

## Renal upregulation of HO-1 reduces albumin-driven MCP-1 production: implications for chronic kidney disease

Narayana S. Murali,<sup>1</sup> Allan W. Ackerman,<sup>1</sup> Anthony J. Croatt,<sup>1</sup> Jingfei Cheng,<sup>2</sup> Joseph P. Grande,<sup>1,2</sup> Shari L. Sutor,<sup>3</sup> Richard J. Bram,<sup>3,4</sup> Gary D. Bren,<sup>5</sup> Andrew D. Badley,<sup>5,6</sup> Jawed Alam,<sup>7</sup> and Karl A. Nath<sup>1</sup>

<sup>1</sup>Division of Nephrology and Hypertension, <sup>2</sup>Department of Laboratory Medicine and Pathology, <sup>3</sup>Department of Pediatrics and Adolescent Medicine, <sup>4</sup>Department of Immunology, <sup>5</sup>Division of Infectious Diseases, <sup>6</sup>Program in Translational Immunovirology and Biodefense, Mayo Clinic College of Medicine, Rochester, Minnesota; and <sup>7</sup>Department of Molecular Genetics, Alton Ochsner Medical Foundation, New Orleans, Louisiana

Submitted 5 July 2006; accepted in final form 5 September 2006

**Murali NS, Ackerman AW, Croatt AJ, Cheng J, Grande JP, Sutor SL, Bram RJ, Bren GD, Badley AD, Alam J, Nath KA.** Renal upregulation of HO-1 reduces albumin-driven MCP-1 production: implications for chronic kidney disease. *Am J Physiol Renal Physiol* 292: F837–F844, 2007. First published September 12, 2006; doi:10.1152/ajprenal.00254.2006.—Proteinuria contributes to chronic kidney disease by stimulating renal tubular epithelial cells to produce cytokines such as monocyte chemoattractant protein-1 (MCP-1). The present study determined whether cellular overexpression of heme oxygenase-1 (HO-1) can influence albumin-stimulated MCP-1 production. In response to bovine serum albumin, NRK-52E cells constitutively overexpressing HO-1 (HO-1 OE cells) exhibit less induction of MCP-1 mRNA and less production of MCP-1 protein compared with similarly treated, control NRK-52E cells (CON cells). In wild-type NRK-52E cells, and under these conditions, we demonstrate that the induction of MCP-1 is critically dependent on intact NF- $\kappa$ B binding sites in the MCP-1 promoter. In response to albumin, CON cells exhibit activation of NF- $\kappa$ B, and this is reduced in HO-1 OE cells. Albumin also activates ERK1/2 and increases ERK activity, both of which are exaggerated in HO-1 OE cells. Studies with an inhibitor of MAPK/ERK kinase (U0126) demonstrate that the inhibitory effects of U0126 on MCP-1 production are attenuated in HO-1 OE cells. We conclude that HO-1 overexpression in the proximal tubule reduces MCP-1 production in response to albumin, and this occurs, at least in part, by inhibiting an ERK-dependent, NF- $\kappa$ B-dependent pathway at a site that is distal to the activation of ERK. These findings suggest that the induction of HO-1 in the proximal tubule, as occurs in chronic kidney disease, may be a countervailing response that reduces albumin-stimulated production of cytokines such as MCP-1.

proteinuria; extracellular signal-regulated kinase 1/2; tubulointerstitial disease; cytoprotection

EMPLOYED AS A DIAGNOSTIC INDEX for intrinsic kidney disease, proteinuria is also a prognostic index of considerable reliability: the severity of proteinuria commonly predicts the risk for progressive kidney disease and the rate at which kidney function is lost (4, 11, 15, 25, 32, 42, 58). This faithfulness of proteinuria as a prognostic index for kidney disease reflects, at least in part, pathogenetic pathways that are predictably entrained when abnormal amounts of protein leak across the glomerular filtration barrier and into the urinary space (4, 11, 15, 25, 32, 42, 58). Such appearance of albumin and other

proteins in the urinary space leads to the incorporation of these proteins by the proximal tubule, and, once within proximal tubular epithelial cells, albumin and other proteins can instigate intracellular signaling and other pathways that culminate in the elaboration of inflammatory and fibrogenic cytokines (4, 11, 15, 25, 32, 42, 58). Intracellular uptake of filtered proteins by the proximal tubule, for example, can impose oxidative stress, recruit the MAPK, JAK/STAT, and other signaling pathways, and activate transcription factors such as NF- $\kappa$ B and activator protein-1, with the attendant elicitation of damaging cytokines, chemokines, and other species (16, 26, 30, 42, 46, 47, 53, 54, 58).

One such chemokine is monocyte chemoattractant protein-1 (MCP-1), the latter present in increased amounts in the kidney and urine in diverse types of kidney disease (10, 13, 27, 40, 52, 57). MCP-1 is one of the most potent chemoattractants ever described for monocytes and is also an inducer of T cells and natural killer cells (5, 6, 8). Independent of its chemoattracting effects on leukocytes, MCP-1 can induce transforming growth factor- $\beta$ 1, a potent fibrogenic cytokine, in resident kidney cells (56), and MCP-1 is itself directly proinflammatory on the proximal tubule, in part, by activating NF- $\kappa$ B (48, 49). Indeed, in assorted types of chronic kidney disease, the expression of MCP-1 in the kidney or the amounts of MCP-1 in the urine predict the severity of tubulointerstitial disease and the risk for loss of kidney function (10, 13, 27, 40, 52, 57).

Our laboratory's prior studies noted that MCP-1 is significantly upregulated under unstressed and stressed states in the kidney in mutant mice unable to express heme oxygenase-1 (HO-1) (36, 39). HO-1 is the inducible isozyme of the major heme-degrading enzyme, HO. This isozyme is induced by diverse stressors and insults to the kidney and other tissues, and HO-1 often confers a protective response in injured tissue by interrupting inflammatory, ischemic, immune-mediated, and other pathways of injury (1–3, 18, 19, 31, 33, 45). In assorted types of human kidney disease and in diverse experimental models of kidney injury, HO-1 is upregulated in the diseased kidney, localizing mainly in the proximal and distal tubules (1–3, 14, 18, 19, 23, 28, 29, 31, 33, 37, 38, 45, 50). Such expression of HO-1 in the proximal tubule in proteinuric human kidney disease correlates with the severity of proteinuria, hematuria, and tubulointerstitial disease (28, 37, 38, 44).

Address for reprint requests and other correspondence: Karl A. Nath, Mayo Clinic College of Medicine, 200 First St., SW, Guggenheim 542, Rochester, MN 55905 (e-mail: nath.karl@mayo.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

However, to date, the functional significance of such expression of HO-1 in the proximal tubule in proteinuric kidney disease is unknown: no data are available that address whether such upregulation of HO-1 by the proximal tubule is a response that vitiate, exacerbates, or bears little if any functional relevance to the injurious effects of proteinuria on the proximal tubule.

The present study was initiated in an attempt to address this issue. This study utilized an established model system whereby the damaging effects of protein, such as albumin, on the proximal tubule can be analyzed. For example, as previously shown by others and as recently demonstrated by us, the exposure of the proximal tubular epithelial cells to bovine serum albumin (BSA) leads to a robust tubular induction of MCP-1 (38, 46, 53, 54). To simulate the sustained upregulation of HO-1 in the proximal tubule, as observed in human kidney disease and in experimental models of kidney disease, we engineered an established proximal tubular epithelial cell line to constitutively overexpress HO-1 (HO-1 OE cells). We then examined the response of these HO-1 OE cells to albumin, using the attendant expression of MCP-1 as a representative, clinically relevant, inflammatory response.

## METHODS

**Cell culture.** NRK-52E cells (ATCC, Manassas, VA) were cultured at 37°C in 95% air–5% CO<sub>2</sub> in DMEM (Invitrogen, Carlsbad, CA) containing 4.5 g/l glucose, 10% heat-inactivated FBS (Atlanta Biologicals, Lawrenceville, GA), and the following supplements (Mediatech, Herndon, VA): 20 mM HEPES, 1 mM sodium pyruvate, 100 U/ml of penicillin, and 100 µg/ml streptomycin. Similarly, HO-1 overexpressing NRK-52E cells (HO-1 OE cells) and corresponding control cells (CON cells) were cultured in the medium above with the addition of 100 µg/ml G418 (Mediatech). Cells were grown to confluence, after which the serum-containing medium was removed. Cells were then exposed to the BSA-containing medium, and cells or conditioned media were studied after the duration of exposure, as indicated for each protocol. For incubation with BSA (15 mg/ml, A8806, Sigma-Aldrich, St. Louis, MO) and other treatments, serum-free DMEM with 20 mM HEPES (test medium) was used. In some studies, the MAPK/ERK kinase (MEK) inhibitor U0126 (46) (Promega, Madison, WI) was employed, with an initial concentrated stock solution dissolved in DMSO, followed by dilution to final concentration with test medium.

**HO-1 overexpression in NRK-52E cells.** Plasmid pSFFV/HO-1 was constructed by insertion of the rat HO-1 cDNA downstream of the Friend spleen focus-forming virus 5' long-terminal repeat in the expression vector pSFFV/neo. NRK 52E cells were stably transfected with pSFFV/neo (CON cells) or pSFFV/HO-1 (HO-1 OE cells), by methods previously described in detail (21). Transfectants were selected in G418, and individual clones were selected by limited dilution. Western analysis was used to confirm HO-1 expression, and HO activity was determined in microsomal preparations from CON and HO-1 OE cells by determining the rate of generation of bilirubin, as previously described (22).

**Northern analysis for MCP-1 mRNA expression.** CON and HO-1 OE cells were washed with PBS, and total RNA was extracted using the TRIzol method (Invitrogen). Ten micrograms of total RNA were separated on an agarose gel and transferred to a nylon membrane. Membranes were hybridized overnight with a <sup>32</sup>P-labeled rat MCP-1 cDNA probe. Autoradiograms were evaluated for loading and transfer by assessing the density of the 18S rRNA on an ethidium bromide-stained membrane, as previously described (34).

**ELISA for MCP-1 protein expression.** Production of MCP-1 protein by CON and HO-1 OE cells in response to BSA was measured in

conditioned media using an ELISA kit (BD Pharmingen, San Diego, CA), according to the manufacturer's protocol. Results were standardized against total cellular protein determined using a Lowry-based assay kit (BioRad Laboratories, Hercules, CA).

**Western analysis.** Cells were washed with cold PBS and lysed in a buffer consisting of PBS, pH 7.4, 1% Igepal CA-630, 0.1% SDS, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, protease inhibitor cocktail (Complete, Roche, Indianapolis, IN), and phosphatase inhibitor cocktail (Sigma-Aldrich). Lysates were collected by scraping, sonicated for 10 s, and centrifuged at 18,000 g to pellet the insoluble debris. Western analysis was performed on the resulting supernatants, as previously described in detail (17, 35). Briefly, 25 µg of protein were separated on 10% Tris·HCl gels (BioRad) and transferred to polyvinylidene difluoride membrane. Polyclonal antibodies, obtained from Cell Signaling Technology (Danvers, MA), were employed as primary antibodies for the analysis of the levels of p42/44 (no. 9102), phospho-p42/44 (no. 9101), JNK/SAPK (no. 9252), phospho-JNK/SAPK (no. 9251), p38 (no. 9212), phospho-p38 (no. 9211), STAT1 (no. 9172), phospho-STAT1 (no. 9171), phospho-STAT5 (no. 9351), and phospho-JAK2 (no. 3771). Additionally, a monoclonal antibody for STAT5 (no. 610192, BD Pharmingen) and polyclonal antibodies for HO-1 (SPA-895, StessGen, Ann Arbor, MI), JAK2 (no. 06–255, Upstate, Lake Placid, NY), and BSA (BioVision, Mountain View, CA) were also used. Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse polyclonal antibodies were used as secondary antibodies, as appropriate, and detection was achieved using a chemiluminescence method (enhanced chemiluminescence, Amersham Pharmacia Biotech, Piscataway, NJ).

**ERK kinase assay.** The ERK kinase assay was performed in CON and HO-1 OE cells exposed to BSA using a previously employed method (55). After incubation for the indicated times at 37°C, the cells were lysed, and postnuclear supernatants were incubated with 15 µl packed protein A-Sepharose beads and 2 µl anti-ERK2 antibody for 1 h at 4°C, and the immunoprecipitates were washed, followed by resuspension in kinase buffer (30 mM Tris·HCl, 0.1 mM EGTA, 10 mM magnesium acetate, 1 mM β-glycerophosphate, pH 7.4, containing 1 mM DTT, and 5 mM *n*-octyl β-D-glucopyranoside). ERK kinase reactions were initiated with 20 µl of kinase buffer containing 10 µM ATP, 5 µg myelin basic protein, and 10 µCi [<sup>32</sup>P]ATP. After 10 min at 30°C, the reactions were terminated with 8 µl 4× SDS sample buffer and heated at 100°C for 5 min. The reaction products were separated by SDS-PAGE through a 15% polyacrylamide gel. The proteins were transferred to Immobilon P, and incorporation of radioactivity into myelin basic protein was assessed by autoradiography.

**MCP-1 promoter analysis with mutant NF-κB sites.** The MCP-1 promoter and flanking region were isolated from rat genomic DNA as described (7). Primers were based on GenBank accession no. AF079313. The forward primer was 5'-TGTGAGAGCTGCTTGCTGTAAC-3' (nucleotide 1220–1242), and the reverse primer was 5'-TCTGGCTTCAGTGAGAGTTGGC-3' (nucleotide 3635–3614). The genomic PCR product was sequenced and subcloned into pGL3-Basic, a promoter-less firefly luciferase reporter (Promega). A truncated MCP-1 construct with deletion of the two NF-κB binding sites in the distal enhancer region (AF079313 base pairs 1293–1302 and 1319–1328) was produced by amplification of base pair 1335–3635 using the forward primer 5'-TTATCCTACTCTGCCTCTG-3' and the reverse primer described above. The PCR products were sequenced and subcloned into pGL3-Basic. NRK-52E cells were cotransfected with the MCP-1 promoter construct (350 ng) or the NF-κB deletion MCP-1 promoter construct (350 ng), and pRG-Basic (45 ng) to control for transfection efficiency. Transfections were performed using FuGENE 6 Transfection Reagent (Roche Molecular Biochemical, Indianapolis, IN), according to the manufacturer's instructions. Eighteen hours after transfection, BSA was added, 24 h after which cells were rinsed and lysed. Luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

**Electrophoretic mobility shift assay.** Activation of NF- $\kappa$ B was assessed by electrophoretic mobility shift assay (EMSA), as previously employed, and currently modified by our laboratory (24, 36, 39). Following incubations, cells were washed, collected by centrifugation, and resuspended in 400- $\mu$ l buffer consisting of 10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM MgCl<sub>2</sub>, and 0.1 mM EDTA, and containing the following protease inhibitors: 0.5 mM DTT, 10  $\mu$ M pepstatin, 1  $\mu$ M PMSF, 0.05  $\mu$ g/ml leupeptin, and 10  $\mu$ M aprotinin. Cells were lysed with the addition of 50  $\mu$ l 10% Nonidet P-40, mixed by inversion, and incubated on ice for 5 min. Nuclei were resuspended in an extraction buffer (50 mM HEPES, pH 7.9, 300 mM NaCl, 50 mM KCl, 10% glycerol, and protease inhibitors as above) and mixed vigorously, after which the nuclei were pelleted. The extracted nuclear protein was quantified using a Bradford-based reagent (Bio-Rad) and used for EMSA. Five micrograms of nuclear extract were added to binding reactions containing the following reagents: 20 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.05 mM EDTA, 0.5 mM DTT, 3% glycerol, 0.2 mg/ml poly(dI+dC), and 50,000 counts/min of [ $\gamma$ -<sup>32</sup>P]ATP-labeled NF- $\kappa$ B probe (catalog no. E3291, Promega) and incubated at room temperature for 30 min. The binding reactions were analyzed by electrophoresis on a 5% non-denaturing polyacrylamide gel and visualized by autoradiography. Supershift analysis was performed using a concentrated anti-p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, catalog no. sc-109): 1  $\mu$ l of antibody was added to 5  $\mu$ g of nuclear extract and allowed to incubate on ice for 15 min before the binding reaction.

**Assessment of apoptosis.** The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay was used to evaluate apoptosis in CON and HO-1 OE cells exposed to BSA for 18 h. The assay was performed employing the Apoptag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA), as previously described in detail (12, 17, 38).

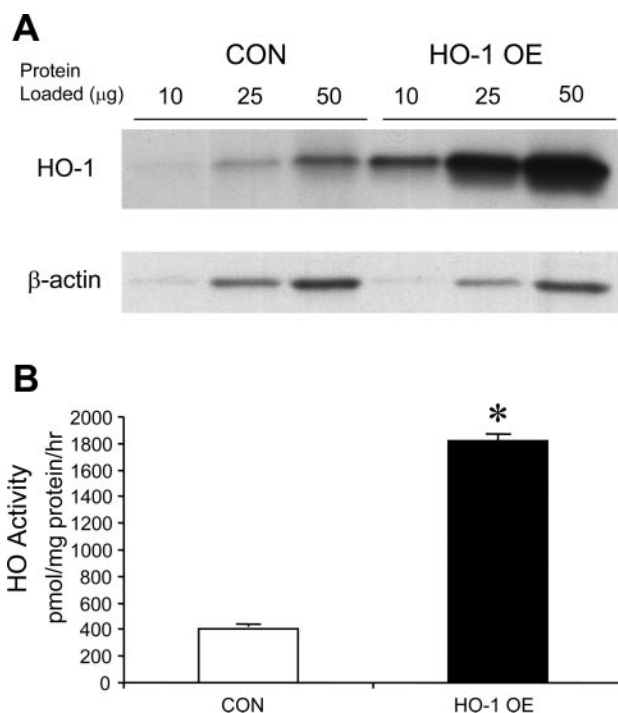


Fig. 1. Characterization of heme oxygenase-1 (HO-1) expression/HO activity in control (CON) and HO-1 overexpressing (HO-1 OE) cells. **A:** Western analysis for the expression of HO-1 in CON and HO-1 OE cells as increasing amounts of protein are loaded onto the gel. Equivalency of loading is confirmed by  $\beta$ -actin. **B:** HO activity as determined by the rate of bilirubin generation by microsomes extracted from CON and HO-1 OE cells,  $n = 4$  in each group. \* $P < 0.05$  vs. CON.

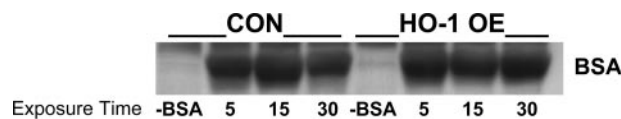


Fig. 2. Assessment of uptake and content of albumin in CON and HO-1 OE cells exposed to bovine serum albumin (BSA). Western analysis for the expression of BSA was performed in cells exposed to BSA-free and BSA-containing media for 5, 15, and 30 min.

**Statistics.** Results are expressed as means  $\pm$  SE and are considered statistically significant for  $P < 0.05$ . For comparison between unpaired groups, the Student  $t$ -test or the Mann-Whitney test was employed as appropriate.

## RESULTS

We first confirmed that the HO-1 OE cells exhibit increased expression of HO-1. As demonstrated in Fig. 1, expression of HO-1 protein along with HO activity were markedly increased in these cells compared with CON cells.

In our studies, we established equivalency of loading of albumin by CON and HO-1 OE cells. As demonstrated in Fig. 2, comparable amounts of albumin were incorporated and detected in these cells, as assessed at three different time points.

Albumin-driven induction of MCP-1 differed in CON and HO-1 OE cells (Fig. 3). Albumin markedly stimulated MCP-1 mRNA expression in CON cells, whereas MCP-1 mRNA expression in HO-1 OE cells following exposure to albumin was reduced by 63%. This stimulation of intracellular MCP-1 mRNA in CON cells was accompanied by markedly increased amounts of extracellular MCP-1 protein; the latter was also significantly reduced in albumin-stimulated HO-1 OE cells (Fig. 4). In these studies, we assessed cell viability by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay; apoptosis was not induced by albumin, either in the CON or HO-1 OE cells (data not shown).

We next sought to determine the mechanisms underlying the basis for such reduction of MCP-1 production. Depending on the experimental setting and stimulus, a number of transcription factors are incriminated in inducing expression of the MCP-1 gene. We first determined the extent to which NF- $\kappa$ B activation determines MCP-1 expression in our model system. Studies were undertaken in which the wild-type MCP-1 promoter or the MCP-1 promoter with mutated NF- $\kappa$ B binding sites were transfected into wild-type NRK-52E cells and exposed to albumin. As a positive control, we assessed the effect of the cytokine, TNF- $\alpha$ , on MCP-1 expression. As demonstrated in Fig. 5, the upregulation of the wild-type MCP-1

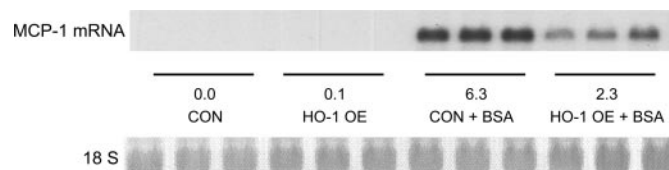


Fig. 3. Northern analysis for the expression of monocyte chemoattractant protein-1 (MCP-1). CON and HO-1 OE cells were exposed to BSA-containing or BSA-free media for 8 h, after which total RNA was extracted and Northern analysis performed for the expression of MCP-1. The numbers below the Northern analysis are the mean standardized densitometric values for each group. Equivalency of loading is assessed by 18S rRNA.

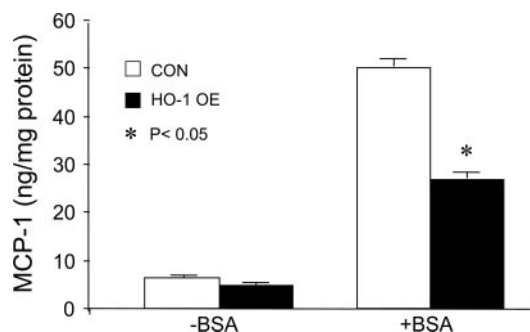


Fig. 4. Production of MCP-1 protein by CON and HO-1 OE cells exposed to BSA-containing (+BSA) or BSA-free (-BSA) media. MCP-1 was measured in conditioned media after 18 h of exposure to either BSA-containing (+BSA) or BSA-free (-BSA) media. Data for CON and HO-1 OE cells are shown in open and solid bars, respectively;  $n = 4$  in each group. \* $P < 0.05$ , BSA-treated CON cells vs. BSA-treated HO-1 OE cells.

promoter by TNF- $\alpha$  was completely dependent on NF- $\kappa$ B. Albumin also elicited upregulation of MCP-1 that was dominantly dependent on activation of NF- $\kappa$ B: the MCP-1 promoter with mutated NF- $\kappa$ B binding sites evinced little, if any, response to albumin.

Since NF- $\kappa$ B is critically required for the induction of MCP-1 in this model system, we examined, using EMSA, the binding capacity of NF- $\kappa$ B in these cells following exposure to albumin. As shown in Fig. 6, albumin induced a time-dependent increase in NF- $\kappa$ B binding in CON cells, most notably after 60 min of exposure, and such binding was reduced in HO-1 OE cells. To confirm the presence of this transcription factor in the shifted band, an anti-p65 antibody was added to the extracts before the binding reaction; in both CON and HO-1 OE cells, this band was supershifted in the presence of the anti-p65 antibody.

Recent studies have incriminated the activation of ERK1/2 (p42/44) and the JAK/STAT pathways in driving cytokine expression in response to albumin (9, 41, 46). Our initial studies demonstrated that, in our model system, activation of ERK1/2 occurred in response to albumin, whereas activation of JAK/STAT did not (data not shown); albumin also failed to upregulate p38 and p46 (data not shown). We thus focused on ERK1/2 and determined whether a differential response with regards to ERK1/2 occurred in CON and HO-1 OE cells. As shown in Fig. 7, in CON cells, albumin provoked an early and

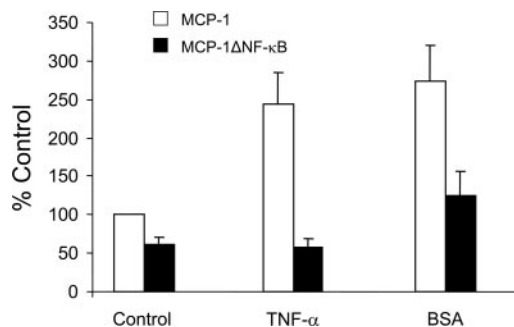


Fig. 5. Activity of wild-type MCP-1 promoter and MCP-1 promoter with mutated NF- $\kappa$ B binding sites (MCP-1 $\Delta$ NF- $\kappa$ B) following exposure to vehicle, TNF- $\alpha$ , or BSA. Luciferase activity in wild-type NRK-52E cells, transfected with either the wild-type or the mutated MCP-1 promoter, was determined under conditions described in the text.  $n = 3$  in each condition.

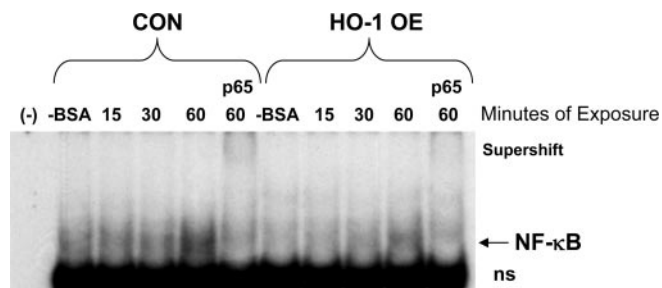


Fig. 6. Electrophoretic mobility shift assay (EMSA) for the expression of NF- $\kappa$ B. EMSA was performed using extracts from CON and HO-1 OE cells treated with BSA-free media, and following exposure to BSA for 15, 30, and 60 min. Supersifting was conducted using a p65 antibody. ns, Nonspecific band.

transient induction of phospho-ERK1/2 (p-ERK1/2), the latter subsiding at subsequent time points. HO-1 OE cells demonstrated a higher baseline expression of p-ERK1/2, and this expression appeared more sustained following the exposure to albumin. Given these unexpected findings, we also undertook measurements of ERK activity so as to corroborate or refute these findings observed on Western analysis. The ERK activity assay, shown in Fig. 8, demonstrated greater augmentation in ERK activity at 5 min and greater persistence of activity at 1 h in HO-1 OE cells following exposure to albumin. Thus these data demonstrate that the reduced expression of MCP-1 in response to albumin in HO-1 OE cells cannot be ascribed to blunted activation of ERK1/2.

To determine the functional contribution of ERK1/2 in inducing MCP-1 in CON and HO-1 OE cells, we examined the effect of an inhibitor of MEK, U0126, on albumin-induced MCP-1 production in these cells. U0126 reduced MCP-1 production by 60% in CON cells (Fig. 9). Production of MCP-1 in vehicle-treated, albumin-stimulated HO-1 OE cells was reduced compared with vehicle-treated, albumin-stimulated CON cells. The inhibitory effect of U0126 on MCP-1 production in albumin-stimulated HO-1 OE cells was blunted compared with its effect in albumin-stimulated CON cells: the mean reductions in MCP-1 achieved by U0126 in albumin-stimulated CON and HO-1 OE cells were  $19.2 \pm 0.4$  vs.  $10.3 \pm 0.5$  ng/mg protein,  $P < 0.001$ . That the inhibition of MCP-1 by U0126 is attenuated when HO-1 is overexpressed, in conjunction with findings regarding ERK expression and activity (Figs. 7 and 8), in aggregate, suggest that the inhibitory effects of HO-1 overexpression reside distal to ERK activation in the ERK-dependent pathway for MCP-1 production.

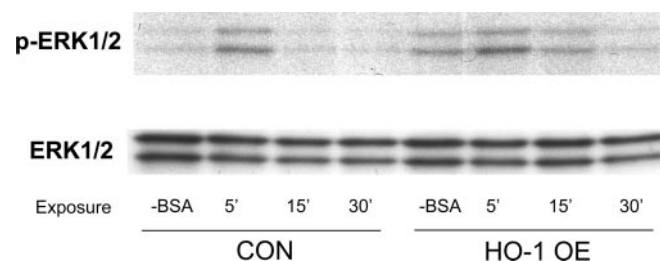


Fig. 7. Western analysis for the expression of ERK1/2. *Top*: expression of phospho-ERK1/2 (p-ERK1/2); *bottom*: total ERK1/2 in CON and HO-1 OE cells in the absence of BSA and following exposure to BSA for 5, 15, and 30 min.

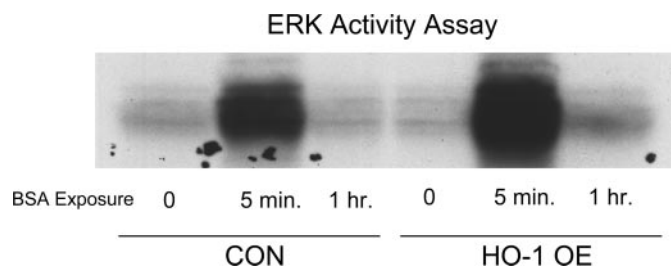


Fig. 8. ERK activity assay following exposure to BSA. ERK activity was determined in CON and HO-1 OE cells under basal conditions and following exposure to BSA for 5 min or 1 h.

## DISCUSSION

A vigorous chemoattractant for monocytes/macrophages, MCP-1 is a critical participant in the pathogenesis of assorted nephropathies, vasculopathies, and inflammatory conditions (5, 6, 8, 49). This broad involvement of MCP-1 in kidney and other diseases reflects numerous other effects of MCP-1, in addition to its capacity to recruit monocytes/macrophages, and these include the following: recruitment of memory T cells and natural killer cells; stimulation of cytokine production by inflammatory and other cells; induction of tissue factor and other procoagulant effects; activation and migration of endothelial cells; and proliferation and migration of smooth muscle cells (5, 6, 8, 49). MCP-1 has direct proinflammatory effects on resident renal cells, inducing, for example, NF- $\kappa$ B and other proinflammatory pathways in proximal tubular epithelial cells (48, 49). MCP-1 participates in a number of reverberating loops that could sustain tissue injury. For example, MCP-1 is induced by oxidant stress, the MAPK system, and by NF- $\kappa$ B, and, in turn, in certain cells, MCP-1 itself induces oxidative stress, the MAPK system, and NF- $\kappa$ B (5, 6, 8, 49).

A substantial body of literature attests to the role of increased expression of MCP-1 in the kidney as a contributor to chronic kidney disease (10, 13, 27, 40, 52, 57). For example, in assorted nephropathies, upregulation of MCP-1 occurs in the kidney, appearing mainly in the renal tubular epithelium and interstitium, and urinary excretion of MCP-1 is also increased in chronic kidney disease. Renal expression of MCP-1 correlates with renal histological injury and recruitment of macrophages, while urinary excretion of MCP-1 correlates with albuminuria, numbers of infiltrating macrophages, renal histological injury, and renal survival (10, 13, 27, 40, 52, 57). In experimental kidney injury, maneuvers that interrupt receptor-mediated actions of MCP-1 markedly reduce renal injury. For example, the administration of a mutant MCP-1 gene (*7ND*), which abrogates the effect of MCP-1 on its receptor (*CCR2*), reduces macrophage recruitment and interstitial fibrosis following urinary tract obstruction (51); *CCR2*<sup>-/-</sup> mice exhibit reduced fibrosis following urinary tract obstruction (20), and the administration of *7ND* significantly reduces the expression of MCP-1, macrophage infiltration, tubular injury, and collagen and transforming growth factor- $\beta_1$  expression in mice subjected to the protein-overload model of tubulointerstitial damage (43).

In view of these considerations, we thus employed MCP-1 as a representative cytokine in examining the effect of cellular overexpression of HO-1 on the proinflammatory influence of albumin on the renal proximal tubule. Our model system

recapitulated previously described findings demonstrating marked expression of MCP-1 mRNA in proximal tubular epithelial cells exposed to albumin, and we also confirmed that such expression is associated with increased production of MCP-1 protein in the extracellular fluid. HO-1 OE cells exhibited reduced expression of MCP-1 mRNA, along with decreased amounts of MCP-1 protein, following exposure to albumin. These findings thus demonstrate that overexpression of HO-1 in the proximal tubule can interrupt the inflammatory response engendered in albumin-exposed renal proximal tubules. However, as shown by our laboratory's prior findings, upregulation of HO-1, assessed either by HO activity, HO-1 mRNA, or HO-1 protein, does not occur in renal proximal tubular epithelial cells exposed to albumin (38). The observed induction of HO-1 in the proximal tubule in chronic kidney disease thus originates from some other stimulus—i.e., cytokines or other proinflammatory species, oxidized lipid, transition metal-bearing proteins, glycosylated or other chemically altered albumin, among other possibilities—rather than albumin per se acting on the proximal tubule. Nonetheless, the induction of HO-1 in the proximal tubule can mitigate the inflammatory effects of albumin on this nephron segment.

To examine the basis for this attenuation of MCP-1 production in HO-1 OE cells, we considered whether reduced activation of NF- $\kappa$ B occurred. NF- $\kappa$ B is a transcription factor that instigates inflammatory cascades by stimulating a number of cytokines and chemokines. In our model system, we first examined the extent to which NF- $\kappa$ B is involved in inducing MCP-1 expression in wild-type NRK-52E cells exposed to albumin. Using a MCP-1 promoter with mutated NF- $\kappa$ B binding sites, we observed that the induction of MCP-1 by albumin was markedly reduced in such mutants, thereby indicating a critical and dominant role for NF- $\kappa$ B in effecting albumin-driven MCP-1 expression in our model. Subsequent studies demonstrated that NF- $\kappa$ B was activated in a time-dependent fashion in CON cells exposed to albumin, and that such activation was significantly attenuated in HO-1 OE cells. From these studies, we conclude that the reduced MCP-1 expression may result from a diminution in the extent to which NF- $\kappa$ B is activated by albumin in HO-1 OE cells.

Recent studies have called attention to activation of ERK1/2 in inducing NF- $\kappa$ B and, in turn, MCP-1 expression in renal tubular epithelial cells exposed to albumin (46). Other signaling pathways that are incriminated in mediating the intracel-

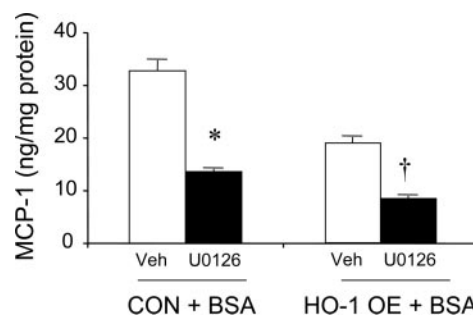


Fig. 9. Effect of U0126 or vehicle (Veh) on MCP-1 production by CON and HO-1 OE cells exposed to BSA-containing media. MCP-1 concentration was measured in the conditioned media;  $n = 4$  in each group. \* $P < 0.05$  vs. vehicle-treated, BSA-exposed CON cells; † $P < 0.05$  vs. vehicle-treated, BSA-exposed HO-1 OE cells.

lular effects of albumin involve the JAK/STAT pathway (30). In our model system, we saw no evidence of activation of JAK/STAT, whereas upregulation of ERK1/2 occurred in CON cells exposed to albumin; we thus explored this pathway. Phosphorylation of ERK1/2 in HO-1 OE cells exposed to albumin was surprising: basal phosphorylation of ERK1/2 was increased in HO-1 OE cells, and such phosphorylation persisted for a longer period in HO-1 OE cells upon exposure to albumin. To examine this further, we undertook measurements of ERK activity, and indeed our studies are the first to demonstrate that, in this model system, increased phosphorylation of ERK1/2, as assessed by Western analysis, is attended by increased ERK activity. Assessment of ERK activity in HO-1 OE cells corroborated the findings on Western analysis, that is, heightened activity along with greater persistence of such activity in HO-1 OE cells after exposure to albumin. Thus the suppressed production of MCP-1 in HO-1 OE cells exposed to albumin cannot be ascribed to lesser induction of ERK1/2 compared with that observed in albumin-exposed CON cells.

Studies were then undertaken using an inhibitor of MEK. The elaboration of MCP-1 in albumin-treated CON cells was reduced by 60% following exposure to the MEK inhibitor, findings that corroborate the previously described dependency of albumin-stimulated MCP-1 expression on p-ERK1/2 (46). In HO-1 OE cells, the effect of the inhibitor of MEK on the production of MCP-1 was blunted. That the inhibition of MCP-1 production by U0126 is attenuated when HO-1 is overexpressed, in conjunction with findings regarding ERK expression and activity, in aggregate, suggests that the inhibitory effects of HO-1 overexpression reside distal to ERK activation in the ERK-dependent pathway for MCP-1 production. Based on prior studies (46) and our present findings, we suggest that the inhibitory effects of HO-1 on albumin-induced MCP-1 production reside, at least in part, at the site in the following pathogenetic cascade marked by the asterisk: albumin  $\rightarrow$   $\uparrow$  p-ERK  $\rightarrow$  \*  $\uparrow$  NF- $\kappa$ B  $\rightarrow$   $\uparrow$  MCP-1. It is also possible, however, that the inhibitory effects of overexpression of HO-1 may involve ERK-independent mechanisms.

Activation of ERK1/2 is a recognized cellular response to stress. The increased upregulation of p-ERK1/2 in the vehicle-treated HO-1 OE cells under basal conditions, and the more sustained expression of p-ERK1/2 in response to albumin, were quite unanticipated; indeed, this was the reason for pursuing additional analysis, as provided by the ERK activity assay. While ERK1/2 is involved in upregulating HO-1 in response to certain stimuli (3, 45), we are unaware of any other study that has addressed the effect of altered expression of HO-1 on p-ERK1/2 expression. The basis for heightened expression of p-ERK1/2 under unstressed and stressed conditions in HO-1 OE cells merits additional exploration, but is beyond the scope of the present study. Whatever the underlying mechanism, these data underscore the capacity of overexpression of HO-1 to dampen the stimulatory effect of ERK1/2 on MCP-1 expression, even when activation of ERK1/2 is increased, as occurs in HO-1 OE cells.

The present findings are complementary to our laboratory's prior studies demonstrating that there is heightened activation of MCP-1 in HO-1-deficient states (36, 39). For example, studies utilizing HO-1<sup>-/-</sup> mice demonstrate that an exaggerated expression of MCP-1 occurs in response to heme proteins, ischemia-reperfusion injury, and aging, and in these instances,

this exaggerated expression of MCP-1 is attended by heightened activation of NF- $\kappa$ B. The present findings, demonstrating that overexpression of HO-1 inhibits albumin-driven activation of NF- $\kappa$ B and induction of MCP-1, provide additional evidence that one of the mechanisms whereby HO-1 inhibits inflammatory responses may involve the suppression of NF- $\kappa$ B and NF- $\kappa$ B-dependent genes such as MCP-1. However, we wish to emphasize that this mechanism would not be restricted to MCP-1, and other NF- $\kappa$ B-dependent genes may be similarly suppressed. An assessment of such genes is beyond the scope of the present study.

In conclusion, we demonstrate that constitutive overexpression of HO-1 by proximal tubular epithelial cells reduces production of MCP-1 by these cells in response to albumin. We conclude that this suppressive effect involves mechanisms that are distal to the activation of ERK1/2 and are associated with a suppressive effect on NF- $\kappa$ B activation. Additionally, as proteins other than albumin, such as IgG, can also activate NF- $\kappa$ B (and presumably NF- $\kappa$ B-dependent genes such as MCP-1) in proximal tubules (26), these suppressive effects of HO-1, conceivably, may also interrupt the inflammatory transformation of the proximal tubule caused by these other proteins. Based on the present findings, we suggest that the sustained induction of HO-1 in the proximal tubule, as observed in human chronic kidney disease, represents a countervailing response that mitigates the inflammatory effects of albumin on the proximal tubule.

#### ACKNOWLEDGMENTS

We gratefully acknowledge the secretarial expertise of Sharon Heppelmann in the preparation of this work.

#### GRANTS

These studies were funded by the following National Institutes of Health Grants: DK47060 (K. A. Nath), DK16105 (J. P. Grande), and AI62261 and AI40384 (A. D. Badley).

#### REFERENCES

1. Abraham N, Drummond G, Lutton J, Kappas A. The biological significance and physiological role of heme oxygenase. *Cell Physiol Biochem* 6: 129–168, 1996.
2. Agarwal A, Nick HS. Renal response to tissue injury: lessons from heme oxygenase-1 Gene Ablation and expression. *J Am Soc Nephrol* 11: 965–973, 2000.
3. Alam J, Igarashi K, Immenschuh S, Shibahara S, Tyrrell RM. Regulation of heme oxygenase-1 gene transcription: recent advances and highlights from the International Conference (Uppsala, 2003) on heme oxygenase. *Antioxid Redox Signal* 6: 924–933, 2004.
4. Anderson S. Mechanisms of injury in progressive renal disease. *Exp Nephrol* 4, Suppl 1: 34–40, 1996.
5. Charo IF, Peters W. Chemokine receptor 2 (CCR2) in atherosclerosis, infectious diseases, and regulation of T-cell polarization. *Microcirculation* 10: 259–264, 2003.
6. Charo IF, Taubman MB. Chemokines in the pathogenesis of vascular disease. *Circ Res* 95: 858–866, 2004.
7. Cheng J, Diaz Encarnacion MM, Warner GM, Gray CE, Nath KA, Grande JP. TGF- $\beta$ 1 stimulates monocyte chemoattractant protein-1 expression in mesangial cells through a phosphodiesterase isoenzyme 4-dependent process. *Am J Physiol Cell Physiol* 289: C959–C970, 2005.
8. Daly C, Rollins BJ. Monocyte chemoattractant protein-1 (CCL2) in inflammatory disease and adaptive immunity: therapeutic opportunities and controversies. *Microcirculation* 10: 247–257, 2003.
9. Dixon R, Brunskill NJ. Albumin stimulates p44/p42 extracellular-signal-regulated mitogen-activated protein kinase in opossum kidney proximal tubular cells. *Clin Sci (Lond)* 98: 295–301, 2000.

10. Eardley KS, Zehnder D, Quinkler M, Lepenies J, Bates RL, Savage CO, Howie AJ, Adu D, Cockwell P. The relationship between albuminuria, MCP-1/CCL2, and interstitial macrophages in chronic kidney disease. *Kidney Int* 69: 1189–1197, 2006.
11. Eddy AA. Proteinuria and interstitial injury. *Nephrol Dial Transplant* 19: 277–281, 2004.
12. Gonzalez-Michaca L, Farrugia G, Croatt AJ, Alam J, Nath KA. Heme: a determinant of life and death in renal tubular epithelial cells. *Am J Physiol Renal Physiol* 286: F370–F377, 2004.
13. Grandaliano G, Gesualdo L, Ranieri E, Monno R, Montinaro V, Marra F, Schena FP. Monocyte chemoattractant peptide-1 expression in acute and chronic human nephritides: a pathogenetic role in interstitial monocytes recruitment. *J Am Soc Nephrol* 7: 906–913, 1996.
14. Haugen EN, Croatt AJ, Nath KA. Angiotensin II induces renal oxidant stress in vivo and heme oxygenase-1 in vivo and in vitro. *Kidney Int* 58: 144–152, 2000.
15. Ibrahim HN, Rosenberg ME, Hostetter TH. Proteinuria. In: *The Kidney: Physiology and Pathophysiology* (3rd ed.), edited by Seldin DW and Giebisch G. Philadelphia, PA: Lippincott Williams & Wilkins, 2000, p. 2269–2296.
16. Imai E, Nakajima H, Kaimori JY. Albumin turns on a vicious spiral of oxidative stress in renal proximal tubules. *Kidney Int* 66: 2085–2087, 2004.
17. Inguaggiato P, Gonzalez-Michaca L, Croatt AJ, Haggard JJ, Alam J, Nath KA. Cellular overexpression of heme oxygenase-1 up-regulates p21 and confers resistance to apoptosis. *Kidney Int* 60: 2181–2191, 2001.
18. Kanwar YS. A dynamic interplay between monocyte chemoattractant protein-1 and heme oxygenase-1: implications in renal injury. *Kidney Int* 68: 896–897, 2005.
19. Kanwar YS. Heme oxygenase-1 in renal injury: conclusions of studies in humans and animal models. *Kidney Int* 59: 378–379, 2001.
20. Kitagawa K, Wada T, Furuichi K, Hashimoto H, Ishiwata Y, Asano M, Takeya M, Kuziel WA, Matsushima K, Mukaida N, Yokoyama H. Blockade of CCR2 ameliorates progressive fibrosis in kidney. *Am J Pathol* 165: 237–246, 2004.
21. Lee PJ, Alam J, Wiegand GW, Choi AM. Overexpression of heme oxygenase-1 in human pulmonary epithelial cells results in cell growth arrest and increased resistance to hyperoxia. *Proc Natl Acad Sci USA* 93: 10393–10398, 1996.
22. Liang M, Croatt AJ, Nath KA. Mechanisms underlying induction of heme oxygenase-1 by nitric oxide in renal tubular epithelial cells. *Am J Physiol Renal Physiol* 279: F728–F735, 2000.
23. Liu H, Bigler SA, Henegar JR, Baliga R. Cytochrome P450 2B1 mediates oxidant injury in puromycin-induced nephrotic syndrome. *Kidney Int* 62: 868–876, 2002.
24. McElhinny JA, MacMorran WS, Bren GD, Ten RM, Israel A, Paya CV. Regulation of I kappa B alpha and p105 in monocytes and macrophages persistently infected with human immunodeficiency virus. *J Virol* 69: 1500–1509, 1995.
25. Meyer TW. Tubular injury in glomerular disease. *Kidney Int* 63: 774–787, 2003.
26. Morigi M, Macconi D, Zoja C, Donadelli R, Buelli S, Zanchi C, Ghilardi M, Remuzzi G. Protein overload-induced NF-kappaB activation in proximal tubular cells requires H(2)O(2) through a PKC-dependent pathway. *J Am Soc Nephrol* 13: 1179–1189, 2002.
27. Morii T, Fujita H, Narita T, Koshimura J, Shimotomai T, Fujishima H, Yoshioka N, Imai H, Kakei M, Ito S. Increased urinary excretion of monocyte chemoattractant protein-1 in proteinuric renal diseases. *Ren Fail* 25: 439–444, 2003.
28. Morimoto K, Ohta K, Yachie A, Yang Y, Shimizu M, Goto C, Toma T, Kasahara Y, Yokoyama H, Miyata T, Seki H, Koizumi S. Cytoprotective role of heme oxygenase (HO)-1 in human kidney with various renal diseases. *Kidney Int* 60: 1858–1866, 2001.
29. Mosley K, Wembridge DE, Cattell V, Cook HT. Heme oxygenase is induced in nephrotoxic nephritis and hemin, a stimulator of heme oxygenase synthesis, ameliorates disease. *Kidney Int* 53: 672–678, 1998.
30. Nakajima H, Takenaka M, Kaimori JY, Hamano T, Iwatani H, Sugaya T, Ito T, Hori M, Imai E. Activation of the signal transducer and activator of transcription signaling pathway in renal proximal tubular cells by albumin. *J Am Soc Nephrol* 15: 276–285, 2004.
31. Nath KA. Heme oxygenase-1: a provenance for cytoprotective pathways in the kidney and other tissues. *Kidney Int* 70: 432–443, 2006.
32. Nath KA. Tubulointerstitial changes as a major determinant in the progression of renal damage. *Am J Kidney Dis* 20: 1–17, 1992.
33. Nath KA, Balla G, Vercellotti GM, Balla J, Jacob HS, Levitt MD, Rosenberg ME. Induction of heme oxygenase is a rapid, protective response in rhabdomyolysis in the rat. *J Clin Invest* 90: 267–270, 1992.
34. Nath KA, Croatt AJ, Haggard JJ, Grande JP. Renal response to repetitive exposure to heme proteins: chronic injury induced by an acute insult. *Kidney Int* 57: 2423–2433, 2000.
35. Nath KA, Haggard JJ, Croatt AJ, Grande JP, Poss KD, Alam J. The indispensability of heme oxygenase-1 in protecting against acute heme protein-induced toxicity in vivo. *Am J Pathol* 156: 1527–1535, 2000.
36. Nath KA, Vercellotti GM, Grande JP, Miyoshi H, Paya CV, Manivel JC, Haggard JJ, Croatt AJ, Payne WD, Alam J. Heme protein-induced chronic renal inflammation: suppressive effect of induced heme oxygenase-1. *Kidney Int* 59: 106–117, 2001.
37. Ohta K, Yachie A, Fujimoto K, Kaneda H, Wada T, Toma T, Seno A, Kasahara Y, Yokoyama H, Seki H, Koizumi S. Tubular injury as a cardinal pathologic feature in human heme oxygenase-1 deficiency. *Am J Kidney Dis* 35: 863–870, 2000.
38. Pedraza-Chaverri J, Murali NS, Croatt AJ, Alam J, Grande JP, Nath KA. Proteinuria as a determinant of renal expression of heme oxygenase-1: studies in models of glomerular and tubular proteinuria in the rat. *Am J Physiol Renal Physiol* 290: F196–F204, 2006.
39. Pittock ST, Norby SM, Grande JP, Croatt AJ, Bren GD, Badley AD, Caplice NM, Griffin MD, Nath KA. MCP-1 is up-regulated in unstressed and stressed HO-1 knockout mice: pathophysiologic correlates. *Kidney Int* 68: 611–622, 2005.
40. Prodjosudjadi W, Gerritsma JS, van Es LA, Daha MR, Bruijn JA. Monocyte chemoattractant protein-1 in normal and diseased human kidneys: an immunohistochemical analysis. *Clin Nephrol* 44: 148–155, 1995.
41. Reich H, Tritschler D, Herzenberg AM, Kassiri Z, Zhou X, Gao W, Scholey JW. Albumin activates ERK via EGF receptor in human renal epithelial cells. *J Am Soc Nephrol* 16: 1266–1278, 2005.
42. Remuzzi G, Bertani T. Pathophysiology of progressive nephropathies. *N Engl J Med* 339: 1448–1456, 1998.
43. Shimizu H, Maruyama S, Yuzawa Y, Kato T, Miki Y, Suzuki S, Sato W, Morita Y, Maruyama H, Egashira K, Matsuo S. Anti-monocyte chemoattractant protein-1 gene therapy attenuates renal injury induced by protein-overload proteinuria. *J Am Soc Nephrol* 14: 1496–1505, 2003.
44. Shimizu M, Ohta K, Yang Y, Nakai A, Toma T, Saikawa Y, Kasahara Y, Yachie A, Yokoyama H, Seki H, Koizumi S. Glomerular proteinuria induces heme oxygenase-1 gene expression within renal epithelial cells. *Pediatr Res* 58: 666–671, 2005.
45. Sikorski EM, Hock T, Hill-Kapturczak N, Agarwal A. The story so far: molecular regulation of the heme oxygenase-1 gene in renal injury. *Am J Physiol Renal Physiol* 286: F425–F441, 2004.
46. Takaya K, Koya D, Isono M, Sugimoto T, Sugaya T, Kashiwagi A, Haneda M. Involvement of ERK pathway in albumin-induced MCP-1 expression in mouse proximal tubular cells. *Am J Physiol Renal Physiol* 284: F1037–F1045, 2003.
47. Tang S, Leung JC, Abe K, Chan KW, Chan LY, Chan TM, Lai KN. Albumin stimulates interleukin-8 expression in proximal tubular epithelial cells in vitro and in vivo. *J Clin Invest* 111: 515–527, 2003.
48. Viedt C, Dechend R, Fei J, Hansch GM, Kreuzer J, Orth SR. MCP-1 induces inflammatory activation of human tubular epithelial cells: involvement of the transcription factors, nuclear factor-kappaB and activating protein-1. *J Am Soc Nephrol* 13: 1534–1547, 2002.
49. Viedt C, Orth SR. Monocyte chemoattractant protein-1 (MCP-1) in the kidney: does it more than simply attract monocytes? *Nephrol Dial Transplant* 17: 2043–2047, 2002.
50. Vogt BA, Shanley TP, Croatt A, Alam J, Johnson KJ, Nath KA. Glomerular inflammation induces resistance to tubular injury in the rat. A novel form of acquired, heme oxygenase-dependent resistance to renal injury. *J Clin Invest* 98: 2139–2145, 1996.
51. Wada T, Furuichi K, Sakai N, Iwata Y, Kitagawa K, Ishida Y, Kondo T, Hashimoto H, Ishiwata Y, Mukaida N, Tomosugi N, Matsushima K, Egashira K, Yokoyama H. Gene therapy via blockade of monocyte chemoattractant protein-1 for renal fibrosis. *J Am Soc Nephrol* 15: 940–948, 2004.
52. Wada T, Furuichi K, Sakai N, Iwata Y, Yoshimoto K, Shimizu M, Takeda SI, Takasawa K, Yoshimura M, Kida H, Kobayashi KI, Mukaida N, Naito T, Matsushima K, Yokoyama H. Up-regulation of monocyte chemoattractant protein-1 in tubulointerstitial lesions of human diabetic nephropathy. *Kidney Int* 58: 1492–1499, 2000.

53. Wang Y, Chen J, Chen L, Tay YC, Rangan GK, Harris DC. Induction of monocyte chemoattractant protein-1 in proximal tubule cells by urinary protein. *J Am Soc Nephrol* 8: 1537–1545, 1997.
54. Wang Y, Rangan GK, Tay YC, Harris DC. Induction of monocyte chemoattractant protein-1 by albumin is mediated by nuclear factor kappaB in proximal tubule cells. *J Am Soc Nephrol* 10: 1204–1213, 1999.
55. Williams BL, Irvin BJ, Sutor SL, Chini CC, Yacyshyn E, Bubeck Wardenburg J, Dalton M, Chan AC, Abraham RT. Phosphorylation of Tyr319 in ZAP-70 is required for T-cell antigen receptor-dependent phospholipase C-gamma1 and Ras activation. *EMBO J* 18: 1832–1844, 1999.
56. Wolf G, Jocks T, Zahner G, Panzer U, Stahl RA. Existence of a regulatory loop between MCP-1 and TGF-beta in glomerular immune injury. *Am J Physiol Renal Physiol* 283: F1075–F1084, 2002.
57. Yoshimoto K, Wada T, Furuichi K, Sakai N, Iwata Y, Yokoyama H. CD68 and MCP-1/CCR2 expression of initial biopsies reflect the outcomes of membranous nephropathy. *Nephron Clin Pract* 98: c25–c34, 2004.
58. Zoja C, Benigni A, Remuzzi G. Cellular responses to protein overload: key event in renal disease progression. *Curr Opin Nephrol Hypertens* 13: 31–37, 2004.

