Expression of the Constitutive and Inducible Forms of Heat Shock Protein 70 in Human Proximal Tubule Cells Exposed to Heat, Sodium Arsenite, and CdCl$_2$

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We determined the expression of the constitutive (hsc 70) and inducible (hsp 70) forms of heat shock protein 70 mRNA and protein in human proximal tubule (HPT) cells exposed to lethal and sublethal concentrations of Cd^{+2} under both acute and extended conditions of exposure. The HPT cells exhibited the classic heat shock response when subjected to a physical (heat) or chemical stress (sodium arsenite); hsc 70 mRNA and protein levels were constant or slightly increased, whereas hsp 70 mRNA and protein were greatly elevated. Acute exposure to 53.4 μM CdCl$_2$ for 4 hr failed to increase either hsc 70 mRNA or protein, a finding similar to that observed under classic conditions of stress. However, under identical conditions of acute exposure to Cd^{2+}, the expected increase in hsp 70 protein level was suppressed as compared to that found under classic conditions of physical or chemical stress. The decrease in hsp 70 protein level correlated to the reduced expression of mRNA from the *hsp 70B* gene. The expression of mRNA from the *hsp 70A* and *hsp 70C* genes was similar to that found when the cells were treated with heat shock or sodium arsenite. We modeled an extended exposure to Cd^{2+} by treating the cells continuously with Cd^{2+} at both lethal and sublethal levels over a 16-day time course. Chronic exposure to Cd^{2+} failed to increase either hsc 70 mRNA or protein levels in the HPT cells at a nonlethal dosage level and decreased hsc 70 mRNA and protein levels late in the time course of lethal exposure. Under identical conditions, the expression of hsp 70 protein remained at basal levels that were only marginally detectable throughout the time course. Hsp 70A and hsp 70C mRNA levels were unaltered by extended exposure to Cd^{2+}, and hsp 70B mRNA was not detected during the 16-day time course. Cd^{2+} is a poor inducer of hsc 70 and hsp 70 in the proximal tubule under both acute and long-term exposure. These results reinforce the fact that the expression of hsp 70 protein does not result from the transcription of a single gene, but is derived from what may be a complex interplay of several underlying genes. *Key words*: cadmium, gene expression, heat shock, heavy metals, hsc 70, hsp 70, proximal tubule, sodium arsenite. *Environ Health Perspect* 107:887–893 (1999). [Online 12 October 1999]
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The heat shock response (stress response) is widely recognized and accepted as a major weapon in the cell's armamentarium for protection against and recovery from environmental insult, both physical and chemical [for review (*1–4*)]. The most obvious feature of the heat shock response consists of alterations in gene expression leading to increased synthesis of heat shock proteins (hsps) and a cessation of other cellular protein synthesis when cells are exposed to stress. The hsps are a large superfamily of proteins with molecular weights ranging from 8 to 170 kD; members are referred to as hsp 27, hsp 60, hsc 70 (constitutive form), hsp 70 (inducible form), and hsp 90, the proteins classically identified to be induced as a result of heat treatment of mammalian cells.

The current study was undertaken for two reasons. First, a goal of this laboratory is to define the role of the stress response in mediating toxicity that the heavy metal pollutant cadmium elicits on the human proximal tubule (HPT) cell. The working hypothesis is that identification of members of the stress response superfamily that do not respond to Cd^{2+} exposure by induction of the corresponding protein might identify cellular responsibilities particularly susceptible to Cd^{2+}-induced damage. The first test of this hypothesis involved determining the expression of metallothionein (MT), a protein thought to mediate heavy metal toxicity by metal sequestration in cultures of HPT cells exposed to both lethal and sublethal concentrations of Cd^{2+} (*5–7*). Garrett et al. (*5,6*) and Hoey et al. (*7*) demonstrated that the HPT cells responded to both lethal and sublethal exposures to Cd^{2+} with a large, rapid, and sustained increase in the level of MT protein, providing strong evidence that the MT stress response was active and not compromised in these cells.

A second test of this hypothesis involved the determination of hsp 27 in HPT cells identically exposed to Cd^{2+} (*8*). In this instance, the results were equivocal because hsp 27 was increased early in the time course of Cd^{2+} exposure, but returned rapidly to values below control despite continued presence of the metal. The finding that hsp 27 expression was reduced in HPT cells chronically exposed to Cd^{2+} suggests that a consequence of chronic Cd^{2+} exposure might involve a loss in the maintenance of microfilament dynamics. This is based on many studies which show that hsp 27 exerts its protective effects on the cell, at least in part, through a chaperone action that stabilizes microfilament dynamics (*9–11*). Studies in the rodent model have shown that enhanced expression of hsp 27 correlates to the protection of the proximal tubule from brief periods of ischemia and the corresponding disruption of the actin filament network (*12–14*).

The present study examines the expression of the hsp 70 family in HPT cells exposed to Cd^{2+} in a manner identical to that described above for MT and hsp 27 (*5,6,8*). The goals of this study are to determine if the classic induction of hsp 70 that occurs ubiquitously for mammalian cells exposed to heat shock also occurs when HPT cells are exposed to sodium arsenite and Cd^{2+}, and if the *hsp 70* isoform genes have distinct patterns of expression when exposed to these agents. The hsp 70 chaperones are the most studied heat shock proteins and, with their co-chaperones, comprise a set of abundant cellular machines that assist a large variety of protein-folding processes in almost all cellular compartments (*15*). Under conditions of stress, they have been shown to prevent aggregation of denatured proteins and assist in refolding of misfolded proteins. Under normal conditions, heat shock proteins serve a number of essential roles: they assist in folding of selected newly translated proteins; they assist in the translocation of proteins across organellar membranes; they disassemble oligomeric protein structures; they facilitate proteolytic degradation of unstable proteins; and they control the biologic activity of folded regulatory proteins (*15*).

Materials and Methods

Cell culture: Stock cultures of HPT cells used in experimental protocols were grown in 75-cm^2 T-flasks (Corning, Corning, NY) using procedures previously described by this laboratory (*16,17*). The growth medium was a serum-free formulation consisting of a 1:1 mixture of Dulbecco's modified Eagles' medium (DME) and Ham's F-12 growth medium supplemented with selenium (5 ng/mL), insulin (5 μg/mL), transferrin (5 μg/mL),

hydrocortisone (36 ng/mL), triiodothyronine (4 pg/mL), and epidermal growth factor (10 ng/mL). The growth surface was treated with a collagen matrix to promote cell attachment and subculture. The cells were fed fresh growth medium every 3 days and, at confluence (normally 3–6 days after subculture), were subcultured using trypsin-EDTA (0.05% trypsin, 0.02% EDTA). For use in experimental protocols, the cells were subcultured at a 1:2 ratio and allowed to reach confluence (6 days after subculture) before initiation of experimental protocols. The cells were fed every 3 days. Three isolates of HPT cells were used; these isolates were derived from normal cortical tissue obtained from kidneys removed for renal cell carcinoma. The kidneys were from a 72-year-old female, a 63-year-old male, and a 58-year-old female. We used HPT cells between passages 5 and 7 in the present study. The total protein and RNA samples used in the present analysis of hsc 70 and hsp 70 expression were obtained from a previous study that examined hsp 27 expression in HPT cells exposed to heat shock, sodium arsenite, and lethal and sublethal concentrations of $CdCl_2$ (8).

The effect of chemical stress on the expression of hsc 70 and hsp 70 was determined by exposing confluent HPT cells from three independent cell isolates to 100 µM sodium arsenite for 4 hr, followed by a recovery period of 48 hr.

We determined the effect of heat shock on HPT cells by exposing the cells to heat shock (42.5°C) for 1 hr. The cells were then returned to 37°C for a 48 hr recovery period.

The effect of acute Cd^{2+} exposure on the expression of hsc 70 and hsp 70 in HPT cells was determined by exposing confluent cell monolayers to 53.4 µM Cd^{2+} for 4 hr; this was followed by a recovery period of 48 hr in Cd^{2+}-free growth media.

We determined the expression of hsc 70 and hsp 70 mRNA and protein after exposing the HPT cells to Cd^{2+} for an extended time. We used three concentrations of Cd^{2+}: 9.0 µM, which produces no cell death over the 16-day time course; 27.0 µM, which produces cell death late in the 16-day time course; and 45.0 µM, which produces cell death early in the 16-day time course (5).

Cell viability, isolation of total RNA, and RT-PCR. The effects of various treatments on the viability of confluent cell monolayers were determined by the automated counting of 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei of cells (8). Total RNA was isolated according to the protocol supplied with TRI REAGENT (Molecular Research Center Inc., Cincinnati, OH) as described previously (5). We determined the concentration and purity of samples using spectrophotometer scan in the ultra violet (UV) region and ethidium bromide visualization of intact 18S and 28S RNA bands following agarose gel electrophoresis.

For reverse transcriptase-polymerase chain reaction (RT-PCR), 500 ng total RNA from cultured HPT cells was reverse transcribed in a 20-µL reaction mixture using 50 units Murine Leukemia Virus Reverse Transcriptase (N808-0143; Perkin Elmer, Foster City, CA) in 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 5 mM $MgCl_2$, 20 units RNase inhibitor, 1 mM each of the dNTPs, and 2.5 µM random hexanucleotide primers. The samples were reverse transcribed for 20 min at 42°C, followed by a 5 min denaturation step at 99°C using a DNA thermocycler (Perkin Elmer 9600). The resulting cDNA was amplified in 100 µL reaction mixture containing 2 mM $MgCl_2$, 1X PCR buffer, 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer), and the specific upstream and downstream primers at a concentration of 0.15 µM each. For the amplification of hsc 70, primers were (upper and lower, respectively) 5′TGTGGCTTC-CTTCGTTATTGG3′ and 5′GCCAG-CATCATTCACCACCAT3′ (StressGen, STM-505, Victoria, British Columbia, Canada). For the amplification of hsp 70A, B, and C, primers were (upper and lower, respectively) 5′TGTTCCGTTTCCAGCC-CCCAA3′ and 5′GGGCTTGTCTCC-GTCGTTGAT3′ (STM-506) for 70A; 5′CTCCAGCATCCGACAAGAAGC3′ and 5′ACGGTGTTGTGGGGGTTC-AGG3′ (STM-507) for 70B; and 5′TTG-AGGAGGTGGATTAGGGGC3′ and 5′AGCCTTTGTAGTGTTTTCGCC3′ (STM-508) for 70C. Primers for the determination of glyceraldehyde 3-phosphate dehydrogenase (*g3pdh*) gene were obtained commercially (Clontech, Palo Alto, CA). The thermocycler was programmed to cycle at 95°C for a 2-min initial step, at 95°C for 30 sec, and at 50°C for 30 sec, with a final elongation step at 50°C for 7 min. Controls for each PCR included a no-template control in which water was added instead of RNA and a no-reverse transcriptase control in which water was added instead of the enzyme. Samples were removed at 30, 35, and 40 PCR cycles to ensure that the reaction remained in the linear region. The final PCR products were electrophoresed on 2% agarose gels containing ethidium bromide along with DNA markers.

For reactions in the linear region of the cycle, integrated optical densities (IOD) of the samples were obtained by input of the ethidium bromide fluorescent image into a Roche Pathology Workstation (AutoCyte, Burlington, NC) configured with KS400 software (Zeiss, Thornwood, NY) using a Kodak DCS 420 CCD camera (Kodak, Rochester, NY). The resulting IOD values were used to generate a relative IOD that is the ratio of the hsc 70 and hsp 70 reaction products to products for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase.

Western analysis. Cells were washed two times with phosphate-buffered saline (PBS) and lysed directly in the flask by addition of 400 µL hot (85°C) 1X SDS buffer [2% sodium dodecyl sulfate (SDS) and 50mM Tris-HCl, pH 6.8]. The cell lysate was heated in a boiling water bath for 10 min. DNA was sheared by repeated passage through a 23-gauge needle. The samples were centrifuged at 10,000*g* for 10 min at room temperature, and the supernatant was transferred to a new tube. The concentration of protein in the samples was determined by the BCA protein assay (Pierce Chemical Co., Rockford, IL). Total cellular protein (10 µg) was separated on 12% SDS-containing polyacrylamide gels and electrophoretically transferred to polyvinylidine difluoride membrane (Bio-Rad, Hercules, CA). Hsc 70 was visualized using primary rat monoclonal antibody (SPA-815; StressGen), whereas hsp 70 was visualized using primary mouse monoclonal antibody (SPA-810; StressGen). Membranes were blocked with 10% (w/v) nonfat dry milk in PBS and incubated with rat monoclonal antibody specific for hsc 70 diluted 1:2000 in PBS containing 0.1% (w/v) bovine serum albumin as carrier or mouse monoclonal antibody specific for hsp 70 diluted 1:2000 in PBS containing 5% (w/v) nonfat dry milk as carrier and 0.05% Tween-20. This was followed by incubation with a goat anti-rat or goat anti-mouse alkaline phosphatase conjugated secondary antibody (Promega, Madison, WI). Colorimetric detection employed an alkaline phosphatase Vectastain ABC-AP kit (Vector, Burlingame, CA). For color reactions in the linear range of analysis, IOD values of the samples were obtained by input of the image into a Roche Pathology Workstation configured with KS400 software using a Kodak DCS 420 CCD camera.

The IOD for an individual band is the sum of OD values × pixels^2. The relative IOD was obtained by dividing the IOD for each hsp 70 and hsc 70 band by the IOD for g3pdh at the appropriate time point.

Nomenclature. In this study, we define the term hsc 70 as the constitutive member(s) of the *hsp 70* gene family (18). The exact number of *hsc 70* genes present in the genome is not known because many processed hsc 70 pseudogenes are present, which complicates analysis. A genomic structure for one active *hsc 70* gene has been determined; this gene is characterized by the presence of eight introns (18). The hsc 70 protein has been purified and an antibody has been generated that does not cross-react

with the inducible forms of hsp 70. We used this commercially available antibody and primers for RT-PCR that span an intron/exon junction of hsc 70 in the present study to define the expression of hsc 70 mRNA and protein. In the present study we use the term hsp 70 to indicate the inducible members of the *hsp 70* gene family (*18*). The exact number of *hsp 70* genes present in the genome is also unknown because there are numerous active and pseudogene sequences, but commercially available RT-PCR primers can specifically determine the mRNA originating from three distinct *hsp 70* genes (*19*). The complete genomic structures of two of these genes have been determined; they are intron-less genes that encode an identical protein product of 641 amino acids and map in the major histocompatability complex class III region (*20*). Originally referred to as *HSP70-1* and *HSP70-2*, in this study we refer to them as *hsp 70A* and *hsp 70C*, respectively, according to commercial

nomenclature. A third *hsp 70* gene has been identified based on a partial sequence that maps to chromosome 1 whose mRNA can be determined by RT-PCR (*18*). We refer to this gene, originally designated *HSP70B*, as *hsp 70B*. While the primers developed for RT-PCR may also monitor the mRNA from other unidentified *hsp 70* genes, they are specific in that they individually distinguish the *hsp 70A*, *hsp 70B*, and *hsp 70C* isoforms from one another. The hsp 70 protein has been purified, and an antibody that does not cross-react with the constitutive hsc 70 has been generated and is commercially available.

Results

Hsc 70 and Hsp 70 expression in HPT cells exposed to sodium arsenite and heat shock.

We anaylzed the effect of chemical stress on the expression of hsc 70 and hsp 70 in confluent HPT cells from three independent cell isolates exposed to 100 μM sodium arsenite for 4 hr, followed by a recovery

period of 48 hr. The results for the expression of mRNA originating from the *hsc 70* and *hsp 70 A*, *B*, and *C* genes are presented as native gel data for one isolate (Figure 1A) and graphically as the mean relative IOD values for all three isolates when expression is compared to mRNA from the *g3pdh* housekeeping gene (Figure 1B). This analysis demonstrated that each mRNA species had a distinct pattern of expression under basal conditions and when the HPT cells were exposed to sodium arsenite. For hsc 70, a high level of basal mRNA was demonstrated that increased approximately 2-fold as a result of exposure to sodium arsenite. This elevation in hsc 70 mRNA occurred between 2 and 4 hr of treatment and returned to control values within 16 hr of recovery. The *hsp 70A* isoform was the only inducible isoform that had a detectable level of basal mRNA that was consistently present in control cells, and this was approximately 4-fold less than hsc 70 expression. The expression of hsp 70A mRNA increased
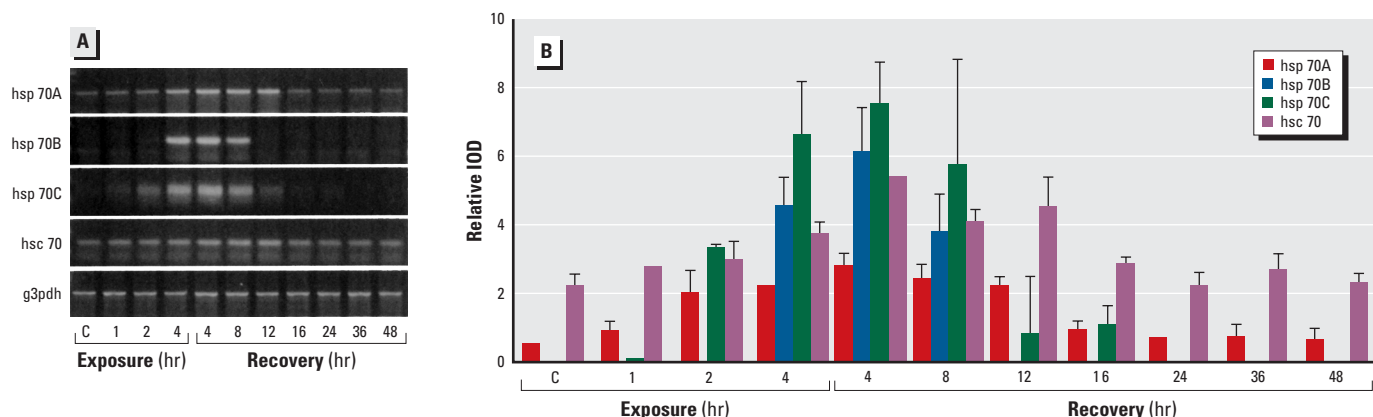
Figure 1. RT-PCR analysis of mRNA from HPT cells exposed to 100 μM sodium arsenite for 4 hr, followed by a 48-hr recovery period. Abbreviations: C, control; g3pdh, glyceraldehyde 3-phosphate dehydrogenase; hsc, constitutive form of hsp; hsp, heat shock protein; HPT, human proximal tubule; IOD, integrated optical density; PCR, polymerase chain reaction. (*A*) Ethidium bromide-stained agarose gels for PCR products representing hsp 70A, hsp 70B, hsp 70C, hsc 70, and g3pdh mRNA for one HPT cell isolate. (*B*) Mean (± standard error) relative IOD of bands representing hsp 70A, hsp 70B, hsp 70C, and hsc 70 mRNA for three HPT cell isolates. See "Materials and Methods" for further details.
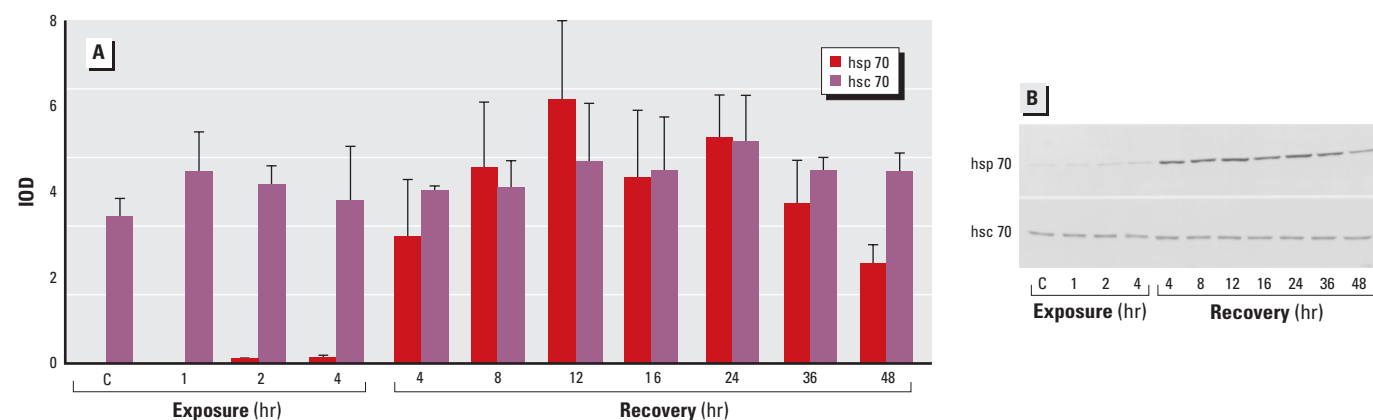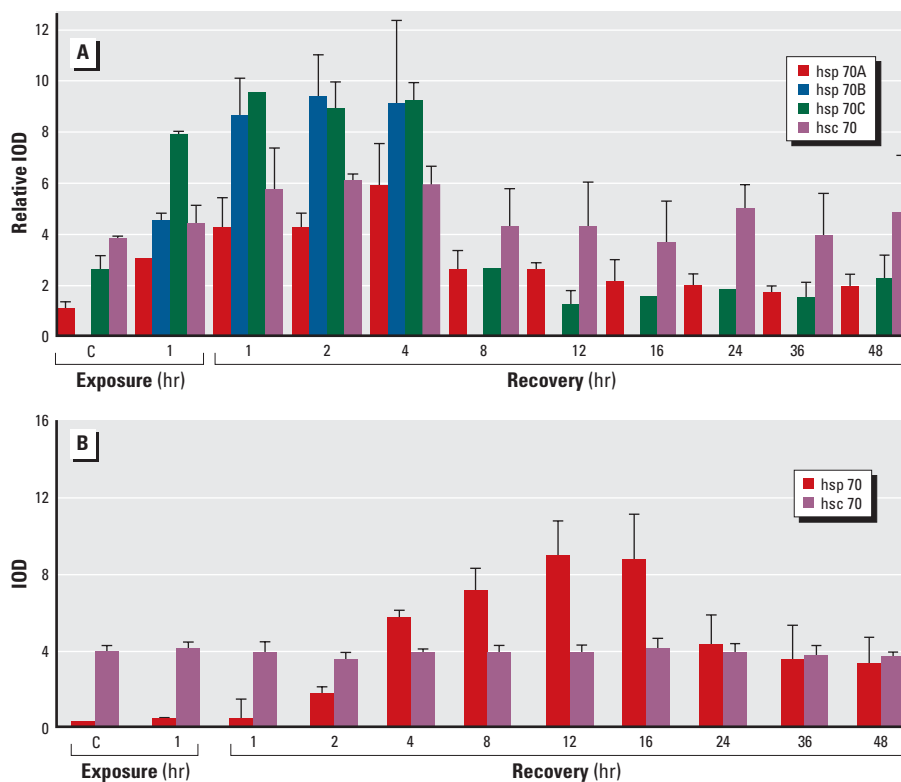

Figure 2. Western analysis of hsp 70 and hsc 70 protein in HPT cells following sodium arsenite exposure and recovery periods. Abbreviations: C, control; hsc, constitutive form of hsp; hsp, heat shock protein; HPT, human proximal tubule; IOD, integrated optical density. (*A*) Mean (± standard error) IOD values for bands representing hsp 70 and hsc 70 protein for three HPT cell isolates. (*B*) Western blots for hsp 70 and hsc 70 protein from a single isolate.

approximately 5-fold as a result of sodium arsenite treatment, with accumulation beginning 2 hr after exposure, peaking at 4 hr of exposure and 4 hr after exposure, and returning to control values by 16 hr after exposure (recovery period). No basal expression of hsp 70B could be detected in total RNA from HPT cells when 500 ng RNA and 40 cycles were used. However, levels of hsp 70B mRNA were detected after 4 hr of sodium arsenite treatment; these levels exceeded those of both hsc 70 and hsp 70A mRNA. The mRNA for hsp 70B remained elevated at 4 and 8 hr after exposure, thereafter falling to undetectable levels. The basal level of expression of hsp 70C mRNA was highly variable from experiment to experiment in the control HPT cells. For the sodium arsenite studies, no basal expression of hsp 70C was noted; however, substantial amounts of hsp 70C mRNA accumulated as a result of sodium arsenite exposure, with peak levels exceeding or equaling those of the other isoforms at 4 hr of exposure and at 4 and 8 hr after exposure. Accumulation of hsp 70C mRNA was apparent after 2 hr of exposure to sodium arsenite and returned to undetectable levels by 24 hr of recovery. Assuming equal RT-PCR efficiencies and mRNA degradation rates, the following induction pattern can be proposed based on both absolute peak values and when a given nonbasally expressed mRNA species first appears following sodium arsenite exposure: hsc 70 < hsp 70A < hsp 70B ≤ hsp 70C.

The levels of hsc 70 and hsp 70 protein were determined by Western analysis of protein extracts prepared simultaneously with the total RNA samples analyzed above (Figure 2A, B). Western analysis disclosed an easily determined basal level of hsc 70 protein expression; however, this level did not increase during or after exposure of the HPT cells to sodium arsenite. This indicates that the 2-fold elevated levels of hsc 70 mRNA demonstrated above do not translate into increased levels of hsc 70 protein. Using Western analysis, we demonstrated that the basal level of hsp 70 protein was at the limit of detection, with convincing bands representing hsp 70 protein being visualized in approximately half the basal samples analyzed throughout the present study. During the 4 hr that the HPT cells were exposed to 100 μM sodium arsenite, hsp 70 protein remained at the limit of detection. However, within 4 hr of sodium arsenite removal, hsp 70 protein was easily detectable, and levels approximately equaled levels for hsc 70. The level of hsp 70 remained elevated for at least 48 hr after removal of sodium arsenite. The antibody used for the Western analysis does not distinguish between the *hsp 70* isoforms.

For heat-shocked HPT cells, the expression patterns of hsc 70 and hsp 70 mRNA and protein were very similar to those obtained for the sodium arsenite-treated cells (Figure 3A, B). Hsc 70 mRNA was increased only moderately by heat shock, with a return to control values after removal of heat stress (Figure 3A). Similar to sodium arsenite-treated cells, the increase in hsc 70 mRNA did not translate into an increase in hsc 70 protein (Figure 3B). The mRNA for the *hsp 70A*, *hsp 70B*, and *hsp 70C* isoforms increased rapidly after heat shock, peaked at 2–4 hr into the recovery period, and then returned to control values (Figure 3A). Identical to that for arsenite-exposed cells, the pattern of induction of mRNA was hsc 70 < hsp 70A < hsp 70B ≤ hsp 70C. A low basal expression of hsp 70 protein was demonstrated in control HPT cells. This basal level increased 1–2 hr after heat shock and reached a peak 12–16 hr into the recovery period, with peak values being approximately twice those attained by hsc 70 (Figure 3B). Thereafter, the expression of hsp 70 decreased but remained elevated over control at the end of the 48-hr recovery period.

Hsc 70 and hsp 70 expression in HPT cells after acute and chronic exposure to CdCl₂. We determined the effect of acute Cd^{2+} exposure on the expression of hsc 70 and hsp 70 in HPT cells by exposing confluent cell monolayers to 53.4 μM Cd^{2+} for 4 hr, followed by a recovery period of 48 hr in Cd^{2+}-free growth media. This represents a level of Cd^{2+} exposure previously shown to be lethal to 15–25% of the cells by the end of the recovery period (8). In contrast to the results obtained previously for HPT cells exposed to both heat and sodium arsenite, hsc 70 mRNA did not increase as a consequence of acute exposure to Cd^{2+} (Figure 4A). However, identical to that described previously for heat shock and sodium arsenite, hsc 70 protein levels were not altered as a consequence of acute Cd^{2+} exposure (Figure 4B). The expression patterns and levels of mRNA for the *hsp 70A* and *hsp 70C* isoforms were also very similar to those obtained when HPT cells were exposed to heat and chemical stress (Figure 4A). Hsp 70A and hsp 70C mRNA increased (3- to 5-fold) following exposure to Cd^{2+}, with a return to control values by the end of the recovery period. However,
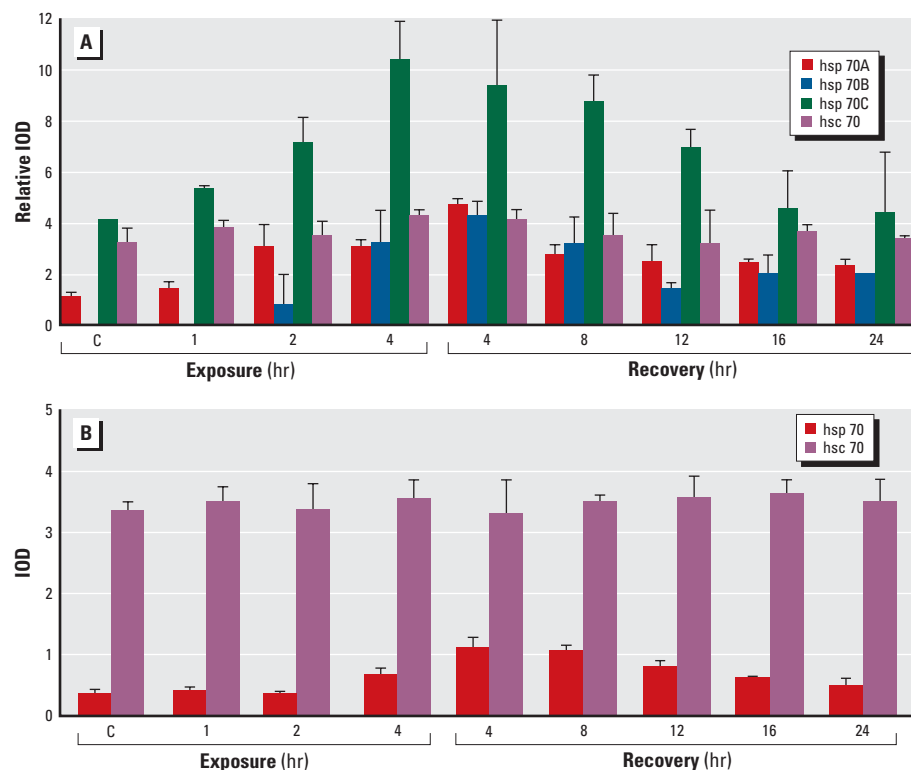

Figure 3. HPT cells exposed to heat shock (42.5°C) for 1 hr and then returned to 37°C for a 48-hr recovery period. Abbreviations: C, control; hsc, constitutive form of hsp; hsp, heat shock protein; HPT, human proximal tubule; IOD, integrated optical density; RT-PCR, reverse transcriptase-polymerase chain reaction; SE, standard error. (*A*) Mean (± SE) relative IOD of bands representing hsp 70A, hsp 70B, hsp 70C, and hsc 70 mRNA for three HPT cell isolates as determined by RT-PCR analysis of heat-shocked HPT cells during exposure and recovery periods. (*B*) Mean (± SE) IOD of bands representing hsp 70 and hsc 70 proteins at various time points for three HPT cell isolates as determined by Western analysis of heat-shocked HPT cells during exposure and recovery periods.

the expression of hsp 70B mRNA was very different from that obtained when HPT cells were exposed to heat or chemical stress (Figure 4A). Whereas hsp 70B mRNA levels increased to IOD values of 7–9 for heat- and arsenite-exposed HPT cells, they peaked at < 4 relative IOD units for the Cd^{2+}-exposed cells (compare Figure 1B, Figure 3A, and Figure 4A). The reduction in hsp 70B mRNA noted for Cd^{2+}-exposed cells is given increased validity by the fact that identical total RNA samples were used for the determination of the three respective *hsp 70* isoforms. Even more striking than the reduced induction of hsp 70B mRNA accumulation in the Cd^{2+}-exposed cells was the decreased accumulation of hsp 70 protein in the HPT cells acutely exposed to Cd^{2+} (Figure 4B). In contrast to heat-shocked or sodium arsenite-treated cells, hsp 70 protein was only marginally increased in the HPT cells by acute Cd^{2+} exposure (compare Figure 2A, Figure 3B, and Figure 4B). In cells acutely exposed to Cd^{2+}, hsp 70 protein reached a maximum of 1.5 IOD units, whereas sodium arsenite and heat shock induced maximum values of 6.5 and 13 units, respectively. Thus, for HPT cells
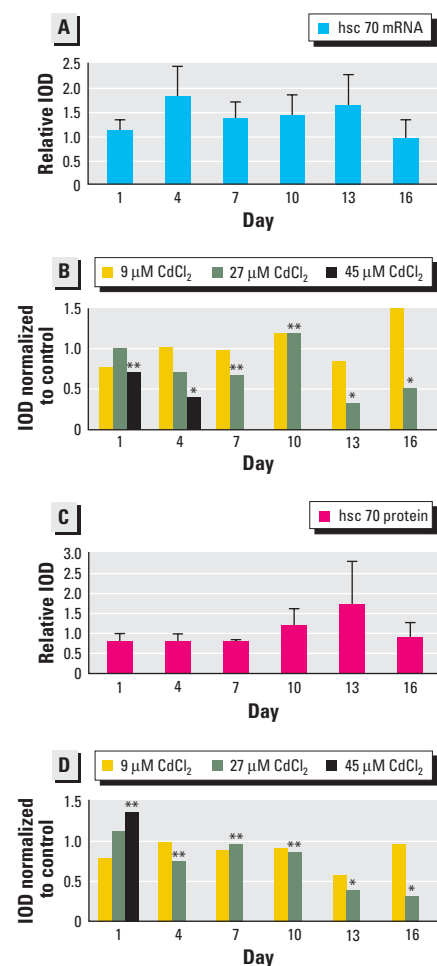
acutely exposed to Cd^{2+}, the expression of mRNA for the *hsc 70*, *hsp 70A*, and *hsp 70C* isoforms and hsc 70 protein was very similar to expression obtained for HPT cells exposed to heat shock and sodium arsenite. In contrast, the expression of hsp 70B mRNA and hsp 70 protein were greatly reduced when compared to levels attained during heat shock or sodium arsenite treatment. A concentration response study demonstrated that Cd^{2+} induced the accumulation of hsp 70 mRNA and protein, albeit at reduced levels, at concentrations as low as 9.2 µM (data not shown).

The expression of hsc 70 and hsp 70 mRNA and protein was also determined when the HPT cells were exposed to Cd^{2+} for an extended time, rather than the acute exposure classically used to define the stress response. Three concentrations of Cd^{2+} were utilized: 9.0 µM, which produces no cell death over the 16-day time course; 27.0 µM, which produces cell death late in the 16-day time course; and 45.0 µM, which produces cell death early in the 16-day time course (5). For hsc 70, the expression of both mRNA and protein in control HPT cells remained relatively constant over the

16-day time course (Figure 5A, C). For data presentation of the expression of hsc 70 in Cd^{2+}-exposed cells, the levels of mRNA and protein were normalized to the control, with the control being assigned a value of 1.0 at each day of the time course. Exposure of the HPT cells to sublethal concentrations
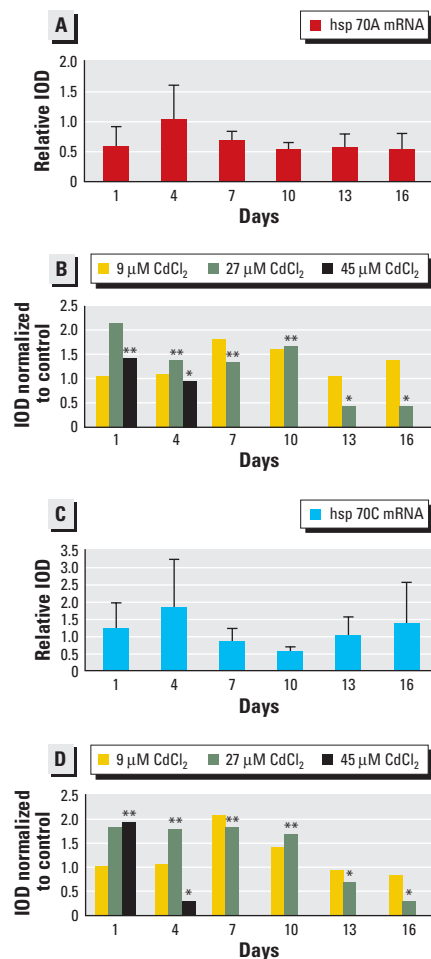

Figure 5. Three HPT cell isolates after continuous exposure to CdCl$_2$ (9, 27, and 45 µM for 16 days). Abbreviations: g3pdh, glyceraldehyde 3-phosphate dehydrogenase; hsc, constitutive form of hsp; hsp, heat shock protein; HPT, human proximal tubule; IOD, integrated optical density; RT-PCR, reverse transcriptase-polymerase chain reaction. (*A*) Mean (± SE) relative IOD of bands representing hsc 70 mRNA in control cells as determined by RT-PCR analysis; the IOD for each hsc 70 band was divided by the IOD for g3pdh at the appropriate time point. (*B*) IOD of hsc 70 bands normalized to control for HPT cells exposed to CdCl$_2$ over a 16-day time course as determined by RT-PCR analysis; bands representing the three Cd^{2+} concentrations were divided by the control values for each time point. (*C*) Mean (± SE) IOD values for bands representing hsc 70 protein in control cells as determined by Western analysis. (*D*) IOD values normalized to controls for HPT cells exposed to CdCl$_2$ as determined by Western analysis.

*There was only one viable sample. **There were only two viable samples.

Figure 4. HPT cells exposed to 53.4 µM CdCl$_2$ for 4 hr, followed by a 48 hr recovery period. Abbreviations: C, control; hsc, constitutive form of hsp; hsp, heat shock protein; HPT, human proximal tubule; IOD, integrated optical density; PCR, polymerase chain reaction; SE, standard error. (*A*) Mean (± SE) relative IOD of bands representing hsp 70A, hsp 70B, hsp 70C, and hsc 70 mRNA for three HPT cell isolates. (*B*) Mean (± SE) IOD of bands representing hsp 70 and hsc 70 protein at various time points for three HPT cell isolates as determined by Western analysis of hsp 70 and hsc 70 protein following Cd^{2+} exposure and recovery periods.

of Cd^{2+} had no effect on the expression of either hsc 70 mRNA or protein (Figure 5B, D). This was most apparent for HPT cells exposed to 9.0 μM Cd^{2+}, a level of exposure that elicited no cell death over the entire 16-day time course. For cells exposed to 27.0 μM Cd^{2+}, there was no change in the expression of hsc 70 mRNA and protein in the early days of the time course. However, once cell death was appreciable (days 13 and 16) there was a marked reduction in the levels of both hsc 70 mRNA and protein.

For the inducible isoform, the expression of hsp 70A and hsp 70C mRNA was constant

Figure 6. RT-PCR analysis of hsp 70A and hsp 70C after three HPT cell isolates were continuously exposed to $CdCl_2$ (9, 27, and 45 μM $CdCl_2$ for 16 days). Abbreviations: hsp, heat shock protein; HPT, human proximal tubule; IOD, integrated optical density; RT-PCR, reverse transcriptase-polymerase chain reaction; SE, standard error. (*A*) Mean (± SE) relative IOD of bands representing hsp 70A mRNA in control cells. (*B*) IOD of hsp 70A bands normalized to control in HPT cells exposed to $CdCl_2$. (*C*) Mean (± SE) relative IOD of bands representing hsp 70C mRNA in control cells. (*D*) IOD of hsp 70C bands normalized to control in HPT cells exposed to $CdCl_2$.
*There was only one viable sample. **There were only two viable samples.

over the 16-day time course for HPT cells unexposed to Cd^{2+} (Figure 6A, C). Likewise, exposure to Cd^{2+} had no effect on the level of hsp 70A or hsp 70C mRNA over the 16-day time course (Figure 6B, D). No expression of mRNA for the *hsp 70B* isoform was demonstrated in either control or Cd^{2+}-exposed cells at any time during the time course. As demonstrated by Western analysis, there was no detectable (or only marginally detectable) hsp 70 protein in the control or Cd^{2+}-treated cells at any point in the time course (data not shown).

Discussion

The first goal of the present study was to define the response of hsc 70 and hsp 70 when HPT cells were acutely exposed to Cd^{2+}. The response expected from such an exposure was defined by both comparison to that reported in the literature and to the response demonstrated when HPT cells were exposed to the classic stimuli of heat shock and sodium arsenite. For hsc 70, acute exposure of HPT cells to Cd^{2+} had no effect on the level of hsc 70 mRNA or protein, either during exposure to Cd^{2+} or during the subsequent postexposure recovery period. That hsc 70 was not induced in HPT cells by Cd^{2+} exposure was not unexpected, being in agreement with the finding that hsc 70 was also not induced in the HPT cells by either heat shock or sodium arsenite treatment. Furthermore, these findings are in agreement with the initial study that described hsc 70 as a constitutive protein with a high level of expression, attaining a level that can comprise 1% of total cell protein under nonstressed conditions (*21*). While the majority of studies on the heat shock response are cell culture based, one study has examined hsc 70 expression in the rat kidney after administration of Cd^{2+} (*22*). In agreement with the present findings, this study demonstrated that Cd^{2+}, when administered intravenously (iv) as $CdCl_2$ (2 mg/kg), had no effect on the level of expression of hsc 70 protein in the kidney 4 hr after exposure. However, this study also modeled chronic exposure to Cd^{2+} by exposing the rats to an equivalent dose of Cd^{2+} for 4 hr, but as the cadmium–cysteine (Cd-Cys) conjugate in place of the $CdCl_2$ salt. Under these experimental conditions, although there was no increase in hsc 70 at any dosage level, a marked reduction in the synthesis of hsc 70 was found at the highest dosage of Cd-Cys (2 mg/kg). In the present study, in addition to the acute 4-hr exposure of the HPT cells to Cd^{2+}, we used an extended time course of 16 days at both lethal and sublethal levels of Cd^{2+} exposure to model a more chronic time course of exposure. Under the conditions of extended

exposure to Cd^{2+}, there was no alteration in the level of hsc 70 mRNA or protein at sublethal levels of Cd^{2+} exposure. However, at lethal levels of Cd^{2+} exposure, a marked decrease in hsc 70 mRNA and protein were evident at 13 and 16 days of exposure. In general, hsc 70 expression in HPT cells exposed to Cd^{2+} was similar to that expected from both the literature and similar determinations performed on HPT cells exposed to the classic inducers of physical and chemical stress.

In contrast, using the level of hsp 70 induction by heat and sodium arsenite in the HPT cells as a comparative guide, the accumulation of hsp 70 protein was very restricted following acute exposure to Cd^{2+}. Specifically, in the HPT cells acutely exposed to Cd^{2+}, hsp 70 protein reached a maximum of 1.5 IOD units, whereas sodium arsenite and heat shock induced maximum values of 6.5 and 13 units, respectively. Although no other studies could be found for comparison that employed renal epithelial cell cultures, studies in other cell types have shown that Cd^{2+} exposure results in the consistent induction of a 70-kDa heat shock protein, indirectly supporting the concept that a marginal induction of hsp 70 following exposure to Cd^{2+} in the HPT cells is an unexpected finding. Induction of a 70-kDa hsp by Cd^{2+} treatment has been demonstrated in murine L929 cells (*23*), human amniotic cells (*24,25*), 9L rat brain tumor cells (*26*), Reuber H35 hepatoma cells (*27,28*), CEM-C12 human T cells (*29*), HepG2 hepatoblastoma cells (*30*), and HeLa cells (*31*). The only *in vivo* data in the kidney detailing hsp 70 expression was the previously detailed study in the rat kidney that examined hsc 70 expression after administration of Cd^{2+} as both the $CdCl_2$ salt and as the Cd-Cys complex (*22*). In this study it was also demonstrated that acute exposure of rats to iv-administered $CdCl_2$ had no effect on the expression of renal hsp 70, whereas identical exposure to the Cd-Cys complex resulted in a modest (2-fold) elevation of hsp 70. This finding suggested that a chronic exposure to Cd may induce a modest increase in the accumulation of hsp 70. This possibility was examined in HPT cells using a 16-day exposure to both lethal and sublethal levels of Cd^{2+}; however, there was no alteration in the level of hsp 70. The difference between the two observations may simply reflect the fact that the *in vivo* analysis measured new synthesis of hsp 70 and the HPT cell culture analysis measured total accumulation. Alternatively, the increased hsp 70 noted in the *in vivo* studies could be derived from a cell type other than the proximal tubule. However, even considering the small increase in hsp 70 found

in vivo, overall, the findings support the concept that Cd^{2+} is a poor inducer of hsp 70 accumulation in the proximal tubule under both acute and long-term conditions of exposure.

An additional goal of the present study was to determine if the individual genes responsible for the expression of hsp 70 have distinct patterns of regulation when HPT cells were acutely exposed to heat shock, sodium, or Cd^{2+}. At present, three genes (*hsp 70A*, *hsp 70B*, and *hsp 70C*) underlying the expression of hsp 70 can be specifically identified using RT-PCR technology. The pattern of *hsp 70* isoform-specific mRNA expression was shown to be very similar when the HPT cells were exposed to either heat shock or sodium arsenite. This similarity included the important finding that mRNA for all three *hsp 70* isoforms was not only increased, but increased to a similar maximum value of peak expression and a period of sustained expression. For HPT cells exposed acutely to Cd^{2+}, the patterns of expression for the *hsp 70A* and *hsp 70C* isoforms were also indistinguishable from the heat-shocked and sodium arsenite-treated cells. In contrast, the increase in the expression of mRNA representing the *hsp 70B* isoform was blunted for Cd^{2+}-treated cells as compared to that shown for the HPT cells exposed to heat shock or sodium arsenite. Furthermore, no hsp 70B mRNA was expressed when HPT cells were exposed to either lethal or sublethal levels of Cd^{2+} for 16 days. The potential significance of the decreased expression of hsp 70B mRNA is heightened by the fact that it correlated with the marked reduction in the expression of hsp 70 protein noted in Cd^{2+}-exposed cells as compared to those treated with heat or sodium arsenite. This finding suggests that Cd^{2+} exposure can inhibit the induction of hsp 70 protein by affecting the expression of only one of the *hsp 70* isoform genes. This observation also provides initial evidence that hsp 70 protein accumulation in stressed cells is derived preferentially from the *hsp 70B* isoform-specific mRNA as compared to those transcripts originating from the *hsp 70A* and *hsp 70C* genes. Overall, these observations highlight the fact that the expression of the hsp 70 protein does not result from the transcription of a single gene, but is derived from what may be a complex interplay of several underlying genes.

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