Increased temperature, not cardiac load, activates heat shock transcription factor 1 and heat shock protein 72 expression in the heart

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Increased temperature, not cardiac load, activates heat shock transcription factor 1 and heat shock protein 72 expression in the heart. Am J Physiol Regul Integr Comp Physiol 292: R432–R439, 2007. First published September 21, 2006; doi:10.1152/ajpregu.00895.2005.—The expression of myocardial heat shock protein 72 (HSP72) postexercise is initiated by the activation of heat shock transcription factor 1 (HSF1). However, it remains unknown which physiological stimuli govern myocardial HSF1 activation during exercise. These experiments tested the hypothesis that thermal stress and mechanical load, concomitant with simulated exercise, provide independent stimuli for HSF1 activation and ensuing cardiac HSP72 gene expression. To elucidate the independent roles of increased temperature and cardiac workload in the exercise-mediated upregulation of left-ventricular HSP72, hearts from adult male Sprague-Dawley rats were randomly assigned to one of five simulated exercise conditions. Upon reaching a surgical plane of anesthesia, each experimental heart was isolated and perfused using an in vitro working heart model, while independently varying temperatures (i.e., 37°C vs. 40°C) and cardiac workloads (i.e., low preload and afterload vs. high preload and afterload) to mimic exercise responses. Results indicate that hyperthermia, independent of cardiac workload, promoted an increase in nuclear translocation and phosphorylation of HSF1 compared with normothermic left ventricles. Similarly, hyperthermia, independent of workload, resulted in significant increases in cardiac levels of HSP72 mRNA. Collectively, these data suggest that HSF1 activation and HSP72 gene transcriptional competence during simulated exercise are linked to elevated heart temperature and are not a direct function of increased cardiac workload.

Heat shock protein 72; heat shock transcription factor 1; working heart; exercise; phosphorylation

THE ABILITY OF CELLS TO ADAPT TO PHYSIOLOGICAL DISTURBANCES is a fundamental requirement of survival. An organism’s response to environmental and physiological stressors includes a highly ordered set of events that is often represented by rapid changes in gene expression followed by the synthesis of proteins involved in adaptation (34). Heat shock proteins (HSPs) are a family of highly conserved stress proteins that play a paramount role in maintaining homeostasis in response to cellular stress (11, 20, 21, 24–26, 31, 42).

Numerous studies reveal that cellular upregulation of the stress-inducible isoform within the 70-kDa family of HSPs, HSP72, occurs in response to heat stress, oxidative stress, pH disturbances, and exercise (11, 20, 21, 24–26, 31, 42). Endurance exercise imposes a unique combination of the aforementioned physiological factors, and the resulting HSP72 expression is beneficial against a variety of cellular insults. The cardioprotection afforded to the myocardium after exercise is potentially mediated by the molecular chaperoning capability of HSP72, which incorporates several functional properties of HSP72, including an active participation in folding proteins by minimizing incorrect interactions within and between molecules, maintaining proteins in their native folded state, and in repairing or promoting the degradation of misfolded proteins (2, 12). Therefore, the induction of HSP72 during exercise may assist in functional restoration of key intracellular proteins damaged by stress, subsequently facilitating recovery from and providing protection against injury and cell death.

The modulation of heat shock transcription factor 1 (HSF1) is purported to occur in response to cellular stress, thereby initiating the cascade of signaling events to upregulate HSP72 protein expression postexercise. The induction of HSP72 is mediated through the interaction of HSF1 with the proximal promoter heat shock element (HSE) on the HSP70 gene (32). During unstressed conditions, HSF1 remains a latent monomeric protein in the cytoplasm. Activation of HSF1 is a complex multistep process involving an oligomerization from inert monomer to active trimer, acquisition of DNA-binding ability, phosphorylation, and nuclear localization and transcriptional activation (33). Although exercise in warm environments increases body temperature and HSP72 expression, recent studies have shown that exercise in the cold attenuated the rise in core temperature and prevented the upregulation of HSP72 expression by an unknown mechanism (15, 43). These data suggest that temperature could be a critical variable mitigating transcriptional competency in myocardial HSP72 expression during exercise.

In addition to an increase in temperature during exercise, it has been shown that mechanical stretch and pressure overload can induce HSP72 expression in a variety of different cells and tissues (8, 46, 47). Specifically, in the heart, HSF1 can be activated in the Langendorff preparation by increasing left-ventricular balloon inflation. Indeed, this mechanical stretch of the left ventricle is followed by a concomitant increase in HSP72 mRNA (6, 17, 19). Moreover, others report that increased preload, but not afterload, directly induced the activation of HSF1 in the left ventricle (36). Hence, it seems possible that increased cardiac workload imposed by exercise may also initiate HSP72 expression.

Given the importance of HSP72 as a cytoprotective protein, understanding the physiological factors that regulate exercise-induced expression of this molecule in the heart is important. Therefore, these experiments tested the hypothesis that thermal stress and mechanical load provide independent stimuli for HSF1 translocation into the nucleus and HSP72 gene expres-

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sion in the heart after simulated exercise in an isolated working heart preparation.

MATERIALS AND METHODS

Animals and Experimental Design

The University of Florida Animal Care and Use Committee approved all of the procedures used in these experiments. Adult male Sprague-Dawley rats (6 mo old) were housed in pairs with access to food and water ad libitum in a room with a 12:12-h reverse light-dark cycle maintained throughout the experimental period. To investigate the independent effect of temperature and cardiac workload on HSP72 expression, hearts from 33 animals were isolated and perfused in an in vitro working heart preparation. Hearts were randomly assigned to one of five experimental conditions using varying temperatures and myocardial workloads: 1) controls (hearts removed from anesthetized animal hearts, rinsed 3 times in ice-cold saline baths, and rapidly frozen) (C; n = 6), 2) buffer temperature (40°C), high load (23-cm preload, 110-cm afterload) (40°C HL; n = 7), 3) 40°C temperature, low load (15 cm preload, 60 cm afterload) (40°C LL; n = 7), 4) 37°C temperature high load (37°C HL; n = 7), and 5) 37°C temperature, low load (37°C LL; n = 6).

Overview of Simulated Exercise Using the In Vitro Working Heart Model

To simulate the thermal and metabolic changes in the heart occurring with both low- and high-intensity exercise, heart temperature and cardiac workload were adjusted in the isolated and perfused working heart preparation. Specifically, buffer temperatures were maintained at either 37°C or 40°C using a circulating water bath, and the cardiac workload was manipulated by altering preload and afterload column heights. Preliminary experiments in our laboratory confirmed that the heart temperature remained at the respective experimental temperature throughout the course of the experiments, as determined by continuous measurement of left ventricular (LV) temperature with thermistor probes (Physiotemp Instruments, Clifton, NJ). Moreover, the temperature of the working heart apparatus was maintained by use of a circulating water bath and insulated, water-jacketed glass chambers throughout these experiments (Harvard Apparatus, Holliston, MA). The selection of 23 and 110 cm of H2O for the high load and pre- and afterloads, respectively, was based on preliminary experiments in our laboratory indicating that these are the highest cardiac workload that can be sustained in an isolated working heart for 60 min at 40°C without a significant loss of ventricular function.

To standardize cardiac workload, each isolated heart was paced at a rate of 400 bpm. The decision to pace hearts at 400 bpm was based on several considerations. First, pacing hearts at the same rate controlled for potential differences in spontaneous heart rates between the experimental groups. Further, we chose 400 bpm because this heart rate consistently exceeded the spontaneous beating rate of unpaced hearts exposed to hyperthermia. Moreover, our preliminary experiments revealed that pacing isolated working hearts beyond 400 bpm compromises the stability of the “hyperthermic” working heart preparation during the final minutes of a 60-min experiment. Hence, it is not possible to complete our hyperthermic experiments with heart rates above 400 bpm in the isolated working heart preparation.

In Vitro Working Heart Protocol

Myocardial function was evaluated during the simulated exercise protocol using an isolated working heart preparation previously described by our group (22, 23). Briefly, animals were anesthetized with pentobarbital sodium at a dose of 100 mg/kg. After reaching a surgical plane of anesthesia, 100 IU of heparin were injected directly into the hepatic vein, and the heart was rapidly excised and placed in cold saline (4°C) to determine heart weight. Excess tissue was trimmed, weighed, and subtracted from the gross weight to determine the heart’s final weight. Next, the aorta was secured to a stainless-steel catheter and perfused in a retrograde, or Langendorff, mode, at 80 cm H2O for 10 min. Hearts then were perfused at 37°C or 40°C, depending on the protocol, with Krebs-Henseleit buffer with pH maintained at 7.4–7.45 containing 1.25 mM CaCl2, 130 mM NaCl, 5.4 mM KCl, 11 mM glucose, 0.5 mM MgCl2, 0.5 mM NaH2PO4, 25 mM NaHCO3, and aerated with 95% O2-5% CO2. Finally, the left atrium was cannulated via the pulmonary veins and sutured securely to the atrial cannula (Harvard Apparatus, Holliston, MA).

After 10 min of retrograde perfusion, the heart was switched to the working heart mode, and function was evaluated at a fixed pre- and afterload (atrial filling pressure). For the high-load settings, preload was set to 23 cm H2O with a 110-cm-high aortic column and 15 cm H2O with a 60-cm-high aortic column was established for the low load. To maintain a constant heart rate across the experimental groups, platinum electrodes were placed directly into the right ventricle (RV), and hearts were paced to 400 bpm using a stimulator set at 5 V and a 0.1-ms pulse duration (Grass, W. Warwick, RI). During the course of the 60-min experiment, heart rate was maintained with hearts enclosed in a sealed, water-jacketed chamber maintained at 37°C or 40°C, depending on the experimental treatment. Upon completion of the 60-min simulated exercise protocol in the organ bath, LV temperature was measured, and sections of the LV were removed and rapidly frozen in liquid nitrogen for subsequent measurement of cytosolic and nuclear HSF1 protein along with HSP72 mRNA.

Cardiac Function Measurements

Cardiac functional measurements were recorded every 10 min throughout the experiment. Specifically, a timed collection of coronary flow (CF) and aortic overflow (AF) were made from the heart effluent and the aortic overflow, respectively. Cardiac output was defined as the sum of these two flows (CF + AF) and normalized to heart weight. Peak systolic pressure, diastolic pressure, the rate of pressure development and decline (±dP/dt), and heart rate were measured via a pressure transducer connected to a T-connector from the aortic cannula (Harvard Apparatus, Holliston, MA). Data were recorded using a customized real-time data acquisition system (LabVIEW 6i, National Instruments, Houston, TX).

Tissue Removal and Preparation

At the conclusion of the 60-min experimental protocol, LV temperatures were assessed using a tissue-implantable thermocouple microprobe inserted directly into the LV wall (Physiotemp Instruments, Clifton, NJ). Next, hearts were removed from the aortic cannula, weighed, and sectioned into LV, RV, and septum, and then they were rapidly frozen in liquid nitrogen prior to storage at −80°C until assay. Samples delineated for RNA isolation and RT-PCR were placed in RNA later and then stored at −80°C (Ambion, Austin, TX).

Biochemical Measures

Protein oxidation. Oxidative damage to myocardial protein was determined by measurement of protein carbonyls. Protein carbonyls were assayed using an enzyme immunoassay (Zentech PC Test; Zenith Technology, Dunedin, NZ). Samples were homogenized in cold 100 mM phosphate buffer (pH = 7.4) plus protease inhibitors (2 mM AEBSF, 1 mM EDTA, 130 mM bestatin, 14 μM E-64, 1 μM leupeptin, 0.3 μM aprotinin) in a 3-ml glass homogenization tube (Sigma-Aldrich, St. Louis, MO). Briefly, all samples, standards, and quality controls were normalized to 1.8 mg/ml. Next, 11 μl of each sample, standard, and control were incubated in 19 μl of dinitrophenylhydrazine (DNPH) for 45 min at room temperature. Following derivitization with DNPH, 7.5 μl of each sample, standard, and control, were diluted with 1 ml of enzyme immunoassay (ELA) buffer provided with the kit. Manufacturer’s instructions were then followed.
starting with the section entitled ELISA procedure #3. All samples, standards, and controls were run in triplicate.

**HSF1 Nuclear Translocation and Phosphorylation**

Myocardial nuclear and cytosolic fractionation. Analysis of HSF1 cellular localization and phosphorylation status was performed as described previously (28, 29, 41, 48). Briefly, nuclear and cytosolic extracts were prepared from LVS using NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL) with the addition of protease (Pierce) and phosphatase (Sigma-Aldrich) inhibitors, as directed by the manufacturer. A section of LV was mixed and homogenized on ice in 100-mM phosphate buffer containing 0.05% bovine serum albumin and EDTA (1:10 wt/vol; pH 7.4) using a glass-on-glass tissue homogenizer. Homogenates were centrifuged at 500 g for 4 min at 4°C. The supernatant was removed and incubated for 10 min on ice in 200 µl CERI solution containing 0.75 mM PMSF, 2.0 mg/ml aprotinin and leupeptin, 20 mM NaF, and 2.0 mM NaN3VO4. CERII solution (11 µl) was then added, and cytoplasmic extracts were collected by centrifugation at 12,000 g for 5 min. Nuclear pellets were resuspended in 100 µl of NER solution containing 2 mM PMSF, 2.0 mg/ml aprotinin and leupeptin. Protein concentrations in the nuclear and cytosolic fractions were determined by methods described by Bradford (3).

**SDS PAGE and Western immunoblot analysis for HSF1.** Nuclear and cytosolic extracts were suspended in Laemmli sample buffer, denatured, loaded (100 µg protein), and separated on 7.5% polyacrylamide gels along with heat-shocked HeLa cell lysate (LYC-HL101F) and a molecular weight standard (Stressgen Biotechnologies, Victoria, BC, BioRad, Hercules, CA). Selected samples were treated with 80 U of calf intestine alkaline phosphatase at 37°C for 60 min before electrophoresis (Sigma-Aldrich). The separated proteins were then transferred to nitrocellulose membranes and blocked with 5% nonfat milk in Tris-buffered saline with 0.01% Tween-20 (TBST) for 2 h. Next, membranes were incubated in anti-HSF1 monoclonal primary antibody (SPA-950) overnight at 4°C (Stressgen Biotechnologies, Victoria, BC). After incubation in the primary antibody, membranes were washed three times for 10 min each and incubated with a secondary antibody conjugated to horseradish peroxidase for enhanced chemiluminescent detection (Amersham, Piscataway, NJ). Quantification of the bands from the immunoblots was performed using computerized densitometry (KODAK Image Station 400MM Digital Imaging System, Rochester, NY). To ensure equal transfer and loading of proteins, the Ponceau staining method was used (Sigma-Aldrich), and three nonspecific protein bands on the membrane were chosen for densitometric analysis. Given that no differences in protein loading were observed, bands were not normalized to Ponceau stained bands.

**RNA isolation and real-time RT-PCR**

Total RNA was extracted from the LV by placing a 50-µg tissue sample directly into 1.5 ml of TRIzol (Invitrogen, Carlsbad, CA) and homogenized on ice using a Polytron homogenizer (PowerGen 125, Fisher Scientific, Pittsburgh, PA) on a medium setting for three pulses, ~15 s per pulse. Homogenized samples were centrifuged at 13,000 g for 10 min at 4°C to remove insoluble material. The RNA portion was transferred to a new tube and extracted with 120 µl of bromochloropropane, vortexed, and centrifuged at 13,000 g for 10 min at 4°C. After transfer of the aqueous phase containing RNA to a new tube, RNA was precipitated with one volume of isopropanol and washed twice with two volumes of 75% ethanol. The RNA was then pelleted via centrifugation and resuspended with RNAse-free water (Sigma-Aldrich). Concentration and purity of the extracted RNA were measured spectrophotometrically at A260 and A280 in 1× TE buffer (Promega, Madison, WI). The integrity of the extracted total RNA was verified by gel electrophoresis on a 1% agarose ethidium bromide-stained Tris-borate-EDTA gel. Purified RNA was then stored at −80°C until later assay.

RT was performed using the SuperScript III First-Strand Synthesis System for RT-PCR according to the manufacturer’s instructions (Life Technologies, Carlsbad, CA). Reactions were carried out using 5 µg of total RNA and 2.5 µM oligo(dT)20 primers. First-strand cDNA was subsequently treated with two units of RNase H and stored at −80°C.

After the addition of 2 µl of GlycoBlue coprecipitant, first-strand cDNA was cleaned of RNA and unincorporated nucleotides by treating the cDNA with an RNAse cocktail, bringing the sample to a volume of 100 µl with water and applying the sample to a NucAway spin column (Ambion, Austin, TX). Samples were then mixed with phenol:chloroform:1 AA (pH = 7.9), and the aqueous phase was recovered using a 1.5-ml heavy-phase lock gel (Eppendorf, Hamburg, Germany). Finally, the cDNA was then precipitated by adding one volume of 5.0 M NaHAc and two volumes of 100% ethanol and stored at −20°C overnight. After centrifugation at 13,000 g for 20 min (4°C), the cDNA was washed with two volumes of 75% ethanol, centrifuged at 13,000 g for 10 min (4°C) and resuspended in 50 µl of 1× TE buffer. The cDNA was subsequently quantified using the Oligreen ssDNA Quantitation Reagent and Kit, according to the manufacturer’s instructions (Molecular Probes, Eugene, OR).

Primers and probes for HSP72 (assay Rn00583013_s1) were obtained from the ABI Assays-on-Demand service and consisted of TaqMan 5’ labeled FAM reporters and 3’ nonfluorescent quenchers. Primer and probe sequences from this service are proprietary and, therefore, are not reported. However, the nucleotide sequence surrounding the probe for HSP72 is available and consists of the following: 5′-GAGGATCTCGTGCACAAGCGGGAGG-3′. Primer and probe sequences also consisting of TaqMan 5’-labeled FAM reporters and 3’ nonfluorescent quenchers for hypoxanthine guanine phosphoribosyl transferase (HPRT) were obtained from Applied Biosystems (Assays-by-Design) forward, 5′-TTGGGATTACGGC-CAGACTTTGT-3′; reverse, 5′-AGTCAAGGGCATATCCAAC-3′; and probe, 5′-ACTGTCTGGAAATTTCA-3′.

Quantitative real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (ABI, Foster City, CA). Each 25-µl PCR reaction, performed in duplicate, contained 1 ng of cDNA template as determined by cDNA quantification described in previous sections. Gene expression was calculated using the relative standard curve method, as described in the ABI, User Bulletin #2. Briefly, amplification of the endogenous control was performed to standardize the amount of sample added to the reaction. For all experimental samples, the target quantity was determined from the standard curve and then divided by the target quantity of the calibrator, or C (see RESULTS). Specifically, the calibrator becomes the 1× sample, and all other quantities are expressed as an n-fold difference relative to the calibrator. In these experiments, HPRT was selected to normalize the mRNA because the expression of this gene in the LV is not significantly altered by exercise in our laboratory (P > 0.05).

**Statistical Analysis**

Comparisons among experimental groups for each dependent variable were made by one-way factorial ANOVA (group × dependent variable) within each experiment using SPSS version 12.0. Planned comparisons were used to determine changes in coronary flow and cardiac work for the working heart experiments. When significant main effects were observed, Tukey’s post hoc analyses were implemented. Significance was established a priori at P < 0.05.

**RESULTS**

**Animal Morphological Characteristics**

The morphological characteristics of the animals included in these experiments are reported in Table 1. Although animals...
were randomly assigned to experimental groups, animal body weights were significantly different between the two temperature groups \((P < 0.05)\). Importantly, although the body weights were different among temperature groups, no differences existed from control animals in heart weight or the heart weight/body weight ratio \((P > 0.05)\).

**Cardiac Functional Measures and Post-Exercise Left Ventricular Temperature**

These experiments were designed to investigate the independent effect of increased temperature and cardiac work on myocardial HSP72 expression. In these experiments, buffer temperature, as well as cardiac preloads and afterloads were controlled using an in vitro working heart model to simulate exercise conditions. The functional characteristics and LV temperature of the simulated exercise in in vitro working hearts are summarized in Table 2. Note that at the conclusion of the 60-min simulated exercise protocol, LV temperature was significantly higher in the 40°C buffer temperature groups compared with 37°C buffer temperature \((P < 0.001)\) but not different within temperature treatment groups \((P > 0.05)\).

Stable rates of CF were maintained in all simulated exercise groups throughout the course of the 60-min experiment \((P > 0.05)\). Moreover, Fig 1A also reveals that compared with all other experimental groups, CF was significantly higher in hearts perfused with 40°C buffer at a high load \((40°C \text{ HL})\; P < 0.001\). Additionally, the rates of CF in the hearts perfused with 37°C buffer at a high load \((37°C \text{ HL})\) were significantly lower than 40°C HL but significantly higher than both low-load groups \((P < 0.001)\). No differences in CF rates were observed between the low-load groups \((P > 0.05)\).

Fig. 1. Changes in coronary flow rates \(\text{(CF)}\) \(A\) and cardiac work \(\text{(B)}\) in response to buffer temperature and workload manipulated in isolated working hearts during 60 min of simulated exercise. HL, high load; LL, low load. \(A\): \(a\) = higher than all other groups \((P < 0.05)\); \(b\) = 40°C HL > 37°C HL > both low-load groups \((P < 0.05)\). \(B\): \(c\) = higher than all other groups \((P < 0.05)\); \(d\) = 40°C HL > 37°C HL > both low load groups \((P < 0.05)\).

### Table 1. Animal morphometric characteristics for the in vitro simulated exercise groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Body Weight, g</th>
<th>Heart Weight, g</th>
<th>Heart/Body Weight, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>396.17±2.40</td>
<td>118.0±0.7</td>
<td>3.02±0.14</td>
</tr>
<tr>
<td>40°C HL</td>
<td>7</td>
<td>390.71±6.19†</td>
<td>124±0.04</td>
<td>3.14±0.08</td>
</tr>
<tr>
<td>37°C HL</td>
<td>7</td>
<td>416.71±5.93§§</td>
<td>132±0.05</td>
<td>3.17±0.08</td>
</tr>
<tr>
<td>37°C LL</td>
<td>6</td>
<td>413.67±1.71‡‡</td>
<td>127±0.06</td>
<td>3.05±0.15</td>
</tr>
</tbody>
</table>

Values are means ± SE. 40°C and 37°C buffer temperature. HL, high load; 23 cm preload, 110 cm afterload; LL, low load, 15 cm preload, 60 cm afterload. †Significantly different from 37°C HL, \(P < 0.05\). ‡Significantly different from 37°C LL, \(P < 0.05\). §Significantly different from 40°C LL, \(P < 0.05\). Note: no differences in heart weight or the body weight/heart weight ratio existed among the groups, \(P > 0.05\).

### Table 2. Functional characteristics and end left ventricular temperature in hearts assigned to the in vitro simulated exercise groups

<table>
<thead>
<tr>
<th>Group</th>
<th>40°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HL ((n = 7))</td>
<td>LL ((n = 6))</td>
</tr>
<tr>
<td>AF, ml (\cdot) min(^{-1}) (\cdot) gww(^{-1})</td>
<td>45.85±4.73(^{b})</td>
<td>33.97±3.86</td>
</tr>
<tr>
<td>CO, ml (\cdot) min(^{-1}) (\cdot) gww(^{-1})</td>
<td>66.74±5.45(^{a})</td>
<td>42.35±4.02(^{a})</td>
</tr>
<tr>
<td>SP, mmHg</td>
<td>125.0±2.61(^{b})</td>
<td>71.92±3.02(^{e})</td>
</tr>
<tr>
<td>RPP, HR (\cdot) Sp</td>
<td>50023±1045(^{b})</td>
<td>28768±1208(^{c})</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>40.64±0.23(^{b})</td>
<td>40.44±0.17(^{b})</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. 40°C and 37°C = buffer temperature. 23 cm H\(_2\)O preload, 110 cm afterload; LL = 15 cm preload, 60 cm afterload, AF, aortic flow; CO, cardiac output; SP, systolic pressure; RPP, rate pressure product. \(^{a}\)Significantly different from all other groups, \(P < 0.05\). \(^{b}\)Significantly different from 37°C HL, \(P < 0.05\). \(^{c}\)Significantly different from 37°C LL, \(P < 0.05\). \(^{d}\)Significantly different from 40°C HL, \(P < 0.05\). \(^{e}\)Significantly different from 40°C LL, \(P < 0.05\).
Biochemical Measures

Protein oxidation. Protein carbonyls, a commonly used marker of protein oxidation, were measured in the LV from animals using an ELISA. Group comparisons revealed that no differences existed across experimental groups in LV protein carbonyl formation \((P > 0.05); C = 0.69 \pm 0.10, 40^\circ C \text{ HL} = 0.73 \pm 0.05, 40^\circ C \text{ LL} = 0.73 \pm 0.07, 37^\circ C \text{ HL} = 0.91 \pm 0.07; 37^\circ C \text{ LL} = 0.70 \pm 0.07 \text{ nmol/mg protein}\). It is important to note, however, that these data do not rule out the possibility that other forms of oxidative stress (i.e., lipid peroxidation) could have occurred in isolated working hearts. Moreover, it is also possible that a small number of cardiac proteins were oxidized in our experiments but this increase went undetected by the analysis of total protein carbonyls.

**HSF1 Nuclear Translocation and Phosphorylation**

To determine the subcellular location and phosphorylation status of HSF1 following exercise, an immunoblot specific to HSF1 was implemented (Figs. 2 and 3). The nuclear fractions of in vitro simulated exercise hearts exhibited a phosphorylated form of HSF1 demonstrated by a higher molecular weight band of HSF1 (Fig. 3). Importantly, this higher-molecular-weight band disappeared with the addition of alkaline phosphatase (\(~85 \text{ kDa}\)), confirming that this band represents phosphorylated HSF1. The nuclear fractions from both high temperature \((40^\circ C)\) group hearts contained significantly more total HSF1 compared with the C and \(37^\circ C\) groups without differences between loads, indicating that temperature elevation, not increased cardiac load, was the responsible stimulus for nuclear translocation (Fig. 2; \(P < 0.05\)). Importantly, the same pattern was observed regarding the accumulation of phosphorylated HSF1 in the nuclei, in which both \(40^\circ C\) groups contained significantly greater HSF1 accumulation than \(C\) and \(37^\circ C\) groups \((P < 0.05)\) suggestive that high temperature increased HSF1 phosphorylation (Fig. 3; \(P < 0.05\)). When expressed as a percentage of total nuclear HSF1, high-temperature groups exhibited a greater percentage of phosphorylated HSF1 compared with low-temperature groups (Fig. 3B; \(P < 0.05\)). Finally, no significant differences existed among groups in the cytosolic fractions of left ventricles (Fig. 4; \(P > 0.05\)).

**HSP72 mRNA Expression**

As depicted in Fig. 5, temperature had a powerful effect on the upregulation of myocardial HSP72 mRNA levels. Strikingly, \(40^\circ C\) HL animals exhibited a 98-fold higher mRNA expression and \(40^\circ C\) LL animals exhibited an 86-fold higher expression compared with low-temperature and control groups \((P < 0.001)\). Notably, the temperature-induced upregulation of myocardial HSP72 mRNA did not differ among the low and high workloads, suggesting that myocardial work does not play a significant role in HSP72 gene expression. Finally, we did not measure HSP72 protein levels in these experiments because of the short time period (i.e., 60 min) available for protein synthesis.

**DISCUSSION**

**Overview of Principle Findings**

To determine the signals responsible for the exercise-induced HSP72 expression in the heart, these experiments investigated the independent effect of myocardial temperature and cardiac workload on HSP72 expression in a simulated exercise model. Specifically, we tested the hypothesis that thermal stress and mechanical load provide independent stimuli for HSF1 activation and HSP72 gene expression in the heart during simulated exercise. The principle finding of the current investigation is that elevated cardiac temperature is a requirement for HSP72 gene expression following simulated exercise, whereas increased cardiac work does not initiate the transcription of HSP72 alone. Further, these data reveal that the acquisition of transcriptional competency in the heart may be due, at least in part, to phosphorylation of nuclear-localized HSF1 after an increase in heart temperature.

**Exercise-mediated temperature increase provides a proximal stimulus for HSF1 activation.** While it is known that HSP72 synthesis is transcriptionally regulated by HSF1, the...
experimental groups (1, 7, 49). Therefore, the possibility exists that heat-induced elevation in cellular temperature is not the direct activator of HSP72 expression, thereby arguing against HSF1 as a direct sensor of heat stress. The temperature at which HSF1 is activated by heat-induced factors, such as circulating hormones, cytokines, or proteins, is not absolute, and the possibility exists that heat-induced elevated temperature is not the direct activator of HSP72 expression. Indeed, some studies indicate that the increased cardiac temperature during exercise could activate HSF1 either by directly sensing heat or indirectly by competing for damaged proteins.

Increased cardiac load is not a primary stimulus to promote HSF1 activation. In addition to temperature elevation, increased cardiac preload and afterload could promote HSP72 expression during exercise. Previously, it has been reported that mechanical stretch and pressure overload can induce HSP72 expression in a variety of different cells and tissues (8, 46, 47). Investigations using a Langendorff heart preparation, whereby balloon inflation mechanically stretches the left ventricle, was followed by a concomitant increase in HSP72 mRNA (6, 17, 19). Additionally, another group of experiments using the Langendorff preparation revealed that increased preload, but not afterload, directly induced the activation of HSF1 in the left ventricle (36).

In the current experiments, we used an in vitro working heart model because of its physiological relevance as a model of cardiac work that closely mimics the cardiac work occurring with exercise. Our findings clearly indicate that increased cardiac work (i.e., increased preload and afterload) does not promote HSP72 expression (37, 39). Regarding temperature and HSF1 activation, the intramolecular mechanism by which HSF1 directly or indirectly senses high temperatures, could be related to a conformational change in HSF1, such that the monomeric form creates the HSF1 trimer (37, 39). Modulation of HSF1 activity by increased cellular temperatures could result from structural damage to cellular proteins exposing hydrophobic regions in these unfolded polypeptides that compete for binding with HSP72. This competition results in HSF1 release from HSP72 and initiates HSF1 activation. Indeed, a variety of environmental and chemical conditions has been linked to the generation of unfolded proteins to activate the HSF1 transcriptional response, particularly in response to temperature elevation (37, 39). Therefore, elevated cardiac temperature during exercise could activate HSF1 either by directly sensing heat or indirectly by competing for damaged proteins.

sensory mechanisms that relay intracellular signals produced during exercise to mediate HSP72 transcription and translation are unclear. Nonetheless, evidence indicates that HSF1 activation involves a complex series of regulatory events, including nuclear localization, oligomerization, and acquisition of HSE-DNA binding, ultimately resulting in the transcription of the HSP70 gene (40). Several reports show that exercise in a warm environment promotes HSP72 expression, whereas exercise in the cold is not associated with increased HSP72 expression (15, 16, 22, 25, 27, 43). These observations led us to hypothesize that the increased temperature is an important proximal stimulus to initiate HSP72 transcription. The postulate that temperature is the critical upstream HSF1 activator is supported by several lines of evidence, including reports indicating that HSF1 can directly and indirectly sense temperature. For example, several studies have shown that HSF1 is directly activated by temperature since purified HSF1 can bind to the HSE when subjected to heat (10, 13, 49). In the current study, we observed that a significant increase in heart temperature was followed by increased HSP72 expression. This finding is consistent with the notion that an increase in cellular temperature is a critical upstream stimulus mediating exercise-induced HSP72 expression in the absence of other exercise-mediated factors, such as circulating hormones, cytokines, or neural components.

In contrast to the present finding that temperature plays a direct role in HSP72 expression, others have argued that elevated temperature is not the direct activator of HSP72 expression (1, 7, 49). Indeed, some studies indicate that the temperature at which HSF1 is activated is not absolute, thereby, arguing against HSF1 as a direct sensor of heat stress (1, 7, 49). Therefore, the possibility exists that heat-induced changes in the cellular environment can indirectly activate HSF1. For example, exposure to heat induces numerous physiological changes in the organism, including the release of stress hormones, free radical production, changes in tissue oxygenation, thermal unfolding of proteins, and an increase in proteolytic activity. Any one of these factors can indirectly promote HSP72 expression (37, 39).

Fig. 4. Temperature and load-induced cytosolic localization of HSF1 following in vitro simulated exercise determined by SDS-PAGE and Western immunoblot analysis. A representative blot (A) and analysis (B) of the total cytosolic HSF1 content in the left ventricle from simulated exercise groups. Values are means ± SE. No significant differences were observed among experimental groups (P > 0.05).

Fig. 5. Real-time quantitative RT-PCR analysis of HSP72 mRNA expression, normalized to HPRT in the left ventricle from in vitro simulated exercise groups. Values are means ± SE. *Significantly higher than control and low temperature groups (P < 0.05).
result in the achievement of HSF1 transcriptional competence, as our euthermic high workload experimental groups did not exhibit increased HSP72 mRNA levels compared with low-workload experimental groups. These findings are in agreement with a previous study, indicating that working hearts showed little activation of HSF1 compared with the Langendorff-perfused hearts (6). Therefore, on the basis of the lack of load-induced activation of HSF1 in the simulated exercise experimental groups, we conclude that increased cardiac workload does not appear to be an independent stimulus regulating myocardial HSP72 expression during exercise.

Heat-induced HSP72 expression in the heart follows nuclear translocation and phosphorylation of HSF1. HSP72 synthesis is primarily regulated at the transcriptional level by the transcription factor HSF1 (5, 14, 38). Activation of HSF1 involves a complex, dynamic series of regulatory events, including oligomerization of HSF1, followed by nuclear localization from the cytosol, phosphorylation of HSF1, and binding of HSF1 to the HSE on DNA (5, 14, 38). By measuring HSF1-HSE binding, the gel-mobility shift assay has generally been used to demonstrate HSF1 activation by us and others (9, 48). However, this assay does not quantitatively measure HSF1 activity (41). Several studies suggest that events downstream of HSE-DNA binding regulate HSF1 transcriptional activity, as it has been reported that HSF1 binding on DNA can occur without the acquisition of transcriptional activity (4, 9, 18, 29, 41, 48, 50). Indeed, it appears that for transcriptional activation to occur after DNA binding, HSF1 trimers must undergo an additional and required stress-induced step such that hyperphosphorylation occurs on specific serine residues to render trimerized HSF1 transcriptionally competent (4, 5, 14, 18, 29, 45, 50). Therefore, our approach to detect HSF1 activation was modeled after the work of Shinohara et al. (41) and involved a quantitative assessment of the translocation and phosphorylation of HSF1 from the cytosolic to the nuclear fraction of the cell.

In agreement with others, our results support the notion that additional transcriptional activating events occur downstream of HSF1-HSE DNA binding (8, 9, 22, 27, 40, 41, 61, 65). The current data demonstrate that increased cardiac temperature, and not cardiac workload, is a key factor that determines both the level of HSF1 accumulation in the nucleus and the degree of HSF1 phosphorylation. Our results reveal that normothermic hearts subjected to increased cardiac preloads and afterloads do not experience an increase in nuclear levels of phosphorylated HSF1; this finding could explain, at least in part, the failure of these hearts to achieve transcriptional competence (Figs. 2, 3, and 5). Moreover, our data show that transcriptional competence is mediated by hyperphosphorylation of HSF1 as evidenced by the finding that hearts in the high temperature groups exhibited higher levels of phosphorylated HSF1 and HSP72 mRNA compared with normothermic hearts. Although it is generally believed that HSP72 synthesis in the heart is primarily regulated via transcriptional control, it should be noted that some evidence suggests that modulation of HSP72 protein synthesis can also be regulated by posttranscriptional processes (20, 30, 44). Under specific in vitro experimental conditions, it appears that the heat shock response in cells can be partially regulated at the posttranscriptional level with control mechanisms regulating both mRNA stability and initiation of mRNA translation through the HSP72 3’-untranslated region (35). Therefore, although transcriptional competence involves several factors such as phosphorylation, posttranscriptional factors may also influence the rate of HSP72 protein accumulation in the cell. Given that the time for these processes to occur exceeds our 60-min experimental protocol, we did not measure HSP72 protein content in the present investigation. Understanding the posttranscriptional factors postexercise presents an interesting area for future research.

In conclusion, these experiments investigated the independent influence of myocardial temperature and cardiac workload on the essential molecular steps leading to HSP72 expression in the heart. The current data implicate elevated heart temperature associated with simulated exercise as a stimulus that is sensed either directly, indirectly, or both by HSF1. Further, our finding that HSF1 activation only occurs in hearts with elevated temperatures indicates that temperature may be the critical mediator of HSP72 in transcriptional signals. Therefore, it seems likely that the increase in heart temperature associated with simulated exercise may impact the activity of HSF1 at multiple levels to render HSF1 transcriptionally competent. Moreover, our data reveal that heat-related acquisition of transcriptional competency follows phosphorylation of nuclear-localized HSF1. Collectively, our results, along with previous investigations, suggest that a potential mechanism to explain the failure of euthermic hearts in exercised animals to increase HSP72 mRNA expression during exercise in the cold is due to the inability to acquire transcriptional competence (15, 16, 43).

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REFERENCES


