Cytosolic Heat Shock Protein 60, Apoptosis, and Myocardial Injury

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Background—Heat shock proteins (HSPs) are well known for their ability to “protect” the structure and function of native macromolecules, particularly as they traffic across membranes. Considering the role of key mitochondrial proteins in apoptosis and the known antiapoptotic effects of HSP27 and HSP72, we postulated that HSP60, primarily a mitochondrial protein, also exerts an antiapoptotic effect.

Methods and Results—To test this hypothesis, we used an antisense phosphorothioate oligonucleotide to effect a 50% reduction in the levels of HSP60 in cardiac myocytes, a cell type that has abundant mitochondria. The induced decrease in HSP60 precipitated apoptosis, as manifested by the release of cytochrome c, activation of caspase 3, and induction of DNA fragmentation. Antisense treatment was associated with an increase in bax and a decrease in bcl-2 secondary to increased synthesis of bax and degradation of bcl-2. A control oligonucleotide had no effect on these measurements. We further demonstrated that cytosolic HSP60 forms a macromolecular complex with bax and bak in vitro suggesting that complex formation with HSP60 may block the ability of bax and bak to effect apoptosis in vivo. Lastly, we show that as cytosolic (nonmitochondrial) HSP60 decreases, a small unbound fraction of bax appears and that the amount of bax associated with the mitochondria and cell membranes increases.

Conclusions—These results support a key antiapoptotic role for cytosolic HSP60. To our knowledge, this is the first report suggesting that interactions of HSP60 with bax and/or bak regulate apoptosis.

Key Words: apoptosis □ cells □ myocytes

The heat shock proteins (HSPs) are a family of endogenous, protective proteins. Two of these HSPs, 27 and 72 (inducible 70), have been shown to have antiapoptotic effects.1–3 HSP60 is a primarily mitochondrial HSP that is expressed at high levels in the normal cell. Although much is known about the function of HSP60 in folding macromolecules, only limited studies have addressed its possible role in apoptosis.4 In mammalian cells, 75% to 80% of HSP60 is in the mitochondria, whereas 15% to 20% of HSP60 is extramitochondrial.5 We postulated that as key proteins and events in the apoptotic cascade involve the mitochondria, HSP60 may have an antiapoptotic role in the cell.

To address this question, we used antisense phosphorothioate oligonucleotides (AS) to reduce HSP60 by 50% in cardiac myocytes. We report here that this decrease in HSP60 precipitated apoptosis as manifested by the release of cytochrome c, activation of caspase 3, and DNA fragmentation. Bax increased, whereas bcl-2 decreased. As the proapoptotic bak is thought to be relatively abundant in the heart, it was also examined and found to undergo changes similar to bax. A control oligonucleotide had no adverse effects on the cells.

We demonstrate that HSP60 interacts with bax and bak, and that bax can be depleted from the cytoplasm by precipitation with anti-HSP60 antibodies. As the HSP60 in the cytoplasmic fraction is decreased by AS treatment, a small fraction of unbound bax can be identified in the cytoplasm. The amount of bax associated with a mitochondrial/membrane fraction increases as HSP60 decreases. Bax alone is sufficient to induce cytochrome c release and apoptosis.6 Thus, this interaction between HSP60 and bax may be critical in preventing apoptosis in the normal cell. To our knowledge, this is the first report of the interaction of HSP60 with bax and bak, and the first report that changes in HSP60 can precipitate apoptosis. We propose that HSP60 has a pivotal antiapoptotic role.

Methods

Isolation of Cardiac Myocytes

Adult cardiac myocytes were isolated from male Sprague Dawley rats (Harlan, Indianapolis, Ind) and cultured as previously described.7 The cells were incubated at 37°C for 2 hours to allow adherence, and then treated with either AS to HSP60 or a scrambled sequence (SCR) as a control for the effects of phosphorothioate oligonucleotide; both were used at a concentration of 5.5 μmol/L (25 μg/mL). Cells without any treatment were used as a second control.

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The primary AS oligonucleotide used throughout this study corresponds to bases 109 to 123 (5'-TAAGGCCTGAGCATC-3'; TriLink). A second AS sequence was also used that corresponds to bases 27 to 41. Similar results were obtained with both AS sequences. The scrambled sequence (5'-GCTCGTGGTCAATAC-3') was used as a control for the effects of the phosphorothioate compound (SCR). All of the oligonucleotide sequences were screened in GenBank.

Further information about Methods is included in the online-only Data Supplement.

**Statistics**

Groups were compared by an ANOVA followed by the Student-Neumann-Keuls test. When appropriate, an ANOVA on ranks was performed followed by a Dunn test. *P*<0.05 was considered significant. All data are expressed as mean±SEM.

**Results**

After 24 hours of treatment, the levels of HSP60 were significantly reduced in the AS group, but not in SCR or control (Figure 1A and 1C). There were no changes in the α-sarcomeric actin levels on the same Western blot (Figure 1B). Examination of cytosolic and mitochondrial cell fractions by Western blotting showed that HSP60 was present in both fractions and that AS treatment decreased the amount of HSP60 in each fraction similarly (Figure 1D). Although the amount of HSP60 appears somewhat similar in the figure (approximately a 2-fold difference), far more total protein is present in the mitochondrial fraction, so as equal amounts of protein were loaded on the gel, far more HSP60 was present in the mitochondria.

With the decrease in HSP60, there was a decrease in cell viability by live/dead staining (Figure 2A), but no release of lactate dehydrogenase (LDH), a marker of necrosis (Figure 2B). As the membrane is relatively impermeable in apoptosis, this method may underestimate apoptotic cell death. We performed a series of experiments to determine whether the reduction of HSP60 was causing apoptosis.

A pivotal point in apoptosis is release of cytochrome c from the mitochondria. As shown in Figure 3A, cytochrome c was released with reduction of HSP60. Neither control cells nor SCR-treated cells had release of cytochrome c. To verify that the release of cytochrome c was due to apoptosis rather than disintegration of the mitochondria, citrate synthase activity was measured in the medium. There was no release of citrate synthase from the mitochondria in any of the groups (Figure 3C). Furthermore, citrate synthase activity was the same for the mitochondria from all groups. Likewise, ATP levels were the same in the three groups after 24 hours of treatment (control [C], 191.0±20.0; AS, 181.3±27.4; and SCR, 178.3±21.5 nmol/mg protein). To confirm activation of the caspase proteases, caspase 3 cleavage was assessed by Western blot and was only present in the AS-treated myocytes (Figure 3D). A terminal deoxynucleotidyltransferase–mediated dUTP nick-end labeling (TUNEL) assay showed that 28.7% of AS-treated cells were positive for DNA fragmentation (*P*<0.05 compared with SCR and control; for details, see the Data Supplement).

After 24 hours of AS treatment, Western blots of whole-cell lysate revealed a significant increase in bak and bax and a significant decrease in bcl-2 in the AS-treated cells compared to controls (Figure 4A).
pared with SCR and control (C) (Figure 4A and 4B). Studies were done to determine the mechanism of the changes in bax and bcl-2. By polymerase chain reaction no difference was observed in GAPDH, bcl-2, or bax mRNA among the three groups (Figure 5A). The primers screened for five different mRNAs, two of which, bcl-xl and bcl-xs, were present at very low levels and are not visible in the figure. Labeling of newly synthesized protein demonstrated a marked increase in bax, but no change in bcl-2, which was barely detectable after 8 hours of labeling (Figure 5B and 5C). To look at degradation, the myocytes were labeled for 12 hours with [S-35]methionine and then returned to their regular media for 8 hours. Bax showed no change, whereas bcl-2 decreased 40% in the AS-treated group (P<0.01 versus C/SCR, Figure 5D and 5E).

Normal cardiac myocytes were collected, sonicated, and immunoprecipitated with an anti-HSP60 antibody to determine whether HSP60 bound bcl-2, bax, or bak. As shown in Figure 6A, both bax and bak coimmunoprecipitated with HSP60, but not bcl-2 (not shown). To further confirm the cytosolic interaction of HSP60 and bax, exhaustive immunoprecipitation (IP) was performed on the cytosolic fraction from the myocytes. As shown in Figure 6B, repetitive IP with either anti-HSP60 (lane B1 to B3) or anti-bax (lane D1 to D3) completely depleted all HSP60 (upper panel) and bax (lower panel) in the cytosolic fraction (no mitochondria, no plasma membrane) of the myocytes. For comparison, lane A shows 40 μg of total cytosol and lanes C and E show 40 μg of protein post-IP with anti-HSP60 or anti-bax, respectively. No HSP60 or bax is detectable in lane B4 or C4. Thus, exhaustive IP with either anti-HSP60 or anti-bax completely depletes the cytosol of both HSP60 and bax. A nonspecific IgG immunoprecipitated neither HSP60 nor bax from control cells (lane F1 to F3, upper and lower panels), and there is no depletion of either HSP60 or bax (lane G, upper and lower panel, compared with lane A). Immunocytochemistry followed by exhaustive photon reassignment (EPR) was done to demonstrate that HSP60 and bax colocalize within the cell. As shown in Figure 7, both HSP60 and bax were widely

Figure 3. A, Western blot showing cytochrome c release from AS-treated myocytes. B, Western blot for α-sarcomeric actin on the digitonin-permeabilized cells to demonstrate that equivalent numbers are present on plates. C, Graph showing citrate synthase activity in media vs cells (mitochondria); mU/μg total protein. n=10/group. D, Western blot for the two cleavage products that occur with activation of caspase 3 (11 and 17 kDa).

Figure 4. A, Western blots for bax, bak, and bcl-2, as labeled. Same samples are shown on all Western blots. B, Graphs summarize 5 experiments. Values are normalized to control. *P<0.05 vs all others.
distributed throughout the cell, but analysis of the processed (by EPR) data from the images shows that the majority of the protein labels colocalize (white in the figure) and little to no bax alone (green) or HSP60 alone (red) is seen. Likewise, subtracting bax signal from HSP60 signal removes most of the signal from the cell (HSP60 less bax). Thus, by immunocytochemistry the two proteins colocalize within the myocyte, and IP studies support the existence of a bax/HSP60 complex within the cytosol of the myocyte.

These experiments show an interaction between HSP60 and bax. Furthermore, they demonstrate that reduction in HSP60 results in apoptosis. The digitonin-permeabilized myocytes were used to delineate further the relation between HSP60 levels and bax distribution by dividing the cell into two fractions, cytosol and mitochondrial/membrane. The cytosol was immunoprecipitated with HSP60 after 24 hours of treatment with increasing amounts of AS. As shown in Figure 8A, bax coprecipitated with HSP60 from this cytosolic fraction. The supernatants from the IP and the remaining mitochondrial fraction (mitochondria, nuclei, membranes, etc) were examined by Western blot for bax. As shown in Figure 8A, as the level of AS increased, a small amount of bax becomes present in the cytosol. The mitochondrial fraction shows increasing amounts of bax present as the amount of AS is increased. Thus, as the AS concentration increases, HSP60 decreases (Figure 8B), and the amount of bax free of HSP60 increases (ie, the bax present in the supernatant after IP with anti-HSP60). Meanwhile, as HSP60 decreases and unbound bax increases, the amount of bax in the mitochondrial fraction increases.

**Discussion**

Within 24 hours of AS treatment, the level of HSP60 was significantly reduced in the cardiac myocytes. In contrast, control and SCR-treated cells showed no change in HSP60 levels. Along with this decrease in HSP60, there was an increase in cell death, but no LDH release, a sign of necrosis. Western blotting showed that the proapoptotic proteins bak and bax were both increased, whereas the antiapoptotic bcl-2 was decreased in AS-treated cells. These changes in bax, bak, and bcl-2 were accompanied by release of cytochrome c from the mitochondria. The citrate synthase assay confirmed that the mitochondria remained intact, as did the preserved ATP levels. The observed changes were accompanied by cleavage of caspase 3 and by DNA fragmentation within the nucleus. Thus, the reduction in HSP60 alone precipitated apoptosis. Decreasing the amount of HSP60 in the cytoplasm fraction resulted in “free” bax in the cytoplasm after IP with HSP60. Furthermore, the amount of bax associated with the mitochondrial/membrane fraction increased markedly as HSP60
decreased, suggesting that unbound bax immediately translocates to the mitochondria. The addition of bax to mitochondria has been shown to be sufficient to trigger cytochrome c release and subsequent caspase activation.6,11 Our data support a model in which HSP60 complexes with bax preventing apoptosis. Reduction in HSP60, or dissociation from HSP60, can trigger apoptosis.

HSP60 is predominantly mitochondrial with 60% in the inner matrix, 20% in the inner membrane, and 15% to 20% extramitochondrial.5 We found that HSP60 complexes with both bax and bak as demonstrated by IP studies in untreated cardiac myocytes. Bax is in the cytosol in the normal cell as is bak, and both associate with the cytosolic fraction of HSP60, as shown by the current study. Immunocytochemistry followed by EPR demonstrated that HSP60 and bax colocalize. The lack of significant HSP60 signal without bax may result from incomplete permeabilization of the mitochondria by the fixation/permeabilization method that was used. Reduction in HSP60 releases bak and bax, and this may allow conformational changes, as well as the oligomerization, insertion into the outer membrane, and mitochondrial pore formation that are thought to occur with both proteins during apoptosis.12-14 Both bak and bax induce the release of cytochrome c from the mitochondria. Binding to HSP60 in the normal cell may prevent bak and bax from oligomerizing and inserting into the mitochondrial membrane. In fact, our experiments show that with reduction in HSP60, bak redistributes from the cytosol to the mitochondria. During apoptosis, bak is found in the mitochondria along with HSP60; however, these two proteins are found in different compartments with bax localizing to the outer membrane and HSP60 associated with the inner membrane and inner matrix.5,15,16 Thus, it is unlikely that the two proteins interact in the mitochondria. Our results suggest that HSP60 has a regulatory role for the activity of these proapoptotic proteins in the normal cell, and that HSP60 is a key antiapoptotic protein in the normal myocardium. This regulatory role in cell signaling is analogous to the role that HSP90 has in the cell.17,18

Treatment with AS to HSP60 was associated with an increase in bax. The balance of antiapoptotic bcl-2 to proapoptotic bax is critical in mitochondrion-induced apoptosis.19 Our data show no change in mRNA for bax or bcl-2 with AS treatment. On the other hand, there was a significant increase in new synthesis of bax. In contrast, bcl-2 had increased degradation, but no change in synthesis.

HSP27 and HSP72 have been found to have antiapoptotic roles, but few studies have examined HSP60 in apoptosis.1-3 Overexpression of HSP27 or HSP72 can inhibit apoptosis.20,21 Experimental data suggest that HSP72 prevents activation of JNK and also that HSP72 may have antiapoptotic effects downstream of caspase 3 activation.22,23 HSP60 has been found to form a complex with pro–caspase 3 and HSP10 in Jurkat cells, and appears to accelerate the activation of pro–caspase 3 during apoptosis.24 In contrast, we observed that reduction of HSP60 resulted in activation of the apoptotic cascade. Our results are consistent with the observation that overexpression of HSP60 can prevent apoptosis.4

Antisense oligonucleotides can have nonspecific effects like any other experimental manipulation.25 Careful sequence selection to avoid known problems and appropriate controls can avoid these problems.26 We observed the same reduction of HSP60 and cell death via apoptosis with two different AS sequences. Neither the control phosphorothioate oligonucleotide used in this experiment nor any of the sequences used in previous work have affected HSP60 levels or caused cell death.27 Thus, the observed changes are specific for the reduction in HSP60.

**Figure 7.** EPR. HSP60 and bax images as labeled. C indicates EPR; CO-LOC, colocalization of signal for HSP60 and bax; HSP60 less bax, signal for bax was subtracted from HSP60 signal (virtually all signal was removed); white color, both signals present; red, HSP60 alone; and green, bax alone.

![HSP60 and bax images](http://circ.ahajournals.org/)

**Figure 8.** Cells were divided into two fractions, cytoplasm and organelle/membrane by gentle digitonin permeabilization. A, Western blot for bax from representative experiment. Each series of samples is with increasing concentrations of AS (μg/mL as shown). Left side, Coimmunoprecipitation of bax with HSP60 from cytoplasmic fraction; center, supernatant from IP. A small but increasing amount of bax is present as HSP60 decreases with higher AS concentration. Right, Mitochondrial/membrane fraction. B, Western blot of intact cytoplasm fraction showing that as AS concentration increases, HSP60 decreases.
HSP60 is an abundant protein about which little is known with regard to its protective properties. A number of intriguing observations have been made about this protein. The heart of the hypoxia-tolerant turtle has very high levels of this protein compared with the hypoxia-intolerant human heart. Increasing evidence supports the hypothesis that apoptosis is a mechanism of myocyte loss in heart disease. HSP60 is primarily a mitochondrial protein, and the mitochondrion is the final common pathway to apoptotic destruction. In this report, we demonstrate that cytosolic HSP60 interacts with the proapoptotic bax and bak, but not with antiapoptotic bcl-2. Furthermore, we show that reduction in HSP60 is sufficient to precipitate apoptosis, and that cytosolic HSP60 has a key antiapoptotic role in the myocyte. Although opinion regarding the importance of mitochondria in apoptosis has waxed and waned, an argument can be made for the central importance of the mitochondria in the myocardium, and our data suggest that mitochondrial damage is a central event.

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