

Proteinuria as a determinant of renal expression of heme oxygenase-1: studies in models of glomerular and tubular proteinuria in the rat

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Pedraza-Chaverri, José, Narayana S. Murali, Anthony J. Croatt, Jawed Alam, Joseph P. Grande, and Karl A. Nath. 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. First published August 23, 2005; doi:10.1152/ajprenal.00230.2005.—Heme oxygenase-1 (HO-1), a cytoprotective gene, is commonly induced in renal tubules in the diseased kidney. Because proteinuria is a hallmark for kidney disease, we examined the relationship between proteinuria and tubular induction of HO-1, specifically questioning whether increased trafficking of protein across the renal tubular epithelium, as a consequence of proteinuria, induces tubular expression of HO-1. We examined a model of glomerular proteinuria induced by daily injections of BSA, which is associated with increased tubular uptake of filtered protein, and a model of tubular proteinuria induced by maleate, the latter exhibiting decreased tubular uptake and trafficking of protein. The BSA model of glomerular proteinuria failed to exhibit induction of HO-1; HO-1 was not induced in proximal tubular epithelial cells exposed to BSA. In contrast, in maleate nephropathy wherein tubular uptake of protein is decreased because of generalized proximal tubular injury induced by maleate, HO-1 was strongly induced in proximal tubules; inhibition of HO activity in maleate nephropathy worsened proteinuria, renal histological injury, and apoptosis. In renal proximal tubular epithelial cells, maleate induced HO-1 and caused apoptosis, the latter increased when HO activity was inhibited. From these studies, we conclude that expression of HO-1 in the diseased kidney cannot be ascribed to the tubular uptake and metabolism of protein such as albumin, and that the expression of HO-1 in a model of tubular proteinuria reflects a functionally significant stress response to toxin-induced proximal tubular injury.

albumin; maleate; cytoprotection; apoptosis

THE DETECTION OF INCREASED amounts of protein in urine provides an index of diagnostic, prognostic, and pathogenetic significance (2, 11, 14, 22, 35, 43). Proteinuria can arise from lesions within the kidney that are glomerular or tubular in origin (11, 22). Glomerular proteinuria occurs as a consequence of defects in glomerular permselective properties that allow plasma proteins to leak into the urinary space; tubular proteinuria results from failure of the injured tubule to reabsorb and degrade the relatively small amounts of protein normally filtered into the urinary space (11, 22).

In addition to such diagnostic utility, proteinuria also provides a prognostic index for renal disease (2, 11, 14, 22, 35, 43): increasing rates of urinary protein excretion in diverse nephropathies predict more rapid decline in renal function and

an increased likelihood of end-stage kidney disease. This prognostic significance reflects, at least in part, the pathogenetic effects of proteinuria: the presence of increased amounts of protein in the urinary space and the attendant trafficking of reabsorbed protein across the tubular epithelium provoke inflammatory, fibrogenic, and other damaging effects that, in aggregate, contribute to the progression of kidney disease (2, 11, 14, 22, 35, 43).

An intriguing, and perhaps underexplored, consideration in proteinuric states is the extent to which cytoprotective responses are recruited in the kidney as inflammatory and other injurious pathways are instigated in the wake of proteinuria. In this regard, our interest was drawn to heme oxygenase (HO), an enzyme increasingly recognized for its anti-inflammatory and cytoprotective properties (1, 16, 17, 38). Heme oxygenase is the rate-limiting enzyme in the degradation of heme, converting heme to biliverdin, in the course of which carbon monoxide is produced and iron is released; biliverdin is subsequently converted to bilirubin (1, 16, 17, 38). HO-1 is the inducible isoform, which is widely expressed in the kidney injured acutely or chronically in relevant rodent models (1, 16, 17, 38) and in diverse types of acute and chronic human nephropathies (23, 30). In some of these conditions, cytoprotective and anti-inflammatory effects have been assigned to such induction of HO-1 (1, 16, 17, 38), and in a signal case of HO-1 deficiency in humans, kidney disease was a salient affliction (30, 42).

In light of the predictable presence of proteinuria in the setting of kidney disease, and increased expression of HO-1 in the diseased kidney, the present study examined whether such expression of HO-1 is linked to proteinuria, questioning, specifically, whether proteinuria per se provokes the induction of HO-1. This possibility was considered especially because the part of the nephron evincing induction of HO-1 most commonly in the diseased kidney, the renal tubular epithelium (1, 16, 17, 23, 38), is the compartment continually exposed to increased amounts of protein and increased trafficking of protein in proteinuric kidney disease.

To explore this question, we employed well-established and widely utilized models of proteinuria, including the protein overload model, to induce glomerular proteinuria (6, 7, 18, 19, 39) and the maleate nephropathy model to induce tubular proteinuria (4, 8, 29, 33). The daily injection of BSA rapidly induces extraordinarily high rates of urinary protein excretion as a consequence of profound and prompt disturbance in the

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glomerular filtration barrier (6, 7, 18, 19, 39). The maleate model induces reversible, generalized tubular injury, which simulates the Fanconi syndrome wherein multiple defects in proximal tubular function occur including the failure to reabsorb filtered protein (4, 8, 29, 33); in this model, and in contrast to models of glomerular proteinuria, tubular uptake and trafficking of protein are significantly decreased. Using these models characterized either by increased or decreased tubular uptake and trafficking of filtered protein, we evaluated renal expression of HO-1 and the functional significance of any such expression.

METHODS

Models of Proteinuria In Vivo

Our studies were approved by our Institutional Animal Care and Use Committee and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health.

BSA model (protein overload) of glomerular proteinuria. The protein overload model of proteinuria was induced by the intraperitoneal injection of BSA (6, 7, 18, 19, 39). Adult female Wistar rats were injected daily for 7 days with 2 g of BSA or an equivalent volume of sterile normal saline. Rats were allowed free access to water and standard rat chow. BSA (A4503, Sigma, St. Louis, MO) was prepared aseptically as a 33% BSA solution containing 87 mM NaOH and 65 mM NaCl in nanopure water. Urine was collected from rats in metabolic cages for 24 h before death on *day 7*, and creatinine clearance and rates for urinary protein excretion were determined. Kidneys were harvested from BSA-treated and vehicle-treated rats for the evaluation of the expression of HO-1 and other indexes.

Maleate model of tubular proteinuria. Maleate nephropathy was induced in female Wistar rats by a single intraperitoneal injection of maleic acid (400 mg/kg, M0375, Sigma) dissolved in sterile saline with the pH adjusted to 7.4 with NaOH (4, 8, 29, 33). Control rats received an equivalent volume of sterile saline solution. Rats were housed in metabolic cages for 24 h following the injection of maleate or vehicle, urinary protein excretory rates were measured, and renal function was assessed by measurement of creatinine clearance. Twenty-four hours after the administration of maleate or vehicle, rats were killed for analysis of renal HO enzyme activity, expression of renal HO-1 mRNA by Northern blot analysis, expression of HO-1 protein by Western blot analysis, and localization of the expression of HO-1 protein in the kidney by immunohistochemistry. Additional cohorts of maleate-treated and vehicle-treated rats were killed at 8 and 18 h for the analysis of HO-1 mRNA expression in the kidney. In subsequent studies, maleate-treated and vehicle-treated rats were injected subcutaneously with an inhibitor of HO activity, tin protoporphyrin (SnPP; 20 μ mol/kg, Porphyrin Products, Logan, UT) or saline vehicle, administered 3 h before, and 5 and 24 h after, the administration of maleate (25). In these studies, urinary protein excretion was assessed daily for 2 days following the injection of maleate or vehicle. At this time point, rats were killed and their kidneys were harvested for histological assessment and detection of apoptosis.

Studies In Vitro

NRK 52E cells (ATCC, Manassas, VA) were cultured in DMEM (Invitrogen, Carlsbad, CA) containing high glucose (4.5 g/l) supplemented with 10% FBS, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μ g/ml streptomycin. For incubation with BSA, cells were exposed to fatty acid-free, low-endotoxin BSA (30 mg/ml, A8808, Sigma) dissolved in serum-free DMEM (3, 41). At time points between 2 and 16 h of exposure to BSA, RNA was extracted for assessment of HO-1 mRNA expression by Northern blot analysis and

real-time RT-PCR, and evaluation of monocyte chemoattractant protein-1 (MCP-1) mRNA expression by Northern analysis.

In other studies, NRK 52E cells were incubated with maleate (4–20 mM). Maleic acid (M0375, Sigma) was dissolved in sterile water at 100 times the desired final concentration to be studied, the pH was adjusted to 7.4 with NaOH, and diluted with incubation media. Incubations were performed in either serum-free basal Eagle's media (BME; Invitrogen) or BME containing 1% FBS depending on the study. At 8- and 18-h time points, RNA was extracted for assessment of HO-1 mRNA expression by Northern blot analysis. In some studies, zinc protoporphyrin (ZnPP; Porphyrin Products), an inhibitor of HO activity, was employed (9, 15). ZnPP was initially prepared as a concentrated stock solution in 0.05 M NaOH and subsequently diluted with the incubation media. In these studies, cells were exposed to ZnPP (5 μ M) or vehicle for 30 min before and during the exposure to maleate (20 mM). After 24 h, the cells were harvested for assessment of apoptosis.

Determination of Concentrations of Urinary Protein and Creatinine in Plasma and Urine

The concentration of urinary protein was determined using the Coomassie method (10), whereas concentrations of creatinine in plasma and urine were determined using a Creatinine Analyzer 2 (Beckman Instruments, Fullerton, CA) (10).

Assessment of HO-1 mRNA Expression In Vivo and In Vitro

Assessment of HO-1 and MCP-1 mRNA expression by Northern blot analysis. Total RNA from rat kidney and from cultured cells was isolated using the TRIzol method (Invitrogen) (27, 28). Twenty micrograms of total RNA from kidney samples, or 10 μ g of RNA from NRK 52E cells, were separated on an agarose gel and transferred to a nylon membrane (15). Membranes were hybridized overnight with a 32 P-labeled mouse HO-1 or a 32 P-labeled rat MCP-1 cDNA probe (27, 28). Autoradiograms were standardized, as previously described, by factoring the optical density of the message for HO-1 with the optical density of the 18S rRNA, the latter obtained on a negative of the ethidium bromide-stained nylon membrane (27, 28).

Assessment of HO-1 mRNA expression by quantitative real-time RT-PCR. Using real-time RT-PCR, expression of HO-1 mRNA and 18S rRNA was determined from total RNA extracted from renal epithelial cells, as previously employed by our laboratory (34). The analysis was performed on an ABI Prism 7000 by a one-step method using *Taqman* One-Step RT-PCR Mastermix reagents (Applied Biosystems, Foster City, CA). The primers and probes were designed using Primer Express software (Applied Biosystems). The sequences of primers and *Taqman* probes are listed in Table 1. Each reaction mix was performed in a total reaction volume of 25 μ l; primer and probe concentrations were 300 and 200 nM, respectively, and reactions contained between 50 and 100 ng of RNA. Conditions of real-time quantitative RT-PCR were as follows: 30 min at 48°C (RT), 10 min at 95°C (RT inactivation and initial activation), and then 40 cycles of amplification for 15 s at 95°C (denaturation) and 1 min at 60°C (annealing and extension). Expression of 18S rRNA was used to correct for differences in quantities of RNA per reaction and to compensate for any possible inhibition of either RT or PCR. Results are expressed as a ratio of HO-1 to 18S expression.

Expression of HO-1 Protein by Western Blot and Immunohistochemical Analyses

Western blot analysis was performed for the detection of HO-1 protein in kidney microsomal preparations as described in our prior publication (27). Briefly, 60- μ g aliquots of microsomal protein were separated on a 15% Tris-HCl gel and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). For analysis of expression of HO-1 protein, a rabbit anti-rat polyclonal HO-1 primary

Table 1. Primers and probes used for quantitative real-time RT-PCR

Gene	Primer/Probe	Sequence
HO-1	Forward	5'-CGTGCTCGCATGAACACTCT-3'
	Reverse	5'-GGGGTCTTAGCCTCTTCTGT-3'
	TaqMan	5'-(FAM) ACCCCCGAGGTCAAGCAGAGG (TAMRA)-3'
18S	Forward	5'-CAGGCCCGGTACAGTAAA-3'
	Reverse	5'-AGAGGAGCGAGCGACCAA-3'
	TaqMan	5'-(VIC) TGCGAATGGCTCATTAATCAGTTATGGTTCC (TAMRA)-3'

HO, heme oxygenase.

antibody (SPA-895, Stressgen, Victoria, BC) and a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody were employed with a chemiluminescent detection system (RPN2109, Amersham). Equivalency of loading was confirmed by Ponceau S staining.

Immunohistochemical analysis was performed, as previously described, on tissue sections fixed in 10% neutral buffered formalin and embedded in paraffin (10). Expression of HO-1 was localized using a monoclonal antibody (OSA-111, Stressgen) as the primary antibody, a horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG as the secondary antibody (SAB-100, Stressgen), and diaminobenzidine as a substrate for localization.

HO Enzyme Activity

Renal HO activity was measured by the generation of bilirubin by microsomes prepared from kidneys as described previously (25). Microsomes were incubated with mouse liver cytosol (a source of biliverdin reductase), hemin (20 μ M), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (4 U/ml), and NADPH (0.8 mM) for 90 min at 37°C in the dark. The formed bilirubin was extracted with chloroform, Δ OD 464–530 nm was measured (extinction coefficient, 40 mM/cm for bilirubin), and enzyme activity was determined (pmol formed bilirubin \cdot h⁻¹ \cdot mg protein⁻¹).

Kidney Ferritin Content

Kidney ferritin content was measured by an ELISA method as previously described, employing a polyclonal rabbit anti-rat ferritin antibody as the primary antibody and a goat anti-rabbit IgG alkaline phosphatase conjugate as the secondary antibody (25). Incubation with *p*-nitrophenyl phosphate (Sigma) formed color, which was quantified using an absorbance plate reader at a wavelength of 405 nm.

Lipid Peroxidation Assay

Lipid peroxidation in kidney homogenates was determined by measuring the content of thiobarbituric acid-reactive substances as previously described (10).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP nick-end labeling Method for Detection of Apoptosis In Vivo and In Vitro

As previously employed by our laboratory, an Apoptag Plus Peroxidase In Situ Apoptosis Kit (Chemicon, Temecula, CA) was used to assess apoptosis in vivo (26) and in vitro (9, 15). This method detects apoptosis-associated DNA fragmentation by labeling of 3'-OH ter-

mini with digoxigenin-nucleotides employing terminal deoxynucleotidyl transferase. In the studies undertaken in vitro, apoptosis was quantitated in five randomly selected, separate fields for each condition of exposure by counting the number of apoptotic cells (cells with brown-staining nuclei) and the total number of cells; apoptotic cells were expressed as a percentage of the total number of cells in this field.

Statistics

Data are expressed as means \pm SE. Values are considered statistically significant for $P < 0.05$. For comparison of two groups, the unpaired Student's *t*-test or the Mann-Whitney *U*-test was used as appropriate. For comparison involving multiple groups, ANOVA and the Student-Newman-Keuls tests were employed.

RESULTS

Glomerular Proteinuria

Studies in vivo: BSA model of protein overload. Daily injections of BSA administered for 7 days, compared with similarly administered vehicle, as expected, provoked markedly increased urinary protein excretory rates (Table 2). At this time point, when proteinuria is established and markedly increased, HO activity in the kidney was unaltered as were indexes of renal oxidant stress, such as lipid peroxidation (thiobarbituric acid-reactive substances), and renal ferritin content, the latter commonly induced when HO activity is increased (Table 3). Additionally, expression of HO-1 in the kidney was not increased, assessed either by expression of mRNA with Northern blot analysis, by protein expression on Western blot analysis, or by immunohistochemical analysis of the kidney (data not shown). Thus, using multiple approaches, HO-1 is not induced in the BSA model of protein overload at this time point.

Studies in vitro: BSA-treated proximal tubular epithelial cells. To determine whether the trafficking of protein across the proximal tubule per se would induce tubular expression of HO-1, we exposed proximal tubular epithelial cells to BSA (Fig. 1). Such exposure elicited marked upregulation of MCP-1 mRNA, thereby demonstrating an expected cellular

Table 2. General parameters in control and BSA-treated rats

	Control	BSA-Treated	<i>P</i> Value
Body weight, g	189 \pm 4	198 \pm 5	NS
Creatinine clearance, ml \cdot min ⁻¹ \cdot 100 g ⁻¹	0.92 \pm 0.05	0.71 \pm 0.10	NS
Urinary protein excretion, mg/24 h	1 \pm 1	574 \pm 114	<0.01

Values are means \pm SE; *n* = 6/group. NS, not significant.

Table 3. HO activity and other indexes in control and BSA-treated rats

	Control	BSA-Treated	<i>P</i> Value
HO activity, pmol \cdot mg protein ⁻¹ \cdot hr ⁻¹	52 \pm 3	57 \pm 8	NS
TBARS, nmol/mg protein	0.82 \pm 0.08	0.76 \pm 0.10	NS
Ferritin content, μ g/mg protein	2.5 \pm 0.2	3.3 \pm 0.4	NS

Values are means \pm SE; *n* = 6/group. TBARS, thiobarbituric acid-reactive substances.

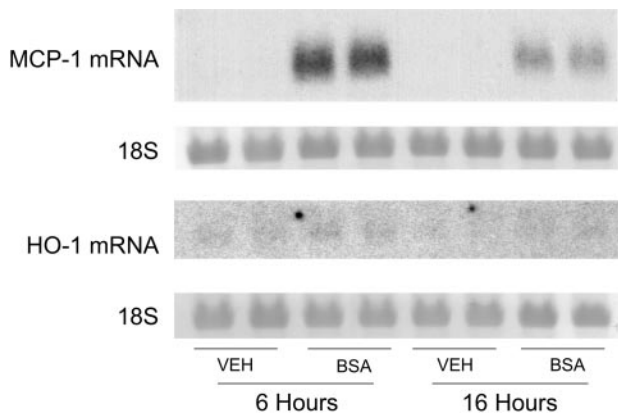


Fig. 1. Expression of monocyte chemoattractant protein-1 (MCP-1) and heme oxygenase (HO)-1 mRNA and 18S rRNA in NRK 52E proximal tubular epithelial cells exposed to vehicle (VEH) or BSA (30 mg/ml) and studied after 6 or 16 h of exposure.

effect of such exposure to BSA. However, under these conditions, expression of HO-1 mRNA was not increased. Assessment of HO-1 mRNA by RT-PCR also failed to reveal increased expression of HO-1 in BSA-treated cells after 2, 4, and 8 h of exposure to BSA compared with vehicle-treated cells at these time points; standardized mean readings of duplicates at each time point for vehicle-treated vs. BSA-treated cells, respectively, were 1.5 vs. 1.1 at 2 h, 1.4 vs. 1.2 at 4 h, and 1.2 vs. 1.7 at 8 h. From these studies, we conclude that trafficking of proteins such as albumin across the tubular epithelium cannot be invoked as a mechanism accounting for induction of HO-1 when it occurs in the injured kidney.

Tubular Proteinuria

Studies in vivo: maleate-induced nephropathy. As shown in Table 4, this model of tubular proteinuria is characterized by low-grade proteinuria and a tendency toward an impaired glomerular filtration rate.

In this model, renal HO activity is significantly increased when measured 24 h after the administration of maleate (67 ± 9 vs. 96 ± 3 pmol·mg protein⁻¹·h⁻¹, $n = 6$ and $n = 7$, respectively, $P < 0.05$). Such an increase in HO activity reflected induction of HO-1. HO-1 mRNA was markedly induced in the maleate-treated kidney at 8 h (Fig. 2) and progressively less so at 18 (Fig. 2) and at 24 h (Fig. 3, top); HO-1 protein was strongly induced in the maleate-treated kidneys when studied at 24 h after the administration of maleate (Fig. 3, bottom). The expression of HO-1 protein, as

Table 4. General parameters in control and maleate-treated rats

	Control ($n = 6$)	Maleate ($n = 7$)	<i>P</i> Value
Body weight, g	190 ± 4	187 ± 3	NS
Kidney weight, g	0.82 ± 0.03	0.93 ± 0.09	<0.05
Creatinine clearance, ml·min ⁻¹ ·100 g ⁻¹	1.3 ± 0.1	0.9 ± 0.2	NS
Urinary protein excretion, mg/24 h	3 ± 1	61 ± 19	<0.01

Values are means ± SE. *n*, No. of rats.

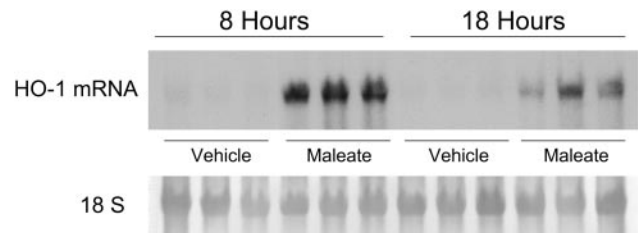


Fig. 2. Expression of HO-1 mRNA and 18S rRNA in the rat kidney 8 or 18 h after the administration of maleate or vehicle. Each lane represents RNA extracted from the kidney of an individual rat treated with vehicle or maleate.

determined by immunohistochemistry, localized mainly to the proximal tubules in maleate-treated rats (Fig. 4).

To determine the functional significance of such induction of HO-1, the effect of the competitive inhibitor of HO activity, SnPP, was studied in maleate-induced nephropathy. Such administration of SnPP exacerbated the course of proteinuria in this model. As shown in Fig. 5, the course of maleate nephropathy in vehicle-treated rats (that is, maleate-treated rats not subjected to SnPP) is characterized by proteinuria, which peaks at *day 1* and subsides by *day 2* after the administration of maleate. However, the administration of SnPP to maleate-treated rats prevented the regression of proteinuria which normally occurs by the second day after the administration of maleate. In rats treated with the vehicle for maleate, SnPP did not significantly alter proteinuria either on *day 1* (5 ± 1 vs. 6 ± 1 mg/day, $n = 6$ in each group, $P =$ not significant) or *day 2* (3 ± 1 vs. 3 ± 1 mg/day, $n = 6$ in each group, $P =$ not significant); in these vehicle-treated rats, SnPP did not alter creatinine clearance on either *day 1* or *day 2* (data not shown). Thus the exacerbatory effect of SnPP on proteinuria in maleate-treated rats is not a nonspecific effect of SnPP because this effect on proteinuria is not seen in vehicle-treated rats in which SnPP is administered.

This exacerbation of proteinuria by SnPP in maleate-treated rats was accompanied by worsened histological injury (Fig. 6) as characterized by more severe tubular dilatation, epithelial cell necrosis, and epithelial cell sloughing (markers of tubular epithelial cell injury), occurring in the juxtamedullary cortex.

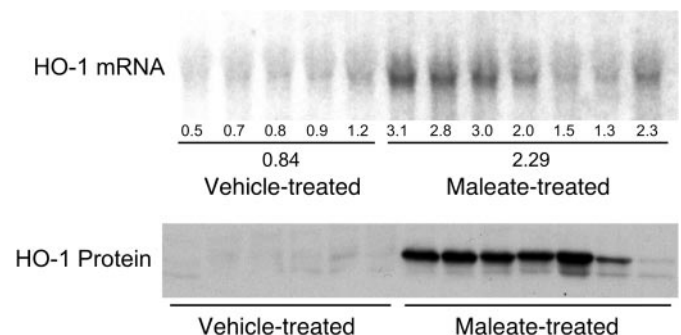
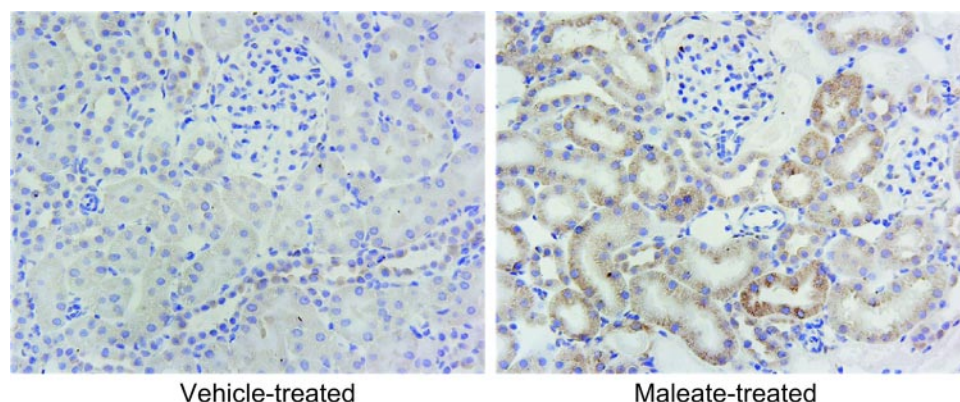


Fig. 3. Top: expression of HO-1 mRNA in the kidney 24 h after the administration of maleate or vehicle. Each lane represents RNA extracted from the kidney of an individual rat. The numbers below each lane represent the standardized densitometric readings, below which are the mean values for the vehicle-treated and the maleate-treated groups. Bottom: expression of HO-1 protein in the kidney 24 h after the administration of maleate or vehicle. Each lane represents protein extracted from the kidney of an individual rat. Equivalency of loading was confirmed by Ponceau S staining (not shown).

Fig. 4. Immunoperoxidase studies for the expression of HO-1 protein in the kidney 24 h after the administration of maleate or vehicle. Original magnification: $\times 400$.



SnPP exerted no histological effect in vehicle-treated rats (Fig. 6).

We also undertook studies of cell death by terminal transferase-mediated dUTP nick end-labeling (TUNEL) staining in maleate nephropathy (Fig. 7). The kidney in vehicle-treated rats showed minimal TUNEL staining, and such staining was not increased with concomitant treatment with SnPP. TUNEL staining was increased in tubular epithelial cells in the kidneys obtained from maleate-treated rats. However, the concomitant administration of SnPP markedly increased TUNEL staining in maleate-treated rats, thus indicating an exacerbatory effect of SnPP on tubular epithelial cell apoptosis in maleate nephropathy (Fig. 7).

The presence of apoptosis as assessed by TUNEL staining in the maleate model, along with the exacerbation of proteinuria and TUNEL staining when HO activity is inhibited by SnPP, led us to examine whether maleate induced apoptosis as well as HO-1 in vitro, and the functional significance of such expression of HO-1 on maleate-induced apoptosis in vitro. We thus studied the effect of maleate on the expression of HO-1 and apoptosis in renal tubular epithelial cells. We first demonstrated that maleate induced HO-1 in renal tubular epithelial cells; shown in Fig. 8 is such an inductive effect achieved with 4 mM maleate. Maleate also caused apoptosis in a dose-dependent fashion in renal tubular epithelial cells, and such an

effect was not due to the osmotic effect of sodium maleate as equimolar amounts of sodium chloride failed to provoke apoptosis (data not shown). The concomitant inhibition of HO activity by the competitive inhibitor, ZnPP, significantly increased apoptosis induced by maleate as shown in Figs. 9 and 10. Thus the induction of HO-1 by maleate exerts a counterbalancing action on the proapoptotic effects of maleate in vitro.

DISCUSSION

In the BSA model of glomerular proteinuria, one exhibiting dramatically increased rates of urinary protein excretion, HO-1 was not detected in the kidney as assessed either by Northern and Western blot analyses, HO activity, or immunoperoxidase staining in studies undertaken at 1 wk. In a complementary model in vitro wherein renal tubular epithelial cells were exposed to large amounts of BSA, and that elicited strong upregulation of a candidate chemokine, MCP-1, HO-1 was not induced as assessed by RT-PCR and Northern blot analysis. From these findings, we conclude that HO-1 is not induced in renal proximal tubular epithelial cells directly exposed to BSA in vitro, or when such cells are exposed to inordinate amounts of proteins (mainly albumin) leaked into the urinary space as a consequence of BSA-induced impairment of glomerular permselectivity in vivo.

The BSA model of glomerular proteinuria at 1 wk (the time point examined in the present study) exhibits interstitial mononuclear cellular infiltration, which tends to progress over the subsequent week and is accompanied by increasing expression of chemotactic peptides such as MCP-1 and osteopontin; interstitial matrix accumulates and is driven by upregulated transforming growth factor- β_1 ; eventually, tubular atrophy, interstitial fibrosis, and scarring appear in the BSA model (6, 7, 18, 39). Cellular injury and tissue scarring are recognized as inducers of HO-1 (1, 16, 17, 38), and it is thus possible that HO-1 may be induced in the later stages of the BSA model when there is clear evidence of tubulointerstitial injury and scarring. However, at these later time points, it would be difficult, if not impossible, to evaluate the role of proteinuria per se as a stimulus for the increased expression of HO-1 in the diseased kidney. This consideration underscores the difficulty in deciphering from available studies of glomerular proteinuria the role of proteinuria in inducing HO-1. For example, tubular expression of HO-1 occurs in such models of glomerular proteinuria as nephrotoxic serum nephritis (24, 40), puromycin

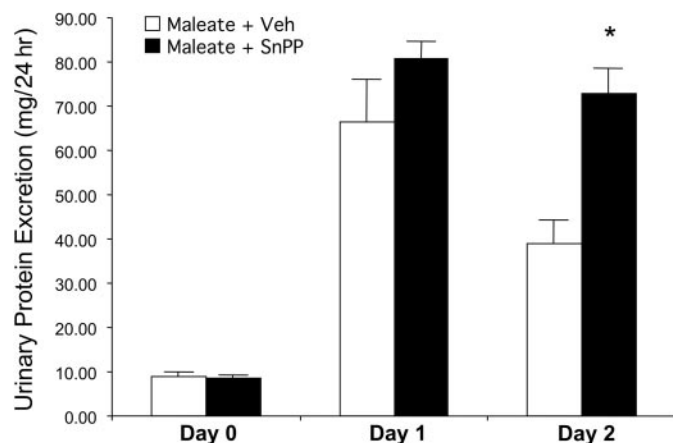


Fig. 5. Urinary protein excretory rates in maleate-treated rats subjected to tin protoporphyrin (SnPP; an inhibitor of heme oxygenase) or vehicle (Veh) determined before the administration of maleate (day 0) and for 2 successive days after the administration of maleate. $*P < 0.05$, maleate+SnPP vs. maleate + vehicle; $n = 8$ /group.

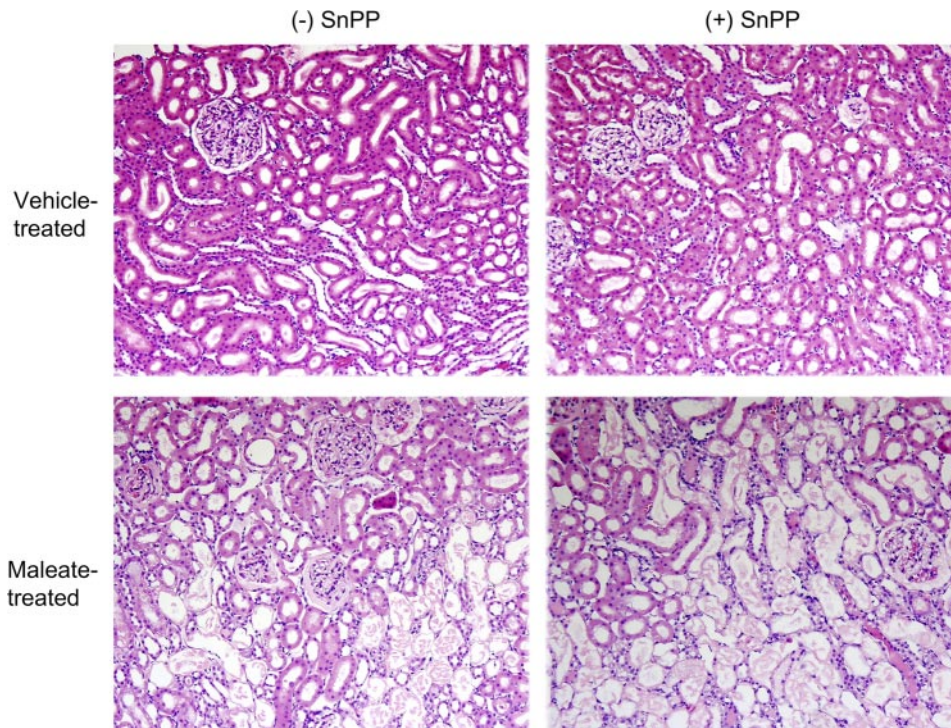


Fig. 6. Histological sections of a kidney from rats subjected to maleate or vehicle and that were treated with SnPP (+SnPP) or saline vehicle (-SnPP); tissues were harvested from rats following studies of urinary protein excretion described in Fig. 5. Kidney sections were stained with hematoxylin and eosin. Original magnification: $\times 200$.

nephropathy (20), and chronic infusion of angiotensin II (10). However, nephrotoxic serum nephritis is associated with intense inflammation and oxidative stress in the glomerular compartment that may extend to the tubulointerstitial compartment as activated leukocytes, exiting from the glomerular microcirculation, traverse the peritubular capillary network, or as cytokines and other proinflammatory and prooxidant species from the inflamed glomeruli leak into the urinary space. Thus tubular induction of HO-1 in this model may result from

inflammatory and oxidant stress, both of which induce HO-1 (1, 16, 17, 38), rather than from proteinuria. Tubular induction of HO-1 also occurs in puromycin- and angiotensin II-induced glomerular proteinuria (10, 20), but either insult provokes oxidative stress. The appeal of the BSA model of glomerular proteinuria, at least at the stage studied, is that it induces massive and precipitous proteinuria in the relative absence of acute inflammation, overt tubular injury, and scarring; in such a setting, we failed to observe induction of HO-1.

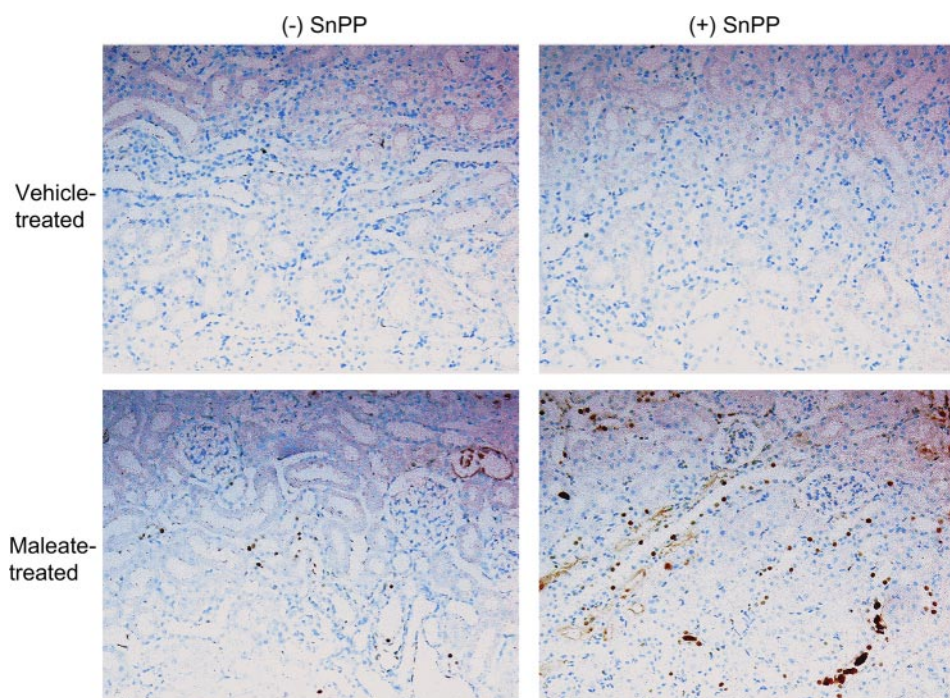


Fig. 7. Assessment of apoptosis by terminal transferase-mediated dUTP nick end-labeling (TUNEL) staining in the kidney in rats subjected to maleate or vehicle and that were treated with SnPP (+SnPP) or saline vehicle (-SnPP); tissues were harvested from rats following studies of urinary protein excretion described in Fig. 5. Maleate-treated rats exhibited the presence of apoptosis, as demonstrated by brown-staining nuclei, and an exacerbation of such staining following treatment with SnPP. Original magnification: $\times 200$.

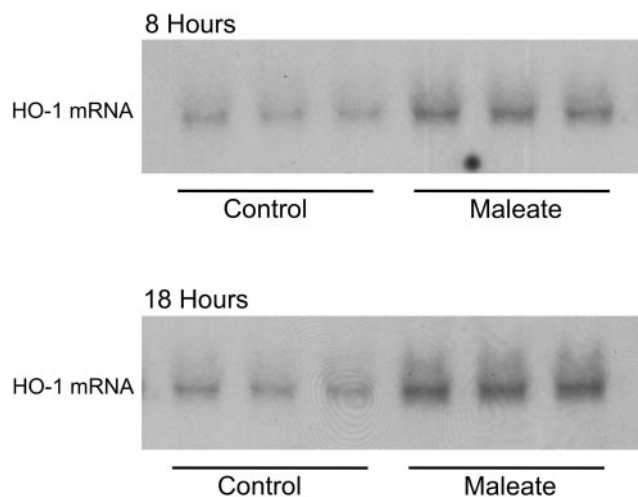


Fig. 8. Expression of HO-1 mRNA in NRK 52E proximal tubular epithelial cells at 8 and 18 h after exposure to media in the absence or presence of maleate (4 mM).

Following escape into the urinary space, albumin and other proteins are incorporated into proximal tubular epithelial cells via the megalin-cubulin receptor system, and are subsequently processed by endosomes and degraded by lysosomes (13, 14, 35, 43). Substantial literature supports the view that such protein degradation stimulates the generation of oxidants, entrains complex signaling pathways, induces inflammatory and other injurious genes, and provokes apoptosis; the culmination of these events is tubulointerstitial inflammation, dropout of tubular epithelial cells, fibrosis, and progressive kidney disease (14, 35, 43). However, the effects of albumin on the proximal tubule are not necessarily adverse, and indeed, evidence has appeared that albumin may not only fail to exert tubular toxicity (5), but indeed may promote the survival of renal

tubular epithelial cells (12, 13). Such protective effects of albumin may arise from the capacity of albumin to carry and proffer to the renal epithelium survival factors such as lysophosphatidic and phosphatidic acids and/or the ability of albumin to directly quench oxidative stress or bind metal catalysts of oxidative stress (12, 13). These divergent effects of albumin, serving, on the one hand, as an instigator of cell injury and inflammation, or on the other, as a survival factor, may be conciliated by the thesis that it is the metabolism by tubules of chemically altered albumin rather than native, unaltered albumin that is likely to prove damaging (12, 13). Glomerular disease may incur the chemical alteration of albumin before its filtration into the urinary space, thereby exposing renal tubules to albumin containing oxidized side chains, advanced glycated end products, or oxidized lipids (3, 12, 13, 43); renal epithelial cell injury and inflammation may more likely occur when chemically altered albumin is metabolized by tubules (13). Because upregulation of HO-1 in tubular epithelial cells is more commonly seen in the setting of cell injury (1, 16, 17, 38), we speculate that the lack of expression of HO-1 in the BSA model *in vivo* and *in vitro* at the stages and time points examined in the present study may reflect, at least in part, the relative absence of injury-inducing modifications of albumin and/or the paucity of tubular cell injury despite severe proteinuria and albuminuria.

The lack of expression of HO-1 in the BSA model, especially if the trafficking of BSA across the proximal tubule in some way suppresses HO-1 expression, may be relevant to the upregulation of cytokines, chemokines, and other inflammatory participants that occurs in this model. In this regard, the failure to express HO-1, as occurs in HO-1^{-/-} mutant mice, is accompanied by exaggerated MCP-1 expression under basal and stressed conditions (28, 34). MCP-1 is upregulated in the BSA model *in vivo* and *in vitro*. We speculate that the failure

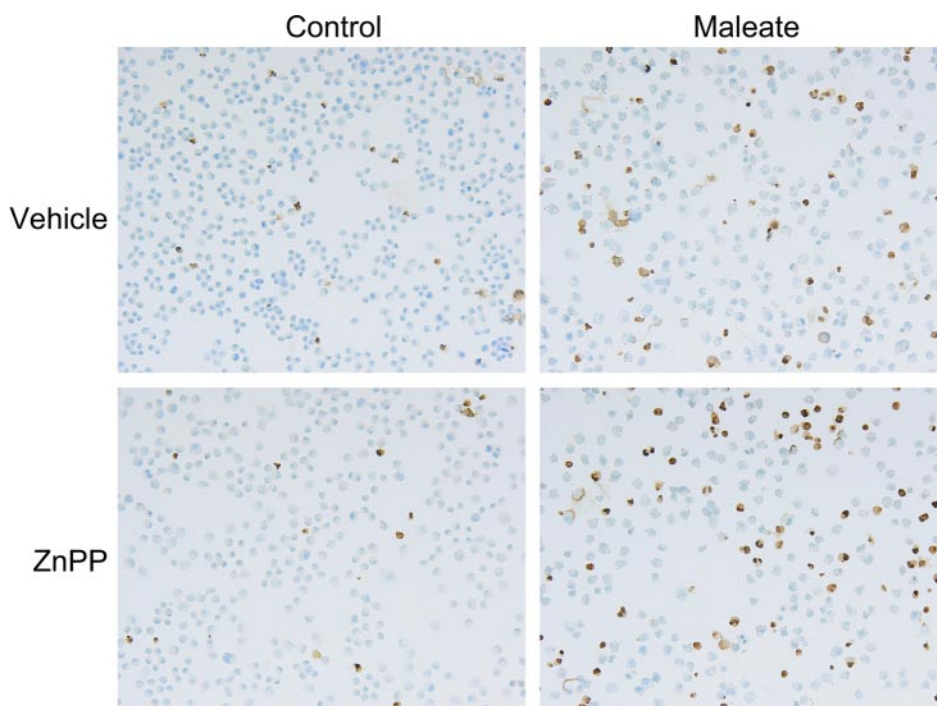


Fig. 9. Representative fields of NRK 52E proximal tubular epithelial cells, examined by the TUNEL method, subjected to media in the absence and presence of maleate (20 mM), and pretreated with zinc protoporphyrin (ZnPP; 5 μ M) or vehicle.

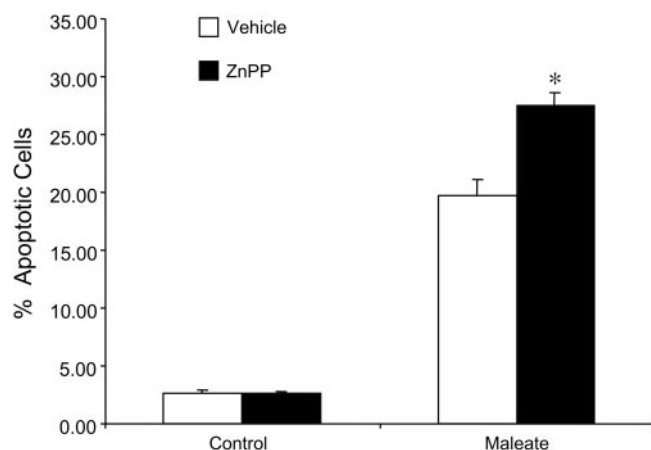


Fig. 10. Quantitation of apoptosis detected by the TUNEL method in NRK 52E proximal tubular epithelial cells subjected to media in the absence and presence of maleate (20 mM) and pretreated with ZnPP (5 μ M) or vehicle. * $P < 0.05$ vs. vehicle-treated maleate-exposed cells.

to express HO-1 may facilitate the upregulation of MCP-1 and other proinflammatory species that occurs in this model.

In contrast to these findings with glomerular proteinuria, HO-1 was markedly induced in a commonly employed model of tubular proteinuria, namely, maleate nephropathy. Maleate nephropathy is characterized by diverse biochemical and structural alterations in the proximal tubule that culminate in impairment of tubular function, including the failure to reabsorb the relatively small amounts of protein filtered into the urinary space (4, 8, 29, 33). In this model, there is increased urinary loss of megalin, the protein that, along with cubulin, contributes to much of the proximal tubular uptake of filtered protein; this model is thus characterized by decreased tubular uptake and trafficking of protein in the proximal tubule (4). Sodium maleate induces numerous metabolic and biochemical abnormalities including the imposition of oxidative stress as glutathione is consumed; inhibition of the TCA cycle; decrease in renal ATP content; inhibition of Na-K-ATPase activity; and decreased tubular content of phosphate, glycine, and other amino acids, and phospholipids (4, 8, 29, 33). Studies of the biochemical effects of diethyl maleate in the kidney reveal increased activity of heme-synthesizing enzymes, heme content, and HO activity (21). In the present model, our data demonstrate that HO-1 is strongly induced and localizes, as demonstrated by immunoperoxidase studies, to proximal tubules, the site along the nephron damaged by maleate; this induction of HO-1 protein is accompanied by increased HO activity. Moreover, maleate directly induces HO-1, as shown in studies in vitro in which renal epithelial cells were exposed to maleate. We suggest that the induction of HO-1 observed in the model of sodium maleate-induced nephropathy reflects the broad-based, injurious effects of sodium maleate on the proximal tubule and, to the extent that biochemical studies of diethyl maleate are germane to the present model, the increased amounts of heme that accrue in the kidney.

That the expression of HO-1 in the maleate model of tubular proteinuria is functionally significant is demonstrated by studies in which HO activity is inhibited: such inhibition of HO activity prevented the subsiding of proteinuria that characteristically occurs at this time point in this model; additionally,

inhibition of HO activity was associated with worsened lethal and sublethal tubular epithelial injury including more severe apoptosis. These studies in vivo were corroborated by studies in vitro. Using rat proximal tubular epithelial cells in culture, we demonstrate that maleate induces apoptosis, the latter increased when HO activity was inhibited. From these studies, we conclude that in the maleate model, induction of HO-1 is functionally significant because the inhibition of HO activity exaggerates the proteinuric response, exacerbates renal structural injury including apoptosis, and the latter findings are recapitulated in vitro. To the best of our knowledge, our present findings in the maleate model are the first to describe the presence of apoptosis; to define the expression of HO-1 in this model by Northern and Western blot analyses and by immunohistochemistry; and to uncover the functional significance of such expression of HO-1.

The presence of apoptosis in the maleate model provides a potentially useful model by which to interrogate the significance of apoptosis in the diseased kidney. We wish to point out that the assessment of apoptosis in the present study utilized the TUNEL technique without the evaluation of nuclear structural changes, and that the TUNEL technique may detect cell necrosis as well as apoptosis in certain types of renal injury. Apoptosis is incriminated as a cause for cell loss following acute insults, as a mechanism involved in the reparative responses following acute renal failure, and as a contributor to nephron dropout in progressive kidney disease (31, 32). A feature of apoptosis in the maleate model is its correlation with proteinuria: it accompanies the appearance of tubular proteinuria and is exacerbated with experimental maneuvers, such as HO inhibition, which increased proteinuria. Moreover, the finding that apoptosis is exacerbated as HO activity is inhibited provides additional support for the antiapoptotic effects of HO-1, a finding first described in cisplatin-induced renal injury (37).

In summary, increased tubular uptake and trafficking of protein, as occur in the BSA model of glomerular proteinuria, fail to induce tubular expression of HO-1; conversely, tubular expression of HO-1 is markedly induced in the maleate model of tubular proteinuria, one in which there is diminished tubular uptake and trafficking of protein. Our studies lead us to conclude that the expression of HO-1 in the diseased kidney cannot be ascribed to the tubular uptake and metabolism of protein leaked by porous glomeruli; rather, the expression of HO-1, at least in the maleate model, seems to reflect tubular injury. In other settings, when overt tubular injury is not apparent, tubular expression of HO-1 may be driven by stimuli that are instigated by or associated with glomerular lesions, including, for example, inflammatory cytokines in the case of glomerulonephritides, and oxidative stress as occurs in glomerulopathies induced by angiotensin II or puromycin. Additionally, tubular expression of HO-1 may arise from specific proteins abnormally present in plasma and that are leaked into the urinary space, such as hemoglobin, as occurs in hemolytic states (28), or possibly, proinflammatory light chains, as occurs in plasma cell dyscrasias (36). Finally, we uncovered the presence of apoptosis in maleate nephropathy, a long-established model of tubular proteinuria, and the protective effect of induced HO-1 in this setting.

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