p38 MAPK/HSP25 signaling mediates cadmium-induced contraction of mesangial cells and renal glomeruli


IN INDUSTRIALIZED countries, cadmium (Cd) is an environmental pollutant causing human health problems, with the kidney being a primary target organ. In a cross-sectional population study, environmental Cd exposure was shown to be associated with renal dysfunction (10). Most studies on Cd-related nephrotoxicity have focused on proximal tubules as the major site of renal injury (37). Although poorly recognized as a target and less well studied, glomeruli were shown to be also a significant target of Cd-related renal dysfunction (26). Detailed analysis of urinary markers of glomerular vs. tubular injury in workers exposed to Cd strongly suggested that glomerular injury represents a significant manifestation of Cd-induced renal disease (45). Occupational exposure to Cd induced irreversible glomerular lesions with a decrease in glomerular filtration (27, 28). Morphometric studies on both isolated glomeruli and mesangial cells, a smooth muscle-like cell type, showed that mesangial cells contract in response to Cd (4, 5, 34). Cd-stimulated contraction of glomeruli was suggested to be the basis for the observed alterations in intrarenal hemodynamics and glomerular filtration rate preceding proteinuria (5, 27, 28, 45). Although literature on the cellular action of Cd is available, the precise mechanisms by which Cd induces cell damage remain obscure. Cd has pleiotropic effects including the induction of metallothioneins, heat shock proteins (6, 8) and proto-oncogenes (60), interference with calcium/calmodulin signaling (8), profound rearrangements of microfilaments (59), and stimulation of signal transduction cascades involving the MAP kinases (MAPK) Erk1/2 (12, 54, 60), JNK (12), and p38 (29, 54).

The p38 MAPK phosphorylates and activates the mitogen-activated protein kinase-activated protein kinases-2/3 (MK-2/3) in vivo (47), which themselves phosphorylate HSP25, one of the mammalian small heat shock proteins (also known as HSP27) (14), at two sites, Ser15 and Ser86, in the mouse sequence (15, 46). Exposure of several cell types to Cd resulted in increased HSP25 phosphorylation (29, 44, 53), suggesting a role for the p38 MAPK/HSP25 signaling pathway in Cd toxicity. Several studies have also indicated that HSP25 and p38 MAPK/HSP25 signaling is involved in regulating the cell contraction: 1) fibroblasts overexpressing HSP25 showed a stronger contraction in response to LPA and PDGF than control cells (21), and inhibition of the p38 MAPK/HSP25 signaling using the specific inhibitor SB-203580 inhibited serum- and thrombin-stimulated fibroblast contraction (20); and 2) agonists (bombesin, endothelin-1, ANG II) that stimulated contraction of smooth muscle and mesangial cells also stimulated HSP25 phosphorylation (9, 39, 41, 62), and inhibition of p38 MAPK/HSP25 signaling inhibited this contraction (39, 41, 62). Recently, a total of 10 mutations in 3 human small heat shock proteins (αB-crystallin, HSP22, HSP25) have been identified, which cause severe neuromuscular and muscular disorders (7, 13, 25, 49, 57). Collectively, these findings prove...
the important role of small heat shock proteins in the proper function of several tissues including muscles and muscle-like cells such as mesangial cells.

In this study, we show that the Cd-induced contraction of both mesangial cells and isolated rat glomeruli occurs concomitantly with activation of p38 MAPK, phosphorylation of HSP25, and association of HSP25 with microfilaments. Phosphorylation of HSP25 occurs in an ordered way with Ser15 being phosphorylated before Ser86. Inhibition of p38 MAPK by a specific inhibitor prevents Cd-induced contraction of both mesangial cells and isolated glomeruli and also blocks phosphorylation of HSP25. These data support the hypothesis that contraction of mesangial cells in response to Cd is mediated, at least in part, by activation of the p38-MAPK/HSP25 signaling pathway and transduction of this signal to the microfilaments. This mechanism is likely to contribute to the glomerular contraction and reduction in glomerular filtration rate seen in Cd nephrotoxicity.

**MATERIALS AND METHODS**

**Stock solutions.** CdCl₂, 100 mM in water; thrombin, 5 U/ml in PBS; LPA, 20 mM in PBS; SB-203580, 25 mM in DMSO; PD-98059, 100 mM in DMSO; bis-indolylmaleimide III-HCl, 1 mM in PBS; chelerythrine·Cl, 15 mM in DMSO; H-89, 10 mM in DMSO; KT-5720, 2 mM in DMSO; KT-5823, 2 mM in DMSO; H1004, 10 mM in PBS; H1077, 50 mM in PBS; ML-7, 50 mM in DMSO; EGTA, 50 mM in 10X PBS (adjusted with NaOH to pH 7.4); BAPTA/AM, 50 mM in DMSO. All reagents were from Calbiochem. Stock solutions were further diluted as necessary. The maximum amount of DMSO added to cell cultures or glomeruli preparations was 0.1%.

**Cell line.** The mesangial cell line MES13 (ATCC) was maintained in F-12/DMEM medium containing 5% FCS and cultured at 37°C in a humidified, 5% CO₂ atmosphere. All substances to be tested were added to the cell cultures in complete medium unless otherwise indicated.

**Viability assay.** Cells were treated with 0, 1, 10, or 50 μM CdCl₂ for 2 h, washed with medium, and then incubated for up to 2 days in complete medium. Toxicity was evaluated after 1 and 2 days by determining total cell number and percentage of dead cells. The numbers of living and dead cells were determined using the Live/Dead Viability/Cytotoxicity kit (Molecular Probes).

**Cell contraction assay.** MES13 cells were treated for 60 min with CdCl₂ with or without pretreatment for 20 min with 20 μM SB-203580. After fixation in 3.7% paraformaldehyde, the planar surface area (PSA) of 100 cells per group was measured from phase-contrast digital images of randomly selected microscopic fields using National Institutes of Health (NIH) Image software. Experiments were performed in quadruplicate in a blinded manner.

**Collagen lattice contraction assay.** Rat tail type I collagen and collagen matrices were prepared as described (17). Cells were cultured in six-well plates in the attached matrix for 4 days. Three hours before the experiment, complete culture medium was replaced with 2 ml of serum-free medium. Ninety minutes after addition of serum or CdCl₂, collagen lattices were released from the dishes, permitting contraction. To some dishes, 20 μM SB-203580 was added 20 min before CdCl₂ treatment. Thirty minutes after detachment, lattices were fixed with paraformaldehyde and lattice area was measured. The decrease of the lattice area was termed contraction. Each group had three lattices assayed and experiments were performed in quadruplicate.

**Analysis of activation of the MAPKs Erk1/2, p38, and JNK.** Cells treated with CdCl₂ were harvested in 50 μl of buffer A (62.5 mM Tris·HCl, pH 6.8, 2% SDS, 10% glycerol, 200 mM DTT, 0.01% bromophenol blue, 1× protease inhibitor mix), and proteins were separated by SDS-PAGE followed by Western blot analysis. Detection of phosphorylated (activated) MAPKs was performed using anti-phospho-Erk1/2 monoclonal antibody (Calbiochem), rabbit anti-phospho-p38 MAPK antibody (Calbiochem), anti-phospho-JNK monoclonal antibody (Santa Cruz Biotechnology), and secondary antibodies conjugated with horseradish peroxidase (Jackson Laboratories).

**Preparation and use of wild-type and dominant-negative p38 MAPK adenovirus.** Recombinant adenoviruses expressing wild-type (wt)p38 MAPK and the corresponding dominant-negative (dn)p38 MAPK mutant (TGY->AGF), driven by a cytomegalovirus promoter, were obtained from Dr. Y. Wang (Los Angeles, CA). Characterization and preparation of these adenoviral vectors were reported previously (58).

For adenovirus infection, MES13 cells were plated 24 h before infection in six-well plates. At time of infection, cells were 60% confluent. After infection with 10¹⁰ plaque-forming units of wt-p38 MAPK or dn-p38 MAPK adenovirus per well, cells were incubated for 24 h in complete medium. Thereafter, the medium was replaced with serum-free DMEM/F-12 medium containing 10 μM CdCl₂. After 1 h, cells were harvested and processed for isoelectric focusing electrophoresis (IEF-PAGE)/Western blotting and HSP25 was visualized using the rabbit anti-HSP25 antibody (Stressgen).

**Cell fractionation.** Cell fractionation was performed as described (50). To extract soluble proteins, cells were treated with buffer B (10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100). Proteins from buffer B-soluble and -insoluble fractions were separated by SDS-PAGE followed by Western blot analysis using the rabbit anti-HSP25 antibody (Stressgen). For controls, the blots were also probed with anti-actin monoclonal antibody (Sigma), which recognizes all actin isoforms. Experiments were repeated three times.

**Immunofluorescence microscopy.** Cells were cultured on collagen-coated glass coverslips. If indicated, cells were pretreated with 20 μM SB-203580 for 20 min or treated directly with 50 μM CdCl₂ for 60 min. Cells were rinsed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. To extract soluble proteins, some cells were treated with buffer B for 2 min before fixation. Fixed cells were stained sequentially with a rabbit anti-HSP25 antibody (Stressgen) and anti-rabbit FITC-conjugated secondary antibody (Jackson) to visualize HSP25 and with rhodamine-phalloidin (Molecular Probes) to visualize F-actin. Cells were imaged using a Zeiss LSM510 confocal microscope with a ×60 1.2 numerical aperture water immersion lens.

**Isolation, incubation, and analysis of glomeruli.** Four kidneys from two female rats (~200 g) were harvested and glomeruli were isolated as described elsewhere (51, 52). Glomerular preparations were assessed by microscopic inspection and routinely contained >95% glomeruli. These preparations were used for both contraction assays and analysis of HSP25 phosphorylation. To assess glomerular contraction, 50 μl of glomeruli suspension were placed onto polylysine-coated 24-well plates and glomeruli were allowed to attach for 5 min. Then, 0.45 ml of Hank’s balanced salt solution (HBSS), containing 20 μM SB-203580 if indicated, were added and the glomeruli were equilibrated at 37°C for 20 min; 0.5 ml of HBSS containing 50 μM CdCl₂ and 20 μM SB-203580 (if indicated) were added, and the glomeruli were incubated at 37°C for an additional 90 min. At times 0 min (control), 30 min, and 90 min, microscopic images of 50 randomly selected glomeruli per group were obtained and PSA was determined. To analyze the effect of Cd on the phosphorylation of glomerular HSP25, 100 μl of glomeruli suspension were incubated for 20 min at 37°C in the absence or presence of 20 μM SB-203580. CdCl₂ was added yielding 50 μM final concentration, and the glomeruli were incubated for 90 min at 37°C. The glomeruli were recovered by centrifugation and processed for IEF-PAGE/Western blotting. HSP25 was visualized using the rabbit anti-HSP25 antibody (Stressgen).
Electrophoretic methods. SDS-PAGE/Western blotting (52) was used to determine the relative amounts of HSP25 in cell fractions and of MAPKs and actin in MES13 cells. IEF-PAGE/Western blotting (51) was used to determine the extent of phosphorylation of HSP25 in MES13 cells and in glomeruli. The size of oligomeric HSP25 complexes was analyzed by native PAGE/Western blotting using 7% nondenaturing Tris-polyacrylamide gels (16). For that purpose, cells were harvested after 60-min treatment in 50 µM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, sonicated, and the centrifuged cell extracts were processed for electrophoresis.

For detection of mouse and rat HSP25 (all isoforms) on PVDF membranes, the rabbit anti-HSP25 antibody (Stressgen) was used. For detection of phosphorylated Ser86 in mouse HSP25, a phosphorylation site-specific antibody (Biosource) was used. For detection of MAPKs and actin, antibodies were used as indicated. Protein bands were visualized using suitable secondary antibodies conjugated to horseradish peroxidase, the ECL chemiluminescence kit (Amersham), and X-ray films. For quantification, the band intensity was determined using the Bio-Rad scanning system (model GS-700) and NIH Image software. The ratio R of the band intensities (1) of the phosphorylated (HSP25/2, HSP25/3) and nonphosphorylated (HSP25/1) HSP25 isoforms was calculated from these intensities according to Eq. 1:

\[ R = \frac{I_{\text{HSP25/2}} + I_{\text{HSP25/3}}}{I_{\text{HSP25/1}}} \]  

Statistical analysis. Data are expressed as means ± SE. Unpaired Student’s t-test was applied to compare results between sample groups. Differences between groups were considered statistically significant if 0.05.

RESULTS

Toxicity of Cd for mesangial cells. To evaluate the toxicity of CdCl₂, mesangial cells (MES13) were treated for 2 h with 1, 10, and 50 µM CdCl₂ and cultured for 2 days in medium without CdCl₂. Toxicity was evaluated after 1 and 2 days by determining the total cell number and the percentage of dead cells (Fig. 1). As expected, 10 and 50 µM CdCl₂ slowed cell growth significantly, but the percentage of living cells after 1 day remained virtually constant (99.64 and 98.3%, respectively). This indicates that all tested doses of CdCl₂ can be considered sublethal, and the responses seen in this study were that of living cells.

Cd-induced contraction of mesangial cells. Cell contraction in response to CdCl₂ was measured using two assays, single-cell contraction and collagen lattice contraction. For single-cell contraction, the cell PSA was measured after 60 min of CdCl₂ exposure. The PSA was significantly reduced after treatment with either 10 or 50 µM CdCl₂ (as compared with the no treatment control), indicating cell contraction (Fig. 2A). To examine the role of the p38 MAPK, MES13 cells were pretreated with the specific inhibitor SB-203580 before the CdCl₂ treatment. No contraction was observed in cells pretreated with SB-203580 before CdCl₂ treatment compared with the no treatment control. Although SB-203580 alone resulted in a certain reduction of the PSA, this was not significant according to the used criteria and probably reflects the variability inherent to the used assay.

Contraction of MES13 cells in response to CdCl₂ was also evaluated using a collagen lattice contraction assay in which the cells are surrounded by extracellular matrix, a setting more similar to their in vivo milieu (17). Contraction of collagen lattices (stressed matrix contraction) is associated with cellular contraction (18) and reflects the collective activity of many cells, as opposed to the single-cell assay. Collagen lattices containing the cells were treated for 90 min with CdCl₂ with or without pretreatment with the specific p38 MAPK inhibitor SB-203580. Thereafter, the lattices were released to permit contraction; 10% FCS was used as positive control that resulted in a strong contraction compared with the negative control (no treatment) (Fig. 2B). Treatment with CdCl₂ caused a significant, dose-dependent contraction of lattices, with 50 µM CdCl₂ being almost as effective as 10% FCS. Lattice contraction induced by 50 µM CdCl₂ was completely inhibited.
by SB-203580. The inhibitor alone had no detectable effect on lattice contraction.

**Cd-induced MAPK activation.** Treatment with CdCl₂ resulted in activation of the three MAPKs analyzed (Fig. 3). Activated Erk1 (p44 MAPK), which was hardly detectable in the controls, was induced within 10 min by either 10 or 50 μM CdCl₂, and activation persisted for at least 90 min. Activated Erk2 (p42 MAPK) was present in controls and increased only slightly between 10 and 90 min after treatment with either 10 or 50 μM CdCl₂. The time course of p38 and JNK (p46/p54) MAPK activation differed from that observed for Erk MAPKs. The amount of activated p38 MAPK, p46 JNK, and p54 JNK increased only after 90 min of treatment with 10 μM CdCl₂ or 60 min of treatment with 50 μM CdCl₂. In summary, the Cd-induced activation kinetics are characteristic for each of the three analyzed MAPKs with regard to the time course, intensity, and isoform pattern.

**Cd-induced phosphorylation of HSP25.** Because SB-203580 pretreatment completely inhibited Cd-induced contraction of mesangial cells, we next examined HSP25 phosphorylation, an event downstream of the p38 MAPK, by IEF-PAGE/Western blotting. First, we tested the effect of three nontoxic agonists (FCS, thrombin, LPA) shown previously to induce lattice contraction associated with cell contraction (18, 20, 21) on HSP25 phosphorylation. As shown in Fig. 4A, in control cells the majority of HSP25 is the nonphosphorylated isoform (HSP25/1), with some mono-phosphorylated isoform (HSP25/2) also present. All agonists induced phosphorylation of HSP25, as judged from the appearance of the bis-phosphorylated isoform (HSP25/3) and the decrease in the relative intensity of HSP25/1 compared with HSP25/2. The amount of HSP25/2 appears to remain relatively constant. In response to FCS, an increase in phosphorylation of HSP25 occurred within 10 min, and longer exposure to FCS (90 min) did not further increase HSP25 phosphorylation. Treatment with CdCl₂ at a concentra-

![Fig. 3. Activation of MAPKs after treatment with CdCl₂. MES13 cells were treated with CdCl₂ for various times as indicated, and kinase activation was analyzed by SDS-PAGE/Western blotting using specific antibodies directed against the phosphorylated isoforms of the MAPKs. Right: positions of MAPKs.](image)

![Fig. 4. Phosphorylation of HSP25 after treatment with CdCl₂. After treatment of MES13 cells as indicated, the cell proteins were analyzed by IEF-PAGE/Western blotting using a specific anti-HSP25 antibody. A: effect of agents known to cause cell contraction on HSP25 phosphorylation. Cells were treated with 10% FCS (10 and 90 min), 0.5 U/ml thrombin (10 min), or 20 μM LPA (10 min). B: effect of various protein kinase inhibitors on the phosphorylation of HSP25 induced by CdCl₂. Cells were treated with 50 μM CdCl₂ for 30 min with or without a 20-min pretreatment with various protein kinase inhibitors as indicated. The applied inhibitor concentrations are given in MATERIALS AND METHODS. C: time course of HSP25 phosphorylation. Cells were pretreated (right) or not (left) for 20 min with SB followed by treatment with CdCl₂ as indicated. D: determination of the phosphorylation sites of HSP25. Cells were treated or not with 10 μM CdCl₂ for 90 min in serum-free DMEM/F-12 medium. Western blots of the IEF gels were developed with an antibody that is specific for phospho-Ser86 (AB2, right) or that recognizes all HSP25 isoforms (AB1, left). The position of the HSP25 isoforms (1, nonphosphorylated; 2, mono-phosphorylated; 3, bis-phosphorylated) is indicated. PD, PD-98059.](image)
or completely (10 M CdCl₂) blocked the increase in HSP25 phosphorylation by an independent method, a dn form of the p38 MAPK was expressed in MES13 cells by using an adenovirus expression system (58). For control, the wt form of the p38 MAPK was also expressed. After treatment of these cells with CdCl₂ (10 μM, 60 min), the phosphorylation of HSP25 was analyzed by IEF-PAGE/Western blotting. From the determined band intensities, the ratio of phosphorylated (HSP25/2 + HSP25/3) vs. nonphosphorylated HSP25 (HSP25/1) isoforms was calculated. In cells expressing dn p38 MAPK, treatment with CdCl₂ does not result in increased phosphorylation of HSP25, whereas in cells expressing wt p38 MAPK (control), the same treatment results in a strong increase in phosphorylation of HSP25 (Fig. 5). As expected, the dn p38 MAPK inhibits the Cd-induced phosphorylation by the endogenous p38 MAPK. This strongly suggests the involvement of the p38 MAPK signaling in the Cd-induced phosphorylation of HSP25 in mesangial cells.

Cd-induced reduction of the oligomeric size of HSP25. Changes in HSP25 phosphorylation are often coincident with changes in its oligomeric state (29, 46). Therefore, we analyzed the effects of CdCl₂ and SB-203580 on the size of HSP25 oligomers in MES13 cells using native PAGE/Western blotting (Fig. 6). In control cells, four HSP25-positive bands were detected, corresponding to molecular masses of ~400, 250, 180, and 150 kDa. Pretreatment with 20 μM CdCl₂ for 60 min with (+) or without (−) a 20-min pretreatment with 20 μM SB and the size of HSP25 complexes was analyzed by nondenaturing PAGE/Western blotting using anti-HSP25 antibody. The positions of molecular mass markers are indicated at right, and the calculated size of the HSP25 complexes (as multiples of the monomer) is indicated at left.

Fig. 6. Oligomeric state of HSP25 after treatment with CdCl₂. MES13 cells were treated with CdCl₂ for 60 min with (+) or without (−) a 20-min pretreatment with 20 μM SB and the size of HSP25 complexes was analyzed by nondenaturing PAGE/Western blotting using anti-HSP25 antibody. The positions of molecular mass markers are indicated at right, and the calculated size of the HSP25 complexes (as multiples of the monomer) is indicated at left.
200, and 150 kDa. Treatment with CdCl₂ resulted in complete loss of the 400- and 250-kDa bands and appearance of lower molecular mass bands. The size reduction of HSP25 oligomers observed after CdCl₂ treatment was blocked by SB-203580.

Cd-induced translocation of HSP25. HSP25 has been reported to associate with the cytoskeleton under stress conditions. To learn about the possible mechanism of the Cd-induced mesangial cell contraction, we analyzed the effect of CdCl₂ with regard to both the amount of HSP25 in the Triton X-100-insoluble fraction and the intracellular localization of HSP25. When cells were treated with Triton X-100-containing buffer, unbound cytosolic proteins were extracted and only the proteins bound to structures resistant to the detergent (e.g., nucleus, cytoskeleton) remained in the insoluble fraction. Most of the HSP25 partitioned into the Triton X-100-soluble fraction in control cells, and only a minor fraction was found to be associated with the cytoskeleton-enriched, Triton X-100-insoluble proteins (Fig. 7A). Treatment with CdCl₂ resulted in increased amounts of HSP25 in the Triton X-100-insoluble fraction, while the amount of HSP25 in the soluble fraction remained apparently unchanged (due to the relatively large amount of cellular HSP25 in this fraction). Pretreatment with SB-203580 prevented this increase in insoluble HSP25. For control purposes, the same blots were reprobed with an anti-actin antibody to confirm equal sample loading.

The localization of HSP25 was analyzed by immunofluorescence microscopy in cells doubly labeled for HSP25 and actin (Fig. 7B). In control cells, HSP25 was evenly distributed throughout the cytoplasm, with little staining in the nuclei. F-actin was observed in subcortical structures and stress fibers, and no colocalization of HSP25 and actin was seen. Actin and HSP25 staining in cells treated with 50 μM CdCl₂ did not appear significantly different from controls. To analyze changes in the small fraction of HSP25 that associated with the cytoskeletal fraction, soluble proteins (including the majority of HSP25) were extracted with buffer B before fixation. In control cells, the remaining fraction of HSP25 labeling was in nuclei and cytosolic foci of undetermined nature. There was no apparent colocalization of Triton X-100-insoluble HSP25 with F-actin in control cells. After treatment with 50 μM CdCl₂, HSP25 localization to nuclei and cytosolic foci remained unchanged; however, there was an obvious colocalization of HSP25 with F-actin. Pretreatment with SB-203580 before treatment with CdCl₂ resulted in less colocalization of HSP25 with F-actin, although a few cells showed some colocalization of HSP25 with F-actin (data not shown). HSP25 did not colocalize with either microtubules or vimentin intermediate filaments in control or CdCl₂-treated cells (data not shown). Together, these data suggest that CdCl₂ treatment of mesangial cells results in an association of a small but significant fraction of HSP25 with microfilaments.

Cd-induced glomerular contraction and phosphorylation of glomerular HSP25. Finally, we examined whether the response of MES13 cells to CdCl₂ is representative of the behavior of mesangial cells in intact glomeruli. Glomeruli contain endothelial cells and podocytes in addition to mesangial cells, and all three cell types contain substantial amounts of HSP25 (41, 42, 51, 52); however, only mesangial cells are contractile cells. Rat glomeruli were isolated according to Smoyer et al. (51, 52) and microscopic inspection of control and treated glomeruli did not reveal any obvious lesion possibly caused by these procedures (representative example is shown in Fig. 8, inset). The isolated glomeruli were treated with 50 μM CdCl₂ with or without pretreatment with 20 μM SB-203580, and the PSA of glomeruli was measured. Treatment of the glomeruli with CdCl₂ for 30 and 90 min caused a significant contraction. Although the treatment (90 min) with SB-203580 alone caused some contraction on its own, it clearly and significantly inhibited the Cd-induced contraction (Fig. 8, left).

The HSP25 fraction that was phosphorylated in untreated glomeruli was somewhat greater than in untreated MES13 cells. However, treatment with 50 μM CdCl₂ resulted in complete phosphorylation of the glomerular HSP25. As in MES13 cells, preincubation of the glomeruli with SB-203580 reduced the HSP25 phosphorylation compared with control or CdCl₂-treated glomeruli (Fig. 8, right).

DISCUSSION

Exposure to Cd has been implicated in renal dysfunction. Initial Cd-induced renal dysfunction is manifest as tubular-type proteinuria, while continued exposure to Cd leads to glomerular damage (26–28). Cd nephrotoxicity is associated with alterations of intrarenal hemodynamics and glomerular filtration rate, and Cd-induced contraction of mesangial cells has been proposed as a mechanism whereby the effective renal filtration surface and size of the glomerular capillaries may be altered (4, 27, 45). Isolated rat glomeruli and cultured mesangial cells that retain their contractile properties in vitro have been shown to be suitable models to study the impact of nephrotoxic agents (4, 5). In the present study, we used two assays to measure the Cd-induced contractile response of mesangial cells, confirming earlier reports (4, 5). The applied doses of CdCl₂ in the current study did not cause significant cell death, suggesting that the contractile behavior of mesangial cells is a specific response of live cells, rather than a general “toxic” response.

Cd is known to interfere with signaling pathways involving intracellular Ca, including PKC and MLCK-related signaling (11). Although these processes are likely to be involved in Cd-induced cell contraction, the fact that the highly specific inhibitor of p38 MAPK alone blocks contraction suggests that this signaling pathway is necessary, although not sufficient, for...
Cd-induced mesangial cell contraction. Using different experimental systems, others have shown that the p38 MAPK is activated by Cd (29, 54) and that p38 MAPK signaling plays a role in contraction of mesangial cells in response to ANG II (41) and of smooth muscle cells in response to ANG II, bombesin, and endothelin-1 (9, 39, 62). Thus our data suggesting an involvement of p38 MAPK in Cd-induced contraction of mesangial cells are in agreement with these findings.

Usually the p38 MAPK signaling pathway is activated by oxidative stress factors (e.g., TNF, H₂O₂) within a short time period of ~10 min (23). In contrast, the Cd-mediated activation took more time (~60–90 min in Fig. 3). This difference is probably due to the complexity of the cellular response to Cd, with several possible, although unknown, Cd-induced signaling events upstream of the p38 MAPK.

Exposing mesangial cells to CdCl₂ resulted in activation of both Erk1/2 MAPK isoenzymes within 10 min (Fig. 3), a finding similar to previously described results (60). PD-98059, a specific inhibitor of MEK, the upstream activator of Erk1/2, also inhibited cell contraction but to a lesser extent than did SB-203580 (not shown), which suggests some role also for Erk1/2 MAPK in mesangial cell contraction. Another protein kinase, MLCK, is also involved in the Cd-induced cell contraction, as the specific inhibitor ML-7 partially inhibited this contraction (not shown). Both inhibitors, PD-98059 and ML-7, however, had no effect on phosphorylation of HSP25 in the current study (Fig. 4B). Similar findings were reported for the contraction of fibroblasts (20). These data suggest that 1) Cd-induced cell contraction involves, to a certain extent, also MEK and MLCK signaling, and 2) these processes are independent of the p38 MAPK/HSP25 pathway. The third tested MAPK, JNK, was also activated by CdCl₂ (Fig. 3). However, no other information is available on a possible involvement of JNK in the contractile response.

A key remaining question is the possible role of HSP25 in cell contraction. Agonists such as FCS, thrombin, LPA, or ANG II, which are known to induce contraction of mesangial cells, also stimulated HSP25 phosphorylation (Fig. 4A). In the current study, three facts strongly suggest the signal transduction from the p38 MAPK via MK2/3 to HSP25: 1) the similarity of the time course for Cd-induced phosphorylation of HSP25 and p38 MAPK activation, 2) the inhibition of HSP25 phosphorylation by the p38 MAPK, and 3) the inhibition of HSP25 phosphorylation by the p38 MAPK. Other known substrate proteins of p38 MAPK (transcription factors ATF2, MEF2C, Elk1; protein kinases PRAK, MNK1/2, MSK1/RLPK, RSK-B) are not known to play a direct role in cell contraction (35) and are unlikely to account for the relatively rapid response to Cd described in this study and by others (4, 5). We are unaware of other MK-2/3 substrates with relevance to the actin-myosin system, which is the known mechanism of smooth muscle contraction. Thus, despite pleiotropic signaling pathways downstream of p38 MAPK and MK-2/3, phosphorylation of HSP25 remains the most likely process accounting for Cd-induced mesangial cell contraction. In support of this conclusion, other reports have also provided arguments for the involvement of HSP25 in contraction of smooth muscle and mesangial cells (9, 24, 41, 62).

It is still difficult to fully explain at the molecular level the involvement of HSP25 in cell contraction. A number of studies report the association of HSP25 with actin. Overexpression of wild-type or mutant HSP25 that mimics phosphorylated HSP25 in cultured cells increased the amount of F-actin at the cell cortex and in stress fibers and enhanced the microfilament dynamics and cell motility (19, 31, 32, 38). HSP25 fractionated with the membrane-associated actin cytoskeleton where dy-
namic actin rearrangements occur continuously (43), and it formed bead-like structures (foci) that alternate with focal actin structures in the cortical submembrane region of Madin-Darby canine kidney cells (50). Various stress treatments lead to a disruption of microfilaments, and overexpression of HSP25 has been shown to protect microfilaments or accelerate recovery (1–3, 22). Phosphorylation of HSP25 has been shown to be essential for the protection of cell architecture (31, 33, 42, 43, 48). In an effort to provide an explanation for this role of HSP25, it was recently suggested that the small phosphorylated HSP25 oligomers would exert a protective effect by coating microfilaments (40). Indeed, a number of observations favor this view. First, with an increase in HSP25 phosphorylation, HSP25 complexes frequently dissociate into smaller complexes (29, 46), as we observed in this study in Cd-treated cells (Fig. 6). Second, HSP25 colocalizes in some cell types with structures containing actin, such as in the stress fibers of Sertoli cells (61) or in sarcomeres of cardiomyocytes (36). HSP25 translocated to microfilaments in renal ischemia (3) and to the I-band in heat-stressed neonatal cardiomyocytes (56). After translocation, this fraction of HSP25 may well modulate the microfilament length.

Using a phosphorylation site-specific antibody, we showed that the mono-phosphorylated isofrom HSP25/2 is essentially not phosphorylated at Ser86, which implies phosphorylation at Ser15. Cd treatment resulted in the formation of the bis-phosphorylated isoform HSP25/3, with both Ser15 and Ser86 being phosphorylated. These results also suggest that HSP25 is phosphorylated in a sequential way: Ser15 is phosphorylated before Ser86. A possible explanation is that phosphorylation of Ser15 induces a conformational change that subsequently exposes Ser86 to the MAPKAPK-2. The consequence of phosphorylation of Ser15 then may be the disassembly of HSP25 complexes (see Fig. 6). This finding is in agreement with previous studies, in which the crucial role of phosphorylation of hamster Ser90 (corresponds to mouse Ser86) in the disassembly of HSP25 complexes has been identified (30, 55).

In conclusion, we found that exposure of mesangial cells to CdCl2, which causes cell contraction, results in phosphorylation of HSP25 (with Ser15 being phosphorylated before Ser86), a decrease in the size of HSP25 oligomers, and translocation of a small but detectable fraction of HSP25 from the cytosolic to the cytoskeletal fraction, where it colocalizes with microfilaments. These Cd-induced effects are mediated by p38 MAPK. The decoration of actin filaments with HSP25 might induce or enhance contraction of the mesangial cells, which could then alter the hemodynamic properties of glomeruli. The schematic drawing of this possible mechanism is shown in Fig. 9. If confirmed, this model would represent a change in paradigm with respect to the action of HSP25: in addition to its well-established cytoprotective function, HSP25 could be also part of a sequence of events that eventually leads to the detrimental effects as seen in response to the metal toxicant Cd. However, to link these findings directly to clinical nephrotoxicity, a separate study is required. These data also suggest that inhibition of the p38 MAPK/HSP25 signaling might be a useful future strategy to reduce Cd-associated renal glomerular toxicity.

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CADMIUM-INDUCED MESANGIAL CELL CONTRACTION


