The expression of hsp 27 mRNA and protein was determined in cultured human proximal tubule (HPT) cells exposed to lethal and sublethal concentrations of Cd\textsuperscript{2+} under both acute and extended conditions. Initial procedures demonstrated that HPT cells display the classic stress response following physical and chemical stress. Heat stress (42.5°C for 1 hr) caused an increase in both hsp 27 mRNA and protein as well as a shift in the protein to a more phosphorylated state. Results were similar when the cells were subjected to chemical stress (exposure to 100 μM sodium arsenite for 4 hr). Acute exposure to 53 μM CdCl\textsubscript{2} for 4 hr also resulted in an increase in hsp 27 mRNA and protein and a shift to the more phosphorylated protein isoform. Extended Cd\textsuperscript{2+} exposure involved continuous treatment with Cd\textsuperscript{2+} at both lethal and sublethal levels over a 16-day time course. The results of this treatment showed that chronic exposure to Cd\textsuperscript{2+} failed to increase either hsp 27 mRNA or protein expression in HPT cells, even at lethal Cd\textsuperscript{2+} concentrations. In fact, hsp 27 protein levels decreased as compared to controls at both lethal and sublethal exposure to Cd\textsuperscript{2+}. These findings imply that hsp 27 expression in human proximal tubule cells may have two distinct modes depending on the nature (acute vs. chronic) of the stress. *Key words* cadmium, gene expression, heat shock, heavy metals, hsp 27, proximal tubule, sodium arsenite. Environ Health Perspect 107:545–552 (1999). [Online 2 June 1999] http://ehpnet1.niehs.nih.gov/docs/1999/107p545-552somji/abstract.html

The kidney, and in particular the proximal tubule, is critically affected by chronic exposure to the environmental pollutant Cd\textsuperscript{2+} in both animals and humans (1,2). Nephrotoxicity results from a slow accumulation of Cd\textsuperscript{2+} in the proximal tubules of the kidney. The earliest markers of chronic Cd\textsuperscript{2+} nephrotoxicity are disorders of proximal tubule transport characterized by low-molecular-weight proteinuria, increased high-molecular-weight protein excretion, and a variety of other proximal tubule ion transport disorders (1,3–6). Selective and direct absorption of Cd\textsuperscript{2+} by proximal tubules has been demonstrated using microinjection techniques (7). In an effort to define the fundamental mechanistic processes underlying human Cd\textsuperscript{2+}-induced nephrotoxicity, we used a cell culture model of the human proximal tubule (HPT). The HPT cell culture model retains important features of proximal tubule cell differentiation (8–10). These retained features are stable with cell passage and include a consistent enzyme histochemical profile, sodium-dependent glucose transport, parathyroid hormone stimulation of cAMP, generation of an apical-negative potential difference, and the presence of gap junctions. When these cells are exposed to sublethal concentrations of Cd\textsuperscript{2+}, they exhibit the transport and ultrastructural alterations expected from *in vivo* knowledge of Cd\textsuperscript{2+}-induced nephrotoxicity (11–13). Also in agreement with *in vivo* findings is the fact that at lethal Cd\textsuperscript{2+} concentrations, the cells undergo necrotic cell death (1). This model system is currently being used to define the roles and interactions of the stress response superfamily of proteins in protection and recovery from Cd\textsuperscript{2+} exposure. Initial examinations centered on the metallothionein gene family because these proteins are known for their ability to bind and sequester heavy metals (14–16). The current study focuses on the role of the stress response protein, hsp 27, as a possible mediator of Cd\textsuperscript{2+}-induced nephrotoxicity. This examination was motivated by recent studies demonstrating that enhanced hsp 27 expression has a role in the protection and recovery of the proximal tubule cell during and after brief periods of renal ischemia (17–19). This finding suggested that hsp 27 expression may also have a role in the protection of the protein tubule proximal tubule from the cytotoxic effects of the environmental pollutant, cadmium.

Hsp 27 is a member of a large superfamily of proteins with molecular weights ranging from 8 to 170 kDa and collectively referred to as the heat shock (hsp) or stress response proteins (20,21). In humans, hsp 27 is encoded by a single active gene located on chromosome 9 (22). Cell lines that over-express hsp 27 protein exhibit an enhanced ability to survive and recover from heat stress (23–29). Increasing hsp 27 expression by transiently or stably transfecting cell lines confers increased cellular resistance to a variety of toxicants including doxorubicin, daunorubicin, actinomycin D, vincristine, colchicine, arsenite, hydrogen peroxide, and tumor necrosis factor (23–25,27,30). Hsp 27 appears to exert its effects on cell survival, at least in part, through a chaperone action that stabilizes microfilament dynamics. Hsp 27 regulates actin dynamics, and hsp 27 overexpression prevents microfilament disruption and enhances mitogen-stimulated actin polymerization (23–25,31,32). Hsp 27 also demonstrates actin capping activity (25,33). Hsp 27 is phosphorylated at serine residues in response to heat shock or mitotic stimuli, suggesting a role in the regulation of signal transduction pathways. Recent studies also indicate that hsp 27 is involved in the regulation of programmed cell death in several cell lines (33–35).

**Materials and Methods**

**Cell culture.** Stock cultures of HPT cells were grown in 75-cm\textsuperscript{2} T-flasks using procedures previously described by this laboratory (8,9). The growth medium was a serum-free formulation consisting of a 1:1 mixture of Dulbecco’s modified Eagles’ medium 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 post subculture) were subcultured using trypsin-EyDTA (0.05%, 0.02%). For use in experimental protocols, the cells were subcultured in six-well plates 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. HPT cell passages between 5 and 7 were used in the present study. The three isolates of HPT cells 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.

**Cell viability.** The effect of the various treatments on the viability of confluent cell...
monolayers was determined by counting cell nuclei of viable cells remaining attached to the culture surface using the nuclear stain DAPI (4′,6-diamidino-2-phenylindole) and Kontron KS400 image analysis software (Zeiss, Thornwood, NY), as described previously (15). At the indicated time points, wells containing the cell monolayers were fixed for 15 min in 70% ethanol, rehydrated with carbonate-buffered saline (PBS), and stained with 10 μL DAPI (10 μg/mL in distilled water). Each well was examined under epifluorescence illumination at 40× magnification on a Zeiss Axiosvert 35 (Zeiss) linked to the computer with an Optronics DEI 470 CCD camera (Optronics, Goleta, CA). For each time point, a minimum of 20 fields per well and three wells per data point were determined. Both nuclear counts and total nuclear area were obtained from the program and yielded equivalent results.

**Isolation of total RNA, RT-PCR, and Northern analysis.** Total RNA was isolated according to the protocol supplied with TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH) as described previously by this laboratory (15). The concentration and purity of the RNA samples were determined using spectrophotometer scan in the ultraviolet (UV) region and ethidium bromide (EtBr) visualization of intact 18S and 28S RNA bands following agarose gel electrophoresis.

For reverse transcriptase polymerase chain reaction (RT-PCR), 500 ng of total RNA from cultured HPT cells was reverse transcribed by incubating for 20 min at 42°C in a 20-μL reaction mixture containing 2.5 μM random hexamers, 5 mM MgCl₂, 1X PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl), 4 mM dNTPs, 1 U/μL RNase inhibitor, and 2.5 U/μL MuLV reverse transcriptase. The resulting cDNA was amplified in 100 μL reaction mixtures containing 2 mM MgCl₂, 1X PCR buffer, 0.025 U/μL Tag polymerase, and 0.015 μM of the respective primers. The primer pair for amplification of hsp 27 cDNA consisted of oligonucleotide sequences 5′-CAGGAGA-GCGGCAGACAGG3′ and 5′-CAGTGA-GCGCCAGCAGGGTG3′ (PCR primer pair STM-500; StressGen, Victoria, British Columbia, Canada). Reaction volumes were incubated at 95°C for 2 min, followed by 30 cycles of 95°C for 30 sec/58°C for 30 sec. PCR products were electrophoresed in 2% agarose gels, stained with EtBr, and photographed under UV light. A DNA ladder (Bio-Rad, Hercules, CA) was included in each gel to verify the size of PCR products. For Northern analysis, 5.0 μg of cellular RNA was separated on 1.2% agarose gels containing 0.003% (w/v) EtBr, 1 × MOPS buffer (0.1 M 3-(N-morpholino)propanesulfonic acid, 40 mM sodium acetate, 5 mM EDTA, pH 8.0), and 0.6 M formaldehyde. After electrophoresis, RNA was transferred to nylon membranes and covalently attached by microwave treatment for 1.5 min. Hsp 27 cDNA produced by reverse transcription PCR of heat-shocked HeLa cell control RNA (provided by StressGen) was purified using a PCR product purification kit (QIAquick kit 28104; QIAGEN, Chatsworth, CA). Thirty-five nanograms of purified PCR product was labeled to > 1.2 × 10⁹ cpm/μg with 50 μCi α²²P-dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) using a random primed synthesis kit (Promega Corp., Madison, WI). Nylon membranes were prehybridized for 2 hr at 50°C in a solution containing 50% (v/v) formamide, 0.25 M NaCl, 0.25 M sodium phosphate (pH 7.2), 0.7% SS, 1 mM EDTA (pH 8.0), and 100 μg/mL sonicated herring sperm DNA. After prehybridization, radiolabeled cDNA probe was hybridized to Northern filters in the same solution as prehybridization for 16 hr at 52°C. Membranes were washed twice in 2 × standard saline citrate 0.1% sodium dodecyl sulfate (SDS) for 15 min at 50°C, twice in 25 mM sodium phosphate (pH 7.2) 1 mM EDTA 0.1% SDS for 15 min at 50°C, and twice in 25 mM sodium phosphate (pH 7.2) 100 mM EDTA 0.1% SDS for 15 min at 50°C, followed by 1 × SSC 0.1% SDS at 50°C for 30 min. After hybridization and washing, membranes were exposed to X-ray films (Kodak) for 3–7 days.
7.2) 1 mM EDTA 1.0% SDS for 15 min at 50°C. Blots were wrapped in plastic and exposed overnight at -76°C for autoradiography. Radioactive probes were removed from membranes by immersion in boiling 0.1% SDS. Stripped blots were reprobed with radiolabeled cDNA complementary to human glyeraldehyde 3-phosphate dehydrogenase mRNA (Clontech, Palo Alto, CA) for loading correction.

Western analysis. Cells were washed twice with PBS and lysed directly in the flask by addition of 400 µL (85°C) 1 x SDS buffer (2% SDS, 100 mM dithiothreitol, and 50 mM 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 10,000g for 10 min at room temperature and the supernatant transferred to a new tube. The concentration of protein in the samples was determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL). Equal amounts of total cellular protein were separated on 12% SDS-containing polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked with 10% (w/v) nonfat milk in PBS, incubated with a mouse monoclonal antibody specific for human hsp 27 (StressGen) diluted 1:2000 in PBS containing 1% (w/v) bovine serum albumin as carrier, followed by incubation with a goat antihouse, alkaline phosphatase conjugated secondary antibody (Promega). Colorimetric detection used an alkaline phosphatase Vectastain ABC-AP kit (Vector, Burlingame, CA).

Analysis of hsp 27 phosphoisoforms. Proteins were extracted from cell monolayers with 9.0 M urea, 1.0 mM phenylmethyl sulfonyl fluoride, 10.0 mM NaF, 2% amphotelines, 5% β-mercaptoethanol, and 2% Triton X-100. The proteins were focused on 4% polyacrylamide capillary tube gels containing 9 M urea, 1.5% 5/7 Biolyte, and 0.5% 3/10 Biolyte amphotelines (Bio-Rad). Capillary tube gels containing focused proteins were placed at the top of 12% polyacrylamide slab minigels, followed by separation of proteins in the second dimension. Resolved proteins were electrotransferred onto PVDF membranes (Bio-Rad). Hsp 27 phosphoisoforms were detected using procedures identical to those described for Western analysis.

Integrated optical density (IOD). IOD values were determined using an image analysis work station configured with Kontron KS 400 software. For IOD evaluations of EtBr-stained gels, inverted images were used. In the heat-shock, acute CdCl₂, and sodium arsenite protocols, IOD values for Northern blots and RT-PCR gels are [(IOD hsp 27 experimental/IOD hsp 27 control)]/[IOD glyeraldehyde-3-phosphate dehydrogenase (GAPDH) experimental/IOD GAPDH control]. IOD values for Western blots are IOD experimental/IOD control. In the 16-day CdCl₂ exposure, the IOD for each Hsp 27 band was divided by the IOD for GAPDH at the appropriate time point and Cd concentration. Bands representing the three Cd²⁺ concentrations were then divided by the control values for each time point.

Results

Hsp 27 expression in HPT cells exposed to heat shock. The classic method used to examine the response of cultured mammalian cells to physical stress is exposure to elevated temperature, usually 42–44°C, followed by a recovery period at normal temperature. To determine the effect of heat shock on hsp 27 expression in HPT cells, confluent cells from three independent cell isolates were exposed to an elevated temperature of 42.5°C for 1 hr followed by a recovery period of 48 hr at 37°C. Exposure to heat shock clearly resulted in an increase in the amount of hsp 27 mRNA for all three HPT cell isolates as determined by Northern analysis (Figure 1A, B). Hsp 27 mRNA was increased at the end of the 1-hr heat shock period, continued to increase during the initial hour of the recovery period, and remained elevated for the next 12–16 hr before returning to control values 24 hr postheat shock. Hsp 27 mRNA levels were also examined on the same total RNA samples using an RT-PCR assay to determine if results would be equivalent to those found with Northern analysis (Figure 1C, D). This is important because RT-PCR analysis consumes a much smaller amount of total RNA than Northern analysis. The relative

![Figure 2](image-url) Western analysis (A, B) and two-dimensional gel electrophoresis (C, D) of heat shocked HPT cells during exposure and recovery periods. Abbreviations: a, unphosphorylated; b, one serine phosphorylated; c, two serines phosphorylated; d, three serines phosphorylated; hsp, heat shock protein; HPT, human proximal tubule; IOD, integrated optical density. (A) Western blot for a single isolate. (B) Relative (normalized to control values) IODs of bands representing hsp 27 protein at the various time points for three HPT cell isolates. (C) Blot showing increasing phosphorylation of hsp 27 protein represented by the a, b, c, and d phosphoisoforms. (D) IOD values for each of the phosphoisoforms in three HPT cell isolates.
amounts of hsp 27 mRNA were similar for both Northern and RT-PCR assay methods.

The expression of hsp 27 protein was also determined at selected time points of the heat shock protocol by Western analysis (Figure 2A, B). Hsp 27 protein was expressed under control conditions and did not increase during the initial 1-hr period at 42.5°C. Hsp 27 protein levels increased in the recovery period, reaching peak values by 8–12 hr. The phosphorylation state of hsp 27 protein was also determined as a function of heat shock and recovery (Figure 2C, D). In the control condition, hsp 27 protein was present in the unphosphorylated and mono-phosphorylated state. There was an increase in phosphorylation during the 1-hr heat shock, as noted by a faint additional immunoreactive spot representing the di-phosphorylated state. There was no loss of cell viability during the treatment and recovery periods (data not shown).

**Hsp 27 expression in HPT cells following acute exposure to arsenite and CdCl₂** The classic method to evaluate the response of cultured cells to chemical stress is exposure to sodium arsenite followed by a recovery period that involves the removal of the chemical stress through a change of the culture medium and renewed incubation at 37°C. This method was used to determine if acute exposure to Cd²⁺ induces hsp 27 expression in HPT cells. Confluent cells were exposed to 53 μM Cd²⁺ for 4 hr, followed by a 48-hr recovery period in Cd²⁺-free growth media. The Cd²⁺ concentration used was established in preliminary experiments to be an exposure level resulting in the death of 15–30% of the cells by the end of the recovery period. The effect of this level of exposure was confirmed by monitoring HPT cell viability over the total time course of exposure and recovery (Figure 3A). Exposure to 53 μM Cd²⁺ clearly resulted in an increase in the amount of hsp 27 mRNA, as determined by RT-PCR analysis (Figure 3B, C). The increase in hsp 27 mRNA was rapid, 5–10-fold over control, occurring largely within the first 4 hr of Cd²⁺ exposure, and was not dependent on a Cd²⁺-free recovery period. This elevated level of hsp 27 mRNA was maintained 4–8 hr into the recovery period and returned to control values by 48 hr of recovery in Cd²⁺-free growth medium.

The level of hsp 27 protein was also increased by acute Cd²⁺ exposure (Figure 3D, E). Hsp 27 protein was maximally elevated following 1 hr of Cd²⁺ exposure, remained elevated 8–12 hr into the recovery period, and returned to control values following 24 hr of recovery in Cd²⁺-free growth medium. Exposure to 53 μM Cd²⁺ resulted in an immediate and prolonged shift of the hsp 27 isoform pattern to an enhanced phosphorylation state (Figure 3F). The shift in the phosphoisoform pattern occurred within the first hr of Cd²⁺ exposure and was retained in the recovery period.

For comparison, confluent HPT cells were also exposed to 100 μM sodium arsenite for 4 hr, followed by a 48-hr recovery period (Figure 4). Exposure to 100 μM sodium arsenite resulted in an increase in the amount of hsp 27 mRNA (Figure 4B, C). The level of hsp 27 mRNA was relatively constant during the initial hours of sodium arsenite exposure and began to increase after 4 hr. Four hours into the recovery period, there was a large increase in the level of hsp 27 mRNA. The increased level of hsp 27 mRNA was sustained for 12 hr of recovery and thereafter rapidly returned to control values. Hsp 27 protein also increased as a consequence of sodium arsenite treatment (Figure 4D, E). Hsp 27 protein levels increased following 1 hr of sodium arsenite treatment and remained elevated 24 hr into the recovery period. The phosphorylation state of hsp 27 protein was also evaluated for HPT cells exposed to sodium arsenite. The unphosphorylated and mono-phosphorylated forms of hsp 27 were evident in the control condition. Sodium arsenite exposure resulted in a shift in the isoform pattern of hsp 27 to a more phosphorylated state following 4 hr of exposure (Figure 4F).

**Hsp 27 expression in HPT cells following chronic exposure to lethal and sub-lethal levels of Cd²⁺** Because the stress response is typically assessed under conditions of acute agent exposure, an experiment was designed to determine if the hsp 27 stress response of
HPT cells is different with extended exposure. To accomplish this, HPT cells were continuously exposed to Cd\textsuperscript{2+} over a 16-day time course. Three concentrations of Cd\textsuperscript{2+} were used: 9 M, which produces no cell death over the 16-day time course; 27 M, which produces cell death late in the 16-day time course; and 45 M, which produces cell death early in the 16-day time course (Figure 5). Hsp 27 mRNA and protein expression were determined after 1, 4, 7, 10, 13, and 16 days of exposure. The level of hsp 27 mRNA expression was constant over the 16-day time course for control HPT cells (Figure 6B). The level of hsp 27 mRNA expression was also not altered as compared to control for any of the Cd\textsuperscript{2+} treatment groups regardless of Cd\textsuperscript{2+} dose or Cd\textsuperscript{2+}-induced cell lethality (Figure 6). The level of hsp 27 protein expression was also relatively constant over the 16-day time course for control cells (Figure 7B). In contrast, the level of hsp 27 protein level was reduced as compared to control at each concentration of Cd\textsuperscript{2+} (Figure 7). The pattern of hsp 27 phosphoisoforms was evaluated after 24 hr of exposure to each Cd\textsuperscript{2+} concentration and was identical to control cells (data not shown).

Discussion
The first goal of the present study was to determine if acute exposure to Cd\textsuperscript{2+} evokes the hsp 27 stress response in cultured HPT cells. This was accomplished by mimicking the classic protocols used to determine a cell's response to physical or chemical stress: a short exposure to a agent, followed by agent removal and monitoring of the response during a recovery period. For the HPT cells, this involved treatment with 53 M Cd\textsuperscript{2+} for 4 hr, followed by removal of the metal through a change in growth medium, and subsequent monitoring of the hsp 27 response during a 48-hr recovery period. The results demonstrated that Cd\textsuperscript{2+} treatment induces the hsp 27 stress response, as evidenced by an increase in both hsp 27 mRNA and protein as well as by a shift in hsp 27 isoforms to a pattern of increased phosphorylation. To confirm that the Cd\textsuperscript{2+}-induced hsp 27 response in HPT cells was similar to the classic response induced by heat and sodium arsenite, these agents were also used to induce hsp 27 expression. It was demonstrated that the hsp 27 response was similar for heat, sodium arsenite, and Cd\textsuperscript{2+}. The only feature of the hsp 27 response in HPT cells that was different from other systems was that a recovery phase was not necessary for induction of the hsp 27 response. In HPT cells the induction of hsp 27 mRNA and protein occurred in the presence of Cd\textsuperscript{2+} and did not require a recovery phase. This implies that acute exposure to lethal concentrations of Cd\textsuperscript{2+} does not immediately inactivate any of the cellular components necessary for transcription, translation, or phosphorylation of hsp 27 in HPT cells. Otherwise, the hsp 27 response to acute Cd\textsuperscript{2+} exposure is what would be expected based on many studies in other cell systems.

Although studies in the renal system are limited, a role for the hsp 27 response in protection of the proximal tubule cell from acute Cd\textsuperscript{2+} exposure can be inferred from recent studies on renal ischemia. In studies using the rodent model, evidence shows that induction of the hsp 27 stress response can attenuate the effects of acute renal ischemia (17–19). The expression and intracellular distribution of hsp 25 (the rodent homologue to human hsp 27) was evaluated in rat renal cortex following 45 min of renal ischemia with subsequent reflow (17). Cortical hsp 25 was induced within 2 hr of reflow, peak values were reached by 6 hr, and elevated levels were maintained after 24 hr of reflow. The shift in hsp 25 between the detergent soluble and insoluble cytoskeletal fractions and the localization of hsp 25 within the proximal tubule cell as a function of ischemia and recovery both suggested specific interactions between hsp 25 and actin during the early postischemic reorganization of the cytoskeleton. The suggestion that hsp 25 provides assistance in the reorganization of the actin cytoskeleton following renal ischemia is consistent with one of the known functions of hsp 27 and the alterations of the actin cytoskeleton that are
known to occur in ischemia. A series of studies demonstrated that the loss of proximal tubule cell structure and transport function associated with renal ischemia involves alterations in the actin cytoskeleton [reviewed by Moltoriis (36)]. The induction of hsp 27 in HPT cells by acute Cd\textsuperscript{2+} exposure would likewise be expected to stabilize the actin filament network and help preserve proximal tubule transport function.

Recent studies in other systems have demonstrated that expression of hsp 27 regulates apoptosis (33–35). In studies using L929 cells that constitutively express the Fas receptor, expression of human hsp 27 inhibits apoptosis mediated by stimulation of the Fas receptor (34). In addition, hsp 27 expression interfered with apoptotic cell death mediated by staurosporine (33,34). A similar finding for a protective effect of the small hsp against apoptosis was also observed in U937 and Wehi-s cells exposed to actinomycin D, camptothecin, and etoposide (35). HPT cells express Fas under normal growth conditions and increased Fas expression occurs after treatment with interferon-\gamma (37). These observations suggest that the rapid induction of hsp 27 protein and phosphorylation early in the time course of acute exposure to Cd\textsuperscript{2+} may protect the proximal tubule cell against programmed cell death.

The classic methods used to examine the stress response in cultured cells evaluate the presence or absence of the response after a short duration of agent exposure. The results discussed for hsp 27 expression in Cd\textsuperscript{2+}-exposed HPT cells can then be categorized as early events within the initial 4 hr of Cd\textsuperscript{2+} exposure. Although this illustrates the hsp 27 stress response following acute exposure to Cd\textsuperscript{2+}, it is likely that actual exposure in many instances is more prolonged and involves lower concentrations. An additional goal of this study was to determine if the Cd\textsuperscript{2+}-induced early increase in hsp 27 level was sustained when HPT cells were exposed over a longer time course. Sustained Cd\textsuperscript{2+} exposure was modeled by continuous treatment of HPT cells with both lethal and sublethal Cd\textsuperscript{2+} levels over a 16-day time course while monitoring cell viability and hsp 27 mRNA and protein levels at days 1, 4, 7, 10, 13, and 16. For hsp 27 mRNA, the level of hsp 27 mRNA did not increase over control levels at any point in the time course regardless of sublethal (9 \textmu M) or lethal (27 and 45 \textmu M) levels of Cd\textsuperscript{2+} exposure. The finding that hsp 27 mRNA was not elevated in HPT cells following 24 hr of exposure to any of the three Cd\textsuperscript{2+} concentrations was somewhat surprising because the described acute exposure experiments showed a large elevation of hsp 27 mRNA following 4 hr of exposure to 53 \textmu M Cd\textsuperscript{2+}. Subsequent examination of hsp 27 mRNA levels in HPT cells within the initial 24 hr of exposure demonstrated that 9 \textmu M Cd\textsuperscript{2+} caused no increase in hsp 27 mRNA levels, 27 \textmu M Cd\textsuperscript{2+} elicited a maximal 2-fold increase in hsp 27 mRNA only between 4 and 12 hr of exposure, and 45 \textmu M Cd\textsuperscript{2+} resulted in a 2- to 4-fold increase in hsp 27 mRNA. 

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; HPT, human proximal tubule; IOD, integrated optical density. Three HPT cell isolates were exposed to 9, 27, and 45 \textmu M CdCl\textsubscript{2} for a period of 16 days. Computer-assisted cell counts are shown for one HPT cell isolate. DAPI-stained nuclei in 20 fields for each triplicate well were counted and results are expressed as percentage of control.

Figure 5. HPT cell continuous exposure to CdCl\textsubscript{2}. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; HPT, human proximal tubule; IOD, integrated optical density. Three HPT cell isolates were exposed to 9, 27, and 45 \textmu M CdCl\textsubscript{2} for a period of 16 days. Computer-assisted cell counts are shown for one HPT cell isolate. DAPI-stained nuclei in 20 fields for each triplicate well were counted and results are expressed as percentage of control.

Figure 6. RT-PCR analysis of HPT mRNA during 16-day exposure to three Cd\textsuperscript{2+} concentrations. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hsp, heat shock protein; HPT, human proximal tubule; IOD, integrated optical density; RT-PCR, reverse transcriptase polymerase chain reaction; SEM, standard error of the mean. (A) Ethidium-bromide-stained agarose gels for PCR products representing hsp 27 and GAPDH mRNA for one HPT cell isolate. Average (±SEM) IOD of (inverted) bands representing (B) hsp 27 mRNA in controls and (C) cell exposed to 9, 27, and 45 \textmu M CdCl\textsubscript{2} for three HPT cell isolates over the 16-day time course. IOD values were divided by the respective GAPDH IOD values and normalized to IODs for control cells. *Only two viable samples.

Figure 7. Western analysis of hsp 27 protein following 16-day exposure to CdCl\textsubscript{2}. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hsp, heat shock protein; HPT, human proximal tubule; IOD, integrated optical density; SEM, standard error of the mean. (A) Western blots for a single isolate. Average (±SEM) relative IOD values for bands representing (B) hsp 27 protein in controls and (C) cells exposed to 9, 27, and 45 \textmu M CdCl\textsubscript{2} for three HPT cell isolates. IOD values were divided by the respective GAPDH IOD values and normalized to IODs for control cells. *Only two viable samples.
increase between 2 and 20 hr of exposure, with all dosage groups returning to control values following 24 hr of exposure (38). There was no sustained elevation of hsp 27 mRNA levels in HPT cells during extended exposure to both lethal and sublethal concentrations of Cd²⁺. Analysis of hsp 27 protein levels over the extended time course demonstrated a marked decrease in hsp 27 protein as compared to control cells. This decrease in hsp 27 protein was evident at both lethal and sublethal levels of Cd²⁺ exposure, although the decrease was greatest at lethal levels of Cd²⁺ exposure. To our knowledge, this is the only example of non-lethal levels of toxicant exposure that reduces the constitutive level of hsp 27 protein expression in the cell.

In a previous study using identical HPT cell lines, Cd²⁺ concentrations, and exposure time course, Cd²⁺ exposure also elicited a sustained induction of metallothionein (MT) protein (15). Within the first 24 hr of exposure to 9 μM Cd²⁺, HPT cells showed a 10- to 20-fold increase in MT protein levels and this increase continued to the end of the time course where MT represented 7 to 10% of total cell protein. Because of the high binding affinity of MT for Cd²⁺, it would be expected that Cd²⁺ is in the unsequestered state for only a brief period following the initial exposure of the cells before complexing with MT. Assuming that hsp 27 induction occurs in response to Cd²⁺ in the unsequestered state, then hsp 27 induction would only be expected to occur immediately after Cd²⁺ exposure—the brief interval before MT protein induction and complexation with MT. This would provide a mechanism to explain the current finding that hsp 27 expression is limited to an early transient induction in Cd²⁺-exposed HPT cells. Because the Cd- sequestering MT protein level continues to increase with time, this explanation is also consistent with the finding that hsp 27 expression is not increased later in the time course. Although the induction of MT may explain the lack of a sustained or long-term induction of hsp 27 expression by Cd²⁺, it does not explain why the levels of hsp 27 protein are decreased by prolonged exposure to lethal and sublethal concentrations of Cd²⁺. Although there is no explanation for this finding at present, it does not appear to be due to a non-specific overall decrease in protein synthesis because MT protein is increased at the same time hsp 27 protein is decreased.

Two distinct roles can be proposed for hsp 27 expression when the HPT cell is exposed to Cd²⁺. Induction early in the time course of Cd²⁺ exposure may protect the cell while the Cd²⁺-binding protein MT is being synthesized. This would serve to stabilize the actin filament network of the cell in a fashion similar to that proposed to occur during renal ischemia and subsequent reflow (17–19). Direct evidence that a transient elevation of hsp 27 expression can provide cellular resistance to Cd²⁺ toxicity comes from recent studies with mouse embryonic stem cells transfected with sense or antisense hsp 27 cDNA (39). In these studies, the level of hsp 27 expression was directly correlated with cellular resistance to the toxicity of CdCl₂, HgCl₂, cis-platinum (II)-diamine dichloride, or sodium arsenite within a 12-hr exposure period. Protection against programmed cell death provided by the early induction of hsp 27 is speculative based solely on hsp 27 involvement in programmed cell death decision in other cell systems (33–35). In contrast, the inability to sustain constitutive levels of hsp 27 protein may potentially have deleterious effects, as was demonstrated when HPT cells were subjected to a longer period of sustained Cd²⁺ exposure. Whereas the induction of hsp 27 protein has been proposed to stabilize actin filament dynamics, the loss of hsp 27 protein would be expected to render the actin filaments susceptible to damage. The demonstration that constitutive levels of hsp 27 protein are not maintained in HPT cells during a chronic course of Cd²⁺ exposure suggests that the cytoskeleton might be a site particularly susceptible to damage in cadmium-induced nephropathy.

References and Notes

1. Bernard A, Roels H, Hubermont G, Buchet JP, Masson PL, Lauwersys R. Characterization of the proteinuria in Cd exposed workers. Int Arch Occup Environ Health 59:19–30 (1976).

2. Kido T, Honda R, Tasuitani Y, Yamada H, Iwasaki M, Yamada Y, Nogawa K. Progress of renal dysfunction in inhabitants environmentally exposed to cadmium. Arch Environ Health 43:213–217 (1988).

3. Fisacator M. Proteinuria in chronic cadmium poisoning. Arch Environ Health 53:25–32 (1982).

4. Lauversys R, Buchet JP, Roels H, Brouwers J, Stanesuc D. Epidemiological survey of workers exposed to cadmium: effect of lung, kidney, and several biological indices. Arch Environ Health 29:145–148 (1974).

5. Gonich HC, Indraprastis S, Rosen VJ, Neustein H, Van de Velde R, Ravaghi SRV. Effect of cadmium on renal tubular function, the ATP-NA-ATPase transport system and renal tubular ultrastructure. Mineral Metab 3:21–35 (1986).

6. Kinter LS. Sudden death and anatomy and physiology of the kidney. In: Toxicology of the Kidney (Hook JB, Goldstein RS, eds). New York: Raven Press, 1992:1–36.

7. Bosone R, Porta N, Diez J. Renal handling of cadmium: a study by tubular microinjections. Arch Toxicol 73:71–373 (1982).

8. Detrichcs CJ, Sens MA, Garvin AJ, Spicher SS, Sens DA. Tissue culture of human kidney epithelial cells of proximal tubule origin. Kidney Int 25:385–390 (1984).

9. Todd JH, Mc Martin K, Sens DA. Enzymatic isolation and serum-free culture of human renal cells retaining properties of proximal tubule cells. In: Methods in Molecular Medicine. Human Cell Culture Protocols (Jones GE, ed). Totowa, NJ: Humana Press, 1996:431–436.

10. Sens DA, Detrichcs CJ, Sens MA, Ross MI, Wenger SL, Todd JH. Tissue culture of human renal epithelial cells using a defined serum-free growth formulation. Exp Nephrol (in press).

11. Hazen-Martín DJ, Sens DA, Blackburn JG, Sens MA. Cadmium nephropathy in human proximal tubule cells. In Vitro Cell Dev Biol 25:794–799 (1989).

12. Hazen-Martín DJ, Sens DA, Blackburn JG, Flath MC, Sens MA. An electrophysiological and freeze fracture assessment of cadmium nephropathy in vitro. In Vitro Cell Dev Biol 25:791–798 (1989).

13. Hazen-Martín DJ, Todd JH, Sens MA, Khan W, Bylander JE, Smyth BJ, Sens DA. Electrical and freeze-fracture analysis of the effects of ionic cadmium on the cell membrane of human proximal tubule cells. Environ Health Perspect 101:510–516 (1993).

14. Hoey JG, Garrett SH, Sens MA, Todd JH. Expression of MT-3 mRNA in human kidney, proximal tubule cell cultures, and renal cell carcinoma. Toxicol Lett 92:149–160 (1997).

15. Garrett SH, Somji S, Todd JH, Sens DA. Exposure of human proximal tubule cells to Cd²⁺, Zn²⁺, and Cd²⁺ induces metallothionein function and accumulation, but not metallothionein isoform 2 mRNA. Environ Health Perspect 106:587–590 (1998).

16. Garrett SH, Somji S, Todd JH, Sens MA, Sens DA. Differential expression of human metallothionein isoforms in human proximal tubule cells exposed to metals. Environ Health Perspect 106:825–832 (1998).

17. Aufricht C, Arldo T, Thulin G, Khashgarden M, Sieger NJ, Van Why SK. Heat-shock protein 25 induction and redistribution during actin reorganization after renal ischemia. Am J Physiol 274:F215–F222 (1998).

18. Schöber A, Müller E, Thurai K, Beck F. The response of heat shock proteins 25 and 70 to ischaemia in different kidney zones. Pflugers Arch Eur J Physiol 434:289–299 (1997).

19. Schöber A, Burger-Kern K, Müller E, Beck F. Effect of ischaemia on localization of heat shock protein 25 in kidney. Kidney Int 54:174–176 (1998).

20. Welch W. Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. Physiol Rev 72:1063–1081 (1992).

21. Georgopoulous C, Welch WJ. Role of the major heat shock proteins as molecular chaperones. Annu Rev Cell Biol 9:601–634 (1993).

22. Ciocca DR, Oesterreich S, Chammess GC, McGuire WL, Fuqua SAW. Biological and clinical implications of heat shock protein 27. J Natl Cancer Inst 85:1558–1570 (1993).

23. Huot J, Houle F, Spitz DR, Landry J. Hsp 27 phosphorilation-mediated resistance against cell fragmentation and cell death induced by oxidative stress. Cancer Res 56:273–279 (1996).

24. Lavoie J, gingras-Breton G, Tanguay RM, Landry J. Induction of Chinese hamster hsp 27 gene expression in mouse cells confers resistance to heat shock. Hsp 27 stabilization of microfilament organization. J Biol Chem 268:3420–3429 (1993).

25. Lavoie JN, Hickey E, Weber LA, Landry J. Modulation of actin microfilament dynamics and fluid phase pinocytosis by phosphorylation of heat shock protein 27. J Biol Chem 268:24210–24214 (1993).

26. Landry J, Chenet P, Lambert J, Hickey E, Weber LA. Heat shock resistance conferred by expression of the human hsp 27 gene in rodent cells. J Cell Biol 109:7–15 (1998).

27. Huot J, Roy G, Lambert H, Chenet P, Landry J. Increased survival after treatment with anticaner agents of Chinese hamster cells expressing the human Mt. 27 heat shock protein. Cancer Res 58:2545–2552 (1991).

28. Lavoie JN, Lambert H, Hickey E, Weber LA, Landry J. Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the microfilament structure of heat shock protein 27. Mol Cell Biol 15:505–516 (1995).

29. Kampiinga HH, Luppens JG, Konings AW. Heat induced nuclear protein binding and its relation to
thermal cytotoxicity. Int J Hyperthermia 3:459–465 (1987).
30. Mehlen P, Pravilla X, Chareyron P, Briolay J, Klemenz R, Arrigo A-P. Constitutive expression of human hsp 27, Drosophila hsp 27, or human αB-crystallin confers resistance to TNF- and oxidative stress-induced cytotoxicity by stably transfected murine L929 fibroblasts. J Immunol 154:363–374 (1995).
31. Miron T, Vancompernolle K, Vanderkerckhove J, Wilchek M, Geiger B. A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein. J Cell Biol 114:256–261 (1991).
32. Arrigo A-P, Landry J. Expression and function of the low-molecular-weight heat shock proteins. In: The Biology of Heat Shock Proteins and Molecular Chaperones (Morimoto R, Tissieres A, Georgopoulos C, eds). New York: Cold Spring Harbor Laboratory Press, 1994:325–333.
33. Arrigo A-P. Small stress proteins: chaperones that act as regulators of intracellular redox state and programmed cell death. Cell 70:19–28 (1992).
34. Mehlen P, Schulte-Osthoff K, Arrigo A-P. Small stress proteins as novel regulators of apoptosis. J Biol Chem 271:16510–16514 (1996).
35. Samali A, Cotter TG. Heat shock proteins increase resistance to apoptosis. Exp Cell Res 223:163–170 (1996).
36. Molitoris BA. Putting the actin cytoskeleton into perspective: pathophysiology of ischemic alterations. Am J Physiol 272:F430–F433 (1997).
37. Boonstra JD, Van Der Voude FJ, Wever PC, Laterveer JC, Daha MR, Van Kooten C. Expression and function of Fas (CD95) on human renal tubular epithelial cells. J Am Soc Nephrol 8:1517–1524 (1997).
38. Sens D. Personal communication.
39. Wu W, Welsh MJ. Expression of the 25-kDa heat shock protein (HSP 27) correlates with resistance to the toxicity of cadmium chloride, mercuric chloride, cis-platinum (II)-diamine dichloride, or sodium arsenite in mouse embryonic stem cells transfected with sense or antisense hsp 27 cDNA. Toxicol Appl Pharmacol 141:330–339 (1996).