Heat-Shock Protein Induction in Rat Hearts
A Direct Correlation Between the Amount of Heat-Shock Protein Induced and the Degree of Myocardial Protection

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Background  Previous studies have demonstrated that heat-shock treatment results in the induction of 72-kD heat-shock protein (HSP72) and a reduction of infarct size after subsequent ischemia and reperfusion.

Methods and Results  To test the hypothesis that the degree of protection from ischemic injury in heat-shocked rats correlates with the degree of prior HSP72 induction, rats pretreated with 40°C, 41°C, or 42°C of whole-body hyperthermia followed by 24 hours of recovery and control rats (n=6 in each group) were quantitatively assessed for the presence of myocardial HSP72 by optical densitometry of Western blots and a primary antibody that is specific for HSP72 and a tertiary antibody labeled with 125I. Although rats heat-shocked to 40°C had no significant induction of myocardial HSP72, rats heat-shocked to 41°C and 42°C demonstrated progressively increased amounts of myocardial HSP72 compared with controls. Separate groups of rats heat-shocked to 40°C (n=16), 41°C (n=37), and 42°C (n=36) with 24 hours of recovery and controls (n=26) were subjected to 35 minutes of left coronary artery occlusion and 120 minutes of reperfusion. Compared with control and 40°C rats, there was progressive infarct size reduction, assessed by triphenyltetrazolium chloride staining, in rats that were heat-shocked to 41°C and 42°C. Furthermore, there was a direct correlation between the amount of HSP72 induced and the reduction in infarct size (r= 0.97, P=0.037).

Conclusions  These results suggest that the improved salvage after heat-shock pretreatment may be related to the amount of HSP72 induced before prolonged ischemia and reperfusion. (Circulation. 1994;89:355-360.)

Key Words  • proteins • ischemia • reperfusion

Heat-shock proteins (HSPs) are a group of proteins produced by most organisms in response to hyperthermia or other environmental insults. Previous investigators have found that the induction of HSPs coincides with a certain amount of protection to the cell. For example, exposure of cells to sublethal hyperthermia results in the induction of HSPs and the acquisition of thermotolerance.1-6

A number of studies have shown that both cardiac and noncardiac cells produce HSPs in response to hyperthermia and various other stimuli, including ischemia.7-12 Furthermore, the induction of HSPs from one insult, such as toxin exposure, may confer protection from a different insult, such as a heat-shock challenge.7 This phenomenon of “cross-tolerance” has led to the hypothesis that HSP induction by hyperthermia may confer protection from ischemia. This potential protective effect of HSPs was shown in the isolated perfused rat heart preparation, in which hyperthermic pretreatment led to an improved functional recovery after global ischemia and reperfusion.11,12

Recently, Donnelly et al13 demonstrated that heat-shock pretreatment resulted in marked myocardial HSP72 induction and was associated with significant infarct size reduction after prolonged coronary artery occlusion and reperfusion in the in vivo rat model. In contrast, a low level of HSP72 induction from ischemic pretreatment resulted in no infarct size reduction after prolonged ischemia and reflow. It was hypothesized that HSP72 induction may have been responsible for reducing infarct size and that the absolute level of HSP72 induction might be related to the presence or absence of protection. The purpose of the present study was to quantify the absolute levels of HSP72 induction by progressive degrees of whole-body hyperthermia and to correlate the degree of myocardial HSP72 induction with the degree of protection from subsequent ischemic injury.

Methods  To determine the correlation between the amount of HSP induced and the amount of myocardial protection, two separate experimental groups were used. The hearts from one group were harvested for protein isolation and quantification of HSP72, and those from the other group were used for infarct sizing after subjection to 35 minutes of ischemia followed by 120 minutes of reperfusion. Both groups were split into four subsets of rats subjected either to pretreatment with whole-body hyperthermia of 40°C, 41°C, or 42°C for 20 minutes or to control conditions. All experiments were approved by and conducted within the guidelines of the Committee on Animal Research at the University of California, San Francisco.

Whole-Body Hyperthermia  Female Sprague-Dawley rats (weight, 225 to 250 g) were anesthetized with sodium pentobarbital (50 mg/kg body wt IP). A catheter was placed in the tail vein of each animal for
saline administration. A rectal thermometer was inserted to monitor core body temperature while the rats were subjected to whole-body hyperthermia of 40°C, 41°C, or 42°C attained with a heating lamp and a warming blanket. The body temperature of the rats was increased slowly until the desired temperature was achieved, and then this temperature was maintained for 20 minutes. The rats were then placed on a cool surface and allowed to recover for 24 hours at room temperature. Each heat-shocked rat was given a total of 10 to 12 mL of saline during the heating and the recovery periods. Control rats were anesthetized at the same time as the experimental rats, but they were not subjected to the whole-body hyperthermia.

Protein Isolation and Western Blot Analysis

After the 24-hour recovery period, the rats were anesthetized, and a small myocardial sample of the anterior left ventricle (LV) of each rat heart was harvested. This sample was minced with a razor blade and immediately placed in a tissue dounce homogenizer (Wheaton, 15 mL) in 1 mL of lysis buffer solution (5% SDS/1% 2-mercaptoethanol). The samples were homogenized and boiled until particles were no longer apparent. After the sample was strained through a 27-gauge needle, the sample solution was quickly frozen in liquid nitrogen.

The samples were then thawed and centrifuged, and any precipitate was discarded. The overall protein concentration was determined for each sample by a modified Lowry procedure.\(^14\) Protein samples were diluted into a 1× Laemmli sample buffer solution. Equal total protein loads of 45 μg were loaded into lanes of 12.5% polyacrylamide gels. Protein samples of rat embryo fibroblasts (REF) heat-shocked to 43°C were used as positive controls. REF cells maintained at 37°C were used as negative controls. Electrophoresis was performed to separate the proteins in the sample on the resolving gel. After the proteins were transferred into nitrocellulose paper, equal total protein loads were confirmed with Ponceau staining as well as Coomassie blue staining of untransferred gels. After the Ponceau stain was washed off, the proteins were probed with either a primary antibody that is specific for HSP72 (C92 antibody) or an antibody that recognizes both the constitutive HSP73 and the inducible HSP72 (N27 antibody).

The nitrocellulose was then blotted with a rabbit anti-mouse second antibody. The blots probed with the N27 primary antibody were developed for visual inspection. Western blots that were probed with the C92 primary antibody were blotted with a third antibody of protein A radiolabeled with \(^32\)P. After unbound antibody was washed off, the radiolabeled nitrocellulose was dried and laid onto film for 24 hours. The film was developed, and densitometry was performed to quantify the levels of HSP72 (Microtek Scanmaker model 600 ZF; PHOTOSHOP software, Adobe Systems, Inc, Mountain View, Calif; Macintosh LC computer, Apple Computer, Cupertino, Calif).

Animal Model of Acute Myocardial Ischemia and Reperfusion

Separate groups of heat-shocked and control rats were subjected to a protocol of ischemia and reperfusion described previously.\(^13,15,16\) Rats were anesthetized (pentobarbital, 50 mg/kg body wt IP), tracheostomized, and placed on a Harvard rodent respirator. With a midline sternotomy, the thoracic cavity was opened and the left coronary artery (LCA) was isolated within a reversible snare occluder. A brief occlusion was performed to visually test the occluder and its positioning and reversibility. After a 20-minute stabilization period, the LCA was occluded for 35 minutes and then released for 120 minutes of reperfusion.

Infarct Sizing

Infarct sizing and ischemic risk area were measured as described previously.\(^15,16\) After the prolonged ischemia and reperfusion, the LCA was reoccluded, and 1 mL of phthalocyanine blue dye was injected into the LV cavity and allowed to perfuse the nonischemic portions of the heart. The entire heart was excised and rinsed of excess dye. After the right ventricle and atria were dissected away, the LV was sliced transversely into sections 2 mm thick. These samples were immersed in triphenyltetrazolium chloride (TTC) for 15 minutes to stain the viable myocardium brick red. The samples were fixed in a 10% formalin solution for 24 hours, after which time the samples were photographed (Olympus OM-2 camera with a 90-mm macro lens and a 2× teleconverter) and weighed (LAE 200 balance).

In each photograph, the ischemic risk area (unstained by blue dye) and the infarcted area (unstained by TTC) were outlined and measured by planimetry. The area from each region was averaged from the photographs of each side for each slice and multiplied by the weight of that tissue section. Infarct size was expressed both as a percentage of total LV mass and as a percentage of the ischemic risk area.

Statistics

All values are expressed as mean±SEM. Comparisons between groups were assessed for significance by one-way ANOVA with post hoc analysis using the Student-Newman-Keuls test. Statistical significance was defined as \(P<.05\).

Results

Stress Protein Levels

Qualitative. In initial experiments, Western blot analysis was performed with the N27 antibody, which recognizes both the constitutive HSP73 and the inducible HSP72. As seen in Fig 1, left, REF 37°C cells, when incubated with N27, exhibited only the constitutive HSP73 (negative control). In contrast, REF cells heat-shocked to 43°C exhibit increased levels of HSP73 along with a faster-migrating band corresponding to the 72-kd HSP (positive control).

LV samples from non–heat-shocked control animals and those heat-shocked to only 40°C and allowed to recover for 24 hours demonstrated significant amounts of HSP73 but no appreciable HSP72. In contrast, rats heat-shocked to 41°C and 42°C demonstrated progressively increased amounts of HSP72 within the LV. In the rat heat-shocked to 42°C, increased levels of HSP73 are apparent as well (Fig 1, left).

To clarify these distinctions, Western blot analysis was performed with C92 antibody, which is specific for the inducible HSP72. As seen in Fig 1, right, REF 37°C cells do not show any HSP72 (negative control), whereas the heat-shocked REF cells (REF 43°C) clearly demonstrate the presence of the HSP72. The proteins from the LV tissue of the nonshocked control rat and the animal heat-shocked to only 40°C do not exhibit significant induction of HSP72. In contrast, LV samples from rats heat-shocked to 41°C and 42°C and allowed to recover for 24 hours demonstrate a stepwise increase in the quantity of HSP72 expressed (Fig 2, right). In the Western blots probed with both the N27 and C92 antibodies, a 42°C half-load sample was included to ensure that the amount of total protein loaded was within the linear range of the assay.
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animals heat-shocked to 42°C (positive control). Left, Proteins were probed with N-27 antibody, which recognizes both the constitutive HSP73 and the inducible HSP72. Left ventricular samples from the control rat and rat heat-shocked to 40°C displayed equivalent amounts of HSP73 (73K) and no appreciable HSP72 (72K). However, left ventricular samples from rats heat-shocked to 41°C and 42°C demonstrate progressive increased amounts of HSP72. In addition, there is an increase in the amount of HSP73 noted in the sample from the 42°C heat-shocked rat. The 42°C half-loaded sample demonstrates significantly less apparent HSP72 and HSP73 present. It should also be noted that, whereas HSP73 is noted in both the REF 37°C and REF 43°C samples, HSP72 is present only in the REF 43°C sample. Right, An identical Western blot that was probed with the C-92 antibody, which recognizes only the inducible HSP72. Although no appreciable HSP72 is detectable on the control and 40°C samples, there was mild induction of HSP72 in the 41°C sample and marked HSP72 induction in the 42°C sample. Similarly, there was no detectable HSP72 in the REF 37°C sample and marked induction of HSP72 in the REF 43°C sample.

Quantitative. To quantify these findings, optical densitometry was performed on films of Western blots that were incubated with a primary monoclonal mouse anti-

body specific for HSP72 (C92 antibody), a rabbit anti-mouse secondary antibody, and an anti-rabbit tertiary antibody labeled with 125I. As seen in Fig 2, the optical densitometry data show that with progressive whole-animal hyperthermia, there was a progressive increase in the expression of myocardial HSP72. Although rats heat-shocked to 40°C had no statistically significant expression of HSP72, rats heat-shocked to 41°C and 42°C demonstrated progressively increased amounts of myocardial HSP72 compared with controls (Fig 2).

Infarct Size

Separate groups of rats subjected to 40°C, 41°C, and 42°C and controls were subjected to 35 minutes of LCA occlusion followed by 120 minutes of reperfusion. As noted in Fig 3 and the Table, rats heat-shocked to 40°C had an infarct size similar to that of controls. However, rats heat-shocked to 41°C and 42°C demonstrated a progressive decrease in infarct size. A separate group of control rats was subjected to 35 minutes of LCA occlusion followed by 6 hours of reperfusion. As demonstrated in Fig 4, there was no difference in myocardial infarct size in control rats that reperfused for 2 hours compared with 6 hours (62.4±3.2%, n=26, versus 62.2±4.1%, n=16; infarct size/ischemic risk area ×100%).

Correlation Between the Amount of HSP72 Induced and Infarct Size Reduction

As demonstrated in Fig 5, there was an inverse relation between the amount of cardiac HSP72 induced by prior heat shock and infarct size after subsequent ischemia and reperfusion. This relation was linear with r=.97 and P=.037.
there was hyperthermia whole-body protection from shocked to 40°C and ischemia and 2 hours of reperfusion. Compared with controls, there was progressive reduction in infarct size in rats heat-shocked to 41°C and 42°C. Infarct size of rats heat-shocked to only 40°C was similar to that of controls. Inf/RA indicates infarct size/ischemic risk area.

Discussion

Earlier studies have shown that stress from many different environmental stimuli leads to the induction of HSPs in almost all organisms. Expression of stress proteins has also been found to be correlated with protection from subsequent adverse environmental stresses. In this study, we investigated the relation between the cardiac expression of HSPs induced by whole-body hyperthermia and myocardial protection from ischemic injury (Fig 2). We found that an increased level of whole-body hyperthermia resulted in greater expression of HSP72. Furthermore, there was an inverse relation between the amount of HSP72 expressed and the degree of protection from prolonged ischemia (Fig 5).

Previous studies have shown a correlation between the levels of stress protein induction and the degree of cell survival after a hyperthermic episode. Subjeck and coworkers\(^{17}\) showed that CHO cells demonstrated marked induction of HSP68, HSP89, and HSP110 during the 12-hour recovery period after a heat-shock challenge. In heat-shocked CHO cells subjected to a second hyperthermic challenge, cell survival correlated with the degree of prior stress protein induction. Similarly, Li and Werb\(^{18}\) demonstrated the induction of thermal tolerance in Chinese hamster fibroblasts (HA-1 cells) that were heat-shocked to 41°C for periods ranging from 1 to 4 hours. The amount of induced thermal tolerance correlated grossly with the degree of HSP70 induction assessed qualitatively by autoradiography of polyacrylamide gels of \(^{35}\)S-labeled proteins. Finally, Landry and coworkers\(^{19}\) demonstrated that both the onset and decay of thermal tolerance in heat-shocked hepatoma 7777 cells paralleled the induction and disappearance of a variety of HSPs. The onset of thermal tolerance was noted at approximately 2.5 hours after the initial heat-shock challenge, at a time when significant \(^{35}\)S-methionine incorporation into HSPs was noted. Approximately 80 hours after the initial heat-shock challenge, the loss of thermal tolerance coincided with the loss of HSPs.

More recently, a number of investigators have noted the potential for prior heat shock and induction of stress proteins to protect the heart from ischemic injury. In isolated perfused rat hearts, Currie et al\(^{11,12}\) and Karmazyn et al\(^{20}\) demonstrated improved functional recovery in heat-shocked isolated perfused rat hearts after low-flow and no-flow ischemia with subsequent reperfusion. Donnelly et al\(^{13}\) showed that heat-shocked rats

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<th>Infarct Size (%) in Heat-Shocked and Control Rats After 35 Minutes of Ischemia and 2 Hours of Reflow</th>
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<tr>
<td>Control, 2 h reflow (n=26)</td>
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<td>Heat shock 40°C (n=16)</td>
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<td>Heat shock 41°C (n=37)</td>
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<td>Heat shock 42°C (n=36)</td>
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LV indicates left ventricular.

*P<.05 vs all other groups.

**P<.05 vs control group.
expressed a high level of myocardial HSP72 coincident with increased myocardial salvage, whereas hearts that expressed lower levels of HSP72 induced by ischemic pretreatment did not exhibit significant myocardial salvage. More recently, this observation by Donnelly was confirmed by Currie et al. and Walker et al., who noted reduced infarct size in rabbits after hyperthermic pretreatment and induction of HSP72. The present study is consistent with those findings but goes on to demonstrate quantitatively the direct correlation between the amount of stress protein induced and the degree of myocardial salvage in the in vivo rat model of ischemia and reperfusion.

Although many have shown a correlation between HSP induction and an increase in cell survival, the exact mechanism of this protection is not well understood. Beckmann et al. showed that HSP72 and HSP73 bind transiently to nascent proteins, acting as intracellular chaperones and helping to stabilize these proteins until they achieve their final conformation. Furthermore, HeLa cells that were stressed with the proline analogue L-azetidine-2-carboxylic acid and immunoprecipitated with anti-HSP72,73 demonstrated precipitated proteins that were bound to HSP72,73 for a prolonged period of time. Potentially, in stress conditions, HSP72,73 may stabilize denatured proteins within the cell, possibly facilitating their removal or repair, leading to protection and/or restoration of cell function during recovery from stress.

Study Limitations

Although these results show a correlation between the amount of HSP expressed and the degree of myocardial protection, a cause-and-effect relation has not been proved. The heat-shock response includes the synthesis of many stress proteins other than HSP72 that may play protective roles. Furthermore, the heat-shock response results in many other cellular changes, including changes in intracellular calcium, pH, ATP, and the level of intracellular catalase, all of which have the potential to affect tissue viability. More experiments will have to be performed to further identify the contributions of these factors and others in the protective nature of the heat-shock response.

Conclusions

The present study indicates that prior heat shock to incrementally higher temperatures results in progressively increased amounts of HSP72 induction and progressively decreased infarct size after subsequent prolonged ischemia and reperfusion. Furthermore, there was a strong correlation between the degree of HSP72 induction and the degree of infarct size reduction in this rat model of ischemia and reperfusion. These observations indicate a potential role for HSP72 induction in protection from ischemic injury.

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References


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