Confocal Microscopic Localization of Constitutive and Heat Shock–Induced Proteins HSP70 and HSP27 in the Rat Heart

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Background—Heat-shock treatment of rats elevates expression of heat-shock proteins, which play a role in improving the contractile recovery and reducing infarct size in hearts after ischemic injury. However, the location of these proteins in the heart is unknown.

Methods and Results—Anesthetized rats were heat-shocked by elevation of body temperature to 42°C to 42.5°C for 15 minutes, followed by 24 hours of recovery. Control and heat-shocked hearts were extirpated and perfused briefly with saline followed by 2% paraformaldehyde in PBS. Confocal immunofluorescence microscopy of control hearts revealed that HSP27 was localized in cardiomyocytes in a pattern reminiscent of Z bands and was colocalized with neuronal markers in somata and axons. No obvious change in HSP27 content or distribution occurred after heat shock. Confocal microscopy revealed little or no HSP70 in control hearts. After heat shock, HSP70 was detected neither in cardiomyocytes nor in neuronal elements within the heart, but HSP70 was abundant in small blood vessels found between the ventricular cardiomyocytes.

Conclusions—Heat shock induces a cell type–specific expression of HSP70 in blood vessels but not myocytes or intrinsic cardiac neurons, suggesting that blood vessels play a primary role in myocardial protection. (Circulation. 2000;102:1703-1709.)

Key Words: immunohistochemistry ■ heat shock proteins ■ nervous system, autonomic ■ endothelium ■ myocytes

Many heat-shock proteins are constitutively expressed and some are highly inducible by a variety of stressors. After heat-shock proteins are induced, cells in such organs as the heart and brain can show remarkable resistance to subsequent stress. For example, heat shock–induced expression of HSP70 is correlated with enhanced recovery of myocardial contractility after ischemic injury. In fact, transgenic overexpression of rat and human HSP70 plays a direct role in protection of the mouse myocardium from ischemic injury. Similarly, HSP27, a member of the small heat-shock protein family, confers thermoresistance to NIH/3T3 cells and increases resistance to oxidative stress and cytotoxicity induced by tumor necrosis factor-α.

Recent work suggests that heat-shock proteins play a role in protecting the function of endothelial cells after ischemia-reperfusion injury. Transfection of an expression vector containing human HSP70 cDNA into endothelial cells resulted in significant protection from hypoxia-reoxygenation injury. In hearts, heat-shock treatment significantly improves myocardial and endothelial functional recovery after cardioplegic arrest. In addition, isolated endothelial cells have higher levels of HSP70 than do myocytes from heat-shocked hearts, suggesting that myocardial protection is mainly a result of expression of HSP70 in endothelial cells. Other heat-shock proteins may also be involved in endothelial cell–related myocardial protection. In cultured endothelial cells, HSP70 is associated with protecting microfilaments from disruption and aggregation in response to ischemia-like injury. However, the specific cell types that express HSP27 and HSP70 in the heart are as yet unknown. Thus, our first objective was to determine the distribution of HSP70 and HSP70 in the atrial and ventricular myocardium of normal rat hearts and in hearts 24 hours after heat-shock treatment.

In the central nervous system, HSP70 is not normally expressed but is highly inducible in neurons by such stressors as ischemia and seizures. HSP27 is constitutively expressed in many sensory and motor neurons of the brain stem and spinal cord of the adult rat. It is therefore likely that both of these proteins are either constitutively expressed or can be induced in the peripheral nervous system. However, the distribution of these proteins in intracardiac neuronal elements has not been determined. Moreover, neither the presence of HSP27 nor that of HSP70 has been reported in neurons with somata intrinsic to the heart. Thus, a second objective was to determine whether HSP27 and HSP70 were constitutively expressed or could be induced by heat shock in elements of the intracardiac nervous system.

Methods

Experimental Animals
Male Sprague-Dawley rats (225 to 300 g; Charles River Inc, St Constant, Québec, Canada) were cared for in accordance with the...
Tissue Preparation and Fixation
At 24 hours after heat shock, animals were given an overdose of sodium pentobarbital (100 mg/kg), and hearts were removed and perfused briefly via the aorta with 100 mmol/L PBS. Once the blood was cleared from the heart, the heart was either frozen (−70°C) for later extraction of proteins or prepared for immunohistochemistry. For immunohistochemistry, the atria were separated from the ventricles and pinned flat. Atria and ventricles were fixed for 24 hours by immersion in a solution of 2% paraformaldehyde in 100 mmol/L PBS. Ventricles were then immersed overnight in 30% sucrose in 100 mmol/L phosphate buffer, then sectioned at 40 μm on a freezing microtome. Sections were stored in Millonig’s buffer until processed for immunofluorescence analysis. Whole-mounts of atria were processed similarly.

2D Gel Electrophoresis and Western Analysis
Ventricular samples from control (n=4) and heat-shocked (n=4) rats were analyzed by 2D gel electrophoresis followed by Western analysis, as previously described.6,17 Briefly, ~1 mg of ventricular protein was loaded onto each isoelectric focusing gel and separated overnight. Then gels were equilibrated in SDS buffer, and the proteins were separated by electrophoresis in the second dimension on either 7.5% (for HSP70) or 12% (for HSP27) SDS-polyacrylamide gels. The proteins were transferred overnight onto Immunobilon PVDF membranes (Millipore). Membranes were incubated in PBS containing 5% skim milk powder and reacted overnight at 4°C with either the primary rabbit polyclonal antibody specific for HSP27 (1:1000 dilution; StressGen) or the primary monoclonal antibody specific for HSP70 (1:1000 dilution; StressGen). After a washing in PBS, membranes were incubated for 1 hour with horseradish-conjugated goat antibody raised against rabbit or mouse IgG in 100 mmol/L PBS. Membranes were reacted in PBS containing 4-chloro-1-naphthol (0.05%). Membranes were digitally photographed, counterstained with amido black, and photographed again. Densitometric analysis of immunoprecipitate on membranes was done with Bio-Rad Molecular Analyst version 1.5 software.

Immunofluorescence
Atrial whole-mounts (n=34) were dissected to remove the endocardium, allowing penetration of antibodies to the intrinsic cardiac nervous system.18 Atria were dehydrated in a graded series of ethanol solutions and cleared in xylene. The tissue was rehydrated and incubated in a 4% solution of Triton-X 100 in PBS for 48 hours to improve antibody penetration. Free-floating whole-mounts were incubated in a blocking solution of 2.5% normal donkey serum to reduce nonspecific staining.

Double-label immunohistochemistry was performed with an initial 48 hours of incubation of the primary antibody directed against either protein gene product 9.5 (PGP 9.5), a general neuronal marker; tyrosine hydroxylase (TH), an enzyme in the synthesis pathway for catecholamines used to identify sympathetic postganglionic projections; or choline acetyltransferase (ChAT), an enzyme in the synthesis pathway for acetylcholine, used to identify parasympathetic preganglionic projections and intracardiac neurons. Atria were incubated in the appropriate secondary antibody conjugated to a fluorescent tag as previously described.18 Double-labeling was performed by a second 48 hours of incubation of primary antibody directed against either HSP27 or HSP70. The atria were then incubated in the appropriate secondary antibody directed against the primary antibody.

Images of whole-mount atria and ventricular sections were captured with a confocal microscope (Zeiss LSM 510), and a 3D view of the tissue was reconstructed from optical sections. Anatomic figures were compiled from confocal images with Adobe Photoshop 6.0 software.

Results
Western analysis of proteins separated by 2D gel electrophoresis revealed a constitutive level of HSP27 in control rat hearts (Figure 1A, 1A’). After heat-shock treatment, the amount of HSP27 appeared to be increased (Figure 1B, 1B’). Two immunoreactive isofoms of HSP27 (Figure 1A, 1B) were apparent among other proteins visualized after the membrane had been counterstained with amido black (Figure 1A’, 1B’). The semiquantitative analysis of the immunoreactive spots revealed a modest (nonsignificant) increase in HSP27 after heat-shock treatment (Figure 1C).

A relatively low constitutive level of HSP70 was revealed in control rat hearts by Western analysis (Figure 2A, 2A’). After heat-shock treatment (24 hours), HSP70 was increased (Figure 2B, 2B’). Immunoreactive HSP70 (Figure 2A, 2B) was apparent among other proteins visualized after the membrane had been counterstained with amido black (Figure 2A’, 2B’). The semiquantitative analysis of the immunoreac-
tive spots revealed a significant increase in HSP70 after heat-shock treatment (Figure 2C).

Confocal microscopy of whole-mount atria revealed intense HSP27 immunoreactivity (HSP27-IR) within neuronal somata and axons (Figure 3). Double-labeling of whole-mount atria from control (Figure 3A, 3A') and heat-shocked (Figure 3B, 3B') rats revealed positive PGP 9.5 immunoreactivity (PGP 9.5-IR; Figure 3A, 3B) and HSP27-IR (Figure 3A', 3B') that was colocalized within neurons and axon bundles. Similarly, in atria from control (Figure 3C, 3C') and heat-shocked (Figure 3D, 3D') rats, positive ChAT immunoreactivity (ChAT-IR; Figure 3C, 3D) and HSP27-IR (Figure 3C', 3D') overlapped in neuronal somata and axons. In all instances of colocalization in neuronal somata, HSP27 appeared to concentrate within the cytoplasm, leaving the nucleus relatively unreactive. PGP 9.5, ChAT, and TH immunohistochemistry has been shown to label all neuronal somata and axons within the heart.18

Immunofluorescence confocal microscopy of whole-mount atria revealed no clear constitutive or inducible HSP70-IR in either cardiomyocytes or neuronal elements of the intrinsic cardiac nervous system (Figure 4). Atria were double-labeled for HSP70 and either PGP 9.5 or TH to show elements of the sympathetic cardiac innervation. In control (Figure 4A, 4A'), 4C, 4C') and heat-shocked (Figure 4B, 4B', 4D, 4D') rat atria, PGP 9.5-IR (Figure 4A, 4B) and TH immunoreactivity (TH-IR; Figure 4C, 4D) were detected in neural elements, but no HSP70-IR was detected in neuronal elements or cardiomyocytes in either control (Figure 4A', 4C') or heat-shocked (Figure 4B', 4D') atria. However, HSP70-IR was apparent in the walls of blood vessels within the atria after heat shock (Figure 5). Double-labeling revealed that after heat shock, PGP 9.5-IR (Figure 5A) was detected in axons adjacent to blood vessels, whereas HSP70-IR was associated with blood vessels in control atria (Figure 5B).

Control and heat-shocked hearts were double-labeled with antibodies against neuronal markers PGP 9.5 (A, B) or ChAT (C, D) and HSP27 (A', B', C', D'). Bar=60 μm (A, A', B, B', C, and C') and 75 μm (D and D').
In ventricular sections, HSP27-IR was present within cardiomyocytes, axons, and terminals (Figure 6C). Sections of heat-shocked ventricles were positive for HSP27-IR (Figure 6C). The HSP27-IR within axons and terminals was colocalized with ChAT-IR (Figure 6C). HSP27-IR in cardiomyocytes occurred in a banding pattern transverse to the long axis of these cells reminiscent of the Z banding of the contractile proteins.

No HSP70-IR was observed within control hearts (Figure 6D, 6E), but neuronal processes and nerve terminals with varicosities were immunoreactive for PGP 9.5 (Figure 6D). After heat shock, intense HSP70-IR was found in blood vessels (Figure 6F, 6G) but was not colocalized with neuronal elements containing PGP 9.5-IR (Figure 6F) or TH-IR (Figure 6G). HSP70-IR showed occasional branching patterns that intermingled with and coursed parallel to the myocytes in sections cut longitudinally in relation to myocyte orientation (Figure 6F). In ventricular tissue cut transversely to the long axis of myocyte orientation, it became more apparent that elements labeled positively for HSP70 were situated between the myocytes (Figure 6G).

Confocal microscopy of ventricular sections revealed HSP70-IR associated with apparent interstitial capillaries (Figure 7). In sections of ventricle from control rats, no positive HSP70-IR was observed in neural elements, cardiomyocytes, or blood vessels (Figure 7A), but after heat shock, intense HSP70-IR was observed in capillaries between the myocytes (Figure 7B; see also Figure 6F). To determine independently the microvascular pattern in the ventricular myocardium, Evans blue dye dissolved in a gelatin-saline solution was perfused into the coronary arteries. In sections cut longitudinally to the long axis of the myocytes, long striations of Evans blue dye-filled vessels were observed with occasional branching (Figure 7C, 7D). In sections transverse to the long axis of the myocytes, small-diameter, dye-filled vessels were seen situated between myocytes (not shown). The overall distribution pattern of HSP70-IR (Figures 6F, 7B) in heat-shocked ventricle was similar to that of the Evans blue dye in microvessels (Figure 7C, 7D).

Finally, ventricles from control and heat-shocked rats were double-labeled with antibodies against HSP27 and HSP70. In control ventricular sections, confocal microscopy revealed HSP27-IR (Figure 8A) in cardiomyocytes, but no HSP70-IR (Figure 8A') was detected. After heat shock, the distribution of HSP27-IR (Figure 8B) was similar to that in control hearts. HSP27-IR appeared to be located in cardiomyocytes, neuronal elements, and possibly blood vessels. After heat shock, HSP70-IR (Figure 8B') appeared to be associated with blood...
vessels coursing between the cardiomyocytes in the ventricles. Double-labeling for HSP25 and HSP70 revealed minimal colocalization of the 2 proteins.

Discussion
Within 24 hours of recovery from hyperthermia, a modest increase in HSP27 and a robust increase in HSP70 were detected in the rat heart by Western analysis. HSP27 was localized primarily in cardiomyocytes and in neuronal somata and processes in both control and heat-shocked hearts. HSP70 was not detected in control hearts, but after heat shock, HSP70 was localized within blood vessels in the atria and ventricles.

HSP27 is a constitutive protein of human, rabbit, and rat hearts. In cultured cardiomyocytes, overexpression of HSP27 is associated with resistance to ischemic injury. Recently, overexpression of HSP27 in cultured cardiac cells has been shown to protect against apoptotic stimuli as well as thermal and hypoxic stress. Our finding that HSP27 was present in cardiomyocytes provides more precise anatomic localization of this protein, in the Z bands of the contractile apparatus.

The finding of HSP27 in neuronal somata and axons in the heart is novel. Constitutive HSP27 is seen in many, but not all, neurons of motor nuclei in the brain stem and spinal cord of the rat. Among these, HSP27 is expressed in preganglionic parasympathetic cholinergic neurons of the nucleus
Heat shock–induced HSP70 in the heart was localized in the smaller vessels (confirming the work of Amrani et al12), but not in cardiomyocytes or neurons. The basis for this expression pattern is unclear at present. Perhaps endothelial cells are exposed to the highest burden of reactive oxygen radicals during the heat-shock treatment. Alternatively, HSP70 mRNA may reach higher concentration followed by greater translation in the smaller endothelial cells than in the larger myocytes or neurons.

Finding HSP70 in blood vessels in the heart is interesting for several reasons. First, it suggests that cells have a cell type–specific response to stress, ie, endothelial cells in the heart appear to be more sensitive to heat shock than are cardiomyocytes or neurons. However, each of these cell types can express HSP70 after metabolic injury.34–36 Second, cells, in this case endothelial, appear to respond to stress according to their local environment. After hyperthermia, cells in blood vessels in the heart express HSP70 (present study; Amrani et al13), whereas in the brain HSP70 is localized not in blood vessels but mostly in glia and in some neurons.37 This suggests that the protective role of HSP70 may be through different cell types and cell–cell interactions in different organs. Third, caution should be used in extrapolating from studies of cells in culture (eg, cardiomyocytes) to intact organs that include vascular and neuronal elements. Although considerable information has been gleaned about the effects of overexpressing HSP70 in cardiomyocytes and the resulting cellular protection, in the intact heart after heat shock, myocardial protection is most likely due to high levels of HSP70 in blood vessels. Fourth, as discussed by Gray et al,38 HSP70 in vascular cells of heart has important implications for myocardial protection. If blood vessels are protected from free-radical injury during reperfusion, then the whole organ may be protected. Finally, our findings suggest that therapies designed to increase protective proteins, such as HSP70, should be directed at the blood vessels.

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References

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