Insulin induces myocardial protection and Hsp70 localization to plasma membranes in rat hearts

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Li, Gefeng, Imtiaz S. Ali, and R. William Currie. Insulin induces myocardial protection and Hsp70 localization to plasma membranes in rat hearts. Am J Physiol Heart Circ Physiol 291: H1709–H1721, 2006. First published May 26, 2006; doi:10.1152/ajpheart.00201.2006.—Insulin induces the expression of the 70-kDa heat shock protein (Hsp70) in rat hearts. In this study, we examined insulin- and heat shock-treated hearts for improved contractile recovery after 30 min of ischemia, activation of the heat shock transcription factor, and localization of the Hsp70 in relation to dystrophin and α-tubulin. Adult male Sprague-Dawley rats were assigned to groups: 1) control, 2) sham control, 3) insulin injected (200 μU/g body wt), 4) heat shock treated (core body temperature 42°C for 15 min), and 5) heat shock and insulin treated. Six hours later, hearts were isolated for Langendorff perfusion to determine cardiac function, or myocardial tissues were collected and prepared for either electrophoretic mobility shift assay, Western blot analysis, or immunofluorescence microscopy. Insulin treatment with 6 h of recovery enhances posts ischemic myocardial recovery of contractile function and increases Hsp70 expression through activation of the heat shock transcription factor. Insulin-treated hearts had elevated levels of Hsp70, particularly in the membrane fraction. In contrast, heat-shocked hearts had elevated levels of Hsp70 in the cytosol, membrane, and pellet fractions. After insulin treatment, Hsp70 was mostly colocalized to the plasma membrane with dystrophin. In contrast, after heat shock, Hsp70 was localized mostly between cardiomyocytes in apparent vascular or perivascular elements. The localization of Hsp70 is dependent on the inducing stimuli of either heat shock or insulin treatment. The cell membrane versus vascular localization of Hsp70 suggests the interesting possibility of functionally distinct roles for Hsp70 in the heart, whether induced by insulin or heat shock treatment.

heat shock; heat shock protein 70; heat shock transcription factor; ischemia/reperfusion

INSULIN IS A CRUCIAL REGULATOR of metabolism, and in cardiac muscle, insulin induces glycogen and protein synthesis, and in fat, lipid storage is favored. Insulin also mediates other diverse effects in a wide variety of cells and tissues (57). Sodi-Pallares et al. (67) initially introduced the concept of infusing a metabolic cocktail of glucose, insulin, and potassium to improve recovery from myocardial infarction. Clinical studies (20, 22, 63) suggest that this cocktail might have an important role in reducing in-hospital mortality after acute cardiac infarction. Initially, two possible mechanisms for the effectiveness of the glucose, insulin, and potassium cocktail were suggested: 1) the promotion of cardiac glycolysis and 2) the uptake of free fatty acids into adipocytes (58). More recently, insulin alone has been shown to be as effective as the glucose, insulin, and potassium cocktail at reducing myocardial ischemia-reperfusion injury (32). The cardioprotective effect of insulin may be mediated via phosphatidylinositol 3-kinase, protein kinase B (PKB/Akt), and p70S6 kinase cell signaling (64) or by increasing nitric oxide bioavailability (24). Nitric oxide is proposed as the “second messenger” of insulin. A physiological dose of insulin activates membrane-bound NO synthase. Injection of L-arginine methyl ester before the administration of insulin to mice inhibited not only the insulin-stimulated increase of nitric oxide in plasma but also the glucose-lowering effect of insulin (33). Another mechanism of insulin-activated cardioprotection may be related to the expression of heat shock proteins (Hsps). Various Hsps have cytoprotective functions (5, 19, 25, 31). As molecular chaperones, Hsps regulate folding of nascent proteins, participate in refolding of misfolded or damaged proteins, stabilize structural proteins, and facilitate translocation of proteins across membranes among cellular compartments. Several Hsps act to prevent aggregation of protein and target unstable or damaged protein for degradation. The inducible 70-kDa and 27-kDa heat shock proteins (Hsp70 and Hsp27) have also been shown to suppress intracellular apoptotic signaling pathways (53, 60).

Hsps and particularly Hsp70 is associated with enhanced recovery of myocardial contractility after ischemic injury (16, 31). In fact, transgenic overexpression of rat and human Hsp70 provided strong evidence for a direct role in protection of the mouse myocardium from ischemic injury (47, 61). Interestingly, insulin induces Hsp70 in the hepatoma cell line Hep3B/T2, and this effect is transient and occurred between 4 and 8 h after insulin treatment (68). We recently reported that the highly inducible Hsp70 is indeed induced by insulin, albeit at a relatively low level, in the rat heart. Insulin appeared to have an additive effect on the expression of Hsp70 after heat shock treatment (41). While we have shown that, after heat shock, Hsp70 is mainly localized to microvessels between cardiomyocytes in the rat heart (40), after insulin treatment, the localization of Hsp70 was unclear.

In this study, we show that insulin improves posts ischemic myocardial contractility and that expression of Hsp70 is elevated by activation of the heat shock transcription factor (HSF), and we report the subcellular distribution and localization of the constitutive form of the 70-kDa heat shock protein (Hsc70) and inducible Hsp70 after insulin treatment and after heat shock treatment.

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MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (250–325 g, Charles River, St. Constant, QC, Canada) were used in these experiments. All animal care, handling, and experimental procedures on animals were in accordance with the Guide to Care and Use of Experimental Animals of the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animals.

Experimental Protocol and Groups

Rats were randomized into five groups: 1) CON, naïve control; 2) SHAM, sham control injected with normal saline and with recovery for 6 h; 3) INS, insulin treated; 4) HS, heat shock treated; and 5) HSINS, heat shock and insulin treated. For all groups, rats were anesthetized with pentobarbital sodium (50 mg/kg ip). Naïve control rats received an injection of pentobarbital sodium but were not heated or injected with insulin. For sham control rats, normal saline was injected intramuscularly, similar to the insulin treatment group. For insulin treatment, rats were injected intramuscularly in the thigh with 200 μU/g body wt. For heat shock treatment, rats were placed on a temperature-controlled heating pad (50°C) until core body temperature reached 42°C, monitored with a rectal thermometer. Core body temperature was maintained between 42°C and 42.5°C for 15 min. For heat shock and insulin treatment, rats were subjected to the heat shock treatment first, and at 10 min after the heat shock treatment, they were injected with insulin (200 μU/g body wt) intramuscularly in the thigh.

At 6 h of recovery, rats were injected with pentobarbital sodium (50 mg/kg) and decapitated. For heart functional analysis, hearts were rapidly extirpated and mounted on a Langendorff perfusion apparatus. For cell fractionation and subsequent Western analysis, hearts were removed, perfused briefly with cold (4°C) normal saline to remove blood, and then homogenized. For electrophoretic mobility shift assay, hearts were rapidly extirpated and immediately freeze clamped. The frozen tissue and cell fractions were stored in liquid nitrogen before protein content determination (45), electrophoretic mobility shift assay, and Western blot analysis. For immunofluorescence microscopy, hearts were collected, perfused with a syringe and rinsed with cold saline (4°C) until clean (~1 min), and then immersed and fixed in 2% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4°C overnight.

Langendorff Perfusion and Cardiac Function

Hearts were perfused in the nonworking Langendorff mode as described previously (16). Modified Krebs-Henseleit bicarbonate buffer (1) (in mM: 118.5 NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂, 0.5 EDTA, and 11 glucose, pH 7.4) gassed with 95% O₂-5% CO₂ at 37°C was perfused into hearts at a constant rate of 10 ml/min with a static roller pump. Hearts were equilibrated with 95% O₂-5% CO₂ at 37°C until core body temperature (LVDP), was calculated. After 30 min of equilibration, all hearts except for SHAM were subjected to 30-min no-flow global ischemia. SHAM hearts were continually perfused at 10 ml/min. After ischemia, hearts were reperfused at 10 ml/min for 60 min. Physiological measurements were taken at 10 min before initiation of ischemia (time = −40 min) during preischemic perfusion and at 5, 30, and 60 min of reperfusion.

Analysis of LV Function and Pressure-Volume Relationship

Analysis of LV function and calculation of pressure-volume relationship were done according to Li et al. (42) and Burkhoff and Sagawa (10) with minor modifications. In brief, analysis of LV performance was derived from the measured LVESP and LVEDP data. The volume in the balloon was increased by using 37°C normal saline in 25-μl increments to a maximum volume of 200 μl. A linear regression analysis of LVESP versus volume was performed to estimate the LV end-systolic pressure-volume relationship. To estimate LV work (LVW), we calculated cumulative areas under the LVESP versus volume curve to produce the diastolic work area (DWA), i.e., the area under the diastolic regression curve and under the LVEDP versus volume curve to produce the diastolic work area (DWA), i.e., the area under the diastolic regression curve. SWA and DWA were inserted into the following formula to find LVW.

\[ \text{LVW (mmHg} \cdot \text{ml)} = \text{SWA} - \text{DWA} \]

Coronary vascular resistance (CR) was derived from constant perfusion flow (10 ml/min) and mean pressure perfusion (MAP) with the following formula.

\[ \text{CR (mmHg} \cdot \text{ml}^{-1} \cdot \text{min}^{-1}) = \text{MAP (mmHg)} / 10 \text{ ml/min} \]

Preparation of Cell and Tissue Extracts and Electrophoretic Mobility Shift Assay

Cell and tissue extracts were prepared as described by Mosser et al. (52) and Locke et al. (43) with minor modifications. U-937 human monocytic leukemia cells (ATCC) were grown in culture flasks in RPMI 1640 medium (95% air-5% CO₂) with l-glutamine (Hyclone, Logan, UT), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For heat shock, the U-937 cells (1 × 10⁶ cells) were placed in an incubator at 42°C for 30 min. After heat shock, cells in culture medium were centrifuged in 10-ml tubes at 1,200 rpm at 4°C for 5 min. Pellets were washed twice with 1× PBS, pH 7.4 (Sigma). One-hundred microliters of extract buffer [20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA (pH 8.0), 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml antipain, and 1 μg/ml aprotinin] were added to the washed pellets, homogenized, and incubated on ice for 30 min. Homogenates were centrifuged at 20,000 g (14,000 rpm) at 4°C for 30 min. Supernatants were collected, frozen in liquid nitrogen, and stored at −70°C. Rat hearts from the five experimental groups and the 0.5-h post-ischemia hearts were centrifuged in 10-ml tubes at 150–200 mg) was homogenized with a Polytron at 4°C in 0.75 ml of extract buffer. The homogenate was incubated on ice for 30 min. The homogenate was centrifuged, and the supernatant was collected and stored at −20°C. Protein concentration was determined by the method of Lowry et al. (45).

Electrophoretic Mobility Shift Assay

Nonradioactive electrophoretic mobility shift assay (EMSA) kit for HSF-1 was obtained from Panomics (AY1329, Redwood, CA). Biotin-labeled or nonlabeled heat shock element (HSE) oligonucleotide sequence (5’-CTGGAATATTCCCGACCTGGCAGCCTCATC) was provided with the kit (6). Our method followed the manufacturer’s instructions. In brief, 10-μg protein samples from U-937 cell extracts and 50-μg rat cardiac extracts were mixed with 2 μl of 5× binding buffer, 1 μl of poly d(I-C), and 1 μl of biotin-labeled HSE oligonucleotide and brought to 10-μl total volume by adding distilled H₂O (dH₂O). After 30-min incubation at room temperature, 1 μl of loading dye buffer was added to stop the reaction. Ten microliters of this mixture were loaded on a 6% natural gel [1 ml of 10× tris-borate EDTA buffer, 4 ml of 30% (29:1) acrylamide/bis-acrylamide, 625 μl of 80% glycerol, 14.375 ml of dH₂O, 300 μl of 10% ammonium]

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persuade, and 20 μl of N,N',N'-tetramethylthymidamine). Gels were prerun for 15 min at 120 V, the reaction mixture was loaded and separated at 120 V for ~55 min in 0.5× tris-borate EDTA buffer on ice on a Bio-Rad minigel electrophoretic unit. Gel reaction products were electrophoretically transferred onto presoaked Bioydyne B membrane (catalog no. 60201, Pall). Reaction products were cross-linked to the membrane with an ultraviolet crosslinker for 3 min. Membranes were incubated in 1× blocking buffer (EMSA kit, AY1329, Panomics) for 15 min with a gentle agitation and then for an additional 15 min with the addition of streptavidin-horseradish peroxidase conjugate (1:1,000). After three 8-min washes in wash buffer, membranes were incubated in 20 μl of 1× detection buffer for 5 min, and then 2 μl of display buffer were added to each membrane for 5 min. Membranes were then exposed to X-ray film (Hyperfilm, Amersham). The supershift assay was done with 66× nonlabeled HSE oligonucleotide sequence added to the sample 10 min before the addition of 1× biotin-labeled HSE oligonucleotide sequence. The supershift assay was repeated with minor modifications (11). In brief, following the manufacturer's instructions, fresh cardiac tissue (50 mg) was collected and sonicated in a probe sonicator using four 10-s bursts followed by 60 s of no sonication in 0.5-ml membrane extraction buffer one (M1). The sample tubes were kept on ice during the procedure. Next, 0.5 ml of prechilled membrane extraction buffer two (M2) was added to the sample and mixed by vortex and then incubated on ice for 10 min. Samples were transferred to a 37°C heating block and incubated for 30 min with occasional (3 to 4 times) mixing. Samples were centrifuged at 16,000 g for 5 min at room temperature. Protease inhibitors of leupeptin, pepstatin A, and aprotinin (1 μg/ml, respectively) were added to buffer one (M1) and buffer two (M2) just before use. Buffer M1 and M2 were kept on ice for 15 min before use. Aqueous phase, detergent-rich phase, and homogenized tissue before phase separation was collected. The aqueous phase is enriched with soluble cytoplasmic proteins. The detergent-rich phase is enriched with proteins anchored to the membrane or proteins containing one or two transmembrane domains. The collected fractions were immediately frozen in liquid nitrogen and then kept at ~70°C until analyzed. All fractions were assayed for membrane alkaline phosphatase assay as a membrane marker (8, 48). Protein concentration in each fraction was determined by the RC DC method recommended by manufacturer of ReadyPrep Membrane I (Bio-Rad).

Plasma membrane separation. Commercially available membrane separation kit was from Bio-Rad (ReadyPrep, Membrane I; Bio-Rad, Hercules, CA). This kit is based on the method of Bordier (7). In brief, following the manufacturer's instructions, fresh cardiac tissue (50 mg) was collected and sonicated in a probe sonicator using four 10-s bursts followed by 60 s of no sonication in 0.5-ml membrane extraction buffer one (M1). The sample tubes were kept on ice during the procedure. Next, 0.5 ml of prechilled membrane extraction buffer two (M2) was added to the sample and mixed by vortex and then incubated on ice for 10 min. Samples were transferred to a 37°C heating block and incubated for 30 min with occasional (3 to 4 times) mixing. Samples were centrifuged at 16,000 g for 5 min at room temperature. Protease inhibitors of leupeptin, pepstatin A, and aprotinin (1 μg/ml, respectively) were added to buffer one (M1) and buffer two (M2) just before use. Buffer M1 and M2 were kept on ice for 15 min before use. Aqueous phase, detergent-rich phase, and homogenized tissue before phase separation was collected. The aqueous phase is enriched with soluble cytoplasmic proteins. The detergent-rich phase is enriched with proteins anchored to the membrane or proteins containing one or two transmembrane domains. The collected fractions were immediately frozen in liquid nitrogen and then kept at ~70°C until analyzed. All fractions were assayed for membrane alkaline phosphatase assay as a membrane marker (8, 48). Protein concentration in each fraction was determined by the RC DC method recommended by manufacturer of ReadyPrep Membrane I (Bio-Rad).

Western analysis. Heart tissues samples containing 5 μg of protein were solubilized in sodium dodecyl sulfate (SDS) sample buffer, boiled for 10 min, and loaded onto a mini-SDS-polyacrylamide gel (2.5% upper gel, 7.5% running gel) according to previously described methods with minor modifications (11). To do densitometric quantitative analysis, all samples on gel were duplicated. Proteins were separated by electrophoresis (75 V for 20 min and 125 V for 45 min) and then electrotransferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore), using 100 V for 1 h in ice-chilled transfer buffer. Membranes were incubated in 5% skim milk in 1× Tris-buffered saline with Tween 20 (0.1% Tween 20) as previously described (11) at room temperature for 1 h to block nonspecific binding of primary antibody. Membranes were incubated at 4°C overnight with primary mouse monoclonal anti-Hsp70 antibody (1: 5,000, SPA-810, StressGen) or primary polyclonal rabbit anti-Hsc70 antibody (1:5,000, SPA-816, StressGen), and rabbit polyclonal anti-actin antibody (1:2,000, A-2066, Sigma, St. Louis, MO) in 5% milk in 1× TBST. Next day, after two quick changes of 1× TBST and three 5-min washes at room temperature, membranes were incubated in secondary horse anti-mouse horseradish peroxidase-conjugated antibody (1:10,000, PI-2000, Vector, Burlingame, CA) and goat anti-rabbit horseradish peroxidase-conjugated antibody (1:10,000, PI-1000, Vector). After two quick washes and three 5-min washes at room temperature, membranes were incubated in ECL Plus solution for horseradish peroxidase-labeled antibody (1 ml per membrane, RPN-2132, Amersham Biosciences) for 5 min and then quickly washed in dH2O. Chemiluminescence was directly detected on a STORM 840 scanner with a fluorescence setting at excitation of 430 nm, emission of 503 nm, Photo Manager Two (PMT) 600 V and pixel size 100 μm (Molecular Dynamics). Densitometric analyses for one-dimensional gels were done with imaging software (ImageQuant TL V.2003, Amersham Biosciences).

Confluent Immunofluorescence Microscopy

Tissue preparation. After overnight fixation in 2% paraformaldehyde in 0.1 M PBS (pH 7.4, 4°C), tissues were cryoprotected in 30% sucrose in 0.1 M PBS (pH 7.4). Tissue sections were then cut at 20 μm with a freezing slicing microtome, and sections were kept in Millicon’s solution at 4°C.

Immunofluorescence. To characterize the localization of Hsp70 and Hsc70 in cardiac tissue and their relationship with components of the cell, sections were double labeled for either dystrophin, a cell membrane protein, or β-tubulin, a cytoskeletal protein. Sections were incubated with primary rabbit polyclonal antibody against Hsp70 (catalog no. SPA-812, StressGen) or Hsc70 (catalog no. SPA-816, StressGen), and either mouse monoclonal anti-dystrophin [1:100 in 0.1 M PBS buffer with 0.1% Triton X-100, pH 7.4 (PBST) and 1% BSA, clone MANDRA, catalog no. D-8043, Sigma] (28), mouse monoclonal anti-α-tubulin (1:1,000 in PBST and 1% BSA, clone B-5-1-2, catalog no. T-5168, Sigma), or mouse monoclonal anti-β-tubulin (1:1,000 in PBST and 1% BSA, clone 2-28-3, catalog no. T-5293, Sigma) antibody (18). For double labeling of Hsp70 or Hsc70 and antibodies described above, tissue sections were processed as previously described (40, 41) with minor modifications. In brief, tissue sections were washed 3 × 10 min in PBST at room temperature and then blocked with 10% normal goat serum (Vector) in PBST for 1 h. Tissue sections were washed 1 × 10 min in PBST. After the wash, sections were incubated in primary antibodies at the concentration mentioned above at 4°C overnight. Next day, the tissue sections were washed with 0.01 M PBS (pH 7.4) three times. Tissue sections were then incubated in secondary antibodies (1:400 in PBS and 1% BSA, Alexafluor 546-conjugated goat anti-rabbit IgG and Alexafluor 488-conjugated goat anti-mouse IgG, Molecular Probes) at room temperature for 2 h. Finally, tissue sections were washed 3 × 10 min in 0.01 M PBS and then mounted onto gelatinized slides. Sections were allowed to dry in the dark overnight at room temperature. Sections were coverslipped with Prolong Gold mounting media (Molecular Probes) and sealed. In each batch of sections stained for confocal fluorescence microscopy, some sections were incubated without primary antibody or secondary antibody to serve as control for nonspecific staining. A Carl Zeiss Axiosvert 200 laser-scanning microscope was used to Z section for confocal imaging at 1 μm. Images were captured with a charge-coupled device camera and LSM 510 META software (version 3.2). Captured images were edited, adjusting only brightness and contrast, and composited with Photoshop (version 7.0, Adobe).

Statistical Analysis

Linear regression analysis was done to derive the constant and slope for the pressure-volume relationship (10, 41). Cardiac function data were normalized to the preischemic values. The normalized indexes were analyzed with the general linear model for repeated measures and Bonferroni test for multicomparison (SPSS,
V13.0, SPSS, Chicago, IL). Values of Hsp70 and actin were calculated from densitometric data of Western blots. Hsp70 densitometric values of each treatment group were compared with the control group. Quantitative data were analyzed with one-way or two-way analysis of variance and Bonferroni multicomparison analysis. All statistical results were confirmed with nonparametric tests. The results are expressed as means ± SE. Significance was set at \( P \leq 0.05 \).

Preischemia control values are means ± SE. HR, heart rate; LVDP, left ventricular (LV) developed pressure; \( +\text{dP/dt} \) and \( -\text{dP/dt} \), maximal first derivative of LV pressure increase and decrease, respectively; CR, coronary vascular resistance; CON, naïve control; SHAM, sham control; INS, insulin treated; HS, heat shocked; HSINS, heat shock and insulin treated. There was no significant differences among groups (\( P > 0.05 \)).

### Table 1. Preischemia and sham values of cardiac function

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>HR, beats/min</th>
<th>LVDP, mmHg</th>
<th>LVW, mmHg/μl</th>
<th>( +\text{dP/dt}, \text{ mmHg/s} )</th>
<th>( -\text{dP/dt}, \text{ mmHg/s} )</th>
<th>CR, mmHg · ml⁻¹ · min</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>7</td>
<td>267 ± 14</td>
<td>71 ± 17</td>
<td>7,377 ± 1,886</td>
<td>2,007 ± 490</td>
<td>1,157 ± 262</td>
<td>5.9 ± 1.2</td>
</tr>
<tr>
<td>SHAM</td>
<td>8</td>
<td>277 ± 9</td>
<td>97 ± 9</td>
<td>10,038 ± 1,370</td>
<td>2,944 ± 392</td>
<td>1,581 ± 167</td>
<td>4.8 ± 0.5</td>
</tr>
<tr>
<td>INS</td>
<td>10</td>
<td>282 ± 6</td>
<td>13 ± 10</td>
<td>7,035 ± 1,254</td>
<td>3,295 ± 338</td>
<td>1,320 ± 199</td>
<td>5.8 ± 0.6</td>
</tr>
<tr>
<td>HS</td>
<td>7</td>
<td>311 ± 15</td>
<td>107 ± 8</td>
<td>10,394 ± 1,342</td>
<td>3,468 ± 469</td>
<td>1,836 ± 203</td>
<td>6.2 ± 0.5</td>
</tr>
<tr>
<td>HSINS</td>
<td>7</td>
<td>294 ± 15</td>
<td>87 ± 14</td>
<td>8,560 ± 1,711</td>
<td>2,396 ± 370</td>
<td>1,439 ± 239</td>
<td>8.4 ± 1.3</td>
</tr>
</tbody>
</table>

Preischemia control values are means ± SE. HR, heart rate; LVDP, left ventricular (LV) developed pressure; \( +\text{dP/dt} \) and \( -\text{dP/dt} \), maximal first derivative of LV pressure increase and decrease, respectively; CR, coronary vascular resistance; CON, naïve control; SHAM, sham control; INS, insulin treated; HS, heat shocked; HSINS, heat shock and insulin treated. There was no significant differences among groups (\( P > 0.05 \)).

Fig. 1. Insulin treatment followed by 6 h improves cardiac function during postischemic reperfusion. Experimental group treatments are described in Experimental Protocol and Groups. Animals were recovered for 6 h before isolation and perfusion of hearts. Hearts were equilibrated to perfusion for 30 min at a flow rate of 10 ml/min. Sham control (SHAM) hearts were continually perfused at 10 ml/min (not subjected to ischemia). IS indicates the 30 min ischemic interval for the naïve control (CON), insulin-treated (INS), heat-shocked (HS), and heat shock- and insulin-treated (HSINS) hearts. Hearts were reperfused at 10 ml/min for 60 min starting at time \( t = 0 \). Data are normalized to preischemia values. A: insulin improved left ventricular (LV) developed pressure (LVDP) during reperfusion. For LVDP, insulin-treated hearts (INS and HSINS) were not different from SHAM (nonischemia) hearts. Significant differences are as follows: at 5 min of reperfusion: SHAM and INS vs. CON, \( P = 0.004 \) and \( P = 0.026 \), respectively; vs. HS, \( P = 0.001 \) and \( P = 0.008 \), respectively; and at 30 min of reperfusion: SHAM and HSINS vs. CON, \( P = 0.006 \) and \( P = 0.012 \), respectively; vs. HS, \( P = 0.018 \) and \( P = 0.037 \), respectively. B: insulin improved LV work (LVW) during reperfusion. For LVW, insulin-treated hearts (INS and HSINS) were not different from SHAM hearts. At 5 min of reperfusion, LVW of SHAM and INS hearts was significantly greater than CON (\( *P = 0.007 \) and \( P = 0.015 \), respectively) and HS hearts (\( P = 0.012 \) and \( P = 0.025 \)). Similarly, at 30 min of reperfusion, LVW of SHAM, INS, and HSINS was greater than CON hearts (\( *P < 0.001 \), \( P = 0.005 \), and \( *P = 0.021 \), respectively). In addition, at 30 min of reperfusion, LVW of SHAM hearts was greater than HS hearts (\( *P = 0.04 \)). C: insulin improved maximal first derivative of LV pressure (\( +\text{dP/dt} \)) during reperfusion. For \( +\text{dP/dt} \), INS hearts were not different from SHAM hearts. At 5 min of reperfusion, \( +\text{dP/dt} \) for SHAM hearts was greater than CON, HS, and HSINS hearts (\( *P = 0.001 \), \( *P = 0.001 \), and \( P = 0.023 \), respectively). At 30 min of reperfusion, \( +\text{dP/dt} \) for SHAM hearts was greater than CON and HS hearts (\( *P = 0.003 \) and \( *P = 0.044 \), respectively). D: insulin improved \( -\text{dP/dt} \) during reperfusion. At 5 min of reperfusion, whereas \( -\text{dP/dt} \) for SHAM hearts was greater than CON, INS, and HSINS hearts (\( *P < 0.001 \), \( *P = 0.004 \), and \( *P < 0.001 \), respectively), \( -\text{dP/dt} \) for INS hearts was greater than HS hearts (\( *P = 0.015 \)).
RESULTS

LV Function

Preischemic values of cardiac function are presented in Table 1. No significant differences \((P > 0.05)\) were observed in the preischemic function between the experimental groups for heart rate, LVDP, LVW, \(\pm dP/dt\), and CR (Table 1). SHAM hearts were not subjected to ischemia, and functional parameters for SHAM hearts did not change during 2 h of perfusion (Figs. 1 and 2). During the 30 min of no-flow global ischemia, contractility decreased quickly to zero for the CON, INS, HS, and HSINS groups. During reperfusion, there were no statistical differences \((P > 0.05)\) between the groups for heart rate.

After ischemia, during reperfusion, INS and HSINS hearts were not different from SHAM (nonischemic) hearts for LVDP (Fig. 1A). On the other hand, during reperfusion, LVDP for CON and HS hearts was significantly different from SHAM \((P = 0.004\) and \(P = 0.001\), respectively) and INS \((P = 0.026\) and \(P = 0.008\), respectively) hearts.

INS and HSINS hearts had the highest level of recovery of LVW during reperfusion and were not different from SHAM hearts (Fig. 1B). CON and HS hearts were significantly different for LVW at 5 min of reperfusion compared with SHAM \((P = 0.007\) and \(P = 0.015\), respectively) and INS \((P = 0.012\) and \(P = 0.025\), respectively) hearts. At 30 min of reperfusion, CON hearts were significantly different from SHAM, INS, and HSINS hearts \((P < 0.001, P = 0.005,\) and \(P = 0.021,\) respectively).

For \(\pm dP/dt\) (Fig. 1C), INS hearts were not different from SHAM hearts during reperfusion. At 5 min reperfusion, CON, HS, and HSINS hearts had significantly less recovery of \(\pm dP/dt\) compared with SHAM hearts \((P = 0.001, P < 0.001,\) and \(P = 0.023,\) respectively). Similarly, at 30 min of reperfusion, CON and HS hearts had significantly less recovery of \(\pm dP/dt\) compared with SHAM hearts \((P = 0.003\) and \(P = 0.044,\) respectively).

At 5 min of reperfusion, \(-dP/dt\) (Fig. 1D) was significantly different for SHAM hearts compared with CON, INS, HS, and HSINS hearts \((P < 0.001, P = 0.042, P = 0.004,\) and \(P < 0.001,\) respectively). At this time, INS hearts had significantly increased \(-dP/dt\) compared with HS hearts \((P = 0.015).\)

CR (Fig. 2) was not different during reperfusion among SHAM, CON, INS, or HSINS hearts. Interestingly, CR was significantly elevated in the HS hearts compared with SHAM, CON, and HSINS at 5 min of reperfusion \((P = 0.009, P < 0.001,\) and \(P = 0.063,\) respectively). At 30 min of reperfusion, CR was significantly elevated in the HS hearts compared with SHAM, CON, INS, and HSINS \((P = 0.003, P < 0.001, P = 0.024,\) and \(P = 0.007,\) respectively). Similarly, at 60 min of reperfusion, CR was significantly elevated in the HS hearts compared with SHAM, CON, INS, and HSINS \((P = 0.002, P < 0.001, P = 0.01,\) and \(P = 0.006,\) respectively).

EMSA for HSF-1 Activity

HSF-1 is considered active when it is competent to bind the HSE. HSF-HSE binding is evident when the mobility of the HSE probe is retarded on electrophoretic migration. On our EMSA, HSF-HSE complexes are evident as two bands (Fig. 3). The top band is considered to be inducible and trimerized HSF-1 binding HSE, and the bottom band is considered to be constitutive binding of HSF-1-HSE. U-937 cell extract showed strong HSF-1-HSE binding and 0.5-h post-hearth shocked rat heart also showed HSF-1-HSE binding. Addition of excess unlabeled oligonucleotide probe successfully abolished binding of the labeled probe. Addition of HSF-1 antibodies compromised the HSF-1-HSE binding. At 6 h after insulin injection, HSF-1 was strongly activated when compared with the CON and SHAM animals (Fig. 3). HS also activated HSF-1. The histogram (Fig. 3) summarizes data for three animals in each group (normalized to the CON group), and statistical analysis revealed no differences between groups (ANOVA, \(P = 0.109).\)

Hsc70 Western Blot and Densitometric Analysis

Homogenized heart samples were separated into aqueous phase (soluble proteins), detergent-rich phase (membrane-bound proteins), and pellet (nuclear proteins). Hsc70 was detected in each of the various heart fractions, and the experimental treatments had no apparent effect \((P > 0.05)\) on the levels of Hsc70 (Fig. 4).

Hsc70 and Dystrophin Immunofluorescence Microscopy

Confocal micrographs of ventricular sections, double labeled with primary anti-Hsc70 and secondary antibody (AlexaFluor 546-conjugated anti-rabbit IgG, red), and anti-dystrophin and secondary antibody (AlexaFluor 488-conjugated anti-mouse, green) are presented in Fig. 5. Dystrophin immunoreactivity was clearly localized to plasma membranes in all experimental groups (Fig. 5, A, D, G, J, M). Hsc70 immunoreactivity was detectable in control (CON; Fig. 5, B and C) and sham (SHAM; Fig. 5, E and F) hearts and appeared to be localized diffusely throughout the cytoplasm and with some small concentrations in cardiomyocytes. After INS (Fig. 5, H and I), HS (Fig. 5, K and L), and HSINS (Fig. 5, N and O) treatments, Hsc70 immunoreactivity was abundant and was localized throughout the cytoplasm and with some concentrations in cardiomyocytes. No obvious change in Hsc70 distribution was noted.
Hsc70 and α-Tubulin Immunofluorescence Microscopy

Confocal micrographs of ventricular sections, double labeled with primary anti-Hsc70 and secondary antibody (AlexaFluor 546-conjugated anti-rabbit IgG, red), and anti-α-tubulin and secondary antibody (AlexaFluor 488-conjugated anti-mouse, green) are presented in Fig. 6. α-Tubulin immunoreactivity was localized throughout the cytoplasm with some concentration with contractile elements and microtubular networks in cardiomyocytes and small blood vessels in all experimental groups (Fig. 6, A, D, G, J, M). Hsc70 immunoreactivity was detectable in CON (Fig. 6, B and C) and SHAM (Fig. 6, E and F) hearts and appeared to be localized diffusely throughout the cytoplasm and with some small...
concentrations in cardiomyocytes. After INS (Fig. 6, H and I), HS (Fig. 6, K and L), and HSINS (Fig. 6, N and O) treatments, Hsc70 immunoreactivity was abundant and was localized throughout the cytoplasm and with some concentrations in cardiomyocytes. No obvious change in Hsc70 distribution was noted.

**Hsp70 Western Blot and Densitometric Analysis**

Little or no Hsp70 was detected by Western blot analysis in the heart fractions from CON and SHAM animals (Fig. 7). In the INS-treated animals, Hsp70 was detected in the homogenate and in the membrane fraction of hearts. In HS- and HSINS-treated animals, abundant levels of Hsp70 were detected in the homogenate and all fractions of hearts (Fig. 7). Statistical analysis revealed differences in the homogenates between CON vs. HS and HSINS ($P = 0.04$); cytosolic fractions between CON vs. HS and HSINS ($P = 0.02$); membrane fractions between CON vs. INS and HSINS ($P = 0.03$); and pellet fractions between CON vs. HSINS ($P = 0.03$). It should also be noted that the magnitude of change in the abundance of Hsp70 after INS, HS, or HSINS treatments is many times greater than was seen for Hsc70.

**Hsp70 and Dystrophin Immunofluorescence Microscopy**

Confocal micrographs of ventricular sections, double labeled with primary anti-Hsp70 (AlexaFluor 546, red) and...
anti-dystrophin (AlexaFluor 488, green) are presented in Fig. 8. Dystrophin immunoreactivity was clearly localized to plasma membranes in all experimental groups (Fig. 8, A, D, G, J, M) and appeared to be unchanged by the experimental treatments. Little or no Hsp70 immunoreactivity was detected in CON or SHAM hearts (Fig. 8, B and E). After INS, Hsp70 immunoreactivity was detected and localized mainly to plasma membranes (Fig. 8, H and I). After HS, Hsp70 immunoreactivity was abundant in cardiac tissue and appeared to be localized mostly in small capillaries and in perivascular compartments between the cardiomyocytes (Fig. 8, K and L). After HSINS, Hsp70 immunoreactivity was mostly localized to capillaries and perivascular compartments between the cardiomyocytes; however, there also appeared to be some minor localization of Hsp70 immunoreactivity in the cytoplasm and on the cell membranes of the cardiomyocytes (Fig. 8, N and O).

**Hsp70 and α-Tubulin Immunofluorescence Microscopy**

Confocal micrographs of ventricular sections, double labeled with primary anti-Hsp70 (AlexaFluor 546, red) and anti-α-tubulin (AlexaFluor 488, green) are presented in Fig. 9. α-tubulin immunoreactivity was localized to the microtubular networks in cardiomyocytes and possibly in vascular elements in all experimental groups (Fig. 9, A, D, G, J, M) and appeared to be unchanged by the experimental treatments. Little or no Hsp70 immunoreactivity was detected in CON or SHAM hearts (Fig. 9, B and E). After...
INSulin induces myocardial protection and Hsp70

DISCUSSION

This study reports that insulin treatment improves postischemic myocardial contractility, activates the HSF-1, and induces a small amount of Hsp70 in the rat heart, and interestingly, this insulin-induced expression of Hsp70 appears to be localized to cell membranes. In contrast, heat shock treatment 6 h before isolation of hearts did not improve the postischemic myocardial contractility but induced strong Hsp70 expression mostly in capillaries and perivascular compartments. Heat shock and insulin treatment caused further apparent increase in Hsp70 expression in the rat heart.

In this study, insulin, but not HS, had a beneficial effect of the postischemic recovery of myocardial function (Fig. 1). Improved myocardial function after ischemia is seen after ischemic preconditioning (39, 46, 56). Interestingly, two windows of protection are evident, the first being rapid and persisting for only ~2 h, and the second being present at ~20 to 48–72 h (13, 14, 50, 63). Improved myocardial function after ischemia is also seen 24–48 h after heat shock treatment (15, 22, 30, 34). As shown in this study, myocardial protection is not evident at 6 h after HS (13, 14, 72) even though considerable Hsp70 is present in the heart. However, as shown here, the heart displays myocardial protection at 6 h after insulin treatment. Whether there are multiple windows of protection or a continuous protection, each window or segment of the protection appears to be stimulus dependent. It is also interesting that insulin treatment appears to rescue heart functional recovery after heat shock treatment (Fig. 1, compare HS vs. HSINS).

The absence of enhanced myocardial recovery in the HS hearts 6 h after heat shock treatment when Hsp70 levels are elevated has been repeatedly observed (13, 14, 72). Newly synthesized Hsp70 may not be fully mature or folded into its functional configuration. Alternatively, the protective function of Hsp70 may be related to its differential localization after HS or insulin treatment (Figs. 7–9). Another intriguing possibility is the elevation of coronary resistance (Fig. 2) seen during reperfusion in the hearts 6 h after HS; the HS hearts had the highest level of CR. The elevated CR may be due to increased plasma catecholamine concentration and myocardial norepinephrine turnover after heat shock (36, 37). This may cause an elevated responsiveness of coronary vessels to reperfusion and gradual vasoconstriction at short recovery times after heat shock. The elevated CR in HS hearts (6 h after heat shock treatment) is in contrast to 24- to 96-h post-heart shock hearts having significantly lower perfusion pressures compared with control (non-heart shock) hearts during reperfusion after 30 min of ischemia (34). Coincidentally, transgenic mouse hearts containing high levels of human Hsp70 also had significantly lower perfusion pressures compared with littermate control nontransgenic hearts during reperfusion after 30 min of ischemia (61).

Part of the protective effect of insulin appears to be controlling the elevation of CR as seen in the HSINS hearts compared with the HS hearts. The elevated CR at 6 h after HS may negate other protective mechanisms.

In this study, Hsc70 expression and distribution were not affected by insulin or by heat shock treatment. Hsc70 is a constitutive member of the 70-kDa family of heat shock proteins. In the rat, Hsc70 has an apparent molecular mass of 73 kDa and is normally expressed in all cells and tissues, and its expression changes minimally with injury compared with the inducible member of this family of Hsps. Hsc70 acts as a molecular chaperone to regulate normal protein folding and helps repair damaged proteins.

In contrast, here we and others have shown that the expression of Hsp70 was elevated after insulin treatment (41, 68). Hsp70 is the highly inducible member of the 70-kDa family of Hsps and in rat has an apparent molecular mass of 71 kDa. Hsp70 is normally at low or undetectable levels in most tissues.

**Fig. 7.** Insulin elevated Hsp70 in the membrane fraction of hearts. Hsp70 was analyzed by Western blot and densitometric analysis in heart homogenate, cytosol, membrane, and pellet fractions (n = 6, each group). Actin was used as a loading control for Western analysis. In the homogenate, Hsp70 appears to be elevated in INS, HS, and HSINS hearts. While there is a modest increase in abundance of Hsp70 after INS treatment as seen in the homogenate compared with HS or HSINS treatment, this increase is most evident in the membrane fraction. Histogram results are relative to the control group (times mean control values and SE) for each fraction. One-way ANOVA Bonferroni test confirmed with a Bonferroni post-test, revealed differences in the homogenate, CON vs. HS and HSINS, P = 0.04; cytosolic fraction, CON vs. HS and HSINS, P = 0.02; membrane fraction, CON vs. INS and HSINS, P = 0.03; pellet fraction, CON vs. HSINS, P = 0.03. **INS, Hsp70 immunoreactivity was detected and localized mainly to cellular membranes (Fig. 9, H and I). After HS, Hsp70 immunoreactivity was abundant in cardiac tissue and appeared to be localized mostly in small capillaries and in perivascular compartments between the cardiomyocytes (Fig. 9, K and L). After HSINS, Hsp70 immunoreactivity was mostly localized to capillaries and perivascular compartments between the cardiomyocytes; however, there also appeared to be some minor localization of Hsp70 immunoreactivity in the cytoplasm and on the cell membranes of the cardiomyocytes (Fig. 9, N and O).**
in rat, but with heat shock or injury, its expression is transiently increased many times (2, 15, 17). Hsp70 maintains the HSF in an inactivated form in the cell cytoplasm (49, 66). Similar to Hsc70, Hsp70 is thought to act as a molecular chaperone and also as a regulator of apoptotic signaling pathways (53).

The insulin-induced Hsp70 appeared to have a different localization compared with HS-induced Hsp70. This localization may be related to the intensity of stimulation used to induce the expression of Hsp70. While we generally consider that Hsp70 is expressed in response to noxious stimuli, it is doubtful that insulin is such a stimulus. Insulin administration of 200 µU/g body wt does not affect blood glucose levels significantly (41). Insulin is an existing circulating hormone ubiquitous in the body that does not normally stress or injure cells. Insulin induces low-level expression of Hsp70, in contrast to the high-level expression of Hsp70 after the noxious heat shock treatment. Thus the membrane localization of Hsp70 after insulin treatment may be related to the physiological induction of its expression by insulin and not a pathological induction. In fact, similar distinct stress-dependent cellular localization of Hsp70 has been reported before in the brain (38).

Localization of Hsp70 to cardiomyocyte membranes after insulin treatment compared with microvasculature elements after heat shock likely has implications for the function of Hsp70. Elevated levels of Hsp70 in microvessels 24 h after heat shock treatment may be refolding damaged proteins caused by reactive oxygen species during ischemia-reperfusion.
to maintain overall cardiac function (40, 61). Localization of 
Hsp70 to cardiomyocyte membranes after insulin treatment 
may stabilize the cell membranes or regulate membrane recep-
tors or even cell signaling pathways. Interestingly, Hsp70 has 
been identified on plasma membrane of several carcinoma cell 
lines (3, 9), particularly after heat shock (55) or treatment with 
anti-inflammatory drugs (4) or γ-irradiation (26). However, 
membrane-bound Hsp70 is not found in normal (noncancer-
ous) cell lines after heat shock treatment (55). In carcinoma 
cells, the role of membrane-bound Hsp70 appears to be related 
to immunological recognition. Tumor cells with high levels of 
membrane-bound Hsp70 have high rates of cell growth and 
metastasis (4) and are more effectively killed by natural killer 
cells (27, 29). Similarly, the hydroxylamine derivative bimo-
cloclomol induced membrane-bound Hsp70 that stabilized mem-
brane fluidity at elevated temperatures (69). Interestingly, 
membrane-bound Hsp70 is abundant and mostly expressed be-
tween cardiomyocytes and contractile elements and cell 
membranes (I). J–L: heat shock treated; Hsp70 immunofluorescence is detectable (H) and appears to be mostly localized with contractile elements and cell 
membranes (K), and some colocalization with α-tubulin immunofluorescence 
is evident (L). M–O: heat shock and insulin treated; Hsp70 immunofluorescence is mostly expressed in perivascular elements (N), and some colocalized with 
α-tubulin immunofluorescence is evident (O). Images are representative of 3 animals in each treatment group. Scale bar = 20 μm.
nonstressed and noncancerous hearts. After insulin treatment, Hsp70 localized to plasma membranes may function to stabilize cell membranes or regulate prosurvival signaling pathways that may be responsible for the enhanced recovery.

Our current study has also shown that a physiological dose of insulin activates the DNA binding activity of HSF-1. Activated HSF-1 is represented by two complexes that can be abolished by excess unlabeled cold probe (Fig. 3). The top and bottom HSF bands are considered to be inducible and constitutive binding activity (43, 44). Heat shock genes are regulated by the HSF-1. HSF-1 is maintained in an inactive form by binding with Hsp70 (49) or Hsp90 (70). Under stressful conditions that denature protein, Hsp70 is recruited to the denatured protein, freeing the transcription factor. HSF-1 is activated by phosphorylation, trimerizes and translocates to the nucleus, binds to the heat shock element, and initiates transcription of various Hsps (35, 50, 51, 65). Insulin is a general regulator of protein synthesis (62). As a general regulator of overall protein synthesis, insulin regulates phosphorylation of S6 ribosomal protein, increases the activity of eukaryotic protein synthesis elongation factor-2 (eEF-2), and regulates the eukaryotic protein synthesis initiation factor-4E (eIF-4E) (54). Thus insulin may stimulate and regulate translation through eIF-4E to increase overall protein synthesis and increase Hsp70 content in cells. Alternatively, insulin inhibits glycosyn synthesis kinase 3 (GSK3) activity by activation of phosphatidylinositol 3-kinase and protein kinase B (PKB/Akt) (54). GSK3 is a negative control regulator of HSF-1. After inhibition of GSK3β, activity of HSF-1 in heat-shocked cells increased, whereas overexpression of GSK3β results in significant reduction in heat-induced HSF-1 activity (71). In this study, it is possible that the insulin-induced inhibition of GSK3 led to the activation of HSF-1 and elevation of Hsp70 expression.

In conclusion, 6 h after insulin treatment, hearts have improved functional recovery from 30 min of ischemia, whereas heat-shocked hearts do not. Insulin increases the DNA binding activity of HSF-1 and induces expression of Hsp70 in cardiomyocytes that appears to be localized to cellular membranes. This is in contrast to the expression of Hsp70 after heat shock, which is mainly localized to vascular and perivascular elements between the cardiomyocytes. This differential localization of Hsp70 in response to insulin and heat shock suggests the interesting possibility of functionally distinct roles for Hsp70 in hearts that are stimulus specific. Insulin-induced Hsp70 being localized on cell membranes may be regulating prosurvival cell signaling pathways that are contributing to the functional recovery of the hearts after ischemic injury.

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