Expression profiles of heat shock protein 27 and αB-crystallin and their effects on heat-stressed rat myocardial cells *in vitro* and *in vivo*

SHU TANG[1], HONGBO CHEN[1], YANFEN CHENG[1], MOHAMMAD ABDEL NASIR[1], NICOLE KEMPER[2] and ENDONG BAO[1]

[1]College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu 210095, P.R. China;
[2]Institute for Animal Hygiene, Animal Welfare and Farm Animal Behaviour, University of Veterinary Medicine Hannover, Foundation, Hannover D-30173, Germany

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Abstract. The present study established a heat-stressed rat heart model, and used an H9c2 myocardial cell line to investigate the expression profiles of heat shock protein (Hsp)27 and αB-crystallin, both *in vivo* and *in vitro*. Rats and myocardial cells were subjected to 42˚C for 0, 20, 40, 60, 80 or 100 min, following which the mRNA and protein expression levels of Hsp27 and αB-crystallin were measured. Following heat shock, the protein expression levels of Hsp27 and αB-crystallin were significantly decreased in the rat heart cells *in vivo*, whereas their mRNA levels were significantly increased. The opposing association between the protein and mRNA expression levels of Hsp27 and αB-crystallin suggests that the progression from mRNA into proteins via translation may delayed, or proteins may exist as either oligomers or in the phosphorylated form under heat stress. *In vitro*, Hsp27 and αB-crystallin exhibited similar reductions in the protein levels at 40 and 60 min, then increased to normal values following 80 min of heat stress. However, the mRNA levels were not consistent with the protein levels. The mRNA levels of Hsp27 and αB-crystallin did however exhibit similar tendencies following 60 min of heat stress. The present study investigated these apparently conflicting results between the *in vitro* cell line and the *in vivo* body system. The results demonstrated that the protein and mRNA expression levels of Hsp27 and αB-crystallin exhibited similar trends *in vivo* and *in vitro*, respectively. These results were confirmed by analysis with STRING 9.1 software, which indicated that Hsp27 and αB-crystallin are co-expressed in rat myocardial cells. However, the individual cell lines and whole body system exhibited different trends in Hsp27 and αB-crystallin levels prior to and following heat stress, thus require further investigation.

Correspondence to: Professor Endong Bao, College of Veterinary Medicine, Nanjing Agricultural University, 1 Weigang Road, Nanjing, Jiangsu 210095, P.R. China
E-mail: b_endong@njau.edu.cn

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Introduction

High environmental temperatures may be fatal and are associated with sudden death in animals and humans (1). The incidence of heat-associated mortality is likely to increase with global warming, and with the predicted increase in frequency and intensity of heat waves (2,3). Scientific reports have indicated that hyperthermia (40-45˚C) is cytotoxic (2,4) and prolonged exposure to temperatures >42˚C causes cellular damage, and protein denaturation and aggregation (5). However, the causes of the progression from heat stress to lethal heat stroke and clinical hyperthermia, and the mechanisms underlying hyperthermia-induced cytotoxicity, remain poorly understood (6,7). A previous study demonstrated that heart attacks caused by high temperatures are associated with high mortality due to heart diseases such as hypertension, coronary vessel occlusion, and atherosclerosis (8).

Heat stress induces the expression of heat-shock proteins (Hsps) (9,10). Hsps are usually divided into small Hsps (sHsps) (molecular mass, <40 kDa), and Hsp40, Hsp60, Hsp70 (68-80 kDa), Hsp90 (83-99 kDa), and Hsp100 protein families. All Hsp families share a common chaperoning function. It is generally accepted that Hsps prevent cells from lethal thermal damage (11). A lack of Hsp synthesis is associated with exponential cell death (12). Furthermore, a previous study reported that stretched and decreased myocyte shortening results in an increase in the expression levels of Hsps in the isolated perfused rabbit heart (13).

sHsps are ubiquitous components of protein quality control cell networks, and can be induced by numerous events including hypoxia, heat shock, ultraviolet light, and toxic free radicals (9,14). As with other chaperones, sHsps have a high capacity to bind unfolded proteins and facilitate substrate refolding (15). Hsp27 and αB-crystallin belong to the sHsp superfamily, and are functional, stress-induced sHsps that are expressed in numerous tissues types, notably in muscles (7). In

humans, there are 10 genes that encode sHsps (16); however, only Hsp27 and αB-crystallin function as molecular chaperones (17,18). The rapid upregulation of sHsps is regulated by transcriptional and translational mechanisms (19). Hsp27 and αB-crystallin possess a homologous α-crystallin domain, which prevents actin microfilament disruption under stress conditions (7). The effects of sHsps on the cytoskeleton may be important not only in individual cell tolerance to stress through cytoskeletal stabilization, but may also be integral to the protection of the whole organism through the maintenance of endothelial and epithelial barrier functions (20).

Presently, it remains uncertain whether Hsp27 and αB-crystallin proteins and genes induce similar changes in heart cells and tissues in response to heat stress *in vitro* and *in vivo*. Therefore, the present study investigated the expression levels of Hsp27 and αB-crystallin, both in rats, and in a myocardial cell line, following exposure to high temperature.

Materials and methods

Animals and experimental design. All experiments were performed in accordance with the guidelines of the Animal Ethics Committee of Jiangsu province (China) and were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China). Sixty-day-old Sprague Dawley rats (n=60) were obtained from Qing Long Shan Company (Nanjing (China) and housed at room temperature (25˚C) for 5 days. The rats were then randomly divided into six groups (n=10): Five heat-stress exposure groups (for 20, 40, 60, 80 and 100 min, respectively) and a control group. All animals were given *ad libitum* access to water and were fed the same feed during the experiments. A controlled-climate chamber (New Jiangnan Instrument Co., Ltd., Ningbo, Zhejiang) was pre-heated to 42˚C with circulating fresh air, and the relative humidity was kept between 55-65%. The control rats were maintained at room temperature. The mental state and activities of the control and heat-stressed rats were observed and recorded. Following each heat-stress period, the rats were sacrificed within 3 min. Blood samples were then collected, and the hearts were divided into two sections: The ventriculus sinister, which was fixed in 4% paraformaldehyde for pathologic observation, and the ventriculus dexter, which was stored in liquid nitrogen until further experimentation.

Cell subculture and preparation. The H9c2 myocardial cells (American Type Culture Collection, Manassas, VA, USA) were subcultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco Life Technologies, Carlsbad, CA, USA), and incubated at 37˚C in an atmosphere containing 5% CO_2, until the fusion rate of the H9c2 cells was >90%. The cells were then divided into six groups: A control group (0 min), and five groups exposed to heat stress for 20, 40, 60, 80 and 100 min. For prompt heat exposure, the temperature in the incubator was raised from 37 to 42˚C in a humidified atmosphere containing 5% CO_2.

Western blot analysis. For protein extraction, all experimental rats were humanely sacrificed by decapitation. A total of ~100 μg heart tissue was collected and homogenized in 1 ml phosphate-buffered saline (PBS) using a 623003 Fluko® Super Fine Homogenizer (Fluko Equipment Shanghai Co. Ltd., Shanghai, China) and centrifuged at 2,000 x g. The cell pellets were resuspended in 200 μl ice-cold radioimmunoprecipitation assay lysis buffer containing 50 mM Tris, (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and l ml phenylmethylsulfonyl fluoride WB-0071 (Beijing Ding Guo Chang Sheng Biotechnology Co. Ltd., Beijing, China). The homogenates were then centrifuged at 14,000 x g for 5 min at 4˚C, and the obtained supernatants were used as total protein extracts. Following 0, 20, 40, 60, 80 and 100 min of heat stress treatment in an incubator at 42˚C, H9c2 cells were washed twice with PBS and lysed in M-PERH mammalian protein extraction reagent (28501; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with Halt™ protease inhibitor cocktail according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.). The cell homogenates were then centrifuged at 14,000 x g for 5 min at 4˚C and the supernatants were used as total protein extracts. All protein concentrations were measured using a Micro-Bicinchoninic Acid™ Protein Assay kit (Thermo Fisher Scientific, Inc.). A total of 20 μg H9c2 protein and heart sample protein (80 μg) were loaded onto a 13% acrylamide gel with a 4% stacking acrylamide gel. Migration was performed using a buffer containing 25 mM Tris, pH 7.6, 0.1% SDS, and 0.2 mg lysine. The proteins in the gels were transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were then blocked for 1 h at room temperature in Tris-buffered saline with Tween 20 (TBST; Sigma-Aldrich, St. Louis, MO, USA) containing 5% milk powder, and incubated overnight at room temperature with αB-crystallin mouse immunoglobulin G monoclonal primary antibody (cat. no. ADI-SPA-222-F) and GAPDH mouse IgG monoclonal primary antibody (cat. no. ADI-CSA-335-E) (Enzo Life Sciences, Inc., Farmingdale, NY, USA). The blots were washed three times for 5 min in TBST and 5% skim milk powder containing goat anti-mouse antibody (cat. no. SN133; Sunshine Biotechnology Nanjing Co. Ltd., Nanjing, China) at room temperature for 1 h. Following three 5 min washes with TBST, the bands were revealed using diaminobenzidine (Sigma-Aldrich) in a 30 ml buffer containing 60 mM Tris, pH 6.8, 0.2% hydrogen peroxide, and 200 ml 0.8% $NiCI_2$. Following staining, the membranes were washed in distilled water and dried at 37˚C in an oven. The bands on the developed film were quantified with Quantity One software, version 4.6.2 (Bio-Rad Laboratories, Inc.). The density of each band was normalized to that of the GADPH protein.

Detection of αB-crystallin and hsp27 mRNA by fluorescence reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in vivo and in vitro

Isolation of total RNA and reverse transcription. Total RNA was extracted from the cultured H9c2 cells using RNAiso Plus reagent (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's instructions. Briefly, after H9c2 myocytes were heat-stressed at 42˚C for various time periods, 1 ml RNAiso Plus reagent was added to 10 mm^2 cell culture plates. For the rat tissue samples, 100 mg heart tissue was homogenized with the 623003 Fluko® Super Fine Homogenizer and 1 ml RNA extraction buffer (TRIzol;

Takara Biotechnology, Co., Ltd.) was added, according to the manufacturer's instructions. RNA concentration was determined using a M200PRO spectrophotometer (Tecan Austria GmbH, Grödig, Austria). RNA samples were synthesized into cDNA using PrimeScript Reverse Transcriptase Master Mix Perfect Real Time (cat. no. DRR036A; Takara Biotechnology Co., Ltd.), according to the manufacturer's instructions, and stored at -80˚C for further analysis.

Design of PCR primers. Primer sets were specifically designed to anneal to each target mRNA. The sequences for Hsp27, αB-crystallin and GAPDH mRNA were obtained from the National Center for Biotechnology Information Genbank (accession nos. NC_005111.4, NC_005107.4 and NC_005103.4, respectively). The primers were designed using the Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA) for conventional and RT-qPCR amplification. The primer sequences for the genes were as follows: Hsp27, forward 5'-CGTGGTGGAGATCACTGGCAAGC-3', and reverse 5'-CGGGCCTCGAAAGTGACCGG-3'; and αB-crystallin, forward 5'-CACGAAGAGCGCCAGGACGA-3', and reverse 5'-CGTCGGCTGGGATCCGGTACT-3'; GAPDH, forward 5'-GGCTCTCTGCTCCTCCCTGTTCTAG-3' and reverse 5'-GGCTCTCTGCTCCTCCCTGTTCTAG-3'. The expected sizes of the Hsp27 and αB-crystallin PCR products were 216 and 153 bp, respectively. Primers were obtained from Invitrogen Life Technologies (Shanghai, China).

RT-qPCR. Using the iQ5 Real-Time PCR Detection system thermocycler (Bio-Rad Laboratories, Inc.), each cDNA sample (2 μl, 10X dilution) was suspended in 2X iQ™ SUPER® Green Supermix (Bio-Rad Laboratories, Inc.) with 0.6 μl of both sense and antisense primers, and double-distilled water to a total volume of 20 μl. The PCR cycling 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 sec, annealing at 60˚C for 30 sec and elongation at 72˚C for 30 sec.

For each run, a negative control tube without cDNA was analyzed alongside the experimental groups. A 4-fold dilution series of the template was used in the PCR amplification reactions. The data were analyzed using Bio-Rad iQ5 software (Bio-Rad Laboratories, Inc.) and the αB-crystallin mRNA levels were normalized using the following formula: Relative quantity of αB-crystallin/hsp27 mRNA = $2^{-\Delta\Delta Ct}$ $\Delta\Delta Ct = [(Ct_{\alpha B\text{-crystallin/hsp27}}$ mRNA - Ct_{GAPDH} mRNA) control group - ($Ct_{\alpha B\text{-crystallin/hsp27}}$ mRNA - Ct_{GAPDH} mRNA) test group].

Analysis of the association between Hsp27 and αB-crystallin. To analyze the association between Hsp27 and αB-crystallin, the STRING 9.1 database (http://string-db. rg/) was used, which aims to provide a global perspective for as many organisms as possible. In the STRING database, known and predicted associations are scored and integrated, resulting in comprehensive protein networks covering >1,100 organisms. This software extends the automated mining of scientific texts for interaction information, and also includes full-text articles (21).

Statistical analysis. Statistical differences between the heat-stressed groups and the control group were analyzed by one-way analysis of variance followed by a least significant difference multiple comparison test, using the SPSS 20.0 for Windows (IBM SPSS, Armonk, NY, USA). The results were expressed as the mean ± standard deviation of at least three independent experiments. All experiments were performed in triplicate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Protein expression levels of Hsp27 and αB-crystallin in vivo in response to heat stress. The protein expression levels of Hsp27 and αB-crystallin were measured *in vivo* in response to various durations of exposure to heat shock, and normalized to GAPDH (Fig. 1). In the heat-stressed rat heart, αB-crystallin decreased significantly ($P<0.01$) after 20 min exposure to heat, but increased by almost 3-fold after 40 min ($P<0.01$), and decreased after 60, 80 and 100 min ($P<0.01$). The protein expression levels of Hsp27 decreased significantly ($P<0.01$) after all durations of exposure to heat stress; however, after 40 min, the expression levels of Hsp27 were higher, as compared with after 20 min.

Protein expression levels of Hsp27 and αB-crystallin in vitro in response to heat stress. The protein expression levels of Hsp27 and αB-crystallin were measured *in vitro* in response to various durations of exposure to heat shock, and normalized to GAPDH (Fig. 2). The expression levels of αB-crystallin decreased significantly ($P<0.01$) after 40 and 60 min of heat stress, as compared with the control group, but increased after 80 and 100 min of exposure to heat stress ($P<0.01$). However, no statistically significant difference in the expression levels of αB-crystallin was observed between the 80 and 100 min groups. The expression levels of Hsp27 decreased after 40 min of heat exposure ($P<0.01$), but after 60, 80 and 100 min the expression levels increased, as compared with the control group.

mRNA expression levels of Hsp27 and αB-crystallin in vivo in response to heat stress. The mRNA expression levels of Hsp27 and αB-crystallin in response to heat shock exposure *in vivo*, normalized to the GAPDH gene, are displayed in Fig 3. The RT-qPCR results demonstrated that compared with the control group, the mRNA expression levels of αB-crystallin and Hsp27 were significantly increased after 20, 40, 60, 80 and 100 min of heat stress in the rat heart ($P<0.01$).

mRNA expression levels of Hsp27 and αB-crystallin in vitro in response to heat stress. The mRNA expression levels of hsp27 and αB-crystallin in response to heat shock exposure *in vitro*, normalized to the GAPDH gene, are displayed in Fig. 4. The mRNA expression levels of αB-crystallin were significantly reduced ($P<0.01$) following 20, 40 and 80 min heat shock exposure, and those of Hsp27 were significantly increased ($P<0.01$) following 20 and 40 min heat shock exposure. However, following 60 min of heat shock exposure, the levels of Hsp27 and αB-crystallin exhibited similar trends.

The association between Hsp27 and αB-crystallin. These preliminary results suggest that the protein and mRNA expression levels of Hsp27 and αB-crystallin exhibit a similar trend in heat-stressed myocardial cells *in vivo* and *in vitro*, respectively. According to the rat database in STRING, αB-crystallin is able to bind Hsp27 in rat cells (Fig. 5A). In

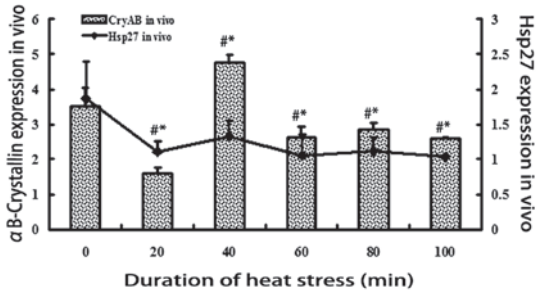

Figure 1. The protein expression levels of heat shock protein 27 (Hsp27) and αB-crystallin (cryAB) in heat-stressed myocardial cells *in vivo*. #P<0.01 (CryAB), and *P<0.01 (Hsp27).

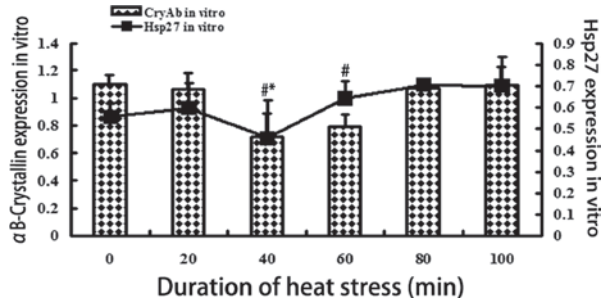

Figure 2. The protein expression levels of heat shock protein 27 (Hsp27) and αB-crystallin (cryAB) in heat-stressed H9c2 myocardial cells *in vitro*. #P<0.01 (CryAB), and *P<0.01 (Hsp27).

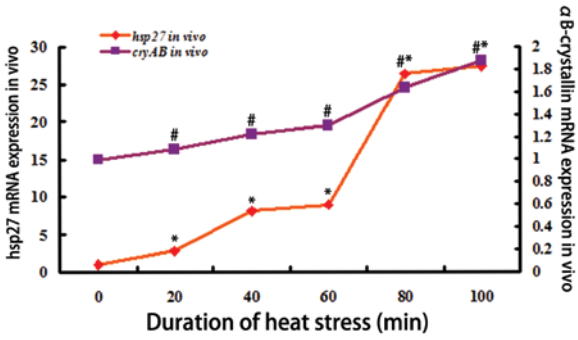

Figure 3. The mRNA expression levels of heat shock protein 27 (Hsp27) and αB-crystallin (cryAB) in heat-stressed myocardial cells *in vivo*. #P<0.01 (CryAB), and *P<0.01 (Hsp27).

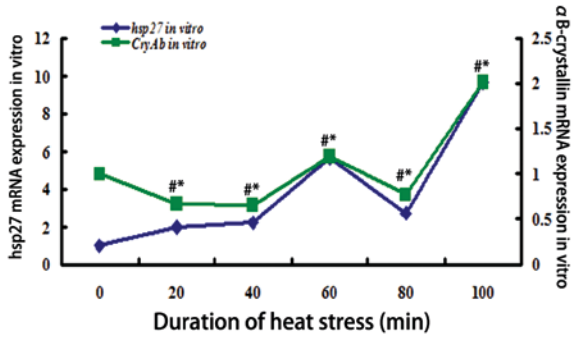

Figure 4.The mRNA expression levels of heat shock protein 27 (Hsp27) and αB-crystallin (cryAB) in heat-stressed H9c2 myocardial cells *in vitro*. #P<0.01 (CryAB), and *P<0.01 (Hsp27).

the evidence view in STRING (Fig. 5B), the different colors represent various sources of evidence from the database and experiments, and confirmed the co-expression of αB-crystallin and Hsp27. Compared with other proteins that are associated with αB-crystallin, such as Hspb2, Hsp27 has the highest co-expression score with αB-crystallin (Fig. 5C). The co-expression view (Fig. 5D) indicated that αB-crystallin and Hsp27 are co-expressed in rats, but not in other species, such as *Homo sapiens*. In the present study, both the protein and mRNA expression levels of Hsp27 and αB-crystallin exhibited similar trends in response to heat shock *in vivo* and *in vitro*, respectively, thus indicating that they may be co-expressed.

Discussion

Heat stress causes extensive cytoskeletal and mitochondrial damage, as well as uncoupling of oxidative phosphorylation. Hsps are thought to limit injury and accelerate recovery by refolding disrupted proteins and preventing deleterious peptide interactions (17,22). sHsps are a widespread and diverse class of proteins. Of the major Hsps, Hsp27 and αB-crystallin have recently been identified as molecular chaperones (9). Stressors that transiently induce Hsp27 and αB-crystallin include heat shock, anticancer drugs, radiation, and oxidative stress. As compared with other Hsps, Hsp27 and αB-crystallin bind to numerous non-native proteins via an oligomeric complex, and thus represent the most efficient chaperones in terms of quantity of substrate binding (14,23). In the present study, the mRNA expression levels of αB-crystallin were significantly reduced (P<0.01) following 20, 40 and 80 min heat shock exposure, and those of Hsp27 were significantly increased (P<0.01) following 20 and 40 min heat shock exposure. However, following 60 min of heat shock exposure, the levels of Hsp27 and αB-crystallin exhibited similar trends. The protein expression levels of Hsp27 decreased after 40 min, as compared with the other groups, whereas αB-crystallin decreased only after 40 and 60 min of heat exposure *in vitro*. After 60 min, both sHsps exhibited an increase in expression levels. The protein expression trends of both sHsps were not concordant with their mRNA expression levels from the initiation of heat stress *in vitro*, however at 100 min sharply increased to mRNA levels consistant with the levels of protein expression. However, in heat-stressed rat heart *in vivo*, both the mRNA expression levels of Hsp27 and αB-crystallin significantly increased after all durations of heat exposure. A previous study suggested that the expression levels of Hsp27 and αB-crystallin may be regulated at the transcriptional level (24). However, in rat hearts *in vivo*, the protein expression levels of Hsp27 and αB-crystallin decreased, except for αB-crystallin after 40 min of heat exposure. Both sHsps exhibited decreased expression level trends after 100 min of heat stress. A previous study demonstrated that Hsp27 and αB-crystallin mRNA is overexpressed in the heart and other smooth muscles following heat stress, but the expression of their corresponding proteins does not follow the same trend (25). The present study investigated the mRNA and protein expression levels of Hsp27 and αB-crystallin in response to heat stress. Hsp27 and αB-crystallin may modulate interactions between cellular factors by forming

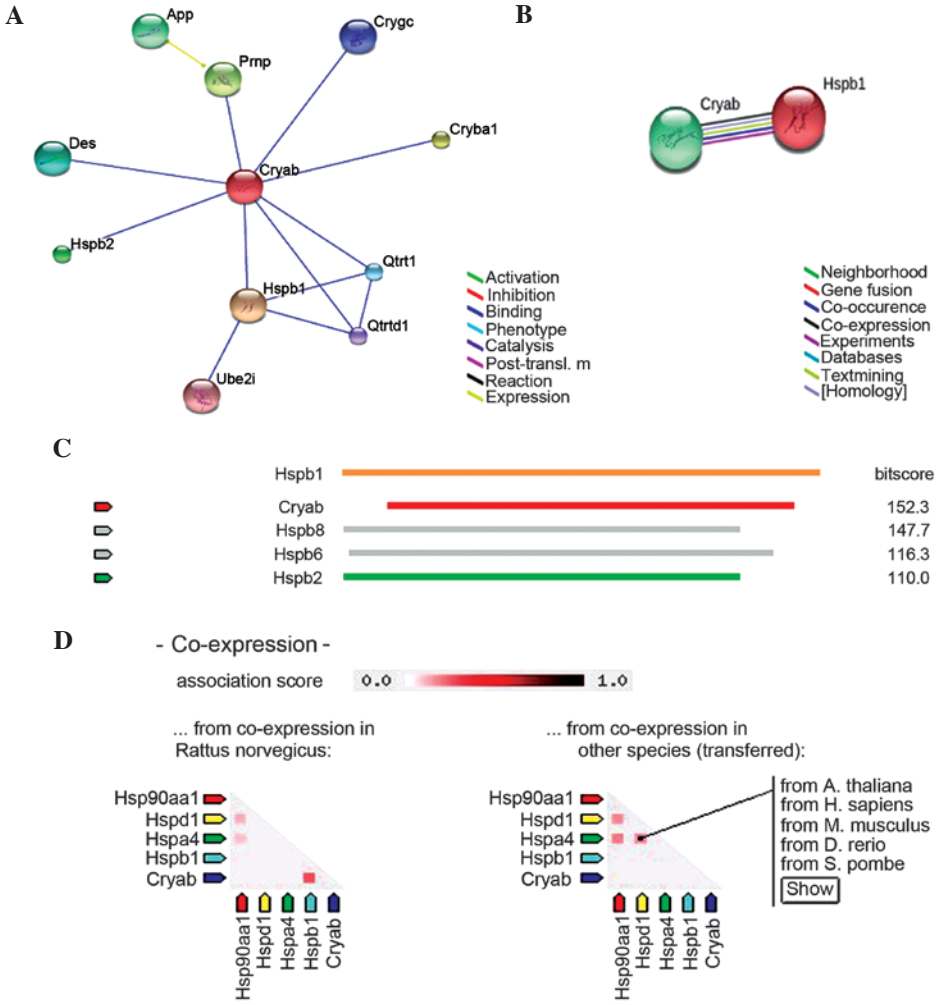

Figure 5. The association between heat shock protein (Hsp)27 and αB-crystallin in rat myocardial cells, as determined by STRING 9.1: CryAB, αB-crystallin; HspB1, Hsp27. (A) The various proteins that are associated with αB-crystallin (Hsp27 binds to αB-crystallin, blue line). (B) The association between Hsp27 and αB-crystallin has been confirmed by previous studies: Various colors indicate various sources of evidence. (C) The co-expression score between Hsp27 and αB-crystallin was calculated, which is higher than that of all the other proteins. (D) Hsp27 and αB-crystallin are not co-expressed in all species (including *Homo sapiens*). App, amyloid beta (A4) precursor protein; Pmp, peripheral myelin protein; Cry, crystallin; Des, desmin.

small or large oligomers, undergoing phosphorylation, and inducing cell-cell contact *in vivo* (7,22,24). A previous study confirmed that Hsp27 and αB-crystallin exhibited a phosphorylation response in the atrial myocardium of patients undergoing valve repair (22). However, the specific mechanism underlying this phosphorylation response remains to be investigated.

In response to stressful conditions, αB-crystallin is able to bind to Hsp27, relocalize to the cytoskeleton, and binds to microtubules via microtubule-associated proteins, which may protect the cells from damaged intracellular proteins by sequestering these proteins on the cytoskeleton (15,26). The present study demonstrated that the expression levels of Hsp27 and αB-crystallin markedly differ in the rat heart *in vivo*, as compared with the H9c2 cells *in vitro*. In the whole rat body, there may be numerous factors that affect the heat-stressed heart. The protein expression levels of Hsps in cardiomyocytes may be regulated by hormones. Thyroid hormones may be involved in the regulation of Hsp27 expression in the rat myocardium (27). However, in the H9c2 cell line, after 60 min exposure to heat stress, the protein expression levels of both sHsps increased to reach a baseline level, which was different from their expression trends *in vivo*. The comparative analysis of the present study suggests that the mechanism underlying this protection is not the same between the individual cell and the whole body. During the experiments, the mortality rate of heat-stressed rats was 100% after 120 min. The low protein expression levels of both Hsps after 80 min indicated that the inherent expression levels of Hsp27 and αB-crystallin may not be enough to resist acute heat stress (28). A previous study demonstrated that overexpression of Hsp27 offers protection via an anti-apoptotic mechanism (15). However, these observations still require confirmation, and pre-induction of sHsps prior to heat stress may be an effective way to reduce the mortality rate.

In the present study, Hsp27 and αB-crystallin were activated by acute heat stress at both the mRNA and protein level *in vivo* and *in vitro*. These results were concordant with those of previous studies that demonstrated that Hsp27 and αB-crystallin have similar roles when subjected to various stressors (29,30). Hsp27 and αB-crystallin are characterized

by a conserved C-terminal region, termed the αB-crystallin domain, a variable N-terminal sequence, and in most cases a short and variable C-terminal tail (16). The results of the present study suggested that the protein and mRNA expression levels of both Hsp27 and αB-crystallin exhibit similar trends in the H9c2 rat cell line. Furthermore, as confirmed by STRING, these results support the evidence that αB-crystallin is able to bind to Hsp27 in rat cells (Fig. 5B). The co-expression score between αB-crystallin and Hsp27 was the highest in rats, but did not occur in all the species analyzed (including *Homo sapiens*). A previous study demonstrated that Hsp27 and αB-crystallin proteins often accumulate as inclusion bodies in numerous protein conformation diseases, and have a similar role to molecular chaperones (19). Investigating the specific mechanism underlying the co-expression of Hsp27 and αB-crystallin will provide the basis for our future experiments.

In conclusion, Hsp27 and αB-crystallin are activated by acute heat stress both at the mRNA and protein level *in vivo* and *in vitro*. However, the expression levels of Hsp27 and αB-crystallin differed between the whole body system and the cell line when subjected to heat stress. When co-expressed, Hsp27 and αB-crystallin may have roles in rat myocardial cells *in vivo* and *in vitro*.

Acknowledgements

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