Combined and Individual Mitochondrial HSP60 and HSP10 Expression in Cardiac Myocytes Protects Mitochondrial Function and Prevents Apoptotic Cell Deaths Induced by Simulated Ischemia-Reoxygenation

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Background—The mitochondrial heat-shock proteins HSP60 and HSP10 form a mitochondrial chaperonin complex, and previous studies have shown that their increased expression exerts a protective effect against ischemic injury when cardiac myocytes are submitted to simulated ischemia. The more detailed mechanisms by which such a protective effect occurs are currently unclear. We wanted to determine whether HSP60 and HSP10 could exert a protection against simulated ischemia and reoxygenation (SI/RO)–induced apoptotic cell death and whether such protection results from decreased mitochondrial cytochrome c release and caspase-3 activation and from the preservation of ATP levels by preservation of the electron transport chain complexes. In addition, we explored whether increased expression of HSP60 or HSP10 by itself exerts a protective effect.

Methods and Results—We overexpressed HSP60 and HSP10 together or separately in rat neonatal cardiac myocytes using an adenoviral vector and then subjected the myocytes to SI/RO. Cell death and apoptosis in myocytes were quantified by parameters such as enzyme release, DNA fragmentation, and caspase-3 activation. Overexpression of the combination of HSP60 and HSP10 and of HSP60 or HSP10 individually protected myocytes against apoptosis. This protection is accompanied by decreases in mitochondrial cytochrome c release and in caspase-3 activity and increases in ATP recovery and activities of complex III and IV in mitochondria after SI/RO.

Conclusions—These results suggest that mitochondrial chaperonins HSP60 and HSP10 in combination or individually play an important role in maintaining mitochondrial integrity and capacity for ATP generation, which are the crucial factors in determining survival of cardiac myocytes undergoing ischemia/reperfusion injury. (Circulation. 2001;103:1787-1792.)

Key Words: ischemia ■ apoptosis ■ reperfusion

Myocardial ischemia and the resulting myocardial infarct are a major cause of mortality and morbidity. More recent findings indicate that cardiac myocytes die by distinct mechanisms, necrosis or apoptosis. Necrosis may be the earliest and most prominent mode of cell death in the infarcted tissues. Apoptotic cardiac myocytes are observed later in the areas of infarct and the surrounding tissues. Myocyte cell death, possibly apoptosis, has been related to the cause of subsequent heart failure among patients with coronary artery occlusions.1,2 It has been shown in in vitro cell culture or in vivo animal models of hypoxia/ischemia with or without reoxygenation that apoptosis occurs in cardiac myocytes.3 One of the major mechanisms for the initiation of apoptosis is provided by the release of mitochondrial factors, such as cytochrome c (cytoC) and precursors of caspases, into the cytoplasm.4 As a result, degradation of cellular proteins, cell shrinkage, and genomic DNA fragmentation occur as the characteristics of apoptosis.5

The mitochondrial chaperonins composed of heat-shock protein 60 (HSP60) and HSP10 are the major sites of protein folding in mitochondria. On the basis of the well-studied Escherichia coli model, GroEL (HSP60 from E coli) and GroES (HSP10 from E coli), the structure and details of the folding/unfolding cycle of chaperonins have become clear, although some mammal-specific functions remain unexplored.6–8 Two ring-like structures composed of 7 HSP60 subunits capped with 1 complex of 7 HSP10 subunits form a bell-shaped chaperonin unit. The requirement for both HSP60 and HSP10 to complete the tasks of folding proteins has prompted our group to generate an adenoviral vector simul-
taneously overexpressing HSP60 and HSP10. Overexpressing both HSP60 and HSP10 protected rat neonatal cardiac myocytes and H9C2 cells against simulated ischemia injury (SI); the mechanism of protection by HSP60 and HSP10, however, is unclear. In the present study, we wanted to determine whether individual HSP60 or HSP10 complexes in addition to the combined HSP60/10 complex exert a protection against SI/reoxygenation (RO) in cardiac myocytes. Our results suggest that HSP60 and HSP10 in combination prevent apoptotic cell death induced by SI/RO very effectively. HSP60 or HSP10 by itself, however, also exerts a protection on certain parameters. The chaperonins appear to mediate their protective effects by maintaining mitochondrial function and integrity.

**Methods**

**Cell Culture**
Neonatal rat cardiac myocytes were cultured and infected with adenoviral vectors expressing HSP60, HSP10, HSP10/60, and inducible HSP70 (HSP70i) as previously described. The infection protocol was similar to that described previously.9

**Lactate Dehydrogenase Release Assay**
LDH activity released to the medium or remaining in the cells was determined with an LDH kit (Sigma Diagnostics). Cytoplasmic enzyme released was shown as a percentage of LDH activity in the medium over the total enzyme activity (medium + remaining activity in the cells). The LDH release by SI/RO is determined as the difference in LDH release in cells kept as control and in cells after SI/RO.

**Flow Cytometry and TUNEL Assay**
A kit using a 2-color staining method for labeling DNA breaks and total cellular DNA by flow cytometry was used to study apoptosis (Apo-BRDU kit, Phoenix Flow System). Apoptotic cells were labeled with bromodeoxyuridine triphosphate nucleotides (Br-dUTP) followed by detection with fluorescein-labeled anti-BrdU monoclonal antibody. Total cellular DNA was stained with propidium iodide. The percentage of BrdU-stained cells in the whole population was then analyzed by a flow cytometer (Beckman Dickinson) and was designated as terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL)–positive or apoptotic cells.

**Cytoplasmic DNA Fragments Determined by ELISA**
The cytoplasmic DNA fragments in apoptotic myocytes were detected with a kit (Boehringer Mannheim 1774425) that detects histone-associated DNA fragments in the cytoplasmic portion of cell lysates. In short, the cytoplasmic portions of cell lysates were incubated for 2 hours in a streptavidin-coated microtiter plate with a monoclonal biotinylated anti-histone antibody and a peroxidase–conjugated anti-DNA antibody. After unbound antibodies had been washed off, the amount of DNA fragments was quantified by the peroxidase retained in the immunocomplex. Peroxidase was determined photometrically (405/490 nm) with ABTS as substrate.

**Caspase-3 Activity Assay**
The caspase-3 activity within the cells was measured with a caspase-3 colorimetric assay kit (Clontech K2027-2). In short, the caspase-3–specific substrate DEVD-pNA was incubated with 20 μg of myocyte lysates at 37°C for 1 hour, and the cleaved product was measured by a spectrophotometer at 405 nm. The specificity of the cleavage reaction was verified by including DEVD-CHO (caspase-3 inhibitor) 10 μmol/L in the incubation, and <10% of activity was detected.

**Protein Analysis**
In Figure 1, rat neonatal cardiac myocytes infected with AdvSR, AdvHSP60/10, AdvHSP60, and AdvHSP10 were lysed by RIPA buffer (Santa Cruz), and proteins were separated by SDS-PAGE with 4% to 20% gradient gel followed by Western blot. Anti-HSP60 and anti-HSP10 antibodies were from Stressgen. In Figure 5, mitochondrial proteins and mitochondrion-free cytoplasmic proteins were separated as described previously with minor modifications.

**ATP Measurement**
The cellular ATP concentration was measured by a modified luciferase assay protocol. Two million cells were lysed by addition of 500 μL lysis buffer containing 0.5% Triton X-100. The average protein concentration in cell lysates from control conditions was between 0.8 and 1 mg/mL, and no significant difference was observed among samples with various virus infections. The substrate buffer contained luciferin (10 mmol/L). Aliquots of 100 μL substrate buffer were ejected automatically by a luminometer into the assay tube containing luciferase 5 μL (0.0125 mg/mL) and 10 μL of cell lysate. The integrated light output was measured for 1 second. Samples with known ATP concentrations were also prepared and measured to generate the standard curves for the assay.

**Polarography to Measure Complex I, Complex III, and Complex IV Activity**
The activity of complex I, complex III, or complex IV was measured according to a previously described protocol with modification as noted. Adenovirus-infected myocytes before or after 5 hours of simulated ischemia were trypsinized and permeabilized by incubation with digitonin (10 μg/mL for 10 minutes) or until ≥95% of the...
cells were permeabilized. Cell number and protein concentration in each sample were counted and measured. Samples with equal amounts of proteins were divided into aliquots and stored at \(-80^\circ\text{C}\). After ischemia, it took \(\sim 3\) hours to finish the above procedures, and this period was considered a period of reoxygenation. These samples (500 \(\mu\text{g}\) each) were used later to measure the activity of the complexes. The complex I, complex III, and complex IV activity was defined as the rate of oxygen consumption in the presence of the specific substrates glutamate/malate for complex I, succinate for complex III, and ascorbate/N,N,N',N'-tetramethyl-p-phenylenediamine for complex IV and was calculated as the fraction that was sensitive to the inhibitors rotenone for complex I, antimycin for complex III, and sodium cyanide for complex IV.

Statistics
ANOVA was performed in all comparisons, followed by post hoc analysis. \(P\) values were determined by Fisher’s protected least significant difference method. The statistical software used was StatView. All error bars are SD.

Results
Increased HSP60/10, HSP60, and HSP10 Expression Protects Against SI/RO Damage Determined by LDH Release
One of the aims of our studies was to determine whether HSP60 or HSP60 by itself could exert a protective effect against SI/RO-induced injury. In our protocol, rat neonatal cardiac myocytes were infected with adenovirus constructs expressing equal amounts of HSP60 and HSP10, HSP60 only, and HSP10 only. Figure 1A demonstrates the level of expression of HSP60 and HSP10 in myocytes 48 hours after adenoviral infection. Both HSP60 and HSP10 were increased in myocytes infected with AdvHSP60/10. Only HSP60 was increased by AdvHSP60 infection, and HSP10 was increased by AdvHSP10 infection. AdvSR– was the control virus without transgene expression.

After SI/RO, the LDH activity in the medium was \(60\pm12\%\) of total LDH activity in the cells infected with control virus (AdvSR–), compared with only \(5\pm2\%\) of total LDH activity found in the medium with untreated cells for the same period of time (data not shown). As shown in Figure 1B, overexpression of HSP60 (AdvHSP60) decreased the amount of LDH released after SI/RO to a level of only 49\% of the release found in AdvSR– infected cells. Overexpression of HSP10 (AdvHSP10) decreased the LDH release further, to only 23\% of that in AdvSR– after SI/RO. Simultaneous overexpression of both HSP10 and HSP60 (AdvHSP60/10) resulted in a decrease in LDH release to only 35\% of that in AdvSR–. Judged by the mean values, overexpression of HSP10 seemed to be most effective in reducing the LDH releases after SI/RO; this protection, however, was not significantly different from that with overexpression of HSP60 or HSP60/10 together. The protection by another major HSP, HSP70i, against SI/RO was also examined. Overexpression of HSP70i was less protective than overexpression of the mitochondrial HSP60 and HSP10 against SI/RO, because it decreased the LDH release to only 66\% of that with AdvSR–.

Preservation of Respiratory Enzymes by Overexpression of HSP60 and HSP10
Generation of ATP in mitochondria by ATP synthase (F\(_{1}\)F\(_{0}\)-ATPase) is coupled to the electron transport chain (ETC), in which NADH is oxidized and oxygen is converted into water. The ETC is composed of NADH-ubiquinone oxidoreductase (complex I), ubiquinol cytoC oxidoreductase (complex III), and cytoC oxidase (complex IV). By polarograph, we measured the activity of complex I, III, and IV in myocytes infected with AdvSR–, AdvHSP60/10, AdvHSP60, or AdvHSP10 before and after SI/RO. As shown in Figure 2, complex I activity in myocytes was not changed by overexpression of HSP60 and/or HSP10 before or after SI/RO. Before SI/RO, complex III activity was significantly higher in myocytes infected with AdvHSP60/10 and AdvHSP60. Overexpression of HSP10 in myocytes did not increase the complex III activity. Complex IV is the complex with the greatest oxidation capacity in the ETC. Overexpression of HSP60 alone increased the complex IV activity by 50\% compared with AdvSR– infected myocytes, whereas overexpression of both HSP60 and HSP10 or HSP10 alone did not significantly increase the complex IV activity (Figure 2A). After SI/RO, complex III activity was increased in cells overexpressing HSP60 and HSP10 by 30\% and was not changed by overexpression of HSP60 or HSP10 alone. The complex IV activity was greater in cells overexpressing HSP60 and HSP10 or HSP60 alone and was less in cells overexpressing HSP10 alone (Figure 2B). Taken together, our data suggest that (1) overexpression of HSP60 alone increases both the complex III and complex IV activity.

Figure 2. HSPs preserve ETC complexes before and after SI/RO. Polargraphy was used to measure activity of complex I, III, and IV in myocytes infected with AdvSR–, AdvHSP60/10, AdvHSP60, or HSP10. Complex activity is represented as rate of oxygen consumption/mg protein. A, Activity in myocytes before simulated ischemia. B, Activity in myocytes after simulated ischemia. Results are from 4 independent experiments. *\(P<0.05\) vs same complex of AdvSR–. #\(P<0.05\) vs same complex of AdvHSP60/10 or AdvHSP60.
Overexpression of HSP10 alone does not have a beneficial effect on these enzymes; nevertheless, it attenuates the increases in complex IV activity by HSP60. (2) The beneficial effect of coexpression of HSP60 and HSP10 on complex III becomes evident after SI/RO, and the detrimental effect of HSP10 on complex IV is also amplified after SI/RO.

Preservation of ATP Levels by HSP60/10 Overexpression

Oxidative phosphorylation and the formation of ATP are essential functions of mitochondria. We therefore determined potential protective effects of HSP60 and HSP10 on ATP levels in myocytes subjected to 8 hours of simulated ischemia followed by 4 and 8 hours of reoxygenation. It is interesting to note that control myocytes not subjected to SI but infected with AdvHSP60/10 versus myocytes infected with control adenovirus (AdvSR) showed a 30% increase in ATP levels. Myocytes infected with adenovirus expressing only HSP60 or HSP10 had ATP levels not significantly different from those of the AdvSR group (Figure 3). Myocytes exposed to an 8-hour period of ischemia showed an ~80% drop in ATP levels. Increased expression of HSP60/10 or HSP10 did not influence the ischemia-mediated decline in ATP. In HSP60-overexpressing cells, ATP levels were slightly but significantly higher than those in other groups. After 4 hours of reoxygenation, ATP levels showed minimal but significant recovery in AdvHSP60/10-infected myocytes but not in other groups. A reoxygenation period of 8 hours showed a significant increase in ATP levels primarily in the HSP60/10-overexpressed cells.

Apoptosis of Cardiac Myocytes Induced by SI/RO Is Decreased by HSP60 and HSP10 Expression

To determine apoptosis in cardiac myocytes after SI/RO, an ELISA-based assay for cytoplasmic DNA fragments was used. Ischemia for 8 hours followed by 4-hour reoxygenation resulted in a >10-fold increase in ELISA signal for cytoplasmic DNA fragments compared with the untreated condition in AdvSR--infected cardiac myocytes (data not shown). As shown in Figure 4A, after the same SI/RO episodes, overexpression of HSP60 and HSP10 together, HSP60 by itself, and HSP10 by itself decreased the percentage of myocytes that stained positive in TUNEL assay from 3% under control conditions to 24% of total cells after simulated ischemia followed by 24 hours of reoxygenation (SI/RO). Results are from 4 independent experiments. *P<0.05 vs AdvSR-- after SI/RO.

Decrease in cytoC Release and Caspase-3 Activation by Overexpression of HSP60 and HSP10 in Myocytes Under SI/RO

The release of mitochondrial cytoC into the cytosol has been shown to be involved in triggering the apoptotic program. We therefore determined the release of cytoC by Western blotting of the mitochondrion-free cytosolic proteins collected from myocytes with altered chaperonin expression submitted to
after reoxygenation by HSP10 together or HSP10 alone decreased the cytoC release-infected myocytes. Overexpression of HSP60 and HSP10 together or HSP60 and HSP10 alone decreased the cytoC release-infected myocytes. Overexpression of HSP60 and HSP10 together or separately did not significantly reduce the ischemia-induced caspase-3 activation (Figure 5B). The subsequent reoxygenation period after ischemia further increased the caspase-3 activity. After 4-hour reoxygenation, the increases in caspase-3 activity were 6.5-fold in AdvSR--infected, 3.9-fold in AdvHSP60/10-infected, 2.9-fold in AdvHSP60-infected, and 4.2-fold in AdvHSP10-infected myocytes. Caspase-3 activation occurs primarily during reoxygenation, and HSP60 or HSP10 by itself exerts an inhibitory effect on caspase-3 activity equal to that seen with the HSP60/10 combination.

Discussion
Cardiac myocytes are specifically rich in mitochondria to generate the high amount of ATP needed for normal cardiac contractile function. To maintain the protein members of the oxidative phosphorylation cascade in a normal folding state, a specific set of mitochondrial HSPs, especially HSP60 and HSP10, is used to form the chaperonin complex for efficient protein folding. The majority of mitochondrial proteins are nucleus-encoded and need to be refolded after mitochondrial import by use of the chaperonin complex made by HSP60 and HSP10. The role that HSP60 and HSP10 play in helping myocytes to recover from SI/RO injury is only incompletely explored. A general protection of combined expression of HSP60 and HSP10 in cardiac myocytes against ischemic injury has been demonstrated. It is currently unclear, however, whether increased expression of HSP60 and HSP10 by themselves exerts a protection, whether this protection results in better preservation or recovery of ATP levels after an ischemia-reperfusion episode, and whether the protection includes prevention of apoptotic cell death. Our results provide answers to these questions with the demonstration that HSP60 and HSP10 by themselves have a protective effect in addition to the HSP60/10 combination. Part of the protective mechanism is due to better preservation and accelerated recovery of ATP levels after SI/RO. In addition, protection against apoptotic cell death occurs that may be mediated by decreased release of cytoC from mitochondria. The beneficial effect that HSP60/10 or HSP60 and HSP10 individually exert on different cellular events linked to SI/RO injury will be discussed in more detail below.

Myocardial ischemia is frequently followed by reperfusion because of therapeutic interventions leading to the opening of occluded cardiac arteries. Reperfusion and the resultant reoxygenation lead to the generation of oxygen radicals that can by themselves cause myocardial damage called reperfusion injury. We used a myocyte culture model of SI/RO to mimic the in vivo events. It is interesting to note that LDH release as a marker of general myocyte injury was markedly reduced in myocytes undergoing SI/RO, with increased expression of the combination of HSP60 and HSP10, but also in myocytes expressing only increased amounts of HSP60 and HSP10 by themselves (Figure 1). Previous studies in yeast have shown that HSP60 or HSP10 by itself can influence the folding state of a certain subset of proteins. It has also been postulated that the sequestered space formed by a ring-like structure...
composed of only HSP60 could serve as a protective holding reservoir for proteins during noxious cell stress.\(^7\)

In this report, we specifically identified protection of increased expression of HSP60/10 or HSP60 as well as HSP10 by itself against apoptotic cell death. This protective effect can be linked to decreased release of cytoC from mitochondria to the cytoplasm (Figure 5). CytoC has a well-established role of combining with Apaf1, leading to caspase-9 activation and thus triggering the apoptotic program.\(^14\) In our study, we found decreased cytoC release, decreased caspase-3 activity, and decreased DNA fragmentation measured by TUNEL and ELISA by overexpressing HSP60 and HSP10. The cell death program is complicated and can be elicited by different pathways\(^14\); cell death induced by SI/RO is an even more complex event. Our data from rescuing myocytes by overexpressing chaperonins against SI/RO serve as an example of this complexity. For some parameters, eg, LDH release and cytoC release, sole HSP10 overexpression appeared most protective, but for other parameters, such as caspase-3 activity and DNA fragmentation, HSP10 protection was not better, if not worse, than combined HSP60/HSP10 expression. Generally, expression of HSP60 alone is most protective against ischemia in most parameters, and expression of HSP60 and HSP10 in combination protects better after a period of reoxygenation.

Several mechanisms can be involved by which increased expression of the mitochondrial chaperonins leads to decreases in cytoC release. Higher ATP levels occur when cardiac myocytes with increased expression of HSP60/10 are submitted to SI/RO. In such HSP60/10-overexpressing myocytes, we also observed a specific protective effect on individual complexes of the ETC. The activity of complex I that contains the largest amount of individual proteins was not influenced by increased mitochondrial chaperonin expression. In contrast, the activities of complex III and complex IV were significantly higher in HSP60- or HSP60/10-overexpressed myocytes, and the protection of complex III and IV was also evident in these cells after SI/RO. Whether better preservation of ETC and ATP synthesis leads to a smaller amount of cytoC leaving the mitochondrial intermembrane space is still unclear. Our data suggest a relation between mitochondrial function (ETC) and integrity (cytoC release). HSP60 and HSP10 are localized in the mitochondrial matrix, whereas cytoC is positioned at the intermembrane space outside the mitochondrial matrix. A direct physical contact between the HSP60/10 ring-like structure and cytoC therefore appears unlikely, and intermediate molecules, such as those that are part of the ETC, will be necessary to lead to less cytoC release after SI/RO.

It should also be noted that a recent article demonstrated that overexpression of HSP60 results in an increase in apoptosis in Jurkat cells.\(^15\) Our present results obtained from myocytes seem at odds with the previous finding. The effect of overexpression of mitochondrial chaperonins (HSP60) may be different in myocytes from that seen in T cells, such as Jurkat cells, which are prone to go into apoptosis. In contrast, cardiac myocytes are particularly rich in mitochondria but relatively resistant to apoptosis. Thus, it is possible that HSP60 and HSP60/10 may be particularly protective in myocytes against apoptosis induced by SI/RO while being proapoptotic in cells already destined to apoptosis.

In summary, our results show that overexpression of HSP60 and HSP10 individually in addition to combined expression of HSP60 and HSP10 results in protection of cardiac myocytes against apoptotic cell death induced by SI/RO. Preservation of oxidative phosphorylation, accelerated ATP recovery after SI/RO, decreased cytoC release, and caspase-3 activation indicating a better preservation of mitochondria by mitochondrial chaperonins contributed to this beneficial effect.

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References
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