HSP72 inhibits apoptosis-inducing factor release in ATP-depleted renal epithelial cells

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Ruchalski, Kathleen, Haiping Mao, Satish K. Singh, Yihan Wang, Dick D. Mosser, Fanghong Li, John H. Schwartz, and Steven C. Borkan. HSP72 inhibits apoptosis-inducing factor release in ATP-depleted renal epithelial cells. Am J Physiol Cell Physiol 285: C1483–C1493, 2003—Inhibition of the mitochondrial release and nuclear translocation of apoptosis-inducing factor (AIF) by heat stress protein (HSP)72 may ameliorate apoptosis in renal epithelial cells exposed to a metabolic inhibitor. To evaluate this hypothesis, cells were transiently exposed to 5 mM sodium cyanide in the absence of medium glucose, a maneuver known to induce apoptosis. ATP depletion for 1–2 h resulted in the progressive accumulation of mitochondrial AIF in the cytosol of samples obtained by selectively permeabilizing the plasma membrane with digitonin. During recovery from ATP depletion, time-dependent nuclear AIF accumulation (but not cytochrome c, an F0F1 ATP synthase subunit, or talin) was observed in isolated nuclei. Nuclear AIF accumulation was associated with peripheral chromatin condensation and DNA degradation. Prior heat stress (HS) significantly reduced AIF leakage into the cytosol, decreased nuclear accumulation of AIF, and inhibited DNA degradation. HS also increased the interaction between AIF and HSP72 detected by immunoprecipitation. In ATP-depleted cells, selective overexpression of human HSP72 reduced the leakage of mitochondrial AIF in a dose-dependent manner (r = 0.997). This study suggests that mitochondrial membrane injury and subsequent AIF release contribute to nuclear injury and apoptosis in ATP-depleted renal cells. HSP72, an antiapoptotic protein, inhibits cell injury in part by preventing mitochondrial AIF release and perhaps by decreasing its nuclear accumulation.

heat stress; adenosovirus; metabolic inhibitors; heat stress protein 60; DNA degradation

apoPTOSIS CAUSES DEATH in renal epithelial cells exposed to metabolic inhibitors (11, 20, 41) and contributes to the acute renal dysfunction that accompanies an ischemic insult (19, 33). Renal epithelial cell apoptosis is characterized by mitochondrial membrane injury, release of cytochrome c, and the subsequent activation of caspases (18, 32). In apoptotic cells, nuclei also undergo characteristic changes including chromatin condensation and DNA fragmentation (11, 20, 41). Release of apoptosis-inducing factor (AIF), a proapoptotic protein that resides in the intermembranous space, causes some of these nuclear events and promotes cell death (5, 9, 21, 38).

AIF is synthesized in the cytosol as a 67-kDa precursor protein that contains a mitochondrial localizing sequence (MLS) (9, 25). After AIF is imported into mitochondria, the MLS is shed, resulting in the accumulation of a mature, 57-kDa protein species (9). Although its role in normal cell function is presently unknown, structural similarity to flavoproteins suggests that AIF may act as an oxidoreductase in mitochondrial electron transport (24). In response to various stressors, AIF exits the outer mitochondrial membrane, traverses the cytosol, and then enters the nucleus (5, 9, 21, 46). AIF activates endonucleases that cause peripheral nuclear condensation and the degradation of DNA into 50 kb-sized fragments in both intact cells and isolated nuclei (9, 16, 31). AIF alone is sufficient to cause cell death in a variety of models (8, 21, 31, 46), including exposure to menadione or staurosporine, serum, or growth factor withdrawal (5) and mediates injury caused by p53 (8). A recent study demonstrated that renal epithelial cells release mitochondrial AIF into the cytosol (18), suggesting that AIF could contribute to nuclear injury and cell death.

Several investigators have demonstrated that heat stress proteins (HSP) inhibit apoptosis at multiple checkpoints in the cell death pathway (reviewed in Refs. 2 and 44). Prior heat stress, as well as the selective overexpression of HSP72, an inducible cytoprotectant protein, decreases apoptosis in renal epithelial cells exposed to metabolic inhibitors (41, 43). Although HSP72 inhibits mitochondrial cytochrome c release and subsequent caspase activation in these cells, the effects of stress proteins on AIF release and translocation have not been evaluated.
Transient exposure to metabolic inhibitors causes death in renal epithelial cells. In this model, most cells die by apoptosis as evidenced by characteristic morphological changes observed by electron microscopy (e.g., chromatin condensation, formation of apoptotic bodies), as well as endonucleosomal DNA degradation, release of both cytochrome c and AIF and caspase activation. In addition, renal cells exhibit minimal evidence of plasma membrane injury assessed by either trypan blue uptake or release of lactate dehydrogenase.

The present data show that mitochondrial AIF, once released into the cytosol, accumulates in nuclei during recovery from transient ATP depletion. Nuclear AIF accumulation is associated with DNA degradation, peripheral chromatin condensation, and morphological evidence of apoptosis. Prior heat stress inhibits mitochondrial AIF release and reduces nuclear AIF accumulation, DNA degradation, and apoptosis. HSP72 co-immunoprecipitates with AIF and, prior heat stress markedly increases this interaction. In a dose-dependent manner, selective overexpression of HSP72 reproduces the cytoprotective effects of prior heat stress on mitochondrial AIF release. These studies suggest that HSP72 might improve renal cell survival, at least in part, by inhibiting AIF-mediated events in the apoptotic pathway.

METHODS

Materials. All reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Cell culture. Opossum kidney (OK) cells were obtained from the American Type Culture Collection (ATCC CRL-1840) and grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS). Cells were used within 72 h of achieving confluence.

ATP depletion. To induce ATP depletion, cells were incubated for 1–2 h at 37°C in glucose-free medium (DMEM; Gibco BRL n° 23800-014) that contained 5 mM sodium cyanide as previously described (34). This maneuver results in rapid, severe ATP depletion (29) similar to that observed in renal cells exposed to both 5 mM sodium cyanide and 2-deoxy-D-glucose, a nonmetabolized glucose inhibitor, as previously reported by our laboratory (42). In control cells, parallel medium changes were performed by using 10 mM glucose-containing DMEM. We have previously established that 1–2 h of exposure to metabolic inhibitors causes apoptosis (18, 42, 43).

Estimation of cytosolic AIF. To assess the cytosolic content of AIF, HSP72, and mitochondrial HSP60 (mtHSP60), intact cells were incubated with digitonin (0.15 mg/ml for 10–20 min at 4°C) as recently described by our laboratory (18). This procedure has been shown to distinguish mitochondrial from cytosolic AIF (18) and does not itself cause mitochondrial dysfunction (42). The identity of the relevant bands (HSP72, mtHSP60, and AIF) was confirmed in whole cell lysates exposed to 2% sodium dodecyl sulfate (SDS) as previously described (18). To exclude injury caused by digitonin, heat stress, or mechanical harvesting, the following criteria were established to ensure the adequacy of each study for further analysis. First, heat stress should increase the content of HSP72. Second, minimal mature AIF (57 kDa) should be detected in digitonin-treated samples obtained from control or previously heated cells before ATP depletion. Third, metabolic inhibition should induce mitochondrial AIF release in control cells. In 2 of 10 rejected experiments, heat stress failed to induce HSP72. In 7 of 10 rejected experiments, AIF release was observed under baseline conditions in either the control or heat stress groups. In only 1 of 14 experiments did ATP depletion fail to induce AIF release. Four of fourteen studies were subjected to formal analysis and are presented in RESULTS.

Immunohistochemistry. To visualize AIF, subconfluent cells grown on glass coverslips were rinsed twice with PBS to remove the DMEM, fixed with 2% paraformaldehyde for 30 min at 4°C, and then washed three times with PBS at pH 7.4. To assess nuclear morphology, live cells were incubated with 1 μg/ml Hoechst dye no. 33342 in PBS (pH 7.40, 10–15 min at 25°C) during the final 10 min of recovery from ATP depletion (41). To improve antibody penetration, cells were permeabilized with 0.2% Triton X-100 before exposure to an anti-AIF antibody (catalog no. sc-13116, 1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibody was detected with a Cy3-conjugated anti-mouse antibody (Jackson Immunoresearch; 1:300 dilution; Jackson Immunoresearch, West Grove, PA). To colocalize AIF within nuclei, fixed cells were stained with Sytox green (Molecular Probes, Eugene, OR), anti-AIF antibody (Santa Cruz) was detected with a Cy3-conjugated secondary antibody, and cells were examined by confocal microscopy (Perkin Elmer Ultraview Scanning Disc Microscope). An observer blinded to the experimental condition (S. K. Singh) obtained six to seven random fields in each group. To quantify AIF uptake, individual AIF particles were counted in all fields in which complete nuclei were visualized. Particles crossing the outer perimeter of nuclear membrane were excluded.

Induction of HSP72. Wild-type HSP72 content was increased either by exposure to transient heat stress (42.5 ± 0.5°C for 45 min) in a temperature-regulated incubator followed by incubation at 37°C for 16–18 h (42) or by coinfecting cells with adenoviruses containing human HSP72 and green fluorescent protein (GFP) (AdTR5/HSP70-GFP) expressed on separate cistrons, as well as a tetracycline-regulated promoter (AdCMV/tTA) as previously described (27). Control cells were coinfectected only with AdTR5/GFP and AdCMV/tTA. To increase HSP72 content, cells were coinfectected with adenovirus for 24 h at 37°C followed by a 24-h incubation period after the virus was removed. Infection efficiency was >80–99% as determined by direct visualization of GFP in cells infected with increasing doses of adenovirus with a multiplicity of infection (MOI) that varied between 30 and 120. The effect of each adenoviral dose on HSP72 content was assessed by immunoblot analysis.

Immunoblot analysis and coimmunoprecipitation. Cells grown in 60-mm² dishes were harvested using a rubber policeman and then resuspended in cell lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1 mM EGTA, and 1% SDS and a mixture of protease inhibitors [5 μM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF-HCl), 10 nM leupentin, 1.5 μM aprotinin, 10 nM E-64, and 5 μM EDTA, pH 7.40; Calbiochem-Novabiochem, San Diego, CA). To obtain positive controls for AIF, mtHSP60, and HSP72, cells were gently sonicated (10 pulses at 4°C), and the whole cell lysates were centrifuged at 10,000 g for 15 min. Commercially available antibodies were used to detect HSP72 (1:2,000 dilution, catalog no. SPA-810; StressGen Biotechnologies, Victoria, BC, Canada), AIF (1:250 dilution, a mouse monoclonal antibody; Santa Cruz), mtHSP60 (1:1,000 dilution; StressGen), histone (1:500 dilution, catalog no. 05-499; Upstate Biotechnology, Lake Placid, NY).
NY), talin (1:1,000 dilution, catalog no. 05-385; Upstate Biotechnology), cytochrome c (cytoC12-abm, 1:1,000 dilution; Research Diagnostics, Flanders, NJ), and the F0F1 ATP synthase α-subunit (A21350, 1:1,000 dilution; Molecular Probes) in whole cell lysates, digitonin-extracted samples, and isolated nuclei. Specific protein bands were detected with a horseradish peroxidase-based enzyme-linked chemiluminescence system (Lumiglow, Kirkegaard & Perry, Gaithersburg, MD). After the image of each immunoblot was digitized (Desk Scan II; Hewlett-Packard), band densities were quantified using NIH Image Quant software.

Immunoprecipitation (IP) was performed in digitonin-treated samples by using an antibody directed against AIF (2 μg/mg protein/ml IP buffer; catalog no. X1106P; Exalpha Biologicals, Watertown, MA) as recently described by our laboratory for cytochrome c (18). Apyrase (10 U/ml), a compound that causes ATP hydrolysis, was used to prevent the ATP-mediated release of AIF from HSP72 (41). Protein interaction was assessed by probing the membranes with antibodies directed against HSP72 (StressGen) and AIF (catalog no. sc-13116; Santa Cruz Biotechnology).

Nuclear isolation. Intact nuclei were separated from “non-nuclear” cell components (cytosol, other organelles, and membrane fragments) by using a commercial kit (BioVision Research Products, Mountain View, CA) as previously reported by our laboratory (29) with minor modifications. To isolate nuclei, cells were subjected to three cycles of freezing (−80°C) and thawing (at 4°C) followed by differential centrifugation. Nuclear content was assessed by visual inspection at each step of purification. The adequacy of nuclear purification was assessed by measuring histone content (a nuclear protein) and the de-enrichment of talin (a non-nuclear protein that resides in multiple cell compartments) by using immunoblot analysis.

DNA degradation. The degree of DNA degradation caused by exposure to metabolic inhibitors was quantified in subconfluent monolayers of cells grown in 12-well trays. The cells were incubated with 1 μCi of [3H]thymidine in 1 ml of DMEM with 10% FCS for 16 h and then assayed using a method previously described by our laboratory (41). In this experimental model, DNA fragmentation correlates with endonucleosomal DNA degradation and morphological evidence of apoptosis as confirmed by electron microscopy (41). This technique permits separation of labeled DNA fragments from free [3H]thymidine and [3H]thymidine-labeled intact chromatin in both attached and detached cells (7). The percentage of DNA fragmentation was calculated as follows: (DNA fragments/[DNA fragments + intact DNA]) × 100. In renal cells exposed to metabolic inhibitors, DNA fragmentation correlates with morphological and biochemical evidence of apoptosis (41).

**RESULTS**

Before ATP depletion, only pro-AIF was detected in the cytosol of controls (Fig. 1). In contrast, exposure to metabolic inhibitors for 1.0–2.0 h (without recovery) was associated with progressive accumulation of both pro- and mitochondrial AIF in the cytosol of renal epithelial cells. After 2 h of ATP depletion, cytosolic accumulation of mitochondrial AIF increased by ~30-fold compared with control as estimated by densitometric evaluation of the 57-kDa bands (data not shown).

Exposure to a metabolic inhibitor caused the appearance of mitochondrial AIF (57 kDa) in digitonin-treated samples obtained from control cells (Fig. 2A). The amount of immunodetectable AIF progressively increased during recovery from transient ATP depletion. A prior study showed that ~10% of mitochondrial AIF and cytochrome c enter the cytosol of renal epithelial cells during ATP depletion and recovery (18). Prior to ATP depletion, only pro-AIF was detected in the cytosol of controls (Fig. 1). In contrast, exposure to metabolic inhibitors for 1.0–2.0 h (without recovery) was associated with progressive accumulation of both pro- and mitochondrial AIF in the cytosol of renal epithelial cells. After 2 h of ATP depletion, cytosolic accumulation of mitochondrial AIF increased by ~30-fold compared with control as estimated by densitometric evaluation of the 57-kDa bands (data not shown).

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heat stress induced a four- to sixfold increase in the content of HSP72 (see Fig. 9B) but did not alter the amount of AIF observed in digitonin-treated samples at baseline compared with control (Fig. 2). Compared with control, heat exposure significantly decreased the leakage of mitochondrial AIF immediately after ATP depletion as well as during recovery (Fig. 2B, P < 0.5, n = 4).

In control renal epithelial cells, AIF staining was detected in a perinuclear pattern typical of a mitochondrial protein previously reported (6, 10) in non-renal cells (Fig. 3A). AIF was not observed in nuclei under these conditions. Before ATP depletion, control nuclei were relatively large and faintly stained with Hoechst dye (Fig. 3B). Three hours after transient ATP depletion, AIF was detected in nuclei (Fig. 3C and inset). AIF-positive nuclei were small, rounded, and intensely stained with Hoechst dye (Fig. 3D). Prior heat stress decreased the leakage of mitochondrial AIF immediately after ATP depletion as well as during recovery (Fig. 2B, P < 0.05, n = 4).

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In control cells examined by confocal microscopy, AIF and Sytox green stains localized in distinct compartments consistent with mitochondria and nuclei, respectively (Fig. 4A). Minimal AIF stain could be visualized in control nuclei (Fig. 4A, left). In contrast, abundant AIF stain could be detected within multiple adjacent nuclei after 1 h of ATP depletion followed by 3 h of recovery (Fig. 4A, right). At higher magnification, these differences in the AIF stain within nuclei could be clearly visualized (Fig. 4B). Quantitative analysis of randomly selected fields of cells assessed by confocal microscopy demonstrated that ATP depletion was associated with a significant increase in nuclear AIF stain (Fig. 4C, P < 0.02).

To confirm that ATP depletion causes selective nuclear AIF accumulation, the content of AIF, cytochrome c, and talin in both nuclear and nonnuclear cell fractions of isolated nuclei was serially examined by immunoblot analysis. As expected, AIF was abundant in the nonnuclear fraction that contains mitochondria both before and 90 min after transient ATP depletion (Fig. 5A, top blot). Although minimal AIF was detected in the nonnuclear cell fraction before ATP depletion, progressive AIF accumulation was observed in isolated nuclei between 15 and 90 min of recovery from 1 h of ATP depletion. In contrast to AIF (57 kDa), minimal cytochrome c (14 kDa), another mitochondrial protein released during ATP depletion (18), was detected in isolated nuclei during recovery from ATP depletion (Fig. 5A, bottom blot). To exclude variable contamination of the nuclear fraction with mitochondria, the F0F1 ATP synthase α-subunit, an integral mitochondrial membrane protein, was also examined. Unlike AIF, the ATP synthase subunit did not accumulate within the nuclear fraction during ATP depletion or recovery.
Both cytochrome c and the ATP synthase subunit were detected in the nonnuclear cell fraction. Nuclear AIF content positively correlated with recovery from ATP depletion. The correlation between recovery time and nuclear AIF content, estimated from densitometric evaluation of cytosolic AIF content, was 0.964 (Fig. 5B). In contrast to AIF, talin content did not increase in the nuclear fraction of ATP-depleted cells during the same time period. To assess the adequacy of the preparation, the content of talin and histone, a nonnuclear protein, was also examined. As expected, histone was markedly increased in the nuclear preparation, whereas talin was markedly less abundant (Fig. 5C).

The observation that mitochondrial AIF, a 57-kDa protein, leaked into the cytosol during ATP depletion suggested that (at least) the outer mitochondrial membrane had been injured. To assess the integrity of the inner mitochondrial membrane, the distribution of mtHSP60, a protein that resides within the inner mitochondrial matrix (10, 35), was examined in digitonin-treated cell samples. Under control conditions, minimal mtHSP60 was detected in the cytosol by immunoblot analysis (data not shown). After 1 h of exposure to metabolic inhibitors, cytosolic mtHSP60 content in-
creased four- to fivefold (Fig. 6A). The pattern of mtHSP60 redistribution, unlike that of AIF, did not continue to increase during recovery. To evaluate the possibility that mtHSP60 was responsible for preventing the leakage of AIF in previously heated cells subjected to ATP depletion, mtHSP60 content was examined before and at several time points during recovery (0–90 min) from 1 h of ATP depletion. Each lane contains 40 μg of total protein separated by 10% SDS-PAGE. B: densitometric evaluation of the major immunoreactive AIF bands in isolated nuclei during recovery from ATP depletion (shown in A) as well as talin (immunoblot not shown). C: adequacy of the separation of nuclear from nonnuclear (non-nuc) cell fractions was estimated by immunoblot analyses for talin (a protein that localizes to several nonnuclear compartments including the cytosol) and histone (a nuclear protein). Each lane in these 3 immunoblots contains 40 μg of protein.

If AIF accumulates within nuclei, then activation of endonucleases and subsequent DNA degradation would be expected. To assess this hypothesis, DNA fragmentation was assessed before and after exposure to metabolic inhibitors (Fig. 7). In both control and previously heated cells, <5% of total, [3H]thymidine-labeled DNA was degraded. Three hours after control cells were subjected to one to two hours of ATP depletion, progressive DNA fragmentation was observed. In addition to preventing cytosolic leakage and nuclear accumulation of AIF, prior heat stress significantly inhibited DNA fragmentation at all time points \( P < 0.05, n = 5 \).

In digitonin-treated samples, some interaction was observed by coimmunoprecipitation (Fig. 8A, lane 1). This interaction is specific, because immunoprecipitates obtained with an equal amount of nonimmune rabbit serum (substituted for the primary antibody) contained minimal HSP72 (data not shown). Compared with baseline, both ATP depletion and recovery (conditions that release mitochondrial AIF into the cytosol) were associated with increased interaction between AIF and HSP72 (Fig. 8A). Two AIF bands with apparent molecular masses of 67 (minor band) and 57 kDa (major band) were detected in each of the immunoprecipitates (Fig. 8B). These bands correspond to the known size of pro- and mitochondrial AIF, respectively (9, 21). In control, greater amounts of pro- and mitochondrial AIF were detected in immunoprecipitates following ATP depletion or recovery than at baseline (Fig. 8B). Relative to control, prior heat stress increased the interaction between HSP72 and AIF at all time points (Fig. 8A). The increase in interaction between these two proteins occurred despite the observation that prior heat stress inhibits AIF leak into the cytosol during ATP depletion and recovery.

Because HSP72 decreases primary mitochondrial membrane injury (18, 27), the effect of this cytoprotec-
tive protein on mitochondrial AIF release was examined. HSP72 was selectively overexpressed by coinfecting cells with adenoviruses encoding GFP and HSP72 (expressed as separate proteins) and a tTA-encoding virus. Infection resulted in >95% GFP positive cells.

![Graph A](image1)

Fig. 6. Redistribution and content of mitochondrial HSP60 (mtHSP60). A: leakage of mtHSP60 into the cytosol was assessed in digitonin-treated samples using 7% SDS-PAGE before (CTL), immediately after 1 h of ATP depletion (ATP deplete), or during recovery (Rec15 and Rec30). Data are expressed as percentages of baseline mtHSP60 content (means ± SE). Each lane contains 16 μg of total protein. *P < 0.05 vs. control, n = 4. B: steady-state mtHSP60 in control and previously heated cells (heat stress) is expressed as the mean density (±SE) of mtHSP60 bands detected in 3 separate experiments. NS, not significant (P > 0.05).

![Graph B](image2)

Fig. 7. Effect of prior heat stress on DNA fragmentation following ATP depletion. The amount of DNA fragmentation was quantified in control (○) and previously heated cells (●) either before (0 h) or 3 h after 1–2 h of ATP depletion. Data are expressed as percentages of DNA fragmentation (means ± SE; SE bars are too small to be visualized). *P < 0.05 vs. control, n = 5.

![Graph C](image3)

![Graph D](image4)

Fig. 8. Interaction between HSP72 and AIF. At baseline (base), after 1 h of ATP depletion (ATP deplete), or after 30 min of recovery (Rec30), digitonin-treated samples of control and previously heated cells containing 300 μg of total protein were immunoprecipitated (IP) with a polyclonal rabbit antibody directed against AIF. After separation by 7% SDS-PAGE, membranes were probed for either HSP72 (IP AIF, IB HSP72; A) or AIF antibodies (IP AIF, IB AIF; B). Immunoglobulin heavy chain (IgG), mitochondrial AIF (mito AIF; 57 kDa), and pro-AIF (67 kDa) are indicated by arrows and matched similar immunoreactive bands in whole cell lysates (not shown). This immunoblot is representative of 2 separate studies.

DISCUSSION

Overwhelming evidence indicates that the mitochondrion is the “Pandora’s box” of death that regulates...
apoptosis (14, 39, 46) and, perhaps, cell necrosis (4, 28, 30, 32). The “intermembranous mitochondrial space,” located between the inner and outer mitochondrial membranes, contains many known regulators of apoptosis such as cytochrome c and AIF (1, 21, 39). AIF and cytochrome c are rate limiting for activating caspases and endonucleases in the apoptotic pathway (21, 39) and represent parallel but complementary mechanisms for promoting cell death (22, 36). In a prior study in our laboratory (41), neither a pan-caspase inhibitor nor a specific caspase-3 inhibitor completely prevented apoptosis in ATP-depleted renal cells. This observation suggests that a portion of cell injury and apoptosis is mediated by non-caspase-dependent factors such as AIF.

The present study shows that mitochondrial AIF, like cytochrome c (18, 32), rapidly translocates from the intermitochondrial membrane space into the cytosol of renal cells during exposure to metabolic inhibitors. AIF leakage is proportional to the duration of ATP depletion (Figs. 1 and 2), suggesting that the mitochondrial membrane undergoes progressive changes in permeability. In addition to mitochondrial (i.e., mature) AIF, pro-AIF also accumulates in the cytosol of ATP-depleted renal cells (Fig. 1). The accumulation of pro-AIF suggests that AIF proteolysis, required to remove the MLS (9), is impaired during metabolic inhibition. During recovery from ATP depletion and after release of mitochondrial AIF, nuclei accumulate AIF, chromatin condenses (especially at the periphery of the nucleus), DNA is degraded, and cells appear apoptotic when stained with Hoechst dye (Figs. 3–5). In addition, AIF accumulation within nuclei appears to be selective, because talin, cytochrome c, and an ATP synthase subunit do not translocate into nuclei from nonnuclear compartments (Fig. 5). These findings suggest that AIF mediates some of the characteristic nuclear features of apoptosis in renal epithelial cells. Although the time course of DNA injury (e.g., chromatin condensation and DNA fragmentation) correlates with nuclear AIF accumulation, this study does not directly show that AIF is responsible for these events. In elegant studies by Ravagnan et al. (31), AIF alone was sufficient to induce peripheral chromatin condensation, nuclear shrinkage, and large-scale DNA fragmentation in isolated nuclei as well as in intact cells (31). Microinjection of purified AIF induced further AIF release and initiated cytochrome c leakage independent of caspase activation (21). AIF also mediated injury in response to poly(ADP-ribose) polymerase-1 (45) and staurosporine (10) in a caspase-independent manner. Finally, a recent study (8) showed that AIF causes cell death in the absence of apoptosis protease activator protein-1, a protein required for activating caspase-9. Taken together, these studies demonstrate that AIF per se is proapoptotic.

In ATP-depleted renal cells, the time course and pattern of AIF release (Figs. 1 and 2) parallel that of cytochrome c (18), with increasing translocation of these proapoptotic proteins during recovery from ATP depletion. In non-renal cells, simultaneous release of AIF and cytochrome c after mitochondrial injury was also observed by Loefler et al. (21). Release of mitochondrial proteins from the intermembranous space requires either membrane permeability transition (MPT) or frank rupture of the outer membrane (9, 37). A recent study in our laboratory (18) demonstrated
that bongkrekic acid, a specific MPT inhibitor, reduces AIF release by 75% in ATP-depleted renal cells. In addition, AIF release is inhibited by Bcl-2, a protein known to antagonize the effect of Bax on MPT (10). It is therefore likely that MPT, rather than membrane rupture, is responsible for AIF release. The increment in the release of mitochondrial proteins during recovery from ATP depletion indicates that progressive injury to the mitochondrial membrane has occurred. Observations by Wang et al. (40) in Caenorhabditis elegans indicate that partial AIF release activates caspase-3, increasing mitochondrial membrane injury and subsequent AIF release. This interpretation is consistent with the marked increase in AIF release after 2 h of exposure to a metabolic inhibitor (Fig. 1).

The degree of mitochondrial injury observed after exposure to a metabolic inhibitor is highly variable between adjacent cells. In monolayers of ATP-depleted cells, approximately 10–15% of total mitochondrial AIF and cytochrome c leak into the cytosol (18). Whether or not the subpopulation of cells with the most marked morphological changes are responsible for AIF and cytochrome c leak is not presently known.

In addition to cytochrome c and AIF (18), metabolic inhibition also releases HSP60 from renal cell mitochondria (Fig. 4). This implies that in some cells, both the inner and outer mitochondrial membranes are damaged (9). Severe membrane injury could result either from cellular necrosis or as a late consequence of AIF or cytochrome c-mediated caspase activation (23, 40). The early, yet stable pattern of mtHSP60 release observed in the present study (Fig. 6) suggests that a fraction of cells has undergone mitochondrial membrane rupture associated with necrosis. This hypothesis is consistent with the modest release of lactate dehydrogenase, a marker of plasma membrane injury, by a fraction of necrotic-appearing renal epithelial cells exposed to metabolic inhibitors (42). mtHSP60 is unlikely to protect mitochondria from ATP depletion, because heat stress failed to increase mtHSP60 content (Fig. 4B). This is likely due to the fact that mtHSP60 is not induced by stress (35).

Prior heat stress (Fig. 2) or the selective overexpression of HSP72 (Fig. 9) significantly decreases mitochondrial AIF release in renal cells subjected to ATP depletion. This cytoprotective effect markedly reduces the potential for AIF to cause subsequent cell injury. Although inhibition of AIF release alone might be not sufficient to prevent apoptosis, recent evidence suggests that HSP72 exerts antiapoptotic effects at multiple steps in the cell death pathway (2), and these findings are summarized in Fig. 10. HSP72 decreases the activation of proapoptotic stress kinases (13, 26), prevents apoptosome assembly in response to cytochrome c release (3), and inhibits caspase-dependent events both proximal (17, 26, 27) and distal (15) to caspase-3. In renal epithelial cells, HSP72 decreases primary mitochondrial membrane injury (18), binds to cytochrome c that translocates into the cytosol (18), and interacts with Bcl-2 (41).

Although HSP72 prevents a substantial fraction of AIF release after ATP depletion (Fig. 9), some AIF escapes into the extramitochondrial compartment. By binding mitochondrial AIF in the cytosol, HSP72 might prevent AIF from entering nuclei. In a single prior study, AIF and HSP72 interacted in cells that were induced to overexpress both proteins (31). In the same study, HSP72 prevented nuclear AIF accumulation, peripheral chromatin condensation, and DNA degradation. The present study extends this seminal observation and shows increased interaction between AIF and HSP72 under pathophysiological conditions (Fig. 8). This interaction is enhanced when the cytosolic content of HSP72 is increased by prior heat stress despite the decrease in mitochondrial AIF released into the cytosol. This interaction is important, because HSP72 prevents the translocation of mitochondrial AIF from the cytosol into the nucleus (31). In contrast to injured cells, interaction between these two proteins in ATP-replete cells suggests that HSP72 might interact with pro-AIF, a role compatible with the chaperone function of HSP72 (12).

The current study identifies a new antiapoptotic mechanism by which HSP72 could inhibit cell death caused by ATP depletion (Fig. 10). By ameliorating mitochondrial membrane injury (primary protection) as well as antagonizing the proapoptotic effects of AIF that escapes the mitochondrial compartment (secondary protection), HSP72 could enhance cell survival. In fact, both prior heat stress (42) and the selective overexpression of HSP72 (43) improve cell survival for 3–6 days after the initial insult. Manipulation of HSP72 could potentially prevent or ameliorate renal cell injury caused by ischemia, glomerulonephritis, urinary tract obstruction, renal transplantation, or exposure to nephrotoxic agents.
diverse insults in which mitochondrial injury and apoptosis contribute to organ dysfunction.

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DISCLOSURES

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