Induction of HSPs in response to ischemia and reperfusion can be mimicked by various stressors. In vivo, HSP induction occurs via heat stress, transfection through molecular means, or in transgenic animals. In vitro, HSP induction can be produced via heat stress or by transfection of inducible protein to greatly increase the levels of stress proteins. These exogenous stressors induce HSPs including HSP90 (8), HSP70 (6, 8, 12), HSP60 (18), HSP25 (6, 19), and HSP10 (18), which have been implicated in protection to the whole heart or cells derived from the heart. Many in vivo and in vitro models have demonstrated that high levels of HSPs protect from injury due to stress. Similarly, Nakano and colleagues (24) have shown that blocking the induction of HSP72 by use of 14-mer phosphorothioate results in an increased susceptibility to hypoxic injury in cardiac myocytes.

Hutter and co-workers (15) showed that not only are HSPs necessary, but also the amount of HSP72 produced by varying degrees of heat stress is directly related to the level of protection that is afforded against ischemia-reperfusion injury. Many groups have shown that whole body hyperthermia can elevate HSPs and afford protection against reperfusion arrhythmias (31) and ischemia-reperfusion injury (9, 12) within a specific time window. The time window currently places protection at 24 h after heat stress with disappearance of protection at 30–40 h (9, 29). Although detectable levels of HSPs can be seen as soon as 2 h after whole body hyperthermia, no protection is associated with this initial rise in HSPs (29). Increased levels of HSPs continue to be detected in heat-shocked animals even after the 24-h time window of protection (29). There seems to be a disparity between protein induction and protection afforded because the time course of HSP induction does not correspond temporally with protection.

Studies involving whole body hyperthermia have not considered the genetic background of the species and strains which may explain some disparity in response to HSPs. It has been proposed by our laboratory that there may be a genetic component to cardioprotection (3), yet examples of specific mechanisms responsible

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Cardioprotection is strain dependent in rat in response to whole body hyperthermia

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Cardioprotection is strain dependent in rat in response to whole body hyperthermia. Am J Physiol Heart Circ Physiol 280: H1208–H1214, 2001.—Previous results showed a genetic component to cardioprotection. Therefore, we investigated the heat shock response in Wistar and Sprague-Dawley (SD) rats at 24 and 48 h. Rats were subjected to whole body hyperthermia achieving colonic temperatures of 40 or 42°C for 20 min. After recovery hearts were excised for protein measurements or were subjected to 30 min of ischemia and then 2 h of reperfusion. Heat shock protein (HSP) expression was determined by Western blotting and infarct size was determined by triphenyltetrazolium staining. All groups of SD and Wistar rats demonstrated HSP72 and HSP90 induction at both time points in response to a heat stress of 42°C. At 24 h there was only a significant reduction in infarct size seen in control vs. small SD (60.0 ± 4.8 vs. 26.5 ± 2.3) rats. However, at 48 h control versus small SD (60.0 ± 4.8 vs. 17.6 ± 3.8) and Wistar (59.4 ± 4.3 vs. 29.8 ± 6.0) and control versus large SD (53.7 ± 2.6 vs. 19.8 ± 4.7) and Wistar (57.3 ± 1.6 vs. 34.5 ± 2.8) rats demonstrated a significant reduction in infarct size with a greater reduction observed in SD rats. We conclude that heat shock-induced cardioprotection in rats is dependent on strain, temperature, time after stress, and size.

heat shock proteins; genetic; infarct size

ISCHEMIA PRODUCES INTRACELLULAR CHANGES that include increased calcium, altered osmotic control, generation of free radicals, membrane damage, decreased pH, problems with energy metabolism, depletion of oxygen, and many others (5). These intracellular changes translate into damage to vital proteins that normally allow for proper functioning of the heart. It is thought that this increase in denatured proteins results in an internal mechanism of enhanced survival related to the induction of heat shock proteins (HSPs) (1). HSPs allow for proper protein folding and assembly, transport (by acting as chaperones), receptor regulation, and stabilization of the cytoskeleton (16, 23). Myocardial ischemia as well as reperfusion after ischemia have been shown to increase mRNA levels for HSP72 in dog (11) and rat (37), and subsequent protein induction is protective to the heart (12).

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for strain-specific responses are lacking. Therefore, we looked at two different strains of rat of various sizes and their responses to whole body hyperthermia to determine whether this trigger of delayed cardioprotection is strain dependent.

**METHODS**

**Study groups.** Rats were divided into different groups based on their strain and size. Male Sprague-Dawley (SD) and Wistar rats were divided into small (200–275 g) and large (350–425 g) groups. Each of these groups was further subdivided into specific time points. Both strains had each size group undergo the general surgical procedure or excision of the heart for protein analysis at 24 and 48 h after whole body hyperthermia. Control groups consisted of nonstressed animals. The rats were fed standardized rodent food and had ad libitum access to water.

**Heat shock protocol.** Rats were anesthetized with 40 mg/kg ip pentobarbital sodium. Anesthetic was supplemented as needed. The rats were placed on a heating blanket and covered completely in terracotta cloth for insulation. Colonic temperature, measured using a rectal probe with a digital output, was elevated to either 40 or 42°C. Colonic temperature was maintained for 20 min with a deviation of ±0.2°C. Once the desired temperature was obtained, the rats were removed from the heating blanket. They were placed on the heating blanket as needed to maintain the proper temperature. After 20 min of hyperthermia, the rectal probe was removed, and the rats were returned to a cage where they had access to food and water. The posthyperthermic status of the rats was monitored until the rats awoke from the anesthetic. Recovery periods lasted for 24 and 48 h at which time the rats underwent the general surgical procedure or hearts were excised for protein measurements.

**HSP measurement.** Rats from each heat shock group underwent protein analysis to determine the expression of specific HSPs. After the specified time point after whole body hyperthermia, hearts were excised for protein measurements. The hearts were homogenized slurry was transferred to a clean tube and centrifuged at 10,000 rpm for 10 min at 4°C (Eppendorf Centrifuge 5801 R). The supernatant was aliquoted to sterile microcentrifuge tubes. Protein concentrations were measured using the Bio-Rad protein assay with bovine serum albumin as the standard. The aliquoted samples were kept at −80°C until used.

Total protein (50 µg) was loaded onto a 10% SDS-PAGE gel and separated by electrophoresis. The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane by electroelution. The efficiency of the transfer was determined by looking at the transfer of prestained molecular markers (Kaleidoscope Prestained Standards, Bio-Rad). Heat-shocked HeLa cell extract (LYC-HL101P, StressGen) was run as a positive control on each membrane. The membranes were blocked in milk overnight and then probed with specific antibodies. The membranes were incubated with primary mouse monoclonal antibodies for HSP70 (detects only inducible HSP72, not the constitutive form HSP73) at a 1:5,000 dilution (SPA-810, StressGen) or HSP90 at a 1:5,000 dilution (SPA-830, StressGen). They were then incubated with an anti-mouse IgG-horseradish peroxidase conjugate secondary antibody. Specific antibody binding was detected using enhanced chemiluminescence (RPN2106, Amersham Pharmacia Biotech) and visualized by exposure to X-ray film.

**General surgical procedure.** Rats were anesthetized using 120–150 mg/kg ip Inactin. The right jugular vein was cannulated for the delivery of saline. The right carotid artery was cannulated for the measurement of blood pressure and heart rate. Pressure and rate measurements were monitored using a Gould PE50 or PE23 pressure transducer connected to a Grass model 7 polygraph. A tracheotomy was then performed. The trachea was intubated with a cannula connected to a rodent artificial ventilator (model CIV-101, Columbus Instruments. Columbus, OH, or model 663, Harvard Apparatus, South Natick, MA). The rats were ventilated with room air at 38–45 breaths/min supplemented with O₂. Atel-ectasis was prevented by maintaining a positive end-expiratory pressure of 5–10 mmHg. Arterial pH, P CO₂, and P O₂ were monitored at control, 15 min of occlusion, and at 60 and 120 min after reperfusion using a blood gas system (AVL 995 pH/blood gas analyzer). Normal values were maintained by adjusting the respiratory rate and/or the tidal volume. Body temperature was maintained at 38°C using a heating pad.

Once heart rate and blood pressure stabilized, a left thoracotomy was performed at the fifth intercostal space. A pericardiotomy was then performed followed by adjustment of the left atrial appendage to locate the left coronary artery. A ligature (6-0 prolene) was passed below the left descending vein and coronary artery from the area immediately below the left atrial appendage to the right portion of the left ventricle. The ends of the suture were threaded through a propylene tube to form a snare. Occlusion for a period of 30 min was elicited by pulling on the snare and clamping the snare onto the epicardial surface using a hemostat. This resulted in left ventricular ischemia. Coronary artery occlusion was confirmed by epicardial cyanosis and a decrease in blood pressure. Reperfusion for a period of 2 h was achieved by unclamping the hemostat and loosening the snare.

**Determination of infarct size.** After the 2-h period of reperfusion, the coronary artery was again occluded using the snare. The area at risk (AAR) was determined by negative staining. Patent blue dye was administered via the jugular vein to stain the nonoccluded area of the left ventricle. The heart was excised and the left ventricle was separated from the remaining tissue and cut into thin cross-sectional pieces. The normal areas were stained blue and the AAR remained pink. The normal area and AAR were separated and placed in different vials containing 1% 2,3,5-triphenyltetrazolium chloride (TTC) in 100 mM phosphate buffer (pH 7.4). These vials were incubated at 37°C for 15 min. TTC is an indicator of viable and nonviable tissue. Tissues were fixed overnight in 10% formaldehyde and the infarcted tissue was dissected from the AAR using a dissecting microscope (Cambridge Instruments). Infarct size (IS) and AAR were determined by gravimetric analysis. IS was expressed as a percentage of the AAR (IS/AAR).

**Statistical measurements.** All values are expressed as means ± SE. For the hemodynamic data, left ventricle mass, and AAR, statistical significance was determined by performing a one-way ANOVA with Dunnett’s multiple comparison test as the post hoc test. For the percent infarct and densitometry, significance was determined by performing an unpaired t-test. Significance was attributed to those groups with P < 0.05.
Table 1. Hemodynamic parameters

<table>
<thead>
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<th>Baseline</th>
<th>15-Min Ischemia</th>
<th>2-H Reperfusion</th>
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<tr>
<td></td>
<td>Heart rate</td>
<td>MAP</td>
<td>RPP</td>
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<tr>
<td>Wistar, small</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>328 ± 13</td>
<td>109 ± 6</td>
<td>46 ± 1</td>
</tr>
<tr>
<td>24-h HS</td>
<td>344 ± 19</td>
<td>102 ± 10</td>
<td>44 ± 5</td>
</tr>
<tr>
<td>48-h HS</td>
<td>309 ± 9</td>
<td>108 ± 4</td>
<td>42 ± 2</td>
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<tr>
<td>Sprague-Dawley, large</td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>338 ± 10</td>
<td>120 ± 9</td>
<td>49 ± 3</td>
</tr>
<tr>
<td>24-h HS</td>
<td>350 ± 15</td>
<td>96 ± 8</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>48-h HS</td>
<td>338 ± 14</td>
<td>97 ± 7</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>Sprague-Dawley, small</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>340 ± 28</td>
<td>96 ± 8</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>24-h HS</td>
<td>327 ± 15</td>
<td>97 ± 8</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>48-h HS</td>
<td>280 ± 9*</td>
<td>84 ± 5*</td>
<td>31 ± 2</td>
</tr>
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Values are means ± SE; n, no. of rats. Rats subjected to 30 min of ischemia then 2 h of reperfusion. Measurements taken at baseline, 15 min ischemia, and 2 h of reperfusion. HS, heat shock; heart rate, beats/min; MAP, mean arterial pressure, mmHg; RPP, rate pressure product, mmHg/1000. *Significant difference from the specific control group.

RESULTS

Hemodynamic data. The hemodynamic data, which include heart rate, mean arterial blood pressure (MAP), and the rate-pressure product (RPP), are summarized in Table 1. There was no significant difference observed in heart rate, MAP, and RPP between any of the experimental groups and comparable control groups at baseline, 15 min of ischemia, or 2 h of reperfusion except for heart rate in the large SD group and in heart rate and RPP in the small group at 48 h after heat shock.

Effect of heat shock on HSP70 and HSP90 expression. Expression of HSP70 was present at both 24 and 48 h for all of the 42°C heat-shocked rats of both sizes and strains. In relation to the HeLa heat-shocked cell lysate control, HSP70 expression was only observed in the small and large 42°C-treated groups with no detectable levels observed in the control and 40°C-treated groups (Fig. 1, A and B, respectively). As expected, there was no constitutive expression of HSP70. Though the densitometry results (expressed as a percentage of HeLa cell control) showed lower levels of HSP70 in each of the 48-h groups in the small and large rats (data not shown), the only significant decrease from the 24-h time point was seen in the large 48-h SD group.

Expression of HSP90 was present in both small and large rats of each strain at 24 and 48 h in control, 40°C heat-shocked groups, and 42°C heat-shocked groups (Fig. 2, A and B, respectively). Densitometry indicated that the levels of HSP90 in the 42°C heat shock groups were not different between the 24- and 48-h groups of
either the small or large rats (data not shown). There was constitutive expression of HSP90; however, levels were increased above control in both the 40°C- and 42°C-treated groups.

Effect of heat shock on myocardial infarct size. There was no significant difference for any group versus control for left ventricle weight and the AAR (data not shown). For the small Wistar rats (Fig. 3), a significant reduction in percent infarct (infarcted area/AAR × 100) was seen at 48 h after heat shock versus control (30.3 ± 3.7 vs. 59.4 ± 4.3; \( P = 0.0003 \)); however, no significant decrease in percent infarct was seen with the 24-h group. Both 24-h (26.5 ± 2.3 vs. 60.0 ± 4.8; \( P < 0.0001 \)) and 48-h (17.3 ± 2.6 vs. 60.0 ± 4.8; \( P < 0.0001 \)) treated small SD groups (Fig. 3) showed a significant decrease in percent infarct compared with control. In the larger rats (Fig. 4), the 48-h Wistar (34.5 ± 2.8 vs. 57.3 ± 1.6; \( P = 0.0001 \)) and 48-h SD (19.8 ± 4.7 vs. 53.7 ± 2.6; \( P < 0.0001 \)) showed a significant decrease in percent infarct size, but the 24-h groups of both large Wistar and SD rats showed no significant reduction in infarct size.

In relation to strain differences, there was a significant difference in infarct size between the SD and the other strains. Fig. 2. HSP90 expression in small and large rats. Protein from control, 40°C, and 42°C treatments at 24 and 48 h for Wistar and SD rats was isolated and run on SDS-PAGE and electroeluted onto PVDF membrane before exposure to monoclonal antibody (HSP90). Secondary antibody was horseradish-conjugated goat antiamouse IgG. A: Western blot for Wistar and SD small rats. First lane contains the positive control, HeLa heat-shocked cell lysate. Subsequent lanes represent control, 40°C, and 42°C for Wistar (24 and 48 h) followed by SD (24 and 48 h) small rats. B: Western blot for Wistar and SD large rats. First lane contains the positive control, HeLa heat-shocked cell lysate. Subsequent lanes represent control, 40°C, and 42°C for Wistar (24 and 48 h) followed by SD (24 and 48 h) small rats.

Fig. 3. Percent infarct for small rats. Rats were either heat shocked or were not heat shocked for control (C) groups. They were allowed to recover (24 or 48 h), at which time they underwent 30 min of ischemia then 2 h of reperfusion. Area at risk (AAR) was determined by injection of patent blue dye and separated from normal tissue. Infarct size was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Percent infarct is expressed as a percentage of the AAR (IS/AAR). Values represent means ± SE. *Significant difference from control groups; †significant difference from Wistar group.

Fig. 4. Percent infarct for large rats. Rats were either heat shocked or were not heat shocked for control groups. They were allowed to recover (24 or 48 h), at which time they underwent 30 min of ischemia then 2 h of reperfusion. AAR was determined by injection of patent blue dye and separated from normal tissue. Infarct size was determined by TTC staining. Percent infarct is expressed as a percentage of the AAR (IS/AAR). Values represent means ± SE. *Significant difference from control groups; †significant difference from Wistar group.
Wistar rats when compared at the specific time and size groups. In the small group the infarct size was significantly smaller in the SD rats at 24 h (P = 0.0008) and at 48 h (P = 0.0078) compared with the Wistar rats. In the larger rats there was no significant difference observed between the strains at 24 h, but at 48 h (P = 0.0150) the infarct size in the SD rats was again significantly reduced in relation to the Wistar rats.

**DISCUSSION**

Our results show that in relation to infarct size, whole body hyperthermia can protect the hearts of small SD rats from damage produced by ischemia and reperfusion at 24 h after heat stress. The protection is enhanced in the small and large groups at 48 h; thus it seems that the apparent window of protection seen after heat shock must be expanded to include later time points. However, this protection has some peculiarities. In larger animals, protection is not observed at 24 h, but only at 48 h after heat stress. There are also strain differences in which the small SD rats show a significant reduction in infarct size at both 24 and 48 h after heat stress compared with Wistar rats, and the large SD rats show a greater reduction at 48 h compared with Wistar rats. The Western blots show that expression of HSPs is present at 24 and 48 h in each of the groups tested at relatively comparable levels. Although the large SD rats in the 48 h group had significantly decreased levels of HSP70 compared with the 24-h time point, infarct size was still reduced. This suggests that protein induction is consistent with afforded protection as long as enough time has passed to allow the HSPs to perform their necessary modification and stabilization.

Many proteins and signaling pathways may potentially be involved in the phenomenon of protection with interconnected pathways. With the peculiarities observed between the different strains, in particular the difference in response of the 24-h groups of Wistar and SD rats, it seems more likely that there is a genetic component that plays a crucial role in mediating protection. It may be that a particular composition of proteins that can interact in a particular way may confer a differential response to heat stress and consequently cardioprotection. HSPs are under genetic regulation and can be regulated by heat shock transcription factors (4, 25, 26). The possibility exists that polymorphisms among different strains may be present in specific genes that contribute to protection. Recent work in collaboration with our laboratory (3) studied myocardial ischemia in five different rat strains to determine whether there was a genetic component to cardioprotection. We observed different responses to global no-flow ischemia in different strains of rats and found that the strains were genetically different. This suggests that the current responses we observed may be due not only to the expression of HSPs and the ability of cells to respond to the HSPs but also to the genetic background within which proteins are expressed and act.

It has generally been accepted that whole body hyperthermia is protective to the heart 24 h after heat stress with the response disappearing at time points beyond 24 h, although the expression of HSPs still exists (9, 29). Our results also suggest a lack of temporal correlation between cardioprotection and the amount of HSP70 and HSP90 induced. It is likely that the HSPs are not the mediators of the response and are an epiphenomenon with some other mechanism being the end effector of protection. Some investigations have suggested that the protection may be a response to the elevation of antioxidants (10, 31, 35), although others have shown that antioxidants may not be the mediators (2, 21). It may be possible that HSPs in some way modulate other end effectors of late protection that have been reported. It has been shown by different groups that opening of the ATP-sensitive K channel may be important in heat shock-mediated protection and that blockade of this channel can attenuate heat shock-induced protection (13, 27). However, there are countless papers that point to each family of HSPs having some influence in contributing to protection. Work by Hutter and colleagues (15) suggests that protection increases as the level of HSPs increases with varying degrees of heat stress. We showed as they did that a submaximal heat stress (40°C) fails to induce HSP70 at either time point. However, a sufficient heat stress (42°C) results in a similar induction of protein at both 24 and 48 h.

Thus HSP induction seems to be associated in some way with protection, and in our model the time after heat shock does not seem to limit the protection that is afforded by heat stress. Yamashita and co-workers (36) have found significant reduction in infarct size at 48 and 72 h in Wistar rats. Fundamentally their protocol was different from ours, but in their model they saw no protection at 24 h after heat stress. Protection was shifted over to 48 and 72 h after heat stress. Incidentally, we have observed in Wistar rats that protection is completely absent at 72 h after heat stress (personal observations). This may be due to the longer ischemic period and shorter reperfusion period in our protocol compared with theirs. Similarly, certain groups have also observed protection at 24 h after stress in SD rats (2, 15, 29). However, we find that protection is limited by the size or age of the animal. Work by Kregel and Moseley (17) suggests that older rats do not have a similar response to heat stress as younger rats. They found that older rats have an attenuated ability to induce HSPs when they were heat stressed (12 vs. 24 mo old). Further work in an older group of rats (24 mo) is necessary to confirm these observations.

In our studies the age response does not account for the protein levels that we observed because the older and younger animals had comparable levels of protein at both 24 and 48 h after heat stress. The important implication is that the response to heat stress in younger and older rats may be different. Younger rats may be able to respond to HSP induction earlier, which is related to the protection we observed at 24 h in the smaller rats. This protection is enhanced at 48 h after...
heat stress, suggesting that modifications by the HSPs are continuing to take place and the cells are protected for long periods of time by this endogenous survival mechanism. This response may be different in larger rats. Although the proteins are elevated as expected with increased protection, the age of the animals may retard the immediate effect of the HSPs so that it takes the same amount of induced protein longer to modify cells for protection. It is possible that older rats have accumulated more cellular damage through such means as generation of reactive oxygen species over time. This would then result in protection being delayed to later time points (48 h) when sufficient time has passed as well as when adequate levels of protein are present.

The important implication of the work of Kregel and Moseley (17) is that the response of different stimuli has differential effects on the induction of HSPs and ultimately the protection that is afforded by these proteins. It is possible that the stimuli rather than the actual proteins induce protection. The induction of proteins may be arbitrary. There is evidence that both supports and argues against this notion. Work by Qian and colleagues (28) shows that ischemic preconditioning does not induce a second window of protection although it induces a comparable level of HSP70 as seen with whole body hyperthermia. Comparatively, Taylor and co-workers (33) show that exercise alone without HSP induction confers protection. This suggests that the stimuli rather than the end product of the stimuli are more important. However, countless studies in vivo and in vitro with numerous stimuli such as whole body hyperthermia, transfection of proteins into animals and cells, and ischemia and reperfusion elevate the levels of HSPs and confer protection (6–9, 14, 18, 20, 22, 30, 32, 34). It cannot be that protection is exclusive of the proteins and is dependent solely on the stimulus. This is supported by the work of Nakano and colleagues (24), who show that an antisense molecule that stops the induction of HSP72 is detrimental to the cardiac cell.

The heat shock response appears to be dependent on multiple factors. We are the first group to show that the whole body hyperthermia response in the rat is strain dependent, and this may prognosticate a possible mechanism for a genetic component to cardioprotection. We are also now challenged to consider the implications of using a specific strain in experiments and investigations are undertaken. This insight has profound implications for model development to look at the genetic component of cardioprotection.

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