Heat Shock Protein 70 Gene Transfection Protects Mitochondrial and Ventricular Function Against Ischemia-Reperfusion Injury

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Background—Upregulation of heat shock protein 70 (HSP70) is beneficial in cardioprotection against ischemia-reperfusion injury, but the mechanism of action is unclear. We studied the role of HSP70 overexpression through gene therapy on mitochondrial function and ventricular recovery in a protocol that mimics clinical donor heart preservation.

Methods and Results—Hemagglutinating virus of Japan (HVJ)-liposome technique was used to transfect isolated rat hearts by intracoronary infusion of either the HSP70 gene (HSP group, n=16) or no gene (CON group, n=16), which was heterotopically transplanted into recipient rats. Four days after surgery, hearts were either perfused on a Langendorff apparatus for 30 minutes at 37°C (preischemia studies [n=8/group]) or perfused for 30 minutes at 37°C, cardiopically arrested for 4 hours at 4°C, and reperfused for 30 minutes at 37°C (postischemia studies [n=8/group]). Western blotting and immunohistochemistry confirmed HSP70 upregulation in the HSP group. Postischemic mitochondrial respiratory control indices (RCIs) were significantly better preserved in HSP than in CON hearts: NADH-linked RCI values were 9.54±1.1 versus 10.62±0.46 before ischemia (NS) but 7.98±0.69 versus 1.28±0.15 after ischemia (P<0.05), and FAD-linked RCI values were 6.87±0.88 versus 6.73±0.93 before ischemia (NS) but 4.26±0.41 versus 1.34±0.13 after ischemia (P<0.05). Postischemic recovery of mechanical function was greater in HSP than in CON hearts: left ventricular developed pressure recovery was 72.4±6.4% versus 59.7±5.3% (P<0.05), maximum dP/dt recovery was 77.9±6.6% versus 52.3±5.2% (P<0.05), and minimum dP/dt recovery was 72.4±7.2% versus 54.8±6.9% (P<0.05). Creatine kinase release in coronary effluent after reperfusion was 0.20±0.04 versus 0.34±0.06 IU · min⁻¹ · g wet wt⁻¹ (P<0.05) in HSP versus in CON hearts.

Conclusions—HSP70 upregulation protects mitochondrial function after ischemia-reperfusion injury; this was associated with improved preservation of ventricular function. Protection of mitochondrial function may be important in the development of future cardioprotective strategies. (Circulation. 2001;104[suppl I]:I-303-I-307.)

Key Words: mitochondria • genes • ischemia • reperfusion • transplantation

Current methods of donor heart preservation are limited by the detrimental effects of ischemia-reperfusion injury. A variety of pathological mechanisms are involved, including generation of free radicals and alterations in metabolic homeostasis.1 Mitochondria, abundant in cardiomyocytes, are key organelles that are damaged by ischemia-reperfusion, resulting in a decrease in respiratory enzyme activity and uncoupling of the oxidative phosphorylation pathways.2

Novel methods of protecting the structural and metabolic components of the heart against ischemia-reperfusion injury include upregulation of intrinsic cardioprotective mechanisms.3 These include an increase in the levels of heat shock proteins, which are a class of highly conserved proteins that act as molecular chaperones during periods of cell stress.4

We and others have shown that the induction of heat shock proteins, especially the 70-kDa protein (HSP70), provides cardioprotection after ischemia-reperfusion injury; this has been demonstrated after heat stress, a nonspecific inducer of stress proteins,5 and after HSP70 gene transfection.6–8 Heat stress–induced cardioprotection has previously been shown to be associated with the preservation of myocardial metabolic function.9,10

We have previously shown that HSP70 gene transfection protects both mechanical and endothelial function in a clinically relevant donor heart preservation model; however, the exact mechanisms by which HSP70 protects cardiac function after ischemia-reperfusion are unknown. The aim of the present study was to investigate whether mitochondrial respiratory function is protected by HSP70 in a clinically relevant...
donor heart preservation model and the relationship to recovery of ventricular function.

Methods

Animals
Male Sprague-Dawley rats were used in all studies. Animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research, the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources (NIH publication No. 86-23, revised 1985), and the European Convention on Animal Care guide. The study was approved by the institutional ethics committee on animal research.

Construction of Expression Vector
Full-length human HSP70 cDNA (donated by Drs S. Fox and R. Morimoto, Northwestern University, Evanston, Ill) was directionally cloned into the EcoRI/BamHI site of pcDNA3, under the control of the cytomegalovirus promoter/enhancer (Invitrogen Corp). 7

Preparation of HVJ-Liposome Complex
The preparation of the HVJ-liposome complex has been described previously. 12 Briefly, 10 mg of lipid mixture (phosphatidylserine, phosphatidylcholine, and cholesterol; Sigma Chemical Co) was deposited on the side of a flask through the removal of tetrahydrofuran in a rotary evaporator. The dried lipid was hydrated in 200 mL of balanced salt solution (137.0 mmol/L NaCl, 5.4 mmol/L KC1, 10.0 mmol/L Tris-HCl, pH 7.6) containing a DNA (200 μg)-HMGI (high-mobility group 1 nuclear protein, 64 μg; Wako) complex.

A liposome-DNA-HMGI complex suspension was prepared through vortexing, sonicating, and shaking to form liposome; the liposome suspension was incubated with 30,000 hemagglutinating units of HVJ, which was inactivated by ultraviolet irradiation, first at 4°C and then at 37°C. Finally, 4 mL of the sucrose gradient layer containing HVJ-liposome was collected for use.

Gene Transfection
Gene transfection was performed as described previously. 13 Donor rats (225 to 250 g) were anesthetized with sodium pentobarbital (50 mg/kg IP), and sodium heparin (1000 IU/kg) was injected via the femoral vein. Laparotomy was performed, and the hearts were arrested with cold cardioplegia (St Thomas' Hospital No.1; Martin- dale) injected retrograde via the abdominal aorta.

Thoracotomy was then performed, and the hearts were rapidly excised. Hearts from the group transfected with the HSP70 gene (HSP, n=16) were infused with 1 mL of HVJ-liposome (containing pcDNA3 with human HSP70 cDNA) via the aorta and thus the coronary arteries; the venae cavae and pulmonary arteries and veins were all ligated, raising intracoronary pressure. The control hearts (CON, n=16) were infused with the same volume of HVJ-liposome (containing pcDNA3 but without human HSP70 cDNA).

After incubation on ice for 10 minutes, hearts were heterotopically transplanted into the abdomens of the recipient rats (300 to 325 g). 14 Recipient rats were killed on the fourth day after gene transfection, allowing the introduced gene to stably express proteins and providing adequate time for endogenous HSP70 induced by surgical stress to decrease to preoperative levels. 7

Experimental Protocol
HSP and CON hearts were studied to determine mitochondrial and ventricular function before and after 4 hours of cardioplegic arrest at 4°C. Rats were anesthetized with diethyl ether and sodium heparin (1000 IU/kg) injected via the femoral vein. Laparotomy was performed, and transplanted hearts were carefully excised and placed in ice-cold Krebs-Henseleit buffer. They were then rapidly attached to a Langendorff apparatus and perfused via the aorta with filtered Krebs-Henseleit buffer (containing [in mmol/L] NaCl 118, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, NaHCO3 24, glucose 11, and CaCl2 1.2, pH 7.4) at a constant pressure of 100 cm H2O and continuously gassed with a 95% O2/5% CO2 mixture at 37°C, as previously described. 8

After an initial stabilization period of 20 minutes of perfusion, preischemic mechanical function was evaluated using an intraventricular balloon catheter inserted into the left ventricle, as previously described. 13 The balloon was inflated to an end-diastolic pressure of 10 mm Hg. Peak-systolic pressures were recorded and used to calculate developed pressure. At the end of 30 minutes of perfusion, some hearts were randomly selected for preischemic studies of mitochondrial function (n=5/group) and Western blotting/immunohistochemistry (n=3/group).

The remaining hearts were arrested by the infusion of 4°C cardioplegia (St Thomas' No.1) via the aorta at a constant pressure of 60 cm H2O for 2 minutes. Hearts were then immersed in cardioplegia (St Thomas’ No.1) and maintained at 4°C with the aid of a temperature probe; after 4 hours of cardioplegic arrest, hearts were reperfused with Krebs-Henseleit buffer at 37°C. Coronary effluent was collected during the first 15 minutes of reperfusion, and creatine kinase leakage was assessed, as previously described. 7 After 20 minutes of reperfusion, postischemic mechanical function was evaluated with the balloon inflated to an end-diastolic pressure of 10 mm Hg. At the end of 30 minutes of perfusion, hearts were removed for postischemic studies of mitochondrial function (n=5/group) and Western blotting/immunohistochemistry (n=3/group).

Recovery of mechanical function was expressed as relative recovery of postischemic versus preischemic developed pressure (relative recovery of developed pressure) and time derivatives of pressure changes (+dP/dt and −dP/dt).

Mitochondrial Function
Perfused rat hearts (n=5/group for preischemia and n=5/group for postischemia) were minced and digested using a protease enzyme (0.5 mg/mL; Nagarse) in ice-cold isolation medium (225 mmol/L mannitol, 75 mmol/L sucrose, 10 mmol/L Tris, 2 mmol/L EGTA, pH 7.2). Cardiomyocyte mitochondria were isolated by homogenization and differential centrifugation, and mitochondrial protein was measured using a modified Lowry method (Lowry DC kit; Bio-Rad). 16 Mitochondrial oxygen consumption was measured as previously described 17 using a Clark-type oxygen electrode (World Precision Instruments) and recorded using Biopaq AcqKnowledge software (Linton). All experiments were carried out at 30°C in 350 μL of respiration medium containing (in mmol/L) KCl 100, mannitol 75, sucrose 25, EDTA (dipotassium salt) 0.05, Tris-HCl 10, and KH2PO4-Tris 10 (pH 7.4). Incubations were carried out using 0.5 mg of mitochondrial protein and 0.125 mg of fat-free bovine serum albumin. State 4 respiration was initiated using 5 mmol/L glutamate plus 5 mmol/L malate or 5 mmol/L succinate plus 1 μmol/L rotenone. State 3 respiration was initiated by the addition of ADP. Respiratory control indices (RCIs) were calculated as state 3 rate divided by state 4 rate.

Immunohistochemistry
Heart samples (n=3/group for preischemia and n=3/group for postischemia) were rapidly embedded in medium (OCT compound; Miles Inc) and frozen in liquid nitrogen, and samples were cut into thin sections (5 μm). After blocking with 5% fetal bovine serum, the sections were incubated first with a 1:1000 dilution of monoclonal mouse antibody to inducible HSP70 (SPA-810; Stressgen) followed by incubation with a 1:180 dilution of FITC-conjugated goat anti-mouse IgG monoclonal antibody (Stressgen). 3 Some slides were analyzed in 1 batch.

Western Blotting
Heart samples (n=3/group for preischemia and n=3/group for postischemia) were freeze-clamped in liquid nitrogen for Western blot analysis, as previously described. 9 Blots were probed with monoclonal antibodies specific to inducible HSP70 (SPA-810; Stressgen) and incubated with secondary horseradish peroxidase–conjugated rabbit anti-mouse antibody (Stressgen). Antibody reac-
activity was visualized using an enhanced chemiluminescence detection system (Amersham).

Statistical Analysis
Values are presented as mean ± SEM. Statistical comparison was performed by unpaired Student’s t test. A value of P < 0.05 was considered a significant difference.

Results

Immunohistochemistry
Immunohistochemical examination showed overexpression of HSP70 in the cytoplasm of cardiomyocytes in HSP, as well as in the cytoplasm of coronary endothelial cells, compared with those from CON. Approximately 60% to 70% of the cardiomyocytes in hearts from HSP were shown to overexpress HSP70 (Figure 1).

Western Blotting
Western blot analysis indicated increased expression of HSP70 in HSP versus CON (Figure 2). Tubulin expression was constant throughout the lanes, indicating equal protein loading on the blot.

Mitochondrial Function
Mitochondrial respiratory control (expressed as RCI ratios) was better preserved in mitochondria isolated from HSP than in those from CON. NAD⁺-linked respiration in HSP versus CON was 9.54 ± 1.1 versus 10.62 ± 0.46 preischemia (NS) but 7.98 ± 0.69 versus 1.28 ± 0.15 postischemia (P < 0.05) (Figure 3A). FAD-linked respiration in HSP versus CON was 6.87 ± 0.88 versus 6.73 ± 0.93 preischemia (NS) but 4.26 ± 0.41 versus 1.34 ± 0.13 postischemia (P < 0.05) (Figure 3B).

Ventricular Function
Postischemic recovery of mechanical function (percent preischemia baseline mean ± SEM values; at 10 mm Hg left ventricular end-diastolic pressure) was greater in HSP than in CON (Figure 4). Recovery of left ventricular developed pressure was significantly higher in HSP than in CON (72.4 ± 6.4% versus 59.7 ± 5.3%, P < 0.05). The maximum dP/dt recovery was significantly higher in HSP than in CON (77.9 ± 6.6% versus 52.3 ± 5.2%, P < 0.05). Likewise, the minimum dP/dt recovery was significantly higher in HSP than in CON (72.4 ± 7.2% versus 54.8 ± 6.9%, P < 0.05). Heart rates did not differ significantly between HSP versus CON after 30 minutes of perfusion (precardioplegia arrest) (241 ± 14 versus 236 ± 12 bpm [NS]) or after 30 minutes of reperfusion (postcardioplegia arrest) (214 ± 17 versus 209 ± 16 bpm [NS]). Creatine kinase release was significantly lower in HSP than in CON (0.20 ± 0.04 versus 0.34 ± 0.06 IU · min⁻¹ · g wet wt⁻¹, P < 0.05).

Discussion
The present study has demonstrated for the first time that overexpression of HSP70 in whole hearts leads to the protection of mitochondrial respiratory function and ventricular function after prolonged cold cardioplegic arrest in a protocol that mimics clinical donor heart preservation. Our results show that postischemic mitochondrial respiratory control, which is consistent with ATP production capacity, is protected in mitochondria isolated from HSP group; this

Figure 1. Immunohistochemical analysis of HSP70 expression in CON heart (A) and HSP heart (B) (magnification ×200), showing overexpression of HSP70 in cardiomyocytes (darker shade) in HSP compared with those in CON.

Figure 2. Western blots of HSP70 (top) and tubulin (bottom) expression in CON hearts (left 3 columns) and HSP hearts (right 3 columns).

Figure 3. Preischemic and postischemic RCIs in CON hearts (n = 5) and HSP hearts (n = 5). Respiratory activity was assessed in presence of glutamate and malate (NAD⁺ linked) (A) or succinate (FAD linked) (B). Values represent mean ± SEM. * P < 0.05 vs preischemia CON. † P < 0.05 vs postischemia CON. I/R indicates ischemia-reperfusion.

Figure 4. Left ventricular developed pressure (LVDP), maximum dP/dt (+dP/dt), and minimum dP/dt (−dP/dt) in CON (n = 8) and HSP (n = 8) groups (percent postischemia recovery). Values represent mean ± SEM. * P < 0.05 vs CON.
correlated with the improved recovery of ventricular function in this group.

We previously demonstrated that heat stress leads to beneficial changes in high-energy phosphate metabolism in the rat heart subjected to cold cardioplegic arrest and ischemia-reperfusion, due mainly to a decreased rate of high-energy phosphate depletion and increased recovery of ATP and phosphocreatine levels during reperfusion. These changes may have been due to protective effects of HSP70 on mitochondrial function resulting from improved preservation of mitochondrial membranes and enzyme complexes during ischemia-reperfusion.

These studies, however, used mild hyperthermic stress as an inducer of heat shock proteins; thus, a variety of other intrinsic mechanisms may also have been induced, including upregulation of MnSOD and catalase. To study the functions of individual HSPs, especially HSP70, we used an efficient method of in vivo gene transfection, with the HVJ-liposome technique, which results in a high level of HSP70 gene transfection in whole rat hearts. We recently showed a significant improvement in postischemic recovery of both endothelial and mechanical function in HSP70 gene-transfected hearts compared with controls in an identical donor heart preservation protocol.

The present study demonstrates that the RCI, which indicates levels of ATP restoration after reperfusion, is much greater in HSP than in CON. The exact mechanisms by which HSP70 may lead to preservation of the RCI are unknown. One potential mechanism by which HSP70 preserves mitochondrial function is by HSP70 acting as a molecular chaperone for proteins encoded by mitochondrial DNA, leading to protection of mitochondrial respiratory chain enzyme activity, resulting in beneficial effects on state 3/state 4 respiration rates, as shown in our study.

Another mechanism may involve HSP70 decreasing apoptosis after ischemia-reperfusion; a recent study has shown that HSP70 gene transfection reduces myocardial apoptosis after ischemia-reperfusion injury. Recent studies have shown that apoptosis predominates in cardiomyocytes after reoxygenation through a mitochondrion-dependent apoptotic pathway and that Bcl-2 prevents reoxygenation-induced apoptosis by inhibiting cytochrome c release from the mitochondria, preventing the activation of caspase-3 and caspase-9. Furthermore, other studies reveal that HSP70 may act in a similar way, by binding and thus reducing cytochrome c loss from mitochondrial membranes, suppressing different types of apoptosis-inducing stimuli. A further study shows that HSP70 may also have antiapoptotic actions downstream of cytochrome c release and upstream of caspase-3 activation. A recent study has shown that combined and individual mitochondrial HSP60 and HSP10 expressions in cardiac myocytes protect mitochondrial function and prevent apoptotic cell death induced by simulated ischemia-reperfusion, again highlighting the protective role of HSPs on mitochondrial function with further evidence to support the antiapoptotic actions of HSPs.

Another mechanism of HSP70-mediated cardioprotection may be the chaperone function of HSP70 on certain cardiac ion channels during ischemia-reperfusion injury. Recent studies reveal that opening of ATP-sensitive K⁺ channels leads to increased protection of the heart after ischemia-reperfusion and that the major part of this protection is due to the mitochondrial rather than the cell membrane or sarcosomal K⁺-ATP channels; furthermore, the opening of mitochondrial K⁺-ATP channels leads to improved ATP-synthesis rates and bioenergetics. By protecting these important ion channels during ischemia-reperfusion, increased HSP70 levels may lead to more favorable ion channel function, resulting in greater cardioprotection.

Increased levels of HSP70 have been shown to be beneficial not only in experimental models but also in the clinical setting; patients with higher levels of HSP70 have been shown to have increased tolerance to ischemia-reperfusion injury after cardiac surgery. Improvements in gene transfection technology may allow this to become a clinically viable method of myocardial protection for donor hearts.

Advantages of the present protocol include the use of a highly efficient in vivo gene transfection technique and established methods for assessment of ventricular and endothelial function.

Gene transfection 4 days before the assessment of cardiac function was required in our protocol; this was partly designed to reduce the transplant-associated HSP70 induction in CON (stress-induced rise in HSP70 levels return to prestress levels by 4 days), and this interval allows optimal levels of HSP70 expression resulting from gene transfection. A similar protocol of infusion of HVJ at the time of donor heart retrieval may provide one possible method of gene delivery. Limitations of our protocol include the use of crystalloid fluid for Langendorff perfusion and the use of an animal rather than a human model.

In summary, the present study shows that increased levels of HSP70 through gene transfection leads to greater protection of mitochondrial function after ischemia-reperfusion in a donor heart preservation protocol. This may be one mechanism by which HSP70 overexpression leads to better myocardial protection; thus, the protection of mitochondria from the effects of ischemia-reperfusion injury may be important in designing future cardioprotective strategies.

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