Heat Shock Protein 72 Enhances Manganese Superoxide Dismutase Activity During Myocardial Ischemia-Reperfusion Injury, Associated With Mitochondrial Protection and Apoptosis Reduction

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Background—Heat shock protein 72 (HSP72) is known to provide myocardial protection against ischemia-reperfusion injury by its chaperoning function. Target molecules of this effect are presumed to include not only structural proteins but also other self-preservation proteins. The details, however, remain unknown. Manganese superoxide dismutase (Mn-SOD) is an enzyme that preserves mitochondria, a key organelle for cellular respiration, from reperfusion injury and limits mitochondria-related apoptosis. We hypothesized that Mn-SOD would play a role in HSP72-mediated cardioprotection.

Methods and Results—Rat hearts were transfected with human HSP72 by intra-coronary infusion of Hemagglutinating Virus of Japan-liposome, resulting in global myocardial overexpression of HSP72. After ischemia-reperfusion injury, cardiac function (left ventricular systolic pressure, maximum dP/dt, minimum dP/dt, and coronary flow) was improved in the HSP72-transfected hearts compared with control-transfected ones, corresponding with less leakage of creatine kinase and mitochondrial aspartate aminotransferase. Postischemic Mn-SOD content and activity in the HSP72-transfected hearts were enhanced in comparison with the controls (content: 96.9±5.4 versus 85.5±3.7%, P=0.038; activity: 93.9±2.2 versus 82.2±3.7%, P=0.022), associated with improved mitochondrial respiratory function (postischemic percent respiratory control index; NAD+-linked: 81.3±3.8 versus 18.5±4.4%; FAD-linked: 71.8±5.5 versus 20.7±5.3%, P<0.001). In addition, incidence of postischemic cardiomyocyte apoptosis was attenuated in the HSP72-transfected hearts (4.0±1.1 versus 10.3±3.3%, P=0.036), correlating with an increased Bcl-2 level and reduced up-regulation of caspase-3.

Conclusions—These data suggest that the enhanced Mn-SOD activity during ischemia-reperfusion injury, which is associated with mitochondrial protection and apoptosis reduction, is a possible mechanism of HSP72-induced cardioprotection. (Circulation. 2002;106[suppl I]:I-270-I-276.)

Key Words: ischemia ■ reperfusion ■ apoptosis ■ gene therapy ■ antioxidants

Reinforcing endogenous self-preservation mechanisms is a promising strategy for advanced myocardial protection.1,2 Overexpression of heat shock protein 72 (HSP72), a major self-preservation protein in the heart, has been extensively reported to result in enhanced myocardial tolerance to ischemia-reperfusion (I/R) injury using transgenic animals3 and gene transfection models.4,5 This molecule is known to act as a molecular chaperone to maintain cellular homeostasis under environmental stress.6,7 Structural and contractile proteins of cardiomyocytes may be major targets of this chaperone function of HSP72, although the details remain unknown. We propose that other self-preservation proteins including superoxide dismutase (SOD) might also be important targets of HSP72. One might expect HSP72 to preserve or enhance the activity of these anti-oxidants during I/R injury by attenuating inactivation/degradation as well as stimulating synthesis/folding/translocation. In turn, such upregulated activity of these self-protective proteins could improve myocardial tolerance to I/R injury by their own mechanisms, playing a role in HSP72-induced myocardial protection.

Mitochondria, abundant in cardiomyocytes, are key organelles involved in myocardial I/R injury. Mitochondrial damage results in decreased respiratory function and ATP supply, and also generates harmful free radicals.8 Further, leakage of cytochrome c as a result of mitochondrial damage activates proapoptotic mechanisms and leads to cardiomyo-
cyte apoptosis. Among 3 distinct SODs in mammals: manganese SOD (Mn-SOD), copper and zinc-SOD and extracellular SOD, a large quantity of Mn-SOD exclusively exists within mitochondria in cardiomyocytes to play a role in attenuating I/R injury in mitochondria. We, in this study, investigated whether HSP72-induced cardioprotection would involve preservation of Mn-SOD activity, and whether this would correspond with mitochondrial protection and reduction in apoptosis of cardiomyocytes.

Materials and Methods

Animal Care
All studies were performed with the approval of the institutional ethics committee of Harefield Heart Science Centre at Imperial College as well as that of the Home Office in the United Kingdom.

Gene Construction and Gene Transfection
Gene transfection to the heart was mediated by intra-coronary infusion of Hemagglutinating Virus of Japan (HVJ)-liposome as described before. Briefly, 200 ng DNA mixed with 64 µg high mobility group 1 nuclear protein (Wako Pure Chemicals, Tokyo, Japan) was enclosed in liposome composed of cholesterol, phosphatidylserine and phosphatidylcholine (Sigma, St. Louis, MO). The liposome was then incubated with 30 000 U of inactivated HVJ, producing 3 mL of HVJ-liposome. Male Lewis rats weighing 200 g were systemically heparinized (200 USP units, IV), the hearts arrested with cold cardioplegia (St. Thomas’ Formula II, Martindale Pharmaceuticals) and removed under anesthesia with sodium pentobarbital (50 mg/kg, IP). The hearts were infused with 1 mL of HVJ-liposome containing pCDNA3 expression vector (Invitrogen), into which had been cloned the full-length cDNA of human HSP72, via the coronary arteries, with the venae cavae and pulmonary arteries ligated. The hearts for the control group were infused with 1 mL of HVJ-liposome containing pCDNA3 without HSP72 cDNA. After 10-minute incubation under increased intra-coronary pressure on ice, the hearts were transplanted into the abdomens of 250 g male Lewis rats. After reperfusion, the surgical wounds were repaired and the rats returned to the cages to await sacrifice.

Isolated Heart Perfusion Protocol
Transplanted hearts were excised through laparotomy under general anesthesia and heparinisation, attached to a Langendorff apparatus and perfused with Krebs-Henseleit buffer (118 mmol/L NaCl; 4.7 mmol/L KCl; 1.2 mmol/L MgSO4; 1.2 mmol/L KH2PO4; 24 mmol/L NaHCO3; 11 mmol/L glucose; 1.2 mmol/L CaCl2, gassed with a 95% O2/5% CO2 mixture, 37 °C), and perfused with Krebs-Henseleit buffer (118 mmol/L NaCl; 4.7 mmol/L KCl; 1.2 mmol/L MgSO4; 1.2 mmol/L KH2PO4; 24 mmol/L NaHCO3; 11 mmol/L glucose; 1.2 mmol/L CaCl2, gassed with a 95% O2/5% CO2 mixture, 37 °C, at a pressure of 100 cm H2O). After 20 minute for stabilization, pre-I/R mechanical function was measured. After 30 minute of reperfusion, the surgical wounds were repaired and the rats returned to the cages to recover. Recipient rats were sacrificed on the fourth day after gene transfection, thus allowing the introduced gene to express HSP72 protein and providing adequate time for endogenous rat HSP70 induced by surgical stress to decrease to preoperative levels.

Mn-SOD Content and Activity
The hearts were freeze-clamped in liquid nitrogen, homogenized, centrifuged, sonicated on ice, and then centrifuged again. The supernatant of these samples was used to measure Mn-SOD content by using a Mn-SOD ELISA system (Nippon-yushi Co, Ltd) and Mn-SOD activity by means of the nitroblue tetrazolium (NBT) method. Briefly, the supernatant was added to the reaction mixture of NBT with xanthine-xanthine oxidase, and the SOD activity measured colorimetrically in the form of inhibitory activity toward blue formazan formation by SOD in the reaction mixture. To determine Mn-SOD activity, the assay was performed in the presence of potassium cyanide (1 mM) to inhibit Cu, Zn-SOD activity.

Mitochondrial Function
Hearts were minced and digested using 1.5 mg/mL Nагarse in ice-cold isolation medium (225 mmol/L mannitol; 75 mmol/L sucrose; 10 mmol/L Tris; 2 mmol/L EGTA; pH 7.2). Mitochondria were isolated by homogenisation and differential centrifugation and mitochondrial oxygen consumption was measured using a “Clark”-type oxygen electrode (World Precision Instruments) and recorded using Biopaq Aknowledge software (Linton). All experiments were carried out at 30°C in 350 μL of respiration medium containing 100 mmol/L KCl, 75 mmol/L mannitol, 25 mmol/L sucrose, 0.05 mmol/L EDTA (dipotassium salt), 10 mmol/L Tris-HCl and 10 mmol/L KH2PO4-Tris. Incubations were carried out using 0.5 mg of mitochondrial protein and 0.125 mg of 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 index (RCI) was calculated as state 3 rate/state 4 rate.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling (TUNEL) Staining
Cryo-sections (6 μm) of the hearts were stained by TUNEL, using a kit from R&D Systems. After fixation, the samples were permeabilized and immersed in 5% H2O2. Residues of digoxigenin-nucleotide were catalytically added to the 3'-OH ends of double- or single-stranded DNA by means of terminal deoxynucleotidyl transferase. After incubation with the anti-digoxigenin-peroxidase, color development was performed with diaminobenzidine substrate/H2O2. Cell type was identified by counter-staining with 1% neutral red. The number of TUNEL-positive cardiomyocytes was divided by the total cardiomyocyte number to determine the ratio of TUNEL-
positive myocytes. Ten different fields for each section were analyzed.

### Statistical Analysis

All values are expressed as mean ± SEM. The differences in the data between 2 groups were determined with Student’s *t* test. Comparison among multiple groups was assessed by one-way analysis of variance followed by Bonferroni’s post hoc test. A value of *P* < 0.05 was considered statistically significant.

### Results

#### Operative Mortality

Rat hearts were transfected with HSP72 or control plasmid, by intra-coronary infusion of HVJ-liposome, and transplanted into syngeneic recipient abdomens. Total of 4 out of 96 recipient animals were lost as a consequence of the postoperative complications. Three died of ileus; the other of infection.

#### Myocardial HSP72-Expression After Gene Transfection

At day 4 after gene transfection, Western blotting demonstrated overexpression of HSP72 in every pre-I/R HSP72-transfected heart compared with the pre-I/R control-transfected hearts. Representative data are shown in Figure 1A. Further, immunohistochemical examination with anti-HSP72 monoclonal antibody demonstrated that extensive cytoplasmic HSP72 expression in cardiomyocytes was globally distributed throughout the HSP72-transfected hearts (Figure 1B) compared with the control-transfected hearts (Figure 1C). These data on HSP72-expression after gene transfection mediated by intra-coronary infusion of HVJ-liposome were comparable with the previous observations.

#### Myocardial Damage and Cardiac Function After I/R Injury

After HSP72- or control-gene transfection, cardiac function before and after I/R was measured using Langendorff crystalloid perfusion. Preischemic cardiac function was not significantly different between the HSP72- and control-transfected hearts (Table 1). After I/R injury, recovery of both systolic (LV developed pressure and maximum dP/dt) and diastolic (minimum dP/dt and LV volume) cardiac function was improved in the HSP72-transfected group, compared with the control-transfected group (Figure 2). Recovery of coronary flow was also improved in the HSP72-transfected group, compared with the control-transfected group. Further, CK leakage was reduced in the HSP72-transfected group. Postischemic recovery of heart rate was not significantly different between groups (94.0 ± 1.2 versus 90.6 ± 1.5%, *P* = 0.078). These data on enhanced myocardial tolerance to I/R injury after HSP72-gene transfection conforms to the previous reports.

#### Myocardial Mn-SOD Content and Activity

Preischemic myocardial Mn-SOD content (Figure 3, left) and its activity (Figure 3, right) tended to be larger (although not significantly) in the HSP72-transfected hearts, compared with the control-transfected ones. Post-I/R Mn-SOD activity of the control hearts was likely to be lower compared with the post-I/R controls. More importantly, both activity and content of post-I/R Mn-SOD were significantly enhanced in the HSP72-transfected group compared with the post-I/R controls. Post-I/R percent content and activity to the respective pre-I/R level were also significantly higher in the HSP72-transfected hearts as compared with the

| Preischemic Cardiac Function Measured with Langendorff Perfusion |
|--------------------|----------------|---------------|----------------|----------------|-----------------|---------------|
|                    | HR (bpm)        | LVDP (mm Hg)  | Max dP/dt (mm Hg/sec) | Min dP/dt (mm Hg/sec) | Balloon Size (µL) | CF (mL/min) |
| HSP                | 268.2 ± 4.4     | 120.8 ± 3.0   | 3552.1 ± 75.2          | −2358.3 ± 68.1        | 199.4 ± 4.5      | 15.0 ± 0.4    |
| CON                | 264.5 ± 3.8     | 116.8 ± 2.6   | 3416.7 ± 70.0          | −2256.2 ± 55.3        | 192.7 ± 4.4      | 14.6 ± 0.5    |
| *P* value          | 0.533           | 0.310         | 0.193                   | 0.251                  | 0.297            | 0.522        |

There was no significant difference in preischemic cardiac performance between the HSP72- and control-transfected hearts. Data were measured with a Langendorff perfusion system at 10 mm Hg of left ventricular end-diastolic pressure. HSP, HSP72-transfected hearts; CON, control-transfected hearts; HR, heart rate; LVDP, left ventricular developed pressure; CF, coronary flow; Values are expressed as mean ± SEM. n = 24 in each group.
controls (content: 96.9±4.1 versus 85.5±2.5%, P=0.038; activity: 93.9±2.2 versus 82.2±3.7%, P=0.022).

**Mitochondrial Damage**

Leakage of m-AST into coronary effluent during reperfusion, as a marker of mitochondrial damage, was reduced in the HSP72-transfected hearts compared with the control-transfected hearts (Figure 4). Pre-I/R mitochondrial respiratory function was not significantly different between 2 groups (P=0.393 for NAD-linked RCI; P=0.510 for FAD-linked RCI). In contrast, post-I/R RCI (both NAD-linked and FAD-linked) was remarkably improved in the HSP72-transfected group (Figure 4). Post-I/R percent RCI to the pre-I/R level was also better preserved in the HSP72-transfected group in comparison with the controls (NAD-linked: 81.3±3.8 versus 18.5±4.4%; FAD-linked: 71.8±5.5 versus 20.7±5.3%, P<0.001).

**Cardiomyocyte Apoptosis Caused by I/R Injury**

After I/R injury, TUNEL-positive cardiomyocytes (black-blue stained nuclei) were found in both groups. Representative observation of the control-transfected hearts is shown in Figure 5A. Incidence of TUNEL-positive cardiomyocytes after I/R injury was significantly reduced in the HSP72-transfected hearts compared with the control ones (4.0±1.1 versus 10.3±3.3%, P=0.036; Figure 5B). The change in
expression of apoptosis-related proteins during the course of I/R injury was analyzed by Western blotting (Figure 6). Bcl-2, Bax, Bak, and caspase-3 were present in the pre-I/R samples of both groups without any significant differences in the expression level between HSP72- and control-transfected hearts. Post-I/R level of Bcl-2 was higher \((P=0.038)\) in the HSP72-transfected group, compared with the post-I/R control samples. Caspase-3 significantly \((P=0.011)\) increased after I/R injury in the control-transfected hearts compared with corresponding pre-I/R samples; however, this postischemic upregulation was significantly \((P=0.029)\) attenuated in the HSP72-transfected hearts. Post-I/R upregulation in the control-transfected hearts was also observed in Bax \((P=0.042)\), but without significant \((P=0.329)\) difference from the post-I/R HSP72-transfected hearts.

**Discussion**

We have demonstrated that myocardial HSP72-overexpression as a result of gene transfection provides improved cardioprotection against I/R injury in a protocol mimicking clinical donor heart preservation for transplantation, and that this is associated with enhanced Mn-SOD content and activity. In addition, these findings corresponded with less mitochondrial damage and improved post-I/R mitochondrial respiratory function, which is consistent with ATP production capacity, and further with reduced apoptosis of cardiomyocytes caused by I/R injury. These data suggest that the enhanced activity of mitochondrial Mn-SOD during I/R injury, which could play a role in mitochondrial protection and apoptosis reduction, is one of the possible mechanisms of HSP72-induced myocardial protection.

HSP72 acts a molecular chaperone, playing an essential role in mediating protein folding, assembly, transport, and degradation, helping to prevent protein denaturation and aggregation, and assisting in the refolding or removal of damaged proteins.\(^6,7\) It has been shown that Mn-SOD activity and content decreases after I/R injury, presumably via inactivation/degradation of mature, active Mn-SOD within mito-
Similar trends were observed in the present study. It is, however, unlikely that HSP72 could prevent I/R injury-induced denaturation or inactivation of mature Mn-SOD within mitochondria, because of the different localization: HSP72 localizes in cytoplasm and nucleus in both normal and stressed conditions, while mature, active Mn-SOD exists and acts in mitochondria. Western blot demonstrated that HSP72 was not found in mitochondrial protein samples that were prepared for measuring mitochondrial function in the present study (data not shown). On the other hand, it has been clarified that HSP72 plays an essential role in keeping newly synthesized mitochondrial proteins (precursor proteins) in their correct unfolding conformation within the cytoplasm and transporting them into mitochondria. Therefore, one could speculate that cytoplasmic HSP72-overexpression could enhance translocation of precursor Mn-SOD into mitochondria, which results in supplementing the pool of mature Mn-SOD within these organelles. In our study, enhanced Mn-SOD activity in the HSP72-overexpressed hearts was observed preischemia (although not significantly; \( P = 0.097 \)) and was seen more remarkably (\( P = 0.006 \)) post-I/R injury. Thus, we suppose that this chaperone mechanism of overexpressed HSP72 might work even under normal (preischemic) conditions, and that this effect could be greatly enhanced in the course of I/R injury.

Cardiomyocytes possess abundant mitochondria that are key organelles involved in myocardial I/R injury. Mitochondrial damage causes decrease of ATP production as well as generation of superoxide via the electron transport chain, leading to death of cardiomyocytes. Crystalloidal perfusion of isolated hearts using Langendorff apparatus, the relative importance of this mitochondria-derived superoxide in myocardial I/R injury might be emphasized because the perfusate does not contain leukocytes which are another major source of superoxide in physiological blood reperfusion. Using this perfusion system, Sammut and his colleagues have shown that prior heat shock treatment enhances mitochondrial respiratory function after I/R injury, associated with HSP70-upregulation. Further, we have demonstrated that post-I/R mitochondrial respiratory function is remarkably enhanced with less m-AST leakage in the HSP72-overexpressing hearts, corresponding with improved Mn-SOD content and activity. This indicates that overexpression of HSP72 provides significant mitochondrial protection against I/R injury, and suggests that enhanced mitochondrial Mn-SOD activity could be an important mechanism of this protection. Enhanced levels of Mn-SOD should be useful in attenuating mitochondrial damage and preserving mitochondrial function by scavenging superoxide produced within mitochondria during I/R injury. In our study, the degree of post-I/R enhancement in mitochondrial function in the HSP72-transfected hearts was more remarkable (NAD"-linked RCI was 5.3-fold higher and FAD-linked RCI was 4.1-fold higher than that of the controls), as compared with the degree of improvement in Mn-SOD activity (1.3-fold). This may suggest the presence of other direct or indirect mechanisms in this HSP72-mediated mitochondrial protection, in addition to indirect effect by enhanced Mn-SOD activity. One possible mechanism might be a chaperoning function of HSP72 directly for mitochondrial membrane proteins or other mitochondrial proteins including respiratory chain enzymes. HSP72 plays an important role in transporting newly synthesized mitochondrial proteins into mitochondria, similar to that described for Mn-SOD. Enhancement of mitochondrial respiratory chain enzyme activity could result in beneficial effects on state 3/state 4 respiration rate. To clarify these direct and indirect effects of HSP72 on mitochondrial function, further investigation will be needed.

Another focus of attention regarding HSP72-mediated cardioprotection may be apoptosis, which is a distinct death pathway from necrosis. This study has shown that myocardial HSP72-overexpression protects mitochondrial function and, at the same time, prevents apoptotic cell death induced by I/R injury, highlighting anti-apoptotic actions of HSP72. The influence of reduction in apoptosis from 10.3% to 4.0%, shown in our study, on global cardiac function is unclear. However, the total number of myocytes that die via apoptosis throughout ischemia-reperfusion can be larger than 10.3%, which is the number of cells that show DNA fragmentation at only 1 time-point: 30 minute of reperfusion. Furthermore, such anti-apoptosis effect of HSP72 could attenuate adverse remodeling processes in the later phase. Recent studies have shown that apoptosis predominates in cardiomyocytes after reoxygenation through a mitochondrion-dependent apoptotic pathway that involves the release of cytochrome c from mitochondria. Cytochrome c binds to Apaf-1 and triggers its oligomerisation. This complex then binds the inactive, unprocessed pro-form of the proteolytic enzyme caspase-9 which is then cleaved to its active form, thereby initiating the apoptotic cascade. HSP72 has been reported to prevent oligomerised Apaf-1 from recruiting pro-caspase-9 by its chaperone function, resulting in attenuation of apoptosis. We also speculate that HSP72-overexpression contributes to reduction in cytochrome c leakage from mitochondrial membranes, resulting from HSP72-induced mitochondrial protection during I/R injury, in which enhanced mitochondrial Mn-SOD activity might play a role. Another possible mechanism of HSP72-induced apoptosis reduction may be a modulation of pro- or anti-apoptotic proteins such as the Bcl-2 family. It is known that Bcl-2 prevents reoxygenation-induced apoptosis by inhibiting cytochrome c release from the mitochondria, preventing activation of caspase-3 and caspase-9. We have shown that the expression of anti-apoptotic Bcl-2 is enhanced in the HSP72-transfected hearts after I/R injury, corresponding with reduced post-I/R upregulation of caspase-3. In addition, HSP72-induced reduction in apoptosis is also likely to involve a caspase-independent pathway, such as through inhibition of c-Jun N-terminal kinase.

There is a variety of possible molecules involved in HSP72’s chaperone function in myocardial I/R injury in vivo, including cytoskeletal or contractile proteins, HSPs and SODs, apoptosis-related proteins or metabolism-related proteins. To clarify this complex pathway, further works are needed and for this purpose, gene transfection models could be useful, since the other methods to upregulate HSP70, such as brief ischemia and heat stress, affect other factors, such as SODs or other HSPs. As we have previously reported, intra-coronary infusion of HSP72-HVJ-liposome mediates...
HSP72-overexpression in 50% to 80% of cardiomyocytes with little cellular damage or inflammation. This HSP72 expression has been previously shown to be approximately double (12 to 20 times higher than control hearts) and stable for a longer period (at least 2 weeks) when compared with heat-stressed hearts (continuing 3 to 4 days only). In conclusion, we have demonstrated that myocardial HSP72-overexpression results in improved myocardial tolerance to I/R injury, associated with enhanced preservation of Mn-SOD activity. Further, this corresponds with enhanced mitochondrial protection, as well as reduced apoptosis of cardiomyocytes. These data suggest that the enhanced activity of Mn-SOD during I/R injury, which could play a role in the mitochondrial protection and reduction in cardiomyocyte apoptosis, is one of the possible mechanisms of HSP72-induced myocardial protection.

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