Cytosolic Heat Shock Protein 60, Hypoxia, and Apoptosis
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Background—Heat shock protein (HSP)60 is an abundant protein found primarily in the mitochondria, though 15% to 20% is found in the cytosol. Previously we observed that HSP60 complexes with bax in the cytosol. Reduction in HSP60 precipitates translocation of bax to the mitochondria and apoptosis. We hypothesized that HSP60 would decrease with hypoxia/reoxygenation and that this would precipitate bax translocation to the mitochondria and release of cytochrome c.

Methods and Results—Adult rat cardiac myocytes were studied at end-hypoxia and at 10 and 24 hours of reoxygenation. HSP60 levels were unchanged at end-hypoxia and decreased 33% and 40% at 10 and 24 hours of reoxygenation, whereas HSP72 increased 80% and 110%. Bax and bcl-2 decreased during reoxygenation. However, cytochrome c release occurred at end-hypoxia, before reoxygenation. Cell fractionation was done to analyze this further. In normal myocytes, bax and HSP60 were present in the cytosol, and bax coimmunoprecipitated with cytosolic HSP60. At end-hypoxia, mitochondrial HSP60 was unchanged, but cytosolic HSP60 had disappeared and was now in the plasma membrane fraction. Concurrently, bax was no longer in the cytosol but now in the mitochondria. Thus, although total HSP60 remained the same, it no longer complexed with bax, and bax was free to translocate to the mitochondria and precipitate apoptosis. Reduction in ATP had a similar effect.

Conclusions—These studies show that hypoxia results in disassociation of the HSP60-bax complex with translocation of cytosolic HSP60 to the plasma membrane and bax to the mitochondria. This is sufficient to trigger apoptosis. (Circulation. 2002;106:2727-2733.)

Key Words: apoptosis ■ hypoxia ■ ischemia ■ myocytes

The heat shock proteins (HSPs) are a family of endogenous, protective proteins. Both HSP27 and HSP72 have been shown to have antiapoptotic effects.1–3 HSP60 is primarily a mitochondrial HSP that is expressed at high levels in the normal cell. Although much is known about the function of HSP60 in folding, only limited studies have addressed its possible role in apoptosis.4 We hypothesized that HSP60 would decrease with hypoxia/reoxygenation, based on previous observations,5 and that this would precipitate translocation of bax to the mitochondria and release of cytochrome c, based on our prior work showing that cytosolic HSP60 binds bax and that reduction in HSP60 precipitates apoptosis.6

In a series of studies on isolated, adult rat cardiac myocytes, we found that HSP60 decreased with reoxygenation despite the accompanying increase in HSP72. Both the proapoptotic bax and the antiapoptotic bcl-2 also decreased during reoxygenation. Cytochrome c release from the mitochondria occurred before reoxygenation, and this was followed by DNA fragmentation (Comet assay). Cell fractionation followed by Western blot analysis demonstrated that the distribution of HSP60 changed during hypoxia, with translocation of cytosolic HSP60 to the plasma membrane. This was accompanied by translocation of bax to the mitochondria. Although the total amount of HSP60 was the same, it was no longer present in the cytosol to complex with bax, and bax was free to move to the mitochondria. Reduction in ATP had similar effects. In summary, these studies show that hypoxia results in dissociation of the HSP60-bax complex, with translocation of cytosolic HSP60 to the plasma membrane and bax to the mitochondria. The interaction between HSP60 and bax may be critical in preventing apoptosis in the normal cell.

Methods

Isolation of Cardiac Myocytes
Adult cardiac myocytes were isolated from 3- to 4-month-old male Sprague-Dawley rats weighing 250 to 300 g and cultured as previously described.6,7 The animal protocol was approved by the Baylor College of Medicine Animal Research committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Western Blotting
Western blotting and analysis was performed as previously described.8 After transfer, all membranes were stained with Ponceau S to verify quality of transfer and equal loading. The same samples were analyzed for HSP60, HSP72, bax, and bcl-2. Where feasible, the same membrane was developed for more than one protein.
Hypoxia Studies
The medium was changed to DMEM base (no glucose, glutamine, or phenol red to prevent switching to glycolysis), and the cells were subjected to hypoxia for 6 hours in an Anaerobic Workstation (model 1025, Forma Scientific; 4.8% CO₂, 10.3% H₂, and 84.9% N₂). A nitrogen-flushed interchange is used to transfer items to and from the work chamber. In the work chamber, a charcoal filter removes H₂S and traces of O₂ and H₂, and a desiccant absorbs H₂O.

Cytochrome c release and LDH levels were detected as described previously.⁵,⁶,⁸

Citrate Synthase Assay
Citrate synthase activity, an index of mitochondrial integrity, was previously reported.⁶,⁹

Separation of Plasma Membrane, Cytosol, and Mitochondria
Plasma membrane, cytosol, and mitochondria were prepared from the myocytes, as previously described with an additional step, collecting the 32%/40% interface on the sucrose gradient to isolate the plasma membrane.⁶,¹⁰ Citrate synthase activity was used as a marker of mitochondria. Plasma membrane had no significant citrate synthase activity, and difference between mitochondrial and plasma membrane citrate synthase activity was 100- to 200-fold. Alkaline phosphodiesterase I activity was assayed as a marker of plasma membrane by a standard method.¹¹ An 80-fold difference in activity was detected between plasma membrane alkaline phosphodiesterase I activity and the trivial activity in the mitochondria.¹² The cytosolic fraction had no significant citrate synthase or alkaline phosphodiesterase I activity (data not shown).

Comimmunoprecipitation Studies
The cytosolic fraction from normal and hypoxic myocytes was used for immunoprecipitation studies. Myocytes were either untreated or collected after 6 hours of hypoxia. The initial cytosol was divided into 3 fractions. An aliquot of the initial cytosol for hypoxia and control cells was set aside for later comparison. An equal amount of protein from the normal myocyte cytosol and cytosol from cells after 6 hours of hypoxia was subjected to exhaustive immunoprecipitation (IP). Thus, the starting protein concentration for the 4 IPs was the same. Details of immunoprecipitation of with anti-bax, anti-HSP60, and a nonspecific IgG1 have been previously detailed.⁶

Comet assay was done to assess DNA fragmentation (R & D Systems). The directions of the manufacturer were followed. Cells were scored as negative or positive for a tail by an observer unaware of treatment.

Mitochondrial Uncoupling
A pilot study performed with a range of concentrations (5 to 50 μmol/L) of CCCP was used to determine the amount necessary to reduce ATP but not release LDH. On the basis of these data, myocytes were treated with 25 μmol/L CCCP for 6 hours and analyzed as above.

Statistical Analysis
Results are expressed as mean±SEM. Groups of data were analyzed by ANOVA or ANOVA on ranks, where appropriate, followed by the Student-Neuman-Keuls test or Dunnett’s test. A value of P<0.05 was considered significant.

Results
HSP60 and HSP72 Levels
We postulated that HSP60 would not increase with hypoxia/reoxygenation; in fact, it might decrease. To test this, adult cardiac myocytes were exposed to near 0 mm Hg oxygen conditions for 6 hours and then reoxygenated. As shown in

Figure 1A, at end-hypoxia, HSP60 levels were unchanged and dropped significantly with reoxygenation. HSP72 has previously been shown to increase in response to hypoxia/reoxygenation, and as shown in Figure 2, HSP72 increased by 80% by 10 hours and 110% by 24 hours of reoxygenation. However, at end-hypoxia, little to no HSP72 could be detected in cells. This probably is secondary to binding of HSP72 to insoluble complexes, even in the presence of detergent, and the resulting failure of HSP72 to enter the gel for analysis. Significantly, these experiments show that in the presence of the expected increase in HSP72, HSP60 decreases.

Indexes of Apoptosis Versus Necrosis
As previously, we had observed that a decrease in HSP60 precipitated apoptosis, cytochrome c release was compared with the changes in HSP60. As shown in Figure 3A, release of cytochrome c occurred by the end of hypoxia, before reoxygenation, even though the decrease in HSP60 was not observed until 10 hours of reoxygenation. 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 media. As shown in Figure 3B, there was no release of citrate synthase from the mitochondria of either control or hypoxic groups. Furthermore, citrate synthase activity was the same for the mitochondria from both groups. Although cytochrome c was released by end-hypoxia, no
changes in the levels of bax or bcl-2 were observed until reoxygenation, when bax and bcl-2 decreased by 40% to 50% (Figure 4, A and B).

The comet assay was used as another index of apoptosis. At end-hypoxia, 65% of cells were positive for DNA fragmentation, compared with <10% of controls. In contrast, LDH media levels, which reflect necrosis rather than apoptosis, rose from a control level of 0.59±0.04 to 2.12±0.14 U/mg of cellular protein (P<0.05). These values were measured after reoxygenation at end-hypoxia. In a single experiment, the media were collected before reoxygenation, and LDH levels were already elevated to 2.06±0.29 versus controls of 0.77±0.08, indicating that LDH release preceded reoxygenation.

**Cell Fractionation Studies**

As we hypothesized that the interaction between HSP60 and bax prevented apoptosis, we theorized that a change in distribution of HSP60 would precipitate apoptosis. The digitonin-permeabilized myocytes were used to divide the cell into two fractions, cytosolic and an organelle/membrane. As shown in Figure 5, top panel, at end-hypoxia less HSP60 was present in the cytosolic fraction and more in the membrane/mitochondria fraction compared with controls. Coincident with this reduction in cytosolic HSP60 at end-hypoxia, bax moved to the mitochondrial/membrane fraction (Figure 5). As a control, levels of actin were analyzed, and they were unchanged (Figure 5, bottom panel).

To further investigate changes in HSP60 localization, discontinuous gradient centrifugation was used to separate the plasma membrane and mitochondria. Western blots of the plasma membrane and mitochondria fractions demonstrated that at end-hypoxia, HSP60 had moved to the plasma membrane and bax was in the mitochondria in contrast to control cells (Figure 6, A and B). Although equal amounts of HSP60 were observed in the plasma membrane and mitochondria (Figure 6A), this is as a percent of total protein. Correcting for total protein (more in mitochondria than in plasma membrane), 88.2±1.3% of the HSP60 was mitochondrial after hypoxia, whereas 11.8±1.3% was associated with the plasma membrane.

**Coimmunoprecipitation**

To further confirm the cytosolic interaction of HSP60 and bax and that this interaction is disrupted by hypoxia, exhaustive immunoprecipitation was performed on the cytosol isolated as part of the cell fractionation studies. As shown in Figure 7, repetitive IP with either anti-HSP60 (lanes B 1 to 3) or anti-bax (lanes C 1 to 3) completely depleted all HSP60 (upper panel) and bax (lower panel) from the cytosolic fraction of the control cells. For comparison, lane A shows 40 μg of total cytosol and lanes B4 and C4 show 40 μg of protein after IP with anti-HSP60 or anti-bax, respectively. No
HSP60 or bax is detectable in lanes B4 or C4. Thus, IP with either anti-HSP60 or anti-bax completely depletes the cytosol of both HSP60 and bax. In contrast, after hypoxia no HSP60 (upper panel) or bax (lower panel) is detected in the cytosol (lane D) and neither is immunoprecipitated with anti-HSP60 (lanes E 1 to 3, upper and lower panels). Furthermore, a nonspecific IgG immunoprecipitated neither HSP60 nor bax (lane F 1 to 3, upper and lower panel), and there was no depletion of either HSP60 or bax (lane F4 upper and lower panel compared with lane A). Thus, HSP60 and bax interact in the cytosol, and as HSP60 decreased in the cytosol as the result of translocation to the plasma membrane during hypoxia, the unbound bax increased and the amount of bax in the mitochondrial fraction increased. Mitochondrial levels of HSP60 were unchanged. Hence, although the total cellular amount of HSP60 and bax were unchanged at end-hypoxia,

Figure 3. A, Representative Western blot shows cytochrome c release into cytosol at end-hypoxia before reoxygenation. B, Citrate synthase activity of cytosol. C indicates control; 10 H Reox or HYP/REOX, 10 hours of reoxygenation; 24 H Reox, 24 hours of reoxygenation.

Figure 4. A, Western data for bax levels. Graph summarizes results of 3 experiments. Values were normalized to control on each blot to allow comparison of different experiments. Representative Western blot for bax in lower panel. B, Western data for bcl-2 levels. Graph summarizes results of 3 experiments. C indicates control; 10 H Reox or 10H, 10 hours of reoxygenation; 24 H Reox or 24H, 24 hours of reoxygenation. *P<0.05 vs controls.
critical changes had occurred in the localization of bax and the cytosolic HSP60. These changes correlated with the release of cytochrome c at end-hypoxia, before reoxygenation (Figure 3A).

**ATP, Acidosis, and HSP60 Localization**

Hypoxia causes a reduction in ATP levels and acidosis. We reasoned that either of these might cause the redistribution of bax and HSP60 and apoptosis. Cells were treated with 25 μmol/L CCCP to reduce ATP levels. A pilot study was done to determine dose and that CCCP treatment did not result in release of LDH. As shown in Figure 8A, CCCP treatment for 6 hours reduced ATP levels to 10% of baseline. This change is similar to that seen with hypoxia (Figure 8B). Reducing ATP levels was sufficient to cause redistribution of HSP60 to the plasma membrane and bax to the mitochondria (Figure 8C). These changes were accompanied by release of cytochrome c (Figure 8D). Media pH remained unchanged, in contrast to the effect of 6 hours of hypoxia, which reduced media pH to 6.9 to 7.0. To mimic the change in pH, cells were placed in media with a pH of 7.0 for 2 hours, reasoning that the change in pH did not occur until the end of hypoxia. Low pH neither caused LDH release nor changed the cellular distribution of HSP60 or bax. Increasing the duration of pH reduction to 3.5, 4, and 6 hours was studied. At 3.5 hours, no changes in cytochrome c or LDH were observed, whereas with longer treatment, LDH release occurred.

**Discussion**

Previously, we have observed that reduction in HSP60 is sufficient to precipitate apoptosis in myocytes and that HSP60 binds to bax. With reduction in HSP60, bax moves from the cytosol to the mitochondria. In the current study, we show that with hypoxia, HSP60 no longer complexes with bax, and that the cytosolic HSP60 relocates to the plasma membrane. In contrast, mitochondrial HSP60 remains unchanged. Coincident with the movement of HSP60 to the plasma membrane, bax moves to the mitochondria, and this is accompanied by a release of cytochrome c. DNA fragmentation ensues, as shown by the comet assay. Thus, whereas the total cellular HSP60 remains unchanged, there is a change in cellular distribution of HSP60 and bax and the precipitation of apoptosis. These changes occur before reoxygenation and the concomitant generation of free radicals.
In mammalian cells, \(15\%\) to \(20\%\) of cellular HSP60 is located in extramitochondrial sites.\(^{12}\) Binding to HSP60 in the normal cell may prevent bax from oligomerizing and inserting into the mitochondrial membrane.\(^{5}\) It is likely that other proteins are present in the cytosolic HSP60/bax complex. We used cell fractionation followed by Western blot analysis to determine the distribution of HSP60 in the myocyte. During hypoxia, the translocation of cytosolic HSP60 to the plasma membrane releases bax, and this may allow the oligomerization and mitochondrial pore formation that are thought to occur as part of apoptosis.\(^{13-16}\) In fact, the current experiments show that with the translocation HSP60 to plasma membrane from the cytosol, bax redistributes from the cytosol to the mitochondria. The addition of bax to mitochondria is sufficient to trigger cytochrome c release and subsequent caspase activation.\(^{17,18}\) In kidney cells, Saikumar et al\(^{19}\) found that during hypoxia there is a translocation of bax from the cytosol to mitochondria. Concurrently, there was cytochrome c release and caspase activation, and if ATP was available during reoxygenation, apoptosis developed, otherwise necrosis occurred. Our results suggest that HSP60 has a regulatory role for the activity of these proapoptotic proteins and that HSP60 is a key antiapoptotic protein in the cell.

In conditions of stress and injury, HSP60 has been reported to be present on the cell surface, as in stressed aortic endothelial cells, where surface expression HSP60 has been suggested as a possible mechanism underlying atherosclerosis.\(^{20}\) Oxidized LDL induced surface expression of HSP60 in monocytes.\(^{21}\) Some investigators have suggested that the membrane state may alter HSP expression and that HSP60 may have membrane-stabilizing properties.\(^{22,23}\) Plasma membrane localization of HSP60 either with or without cellular stress/injury has not previously been reported in the heart. Patients with both ischemic and dilated cardiomyopathy have been found to have antibodies to HSP60.\(^{24,25}\) The surface presentation of HSP60 on the myocyte combined with serum antibodies to this protein may be one mechanism fueling the downward spiral in heart failure. It is possible that membrane HSP60 may be recognized by macrophages and thus mark the myocyte for destruction. Whether the translocation of HSP60 to the membrane might stabilize its structure and have a protective effect is unknown, as is the mechanism(s) controlling this translocation.

HSP60, bax, and bcl-2 all decreased with reoxygenation. HSP60 decreased, even though HSP72 showed a marked increase with reoxygenation. The lack of an increase in HSP60 is not surprising; others have observed little or no change in HSP60 mRNA after in vivo ischemia.\(^{26}\) Knockout of HSF-1 has little effect on HSP60 levels, in contrast to the marked effect on HSP72.\(^{2}\) A reported increase in HSP60 after repetitive in vivo ischemia in the rabbit heart may reflect changes in other cells types or an influx of inflammatory cells.\(^{27}\) It is unclear why there is a late drop in HSP60 levels. We measured media HSP60 but found no clear release of HSP60 (data not shown). Likewise, the decrease in bcl-2 and bax remains unexplained. We have previously observed that

| Figure 8. A, ATP levels after 6 hours of CCCP treatment. B, ATP levels after hypoxia. C, Cell fractionation studies. Upper panel, HSP60 present in mitochondria (MIT) in both control (C) and ATP-depleted (CCCP) cells. HSP60 found in plasma membrane (PM) only with ATP depletion. Lower panel, bax not found with PM but found with MIT after ATP depletion. D, Cytochrome c release after ATP depletion. C indicates control; CCCP, ATP depletion. 4H and 6H, 4 and 6 hours of hypoxia. *\(^{0.05}\) vs control. |
reduction in HSP60 alone results in a 50% decrease in bcl-2 and an increase in bax. The setting of hypoxia and reoxygenation is more complex than the reduction of HSP60 by antisense treatment, and other mechanisms must be involved in the change in these critical proteins.

Apoptosis Versus Necrosis
Although apoptosis and necrosis have been viewed as distinct cascades, our findings indicate that both occurred in the myocytes with hypoxia and reoxygenation. The findings suggest that even before reoxygenation, the cells crossed over from apoptosis, already apparent with the release of cytochrome c, to necrosis with the release of LDH. This crossover may be more common than has been assumed.

In summary, we demonstrate that cytosolic HSP60 during hypoxia translocates to the plasma membrane. Furthermore, we show that reduction in cytosolic HSP60 is accompanied by apoptosis. Our data further strengthen the importance of the mitochondria in apoptosis, suggesting that mitochondrial damage is a central event in cell injury during hypoxia. These results support a key role for HSP60 in the regulation of apoptosis.

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