

Heat Shock Protein Induction in Rat Hearts

A Role for Improved Myocardial Salvage After Ischemia and Reperfusion?

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Background. To test the hypothesis that the heat shock response is associated with improved myocardial salvage after myocardial ischemia and reperfusion, rats treated with prior whole-body hyperthermia and 24 hours of recovery ($n=26$) or 20 minutes of ischemic pretreatment and 8 hours of recovery ($n=24$) and control rats ($n=27$, $n=24$, for hyperthermic and ischemic pretreatment, respectively) were subjected to 35 minutes of left coronary artery (LCA) occlusion and 120 minutes of reperfusion.

Methods and Results. Although ventricular samples from rats subjected to either hyperthermia ($n=7$) or ischemic pretreatment ($n=6$) all showed induction of HSP72 (heat shock protein), Western blot analysis revealed significantly greater amounts of HSP72 in samples obtained from rats subjected to hyperthermia compared with those from rats subjected to ischemic pretreatment. Control rats ($n=7$) showed no significant presence of myocardial HSP72. After 35 minutes of LCA occlusion and 2 hours of reperfusion, infarct size was significantly reduced in heat-shocked rats compared with controls ($8.4 \pm 1.7\%$, $n=26$ versus $15.5 \pm 1.9\%$, $n=27$; $p=0.007$; mean \pm SEM; infarct mass/left ventricular mass $\times 100$). There were no significant differences in left ventricular (LV) systolic pressure, heart rate, LV dp/dt, or rate-pressure product between heat-shocked ($n=11$) and control ($n=14$) rats during the ischemic period. There were no differences in infarct size between ischemically pretreated and control rats subjected to 35 minutes of ischemia and reperfusion ($9.7 \pm 2.1\%$, $n=23$ versus 10.0 ± 2.1 , $n=24$; $p=NS$).

Conclusions. In this model of ischemia and reperfusion, prior heat shock was associated with significantly improved myocardial salvage after 35 minutes of LCA occlusion and reperfusion. This improved salvage was correlated with marked HSP72 induction and was independent of the hemodynamic determinants of myocardial oxygen supply and myocardial oxygen demand during the ischemic period. In contrast, mild HSP72 induction by ischemic pretreatment was not associated with improved myocardial salvage after myocardial ischemia and reperfusion. Thus, the absolute levels of HSP72 may be important in conferring protection from ischemic injury in this animal model. (*Circulation* 1992;85:769-778)

Most organisms react to hyperthermia and other environmental stresses by increased synthesis of a group of proteins called stress or heat shock proteins (HSPs).¹⁻⁶ A number of studies indicate that prior exposure to a sublethal stress with induction of HSP confers protection to the cell.¹⁻⁸ For example, prior exposure of cells to mildly elevated temperatures and induction of increased HSP synthesis results in greater survival after

reexposure to what would otherwise be a lethal heat shock challenge, a phenomenon known as acquired thermotolerance.⁶ That HSPs themselves are associated with the thermotolerant state is indicated by the fact that a loss of tolerance correlates with the half-life of the HSPs.⁷⁻⁹ The association between thermotolerance and HSPs is further supported by the fact that the microinjection of rat fibroblasts with specific monoclonal antibodies to HSP72/73 renders

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TABLE 1. Experimental Protocols

Pretreatment	Ischemic protocol	Groups
Heat shock	35-Minute ischemia, 2-hour reflow	Experimental (group 1, <i>n</i> =26) Control (group 2, <i>n</i> =27)
Ischemia	35-Minute ischemia, 2-hour reflow	Experimental (group 3, <i>n</i> =24) Control (group 4, <i>n</i> =24)

the cells incapable of surviving even a brief heat shock challenge.¹⁰

In addition to hyperthermia, exposure to other environmental stresses, including oxygen deprivation^{1,11–15} and toxic substances,¹⁶ induces HSP synthesis, suggesting that the response may represent a generalized cellular defense mechanism. For example, Li¹⁶ has demonstrated that exposure of Chinese hamster fibroblasts to sodium arsenite and ethanol results in the increased synthesis of HSP and renders the cells tolerant to a subsequent heat shock challenge. This phenomenon of cross-tolerance is further evidence of a generalized protective role for the stress proteins.

There is mounting evidence that cardiac cells synthesize HSPs in response to a variety of stresses and that this stress response may result in protection from subsequent ischemic exposure. For example, several investigators have demonstrated increased cardiac HSP synthesis in response to hyperthermia,^{14,17–19} hemodynamic overload,²⁰ cardiac transplantation,²¹ hypoxia, and ischemia.^{14,15} The potential for the heat shock response to confer protection from ischemia was demonstrated in an isolated perfused rat heart preparation in which hearts from rats subjected to prior hyperthermia exhibited improved functional recovery after ischemia and reperfusion.^{17–19} The purpose of the present study was to investigate the induction of HSP72 by both whole-body hyperthermia and pretreatment with a sublethal ischemic episode and to see if either stress response (heat shock or sublethal ischemia) was associated with infarct size reduction after a prolonged episode of coronary artery occlusion and reperfusion.

Methods

Animal Model of Acute Myocardial Ischemia and Reperfusion

A rat model of reversible myocardial ischemia and reperfusion was used as previously described.^{22–24} After induction of anesthesia (pentobarbital 50 mg/kg i.p.), a tracheostomy was performed, and the animal was ventilated on a Harvard rodent respirator. A reversible snare occluder was placed around the proximal left coronary artery (LCA) via a midline sternotomy. It should be noted that the LCA in the rat does not supply the interventricular septum.^{22,23} After visually testing the coronary artery occluder by a brief period of occlusion and reperfusion, the thoracotomy was closed. The animals were allowed to stabilize for a 20-minute stabilization period and were then sub-

jected to one of the experimental protocols (Table 1). All experiments were approved by and conducted within the guidelines of the animal research committee at the University of California, San Francisco.

Whole-Body Hyperthermia

Before surgical preparation, female Sprague-Dawley rats (weight, 225–250 g) were anesthetized (pentobarbital 50 mg/kg i.p.) and an intravenous catheter was inserted into the tail vein for fluid administration. After a rectal thermometer was inserted, animals were placed on a warming blanket and under heating lamps and gradually heated to 42°C for 20 minutes. They were then removed from the heating area to a cool surface and allowed to recover for 24 hours. The animals received normal saline (10–12 ml i.v.) during the heating and recovery periods. Control animals were anesthetized and left at room temperature. The great majority of animals awoke, ate, drank, and behaved normally. Any animal that appeared morbid after the recovery period was quickly euthanized.

To confirm that hyperthermia induces a significant increase in HSP72, right ventricular and left ventricular (LV) samples were taken from selected animals after the hyperthermia/24-hour recovery protocol (*n*=6) and analyzed for HSP72 as described below.

Ischemic Pretreatment

Animals subjected to ischemic pretreatment were surgically prepared with the reversible snare occluder as above. They were then subjected to 20 minutes of LCA occlusion followed by 8 hours of reperfusion. The 8-hour recovery period was chosen after preliminary experiments showed the reliable induction of HSP72 after this period of recovery.

To determine if this relatively short, nonlethal ischemic period induces significant amounts of HSP72 synthesis, myocardial samples were then taken from the ischemic left ventricle, nonischemic left ventricle, and right ventricle in selected animals (*n*=6) after the 8-hour recovery period and analyzed for HSP72 as described below. To confirm that 20 minutes of ischemia results in no significant irreversible damage, animals subjected to 20 minutes of ischemia followed by 2 hours (*n*=9) and 8 hours (*n*=6) of reflow were analyzed for infarct size by triphenyltetrazolium chloride (TTC) staining as described below.

Pretreatment:**Heat Shock**

Heat Shock (20 min)	Recovery (24 hrs)	Ischemia (35 min)	Reflow (2 hrs)
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Ischemia

Ischemia (20 min)	Recovery (8 hrs)	Ischemia (35 min)	Reflow (2 hrs)
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FIGURE 1. Summary of the heat shock and ischemic pretreatment protocols. Heat-shocked rats (group 1) were subjected to whole-body hyperthermia until the core body temperature was elevated to 42°C for 20 minutes. The rats were then allowed to recover for 24 hours. Ischemically pretreated rats (group 3) were subjected to left coronary artery (LCA) occlusion for 20 minutes followed by 8 hours of reperfusion. Both experimental groups and corresponding controls were then subjected to 35 minutes of LCA occlusion followed by 2 hours of reperfusion.

Experimental Protocols

Heat-shocked, ischemically pretreated, and control animals were divided into four experimental groups (Table 1). Groups 1 ($n=26$) and 2 ($n=27$) were hyperthermic and control animals subjected to a 35-minute LCA occlusion followed by 2 hours of reflow. Groups 3 ($n=24$) and 4 ($n=24$) were ischemically pretreated and control rats subjected to a 35-minute LCA occlusion followed by 2 hours of reflow. These experimental protocols are summarized in Figure 1. In preliminary studies, heat-shocked ($n=34$), ischemically pretreated ($n=12$), and respective control animals ($n=33$, $n=9$) were subjected to 45 minutes of LCA occlusion followed by 2 hours of reflow.

Protein Isolation and Western Blotting

After isolation, myocardial samples for protein analysis were placed in a lysis buffer of 5% sodium dodecyl sulfate and 1% 2-mercaptoethanol. The samples were dounced in a tissue homogenizer (VWR, South San Francisco, Calif.) and boiled alternately until particles were no longer visible. The samples were then passed through a 27-gauge needle, boiled again, and frozen in liquid nitrogen. At a later time, the samples were thawed, centrifuged, and placed in Laemmli sample buffer.

Protein concentrations were determined by using a modified Lowry technique.²⁵ Equal amounts of protein were loaded onto 12.5% polyacrylamide gels and electrophoresis was performed as previously described.²⁶ Verification of equivalent total protein loads was confirmed visually by Ponceau staining (Sigma P-7170) of the gels before antibody reactions. The Ponceau stain was then washed off the gels. Proteins were then transferred to nitrocellulose and probed by incubation with antibodies specific for HSP72 and HSP73 (described below). Protein samples from rat embryo fibroblasts (REF) kept at 37°C (REF 37) were used as negative controls. Protein samples from rat embryo fibroblasts heat shocked to 43°C (REF 43) were used as positive controls.

Primary antibodies used in these studies (C92, N27) are mouse monoclonal IgGs produced against

purified human (HeLa cell) HSP70 related proteins. C92 appears to recognize the stress-inducible form of this protein (HSP72), whereas N27 has a wider specificity, apparently recognizing both inducible and constitutively expressed HSP70-related proteins (HSP72 and HSP73).⁵ Alkaline phosphatase-conjugated goat anti-mouse IgGs (BioRad 170-6520) were used as secondary antibodies.

Infarct Sizing

Infarct size and ischemic risk area were determined by methods described previously.^{24,27} At the end of the experimental protocols, the thoracotomy was reopened, the LCA was reoccluded, and phthalocyanine blue dye was injected into the left ventricular cavity, causing the dye to distribute into the nonischemic regions. The heart was excised and rinsed of excess dye and sliced transversely from apex to base in 2-mm thick sections. The left ventricle was dissected away from the right ventricle and atria and incubated in a 1% solution of TTC until viable myocardium stained brick red.²⁷ (Infarcted myocardium fails to stain with TTC.) Tissue samples were then fixed in a 10% formalin solution and weighed (Mettler LAE 200 balance, Mettler Instrument Corp., Hightstown, N.J.). Color photographs of both sides of transverse slices were obtained using an Olympus OM-4T camera with a 90-mm macro lens and a 2× teleconverter. The regions possessing stained (viable) and unstained (infarcted) tissue were outlined on each color photograph and measured by planimetry. On each side, the fraction of LV area representing infarcted tissue (average of two photographs) was multiplied by the weight of that section to determine the absolute weight of infarcted tissue. The infarct size for each heart was expressed as

$$\text{Infarct size (\%)} = \frac{\sum \text{infarct weight in each slice}}{\text{total LV weight}} \times 100$$

The risk area, unstained by blue dye, was determined by planimetry and expressed as

TABLE 2. Infarct Sizes of Heat-Shocked, Ischemically Pretreated, and Control Rats After 35 Minutes of Ischemia and 120 Minutes of Reperfusion

Group	Infarct size/LV mass	Risk area/LV mass	Infarct size/risk area
1 (n=26)	8.37±1.67 (p=0.007 vs. control group 2)*	50.4±1.6 (p=NS vs. control group 2)*	15.9±3.0 (p=0.001 vs. control group 2)*
2 (n=27)	15.5±1.9	48.4±1.4	33.4±4.2
3 (n=24)	14.0±2.1 (p=NS vs. control group 4)*	46.3±1.9 (p=NS vs. control group 4)*	28.5±3.7 (p=NS vs. control group 4)*
4 (n=24)	11.6±2.0	48.1±2.5†	24.8±5.0†

Group 1, heat-shocked rats; groups 2 and 4, control rats; group 3, ischemically pretreated rats. LV, left ventricular. Infarct size measured by absence of triphenyltetrazolium chloride staining. Risk area measured by absence of phthalocyanine blue staining.

All values expressed as mean±SEM.

*Denotes probability value by unpaired *t* test.

†n=22 for risk area measurement.

$$\text{Risk area (\%)} = \frac{\sum \text{risk area weight in each slice}}{\text{total LV weight}} \times 100$$

The infarct size as a percentage of risk area was then calculated as

$$\text{Infarct size/risk area (\%)} = \frac{\sum \text{infarct weight in each slice}}{\sum \text{risk area weight of each slice}} \times 100$$

Infarct size as a percentage of LV mass was determined in group 1 (n=26), group 2 (n=27), group 3 (n=24), and group 4 (n=24). Risk area and infarct size as a percentage of risk area was determined in group 1 (n=26), group 2 (n=27), group 3 (n=24), and group 4 (n=22) animals. It should be noted that phthalocyanine blue dye and TTC staining was not performed on hearts used for Western blot analysis of stress proteins.

Hemodynamic Measurements

To determine whether the heat shock response might influence the hemodynamic determinants of myocardial oxygen supply or oxygen demand, hemodynamic measurements (Table 2) were obtained in a subset of animals from groups 1 (n=14) and 2 (n=11). A micromanometer (Millar) catheter was introduced into the left atrium via a small incision in the left atrial appendage and advanced to the left ventricle. After the thoracotomy was repaired and the animal was allowed to stabilize for 20 minutes, baseline measurements of left ventricular systolic pressure (LVSP), heart rate (HR), and maximum rate of left ventricular pressure development (LV dP/dt) were recorded on a Gould RS-3200 physiological recorder (Gould Inc., Cleveland, Ohio). The rate pressure product (RPP) was calculated as the HR×LVSP. At the conclusion of 120 minutes of reperfusion, the risk area and infarct size were measured as described in "Infarct Sizing." It should be noted that in three animals in group 1 and one animal in group 2 that underwent hemodynamic monitoring, infarct size data were not available because of inadequate TTC staining. These animals were included in the hemodynamic data section, however.

Statistics

All values are expressed as mean±SEM. Comparisons between the hemodynamic time points were assessed for significance with a one-factor analysis of variance (ANOVA) with repeated measures using the Scheffe's test. At specific time points, differences between heat-shocked and control animals were assessed by the unpaired *t* test. Statistical significance was defined as *p*≤0.05.

Results

Stress Protein Levels

Initial experiments analyzed the relative induction of HSP72 in rat cardiac tissue after whole-body hyperthermia or ischemia. In our first experiment, we analyzed the relative levels of both the constitutive HSP73 and the highly stress-inducible HSP72 by Western blot analysis using the N27 antibody. Rat embryo fibroblasts grown in tissue culture and incubated at either 37° or 43°C for 2 hours were used as controls. As shown in Figure 2A, rat fibroblasts incubated at 37°C exhibited modest levels of the constitutive HSP73. After heat shock, the levels of HSP73 increased along with a second, faster migrating band, which corresponded to the inducible HSP72. Analysis of LV tissue from control animals revealed significant levels of HSP73, as shown in Figure 2A. After whole-body hyperthermia (42°C for 20 minutes followed by 24 hours of recovery), the left ventricle exhibited induction of HSP72 (Figure 2A). In the case of a 20-minute ischemic episode and 8 hours of reperfusion, there occurred a slight induction of HSP72 (Figure 2A). Similar to the control nonheat-shocked animals, both the nonischemic portion of the left ventricle and right ventricle obtained from animals subjected to 20 minutes of ischemia followed by reperfusion showed no induction of HSP72 (Figure 2A).

To more clearly delineate these differences, the same analysis was performed but this time using the C92 antibody, which appears specific for only the stress-induced HSP72. As is shown in Figure 2B, in rat embryo fibroblasts incubated at 37°C, no HSP72

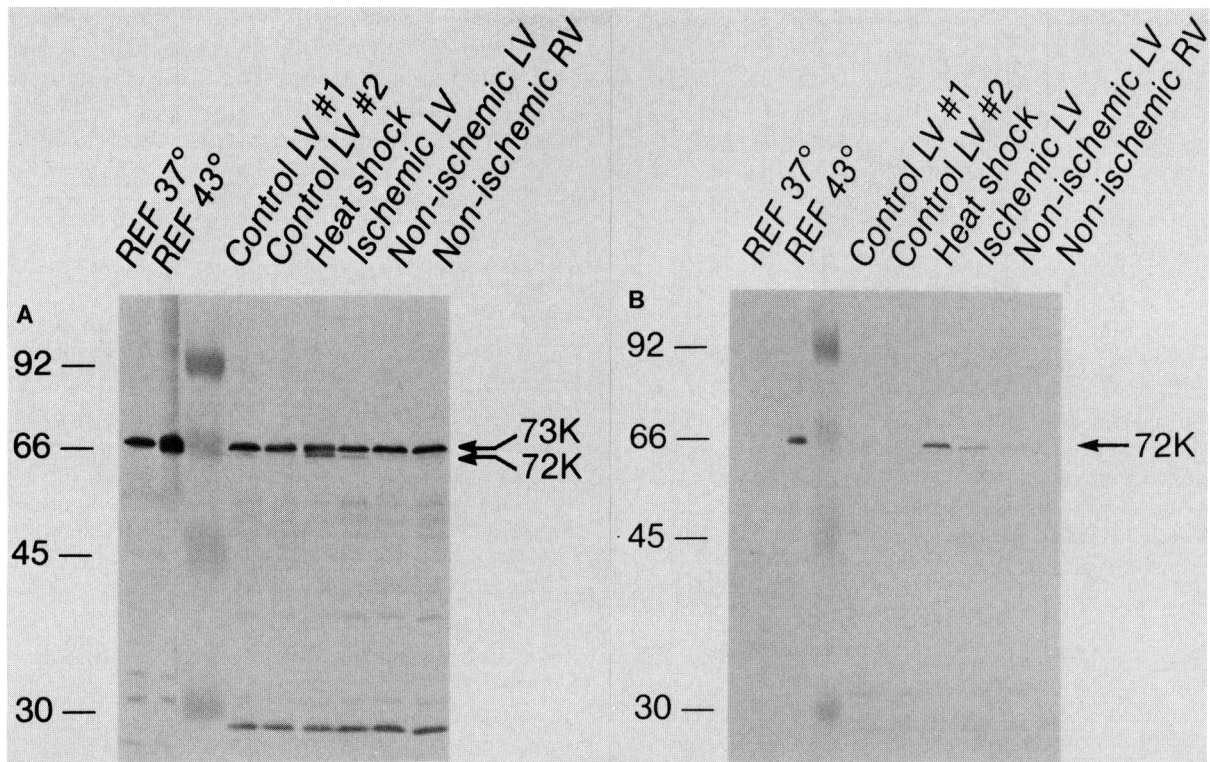


FIGURE 2. Immunoblot analysis of protein obtained from the left ventricle (LV) of two control rats (control LV 1 and LV 2), a rat treated with whole-body hyperthermia (42°C for 20 minutes) and 24 hours of recovery (heat shock), and a rat subjected to 20 minutes of left coronary artery (LCA) occlusion and 8 hours of reperfusion (ischemic LV). In addition, a sample is included from the septum (non-ischemic LV) and right ventricle (non-ischemic RV), two areas of the heart that were not subjected to ischemia during the 20-minute period of LCA occlusion. REF 37° indicates protein from rat embryo fibroblasts maintained at 37° (negative control). REF 43° indicates protein from rat embryo fibroblasts heat-shocked to 43°C (positive control). Prestained markers weighing approximately 30, 45, 66, and 92 kDa were loaded in lane 3 (unlabeled). Equal amounts of total protein were loaded in each lane as determined by the modified Lowry technique and confirmed by Ponceau staining. Panel A: Proteins reacted with N-27 antibody, which recognizes both the inducible HSP72 (HSP, heat shock protein) as well as the constitutive HSP73. Note relatively equivalent amounts of HSP73 present in all samples. In comparison with control hearts (control LV 1 and LV 2), there was a marked induction of HSP72 in rats subjected to prior heat shock and only mild HSP72 induction in rats subjected to ischemic pretreatment (ischemic LV). In ischemically pretreated rats, induction of HSP72 was confined to the ischemic portion of the LV and did not occur in the nonischemic portion of the LV or the right ventricle (non-ischemic RV). Note also that whereas HSP73 was present in both REF 37° and REF 43° samples, HSP72 was only detected in the REF 43° sample. Panel B: Immunoblot similar to above that was reacted with the C92 antibody, which recognizes only the inducible HSP72. Again note that a marked induction of HSP72 was present in the heat-shocked rat and only mild induction of HSP72 was present in the ischemic sample of the rat subjected to 20 minutes of LCA occlusion followed by reperfusion. Also note that whereas HSP72 was prominent in the rat embryo fibroblasts that were heat shocked at 43°C (REF 43°), it was not found in fibroblasts that were kept at 37°C (REF 37°).

was evident. In contrast, after heat shock treatment, rat embryo fibroblasts demonstrated significant induction of HSP72. Similarly, in the animals subjected to whole-body hyperthermia and subsequent recovery at normal temperatures for 24 hours, there occurred a marked induction of HSP72. This was a consistent finding in all LV samples tested ($n=8$). Furthermore, marked induction of HSP72 was noted in right ventricular samples ($n=5$) obtained from heat-shocked rats (data not shown). No significant induction of HSP72 was noted within LV samples of the control rats (see Figure 2B, control LV1 and LV2). This was a consistent finding in all LV samples ($n=5$) and right ventricular samples ($n=3$) tested. Again, in animals subjected to 20 minutes of ischemia

followed by 8 hours of reperfusion, a slight induction of HSP72 was seen (Figure 2B). Little or no HSP72 was observed in the control animals or in the non-ischemic portions of the left and right ventricle in animals subjected to ischemia and reperfusion (Figure 2B). This again was a consistent finding in all animals tested ($n=5$).

Infarct Size

The infarct sizes of heat-shocked (group 1) and control rats (group 2) are summarized in Table 2 and Figure 3. Infarct size was significantly reduced in heat-shocked rats subjected to 35 minutes of LCA occlusion and 120 minutes of reflow (group 1) com-

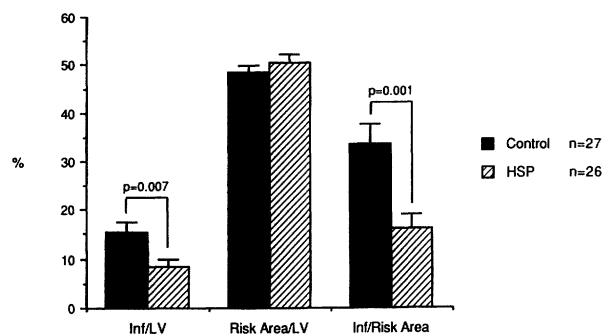


FIGURE 3. Bar graph shows infarct size in heat-shocked and control rats after 35 minutes of ischemia and 2 hours of reperfusion. In comparison with control animals, heat-shocked rats demonstrated a significant reduction in infarct size measured as a percentage of total left ventricular mass and a percentage of the ischemic risk area. Inf, infarct mass; LV, left ventricular mass; risk area, mass of ischemic risk area; HSP, heat-shocked rats.

pared with controls (group 2). There was no difference in the risk area between groups 1 and 2.

The infarct sizes of ischemically pretreated animals (group 3) and controls (group 4) subjected to 35 minutes of LCA occlusion and reperfusion showed no difference in infarct size when expressed as a percentage of LV mass or as a percentage of the risk area (Table 2). In preliminary studies, the infarct size in both heat-shocked ($n=34$) and ischemically pretreated rats ($n=9$) subjected to a longer 45-minute LCA occlusion followed by reperfusion were similar to respective control groups ($16.9 \pm 1.8\%$, $n=34$ versus $19.2 \pm 1.6\%$, $n=33$; $p=NS$; infarct size/LV mass $\times 100$ and $19.8 \pm 3.9\%$, $n=9$ versus $16.5 \pm 3.9\%$, $n=9$; $p=NS$; infarct size/LV mass $\times 100$).

It should be noted that the 20-minute period of ischemic pretreatment that was given to groups 3 and 4 animals was a nonlethal stress for the most part. Animals subjected to 20 minutes of LCA occlusion followed by 2 hours and 8 hours of reflow did not have significant myocardial damage, as determined by TTC staining ($1.4 \pm 0.7\%$, $n=6$; $1.5 \pm 0.8\%$, $n=7$; infarct size/LV mass $\times 100$).

Hemodynamic Data

To determine if heat shock response might alter the determinants of myocardial oxygen supply or demand, hemodynamic data were obtained from heat-shocked and control animals subjected to 35 minutes of ischemia and reperfusion (groups 1 and 2) and are displayed in Figure 4 and Table 3. At baseline, heat-shocked animals had a significantly higher LVSP and RPP compared with control rats (Figure 4 and Table 3). During the 35-minute ischemic period, the LVSP and HR were not significantly different between the two groups. However, there was a trend toward a higher LVSP and RPP in the heat-shocked rats compared with controls. During reperfusion, both the LVSP and RPP were significantly higher in the heat-shocked animals after 15, 30, and 60 minutes of recovery (Figure 4 and Table 3). HR and LV dP/dt were not significantly different between heat-shocked and control animals at baseline or at any time during coronary artery occlusion or reperfusion (Table 3). (Also see Figure 5.)

Relation Between Infarct Size and Hemodynamics

Because there was a trend toward a lower LVSP during the period of coronary occlusion in control versus heat-shocked animals undergoing 35 minutes of ischemia and reperfusion, one might conjecture

TABLE 3. Hemodynamic Changes in Control and Heat-Shocked Rats During 35 Minutes of Ischemia and 120 Minutes of Reperfusion

	Baseline	Occlusion (minutes)				Reperfusion (minutes)			
		5	15	30	35	15	30	60	120
Heart rate (bpm)									
C	361±12	355±14	339±20	341±17	340±17	327±16	329±16	321±14*	310±16*
H	356±15	348±15	343±14	342±12	345±12	339±11	337±10	330±11	314±13*
LVSP (mm Hg)									
C	139±12	82±12*	78±11*	84±11*	86±10*	94±10*	86±8*	100±8*	97±10*
H	187±16†	92±8*	98±7*	111±13*	121±17*	130±13*†	141±17*†	153±19†	129±14*
Heart rate×LVSP (bpm×mm Hg/1,000)									
C	50.6±5.2	30.0±5.6*	27.6±4.9*	29.3±4.6*	29.6±3.8*	30.9±3.7*	31.8±3.2*	32.4±3.3*	30.8±3.7*
H	67.1±6.3†	32.2±3.1*	33.5±2.5*	37.6±3.9*	41.3±5.1*	44.2±4.4*†	47.7±5.5*†	50.7±5.9*†	41.0±4.0*
LV dP/dt (mm Hg/second)									
C	5,567±491	3,853±691*	3,837±564*	3,984±523*	4,172±452*	3,712±423*	3,537±363*	4,038±463*	3,820±434*
H	5,809±669	3,968±448*	4,169±400*	4,662±409*	4,977±480	4,195±437*	4,690±558*	5,168±614	4,580±594*

bpm, Beats per minute; C, control rats ($n=14$); H, heat-shocked rats ($n=11$); LVSP, left ventricular systolic pressure; LV dP/dt, maximum rate of left ventricular pressure development.

* $p<0.05$ vs. baseline value by ANOVA.

† $p<0.05$ vs. control group by unpaired t test.

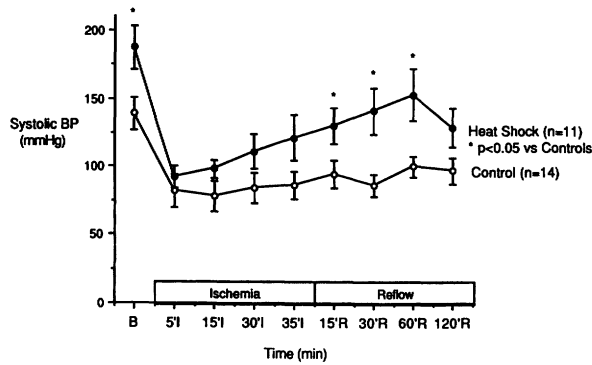


FIGURE 4. Graph shows left ventricular (LV) systolic pressure (BP) in heat-shocked and control rats measured serially at baseline, during 35 minutes of left coronary artery occlusion (ischemia), and during 120 minutes of reflow. Before coronary artery occlusion, heat-shocked rats had significantly higher systolic pressures compared with control rats. During the ischemic period, LV systolic pressures were not significantly different between the two groups, although there was a trend toward higher pressures in heat-shocked animals. LV systolic pressures recovered significantly in heat-shocked animals after 15, 30, and 60 minutes of reperfusion but did not recover in control rats.

that infarcts were larger in the control animals because of a reduced coronary perfusion pressure and decreased collateral blood flow to the ischemic zone in this group. However, there was no relation between infarct size and LVSP after 35 minutes of LCA occlusion in either heat-shocked or control rats (Figure 5A). Similarly, there was no relation between infarct size and the LV RPP, the major hemodynamic determinant of myocardial oxygen demand, after 35 minutes of ischemia in heat-shocked or control rats (Figure 5B).

Discussion

Previous investigators have shown that, in response to a variety of environmental stresses, an increased

synthesis of heat shock or stress proteins occurs in most cells. Coincident with their expression is the acquisition of tolerance to reexposure to a subsequent stress without loss of viability.¹⁻¹⁶ In this study, we investigated the effect of two environmental stresses, hyperthermia and sublethal ischemia, on cardiac tissue in the *in vivo* rat model. We found that both hyperthermia and sublethal ischemia induce myocardial production of HSP72 in this model. Because hyperthermia treatment was systemic, all areas of the heart sampled showed increased HSP72 levels, whereas the ischemically pretreated animals had elevated HSP72 levels in only the stressed region of the heart. The hyperthermia protocol used (i.e., 20 minutes of hyperthermia and 8 hours of recovery) was a much more potent inducer of HSP72 than the ischemic pretreatment used (20 minutes of ischemia and 8 hours of recovery), as noted in Figures 1A and B. Furthermore, rats subjected to the prior whole-body hyperthermia exhibited improved myocardial salvage after 35 minutes of coronary artery occlusion and reperfusion, whereas rats pretreated with a sublethal ischemic stress had no reduction in infarct size despite a mild increase in HSP72 levels. Furthermore, the reduced infarct size noted in heat-shocked animals was accompanied by enhanced recovery of hemodynamic function. Finally, the protective effect of prior heat shock treatment was independent of the hemodynamic determinants of both myocardial oxygen supply and demand during the ischemic period.

Our results are consistent with those of other investigators. Apparent HSP-mediated crosstolerance has been reported previously by Barbe et al²⁸ in rat retina and by Currie et al^{17,18} and Karmazyn et al¹⁹ in the isolated rat heart. Currie and coworkers^{17,18} and Karmazyn and coworkers¹⁹ demonstrated improved functional recovery in *in vitro* rat hearts after low flow and global ischemia followed by reperfusion. However, these did not directly evaluate myocardial salvage.

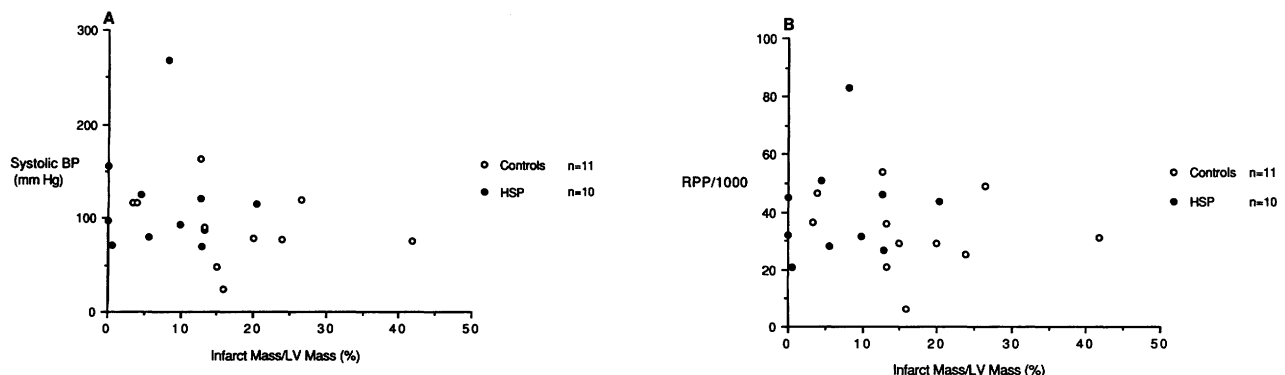


FIGURE 5. Scatterplots show infarct size versus systolic left ventricular pressure and rate pressure product at 35 minutes of coronary occlusion. Panel A: Infarct size versus systolic left ventricular pressure. Note there is no relation between infarct mass measured as a percentage of left ventricular (LV) mass and the systolic left ventricular pressure (BP) during ischemia in either heat-shocked (HSP) or control rats subjected to 35 minutes of coronary artery occlusion and 2 hours of reperfusion. Panel B: Infarct size versus rate pressure product. Note there was no relation between infarct mass measured as a percentage of LV mass and the rate-pressure product (RPP) during ischemia in HSP or control rats subjected to 35 minutes of coronary artery occlusion and 2 hours of reflow.

In the current study, it should be noted that hyperthermia-mediated HSP induction was associated with delaying the onset of irreversible ischemic injury. Although increased myocardial salvage was seen after 35 minutes of ischemia followed by reperfusion in heat-shocked rats, no significant improvement in salvage was observed after 45 minutes of ischemia followed by reflow. Thus, it appears that in this model, HSP induction is associated with a delay in irreversible myocardial injury, but improved salvage is dependent on timely reperfusion.

The improved myocardial salvage after coronary occlusion and reperfusion in heat-shocked animals with a marked cardiac HSP72 induction suggests a potential protective role for this protein. The fact that cardiac protection did not occur in animals pretreated with 20 minutes of ischemia, a milder inducer of HSP72, suggests that a critical amount of protein may be necessary for protection. These observations are analogous with those of other investigators concerning the apparent role of HSPs in protecting cells from hyperthermia. Several studies have shown a correlation between the level of HSP present and cell survival after a hyperthermic episode.^{7-9,29} That HSPs are responsible for thermotolerance is further indicated by the fact that the loss of tolerance correlates with the decline in HSP levels⁷⁻⁹ coupled with the fact that microinjection of antibodies specific for HSP72/73 causes cells to be incapable of surviving even a mild episode of hyperthermia.¹⁰

Despite the apparent role of HSP72 and other stress proteins in protecting cells from adverse environmental stresses, the mechanism of this protective effect is unclear. After an environmental challenge such as hyperthermia, HSP72, the major translational product of the cell, localizes within the nucleus²⁶ and around the nucleolus³⁰ at a time when nucleolar function appears to be compromised.³¹ During subsequent recovery, the nucleoli regain normal morphology and the HSP72 moves to the cytoplasm. Evidence for a protective role of HSP72 follows from studies in which prior elevation of HSP72 levels results in significantly faster repair and recovery of nuclear function after a severe heat shock challenge.^{30,32} These observations and others³³ suggest the possibility that HSP72 and other related HSPs may stabilize or solubilize damaged proteins within the nucleus, nucleolus, and/or the ribosomes after an environmental stress, potentially facilitating the removal or repair of these damaged proteins during recovery.

The results of this study and others indicate that both hyperthermia and ischemia are inducers of HSP72 and that pretreatment with hyperthermia is protective.^{1,32,34} However, it is not clear that HSP72 is the sole protective protein. Hyperthermia is known to induce a variety of proteins, the functions of which are still being characterized. For example, HSP73, a constitutive cytoplasmic protein that is highly homologous with HSP72, migrates to the nucleus and nucleolus after stress. Similarly, the HSP70-related, 75 kDa, and 80

kDa glucose-regulated proteins located in the mitochondria and endoplasmic reticulum, respectively, may have similar protective roles.^{1,34,35} Furthermore, it has recently been shown that another HSP, HSP104, is essential for acquired thermotolerance in yeast.³⁶

The etiology of the higher observed LVSP and RPP after whole-body hyperthermia is unclear. Currie and coworkers¹⁷ observed a trend toward a higher baseline force development in isolated hearts from heat-shocked rats and a significantly higher force development on postischemic recovery. One known consequence of hyperthermia is an increase in cytosolic calcium levels.^{37,38} Potentially increased cytosolic calcium in vascular smooth muscle could increase vascular resistance and raise the systolic blood pressure. Alternatively, increased cytosolic calcium in myocytes could potentially enhance myocardial contractility. There is no evidence, however, that HSP72 per se is directly involved in calcium homeostasis.

This study also demonstrates that a nonlethal ischemic stimulus induces the production of HSP72 in the heart. Consistent with this finding is the study of Mehta et al,¹⁵ which showed an induction of HSP72mRNA in a dog model of prolonged ischemia without reperfusion. However, they did not observe increased production of HSP. This finding of Mehta et al¹⁵ suggests that reperfusion may be required for HSP synthesis to occur and that a substantial recovery period may be necessary for HSP72 levels to accumulate. Sciandra et al¹¹ showed that HSP72 was transiently induced by anaerobic conditions in cell culture and then decreased to baseline levels. It was only with reoxygenation that HSP72 synthesis increased again. Our results demonstrate that an appreciable amount of HSP72 accumulates in myocardium after ischemia and reflow in the *in vivo* rat model of reversible ischemia.

Study Limitations

Although our study demonstrates an association between myocardial protection after hyperthermia and HSP72 production, no cause-and-effect relation can be established. The heat shock response causes changes in intracellular calcium,^{38,39} pH,^{38,39} ATP,⁴⁰ and the level of intracellular catalase,¹⁷⁻¹⁹ all of which can potentially affect tissue viability. Future studies that correlate the degree of protection with the amount of HSP72 production and evaluate the independent contribution of these other cellular changes to cell viability are needed. Also, although we could not demonstrate a protective effect associated with our nonlethal ischemic pretreatment, we cannot rule out the possibility that cellular damage not detected by TTC staining may have occurred after the ischemic pretreatment. This might negate a potential protective effect caused by HSP72 that was induced subsequently.

Significance

This is the initial study showing that prior heat shock treatment improves organ viability after ischemia and reperfusion in an *in vivo* model of cardiac

ischemia and reperfusion. Furthermore, the protective effect of heat shock therapy appears to correlate with the marked degree of HSP72 induction. It is also the initial study that demonstrates myocardial induction of HSP72 after a nonlethal ischemic stimulus in an in vivo model.

Conclusions

The present results indicate that in this animal model of myocardial ischemia and reperfusion, marked hyperthermia-induced HSP72 production is associated with significantly improved myocardial salvage and enhanced functional recovery after a prolonged 35-minute episode of coronary artery occlusion and reperfusion. This improved salvage appears to be independent of the hemodynamic determinants of myocardial oxygen supply and demand during the ischemic period. Our results also indicate that, although HSP72 is produced after a nonlethal ischemic pretreatment with 8 hours of recovery, the amount of HSP72 produced was less than that seen in heat-shocked rats and was not associated with improved myocardial salvage after a subsequent episode of prolonged ischemia and reperfusion. This may be related to the relatively lower amount of myocardial HSP72 induced by ischemic pretreatment compared with hyperthermia.

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