Heat-Shock Response and Limitation of Tissue Necrosis During Occlusion/Reperfusion in Rabbit Hearts

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**Background.** Induction of stress proteins, such as heat-shock protein 71 (HSP71), is associated with cardioprotection in isolated ischemic myocardium. We tested this hypothesis in rabbits pretreated with whole-body hyperthermia and then subjected to 30 or 45 minutes of regional coronary occlusion (CO) followed by 3 hours reperfusion (Rep).

**Methods and Results.** Control rabbits did not undergo whole-body hyperthermia; heat-shocked (HS) rabbits were subjected to whole-body hyperthermia at 42°C for 15 minutes. Rabbits were allowed to recover from whole-body hyperthermia for 24 or 40 hours and were then subjected to CO/Rep. Heart rate and arterial blood pressure were recorded during the experiments. Area of necrosis (tetrazolium staining) was normalized to anatomic risk zone size (microsphere autoradiography). In rabbits treated with whole-body hyperthermia and 24 hours of recovery, infarct size was significantly reduced in HS rabbits compared with control rabbits \((41.2 \pm 7.8\% \text{ versus } 23.2 \pm 6.6\%; p \leq 0.05; \text{ mean } \pm \text{ SD})\) after 30 minutes of CO and 3 hours of Rep. Risk zone size was similar for the two experimental groups. In rabbits treated with whole-body hyperthermia and 40 hours of recovery, infarct size was similar for control and HS animals with either 30 or 45 minutes of CO \((p = \text{NS})\) and 3 hours of Rep. Risk zone size and area of necrosis were similar for these experimental groups. Biopsies from ischemic and nonischemic myocardium were obtained from rabbits at 24 and 40 hours after heat shock and control rabbits to verify expression of HSP71; expression was determined by Western blot analysis.

**Conclusions.** Our findings demonstrate a considerable increase in expression of HSP71 in myocardium from hyperthermia-treated rabbits. Infarct size was significantly reduced after 30 minutes of CO and 3 hours of Rep in hearts at 24 but not 40 hours after heat shock compared with control hearts. We conclude that heat shock–induced cardioprotection is transient and delays the onset of irreversible myocardial injury caused by ischemia. (*Circulation* 1993;87:963–971)

**Key Words:** ischemia • reperfusion • heat shock • proteins, stress • proteins, heat-shock

Metabolic stress, including hyperthermia, promotes the synthesis and accumulation of a class of proteins referred to as heat-shock or stress proteins.\(^1\) In hearts, increased synthesis of these stress proteins occurs in response to hyperthermia\(^2\) and regional myocardial ischemia.\(^3,4\) Heat shock–induced proteins (HSP) play an important role in renaturation of denatured protein, in maintaining newly synthesized proteins in a translocational configuration (linear or unfolded), and in facilitating folding and targeting of newly synthesized proteins to organelles (for a recent review, see Reference 5). Cells with an increased ability to carry out these functions may have an enhanced ability to survive noxious stresses. In fact, there is evidence to suggest that some of the HSPs can afford cell protection.\(^6\)

In an earlier report, Currie et al\(^7\) documented that significant cardioprotection could be obtained in isolated rat hearts subjected to ischemia/reperfusion injury after hyperthermia. In hearts from hyperthermia-treated animals, contractile recovery was enhanced, creatine kinase efflux was reduced, and catalase activity was increased during reperfusion. Inhibition of catalase activity with 3-amino triazole was subsequently shown to abolish the cardioprotection to ischemia/reperfusion injury. Similarly, Knowlton et al\(^8\) suggested that HSP70 might play a role in myocardial stunning and preconditioning, because brief periods of heat stress enhanced posts ischemic ventricular recovery in rabbits. Donnelly et al\(^9\) recently documented myocardial salvage in vivo in rat hearts subjected to 35 minutes of coronary occlusion and reperfusion after whole-body hyperthermia. They suggested that cardioprotection after an ischemic insult was dependent on the degree of induction of HSPs (i.e.,
HSP72). However, Yellon et al. documented that despite a significant increase in HSP72 in rabbit myocardium 24 hours after heat shock, no protective effect of heat stress was observed when infarct size was used as the end point after 45 minutes of regional ischemia and 3 hours of reperfusion.

To test the hypothesis that induction of the heat-shock response before ischemia may have an important cardioprotective effect in vivo, rabbit hearts were challenged with 30 or 45 minutes of regional myocardial ischemia and 3 hours of coronary reperfusion 24 or 40 hours after whole-body hyperthermia. Induction of the heat-shock response in ventricular myocardium was assessed by Northern and Western blot analysis of ventricular tissue. Our results demonstrate significant cardioprotection in rabbit myocardium after 30 minutes of acute regional ischemia and coronary reperfusion 24 hours after induction of HSPs by whole-body hyperthermia. Infarct size was not reduced in heat-shocked rabbits subjected to either 30 or 45 minutes of regional ischemia after 40 hours of recovery from whole-body hyperthermia.

Methods

Male New Zealand White rabbits (2.2–3.0 kg body weight) were used for all studies. Rabbits used in these experiments were cared for in accordance with the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care. They were premedicated with intramuscular acepromazine maleate (Austin Laboratories) and lightly anesthetized with pentobarbital sodium (MTC Pharmaceuticals; 10 mg/kg i.v.). Whole-body hyperthermia was induced by placing the rabbits between two temperature-controlled heating pads set at 50°C until the body temperature reached 42°C. Body temperature was monitored with a rectal thermometer and maintained between 42 and 42.5°C for 15 minutes.2 Rabbits were allowed to recover from whole-body hyperthermia for 24 or 40 hours before reanesthesia for the ischemia/reperfusion studies. Sham control animals included rabbits that were lightly anesthetized and covered with heating blankets for 30–45 minutes but were not heated and were subsequently returned to their cages for either 24 or 40 hours. To confirm that whole-body hyperthermia induces significant expression of messenger RNA (mRNA) coding for stress proteins and accumulation of these stress proteins, rabbits (n = 12) were killed after 1.5, 3, 6, 24, or 48 hours of recovery from whole-body hypothermia treatment. Ventricular biopsies were also obtained from these animals for analysis of high-energy phosphates. Five experimental groups were studied: 1) sham control rabbits (i.e., 24 hours of recovery without whole-body hyperthermia) subjected to 30 minutes of regional ischemia; 2) 24 hours of recovery after whole-body hyperthermia and 30 minutes of regional ischemia; 3) sham control rabbits (i.e., 40 hours of recovery without whole-body hyperthermia) subjected to 45 minutes of regional ischemia; 4) 40 hours of recovery after whole-body hyperthermia and 30 minutes of regional ischemia; and 5) 40 hours of recovery after whole-body hyperthermia and 45 minutes of regional ischemia. All rabbits were reperfused for 3 hours after regional ischemia.

Ischemia and Reperfusion

The surgical procedure for the ischemia/reperfusion studies has been described previously.10 Briefly, rabbits were premedicated with intramuscular acepromazine maleate (5 mg/kg) and anesthetized with pentobarbital sodium (25 mg/kg i.v.). Additional anesthetic was administered as required. The trachea was cannulated and rabbits were mechanically ventilated with room air. The right jugular vein was cannulated for administration of drugs and fluids. Saline was administered throughout the experimental protocol except during administration of drug to maintain vascular volume (±200 ml infused over experimental time period). We have previously shown11 that administration of saline during this time period does not adversely affect total fluid volume in this preparation of acute myocardial infarction (hematocrit levels remained at ±41 vol% during the studies). Arterial pressure was obtained with a Gould-Statham pressure manometer connected to a fluid-filled catheter (PE 90) in the right carotid artery. The heart was exposed via a left thoracotomy. A silk snare (4-0) was placed around the first anterolateral branch of the left circumflex coronary artery midway between the atrioventricular groove and the apex of the heart. The silk suture was passed through a length of plastic tubing to provide a snare for coronary occlusion. Cardiac hemodynamics were allowed to stabilize for 10 minutes.

Heparin sodium (50 IU i.v.) and lidocaine sodium (10 mg/kg i.v.) were administered to prevent thrombus formation proximal to the site of coronary occlusion and to limit the incidence of ischemia- or reperfusion-induced ventricular dysrhythmias. Regional ischemia was induced 5 minutes later by pulling the suture through the plastic tubing and clamping tightly with a mosquito clamp. Myocardial ischemia was verified visually by the appearance of regional epicardial cyanosis and ST segment elevation on the ECG. Ten minutes after the onset of regional ischemia, 141Ce-labeled microspheres (1×106; 15 μm) were injected into the left atrium to allow delineation of the ischemic zone. In hearts that developed ventricular fibrillation, attempts were made to restore normal sinus rhythm; hearts that did not return to normal sinus rhythm were excluded from the data analysis. Regional ischemia was maintained for either 30 or 45 minutes, after which the mosquito clamp was released and the ischemic zone reperfused for 3 hours.

Analysis of Infarct Size

At the end of the experimental protocol, hearts were arrested in diastole by intravenous injection of 10 ml of saturated potassium chloride, quickly excised, and assigned to the histochemical or biochemical aspect of the study. For the histochemical portion of the study, hearts were cannulated via the aorta on a Langendorff perfusion apparatus and perfused via an aortic cannula at 75 mm Hg with 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 30 minutes. Hearts were then removed from the perfusion apparatus, the atria and right ventricle were trimmed away, and the left ventricle was weighed and fixed by immersion in buffered 10% formalin. In hearts used for the biochemical part of the study, Evans blue dye was injected into the left atrium before saturated KCl was administered (the coronary
artery ligation was tied immediately before the animal was killed. Hearts were quickly extirpated and placed in ice-cold saline. The atria and right ventricle were trimmed away, and the ischemic and nonischemic portions of the left ventricle were freeze-clamped in liquid nitrogen.

Postmortem Studies

The primary experimental end point of this study was the effect of whole-body hyperthermia on infarct size (normalized to risk zone size), assessed by the TTC histochemical staining technique. Coronary collateral flow was not determined in these studies, because we and others have shown it to be relatively nonexistent in rabbit myocardium. Hearts were sectioned into 2-mm slices, and the outline of the left ventricular slices and the tetrazolium-negative (i.e., infarct) areas were traced onto clear acetate sheets. Tissue sections were then placed on a glass plate and covered with plastic and radiographic film to produceautoradiograms for delineation of the anatomic risk zone (identified by the absence of trapped radionabeled microspheres). The area of necrosis was normalized to risk zone size for each heart. Total left ventricular cross-sectional area, area at risk, and area of necrosis were determined from enlarged tracings by computerized planimetry with Sigma Scan (Jandel Scientific Inc., Calif.) using a Summographics Summasketch Plus Bitpad connected to an IBM PS/2 computer. The volume of the risk zone, the volume of necrosis, and the volume of the left ventricle in each slice were calculated as the sum of the area obtained from computerized planimetry and thickness of each ventricular slice (2 mm). The volumes from the sequential slices were summed to provide the total volume of the risk zone, infarct zone, and left ventricle.

High-Energy Phosphate Analysis

Tissue high-energy phosphates were assessed in myocardial biopsies by a modified reversed-phase high-performance liquid chromatography (HPLC) procedure described previously. Briefly, tissue biopsies were washed free of blood in ice-cold saline, frozen in liquid nitrogen (±10 seconds required for this procedure), and stored at −80°C for later analysis. Tissue samples (30 mg) were homogenized in 0.3 ml ice-cold 0.6 M perchloric acid. The homogenate was centrifuged and the supernatant neutralized with 2 M KOH. The pellet was solubilized in 1 ml of 1N NaOH, and total tissue protein was determined by the technique of Lowry et al. ATP, ADP, AMP, adenosine, inosine, hypoxanthine, and nicotinamide adenine dinucleotide (NAD) were determined by HPLC. The chromatographic system comprised a Waters model 510 solvent delivery pump, a Waters model 484 absorbance detector set at a wavelength of 254 nm, and an analytic column (15 cm×4.6 mm i.d.) packed with 3-μm Hypersil ODS (Hichrom, Reading, UK). A guard column packed with the same matrix as the separation column was connected between the injector and the analytic column. A 20-ml aliquot of supernatant to be analyzed was introduced into the HPLC system through a Rhедyne 7125 injection valve equipped with a 20-μl sample loop. Sample peaks were integrated and quantified by use of a CR501 Chromatopac Integrator (Shimadzu). The mobile phase was controlled by a low-pressure gradient mixer; buffer A was 150 mM potassium dihydrogen orthophosphate containing 150 mM potassium chloride (pH 6.0 adjusted with KOH); buffer B was 15 vol% acetonitrile in buffer A. The flow rate was 0.9 ml/min. A linear gradient with buffer B was produced at 0 minutes, 0% B; 0.1 minute, 3% B; 3.5 minutes, 9% B; 5 minutes, 100% B; 7 minutes, 100% B; 7.1 minutes, 0% B. A reequilibration time of 4.9 minutes was allowed, resulting in a total cycle time of 12 minutes between injections. Elution times for tissue high-energy phosphates were compared with standards containing known concentrations of these compounds. Tissue nucleotide, nucleoside, and base contents are expressed as nanomoles per milligram tissue protein; total adenine nucleotides were calculated as the sum of ATP, ADP, and AMP.

RNA Isolation and Northern Analysis

Total RNA was extracted from 1 g of heart as described by Chirgwin et al. The RNA was kept frozen at −80°C until prepared for electrophoresis. For Northern analysis of RNA, 1 μg of denatured total RNA was loaded per lane on gels in a mini sub cell apparatus. RNA was blotted onto Zeta probe membrane using 10 mM phosphate buffer and fixed to the membrane by UV irradiation for 3 minutes. The membranes were washed in 1% sodium dodecyl sulfate (SDS) to remove bromphenol blue and xylene cyanol FF and dried under vacuum.

Two complementary DNA (cDNA) inserts obtained by digestion of plasmids with restriction enzymes were nick translated. pBB5, a pUCS plasmid containing a Bgl II 924-base pair fragment of human cDNA coding for the inducible HSP70 (HSP71), was cut with Sal I and EcoRI. p521, a pUCS plasmid containing a complete human HSP70 gene (HSP73) was cut with Pst I. Membranes were prehybridized at 42°C for 4–6 hours in a buffer of 50 mM Tris pH 7.5, 0.1% Na pyrophosphate, 1 M NaCl, 50% formamide, 10× Denhardt’s, 1% SDS, and 50 μg/ml herring sperm DNA. The cDNA probes were nick translated using the Multiprime DNA labeling system of Amersham and [α-32P]deoxycytidine 5′-triphosphate (specific activity >3,000 Ci/mmol). Membranes were hybridized with radiolabeled probes containing 20×106 cpm in 10 ml of fresh hybridization solution at 42°C for 16 hours, washed with 2× standard saline citrate (SSC)/0.1% SDS at 65°C for 3 minutes, 1× SSC/0.1% SDS at 65°C for 30 minutes, and 0.1× SSC/0.1% SDS at 65°C for 30 minutes. The membranes were exposed to Kodak X-Omat AR film for detection of probes and then stripped by two incubations in 0.1× SSC/0.5% SDS at 95°C for 20 minutes. Stripped membranes were exposed to film to confirm removal of the first probe and then rehybridized with a second probe.

Protein Analysis

Protein samples from heart ventricular muscle were analyzed by two-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE)19 as previously described. For Western blotting, proteins were transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Toronto) at 200 mA overnight, according to the method of Towbin et al. Blots were incubated in phosphate-buffered saline containing 5% skim milk powder to block nonspecific binding sites on the membranes. Blots were immunoreacted with a
TABLE 1. Summary of Heart Rate and Cardiac Hemodynamics in Rabbits Subjected to Acute Ischemia/Reperfusion Injury

<table>
<thead>
<tr>
<th></th>
<th>Preoclusion</th>
<th>30 Minutes of ischemia</th>
<th>30 Minutes of reperfusion</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HR</td>
<td>MAP</td>
<td>RPP</td>
</tr>
<tr>
<td>24-Hour recovery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con 30 minute</td>
<td>243±26</td>
<td>93±16</td>
<td>21±3</td>
</tr>
<tr>
<td>HS 30 minute</td>
<td>266±46</td>
<td>99±19</td>
<td>27±9</td>
</tr>
<tr>
<td>40-Hour recovery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con 45 minute</td>
<td>245±41</td>
<td>73±16</td>
<td>21±6</td>
</tr>
<tr>
<td>HS 30 minute</td>
<td>293±33†</td>
<td>91±16</td>
<td>30±7†</td>
</tr>
<tr>
<td>HS 45 minute</td>
<td>268±43</td>
<td>74±16</td>
<td>24±7</td>
</tr>
</tbody>
</table>

HR, heart rate (beats per minute); MAP, mean arterial pressure (mm Hg); RPP, rate-pressure product (HR×MAP/1,000); Con, controls; HS, heat shock. Values are mean±SD.

* p≤0.05 vs. Con 30 minute; † p≤0.05 vs. Con 45 minute.

1:500 dilution of a monoclonal anti-72 kd heat-shock protein antibody (code RPN.1197; Amersham, Mississauga, Ont.) or with a 1:7,500 dilution of a rabbit (No. 799) polyclonal to the highly inducible human HSP71 (Y. Wu and R.M. Tanguay; manuscript in preparation). Secondly, blots were incubated in a 1:500 dilution of a peroxidase-conjugated goat anti-mouse IgG or a peroxidase-conjugated goat anti-rabbit IgG. 4-Chloro-1-naphthol was used as a substrate for the visualization of the immunoreaction. Blots were counterstained with Ponceau rouge or amido black to show other proteins.

Data Analysis

Differences in hemodynamic data before and after coronary occlusion were examined by paired Student's t tests. Variables measured once, including infarct area, risk zone, percentage tissue necrosis, and left ventricular cross-sectional area, were compared by a one-way ANOVA model. A Duncan's test was done on all main effect means to determine statistical differences between experimental groups. A value of p≤0.05 was considered indicative of a statistically significant difference.

Results

Eighteen rabbits underwent 30 minutes of ischemia and 3 hours of reperfusion 24 hours after whole-body hyperthermia (two heat-shock rabbits died during the second anesthesia); 16 rabbits survived the experimental protocol and were included in the statistical analyses. Cardiac hemodynamics and rate-pressure product for the 24-hour recovery experiments are summarized in Table 1. Heart rate and mean arterial pressure were similar before coronary occlusion in both experimental groups in the 24-hour recovery post-whole-body hyperthermia groups; the higher rate-pressure product reflects the slightly but not significantly higher heart rate and mean arterial pressure in the heat-shock animals. Heart rate was also increased in heat-shocked rabbits during ischemia (p≤0.05 versus controls), but mean arterial pressure was not different. During reperfusion, cardiac hemodynamics were higher in the heat-shock group; similarly, rate-pressure product was increased compared with controls (p≤0.05).

Twenty-seven rabbits underwent 45 minutes of ischemia and 3 hours of reperfusion 40 hours after whole-body hyperthermia. Eight died and were excluded from further study (two control rabbits died of ischemia-induced ventricular fibrillation, and six rabbits died 40 hours after heat shock, two of nonconvertible ventricular fibrillation and the four others during the second anesthesia); therefore, 19 hearts were included in the statistical analyses. Ten rabbits underwent 30 minutes of ischemia and 3 hours of reperfusion 40 hours after whole-body hyperthermia (two died during the second anesthesia, and two died as a result of respiratory complications several hours after whole-body hyperthermia); therefore, six rabbits were included in the statistical analysis for this experimental group. Cardiac hemodynamics for the 40-hour-recovery groups are summarized in Table 1. Heart rate before the onset of ischemia was higher in the 30-minute heat-shock group (i.e., HS 30 minute) compared with the control and HS 45 minute groups; similar results were obtained during the first 30 minutes of regional ischemia. The increased rate-pressure product in this experimental group reflects the higher heart rate, since mean arterial pressure was not different between groups. Cardiac hemodynamics and rate-pressure product were not different between groups during coronary reperfusion. No statistical differences were observed between the control and 40-hour post–heat-shock groups with regard to the incidence of ischemia-induced ventricular fibrillation. Ventricular fibrillation did not occur during coronary reperfusion in any hearts.

No correlation was discernable between the extent of tissue necrosis (normalized to risk zone size) and hemodynamic parameters, including mean arterial pressure, heart rate, and rate-pressure product (the principal hemodynamic determinant of myocardial oxygen demand) for any of the experimental groups.

Assessment of Tissue Necrosis

Infarct size normalized to risk zone size for each of the experimental groups is summarized in Table 2. Risk zone size was similar for both control and heat-shocked rabbits in these studies. In the 24-hour recovery post–heat-shock groups subjected to 30 minutes of ischemia with 3 hours of reperfusion, infarct size was significantly reduced in heat-shocked rabbits compared with controls (23.2±6.6% versus 41.2±7.8%; p≤0.05). We compared infarct size as a percent of risk area (MI/risk) for the experimental groups, because earlier studies documented that MI/risk augmented as risk zone size increased. We also correlated infarct weight versus risk weight; infarct weights in the 24-hour heat-shock recov-
ery group subjected to 30 minutes of regional ischemia were significantly smaller than in control rabbits subjected to a similar duration of coronary occlusion. No statistical differences between groups were detected for MI/risk or infarct weight versus risk zone weight in 40-hour post–heat-shock rabbits regardless of the duration of regional ischemia.

In the 40-hour recovery post–heat-shock groups, infarct size (normalized to risk zone size) was similar (p = NS) between control animals and heat-shock animals subjected to either 30 or 45 minutes of regional ischemia. Infarcts in the 45-minute regional ischemia group were slightly larger than in the control group, but this difference was not statistically significant.

**High-Energy Phosphates**

Levi et al.\(^\text{24}\) recently demonstrated that heat-acclimated rat hearts were able to maintain tissue ATP levels and suggested that improved systolic and diastolic ventricular function and reduced ischemic contracture resulted from this mechanism. Therefore, we assessed tissue high-energy phosphate levels in a separate group of 12 hearts from rabbits subjected to the heat-shock protocol described earlier. High-energy phosphate levels in myocardial biopsies from control rabbits and after 0, 1.5, 3, 6, 24, and 48 hours of heat shock are summarized in Table 3. Tissue ATP and ADP levels increased in myocardium from heat-shocked rabbits compared with controls. Tissue AMP levels decreased immediately after heat shock compared with controls but increased progressively during the 48-hour recovery period after heat shock. Total adenine nucleotides (i.e., ATP + ADP + AMP) in myocardium from heat-shocked rabbits increased progressively beginning about 4 hours after heat shock compared with control myocardium. Tissue hypoxanthine and inosine levels were similar to control myocardium; at 48 hours after heat-shock, inosine levels were lower than in control myocardium. Tissue adenosine levels were reduced after heat shock and progressively increased by 48 hours of whole-body hyperthermia. Tissue NAD levels were also increased in heat-shocked rabbits compared with controls.

**HSP Expression**

Total RNA was isolated from hearts of untreated control rabbits and at 1.5, 3, and 6 hours after heat shock. The methylene blue staining confirmed that each lane was loaded with approximately equal amounts of total RNA (Figure 1). The membranes were probed for mRNA encoding for HSP71, the highly inducible member of the 70-kd family of HSPs, and HSP73, the constitutively synthesized member of the 70-kd family of HSPs. Transcripts for HSP71 were undetectable in control hearts, were abundant at 1.5 and 3 hours after heat shock, and decreased to an undetectable level at 6 hours after heat shock. Peak expression of HSP71 transcripts occurred between 1.5 and 3 hours (Figure 1). The transcripts for the cognate HSP73 were barely detectable in the control hearts; at 1.5 and 3 hours after heat shock, there was a modest increase in HSP73 transcripts, and by 6 hours after heat shock, the abundance of transcripts returned to control levels (Figure 1).

Proteins in samples of hearts were separated by two-dimensional PAGE and transferred to membranes for Western blot analysis. In nonsurgical rabbits, HSP71

### Table 2. Summary of Infarct Size Data for Rabbits Subjected to Ischemia/Reperfusion Injury

<table>
<thead>
<tr>
<th>24-Hour recovery</th>
<th>n (%) AN</th>
<th>AR (%)</th>
<th>AN/AR</th>
<th>LV weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con 30 minute</td>
<td>9</td>
<td>12.4±2.1</td>
<td>30.9±7.1</td>
<td>41.2±7.8</td>
</tr>
<tr>
<td>HS 30 minute</td>
<td>7</td>
<td>6.2±2.7*</td>
<td>27.4±8.5</td>
<td>23.2±6.6*</td>
</tr>
<tr>
<td>40-Hour recovery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con 45 minute</td>
<td>10</td>
<td>21.1±6.6</td>
<td>31.6±9.4</td>
<td>68.6±14.8</td>
</tr>
<tr>
<td>HS 30 minute</td>
<td>6</td>
<td>11.8±2.2</td>
<td>20.7±10.1</td>
<td>63.8±19.5†</td>
</tr>
<tr>
<td>HS 45 minute</td>
<td>9</td>
<td>22.8±6.0</td>
<td>32.2±5.6</td>
<td>73.5±22.7</td>
</tr>
</tbody>
</table>

AN, area of necrosis; LV, left ventricle; AR, area at risk; Con, controls; HS, heat shock. Values are mean±SD.

* p≤0.05 vs. Con 30 minute; † p≤0.05 vs. Con 30 minute.

### Table 3. Myocardial Tissue High-Energy Phosphates From Control and Whole-Body Hyperthermia Rabbits Without Ischemia/Reperfusion

<table>
<thead>
<tr>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>TAN</th>
<th>Hypox</th>
<th>Ino</th>
<th>Ado</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>22.7±2.7</td>
<td>8.0±3.2</td>
<td>54.9±5.9</td>
<td>85.7±10.6</td>
<td>0.06±0.04</td>
<td>1.73±0.48</td>
</tr>
<tr>
<td>HS 0 hours</td>
<td>40.0±2.5</td>
<td>15.9±1.1</td>
<td>28.8±2.3</td>
<td>84.6±5.8</td>
<td>0.26±0.03</td>
<td>1.58±0.15</td>
</tr>
<tr>
<td>HS 1.5 hours</td>
<td>36.3±5.1</td>
<td>11.0±1.6</td>
<td>31.2±5.8</td>
<td>78.5±12.5</td>
<td>0.04±0.03</td>
<td>1.04±0.04</td>
</tr>
<tr>
<td>HS 3 hours</td>
<td>50.9±1.3</td>
<td>15.0±0.4</td>
<td>37.0±1.4</td>
<td>102.9±2.7</td>
<td>0.13±0.01</td>
<td>2.21±0.02</td>
</tr>
<tr>
<td>HS 6 hours</td>
<td>39.8±2.0</td>
<td>19.1±1.2</td>
<td>68.3±5.1</td>
<td>127.3±8.3</td>
<td>0.05±0.01</td>
<td>1.14±0.02</td>
</tr>
<tr>
<td>HS 24 hours</td>
<td>36.7±1.3</td>
<td>20.4±0.8</td>
<td>60.1±2.9</td>
<td>117.2±4.9</td>
<td>0.03±0.02</td>
<td>1.20±0.08</td>
</tr>
<tr>
<td>HS 48 hours</td>
<td>32.1±0.9</td>
<td>21.2±1.6</td>
<td>84.6±15.2</td>
<td>137.9±17.4</td>
<td>0.02±0.03</td>
<td>0.58±0.19</td>
</tr>
</tbody>
</table>

ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; TAN, total adenine nucleotides (ATP+ADP+AMP); Hypox, hypoxanthine; Ino, inosine; Ado, adenosine; HS, whole-body hyperthermia rabbits killed at the indicated times after heat shock. Values are mean±SD. Data are expressed as nanomoles per milligram cardiac protein. n=2 rabbits/group.
FIGURE 1. Northern blot analysis of RNA from rabbit hearts. Total heart RNA was isolated from control (C) and hyperthermia-treated rabbits with 1.5, 3, and 6 hours of recovery from heat shock. Approximately 1 µg of total RNA was loaded on each lane. Methylene blue stain of the membrane revealed the molecular weight markers, 23s and 16s ribosomal RNA of Escherichia coli, and the 28s and 18s ribosomal RNA of the hearts. Transcripts for the heat-shock protein HSP71 were undetectable in control hearts, were readily detected at 1.5 and 3 hours, and were undetectable at 6 hours of recovery from heat shock. The highly inducible nature of the transcript for HSP71 is evident. Reprobing the same membrane revealed transcripts for HSP73 in the control hearts and after heat shock. Peak expression of transcripts for HSP73 occurred at 1.5 hours of recovery.

FIGURE 2. Western blot analysis of heat-shock protein HSP71 in nonsurgical rabbit hearts. Approximately 1 mg of protein was loaded on each gel. After proteins were transferred to membrane, they were immunoreacted with a mouse monoclonal anti-71-kd HSP71 antibody. The secondary antibody was a peroxidase-conjugated goat anti-mouse IgG. 4-Chloro-1-naphthol was used as a substrate for the visualization of the immunoreaction. Blots were counterstained with Ponceau rouge to show other proteins. Arrows indicate the position of HSP71. HSP71 was not detectable in control nonsurgical hearts (panel a). HSP71 was detectable in nonsurgical hearts 48 hours after hyperthermic treatment (panel b).

was not detectable in control left ventricle (Figure 2a) but was easily detectable in 48-hour post–heat-shock left ventricle (Figure 2b). In control hearts after 30 minutes of ischemia and 3 hours of coronary reperfusion, HSP71 was not easily detectable (Figure 3a) in the nonischemic zone of the left ventricle. In 24-hour post–heat-shock hearts, after 30 minutes of ischemia and 3 hours of reflow, HSP71 was detectable in the nonischemic left ventricular tissue (Figure 3b) and in ischemic tissue obtained within the risk zone (Figure 3c).
Discussion

In the present study, we document that whole-body hyperthermic treatment of rabbits to induce a heat-shock response followed by 24 hours of recovery reduced infarct size in rabbits subjected to 30 minutes of ischemia and 3 hours of reperfusion. Yellon et al recently reported lack of cardioprotection in rabbit myocardium when the duration of ischemia was extended to 45 minutes (in 24-hour post–heat-shock rabbits). As a result, we did not include this experimental group in the present study. We also observed a lack of cardioprotection when the recovery period from whole-body hyperthermia was extended to 40 hours (with a 30-minute ischemic period). Thus, it appears that cardioprotection during this period is transient, being present at 24 hours but not at 40 hours after whole-body hyperthermic treatment. These results were unexpected, because previous studies in isolated and perfused rat hearts reported that heat-shock pretreatment significantly improved postsischemic contractile recovery and cell viability even after 48 hours of recovery after hyperthermic treatment; none of these studies examined the effect of previous whole-body hyperthermia on infarct size.

In this study, we directly assessed tissue injury with TTC staining. There has been considerable controversy regarding use of this histochemical staining technique for determination of tissue necrosis; however, despite this controversy, its use is widespread for infarct size studies. The duration of regional coronary occlusion used in the present experiments was based on earlier studies from our laboratory and others that examined the effects of regional myocardial ischemia on infarct size.

Studies that attempt to quantify the efficacy of a particular surgical intervention or therapeutic intervention on infarct size caused by acute myocardial infarction must take into account the principal baseline predictors of infarct size. These include anatomic risk zone size, coronary collateral flow to the ischemic area, and myocardial oxygen demand. In the present study, infarct size is expressed as a percent of anatomic risk zone size to account for variations in vascular anatomy or occlusion site. Infarct size was also normalized to percent risk zone size (MI/risk), since infarct size may increase with larger risk areas, and infarct weight was correlated to risk zone weight (i.e., infarct weight is greater with increased risk zone weight); however, infarct size limitation was observed only in the 24-hour post–whole-body hyperthermia group subjected to 30 minutes of regional ischemia and 3 hours of reflow. Coronary collateral circulation was not assessed here because it has been shown to be negligible in rabbits. No correlations were detected between infarct size and body was a peroxidase-conjugated goat anti-rabbit IgG. 4-Chloro-1-naphthol was used as a substrate for the visualization of the immunoreaction. Blots were counterstained with amido black to show other proteins. In control hearts, HSP71 was not easily detected after ischemia and reperfusion in the nonischemic zone of the left ventricle (panel a). In hearts 24 hours after heat shock subjected to ischemia and reperfusion, HSP71 was detectable in the nonischemic zone (panel b) and in the ischemic zone (panel c) of the left ventricle.

FIGURE 3. Western blot analysis of heat-shock protein HSP71 in rabbit hearts after 30 minutes of regional ischemia and 3 hours of reflow. Blots were immunoreacted with a rabbit polyclonal anti–71-kd HSP antibody. The secondary anti-
rate–pressure product for any of the experimental groups.

In these studies, we examined early effects of the heat-shock response on tissue high-energy phosphate levels. Myocardial ATP levels increased immediately after heat shock and continued up to 48 hours. Tissue AMP levels declined immediately after heat shock and subsequently increased at 6, 24, and 48 hours after heat shock. Total tissue adenine nucleotides (i.e., sum of ATP+ADP+AMP) were higher in myocardium from heat-shocked rabbits beginning at 3 hours after whole-body hyperthermia; at 48 hours after heat shock, total tissue adenine nucleotides were considerably augmented compared with controls. This would suggest increased availability of tissue high-energy phosphates, possibly triggered by the heat-shock response. Minor changes were observed regarding total tissue purine levels, including inosine and hypoxanthine, after heat shock. Since de novo synthesis of adenine nucleotides in myocardium is slow,38–40 resynthesis of ATP occurs preferentially via the salvage pathways (i.e., rephosphorylation of nucleosides). Thus, maintenance of tissue high-energy phosphate levels after heat shock is important, because the tissue ATP content plays an important role in ventricular contractile performance and cell viability. Our findings suggest that whole-body hyperthermia affords an ATP-sparing effect; however, these findings should be interpreted with caution, because they are based on relatively low numbers of animals per study group. Our data are also similar to the recent findings of Levi et al.,24 who documented an ATP-sparing effect in ischemic hearts from heat-acclimatized rats. Whether the relative maintenance of myocyte high-energy phosphate levels is responsible for the cardioprotective effect of whole-body hyperthermia is uncertain.

Northern analysis of RNA revealed the highly inducible nature of the mRNA encoding for HSP71. mRNA levels for HSP71 and HSP73 were elevated at 1.5 and 3 hours after heat shock and appeared to have returned to control levels by 6 hours after heat shock. Western blot analysis of proteins revealed HSP71 in hearts of nonsurgical rabbits 48 hours after heat shock. This reveals the relatively stable nature of the protein in the heart once it has been induced. In fact, in rat hearts, HSP71 is detectable for more than 8 days after hyperthermic treatment. After 30 minutes of ischemia and 3 hours of coronary reperfusion, HSP71 was easily detectable in both nonischemic and ischemic zones of the left ventricle of 24-hour post–heat-shock rabbit hearts. Curiously, the ischemic zones appeared to have smaller accumulations of HSP71 compared with the nonischemic zone of the same heart. This may be indicative of cell death and washing out of proteins during coronary reperfusion, but these hearts were protected and had a decrease in infarct size. Alternatively, the lesser amount of HSP71 in the ischemic zone compared with the nonischemic zone may indicate a more rapid degradation of the protein after it has functioned to maintain protein solubility during and after metabolic injury.

In this study, induction of the heat-shock response reduced infarct size in hearts 24 hours after heat shock subjected to 30 minutes of ischemia and 3 hours of coronary reperfusion but not in hearts 40 hours after heat shock subjected to 30 minutes of ischemia. At first, our results appear to add to the controversy of whether induction of the heat-shock response can protect myocardium from ischemic injury. Although there is good evidence for cardioprotection in isolated, buffer-perfused hearts after myocardial ischemia,9 in live animal models, cardioprotection after heat shock is less certain. Yellon et al.9 recently documented that whole-body hyperthermia in rabbits failed to limit infarct size after 45 minutes of coronary occlusion when initiated 24 hours after heat shock. In contrast, Donnelly et al.8 documented significant cardioprotection in rat hearts subjected to 35 minutes of ischemia 24 hours after whole-body hyperthermia. These differing results may be due to species differences or the shorter duration of coronary occlusion. Interestingly, Donnelly et al.8 also reported a lack of cardioprotection in heat-shocked rat hearts subjected to 45 minutes of ischemia followed by coronary reflow. Data from the present study suggest that there is a window of opportunity (i.e., 24 hours after heat shock) during which there is cardioprotection from ischemic injury. Induction of the heat-shock response may be associated with a delay in irreversible myocardial injury.

The mechanism for this cellular protection after induction of HSPs has not been established. The heat-shock response has been documented to alter intracellular calcium, pH,41 ATP,2,42 and the level of intracellular catalase7,25,26; all of these changes potentially affect tissue viability. After hyperthermia, HSPs localize within the cell nucleus43 and around the nucleolus even though nucleolar function is compromised.44 During recovery after hyperthermia, nucleoli regain normal morphology and HSPs are localized within the cytoplasm. These observations indicate that HSPs may stabilize or solubilize damaged proteins found within the nucleus, nucleolus, and/or ribosomes after an acute stress (such as ischemia); as a result, HSPs may facilitate removal or repair of damaged proteins during recovery from the stress event. Donnelly et al.8 suggest a possible correlation between the degree of induction of HSP and myocardial salvage; however, further studies are necessary.

In summary, although heat shock–induced protection of isolated hearts has been demonstrated clearly in isolated buffer-perfused hearts and more recently in vivo in rat hearts subjected to pretreatment with whole-body hyperthermia, the present findings document that cardioprotection in rabbit hearts is transient, decaying by 40 hours after heat-shock.

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