Induced Hsp70 is in small, cytoplasmic complexes in a cell culture model of renal ischemia: a comparative study with heat shock

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Abstract A number of clinical conditions are known to result in the induction of heat shock proteins, but detailed studies on stress response have focused mostly on heat shock as a model. We have analyzed the induction and intracellular distribution of heat shock proteins in a reversible adenosine triphosphate (ATP) depletion model of renal ischemia. Two Hsp70 homologues, Hsp70 in the cytoplasm and BiP in the endoplasmic reticulum (ER) lumen, were found significantly induced during the recovery phase of ATP depletion. Other members of the heat shock protein family, such as Hsp90, constitutive Hsc70, and a related protein Hop60, were not induced. The induction of stress proteins on ATP depletion differed from that after heat shock in the kinds of proteins elaborated, their induction kinetics, and their intracellular distributions. Biochemical fractionation and indirect immunofluorescence experiments indicated that Hsp70 was predominantly cytoplasmic in the recovery phase of ischemia-like stress. Velocity sedimentation on sucrose gradients showed that induced Hsp70 sedimented as small, soluble complexes, ranging in size from $4S_{20,w}$ to $8S_{20,w}$. The results suggest a role for induced Hsp70 that may be different from one of protecting aggregated proteins as under heat shock and emphasize the need for their characterization in other clinical conditions that result in stress response.

INTRODUCTION

All living cells respond to environmental stress by inducing a common group of proteins called heat shock proteins. Originally identified in response to heat stress (Ritossa 1962), heat shock proteins are now known to be synthesized in response to diverse stress conditions (Gabai et al 1998; Samali and Orrenius 1998). The heat shock protein families Hsp60, Hsp70, and Hsp90 have been extensively studied in a large group of organisms and are implicated in cytoprotective functions that provide tolerance to a variety of stress conditions and promote survival (Mosser et al 1997; Gabai et al 1998; Samali and Orrenius 1998). Most information about the biochemical functions of heat shock proteins, their intracellular localization, and their substrates has come from studies on heat stress. The chaperoning ability of heat shock proteins, allowing them to reversibly interact with heat de-

natured proteins, is believed to be responsible for their protective role during heat shock. Such a protein damage control role of heat shock proteins has been generalized to other stress conditions that result in their induction. More recently, novel roles of heat shock proteins have come to light. These include their ability to act as antiapoptotic factors (Gabai et al 1995; Mosser et al 1997; Buzzard et al 1998; Jaattela et al 1998; Samali and Orrenius 1998), their ability to regulate signal transduction cascades (Gabai et al 1997; Buzzard et al 1998; Gabai et al 1998), and their possible role in facilitating membrane protein relocalization (Aufricht et al 1998) following stress. There is growing awareness about the diverse roles that heat shock proteins are capable of performing.

A variety of clinical conditions have been described that result in increased synthesis of heat shock proteins, and there is interest in exploring their therapeutic potential in these instances (Dhillon et al 1993; Vigh et al 1997; Schett et al 1998). However, very few detailed studies of the induction and characterization of heat shock proteins under such physiological stresses have been done. In this

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study, we report characterization of stress proteins induced in response to ischemic shock to kidneys. Renal ischemia is a term used to describe transient block in blood supply to kidneys, resulting in a clinical condition called acute renal failure (Thadani et al 1996). Earlier studies, using ischemia-reperfusion models in experimental animals, have shown an induction of stress proteins during recovery from renal ischemia (Emami et al 1991; Van Why et al 1992; Morita et al 1995). Because the time of induction of heat shock proteins in these studies coincided with the recovery phase, it was proposed that heat shock proteins might be involved in the cellular restructuring process. There is an intense interest in understanding the possible roles of induced heat shock proteins in the recovery process.

Using a widely accepted cell culture model for renal ischemia (Canfield et al 1991; Kuznetsov et al 1996; Tsukamoto and Nigam 1997), we analyzed the types of stress proteins induced and their cellular localization during recovery from ischemia-like stress. Our results show that 2 heat shock proteins of the Hsp70 class, cytosolic Hsp70 and BiP of the ER lumen, are major stress proteins induced early in response to ATP depletion. The induced Hsp70 is present in small soluble complexes in the cytoplasm during the recovery and restructuring phase. The results suggest a role for the induced Hsp70 that may be different from interacting with aggregated proteins and also highlight important differences in the characteristics of heat shock proteins under ischemic stress as opposed to heat stress.

METHODS

Metabolic labeling, ATP depletion, and cell lysis

LLC-PK1 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. For metabolic labeling, typically 100–200 μCi of [^{35}S]-methionine and -cysteine per dish was used. In case of recovery in the presence of radiolabeled amino acids, a 30-minute starvation period in cysteine- and methionine-free RPMI was included before addition of label in the same medium.

ATP depletion was done using antimycin A (AA), an inhibitor of mitochondrial oxidative phosphorylation, at a concentration of 0.1 μM in phosphate-buffered saline (PBS). Cellular ATP was estimated using a luciferase assay-based kit obtained from Sigma Chemical Company (St Louis, MO, USA). For recovery, cells were washed twice in PBS before addition of complete Dulbecco's modified Eagle's medium. Heat shock was given by transferring the dishes containing cells to a 42°C water bath for various times before transferring back to 37°C to allow recovery for various periods.

For detergent lysis, cells were washed with PBS and

placed on ice before addition of 0.5 mL per dish of the lysis buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid [HEPES], 100 mM sodium chloride, 1 mM ethylenediamine-tetraacetic acid, 0.5% Triton X-100, 2 mM phenylmethylsulfonylfluoride, 1 mM each of antipain, pepstatin, and leupeptin). Cells were scraped and allowed to stand in lysis buffer for 15 minutes on ice. Postnuclear supernatant was prepared by centrifuging the lysate at $1200 \times g$. The detergent-insoluble pellet was solubilized in Laemmli sample buffer (Laemmli 1970). The postnuclear supernatant was used for trichloroacetic acid (TCA) precipitation and sedimentation analysis. TCA precipitation of proteins from the cell lysates was performed by addition of equal volumes of ice cold 20% TCA and incubation for 30 minutes on ice. The precipitate was obtained by centrifuging at $1200 \times g$ for 5 minutes. The pellet was solubilized in Laemmli sample buffer (Laemmli 1970).

To determine the total counts per minute of [^{35}S]-methionine and -cysteine incorporated in cells recovering from AA treatment, 0.1 mL of cell lysate was subjected to TCA precipitation. The precipitate was filtered through glass fiber filters (Whatman, Maidstone, UK). The filters that contained the protein precipitate were washed thrice with ice cold acetone, dried, placed in 10 mL of liquid scintillation cocktail, and counted in a liquid scintillation counter (LKB-Wallac, Turku, Finland).

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed by the method of O'Farrell (1975). In brief, the protein samples were subjected to isoelectric focusing in the first dimension using an ampholine range of 3.5–9.5 (Amersham Pharmacia Biotech, New Jersey, USA). They were then subjected to 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. The gels were processed for autoradiography.

Sedimentation analysis

Sedimentation analysis was performed by layering 450 μL of cell lysate on a 11-mL linear gradient of 5–25% sucrose in buffer A (50 mM HEPES [pH 7.4], 100 mM sodium chloride, 0.1% Triton X-100). Centrifugation was carried out in a Beckman SW-41 rotor for 15 hours at 40 000 rpm ($200\,000 \times g$) and 4°C. Proteins from 0.5-mL fractions were TCA precipitated (as described above), and protein pellets were solubilized in Laemmli sample buffer before electrophoresis and immunoblotting. Bovine serum albumin (4.5 $S_{20,w}$), immunoglobulin G (7 $S_{20,w}$), and catalase (11 $S_{20,w}$) were used as sedimentation markers.

Antibodies and immunoprecipitation

Monoclonal antibodies to Hsp70 (SPA810) and Hsc70 (SPA815) were from Stressgen (Ontario, Canada). Antibodies to Hsp90 were a kind gift of Dr Peter Csermely, Semmelweis University School of Medicine, Budapest, Hungary. The monoclonal antibody to Hsp60 was a kind gift of Dr David F. Smith, University of Nebraska Medical Center, Omaha, Nebraska, USA. Alkaline phosphatase-conjugated secondary antibodies were obtained from Bangalore Genie (Bangalore, India). Fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) conjugates for indirect immunofluorescence were obtained from Sigma.

All immunoprecipitations were performed under non-denaturing conditions. The lysates were precleared with protein A-agarose beads and rabbit anti-mouse secondary antibodies for 1 hour at 4°C. The precleared lysates were incubated with the various primary antibodies, rabbit anti-mouse secondary antibodies, and protein A-agarose beads for 10 hours at 4°C. The immunoprecipitates were washed with a washing buffer (50 mM HEPES [pH 7.4], 100 mM sodium chloride, 0.1% Triton X-100) thrice before releasing bound proteins from the complex by boiling in Laemmli sample buffer for 5 minutes. The samples were analyzed by SDS-PAGE and fluorography.

Quantitation of the bands from autoradiograms was done using a laser densitometer (LKB-Bromma, Sweden). To correct for protein loading in cases where total protein profile was examined, the intensity of each band was normalized to that of actin in the same lane. In immunoprecipitated samples, the intensity of the corresponding bands was normalized to the counts per minute of label incorporated in that sample. Normalization was done by first determining the factor by which total counts per minute incorporated in ATP-depleted cells differed from that of control cells in each time point. The intensities of Hsp70 band in lanes corresponding to ATP-depleted cells were then multiplied by the factor for each time point. Quantitation from immunoblots was done from scanned images of the blots, using an image analysis software from Kodak (1D).

Immunofluorescence

For immunofluorescence, cells were grown on 12-mm glass coverslips in 24-well tissue culture plates (Nunc) to 50–60% confluence before subjecting to various treatments. Cells were washed with PBS before fixing with 4% paraformaldehyde in PBS for 20 minutes. Free aldehyde groups were quenched with 50 mM ammonium chloride in PBS for 15 minutes. Cells were then permeabilized in 0.1% Triton X-100 in PBS for 10 minutes and blocked with 10% fetal calf serum in PBS for 30 minutes

before incubating with primary antibody for 1 hour. Three washes with PBS were performed before incubating with TRITC- or FITC-conjugated anti-mouse secondary antibody for 45 minutes. All treatments were carried out at room temperature. After washing thrice with PBS, the coverslips were mounted on slides in 50% glycerol and PBS. The slides were viewed in a laser confocal microscope (Leica, Heidelberg, Germany), and images were captured using the TCS software.

RESULTS

Protein profile of LLC-PK1 cells during recovery from ATP depletion

To identify proteins induced during recovery from ATP depletion, we examined the profile of newly synthesized proteins by metabolic labeling. Of 8 confluent dishes of LLC-PK1 cells, 4 were treated with AA for 2 hours, and the other 4 dishes were mock treated to serve as controls. After 2 hours, cells were allowed to recover in complete medium for 2.5, 4.5, 6.5, and 8.5 hours. Cells were labeled with [³⁵S]-cysteine and -methionine for the last 2 hours of recovery and then lysed with buffer containing 0.5% Triton X-100 as described in the "Methods" section. The lysates were TCA precipitated and analyzed by 7.5% SDS-PAGE and fluorography.

As shown in Figure 1A, the control cells showed a variety of labeled proteins after 2 hours of labeling (lane 1). On the other hand, there were only a few labeled bands visible in cells recovered from ATP depletion for 2 hours (lane 2). The difference was probably due to inhibition of protein synthesis as a result of ATP depletion in AA-treated cells. Among the limited number of labeled proteins seen in cells recovered from ATP depletion, a p70 and a p80 band were the most obvious. Both these proteins increased in intensity at 4.5 hours of recovery (lane 4). Their intensities did not change significantly in control cells (lane 3). At the end of 6.5 (lanes 5 and 6) and 8.5 hours (lanes 7 and 8) intensity of the p80 protein increased further, whereas the level of the p70 species diminished, as shown by quantitation of the bands (bottom panel). The results indicated that although overall protein synthesis was down-regulated, specific proteins were synthesized, preferentially, at elevated levels during recovery from ATP depletion. These included p70 and p80. As shown by the quantitation of the bands, the induction of these proteins was temporally distinct, with p70 induction preceding p80 (bottom panel).

To identify proteins p70 and p80 induced in response to ATP depletion, we used 2-dimensional gel electrophoresis. The samples were first separated based on their pIs and then resolved on SDS-PAGE in the second dimension. We used samples corresponding to lane 3 (control) and

Fig 1. Induction of stress proteins in response to ATP depletion or heat shock. (A) Protein profile during recovery from AA treatment. Eight dishes of LLC-PK1 cells were used. Of these, 4 were treated with AA for 2 hours (lanes 2, 4, 6, and 8), and the remaining 4 were mock treated (lanes 1, 3, 5, and 7). All the dishes were allowed to recover for the different times as indicated in the figure. Label was added to different dishes in the last 2 hours of recovery. Cell lysates were prepared as described in the "Methods" section. The lysates were TCA precipitated and analyzed by fluorography of SDS-PAGE. (B) Protein profile during recovery from heat shock. In an experiment similar to Figure 1A, cells were given a heat shock (43°C for 90 minutes) instead of ATP depletion. Labeling was done in the last 2 hours of recovery. Lanes 1 and 3 are mock-treated controls, whereas lanes 2 and 4 show cells recovered from heat shock. Quantitation of the p70 and p80 bands is shown in the bottom panels. (C) Two-dimensional electrophoresis profile of proteins synthesized during recovery from ATP depletion. In an experiment similar to Figure 1A, samples corresponding to the mock-treated control (top panel) and cells recovered from AA treatment for 4 hours (bottom panel) were subjected to 2-dimensional electrophoresis as described in the "Methods." Spots corresponding to BiP (▼), Hsc70 (→), Hsp70 (↑), and actin (see label) were identified based on their known pIs and relative molecular weights.

lane 4 (4-hour recovery) of Figure 1A on 2-dimensional gel and analyzed the labeled proteins by fluorography. As shown in Figure 1C, several spots of labeled proteins were seen in the control, untreated cells. Based on their reported pIs and molecular weights, we could identify actin (see label, pI 5.3, 43 kDa), constitutive Hsc70 (horizontal arrow, pI 5.5, 70 kDa), and BiP (arrow head, pI 4.8, 78 kDa). In cells recovered from ATP depletion for 4.5 hours, we found the following changes: (1) the spot corresponding to BiP was significantly more intense (arrow head, compare actin spots for comparison), and (2) a spot with a molecular weight of 70 kDa and pI slightly more acidic than Hsc70 appeared (vertical arrow). This spot corresponded to the reported migration of inducible Hsp70 on 2-dimensional gels (Brown et al 1993).

Based on these observations, we tentatively concluded that the p80 band corresponded to BiP of the ER lumen, whereas p70 corresponded to cytosolic, inducible Hsp70. The identity of p80 to be BiP was further confirmed by Western blotting using polyclonal antibody specific to BiP (not shown). Western blotting and immunoprecipitation

using monoclonal antibodies specific to Hsp70 (see below) confirmed the identity of p70 as inducible Hsp70.

We performed an experiment similar to that shown in Figure 1A with heat shock as a stress to compare the kinds of proteins induced and their kinetics of induction. Four confluent dishes of LLC-PK1 cells were used. Two of the dishes were given a heat shock at 42°C for 1 hour. After the heat shock, all the dishes were labeled with [³⁵S]-cysteine and -methionine for the indicated times before cell lysis and TCA precipitation. As shown in Figure 1B, in addition to bands corresponding to p70 and p80, few other bands were found induced after 2.5 hours of recovery from heat shock (lane 2). It is well known that heat shock results in the induction of a panel of heat shock proteins, including Hsp70, Hsp90, BiP, and GRP94 (Ashburner and Bonner 1979; Bansal et al 1991; Gething and Sambrook 1992). After 4.5 hours of recovery, these bands were somewhat diminished in intensities. Quantitation of these bands indicated that at 2.5 hours of recovery from heat shock the levels of heat shock proteins were already at the peak. At recovery times longer than

Fig 2. Hsp70 induction following ATP depletion in LLC-PK1 cells. (A) Cells were subjected to AA treatment for 2 hours before being allowed to recover for various lengths of time as indicated (lanes 2–6). Cells treated with PBS for 2 hours served as controls (lane 1). Cells were then lysed in the presence of detergent, and proteins were TCA precipitated from the lysate. Ten micrograms of protein from the precipitates was immunoblotted for the presence of Hsp70 (upper panel) and Hsc70 (lower panel). (B) Levels of ATP were estimated as described in the “Methods” section from cell lysates obtained from cells treated with 0.1 μ M AA for 2 hours and allowed to recover for the indicated times. ATP levels are expressed as percentage of controls treated with PBS for the same duration.

2.5 hours (lane 4), the intensities of these bands had diminished. It was clear that heat shock-induced stress protein induction was qualitatively, quantitatively, and kinetically different from stress protein induction after ATP depletion.

Hsp70 induction in response to ATP depletion

Earlier studies on ischemia-reperfusion performed with experimental animals have shown that heat shock proteins of the Hsp70 class are induced in the kidneys (Emami et al 1991; Van Why et al 1992). We examined whether the p70 protein found to be induced in our in vitro model is Hsp70. Using monoclonal antibodies specific to the inducible form of Hsp70, we performed Western blotting of TCA precipitates from an experiment similar to Figure 1. As shown in Figure 2A (top panel), a band corresponding to p70 in Figure 1 did light up with antibodies to stress-inducible Hsp70 and was found to be induced with increasing time of recovery (lanes 2–6). It reached maximal levels between 6.5 and 8.5 hours during recovery. The untreated control, on the other hand, showed a very small amount of Hsp70 (Fig. 2A, top panel, lane 1). Probing the

blot with antibodies specific to constitutive Hsc70 showed that the constitutive homologue of Hsp70 did not change in this experiment (Fig 2A, bottom panel). A similar blot using a polyclonal antiserum to BiP showed that the p80 is BiP (not shown). For all the subsequent experiments, we have focused mainly on the inducible form of Hsp70.

We monitored the levels of ATP in LLC-PK1 cells at the end of 2 hours of AA treatment and during the recovery phase. As shown in Figure 2B, immediately after 2 hours of AA treatment, the levels of ATP dropped to about 5% of the control. At different times of recovery, there was a gradual increase in the ATP levels, with the maximum increment found between 2 and 4 hours of recovery. At the end of 8 hours of recovery, ATP levels reached about 60% of the control.

To further analyze the induction of Hsp70 during recovery, we performed immunoprecipitation experiments using antibodies specific to the inducible Hsp70. Six confluent dishes of LLC-PK1 cells were used. Three dishes were treated for 2 hours with 0.1 μ M AA as above, and the remaining 3 dishes were mock treated. At the end of 2 hours of ATP depletion, cells were recovered in com-

plete medium containing [^{35}S]-cysteine and -methionine to label proteins synthesized during recovery. Recovery and labeling were performed for 60, 120, and 240 minutes, after which the cells were lysed and the lysates were immunoprecipitated using antibodies to Hsp70 as described in the "Methods" section. An equal aliquot of the cell lysate from cells labeled for 120 minutes was incubated with protein A alone to see nonspecifically binding labeled proteins (Fig 3A, lane 7). A small aliquot of each lysate was used for scintillation counting to measure the radioactivity incorporated during recovery. The immunoprecipitates were analyzed by 7.5% SDS-PAGE and fluorography.

As shown in Figure 3A, newly synthesized Hsp70 was barely detectable in untreated controls (lanes 1–3), whereas there was steady increase in the amount of Hsp70 evident in cells recovering from ATP depletion (lanes 4–6). The protein A control did not show any labeled bands (lane 7). It is noteworthy that in untreated cells, despite the low levels of newly synthesized Hsp70, a number of labeled bands were found coprecipitating with it. This was most obvious in cells labeled for 240 minutes (lane 3). The coprecipitating labeled bands were missing in AA-treated cells (lane 6). This may be due to inhibition of general protein synthesis in AA-treated cells (see below). Although the levels of Hsp70 were significantly induced in ATP-depleted cells, the total radioactivity incorporated was much lower than in control cells, indicating that ATP depletion results in inhibition of protein synthesis (Fig 3D). Even after a recovery of 240 minutes, total incorporation of radioactivity in cells treated with AA was only about 50% of controls. To compensate for the difference in counts per minute incorporated in control and AA-treated cells, levels of Hsp70 were normalized to total counts per minute incorporated under the 2 conditions. Since the recovery was initiated by adding fresh medium containing label, there was an increase in protein synthesis (total counts per minute incorporated) and increase in recovery times in both control and AA-treated cells.

We also examined whether other related members of the heat shock protein family, namely Hsp90 and Hop60, were induced as a result of ischemia-like stress to LLC-PK1 cells. In 2 independent experiments similar to the above, we used 6 confluent dishes of LLC-PK1 cells. Three of these dishes were treated with AA to mimic ischemia, whereas the other 3 served as controls. Cells were allowed to recover in complete medium containing [^{35}S]-labeled cysteine and methionine for 60, 90, and 120 minutes after which they were lysed in a buffer containing Triton X-100. The lysates were immunoprecipitated with antibodies to Hsp90 and Hop60. The immunoprecipitates were analyzed by 7.5% SDS-PAGE and fluorography.

As shown in Figure 3B, in Hsp90 immunoprecipitates, a major band corresponding to Hsp90 was seen in both control and ATP-depleted cells (in addition, a few non-specific bands were also seen, but they were also found in protein A controls [lane 7]). In contrast to the immunoprecipitates of Hsp70, shown in Figure 3A, the levels of Hsp90 were much higher in untreated cells (lanes 1–3) compared with cells treated with AA (lanes 4–6). Even after a recovery of 120 minutes, there was no significant increase in the levels of Hsp90. The results suggested that unlike Hsp70, Hsp90 is not induced during recovery at early time points examined in this experiment. A similar result was seen with Hop60 (Fig 3C). The 90-kDa protein coprecipitating in Hop60 immunoprecipitations was found to be Hsp90, as confirmed by blotting with antibodies to Hsp90 (data not shown). This was in agreement with reports from several other laboratories (Smith et al 1993; Frydman and Hohfield 1997; Johnson et al 1998). It was clear that, although the overall protein synthesis was turned down, a few specific proteins were induced in response to ATP depletion in renal proximal tubule cell line. These included cytosolic Hsp70 and BiP of the ER lumen.

Sedimentation properties of induced Hsp70

To examine the kinds of substrates the induced Hsp70 may be associated with, we studied its sedimentation on sucrose gradients. We chose to examine its sedimentation at a time when it was induced maximally, ie, at 4.5 hours of recovery (Fig 1A). Two different approaches were used: (1) newly synthesized proteins, including Hsp70, were labeled with radiolabeled amino acids in the last 2 hours of recovery and (2) total cellular levels of Hsp70 were detected by Western blotting using specific antibodies.

For the labeling experiment, 2 confluent dishes of LLC-PK1 cells were used. One dish was treated with AA as above, and the other dish was used as an untreated control. After 2 hours of AA treatment, both dishes were incubated for an additional 2.5 hours in complete medium for recovery followed by labeling for 2 hours with [^{35}S]-methionine and -cysteine to label newly synthesized proteins. At the end of the labeling, cells were lysed in buffer containing Triton X-100, and the cell lysates were sedimented on 5–25% sucrose gradients at $200\,000 \times g$ for 15 hours. The gradients were fractionated from the top, and the fractions were precipitated with TCA as described in the "Methods" section. The precipitates were analyzed by 7.5% SDS-PAGE and fluorography to view the radiolabeled proteins.

The top panel of Figure 4A shows the sedimentation profile of radiolabeled proteins from control cells, whereas the bottom panel shows the same in AA-treated and

Fig 3. Hsp70 is induced early during recovery from ATP depletion. (A) Immunoprecipitation of induced Hsp70 from cells recovering from ATP depletion. LLC-PK1 cells were treated with AA for 2 hours and allowed to recover for various lengths of time (lanes 3–6) in the presence of radioactive cysteine and methionine. Cells treated with PBS served as controls (lanes 1–3). The labeling was stopped in cold PBS, and the cells were lysed with Triton X-100-containing buffer. The cell lysates were immunoprecipitated with antibodies to Hsp70 and analyzed by SDS-PAGE and fluorography as described in the “Methods” section. An aliquot of 120-minute, labeled lysate was incubated with protein A alone to serve as a nonspecific control. In 2 similar but independent experiments, after 2 hours of AA treatment, the recovery was done for 60, 90, or 120 minutes, and the cell lysates were immunoprecipitated with antibodies to Hsp90 (B) or Hop60 (C). The precipitates were

Fig 4. Sedimentation analysis of induced Hsp70. (A) Sedimentation profile of proteins synthesized during recovery from ATP depletion. Cells were subjected to ATP depletion for 2 hours and allowed to recover for 4.5 hours during which time they were metabolically labeled for the last 2 hours of recovery. The cells were lysed in the presence of a detergent, and sedimentation analysis of the cell lysate was performed as described in the "Methods" section. The lanes labeled total show the TCA precipitate of total cell lysates (fractions 15–22 did not show any radiolabeled bands). Sedimentation markers used are bovine serum albumin (4S_{20,w}), immunoglobulin G (7S_{20,w}), and catalase (11S_{20,w}). The arrowhead indicates bands corresponding to Hsp70 in both the panels. (B) In a similar but independent experiment, TCA precipitates from various fractions obtained as in A were immunoblotted with antibodies specific to Hsp70 (upper panel) and Hsc70 (lower panel). (C) Sedimentation profile of proteins synthesized during recovery from ATP depletion was determined as in A from detergent-free hypotonic lysates (see the "Methods" section). The arrowhead indicates the band corresponding to Hsp70, which was found induced in cells recovered from ATP-depleted cells. (D) To confirm the sedimentation of Hsp70 in the experiment described in C, immunoblot analysis of fractions from an experiment similar to C was performed using antibodies specific to Hsp70.

recovered cells. Most radiolabeled proteins in both the samples were confined mainly from the 4S_{20,w} to about the 10S_{20,w} region of the gradient (see markers). The overall profile of proteins in these fractions was similar except that specific bands were represented at higher levels in cells recovering from AA. As in Figure 1, these correspond to Hsp70 (see arrowhead) and BiP.

In a similar experiment, cells treated with AA were allowed to recover continuously for 4.5 hours without any labeling. After this, the cells were lysed, and the lysates from both untreated and AA-treated cells were sedimented on sucrose gradients as above. The fractions from

the gradient were TCA precipitated. The precipitates were analyzed by SDS-PAGE and Western blotting, using antibodies to inducible Hsp70 and constitutive Hsc70. As shown in the top panel of Figure 4B, although the total amount of Hsp70 in AA-treated cells was higher, its sedimentation pattern was similar to that in untreated cells. It was found to sediment from the 4S_{20,w} to about the 8S_{20,w} region of the gradient. Hsc70, on the other hand, did not show a significant qualitative or quantitative difference between the AA-treated and untreated cells (Fig 4B, bottom panel).

To rule out the possibility that detergent lysis was dis-

Fig 3. Continued. analyzed as in A. The bottom panels show quantitation of Hsp70, Hsp90, or Hop60 bands in each experiment. (D) The amount of [³⁵S]-methionine and -cysteine incorporated was determined as described in the "Methods" section in cells treated with AA for 2 hours and allowed to recover for the indicated times in medium containing radiolabeled amino acids as in A, B, and C. Cells treated with PBS for the same duration served as controls.

rupting complexes of induced Hsp70 with its substrates, we repeated the sedimentation analysis with detergent-free, hypotonic lysates. As shown in Figure 4C, labeled band corresponding to induced Hsp70 was still present in complexes of 4 to 8S, similar to that in detergent lysates. Some other labeled bands, including BiP, were missing in the hypotonic lysate, because they fractionate in the membrane pellet on hypotonic lysis. As shown in Figure 4D, the presence of Hsp70 was confirmed by Western blotting using monoclonal antibodies specific to Hsp70.

The results indicated that at a time of its maximal induction Hsp70 was found in small, soluble complexes that ranged in sedimentation coefficient from 4S_{20,w} to about 8S_{20,w}. It must be emphasized that in this experiment we were looking only at the detergent soluble form of Hsp70. Based on this experiment, we could not rule out the possibility that a fraction of Hsp70 may become detergent insoluble during recovery from ATP depletion. We have separately examined this possibility (see below).

Detergent solubility of heat shock proteins

Following heat shock, both induced Hsp70 and constitutive Hsc70 are known to translocate into the nucleus, localizing specifically in the nucleoli. To examine the distribution of these proteins after ATP depletion, we adopted 2 different approaches. We determined the distribution of Hsp70 and Hsc70 in the detergent-soluble and insoluble fractions following ATP depletion. Two time points were examined after ATP depletion: (1) at the end of 4 hours of AA treatment and (2) after 4 hours of recovery from AA (when Hsp70 is found significantly induced).

Three dishes of LLC-PK1 cells were used. Two dishes were treated with AA for 4 hours, and the third dish was used as an untreated control. One of the 2 AA-treated dishes was allowed to recover in the absence of the drug for 4 hours. All 3 dishes were lysed in Triton X-100-containing buffer and centrifuged to obtain a detergent-soluble and an insoluble fraction (see "Methods" section). The fractions were divided into 2 and analyzed for the presence of Hsp70 and Hsc70 by Western blotting.

As shown in Figure 5A, the small amount of Hsp70 found in control cells was mostly present in the detergent-soluble form (top panel). After 4 hours of AA treatment, there was little change in the distribution of Hsp70 (middle panel). After recovery from AA for 4 hours, the total amount of Hsp70 increased significantly, but most of it (80%) was found in the detergent-soluble fraction (bottom panel). Hsc70 showed somewhat different behavior in terms of its detergent solubility after AA treatment (Fig 5B). Although in control cells most Hsc70 was found in the detergent-soluble form (~80%) (Fig 5B, top panel), after 4 hours of AA treatment, a significant

amount moved into the detergent-insoluble fraction (about 50%, middle panel). During recovery, however, there was a reduction in insoluble Hsc70, and it was found predominantly in the detergent-soluble state (bottom panel).

The results indicated that although Hsp70 was mostly found in the detergent-soluble state after ATP depletion, and during recovery, a significant amount of Hsc70 was detergent insoluble after AA treatment and became detergent soluble during the recovery phase. Detergent insolubility may indicate either the presence of Hsc70 in the nuclear fraction or its association with cytoskeletal proteins. To distinguish between the 2 possibilities, we examined the intracellular localization of Hsp70 and Hsc70 by indirect immunofluorescence experiment.

Localization of heat shock proteins

To determine the localization of induced Hsp70, we treated LLC-PK1 cells with AA and allowed recovery as in Figure 5. Four sets of coverslips were given the following different treatments: (1) no treatment, (2) 2 hours of AA treatment, (3) 2 hours of AA treatment followed by 4 hours of recovery, and (4) heat shock at 42°C for 2 hours. After these treatments, inducible Hsp70 (TRITC) and Hsc70 (FITC) were visualized by indirect immunofluorescence as described in the "Methods" section. As shown in Figure 6A, in untreated cells and cells treated with AA for 2 hours, there was no detectable staining corresponding to Hsp70. This was expected, since the levels of Hsp70 under these conditions are very low, and Hsp70 induction is most obvious only during the recovery phase. Accordingly, at 4 hours of recovery, a distinct signal corresponding to induced Hsp70 was detected (Fig 6A, AA→4h recovery). The staining was seen throughout the cell most predominantly found in the cytoplasm and was absent from the nucleoli. Being constitutive in nature, Hsc70 was detected even in untreated cells and was uniformly distributed. After 2 hours of AA treatment, Hsc70 was found to translocate in the nucleus but was excluded from the nucleoli (Fig 6B, AA). Further, after 4 hours of recovery, it was predominantly cytoplasmic (Fig 6B, AA→4h recovery). Unlike AA treatment, in cells that received heat shock, both Hsp70 and Hsc70 were localized specifically in the nucleoli (Fig 6 A, B, HS). The distribution of Hsp70 and Hsc70 following ATP depletion was in agreement with the detergent solubility data shown in Figure 5. Under unperturbed conditions and following recovery from AA treatment, the cytoplasmic distribution of both Hsc70 and induced Hsp70 correlated with their detergent-soluble state.

Unlike heat shock, the induction of Hsp70 following ATP depletion is most obvious only during the recovery phase. The low basal amount of Hsp70 found in unper-

Fig 5. Detergent solubility of Hsp70 and Hsc70 in response to ATP depletion and recovery. Cells were subjected to AA treatment for 4 hours after which they were allowed to recover for 0 hours (+AA) or 4 hours (+AA + recovery). Cells treated with PBS for the same duration served as controls (control). Following these treatments, the cells were lysed in Triton X-100-containing buffer. The detergent-soluble lysate (soluble fraction) was TCA precipitated and was subjected to SDS-PAGE and immunoblot analysis with antibodies to Hsp70 (A) and Hsc70 (B). The detergent-insoluble pellet (insoluble fraction) was solubilized in Laemmli sample buffer and used for immunoblot. The bottom panels show quantitation of the bands in each fraction.

turbed cells is undetectable by immunofluorescence. To examine the distribution of Hsp70 during the period of ATP depletion, we, therefore, preinduced Hsp70 by giving a heat shock as described in the "Methods" section. Cells were allowed to recover from heat shock for 8 hours to allow full recovery of its effects on cell morphology before further treatment (Welch and Suhan 1986). Two sets of coverslips were used, and each set was given 4 different treatments as follows: (1) no treatment, (2) 4 hours of AA treatment, (3) 4 hours of AA treatment followed by 4 hours of recovery, and (4) heat shock at 42°C for 2 hours. After these treatments, Hsp70 (TRITC) and

Hsc70 (FITC) were visualized by indirect immunofluorescence as described in the "Methods" section.

In AA-untreated cells, both Hsp70 (Fig 6C, control, red) and Hsc70 (Fig 6D, control, green) were found mainly in the cytoplasm. After 4 hours of AA treatment, both Hsp70 and Hsc70 were found to translocate in the nucleus. Nuclear translocation of both Hsp70 and Hsc70 after heat shock is well documented (Welch and Suhan 1986; Brown et al 1993; Morcillo et al 1997; Fig 6 C, D, bottom panels). Interestingly, the intranuclear distribution of Hsp70 and Hsc70 seen after AA treatment was different from that after heat shock. Although after heat shock

Fig 6. Localization of Hsp70 and Hsc70 following ATP depletion in LLC-PK1 cells. (A) LLC-PK1 cells were treated with AA for 2 hours. A separate set of coverslips treated with PBS alone served as controls. Cells were then washed and allowed to recover for 0 hours (AA) or 4 hours (AA→4h recovery). Another set of coverslips underwent a heat shock (42°C) for 2 hours (HS). Indirect immunofluorescence was then performed as described in the "Methods" section to localize Hsp70. (B) LLC-PK1 cells were subjected to all the treatments as in A. Cells were then processed for indirect immunofluorescence to localize Hsc70. (C) LLC-PK1 cells were given a heat shock (42°C) for 90 minutes and allowed to recover for 8 hours to preinduce Hsp70. Cells were then treated with AA for 4 hours, and a separate set of coverslips treated with PBS alone served as controls. Cells were washed and allowed to recover for 0 hours (AA) or 4 hours (AA→4h recovery). Another set of coverslips underwent a second heat shock (42°C) for 2 hours (HS). Indirect immunofluorescence was then performed as described in the "Methods" section to localize Hsp70. (D) An experiment similar to that described in C was performed, and Hsc70 was visualized using indirect immunofluorescence. (E) Hsp70 was preinduced in LLC-PK1 cells as described in C. Cells were then given a second heat shock (42°C) for 2 hours either in the absence (top panel, HS) or presence (bottom panel, HS + AA) of AA. Cells were fixed, and Hsp70 was visualized by indirect immunofluorescence.

Hsp70 and Hsc70 were distinctly localized within the nucleoli, they were completely excluded from the nucleoli in AA-treated cells (Fig 6 C, D, AA and HS panels). During recovery from AA treatment, both Hsp70 and Hsc70 were found in the cytoplasm, with a residual signal seen in the nucleoplasm (Fig 6 C, D, AA→4h recovery).

The use of a prior heat shock to preinduce heat shock proteins to detectable levels has been used previously (Welch and Suhan 1986; Brown et al 1993; Kabakov and Gabai 1995; Wang and Borkan 1996). We find that the recovery of 8 hours from the first heat shock allows full reversal of its effects on cell morphology and the distribution of heat shock proteins. Furthermore, a subsequent heat shock (as shown in Fig 6 C, D, HS panels) induces translocation of cytosolic Hsp70 and Hsc70 into the nucleus as expected of a naive, untreated cell, validating our approach of using a prior heat shock.

To further study the differences in the intranuclear localization of the Hsp70 under AA treatment or heat shock, we examined the effect of heat shock on Hsp70 localization in the presence or absence of AA. Cells were

given a prior heat shock to preinduce Hsp70 as in Figure 6C. Cells were then given a heat shock at 42°C for 2 hours either in the presence or absence of AA (Fig 6E). As seen in Fig 6E (HS), heat shock alone resulted in localization of Hsp70 specifically in the nucleoli. However, when heat shock was combined with AA treatment, Hsp70 was retained in the nucleoplasm and was precluded from the nucleoli (Fig 6E, HS + AA). The results suggest that translocation of Hsp70 from the nucleoplasm to the nucleoli may be ATP dependent. Alternatively, the differences in the localization of Hsp70 under AA treatment or heat shock may reflect different sites of damage under these 2 different stress conditions.

DISCUSSION

A widely accepted cell culture model of renal ischemia was used to characterize heat shock proteins induced during the recovery phase of ischemic stress. To simulate conditions of transient ischemic shock in kidneys, we depleted cellular ATP from a pig kidney cell line (LLC-PK1),

using an inhibitor of mitochondrial oxidative phosphorylation, AA. The inhibitor was used at a sublethal concentration that dropped the cellular ATP levels to those reported in ischemic kidneys of animal models (Stromski et al 1986; Canfield et al 1991).

Our results indicated that cytosolic Hsp70 is the predominant heat shock protein induced early during recovery from ischemia-like stress. It reached maximal induction at 4.5 hours of recovery. A homologue of Hsp70 found in the ER lumen BiP was also induced, albeit somewhat slower. BiP showed maximum induction at 6.5 hours of recovery. As expected, the constitutive homologue of Hsp70 (Hsc70) was not induced, and unlike heat shock, other members of the heat shock protein family, such as Hsp90, GRP94, and Hop60, were also not induced under recovery times we examined. The synthesis of these proteins was in fact repressed, as also seen for most other cellular proteins. This was probably due to the inhibition of protein synthesis as a result of ATP depletion (Iwata et al 1995).

Coimmunoprecipitation experiments after metabolic labeling showed several newly synthesized proteins associated with Hsp70 under normal, unperturbed conditions. This was in agreement with its role in the folding of newly synthesized proteins in the cytosol (Eggers et al 1997; Thulasiraman et al 1999). The levels of newly synthesized Hsp70 itself were very minimal under these conditions. In contrast, on ATP depletion, although the levels of Hsp70 increased severalfold, the coprecipitating proteins were significantly reduced. This was partly explained by the low levels of proteins being synthesized under these conditions. The result implied that the induced Hsp70 might have a role different from that of assisting folding of newly synthesized proteins during recovery from ischemia-like stress.

Sedimentation analysis showed that at a recovery time of 4.5 hours, when Hsp70 was induced maximally, Hsp70 was present in soluble complexes that ranged in size from $4S_{20,w}$ to $8S_{20,w}$. There was no indication of its association with aggregated proteins at this time of recovery. It is known that on ATP depletion proteins in the cytosol and the ER lumen undergo aggregation and remain bound to ATP-dependent chaperones (Braakman et al 1992; Tatu et al 1993; Nguyen and Bensaude 1994; Stege et al 1995; Kuznetsov et al 1996; Glover and Lindquist 1998; Netzer and Hartl 1998). Unlike these studies, our focus was mainly on the recovery phase of ATP depletion, when active cellular restructuring is in progress and Hsp70 is found maximally induced. Clearly, the induced Hsp70 was not associated with aggregated proteins under these conditions.

The sedimentation profile of induced Hsp70 remained unchanged in detergent-free, hypotonic lysate, ruling out the possibility of disruption of aggregates by the deter-

gent. The sedimentation coefficient of $4S_{20,w}$ to $8S_{20,w}$ indicated that the induced Hsp70 is either monomeric ($\sim 4S_{20,w}$) or associated with proteins in the range of 70 kDa to 80 kDa (giving a complex of $\sim 8S_{20,w}$). We cannot also rule out the possibility that (1) it was self-associated to form homodimers or (2) it was associated with a small amount of lipid or fatty acids as suggested previously by Guidon and Hightower (1986). The constitutive homologue, Hsc70, was found in detergent-insoluble complexes under these conditions.

Earlier studies on renal ischemia using experimental animals have shown induction of Hsp70 during reperfusion of ischemic kidney (Emami et al 1991; Van Why et al 1992). Because the induction of Hsp70 coincides with the recovery from ischemic injury, it was proposed that induced Hsp70 may actively participate in cellular restructuring during the recovery phase (Van Why et al 1992). It was also suggested that Hsp70 may be associated with mislocalized membrane proteins, such as Na^+/K^+ -adenosine triphosphatase during recovery (Aufrecht et al 1998). The presence of Hsp70 in complexes of 4 to $8S_{20,w}$ during recovery, as shown in this study, is in agreement with these possibilities.

It is known that Hsp70 induced in response to heat shock is translocated to nucleus, localizing specifically within the nucleoli (Welch and Suhan 1986; Brown et al 1993; Morcillo et al 1997). Nucleoli are known to be very sensitive to thermal damage (Pelham 1984), and it is believed that Hsp70 serves the function of protecting the ribosomal RNA synthesis machinery here during stress. Under these conditions, the constitutive homologue, Hsc70, is known to colocalize with Hsp70 in the nucleolus. Unlike heat shock, the induction of Hsp70 following ATP depletion is detectable only during the recovery phase, and under this condition, the induced Hsp70 was found predominantly in the cytoplasm. To examine the distribution of Hsp70 during ATP depletion, we had to preinduce Hsp70 by giving a heat shock. The preinduced Hsp70 and the constitutive Hsc70 both localized in the nuclei during ATP depletion. Most remarkably, in contrast to heat shock, they both were excluded from the nucleoli.

The results suggest that nucleolar translocation of Hsp70s after heat shock may be ATP dependent. Alternatively, it cannot be ruled out that there are different targets for heat damage and damage due to ATP depletion, and consequently Hsp70s localize to these different sites of damage. More experiments are required to clarify this issue. It is noteworthy that Roti Roti et al (1998) have shown that in thermotolerant cancer cells (NSY 42129) Hsp70 induced in response to heat shock translocates in the nucleus but is excluded from the nucleoli. This is similar to the distribution of Hsp70 seen following ATP depletion as shown in this study. It is likely that in thermotolerant NSY 42129 cells the nucleolar machinery is

heat resistant, and consequently Hsp70 is diverted to other, more susceptible sites of heat damage, namely, the nucleoplasm. The observation is in line with the possibility that induced Hsp70 is recruited to different sites of damage under different stress conditions.

In addition to providing clues about the role of stress proteins in renal ischemia, the results of this study also highlight important differences in their behavior from the most well-studied model of stress, the heat shock. Several differences were observed. In case of heat shock, the induction of heat shock proteins reaches maximal levels during the conditions of stress, whereas maximum induction of heat shock proteins in response to ATP depletion was found only during the recovery phase, after the stress was removed. Further, unlike heat shock, which results in the induction of a battery of different heat shock proteins during the shock, ischemia-like stress elaborated only Hsp70 homologues at early times of recovery. Nuclear localization of Hsp70s was also different in these 2 conditions. Using biochemical fractionation, Vincent and Tanguay (1982) have reported different localization of Hsp70 under different stress conditions. These differences in the kinds of proteins induced, their induction kinetics, and their intracellular localization may reflect the different roles played by heat shock proteins under different stress conditions.

There are a host of clinical conditions under which heat shock proteins have been shown to be induced. Examples include rheumatoid arthritis, systemic lupus erythematosus, and ischemic injury (Dhillon et al 1993; Schett et al 1998). Our results emphasize the need for detailed characterization of such physiological perturbations that result in the induction of stress proteins. This will not only help in exploring their potential as new-generation therapeutics but also increase our understanding of the diverse roles that they are capable of performing.

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