HSC73-tubulin complex formation during low-flow ischemia in the canine myocardium

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Decker, Robert S., Marlene L. Decker, Sakie Nakamura, Yu-Sheng Zhao, Sascha Hedjbeli, Kathleen R. Harris, and Francis J. Klocke. HSC73-tubulin complex formation during low-flow ischemia in the canine myocardium. Am J Physiol Heart Circ Physiol 283: H1322–H1333, 2002. First published June 6, 2002; 10.1152/ajpheart.00062.2002.—Canine myocardium was exposed to bouts of low-flow ischemia to identify the interactions that develop between the microtubule-based cytoskeleton and the heat shock protein 70 (HSP70) family of heat shock proteins in viable cardiomyocytes. “Moderate” or “severe” low-flow ischemia was produced in chronically instrumented dogs by reducing circumflex coronary flow by 50% for 2 h or by 75% for 5 h followed by reperfusion for 2 and 24 h, respectively. Electron and immunofluorescence microscopy demonstrated either partial or nearly complete depolymerization of the intermyofibrillar microtubules in areas of myofibril disruption and partial dissolution of the perinuclear microtubule girdle. In contrast, centrosomal tubulin arrays appeared to remain intact following low-flow ischemia. In cardiomyocytes displaying myofibril disruption, constitutively expressed HSP73 (HSC73) co-localized with intact but not disrupted microtubules and with perinuclear and centrosomal tubulin following moderate ischemia. Microtubule depolymerization and high molecular weight tubulin-HSC73 complexes were present in more severely ischemic tissue. These results suggest that HSC73 directly interacts with tubulin and may protect selected elements of the microtubule network and limit myofibril disruption during reversible low-flow ischemia.

microtubule network; myofibril disruption; contractile dysfunction

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MATERIALS AND METHODS

Experimental Animal Preparation

Experiments were conducted with mongrel dogs of both sexes using protocols that conform to the principles in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, 1986). Experimental procedures also were approved by Northwestern University's Institutional Animal Care and Use Committee. Animals weighing 23–36 kg were instrumented following an overnight fast and a 3-wk period of on-site conditioning. Briefly, all animals were surgically instrumented with a Konigsberg micromanometer, ultrasonic volumetric transit-time flow probes, a hydraulic occluder, and downstream catheters to measure pressure. Regional subendocardial segment shortening and/or wall thickening was monitored by placing ultrasonic crystal pairs in the central portion of the distributions of the left circumflex (LCX) and left anterior descending (LAD) vascular beds. Seven to ten days after surgery, the dogs were lightly sedated with Innovar-Vet (fentanyl, 0.4 mg/ml and droperidol, 20 mg/ml) and placed upright in a sling to which they had been previously acclimated. Regional coronary flow in the LCX (i.e., test region) was gradually reduced by injecting saline into the hydraulic occluder until the desired reduction in coronary flow and segmental function was achieved. The LAD region of the left ventricle, where neither coronary flow nor function was altered, represented the remote myocardial site and was examined in parallel with the paired LCX test area. Details of the instrumentation procedure have been fully reported previously (23, 24).

In this investigation, 10 animals were subjected to a moderate reduction in coronary flow of 50% for 2 h that resulted in a 50% decrease in regional wall function. Five animals were euthanized after low-flow ischemia, whereas the other five were reperfused for an additional 2 h before being euthanized. A second group of 12 dogs was exposed to a more severe, sustained 5-h reduction in regional coronary flow sufficient to reduce regional segment function by ~75%. Five animals were euthanized after low-flow ischemia, and seven dogs were reperfused for 24 h. Four additional fully instrumented dogs were conditioned and hemodynamic data collected, but coronary flow was not reduced; these animals represented sham-instrumented, control preparations. All animals were brought to the laboratory for final physiological measurements and then euthanized with an overdose of pentobarbital sodium and potassium chloride. Hearts from the euthanized animals were biopsied in situ, and test and remote tissue specimens were fixed immediately in glutaraldehyde (24). The hearts were then rapidly removed and placed in ice-cold isotonic saline. Additional test, remote, and sham samples were either quickly frozen in liquid nitrogen, preserved in a paraformaldehyde-lysine-periodeate fixative, or processed immediately for the isolation of HSC73/HSP70-tubulin complexes.

Immunofluorescence Microscopy

The distribution of tubulin isoforms was examined in 4-μm frozen sections of tissue that had been fixed in 4% paraformaldehyde, 100 mmol/l L-lysine, and 10 mmol/l sodium periodeate prepared in PBS (pH 7.4) at the time of euthanasia (21). The sections were blocked with a 1:10 dilution of normal goat serum in PBS for 4 h at room temperature to minimize nonspecific adsorption of primary antibodies. α-, β-, and γ-tubulin distribution was then monitored in frozen sections by using mouse monoclonal antibodies (Sigma Chemical; St. Louis, MO). Nonimmune mouse IgG1 was employed to control for the nonspecific binding of the primary anti-tubulin antibodies. The antibodies were diluted to a final concentration of 1 μg/ml with PBS supplemented with 1 mg/ml of BSA, and the sections were incubated with each of the primary antibodies overnight at 4°C before being washed in PBS (3 × for 30 min). Sections were then stained for 1 h at 37°C with a secondary, affinity-purified, goat anti-mouse IgG (Cappel, ICN; Aurora, OH) labeled with either FITC or tetramethyl rhodamine that was diluted 1:300 in PBS plus BSA. After being washed in PBS-BSA, the sections were mounted with gelvatol and Z sectioned with a Zeiss 510 laser scanning confocal microscope. Some sections were doubly stained with affinity-purified, goat polyclonal antibodies (1 μg/ml) produced against highly conserved peptide sequences derived from human HSC73 and HSP70 (Santa Cruz Biotechnology; Santa Cruz, CA). Affinity-purified rabbit anti-goat IgG labeled with FITC (Cappel, ICN) was employed to visualize the distribution of the stress proteins. To monitor the specificity of these antipeptide antibodies, sections were incubated with either nonimmune goat IgG1 or supplemented with 100-fold excess of a blocking peptide that was used to produce the polyclonal anti-HSC73 or HSP70 antibodies (Santa Cruz Biotechnology).

In an effort to quantify the degree of heat shock protein redistribution to the microtubule network, the fluorescence emission spectra of HSC73 and HSP70 colocalized to microtubules was measured after introduction of an electronic raster across the long axis of a cardiomyocyte labeled for tubulin and each of the stress proteins. The fluorescence emission intensity was tabulated from the tubulin window (i.e., rhodamine channel) and from the stress protein window (i.e., fluorescein channel), and a ratio of the two fluorescence emission intensities (in arbitrary units) was calculated at sites where the raster intersected with a microtubule. The ratio (rhodamine/fluorescein intensity) was expressed per microtubule (means ± SE) and was derived from 20 myocytes from each experiment. Significant differences in the fluorescence emission ratios were determined using either a one- or two-way ANOVA followed by post hoc analysis with the Student-Newman-Keuls test; a P < 0.05 was considered statistically significant.

Transmission and Immunogold Electron Microscopy

Biopsied samples of test, remote, and sham subendocardial myocardium were preserved in either 2% glutaraldehyde buffered in sodium cacodylate (pH 7.4) or in the formaldehyde-lysine-periodeate (FLP) fixative used for immunofluorescence microscopy. Glutaraldehyde-fixed tissue was postfixed in 2% osmium tetroxide, dehydrated, and embedded in an epoxy resin as outlined previously (24) and viewed with a JOEL 100CX electron microscope. The ultrastructural distribution of tubulin was monitored in FLP-fixed frozen sections using a postembedding technique. Washed, free-floating 4-μm sections were incubated in a monoclonal anti-α-tubulin antibody (Sigma Chemical) diluted 1:100 in PBS overnight at 4°C. The sections were then washed in PBS and incubated overnight at 4°C in a 1:20 solution of donkey anti-mouse IgG1 conjugated to 6-nm colloidal gold particles (Jackson Laboratories; West Cove, PA). The sections were then processed as described above.

Biochemical Distribution of Tubulin and HSC73/HSP70 Stress Proteins

Relative changes in the total content of tubulin isoforms and HSC73/HSP70 that developed during low-flow ischemia
and reperfusion were obtained from frozen sham, test, and remote subendocardium (200 mg) that was solubilized in five volumes of Laemmli sample lysis buffer [200 mmol/l DTT, 4% SDS, 160 mmol/l Tris-HCl (pH 6.8), and 20% glycerol] and heated at 95°C for 5 min. Other paired samples were solubilized in Laemmli buffer for 15 min at 37°C. The solubilized tissue extracts were clarified by centrifugation, and the protein concentration of the supernatants was determined using the Pierce microprotein assay (Pierce; Rockford, IL). Equal amounts of protein (1 μg/lane) were loaded on 10% SDS-PAGE minislab gels, and tubulin isoenforms and HSC73/HSP70 were separated from one another. Protein was then transferred to a polyvinylidene fluoride membrane for Western blotting. Sample loading and transfer efficiency was evaluated by Coomassie blue brilliant staining. Membranes were blocked in TBST solution [150 mmol/l NaCl, 10 mmol/l Tris-HCl (pH 8.0), 0.05% Tween 20, and 3% nonfat dry milk] and incubated in a solution of tubulin antibody (1:5,000 dilution of α-, β-, and γ-tubulin monoclonal mouse IgG1; Sigma Chemical) and in a 1:2,500 dilution of affinity-purified anti–HSP70 polyclonal HSC73/HSP70 antibodies (Santa Cruz Biotechnology). The membranes were then incubated with a secondary goat anti-mouse/rabbit anti-goat IgG conjugated to horseradish peroxidase, and the Western blots were visualized by reacting the blotted membranes with Supersignal West Pico Luminal Enhancer (Pierce) followed by autoradiography. Metamorph image software was employed to digitize the gel bands, and the protein content of each myocardial sample was evaluated by using purified tubulin isoenforms and HSC73 and HSP70 standards (i.e., 200 ng-2 μg) and catalase (232 kDa) as molecular mass standards (Sigma Chemical). Each fraction also was assayed qualitatively for the presence of protein (means ± SE). Protein contents were compared with values derived from sham-instrumented myocardium using a one- or two-way ANOVA followed by post hoc testing with the Student-Newman-Keuls test, with a P < 0.05 being considered statistically significant.

Tubulin-HSC73/HSP70 Interactions

Putative interactions between tubulin and HSC73/HSP70 were identified in sarcoplasmic and myofibrillar fractions prepared after homogenizing 100 mg of fresh myocardial tissue in 10 volumes of low-salt buffer (40 mmol/l NaCl, 1 mmol/l DTT, 0.1 mmol/l EGTA, and 0.1% Triton X-100, pH 7.4) with a Polytron PC-1. The homogenate was centrifuged at 11,000 g for 30 min at 4°C, and the supernatant was saved as the sarcoplasmic fraction, whereas the pellet (myofibril fraction) was washed five times in ice-cold, low-salt buffer. Sham, test, and remote sarcoplasmic and myofibrillar fractions were diluted/homogenized with 0.5 ml of extraction buffer [50 mmol/l Tris-HCl (pH 8.5), 150 mmol/l NaCl, 20 mmol/l EDTA, 1% Triton X-100, 4 mol/l urea, and 0.5 mmol/l PMSF], and both extracts were clarified by centrifugation. Aliquots of the myofibrillar and sarcoplasmic fractions were layered over a 10–40% linear sucrose gradient and centrifuged for 16 h at 100,000 g in a Beckman L8–80M Ultracentrifuge at 4°C. The gradients were calibrated by using thyroglobulin (669 kDa) and catalase (232 kDa) as molecular mass standards (Sigma Chemical). Each fraction also was assayed qualitatively for its protein composition by immunodot blotting with antibodies directed against actin, desmin, myosin, and tubulin in addition to HSC73/HSP70, HSP27, and αB-crystallin. In those fractions where stress and cytoskeletal proteins were detected, aliquots of each fraction were loaded on to a 4% native PAGE gel and electrophoresed in the cold; thyroglobulin and catalase (Sigma Chemical) standards were run in parallel. Similarly, samples of each fraction also were subjected to denaturing gel electrophoresis in 10% SDS-PAGE as described above.

Characterization of Tubulin-Stress Protein Complexes

In those fractions that disclosed high-molecular-weight complexes containing tubulin and/or HSC73/HSP70, anti-HSC73/HSP70 antibodies were employed to immunoprecipitate those protein(s) from the suspected complexes. Aliquots of protein (100 μg) derived from each of the sucrose gradient fractions were mixed with 10 μg of HSC73/HSP70 antibody (clone 92-mouse IgG1, StressGen). This antibody recognizes both the constitutive (HSC73) and inducible (HSP70) forms of the stress protein with the same degree of affinity. Protein G-agarose (20 μl, Santa Cruz Biotechnology) was added to the incubation mixture and incubated for an additional 30 min at 4°C with gentle agitation. The solution was then centrifuged, and the pellet was solubilized in Laemmli buffer at 95°C and loaded on a 10% SDS-PAGE gel and immunoblotted as described above. The supernatant, known amounts (i.e., 100 ng–1 μg) of purified tubulin and HSP70, and molecular weight standards were run in adjacent lanes with the immunoprecipitates. The amount of tubulin and HSC73/HSP70 in the immunoprecipitates was estimated from a standard curve created with the purified proteins, and tubulin and stress protein content were expressed as micrograms tubulin and/or HSC73/HSP70 per 100 μg of total fraction protein (means ± SE). Significant differences were determined using a one-way ANOVA followed by post hoc testing with the Student-Newman-Keuls test, with a P < 0.05 being significant.

RESULTS

Hemodynamic Parameters During Low-Flow Ischemia

When coronary blood flow was reduced moderately for 2 h, both flow and regional systolic function (i.e., segmental shortening or wall thickening) decreased in parallel. Segmental function in LCX region was reduced on average 50 ± 2%, and coronary flow decreased 54 ± 3% compared with baseline values (Table 1). Coronary flow and segmental function in the remote, left ventricle (LAD) remained at values observed before occlusion. In animals reperfused for 2 h, coronary flow returned to 86 ± 4% of baseline flow, and segment function measured 79 ± 2%, indicating that the LCX (i.e., test) subendocardium displayed a short-term hibernating phenotype (23, 24). Conversely, severely reducing coronary flow for 5 h depressed segmental function 76 ± 2% and reduced coronary flow about 73 ± 5% in the LCX bed (Table 1), whereas flow and function continued to remain unperturbed in the remote LAD myocardium. When the dogs were reperfused for 24 h, flow returned to values seen before occlusion (i.e., 109 ± 6% of baseline), whereas wall function remained markedly depressed (54 ± 8% of the preocclusion baseline segment shortening/wall thickening; see Table 1). The flow-function mismatch was indicative of the development of myocardial stunning (23). The hemodynamic parameters in the remote left ventricle supplied by the LAD were not altered following the severe partial coronary occlusion.
Distribution of Tubulin and HSC73/HSP70 in Normal Cardiomyocytes

The microtubule network assumes a rather complex distribution in the heart cell, forming a densely woven basket of 25-nm tubules surrounding each nucleus and a somewhat less extensively arrayed sheath of tubules enveloping each myofibril (Fig. 1a). A better appreciation of tubulin distribution between neighboring myofibrils was achieved by employing immunogold electron microscopy. Anti-tubulin-conjugated gold particles decorated the 25-nm microtubules that weave between adjacent myofibrils and parallel to the Z line (Fig. 1d). α- and/or β-Tubulin-positive perinuclear arrays also were readily visible in most cardiocytes (Fig. 1a); moreover, γ-tubulin also could be observed at the center of these sites (Fig. 2a). Many of the canine cardiocytes appeared to disclose multiple perinuclear centrosomal sites (Fig. 1a), and fine structural observations confirmed that as many as three centrioles could be visualized in these perinuclear areas (Fig. 2, b and c). In

Table 1. Hemodynamics and myofibril disruption following low-flow ischemia and reperfusion

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Baseline (n = 4)</th>
<th>Moderate 2 h (n = 5)</th>
<th>Moderate 2 h + 2 hR (n = 5)</th>
<th>Severe 5 h (n = 5)</th>
<th>Severe 5 h + 24 hR (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary flow, ml/min</td>
<td>34 ± 5.3</td>
<td>17.1 ± 2.2*</td>
<td>29 ± 4.7</td>
<td>9.1 ± 1.8†</td>
<td>38 ± 6.5</td>
</tr>
<tr>
<td>Segmental function, %shortening</td>
<td>17.5 ± 0.9</td>
<td>8.7 ± 2.1*</td>
<td>13.8 ± 1.7</td>
<td>4.6 ± 0.5†</td>
<td>8.9 ± 1.7*</td>
</tr>
<tr>
<td>Myofibril disruption (+ or −)</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>+</td>
<td>+/−</td>
</tr>
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</table>

Values are means ± SE; n = 4–7 animals for each experiment. Baseline refers to preocclusion values. Moderate, low-flow ischemia for 2 h and reperfusion (R) for 2 h; Severe, low-flow ischemia for 5 h and R for 24 h; + or −, relative measure of thick filament disruption. *P < 0.05, †P < 0.01 one-way ANOVA vs. baseline.

Fig. 1. Distribution of α-tubulin (a) and constitutive heat shock protein (HSC73) (b) in subendocardial cardiomyocytes derived from a sham-instrumented animal. α: α-Tubulin antibody staining of the perinuclear (N) girdle, arrays (arrowheads), and intermyofibrillar microtubules (arrows). HSC73 is diffusely distributed throughout the sarcoplasm and in the perinuclear (N) arrays (b, arrowheads). HSC73 and α-tubulin colocalize to the perinuclear arrays (c, arrowheads) and weakly stain some peripheral microtubules. d: Immunogold (arrows) labeling reveals a complex pattern of α-tubulin-positive microtubules between neighboring myofibrils (M); some Z lines (arrowheads) also display gold-labeled tubulin. m, Mitochondrion; a–c, bar = 25 μm; d, bar = 0.1 μm.
subendocardial myocytes derived from sham-instrumented animals, HSC73 was distributed uniformly throughout the sarcoplasm (Fig. 1b). Anti-HSC73 antibody also stained the central areas of these perinuclear tubulin-positive arrays (Fig. 1, b and c, arrowheads) and weakly labeled some intermyofibrillar microtubules (Fig. 1, a and c). In contrast, there was no evidence that HSP70 stained either intermyofibrillar or perinuclear microtubules, and little HSP70 colocalization of the centrosomes could be observed in “normal” cardiocytes (data not shown).

**Integrity of Microtubules During Low-Flow Ischemia-Reperfusion**

*Moderate low-flow ischemia.* When coronary flow and function were reduced moderately (in the LCX test area), the perinuclear microtubule girdle and microtubular arrays remained intact (Fig. 3a); however, areas within individual myocytes revealed partially disrupted intermyofibrillar microtubules (Fig. 3a). In preparations double labeled for tubulin and HSC73, the structurally intact, peripheral intermyofibrillar microtubules stained intensely for the presence of HSC73 (Fig. 3, b and c) but not HSP70 (data not shown). In those regions of the cardiocyte that displayed poorly ordered (depolymerized/disrupted) microtubules or punctate deposits of tubulin, few of the recognizable tubulin-positive elements stained for HSC73 (Fig. 3, b and c) or HSP70 (data not shown). Anti-HSC73 also weakly stained cardiocyte nuclei following low-flow ischemia (Fig. 3b). Biopsied samples derived from remote, normally perfused myocardium showed only a minimal redistribution of HSC73 (Fig. 4b) to cardióyte intermyofibrillar microtubules (Fig. 4a). Only the centrosomes and the proximal portions of microtubules radiating away from these structures displayed HSC73 immunoreactivity (Fig. 4c). Only small amounts of HSP70 were expressed in the canine myocardium, and no redistribution of the inducible form of the stress protein was observed in the cardiomyocytes following a moderate reduction of coronary flow in the LCX or in the normally perfused (LAD area) myocardium (data not shown).

When the hearts were reperfused, there was little change in the distribution of tubulin in subendocardial cardiocytes. However, intermyofibrillar microtubules stained less intensively for HSC73 than did their counterparts from the ischemic myocardium (Table 2). Sites of depolymerized intermyofibrillar microtubules could be observed in cardiocytes from reperfused hearts...
although less frequently than in tissue sampled immediately following low-flow ischemia. In the remote regions of the left ventricle, no changes in the distribution of tubulin were apparent, and the pattern of HSC73 staining resembled that observed before reperfusion (data not shown).

Severe low-flow ischemia. When coronary flow was reduced more severely for a prolonged period, intermyofibrillar microtubules then appeared to completely depolymerize in cardiocytes revealing myofibril disruption. Intermiyo fibrillar microtubules appeared to be replaced by a punctate tubulin-staining pattern (Fig. 5a) that was somewhat reminiscent of the particulate tubulin pattern reported in ischemic/stunned canine cardiac myocytes (13, 21). The punctate tubulin aggregates generally lacked evidence of stress protein labeling as well (Fig. 5, b and c). Portions of the perinuclear girdle also appeared disrupted (Fig. 5, a and d); however, the remaining intact elements of the girdle stained positively for HSC73 (Fig. 5b). Intranuclear localization of HSC73 also was observed in cardiocytes disclosing intermyofibrillar microtubule depolymerization; moreover, HSC73 appeared closely associated with the inner aspect of the nuclear envelope directly adjacent to the remaining intact, HSC73-positive portions of the microtubule girdle (Fig. 5, b and d). Whereas the centrosomes remained structurally intact following severe low-flow ischemia (Fig. 5, a, c, and d), no microtubules were observed radiating away from them (Fig. 5a; compare with Figs. 3a and 4a); instead, only “blunt-ended” structures projected from some of the centrosomes (Fig. 5a). Even though coronary flow was not compromised in the remote myocardium, some remote cardiac myocytes also displayed microtubule depolymerization, whereas neighboring cardiocytes disclosed a relatively intact intermyofibrillar microtubule network (Fig. 6a) that stained for both HSC73 (Table 2) and HSP70 (Fig. 6, b and c).

Intermyofibrillar microtubules continued to remain depolymerized following 24 h of reperfusion, especially in severely stunned myocardium (Table 2). Likewise, the perinuclear centrosomal arrays remained intensely labeled with HSC73; however, no microtubules radiated away from these structures, implying little microtubule reassembly transpires in these “stunned” cardiocytes (data not shown). In remote regions of the myocardium, the organization of the microtubule network was not influenced by reperfusion with the exception that intermyofibrillar microtubules and cardiocyte

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**Fig. 3.** α-Tubulin (a) and HSC73 (b) redistribution in subendocardial myocytes after 2 h of low-flow ischemia. Both intact intermyofibrillar (arrowhead) and disrupted (*) microtubules are present in the cardiocyte (a). HSC73 stains intact microtubules (arrowhead, b and c) but not disrupted microtubules (*, a–c). Perinuclear girdle, nucleoplasm, and tubulin arrays (arrows) also display enhanced HSC73 staining (b and c). Note intensely stained endothelial cell (e). N, nucleus; a–c, bar = 2 μm.
nuclei were labeled with HSP70 (Fig. 6b) as well as HSC73 (Table 2).

**Tubulin-HSC73/HSP70 Interactions in Canine Myocardium**

*Distribution of tubulin and HSC73/HSP70.* Western immunoblotting was used to monitor the fate of tubulin and the heat shock proteins in test and remote myocardial tissue samples following low-flow ischemia. Neither tubulin nor HSC73/HSP70 could be detected in the low-salt, partially purified myofibrillar fraction; instead, both protein families were present in the soluble, sarcoplasmic fraction of myocardial homogenates, regardless of the degree or duration of low-flow ischemia-reperfusion. When the sarcoplasmic extracts were diluted into Laemmli buffer and solubilized at 95°C, the relative content of α-tubulin was reduced significantly (32.7 ± 6.9%, P < 0.05, n = 5) in the test regions (Fig. 7A) compared with extracts prepared from either remote (Fig. 7A) or sham myocardium (Fig. 7A). γ-Tubulin content declined somewhat more modestly (17.6 ± 3.5%, P < 0.1, n = 5) in extracts of test tissue (Fig. 7A). Although no significant change in total HSC73 content could be detected in gels scanned from Fig. 4.

**Fig. 4.** α-Tubulin (a) and HSC73 (b) distribution in the remote subendocardial myocytes after 2 h of low-flow ischemia. HSC73 stains few of intermyofibrillar microtubules or the perinuclear (N) girdle (a and b); instead HSC73 colocalizes with centrosomes (c, arrows) or remains diffusely distributed throughout the sarcoplasm (*). Note that HSC73-positive microtubules radiate from the centrosomes (arrows, a–c); a–c, bar = 2 μm.

**Table 2.** HSC73/HSP70-tubulin immunofluorescence staining ratios of intermyofibrillar microtubules

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Sham (n = 4)</th>
<th>2 h (n = 5)</th>
<th>2 h + 2 hR (n = 5)</th>
<th>5 h (n = 5)</th>
<th>5 h + 24 hR (n = 7)</th>
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<tbody>
<tr>
<td>HSC73/tubulin</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Remote (LAD area)</td>
<td>0.23 ± 0.06</td>
<td>0.31 ± 0.05</td>
<td>0.28 ± 0.05</td>
<td>0.38 ± 0.09</td>
<td>0.42 ± 0.1*</td>
</tr>
<tr>
<td>Test (LCX area)</td>
<td>0.27 ± 0.06</td>
<td>0.59 ± 0.03*</td>
<td>0.43 ± 0.07*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HSP70/tubulin</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Remote (LAD area)</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.03</td>
<td>0.02 ± 0.01</td>
<td>0.28 ± 0.06†</td>
<td>0.32 ± 0.1†</td>
</tr>
<tr>
<td>Test (LCX area)</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.03</td>
<td>0</td>
<td>0</td>
</tr>
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</table>

Values are means ± SE; n, number of experiments (20 cells/experiment). Sham, preconditioning staining; HSP70, heat shock protein 70; HSC73, constitutive heat shock protein 73; LAD, left anterior descending; LCX, left circumflex. *P < 0.05, †P < 0.01 one-way ANOVA vs. sham measurements.
test, remote, or sham tissue, the amount of the constitutively expressed HSC73 (6.7 ± 1.6 μg/mg total protein, n = 5) was 9.3-fold greater than HSP70 (0.7 ± 0.3 μg/mg total protein, n = 5), the inducible form of the stress protein (P < 0.05), regardless of the changes in coronary flow and contractile function (Fig. 7B). However, in remote regions of the myocardium, an apparent upregulation of HSP70 expression was observed, especially following severe low-flow ischemia (Fig. 7B). HSP70 content increased from 0.7 ± 0.3 to 2.3 ± 0.4 μg/mg of total protein (P < 0.05, n = 5) in the remote regions of the left ventricle where flow and function remained normal. HSC73 content also was elevated slightly (i.e., ~16%, n = 7, P < 0.2) in the remote myocardium. The amounts of HSC73 extracted from canine myocardium were comparable to levels expressed in rodent and human hearts, but HSP70 protein content was somewhat lower than that found in other mammals (15).

Tubulin, HSP70, and HSC73 interactions could be demonstrated in severely ischemic myocardial extracts solubilized at 37°C but not in moderately ischemic tissue preparations (Fig. 7, A and B). Under these conditions, immunoblots prepared from subendocardial sarcoplasmic fractions obtained from test areas of the heart revealed that α- and/or γ-tubulin as well as HSC73 and HSP70 failed to enter 10% SDS-PAGE gels (Fig. 7, A and B). Conversely, extracts prepared at 37°C from remote regions of the left ventricle and sham-instrumented animals displayed all forms of both protein families (Fig. 7, A and B). When severely ischemic myocardium was reperfused for 24 h, the stress proteins and the tubulin isoforms remained aggregated with neither class of proteins being dissociated from one another after incubation at 37°C (data not shown). No evidence of aggregation was ever detected in the remote myocardium; furthermore, no tubulin-stress protein aggregation could be documented in extracts prepared from tissue derived from moderately ischemic-reperfused hearts (data not shown).

Properties of heterooligomeric tubulin-HSC73/HSP70 complexes. Putative high molecular complexes between tubulin and HSC73/HSP70 were identified in sarcoplasmic fractions of the ischemic heart fractionated by linear sucrose density gradient centrifugation. Although presence of both HSC73 and small amounts HSP70 was apparent in the six heavier fractions of the gradient, the heat shock proteins were found concentrated in fractions 3 and 4 of the gradient. When aliquots of each of these fractions were separated on 4% native gels, only HSC73 could be identified in two high molecular aggregates that ranged in size from...


\( \approx 400 \) to \( \approx 800 \) kDa (Fig. 8). Aliquots of fraction 3 solubilized at 95°C revealed the presence of the HSC73 and \( \alpha \)- and \( \beta \)-tubulin (Fig. 9, lanes 1-3) and small amounts of \( \gamma \)-tubulin as well (not illustrated). Aggregates isolated from fraction 4 displayed reduced amounts of \( \beta \)-or \( \gamma \)-tubulin (Fig. 9, lanes 2 and 3). In addition, a 30-kDa peptide also could be immunoblotted from fractions 3 and 4 with anti-HSC73 antibody (Fig. 9, lane 1). The other HSC73/HSP70-positive fractions subjected to denaturing gel electrophoresis lacked tubulin. “Test” fraction 3 consistently disclosed large amounts of the 800-kDa tubulin-HSC73/HSP70 aggregate (Fig. 8, \( \approx 60\% \) vs. \( \approx 40\% \) in fraction 3). To confirm that the aggregates reflected specific protein-protein interactions between tubulin and the HSP70 family of stress proteins, anti-HSC73/HSP70 antibodies were employed to immunoprecipitate proteins that interacted with the stress proteins in fraction 3. The immunoprecipitates yielded only \( \alpha \)- (Fig. 10, lane 2), \( \beta \)- and \( \gamma \)-tubulin (data not shown) and HSC73 (Fig. 10, lane 5). The anti-HSP70/HSC73 antibody routinely immunoprecipitated a constant amount of HSC73 from fraction 3; however, the antibody only brought down significant amounts of tubulin following severe low-flow ischemia and reperfusion (Table 3). Whereas anti-HSP70/HSC73

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**Fig. 6.** Remote subendocardial myocytes derived from 5-h low-flow ischemic tissue. \( \alpha \)-Tubulin (a) and heat shock protein 70 (HSP70, b) colocalize to the microtubules (arrowheads), the perinuclear (N) girdle, and centrosomes (arrows); other myocytes display few intact microtubules (*, a) and no tubulin-HSP70 colocalization (*, c); a–c, bar = 5 \( \mu \)m.

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**Fig. 7.** Temperature-sensitive (37°C/95°C) aggregation of tubulin isoforms (A) and HSC73/HSP70 (B) is depicted in these representative Western blots derived from 5-h test, remote, and sham tissue samples. Note that neither tubulin nor stress protein isolated from test extracts (A and B) entered the 10% SDS gel when samples were solubilized at 37°C; however, when solubilized at 95°C, all isoforms of tubulin and HSC73 and HSP70 can be identified in the test extracts (A and B). Each lane was loaded with 2 \( \mu \)g of protein from sham, test, and remote sarcoplasmic extracts.

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**AJP-Heart Circ Physiol • VOL 283 • OCTOBER 2002 • www.ajpheart.org**
HSC73 antibody immunoprecipitated tubulin, anti-tubulin antibodies failed to immunoprecipitate either HSC73 or HSP70, suggesting that the tubulin was sequestered within these heterooligomeric complexes. Furthermore, the 30-kDa HSC73-positive peptide that was observed in immunoblots from fractions 3 and 4 (Fig. 9) was not present in the immunoprecipitates. The origin of this HSC73 cross-reactive peptide remains unresolved at this juncture, although a HSC73-positive peptide of similar size has been identified in “heat shocked” rat myocardium and is believed to represent a degradation fragment of HSC73 (9). The presence of these high molecular aggregates also could be identified in extracts prepared from reperfused hearts (not illustrated). The content and size of the aggregates was similar to those complexes isolated immediately following low-flow ischemia (Table 3).

**DISCUSSION**

A graded reduction in coronary flow to the subendocardium induced a complex redistribution of the constitutively expressed heat shock protein, HSC73, to elements of the microtubule network in both test and, to a lesser degree, remote canine cardiocytes. Although HSC73 stained intermyofibrillar microtubules, no HSC73-tubulin aggregates could be isolated following moderate low-flow ischemia, implying the development of relatively “weak” interactions between tubulin and the stress protein. However, when coronary flow was reduced more severely, microtubules depolymerized and high molecular weight tubulin-HSC73 aggregates could be isolated from extracts of ischemic tissue. Furthermore, these aggregates persisted for as long as 24 h following reperfusion. The results of these experiments have demonstrated that HSC73 and tubulin directly interact with one another following the reduction of coronary flow and “weak” as well as “strong” interactions appear to exist. Selective preservation of centrosomal tubulin following severe low-flow ischemia further suggests the existence of at least two pools of tubulin, one of which appears to form complexes with HSC73. The presence of some microtubule depolymerization in the remote myocardium also argues that low-flow ischemia, in-and-of-itself, may not be the sole stimulus responsible for the disruption of the microtubules (24). Such observations strengthen the argument that tubulin-HSC73 interactions may serve to stabilize portions of the microtubule network under conditions recognized to disrupt microtubules (13, 21).

Although the subcellular mechanism(s) that afford myocardial “protection” remain largely unknown, heat shock/stress proteins are believed to mediate cardioprotection, in part, as molecular chaperones that target, fold, and stabilize cardiocyte cytoskeletal proteins (3, 17, 30). Nevertheless, only a single report has identified the cardiocyte’s microtubule network as a poten-

### Table 3. $\alpha$-Tubulin and HSP70/HSC73 content in immunoprecipitates isolated from test fraction 3 of 5 h ischemic myocardial extracts

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Sham ($n = 6$)</th>
<th>5 h ($n = 6$)</th>
<th>5 h + 24 ($n = 6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-Tubulin</td>
<td>0.1 ± 0.04</td>
<td>7.4 ± 2.2*</td>
<td>8.9 ± 2.8*</td>
</tr>
<tr>
<td>HSC73</td>
<td>5.6 ± 1.1</td>
<td>6.2 ± 1.4</td>
<td>6.6 ± 0.7</td>
</tr>
<tr>
<td>HSP70</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.2</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$, number of experiments. Experimental groups represent $\mu$g of $\alpha$-tubulin/HSC73/HSP70 immunoprecipitated per 100 $\mu$g of fraction 3 protein with anti-HSC73/HSP70 antibody (clone 92 mouse IgG1, StressGen). Sham, instrumented, control preparations. Severe low-flow ischemia for 5 h and reperfusion (R) for 24 h are presented. *$P < 0.01$, one-way ANOVA vs. sham preparation.
tial target for protection by members of the HSP70 family of stress proteins (5, 8). Furthermore, overexpression of the constitutive (HSC72/73), not the inducible (HSP70) form, of the stress protein appeared to be responsible for stabilizing microtubule integrity in “ischemic” neonatal rat heart cells (5). Brown and colleagues (6) reported that the constitutive form of HSP70 family of stress proteins (i.e., HSC73) acts as a chaperone, which assists the folding of nascent tubulin at perinuclear centrosomes in HeLa cells. The microinjection of HSC73 into cultured HeLa cells also was demonstrated to “protect” centrosomes by illustrating that the cells could nucleate new microtubule assembly immediately following heat shock rather than requiring de novo synthesis of the stress protein (7). From such experiments, the hypothesis has evolved that HSC73 must directly interact with tubulin to facilitate assembly and stabilization of the nascent microtubule network following heat shock and that the centrosome is the likely site of such interactions.

The results from the present investigation provide additional evidence supporting the contention that constitutively expressed HSC73 directly interacts with tubulin in the ischemic myocardium. Two forms of HSC73-tubulin interaction appear to develop following low-flow ischemia. The colocalization of HSC73 and tubulin infers that some form of “weak” interaction was established (Table 2) in cardiocytes following moderate low-flow ischemia (Table 1), because high-molecular-weight HSC73-tubulin complexes could not be isolated from either the ischemic or reperfused myocardium. The nature of these weak interactions has not been defined, but such colocalization may serve to stabilize the structure of intermyofibrillar microtubules, which like cytoplasmic microtubules (6, 7), appear most sensitive to heat shock and ischemic stress. In contrast, tubulin-HSC73 complexes that have been isolated from severely ischemic tissue (Figs. 8–10) appear similar in size and composition to those recently identified in the ciliated protozoan, *Tetrahymena thermophila*, following heat shock (28). The stress protein appears to preferentially target centrioles, which are central components of centrosomes and basal bodies (7, 28). Perhaps the presence of HSC73 at these sites facilitates the oligomerization of cytosolic HSC73 during ischemic stress, creating the high-molecular-weight complexes that sequester and protect centriolar tubulin. Nevertheless, following reperfusion, the centrosome appears incapable of promoting microtubule assembly from existing depolymerized tubulin for at least 24 h, so it seems plausible that the formation of such HSC73-tubulin complexes may limit the ability of anti-tubulin antibodies to immunoprecipitate HSC73. The formation of such oligomeric complexes may ensure that intermyofibrillar microtubules ultimately can be reassembled from the “protected” centrosome during recovery from reversible cell injury. The presence of heterogeneous complexes, depolymerized tubulin, and disrupted myofibrils in stunned myocardium further suggests that the three phenomena may be causally linked. Future experiments must be extended to include prolonged reperusions to test this hypothesis.

Another intriguing scenario regarding the functional significance of the HSC73-positive centrosomal complexes has emerged from several cell culture experiments. Ciechanover’s laboratory (4) has provided evidence demonstrating that HSC73 also can function as a chaperone for the ubiquitination of abnormal/damaged proteins, including cytoskeletal proteins, and others have reported that this activity occurs at the centrosome (1). The colocalization of HSC73 and tubulin also could be indicative of the ubiquitination of “damaged” tubulin and, perhaps, other proteins (4, 14) at the centrosome. The presence of the multicatalytic proteasome in enriched centrosomal fractions is consistent with these observations (1, 14, 27). Because a modest but significant decrease in tubulin develops following low-flow ischemia and reperfusion, future experiments will have to address the relationship between HSC73, tubulin turnover, and proteasome activity during low-flow ischemia to test the validity of this hypothesis. Conversely, others (12, 16, 30) have proposed that the formation of high-molecular-weight heterooligomeric complexes of heat shock proteins actually may limit complex breakdown, thereby conferring a degree of protection to subcellular organelles during stressful circumstances. Perhaps the high levels of HSC73 expression in the canine myocardium reflect the multiple functions that this stress protein must subserve in maintaining cardiomyocyte homeostasis.

In summary, the present experiments have demonstrated that depolymerization of intermyofibrillar microtubules parallels myofibril disruption during low-flow ischemia. Gradually reducing coronary flow has provided evidence for two forms of HSC73-tubulin interactions in the canine cardiocyte. It is conceivable that the weak and strong interactions that develop may reflect the severity of the ischemic insult. Because the intermyofibrillar site has been recognized as a site of thick filament disruption (24), the redistribution of heat shock proteins to microtubules at these sites appears compatible with the hypothesis that the HSC73 stabilizes portions of the microtubule network and theoretically “protects” the myofibril. Conversely, severe low-flow ischemia depolymerizes intermyofibrillar microtubules, leaving only the centrosomal microtubule sites intact. The development of high-molecular-weight complexes that are composed of HSC73 and all three tubulin isoforms demonstrates the highly specific nature of these protein-to-protein interactions. These results support the contention that this constitutively expressed stress protein may represent a first line of defense in preserving selected elements of the cardiocyte’s microtubule network. Because myofibrillar disruption parallels microtubule depolymerization, these results also provide additional evidence supporting the contention (2, 20) that an intact microtubule-based cytoskeleton may contribute to the stabilization of myofibril structure in the cardiac myocyte.
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