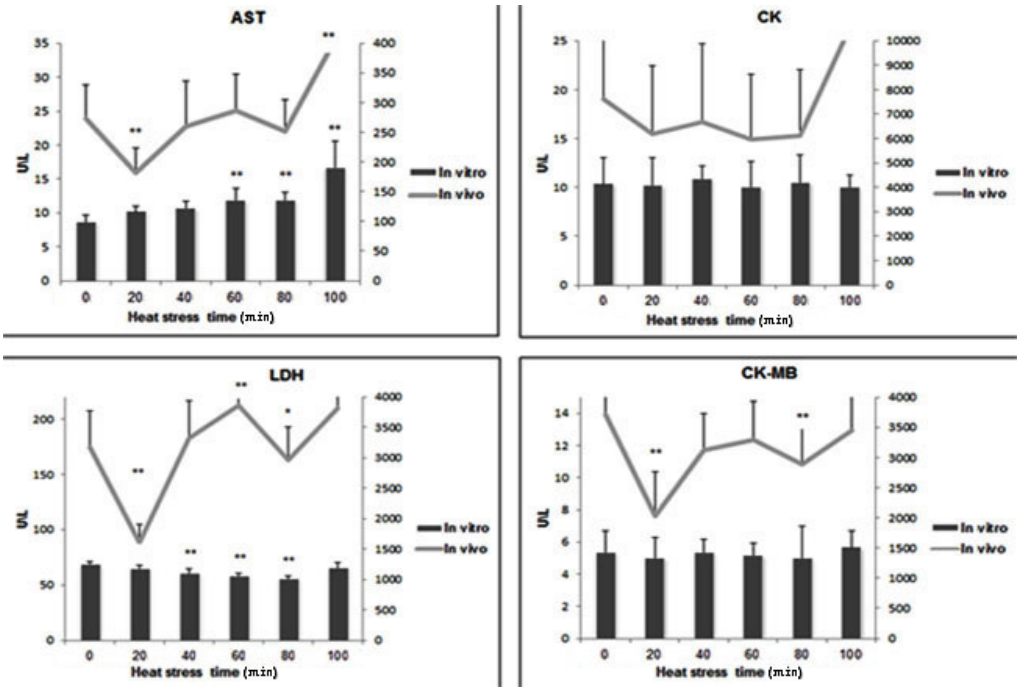

Figure 1. Levels of cell injury-related enzymes in the supernatant of H9c2 cells and serum of rats after exposure to heat stress. Values are reported as means ± SD. *$P < 0.05$; **$P < 0.01$ ($N = 6$).

Although there were no significant changes in the enzymes CK and CK-MB in the supernatant of H9c2 cells, the levels of AST, LDH, and CK-MB in the serum of rats showed the same patterns during the various periods of heat stress. Compared to the corresponding enzyme levels observed *in vitro* in the supernatant of H9c2 cells, *in vivo* changes in heart damage-related enzymes such as AST, LDH, and CK-MB in the rat serum were much more sensitive to heat-stress exposure.

Cytopathological and histopathological changes in heat-stressed myocardial cells *in vitro* and *in vivo*

Cytopathological changes in H9c2 cells exposed to different periods of heat stress are shown in Figure 2. No obvious cytopathological changes were observed in control myocardial cells (Figure 2A). After 20 min of heat stress, H9c2 cells showed acute changes such as granular degeneration in

the cytoplasm (Figure 2B). Acute degeneration was dramatically increased at 40 and 60 min of heat stress (Figure 2C and D). Necrotic H9c2 cells with pyknotic nuclei and acidophilic cytoplasm were clearly observed at 80 and 100 min of heat-stress treatment (Figure 2E and F).

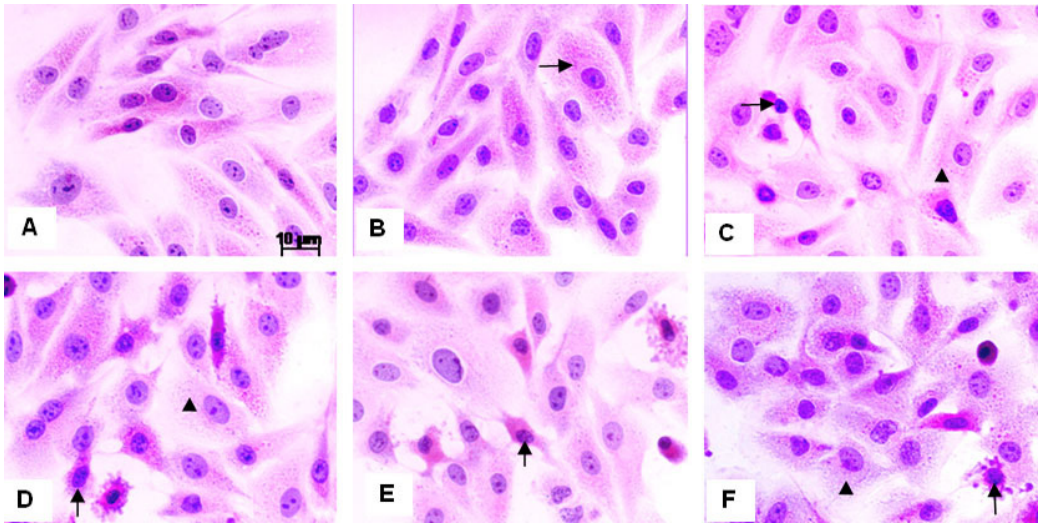

Figure 2. Photomicrographs of H&E-stained heat-stressed H9c2 cells. **A.** No obvious cytopathological changes were observed in myocardial cells of the control group. **B.** After 20 min of heat stress, H9c2 cells showed acute cellular changes characterized by granular degeneration (arrow) in the cytoplasm. **C.** Granular degeneration (arrowhead) and pyknotic nuclei (arrow) were observed at 40 min of heat stress. **D.** Acute lesions characterized by microvacuolation and granular degeneration (arrowhead) in the cytoplasm and necrotic cells (arrow) were observed after 60 min of heat stress. **E.** Necrotic cells (arrow) with pyknotic nuclei and acidophilic cytoplasm were observed at 80 min in heat-stressed H9c2 cells. **F.** Acute lesions characterized by necrotic cells (arrow), microvacuolation and granular degeneration (arrowhead) in the cytoplasm after 100 min of heat stress. Scale bar = 10 μm.

Histopathological changes in rat myocardial cells exposed to different periods of heat stress *in vivo* are shown in Figure 3. No obvious lesions were observed in the myocardial cells of the control group (Figure 3A). Acute degenerative changes of rat myocardial cells were observed, as shown by light pink staining, loss of striations in the sarcoplasm, and light hyperemia and edema in the interstitial tissue (Figure 3B). However, marked degenerative changes, characterized by enlarged cell size, loss of striations in the sarcoplasm, obvious granular and microvascular degeneration in the cytoplasm, and hemorrhage, were severe after 40 min of heat stress (Figures 3C-F).

Transcriptional levels of *hsp47* mRNA in heat-stressed myocardial cells *in vitro* and *in vivo*

Representative transcriptional levels of *hsp47* mRNA after heat stress exposure of H9c2 cells *in vitro* and of heart cells *in vivo* at different time periods are shown in Figure 4. In H9c2 cells, qRT-PCR analysis demonstrated that *hsp47* mRNA was increased at 20 min of heat stress, but the level elevated significantly at 60 min ($P < 0.01$) and 100 min ($P < 0.01$) compared to the control group; additionally, *hsp47* mRNA levels decreased at 40 ($P > 0.05$) and 80 min ($P > 0.05$) of heat stress.

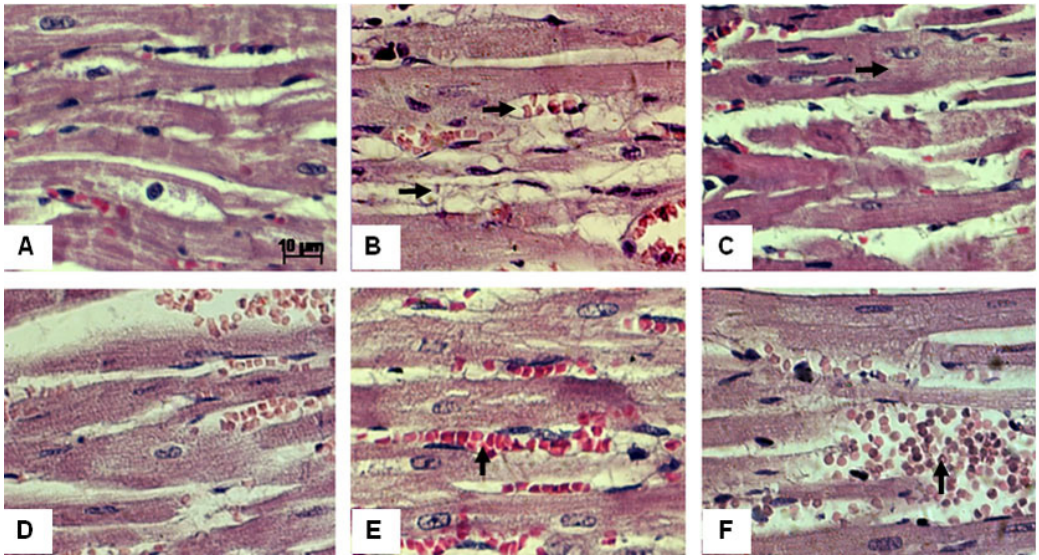

Figure 3. Photomicrographs of H&E-stained heat-stressed rat heart tissues. **A.** No obvious histopathological changes were observed in the rat heart tissue of the control group. **B.** After 20 min of heat stress, acute degeneration characterized by light pink staining, fine particles and loss of striations in the cytoplasm, and light hyperemia and edema (arrow) in the interstitial tissue were observed. **C.** Acute degeneration, characterized by enlarged cell size and loss of striations in the cytoplasm (arrow), was seen after 40 min of heat stress. **D.** Marked degenerative changes, recognized by enlarged cell size with fine particles and microvacuolation and loss of striations in the sarcoplasm, were severe after 60 min of heat stress. **E.** Obvious granular and microvaculation degeneration in the cytoplasm of enlarged myocardial cells and hyperemia (arrow) were observed after 80 min of heat stress. **F.** Marked acute degenerative changes in the enlarged cells and hemorrhage in the interstitial tissue (arrow) were observed after 100 min of heat stress. Scale bar = 10 μm.

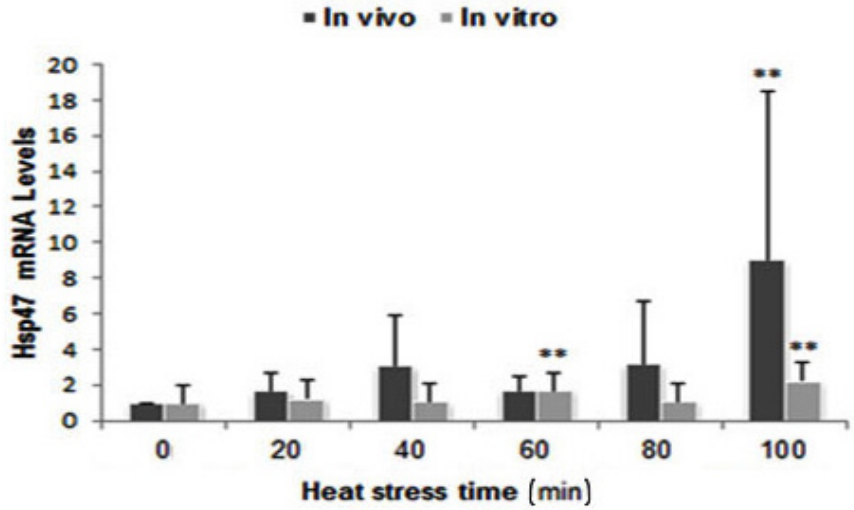

Figure 4. Transcriptional levels of *hsp47* mRNA in heat-stressed rat myocardial cells *in vitro* and *in vivo*. **P < 0.01 compared to control. Values are reported as means ± SD (N = 5).

Transcriptional levels of *hsp47* mRNA in the heart tissues over the 100-min course of heat-stress treatment *in vivo* displayed similar variations to those in H9c2 cells, with transcription significantly induced ($P < 0.01$) at 100 min of heat stress.

Variations in expression of Hsp47 in heat-stressed myocardial cells *in vitro* and *in vivo*

The expression levels of Hsp47 in H9c2 cells *in vitro* and in heart cells *in vivo* after heat stress are shown in Figure 5. Hsp47 was constitutively expressed in both H9c2 cells and in rat myocardial tissues of control and heat-stressed groups. Hsp47 protein levels in rat myocardial cells showed slight variations after 20 min of heat stress, but no significant change was observed compared to the control group. Over the 100-min course of heat shock *in vivo*, Hsp47 expression levels displayed some variations, but no significant changes compared to the control group.

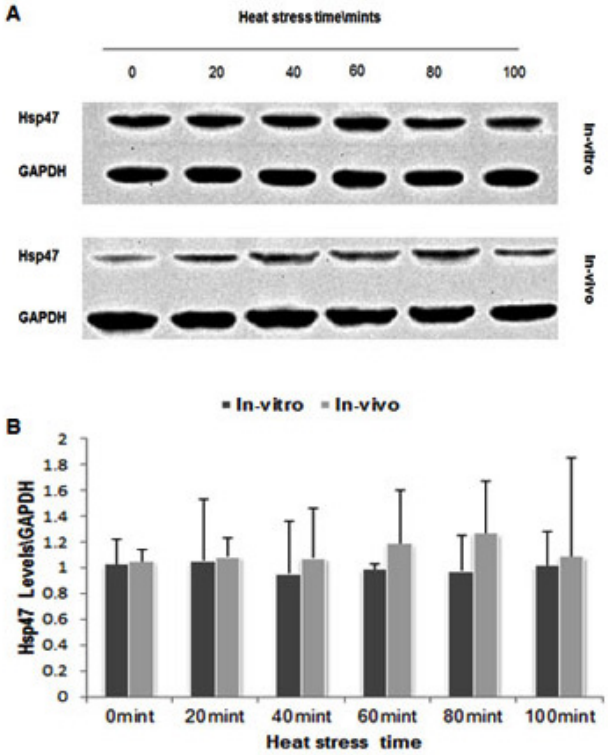

Figure 5. Western blot analysis of Hsp47 expression levels in heat-stressed rat myocardial cells *in vitro* and *in vivo*. **A.** Proteins (50 µg heart tissue and 10 µg H9c2 cells per lane) were electrophoretically separated, blotted onto polyvinylidene difluoride membranes, and hybridized with a mouse monoclonal antibody against rat Hsp47. **B.** Densitometry analysis of the Hsp47 protein. The density of each band was normalized to that of the glyceraldehyde-3-phosphate dehydrogenase protein. Values are reported as means ± SD (N = 6).

Immunofluorescent staining of heat-stressed myocardial cells *in vitro* and *in vivo*

Images showing the localization of Hsp47 in H9c2 cells *in vitro* and in the heart cells of rats *in vivo* after heat stress are shown in Figures 6 and 7, respectively. Immunofluores-

cence analysis showed that green signals representing Hsp47 were localized in the cytoplasm of H9c2 cells both in control and in the heat-stressed groups (Figure 6). While the density of Hsp47 in the cytoplasm of H9c2 cells was reduced at 20 min of heat stress (Figure 6B), it was similar to that of the control group from 40-100 min of heat stress (Figure 6C-F). Similar Hsp47 signal intensities were observed in the sarcoplasm of rat myocardial cells during different heat-stress periods *in vivo* (Figure 7).

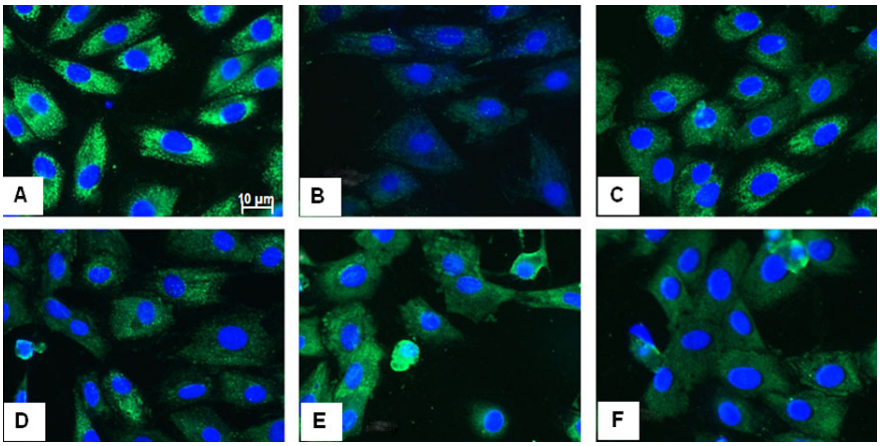

Figure 6. Subcellular localization by immunofluorescent staining of Hsp47 in heat-stressed myocardial cells *in vitro*. **A.** Small particles of Hsp47 signals were localized in the cytoplasm of H9c2 cells in the control group. **B.** Weak Hsp47 signal were distributed in the cytoplasm of H9c2 cells after 20 min of heat stress. **C.-F.** Densities of positive Hsp47 staining in the cytoplasm of H9c2 cells after 40-100 min of heat stress were similar to that of the control group. Scale bar = 10 µm.

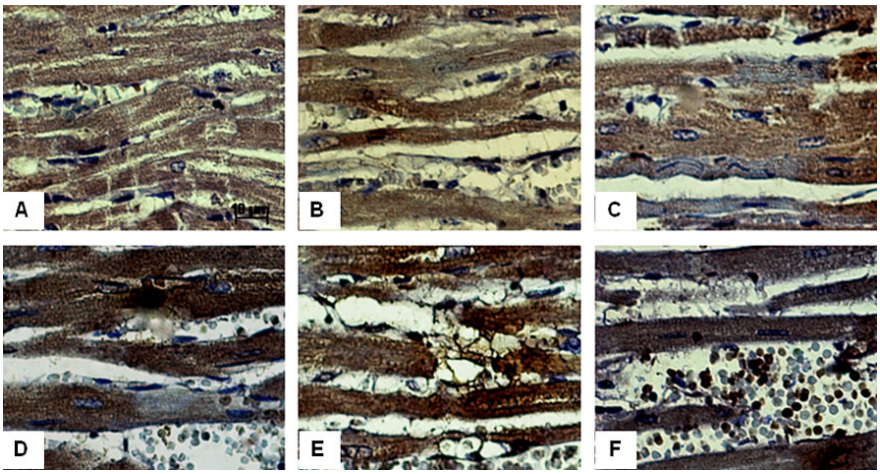

Figure 7. Photomicrographs of immunohistochemically stained sections of representative heart tissues from rats exposed to different periods of heat stress at 42°C. Hsp47 expression was assessed by reacting a monoclonal antibody with Hsp47 and visualization with the DAB substrate (brown color). **A.** Small particles of Hsp47 signals were constitutively expressed and distributed in the cytoplasm of heart tissue cells of control rats. **B.-F.** No obvious differences in distribution of Hsp47 signal intensities were identified in the cytoplasm of heart cells within 100 min of heat stress. Scale bar = 10 µm.

DISCUSSION

In this study, the expression levels of Hsp47 and its corresponding mRNA in H9c2 cells *in vitro* and rat heart tissue *in vivo* in response to different time periods of heat stress were evaluated. We observed constitutive patterns of Hsp47 expression both *in vitro* and *in vivo*, while the corresponding mRNA levels were significantly upregulated ($P < 0.01$). A correlation between Hsp47 expression and pathological changes in H9c2 cells *in vitro* and in the heart tissue *in vivo* during heat stress was also investigated. Expression of the *hsp47* transcript increased at later periods of heat stress concurrently with cyto-histopathological changes, suggesting an association with pathophysiological effects on myocardial cells under heat stress.

Since heart cell injury-related enzymes such as AST, CK, CK-MB, and LDH are intracellularly localized, increases in the activities of these enzymes in the serum or cell culture supernatant are indicators of myocardial cell damage. Several studies have shown that these enzymes can be expressed in response to different stress conditions (e.g., transport stress induced tissue damage to the heart, liver, and kidney), and in piglets, the damage is characterized by histopathological changes, such as acute degeneration of the liver and kidney and granular degeneration of myocardial cells (Zhu et al., 2009; Bao et al., 2009). Due to the cellular damage, enzymes are released into the serum from necrotic cells, which are important measures of cell and tissue injury. Although these enzymes are not individually specific for heart damage, evaluating their levels together may be an indicator of myocardial injury (Chopra et al., 1995; Al-Shabanah et al., 1998). Cardio-specific marker enzymes have been shown to be released from the heart into the blood stream during myocardial damage because of degeneration of myofibrils and myocytes (Senthil et al., 2007). An *in vitro* study showed significantly increased enzymes such as AST, LDH, and CK in the culture medium of primary rat myocardial cells, suggesting that the integrity of these cells was altered because of heat stress (Liu et al., 2012). Our results showed that diagnostic marker enzymes clearly were elevated. For instance, AST levels gradually increased in the supernatant of heat-stressed H9c2 cells, with significant induction levels after 60 min followed by continued elevation at 80 and 100 min of heat stress. Additionally, AST levels in serum of heat-stressed rats gradually increased, reaching maximum levels at 100 min of exposure to high temperature, except for a brief reduction at 20 min. Collectively, these results indicate that enzyme activities were coupled with changes in the H9c2 cells *in vitro* and heart tissue *in vivo* exposed to various periods of heat stress. Plasma CK has been shown to be elevated in broiler chickens after 3 h of heat stress (Yu et al., 2008). In contrast, *in vivo* ischemia-reperfusion in the rat myocardium was reported to cause a significant increase in serum CK level, while pretreatment with heat stress significantly reduced CK release during reperfusion (Hu et al., 2003). In the present study, however, the results suggested that the CK enzyme activity may not be useful as a heart tissue damage marker during the early period of heat stress. Levels of AST and LDH in the serum of rats and in the supernatant of H9c2 cells were similar, with significant induction between the various periods of heat stress compared with the control.

HSPs occupy nearly all subcellular locations where they perform a variety of chaperoning functions, including folding and unfolding of nascent polypeptides, proteins, transport of proteins, and support of antigen presentation processes (Sherman and Multhoff, 2007). Hsp47 expression was constitutively expressed in the cytoplasm of H9c2 cells and rat heart tissue, both in heat-stressed rat heart cells and control myocardial cells. Additionally, the corresponding *hsp47* mRNA transcripts were significantly upregulated. These observations sug-

gest that consumption of Hsp47 exceeds its production under different heat-stress periods. Immunocytochemical studies have shown that Hsp47 is located exclusively in the ER in certain cells (Saga et al., 1987). Hsp47 has been reported to be expressed by various collagen-producing cells such as fibroblasts, smooth muscle cells, chondrocytes, and endothelial cells (Miyaishi et al., 1992). In the present study, immunofluorescence analysis showed that Hsp47 expression was predominantly localized to the cytoplasm of H9c2 cells and heart tissue exposed to heat stress for up to 100 min. These results agree with previous reports showing that Hsp47 is present in the ER of collagen-secreting cells and confirming the pathophysiological significance of this chaperone (Nagata, 1996; Taguchi and Razzaque, 2007).

In the present study, the Hsp47 protein was found to be constitutively expressed in heat-stressed H9c2 and rat heart tissues; *hsp47* mRNA showed obvious increases after heat stress both *in vitro* and *in vivo*, suggesting that the abundance of *hsp47* mRNA transcripts during later periods of heat stress may have a pathophysiological role in myocardial cells. Exposure of H9c2 cells to sub-lethal heat stress at 43°C for 30 min has been shown to elevate Hsp70, Hsp90, and Hsp60 to different levels and conferred different levels of protection against subsequent lethal heat stress at 47°C for 2 h (Heads et al., 1995). In contrast, our results showed that Hsp47 was constitutively expressed without clear variations over the 100-min heat stress period both *in vitro* and *in vivo*, while the corresponding *hsp47* mRNA was significantly upregulated. These observations were not consistent with classical regulatory mechanisms linking transcription and translation. The quantitative relationship between mRNA and its encoded protein not well-understood (de Sousa Abreu et al., 2009). The abundance of an *mRNA* transcript can help to explain protein levels. mRNA and protein expression are correlated, i.e., by associating expression levels across a set of conditions between the mRNA and its corresponding protein. However, if the mRNA level is generally higher than the protein level, a large amount of energy may be spent on regulating mRNA synthesis (Östlund and Sonnhammer, 2012). In a similar study of a collagen-binding HSP in gp46 cells, protein levels showed no correlation with the corresponding mRNA during rat postnatal development, suggesting the presence of complex pre- and post-translational regulatory mechanisms as well as variations in gp46 transcript and protein stability and degradation (Pak et al., 1996). Hsp47 has been found to be localized in the ER of collagen-producing cells and to facilitate the proper formation of the collagen quaternary structure (Nagata, 2003).

Elevated expression of Hsp47 has been correlated with different types of pathological conditions, such as collagen damage caused by hyperthermia, pulmonary fibrosis, and gastric ulcers (Razzaque et al., 1999; Guo et al., 2002; Kakugawa et al., 2013). Our observation of Hsp47 expression resulting from heat stress and its associated pathological conditions agreed with the results of previous studies. Localization of HSPs may be related to the protection of molecular chaperones (Georgopoulos and Welch, 1993). Western blotting results in the current study showed no obvious variations in Hsp47 protein levels in the cytoplasm of myocardial cells. However, the *hsp47* mRNA transcript levels increased after different periods of heat stress, indicating that myocardial cells consumed much more Hsp47 protein or that the consumption of Hsp47 exceeded its production during periods of heat stress in myocardial cells.

In summary, we demonstrated that although levels of the *hsp47* mRNA transcript increased after different periods of heat stress *in vitro* and *in vivo*, Hsp47 was expressed constitutively during different periods of heat stress, as confirmed by immunofluorescent and immunocytochemical analysis. However, the detailed mechanism of *hsp47* mRNA and its corresponding expression during heat stress remains unclear, and further studies are needed.

ACKNOWLEDGMENTS

Research supported by grants from the National Key Basic Research Program of China ("973" Program); (#2014CB138502), the National Natural Science Foundation of China (#31372403), the National Department Public Benefit Research Foundation (Agriculture) (#201003060-11), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the Graduate Research and Innovation Projects in Jiangsu Province, and the Sino-German Agricultural Cooperation Project of the Federal Ministry of Food, Agriculture and Consumer Production, Berlin, Germany.

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