View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Kidney International, Vol. 48 (1995), pp. 1298–1307

CLINICAL INVESTIGATION

Induction of heme oxygenase in toxic renal injury: A protective role in cisplatin nephrotoxicity in the rat

ANUPAM AGARWAL, JÓZSEF BALLA,[1] JAWED ALAM, ANTHONY J. CROATT, and KARL A. NATH

Department of Medicine, University of Minnesota Medical School, Minneapolis, Minnesota and the Department of Molecular Genetics, Alton Ochsner Medical Foundation, New Orleans, Louisiana, USA

Induction of heme oxygenase in toxic renal injury: A protective role in cisplatin nephrotoxicity in the rat. Cellular content of heme is regulated by heme oxygenase, the rate limiting enzyme in the degradation of heme. Induction of heme oxygenase is a protective response in an *in vivo* model of heme protein mediated renal injury, the glycerol model of acute renal failure. In addition to heme, heme oxygenase is induced by diverse forms of oxidative stress, the functional significance of which is currently unknown. We examined whether heme oxygenase is induced, and the functional significance of such induction, in two *in vivo* models of oxidant-induced toxic nephropathy, namely, cisplatin and gentamicin nephropathies; nephrotoxicity in these models is not dependent on the delivery of a burden of heme proteins to the kidney as occurs in the glycerol model. We demonstrate induction of heme oxygenase mRNA and protein in the kidney as early as 6 and 12 hours after a single dose of cisplatin (6 mg/kg i.v.). Pretreatment with tin protoporphyrin, a competitive inhibitor of heme oxygenase, led to higher serum creatinine values on days 3 through 5 and lower inulin clearances on day 5; tin protoporphyrin also exacerbated renal injury in this model. Renal hemodynamics studied at day 2 after cisplatin demonstrate reduced renal blood flow rates, increased renal vascular resistance and increased fractional excretion of sodium in rats treated with tin protoporphyrin. Tin protoporphyrin alone had no significant effect on serum creatinine and renal hemodynamics in rats with intact, disease-free kidneys. We confirmed that tin protoporphyrin prevented the increase in heme oxygenase activity induced by cisplatin. Induction of heme oxygenase by cisplatin was associated with increased kidney heme content and ferritin content. Induction of heme oxygenase, as measured by mRNA and protein content, also occurred in gentamicin nephropathy, though less than that observed in cisplatin nephropathy. Inhibition of heme oxygenase did not influence sequential serum creatinine determinations in gentamicin nephropathy, thus indicating heterogeneity in the functional significance of induction of heme oxygenase in oxidative stress. We conclude that the induction of heme oxygenase is a protective response in toxic nephropathy, specifically, in cisplatin nephropathy. Ferritin content is also increased by cisplatin, and by sequestering iron, may subserve a beneficial role in this model. Endogenous heme, released from heme proteins, is potentially toxic and may contribute to cisplatin nephrotoxicity.

Heme oxygenase is the rate-limiting enzyme in the degradation of heme [1–3]. This enzyme catalyzes the opening of the heme ring and its conversion to biliverdin, a process that concomitantly releases iron from heme and yields carbon monoxide; subsequently biliverdin is converted to bilirubin [1–3]. Abundantly present in tissues of the reticulo-endothelial system, heme oxygenase enables these tissues to degrade hemoglobin released from senescent red blood cells. The importance of this enzyme in the degradation of heme is underscored not only by its copious presence in these hemoglobin-degrading tissues but also by its rapid and often fulminant induction in a wide variety of mammalian cells exposed to heme [2, 4–7]. Such a response in cells will eventually lower the intracellular levels of heme and thus safeguard against the potential toxicity of heme, a metabolite that can attack and impair, in part through its prooxidant actions, a number of subcellular targets [8]. That the induction of heme oxygenase is a protective response *in vivo* against an inordinate burden of heme was shown *in vivo* in studies involving the kidney, an organ which expresses heme oxygenase poorly or not at all in its intact, disease-free state [9]. The intramuscular injection of hypertonic glycerol induces marked hemolysis and rhabdomyolysis, thereby exposing the kidney to a large burden of heme proteins and precipitating acute renal damage [9]. In this model heme oxygenase is rapidly and prominently expressed in the kidney, and inhibiting such activity by a specific competitive inhibitor, tin protoporphyrin, worsens renal function while the induction of heme oxygenase prior to the administration of hypertonic glycerol protects against renal failure [9].

In addition to heme, a variety of other stimuli induce heme oxygenase *in vitro* in mammalian cells, and these include hydrogen peroxide, UV radiation, metals, endotoxin, cytokines and glutathione depletion [2–7]. While the cellular mechanisms underlying such induction of heme oxygenase are complex, one pathway common to most of these stimuli is oxidative stress [2–7]. The speculation has been offered that the expression of heme oxygenase in these circumstances represents an adaptive response to oxidative stress that mitigates the severity of oxidative damage [5, 6]. The mechanisms underlying the putative protective effects of heme oxygenase in these circumstances are uncertain but involve increased provision of bilirubin, an antioxidant metabolite capable of effectively scavenging peroxy radicals and inhibiting lipid peroxidation *in vitro* [10–11]. However, enhanced heme oxygenase activity, in the absence of imposed heme or heme proteins, may need not necessarily be beneficial, and in fact may have harmful cellular actions: the liberation of iron provides a potential catalyst to oxidative reactions while carbon monoxide in excessive amounts may impair organelles such as the mitochondrion [12].

[1] Present address: Department of Medicine, University of Debrecen, Debrecen, Hungary.

Received for publication March 15, 1995
and in revised form May 18, 1995
Accepted for publication May 18, 1995

© 1995 by the International Society of Nephrology

Thus, in circumstances characterized by heme-independent oxidative stress, contrary to current hypotheses, induction of heme oxygenase could conceivably exacerbate cellular injury.

We attempt to resolve this issue by utilizing *in vivo* models of toxic oxidant-induced nephropathy that are independent of the imposition of a burden of heme proteins on the kidney. We examined whether renal heme oxygenase is induced and the functional significance of such induction in nephropathies incurred by cisplatin and gentamicin. The utility of these agents is often limited by nephrotoxicity, and a considerable amount of evidence substantiates the involvement of oxidative stress in the pathogenesis of cisplatin and gentamicin nephropathies. Cisplatin decreases kidney glutathione content and increases lipid peroxidation [13–16]; cisplatin interacts with DNA in a cell free system and generates superoxide anion [17]; oxidant-scavenging enzymes and assorted antioxidants protect against cisplatin-induced renal injury [16, 18–20], while iron chelation with deferoxamine prevents cisplatin-induced lipid peroxidation in rat kidney cortical slices [21]. A similarly impressive body of work support the view that gentamicin exerts nephrotoxic actions via oxidant pathways. Gentamicin stimulates the production of hydrogen peroxide by mitochondria *in vitro* [22] by the kidney *in vivo* [23], while the severity of gentamicin-induced nephrotoxicity is attenuated by a variety of antioxidants [24, 25]. Nephropathies induced by cisplatin or gentamicin thus provide *in vivo* oxidant states, independent of heme protein delivery to the kidney, wherein the induction of heme oxygenase can be examined and its functional significance—be it a protector against or a perpetrator of renal injury—in such states elucidated.

Methods

Male Sprague-Dawley rats (225 to 275 g, Harlan, Madison, WI, USA) were treated with a single intravenous dose of cisplatin, 6 mg/kg (1.5 mg/ml solution in sterile normal saline; Sigma Chemical Co., St. Louis, MO, USA). With this dose, elevations in serum creatinine were observed starting on day 3 and progressively increased thereafter. In some protocols, tin protoporphyrin, 20 μmol/kg body wt, pH 7.5, (Porphyrin Products, Inc., Logan, UT, USA), a specific competitive inhibitor of heme oxygenase, or vehicle (normal saline) was administered subcutaneously, three hours prior to cisplatin treatment. Identical doses of the inhibitor were administered 8 and 24 hours after the first dose. Rats were maintained on standard Purina rat chow (Purina, St. Louis, MO, USA) and tap water *ad libitum*. Renal function was assessed by serum creatinine values on a tail vein sample and measured by the Jaffe reaction (Creatinine Analyzer II, Beckman Instruments, Inc.). Kidneys were processed for histology, Western and Northern analyses.

Renal clearance studies

We performed renal clearance studies in this model at day 2 following cisplatin administration, prior to which animals received tin protoporphyrin or vehicle. Studies were done under euvolemic conditions, achieved with bovine serum albumin in normal saline, in rats anesthetized with an intraperitoneal injection of 5-sec-butyl-5-ethyl-2-thiobarbituric acid (Inactin, 100 mg/kg body wt) as previously described [26]. Arterial pressure was continuously monitored by a femoral arterial catheter. Glomerular filtration rate (GFR) and renal plasma flow rates for both kidneys were determined by the clearances of inulin and para-aminohippurate, respectively. Clearance data represented the mean of values obtained in two consecutive clearance periods. Urinary and plasma sodium, hematocrit and total protein concentration was also determined. Renal blood flow, renal vascular resistance, fractional excretion of sodium were calculated. To determine the renal hemodynamic effects of the inhibitor, tin protoporphyrin, in rats with normal kidneys, additional clearance studies were also performed at day 2, in rats treated with similar doses of the inhibitor or vehicle alone. To determine whether the elevation in serum creatinine induced by tin protoporphyrin in rats with cisplatin nephropathy was accompanied by lower GFRs, we also performed inulin clearances at day 5, under euvolemic conditions in rats with cisplatin nephropathy treated with tin protoporphyrin or vehicle.

Morphometric studies

Histologic injury was quantified by morphometry on day 5 after cisplatin administration by methods described previously [26, 27]. Morphometric analyses were performed in kidneys fixed by perfusion at mean arterial pressure of the rat with 1.25% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4, osmolality 300 mOsm/kg) followed by immersion in fixative for one hour. The perfused kidney was sliced into coronal sections, and all slices except the terminal slice lacking the corresponding right-side cut surface were placed into cassettes, embedded in paraffin, and sections were cut, 5 μm thick. Analyses were performed in a blinded fashion. Two histologic indices were selected that can be readily and clearly identified: (i) intratubular casts and (ii) tubules with frank necrosis as previously described. The volume densities of tubular casts and necrotic tubules were then calculated.

Northern analysis

RNA extraction, Northern analysis and hybridization against cDNA probes were performed as detailed by Rosenberg, Chmielewski and Hostetter [28]. The cDNA probe for human heme oxygenase was kindly provided by Dr. Rex Tyrrell and the cDNA probe for H-ferritin and L-ferritin were obtained from Dr. Hamish Munro. Autoradiograms were quantified by video densitometry and expressed as corrected O.D. units by previously described methods [29]. To standardize the Northern blots for variability due to loading and transfer we performed video densitometry, factoring the OD of the message for heme oxygenase by the OD of the 18S rRNA on a negative of the ethidium bromide stained nylon membrane. We employed this method in preference to the use of housekeeping genes such as actin and GAPDH for standardization of the Northern blots, since expression of these housekeeping genes *per se* may be altered by the injurious insult [29].

Western analysis

Kidneys were harvested from rats 6, 12, 24 hours, two and five days following cisplatin treatment (6 mg/kg i.v.) and on days 1, 3, 6, and 8 after the administration of gentamicin. Microsomal membrane fractions were prepared as previously described [30] and portions equivalent to 10 μg of protein were electrophoresed on a denaturing 12% polyacrylamide gel. The protein samples were electrophoretically transferred to nitrocellulose membranes and Western blot analysis was performed using a rabbit anti-rat heme oxygenase-1 antibody (Stress Gen; Victoria, Canada) and a

chemiluminescent detection system (Tropix, Inc; Bedford, MA, USA) according to the manufacturer's recommendation.

Heme oxygenase enzyme activity

Heme oxygenase activity was measured by bilirubin generation in kidney microsomes as described previously [9, 31]. Kidney microsomes were incubated with rat liver cytosol, a source of bilirubin reductase (3 mg), hemin (20 μM), glucose-6-phosphate (2 mM), glucose-6-phosphate-dehydrogenase (0.2 U), and NADPH (0.8 mM) for one hour at 37°C in the dark. The formed bilirubin was extracted with chloroform and ΔOD 464 to 530 nm was measured (extinction coefficient, 40 $mM^{-1} \cdot cm^{-1}$ for bilirubin) and enzyme activity expressed as pmol of bilirubin formed/60 min/mg protein.

Kidney cytosolic ferritin

Kidney cytosolic ferritin content was determined using an ELISA method as previously described [9, 31]. Kidney cytosol was diluted 1:10000 and loaded into a 96-well ELISA plate overnight. After incubation with 2% bovine serum albumin, the plates were incubated with the primary antibody, anti-rat ferritin [9]. Anti-rabbit IgG alkaline phosphatase conjugate was used as a secondary antibody. Rat ferritin was used as standard for the measurement.

Heme content

For heme determination, the left kidney was perfused *in situ* with phosphate buffered saline until the venous effluent was clear. The kidney was then homogenized in 0.01 M Tris-HCl with 0.25 M sucrose (1:5 wt/vol) and sonicated. An aliquot of the homogenate was frozen at −70°C for determination of total kidney heme content. To separate different subfractions the homogenate was centrifuged first at 3000 g for 20 minutes at 4°C, followed by a 12,000 g centrifugation for 20 minutes at 4°C of the supernatant and a final 105,000 g centrifugation of the 12,000 g supernatant for one hour at 4°C. The resulting cellular subfractions consisting of mitochondria, microsomes and cytosol were resuspended in phosphate buffer and aliquoted for heme measurement by the pyridine hemochromogen method [32, 33]. Sodium dithionite (2 to 3 mg) was used as a reducing agent and potassium ferricyanide (3 μl, 0.1 M) was used as an oxidizing agent in a final reaction mixture consisting of about 1 mg protein of the sample. The absorbances of the reduced sample were scanned between 570 and 520 nm wavelength using the oxidized samples as blanks. From the reduced minus oxidized spectrum of heme, the difference between the α-maximum and the minimum was measured (557 and 541 nm, respectively), and using the extinction coefficient of 20.7 ml/μmol · cm, heme concentration was expressed as nmol of heme/mg protein.

Total thiols, glutathione peroxidase and catalase activity

Kidneys were homogenized and determination of total thiols was performed as previously described [34]. Glutathione peroxidase and catalase activity was determined in kidney cytosol by the methods of Lawrence and Burk [35] and Aebi [36], respectively.

Gentamicin nephrotoxicity

Gentamicin (40 mg/ml; Gensia Laboratories, Ltd. San Diego, CA, USA) was administered at a dose of 160 mg/kg body wt/day daily for eight days, subcutaneously to male Sprague-Dawley rats (225 to 275 g). Animals received three doses of tin protoporphyrin or vehicle as above, the first dose prior to gentamicin. The inhibitor was again administered at days 5 to 6 during the course of gentamicin nephrotoxicity to maintain optimum inhibition of heme oxygenase activity. Serum creatinine was monitored daily. In a separate experiment, animals received gentamicin and kidneys were removed at sequential time points, one, three, six and eight days during gentamicin treatment and processed for Western and Northern analyses.

Ischemia-reperfusion induced renal injury

Ischemic renal failure was induced as previously described [37]. Under methohexital anesthesia (5 mg/100 g body wt by i.p. injection), male Sprague-Dawley rats (225 to 275 g) underwent right nephrectomy and occlusion of the left renal artery for 45 minutes using a non-traumatic vascular clamp. Serum creatinine was determined 24 and 48 hours following the procedure.

Statistical analysis

Data are expressed as mean ± SEM. For comparisons involving two groups, the unpaired *t*-test was used. For comparisons involving more than two groups ANOVA and the Student-Newman-Keuls test were applied. All results are considered significant at $P < 0.05$.

Results

Induction of heme oxygenase in cisplatin nephropathy

Following the administration of a single intravenous dose of cisplatin (6 mg/kg) significant elevations in serum creatinine occurred at day 3 and increased thereafter. At day 5, mean serum creatinine values in the cisplatin-treated and vehicle treated rats were 2.3 ± 0.1 and 0.3 ± 0.01 mg/dl, respectively, $N = 4$ in each group, $P < 0.05$. Cisplatin treated animals exhibited significant weight loss (246.0 ± 4.5 g and 213.3 ± 7.6 g on days 1 and 5 respectively, $P < 0.05$) while the vehicle-treated rats gained weight (248 ± 2.1 g and 266.0 ± 2.5 g on days 1 and 5, $P < 0.05$).

In this model of cisplatin nephropathy heme oxygenase mRNA was induced in the kidney (Fig. 1). As shown in this Northern analysis, there was a time-dependent induction of heme oxygenase mRNA. mRNA for heme oxygenase was induced as early as 6 and 12 hours following the administration of cisplatin and decreased thereafter. The mean densitometric value, factored for the amount of RNA transferred onto the nylon membrane, for each group is as follows: (a) control, 0.9 ± 0.8; (b) cisplatin one hour, 0.3 ± 0.1; (c) 3 hours, 0.5 ± 0.1; (d) 6 hours 41 ± 7; (e) 12 hours, 40 ± 3; (f) 24 hours, 19 ± 7; (g) 2 days, 8 ± 6; (h) 5 days, 6 ± 2 corrected OD units. In addition to heme oxygenase mRNA, we also confirmed by Western blot analysis, the presence of heme oxygenase protein in the kidney. As shown in Figure 2, increased expression of heme oxygenase occurred at six hours, with maximal expression at 12 hours. Heme oxygenase protein was also detectable at 24 hours and two days, and was decreased by day 5. Thus, these results indicate that heme oxygenase is induced in the kidney and such induction occurs as early as six hours following cisplatin administration.

We also explored whether induction of heme oxygenase was accompanied by induction of other antioxidant enzymes in the kidney such as glutathione peroxidase and catalase. Glutathione peroxidase and catalase activities six hours after cisplatin were not

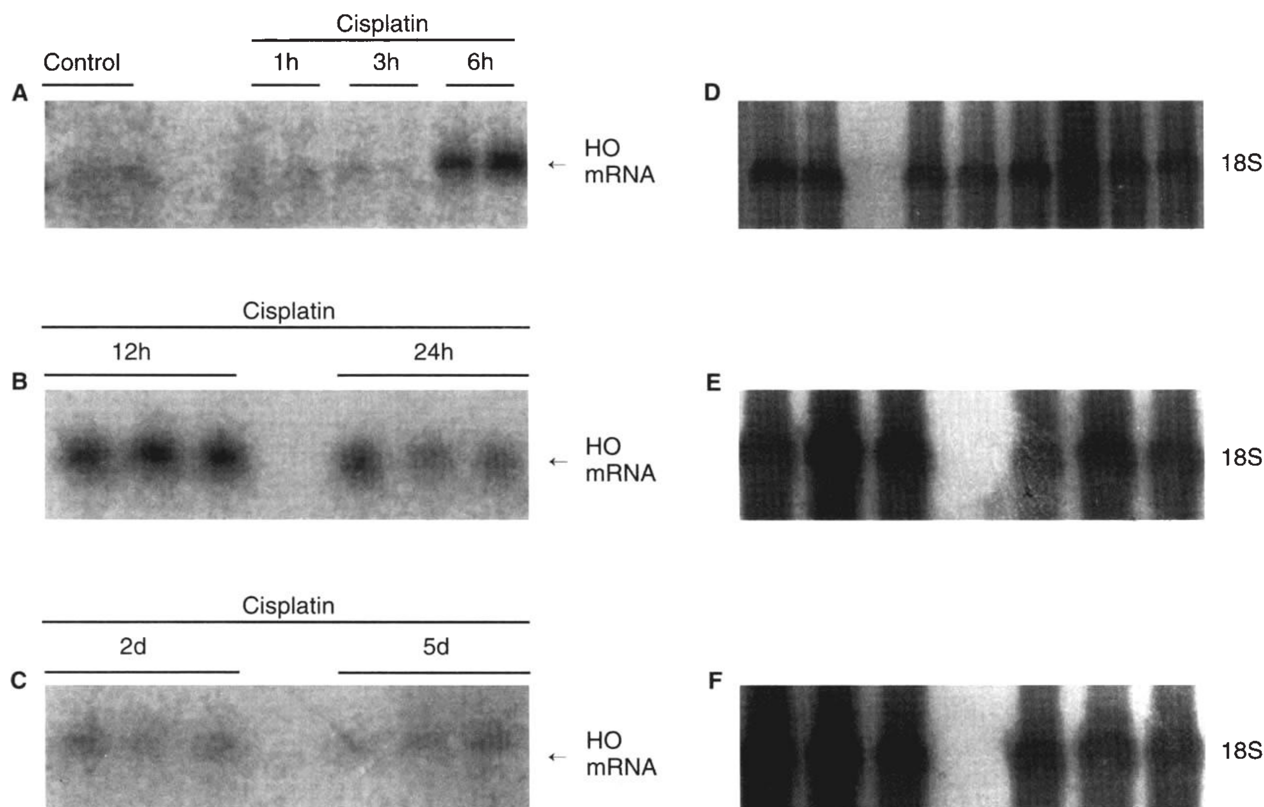

Fig. 1. *Heme oxygenase mRNA in rat kidney at 1, 3, and 6 hours* (**A**), *12 and 24 hours* (**B**), *and 2 and 5 days* (**C**), *following cisplatin (6 mg/kg body wt, i.v.).* Each lane represents RNA (20 μg) from one kidney from an individual animal. Ethidium bromide stained nylon membrane with 18 s RNA for corresponding autoradiogram on the left panel (**D**, **E** and **F**).

statistically different in the cisplatin and the vehicle groups [glutathione peroxidase activity: 445.0 ± 47.6 vs. 448.0 ± 35.0 nmol/min/mg protein; catalase activity: 0.33 ± 0.01 vs. 0.34 ± 0.01 k/mg protein, control ($N = 5$) versus cisplatin ($N = 4$) respectively; P = NS]. Thus the induction of heme oxygenase was not accompanied by induction of other antioxidant enzymes, such as glutathione peroxidase and catalase.

Functional effect of inhibition of heme oxygenase in cisplatin nephropathy

To examine the functional significance of induction of heme oxygenase, we employed a specific competitive inhibitor, tin protoporphyrin. Inhibition of heme oxygenase significantly worsened renal function; by day 3 after administration of cisplatin, serum creatinine was significantly higher in tin protoporphyrin treated rats, and this difference progressively increased thereafter (Fig. 3). While both groups had identical body wts on day 1 (256 ± 3 vs. 257 ± 4 g), cisplatin treated rats subjected to tin protoporphyrin exhibited lower body wts by day 5 (201 ± 7 vs. 219 ± 6 g, $P < 0.05$). Gastrointestinal toxicity, a recognized effect of cisplatin, was observed in both groups of rats. Tin protoporphyrin treatment in rats with cisplatin nephropathy led to similar hematocrits on days 1 and 2, significantly higher values on days 3 (55 ± 1 vs. 49 ± 1%) and day 4 (53 ± 1 vs. 49 ± 1%) and numerically lower values on day 5 (46 ± 1 vs. 48 ± 1%).

We confirmed that the administration of tin protoporphyrin resulted in inhibition of heme oxygenase enzyme activity in the kidney. These studies were undertaken at day 3 after cisplatin. As shown in Figure 4, cisplatin induced a fivefold increase in heme oxygenase activity which was totally prevented by tin protoporphyrin.

We also studied the effect of tin protoporphyrin on renal function in rats with intact, disease-free kidneys. Tin protoporphyrin did not impair renal function in rats with disease-free kidneys: sequential serum creatinine values were unchanged from control values determined up to five days after the administration of tin protoporphyrin or vehicle alone (data not shown). To examine further the specificity of our observations in cisplatin induced nephropathy, and to exclude any possible nonspecific effects that may occur indiscriminately in the injured kidney, we studied the effects of inhibition of heme oxygenase in an ischemia-reperfusion model, one in which heme oxygenase is not induced [38]. Tin protoporphyrin had no effect on renal function as assessed by serum creatinine on day 1 and 2 in this model (vehicle-treated vs. tin protoporphyrin- treated: baseline, 0.31 ± 0.01 vs. 0.32 ± 0.01; day 1, 0.81 ± 0.14 vs. 0.85 ± 0.12; day 2, 0.68 ± 0.08 vs. 0.60 ± 0.08 mg/dl; $N = 6$ in each group, P = NS). Thus, the exacerbatory effect of tin protoporphyrin in cisplatin nephropathy was not due to the effect of tin protoporphyrin *per se* but reflects the effect of the inhibitor superimposed on cisplatin-mediated renal injury.

We performed renal clearance studies in rats with cisplatin nephropathy subjected to tin protoporphyrin or vehicle. These studies were performed at day 2 following cisplatin, a time point

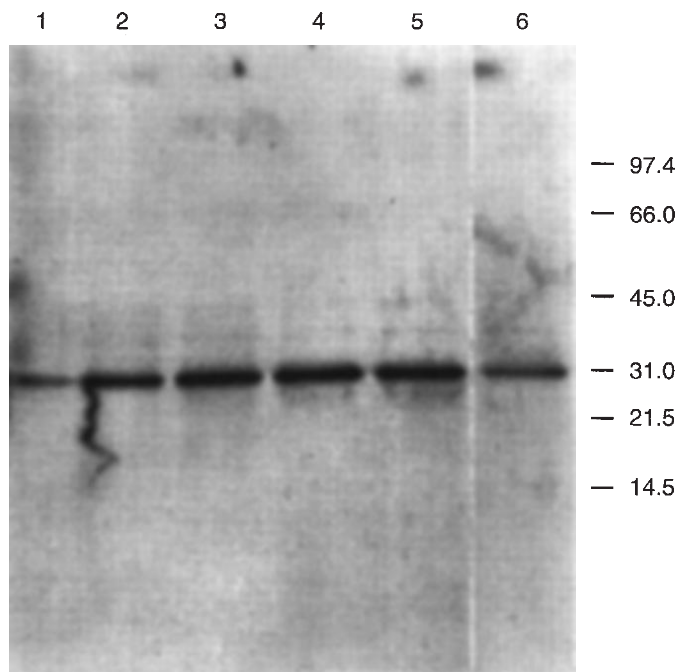

Fig. 2. *Western blot demonstrating induction of heme oxygenase (HO-1) protein in rat kidney six hours (lane 2), 12 hours (lane 3), 24 hours (lane 4), two days (lane 5) and five days (lane 6) following cisplatin treatment.* Lane 1 represents a control animal. The migration and size (kDa) of the molecular weight standards are indicated.

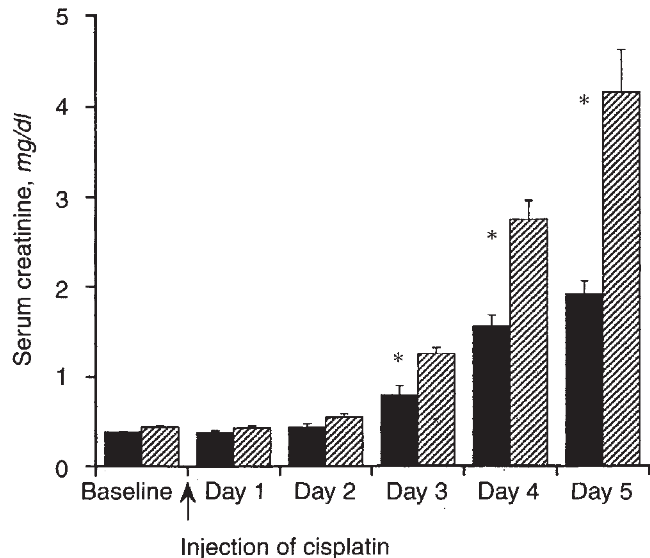

Fig. 3. *Functional significance of induction of heme oxygenase activity in cisplatin nephrotoxicity: Effect of inhibition of heme oxygenase with tin protoporphyrin.* ■, cisplatin with vehicle (N = 6); ▨, cisplatin with tin protoporphyrin (N = 7) * $P < 0.001$.

at which we did not observe any significant difference in serum creatinine values. Body weights (218 ± 2 vs. 219 ± 3 g), basal hematocrit (44 ± 1 vs. 45 ± 1%) and basal total plasma proteins (6.1 ± 0.1 vs. 6.0 ± 0.1 g/dl) were not significantly different between these two groups. GFR was numerically but not statistically lower in animals pretreated with tin protoporphyrin compared to those pretreated with vehicle (0.90 ± 0.09 vs. 1.02 ± 0.09 ml/min, N = 14 in each group, P = NS). Mean arterial pressures during clearances were comparable in the two groups (cisplatin with tin protoporphyrin, 97 ± 3 and cisplatin with vehicle, 94 ± 1 mm Hg, P = NS). However, renal blood flow was significantly reduced in cisplatin treated rats subjected to concomitant treatment with tin protoporphyrin (Fig. 5); pretreatment with tin protoporphyrin markedly and significantly reduced renal blood flow rates to a value approximately 60% of the renal blood flow in cisplatin treated rats administered vehicle only. Calculated renal vascular resistances doubled following prior administration of tin protoporphyrin in this model (cisplatin with tin protoporphyrin vs. cisplatin with vehicle: 22.7 ± 4.4 and 11.0 ± 1.5 mm Hg · min/ml, respectively, $P < 0.02$). Renal clearance studies were also performed in a separate experiment in rats with normal kidneys treated with tin protoporphyrin or vehicle alone (N = 4 in each group); tin protoporphyrin did not significantly alter glomerular filtration rate (GFR, 2.31 ± 0.24 vs. 2.67 ± 0.18 ml/min), mean arterial pressure (MAP, 113 ± 8 vs. 111 ± 4 mm Hg) and renal blood flow rates (RBF, 20.0 ± 2.6 vs. 22.9 ± 1.6 ml/min) in rats with intact, disease free kidneys. Thus, the differences we observed with tin protoporphyrin in cisplatin nephropathy cannot be ascribed to the effects of tin protoporphyrin *per se* on renal function.

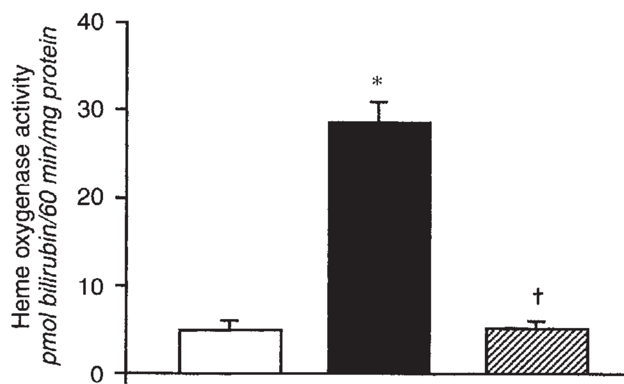

Fig. 4. *Heme oxygenase enzyme activity in cisplatin nephrotoxicity three days after the administration of cisplatin (6 mg/kg): Effect of tin protoporphyrin pretreatment.* Values expressed as mean ± SEM. Symbols are: (□) control; (■) cisplatin with vehicle; (▨) cisplatin with tin protoporphyrin. N = 5 in each group. * $P < 0.001$, cisplatin with vehicle versus control and versus cisplatin with tin protoporphyrin; † P = NS, cisplatin with tin protoporphyrin versus control.

Fractional excretion of sodium (FE_{Na}) was employed to assess tubular function. FE_{Na} was significantly higher in rats with cisplatin nephropathy pretreated with the inhibitor, tin protoporphyrin, attaining an elevenfold increment (cisplatin with vehicle, 0.0010 ± 0.0002; cisplatin with tin protoporphyrin, 0.011 + 0.004, $P < 0.01$). Thus inhibition of heme oxygenase worsens tubular dysfunction as measured by this index. It should be pointed out that the treatment of rats with normal kidneys with tin protoporphyrin as compared to vehicle led to a nonsignificant numerical fourfold increase in FE_{Na} (0.004 ± 0.004 and 0.001 ± 0.0002, respectively, N = 4 in each group, P = NS). Thus, the elevenfold significant increase in FE_{Na} in cisplatin treated rats subjected to

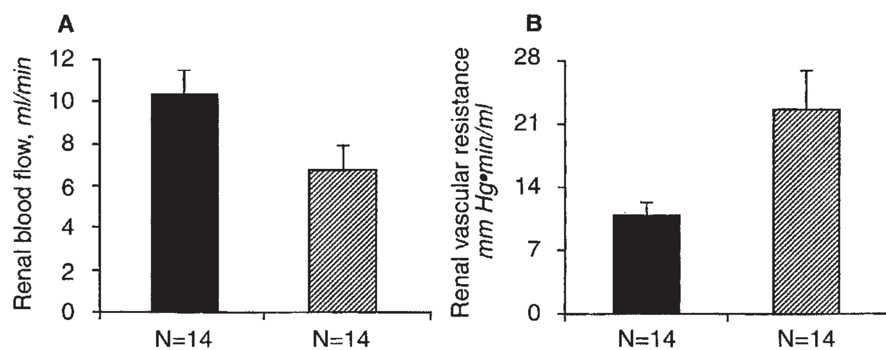

Fig. 5. *Effect of tin protoporphyrin on renal hemodynamics in cisplatin nephrotoxicity: effects on (**A**) renal blood flow ($P < 0.03$) and (**B**) renal vascular resistance ($P < 0.02$) on day 2.* Symbols are: (■) cisplatin with vehicle; (▨) cisplatin with tin protoporphyrin; $N = 14$ in each group.

Table 1. Renal clearance data in rats treated with cisplatin (6 mg/kg i.v.) with vehicle and cisplatin with tin protoporphyrin, studied at day 5

	Cisplatin + vehicle ($N = 6$)	Cisplatin + tin protoporphyrin ($N = 8$)
Body weight *g*	211 ± 4	204 ± 3
Total plasma proteins *g/dl*	6.0 ± 0.1	6.0 ± 0.1
Hematocrit *%*	51 ± 1	49 ± 1
Basal MAP *mm Hg*	101 ± 3	93 ± 2
MAP during clearance *mm Hg*	116 ± 3	103 ± 3[a]
GFR *μl/min/100 g body wt*	76 ± 21	30 ± 5[a]
GFR *μl/min*	162 ± 51	61 ± 11[a]

Values expressed as mean ± SEM. Abbreviation is MAP, mean arterial pressure.

[a] $P < 0.05$, cisplatin with vehicle vs. cisplatin with tin protoporphyrin (day 5)

tin protoporphyrin pretreatment cannot be ascribed to the effects of tin protoporphyrin *per se.*

We also determined GFR by inulin clearance under euvolemic conditions five days after the administration of cisplatin in rats that were treated with tin protoporphyrin or vehicle. This was to assess whether the higher serum creatinine values observed in rats with cisplatin nephropathy treated with tin protoporphyrin reflected lower GFR. These data are summarized in Table 1. In this study, body weights in these two groups were not significantly different, nor were there any significant differences in hematocrit, total plasma protein concentrations or mean arterial pressure in the basal state prior to the commencement of the clearance studies. While marked reductions occurred in both groups, GFRs measured by inulin clearance under euvolemic conditions and expressed either in absolute values or per unit body wt were significantly lower in rats with cisplatin nephropathy concomitantly treated with tin protoporphyrin.

Structural effect of inhibition of heme oxygenase in cisplatin nephrotoxicity

Exacerbation of renal function was accompanied by worsening of structural injury as demonstrated by morphometric analysis performed on day 5 after cisplatin treatment. A higher volume density of tubular necrosis and casts occurred in the animals pretreated with tin protoporphyrin (Fig. 6). Thus inhibition of heme oxygenase worsens structural derangements in this model.

Effect of inhibition of heme oxygenase on ferritin content in cisplatin nephropathy

Prior studies from our laboratory have demonstrated that the induction of heme oxygenase is linked to increased ferritin content [9]. Such increased ferritin content in states characterized by increased amounts of heme or oxidants may provide a storage site for iron released from heme proteins as a consequence of heme oxygenase activity. We therefore determined ferritin content in kidneys subjected to cisplatin nephrotoxicity and treated concomitantly with tin protoporphyrin or vehicle.

A twofold increase in cytosolic ferritin occurred in kidneys exposed to cisplatin and vehicle. Such increase in ferritin in response to cisplatin was unabated when the activity of heme oxygenase was inhibited by tin protoporphyrin (Fig. 7). Thus, the exacerbatory effects of tin protoporphyrin was not due to the suppression of ferritin synthesis elicited by cisplatin in the kidney. This increase in ferritin content in response to cisplatin was not accompanied by enhanced expression of H- or L-ferritin mRNA (data not shown), thus suggesting that this was due to post-transcriptional mechanisms, as has been noted for other instances where ferritin is induced by iron or heme.

Alterations in heme content and total thiol content in cisplatin nephropathy

To determine the mechanisms that may account for the induction of heme oxygenase by cisplatin, we examined two potential pathways that are recognized as inducers of heme oxygenase: increased tissue content of free heme and thiol depletion. The purpose of measuring these parameters at six hours after cisplatin was to determine if significant alterations in heme or thiol content occurred at a time period when heme oxygenase induction was observed. Heme was determined in total kidney homogenate and assorted subcellular fractions. The results are summarized in Table 2. Free heme content increased with cisplatin administration in total kidney and cytosolic fractions, and tended to increase in mitochondria, whereas microsomal heme content was not significantly different. The results of total thiols (predominantly comprising of glutathione) in the kidneys of rats treated with cisplatin (6 mg/kg) revealed that total thiols in the kidney were not decreased, but were significantly higher in cisplatin treated rats compared to the vehicle treated rats (30.6 ± 1.2 vs. 39.4 ± 0.9 nmol/mg protein, $P < 0.05$). Thus, it is unlikely that alterations in kidney thiol content served as a stimulus for heme oxygenase whereas it is possible that increased amounts of free heme may induce heme oxygenase in the cisplatin treated kidneys.

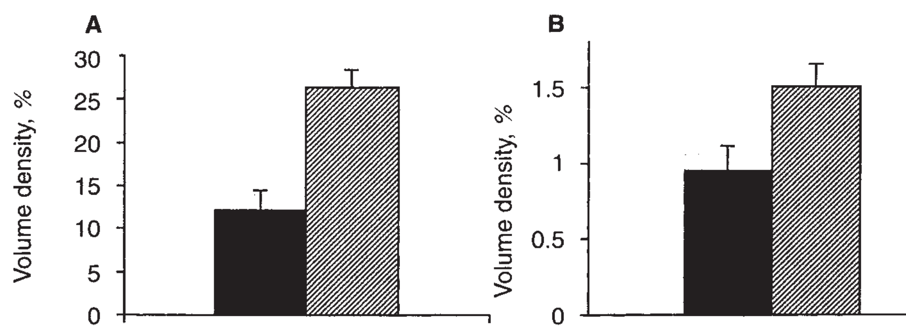

Fig. 6. *Morphometric indices in cisplatin nephrotoxicity with and without tin protoporphyrin pretreatment, five days after the administration of cisplatin.* (**A**) Necrotic tubules ($P < 0.001$). (**B**) Tubular casts ($P < 0.01$). Symbols are: (■) cisplatin with vehicle ($N = 6$); (▨) cisplatin with tin protoporphyrin ($N = 7$).

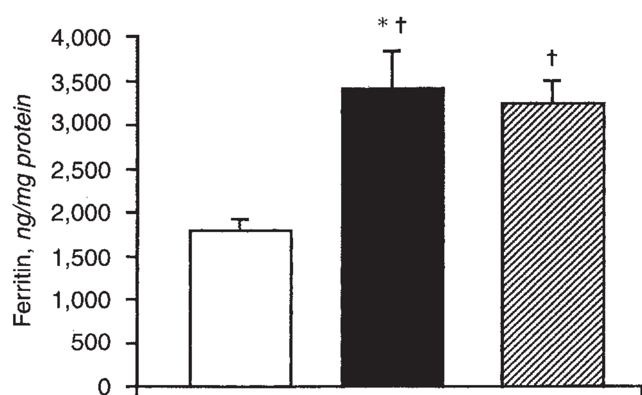

Fig. 7. *Kidney cytosolic ferritin content at day 2 after cisplatin (6 mg/kg).* Symbols are: (□) control ($N = 6$); (■) cisplatin with vehicle ($N = 9$); (▨) cisplatin with tin protoporphyrin ($N = 8$). * P = NS, cisplatin with vehicle versus cisplatin with tin protoporphyrin; † $P < 0.05$, control versus cisplatin with vehicle or cisplatin with tin protoporphyrin.

Table 2. Heme content in the kidney and subcellular fractions, 6 hours after administration of cisplatin (6 mg/kg)

	Control ($N = 5$)	Cisplatin ($N = 4$)	P value
Total kidney	0.49 ± 0.01	0.55 ± 0.01	0.008
Cytosol	0.27 ± 0.01	0.47 ± 0.03	0.0004
Mitochondria	0.72 ± 0.01	0.77 ± 0.03	0.06
Microsomes	0.84 ± 0.05	0.95 ± 0.04	0.11

Values expressed as mean ± SEM, nmol/mg protein for all fractions.

Induction of heme oxygenase in gentamicin nephropathy and effect of inhibiting heme oxygenase

To test the specificity of our observations in cisplatin nephrotoxicity, we studied another model of toxic nephropathy, gentamicin induced nephrotoxicity. Previous studies have demonstrated that oxygen-derived free radicals play a significant role in this model [23–25]. Induction of heme oxygenase mRNA and protein occurred at day 3 in this model of gentamicin nephrotoxicity (Fig. 8 A, B). The mean (of duplicates) densitometric value, factored for the amount of RNA transferred onto the nylon membrane, for each time period is as follows: (a) control, 0.9 ± 0.2; (b) gentamicin one day, 1.43 ± 0.2; (c) 3 days, 10.5 ± 2; (d) 6 days, 3 ± 0.4; (e) 8 days, 1.3 ± 0.8 corrected OD units. The nature of the ~45 kD polypeptide seen in Figure 8B, that reacts with the heme oxygenase antibody is unclear. This protein is present abundantly in several cell lines, but the expression is not inducible by agents that stimulate heme oxygenase synthesis. Sequential assessment of renal function by serum creatinine measurement, after pretreatment with the heme oxygenase inhibitor, tin protoporphyrin, failed to demonstrate any significant functional effect of tin protoporphyrin in this model (Table 3). Thus, inhibition of heme oxygenase in the gentamicin model, one in which heme oxygenase is induced, did not impair renal function, at least as measured by serum creatinine determinations.

Discussion

Alterations in cellular redox and/or increases in cellular heme content are features common to stimuli that induce heme oxygenase [2–7]. While the functional consequences of the induction of heme oxygenase in heme-dependent oxidant stress reside in the ability of this enzyme to reduce intracellular levels of heme, the functional significance of its induction in heme-independent oxidant stress is puzzling. To attempt to elucidate the role of heme in this latter form of oxidative stress, we utilized a toxic nephropathy model, cisplatin nephropathy, one in which multiple lines of evidence attest to the involvement of oxidative stress in the pathogenesis of renal injury [14–17]. This study demonstrates that heme oxygenase is induced in the kidney following the administration of cisplatin. Such induction is characterized by increased amounts of heme oxygenase mRNA and protein, each detectable as early as six hours after the administration of cisplatin, and associated with increased enzyme activity. Inhibition of this enzyme by a specific competitive inhibitor, tin protoporphyrin, worsened the course of cisplatin nephropathy as assessed by functional and structural indices. Renal hemodynamic studies performed at day 2—a time-point at which significant elevations in serum creatinine had not yet occurred in tin protoporphyrin-treated rats—revealed a hemodynamic effect of tin protoporphyrin in rats with cisplatin nephropathy. In this latter group, renal vascular resistance was increased and renal blood flow rates were decreased; additionally, tin protoporphyrin worsened tubular function as reflected by higher fractional excretion of sodium. Tin protoporphyrin did not adversely affect renal function in states in which heme oxygenase is not induced, either in rats with intact, disease-free kidneys, or in rats with postischemic renal disease. Therefore, tin protoporphyrin does not indiscriminately and nonspecifically worsen renal function. We confirmed that tin protoporphyrin effectively inhibited heme oxygenase activity, and indeed prevented the fivefold increase in enzyme activity induced by cisplatin. In aggregate these data provide the first evidence that the induction of heme oxygenase *in vivo* in a model of oxidant-induced, toxic nephropathy subserves a protective role.

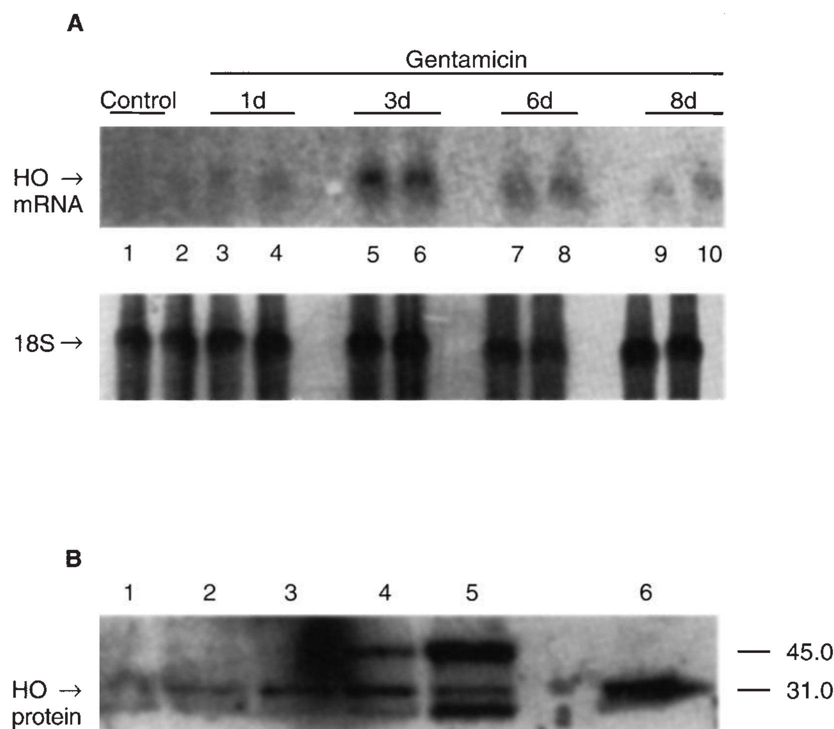

Fig. 8. *Induction of heme oxygenase mRNA (A) and protein (B) in gentamicin nephrotoxicity.* (A) Northern analysis demonstrating induction of heme oxygenase mRNA in the kidney: vehicle-treated (lanes 1, 2), gentamicin treated kidney day 1 (lanes 3, 4), day 3 (lanes 5, 6), day 6 (lanes 7, 8), and day 8 (lanes 9, 10). Each lane represents RNA (20 μg) from one kidney from an individual animal. The ethidium bromide stained nylon membrane for the above autoradiogram is shown in the lower panel. (B) Western analysis demonstrating induction of heme oxygenase (HO-1) protein in the rat kidney at day 1 (lane 2), day 3 (lane 3), day 6 (lane 4), day 8 (lane 5) in gentamicin nephrotoxicity. Lane 6 represents a cisplatin treated kidney at 24 hours. Lane 1 represents a control animal. Heme oxygenase migrates as a 31 kD size protein. The migration and size (kD) of the molecular weight standards are indicated.

Table 3. Effect of tin protoporphyrin on renal function following daily gentamicin (160 mg/kg body wt)

	Control (N = 4)	Vehicle + gentamicin (N = 6)	Tin protoporphyrin + gentamicin (N = 7)
Day 1	0.3 ± 0.05	0.4 ± 0.06	0.5 ± 0.03
Day 3	0.3 ± 0.00	0.4 ± 0.04	0.4 ± 0.03
Day 5	0.3 ± 0.00	0.9 ± 0.17	1.1 ± 0.08
Day 7	0.3 ± 0.01	2.1 ± 0.31	2.5 ± 0.17

Serum creatinine (mg/dl) values expressed as mean ± SEM. P values are not significant.

To explore potential mechanisms accounting for the induction of heme oxygenase in cisplatin nephropathy, we focused on the substrate for heme oxygenase, namely, heme. Thiol depletion, another potent stimulus for the induction of heme oxygenase, was not present in the kidney six hours after the administration of cisplatin, and thus cannot be incriminated as the trigger for such induction. Our results demonstrate that six hours after cisplatin, total kidney and cytosolic heme contents were higher in cisplatin-treated animals compared to controls. We suggest that such increments in heme may drive expression of heme oxygenase. Measurements of heme and thiol contents at earlier time points would be of interest since one would anticipate that alterations in these parameters may antedate the induction of heme oxygenase. While we did not examine the mechanism accounting for increase heme content in the kidney, we speculate that such increased amounts of heme may originate from destabilized intracellular heme proteins. Heme-containing proteins are ubiquitous in cells: besides hemoglobin and myoglobin, the heme prosthetic group is found in such diverse proteins as mitochondrial and microsomal cytochromes, nitric oxide synthase, prostaglandin endoperoxide synthase, catalase, peroxidases, certain oxygenases, respiratory burst oxidase and pyrrolases [3, 5]. Oxidative stress may destabilize heme proteins [39], and as the union of the heme and protein moieties is weakened, the release of heme from its binding protein may occur. We speculate that oxidant stress resulting from cisplatin may unfetter the heme prosthetic group from its allied protein. The heme prosthetic group, either directly or via the effects of its released iron, can damage a number of cellular targets including lipid bilayers, cytoskeleton, mitochondria, enzymatic proteins, the antioxidant reserve and the nucleus [8, 40]. Interestingly, many of these targets are also recognized as sites that are injured in cisplatin nephropathy [14–17, 41, 42]. Induction of heme oxygenase following the administration of cisplatin thus represents a response that restrains the increment in cellular heme content that would otherwise occur. We wish to point out that while prior studies demonstrate increased renal content of heme, such changes in heme were observed at a delayed timepoint, that is, seven days after the administration of cisplatin, a point at which the kidney is profoundly damaged [43]. Such increments in heme at this much later time point are attributed to increased heme synthesis effected by increased ferrochelatase activity. Our studies uncover increments in heme as early as six hours after the administration of cisplatin, a timepoint prior to the occurrence of overt renal injury, and one that is accompanied by induction of heme oxygenase.

In addition to the induction of heme oxygenase, the administration of cisplatin leads to increased ferritin content of the kidney. In previous studies increased ferritin content accompanied the induction of heme oxygenase in an *in vivo* model of heme protein-mediated renal injury [9]. By chelating iron released as a consequence of increased heme oxygenase activity, such increased ferritin content likely subserves a protective role [9, 31]. Therefore, the extent to which catalytic "free" iron contributes to renal

injury induced by cisplatin, increased amounts of ferritin, which is linked to increased heme oxygenase activity, may provide an additional renal response that serves to protect the kidney in cisplatin nephropathy. Other mechanisms by which induction of heme oxygenase may offer protection include increased generation of bilirubin, a metabolite with potent antioxidant capabilities *in vitro* [10, 11]. Our studies raise the possibility that effects of enhanced heme oxygenase activity on renal hemodynamics may also be beneficial in this model. In rats with cisplatin nephropathy the administration of tin protoporphyrin decreased renal blood flow and increased calculated renal vascular resistances at a relatively early timepoint in the course of progressive renal failure, and prior to the emergence of differences in GFR as reflected by serum creatinine determinations. Such augmentation in renal vasoconstriction in cisplatin nephropathy, incurred by ablating the increase in heme oxygenase activity that normally occurs, indicates that the maintenance of renal blood flow is dependent on enhanced heme oxygenase activity. In this regard we offer two speculations. Firstly, increased heme oxygenase activity generates carbon monoxide, a metabolite with potent vasodilatory effects mediated in part through cGMP [44, 45]; in addition, carbon monoxide is also recognized as an important intracellular signaling molecule in neural tissues [46]. Generation of this vasodilator in increased amounts by enhanced heme oxygenase activity in the cisplatin treated kidney may serve to dilate the renal vasculature. Secondly, increased intracellular heme, which we detect in cisplatin nephropathy, may contribute to renal vasoconstriction since heme is recognized as a binding site for nitric oxide [47]. Basal renal vasodilatation is critically dependent on nitric oxide and quenching of nitric oxide, as occurs in assorted disease states, contributes to renal vasoconstriction [48–50]. We speculate that by degrading such increased amounts of intracellular heme, and thus removing a sump for nitric oxide, enhanced heme oxygenase activity may thus maintain renal blood flow.

We also selected for examination another model of toxic nephropathy, gentamicin nephropathy, one in which enhanced generation of reactive oxygen species is incriminated in the pathogenesis of renal injury. This latter model of renal injury is characterized by increased generation of hydrogen peroxide [22, 23] and assorted scavengers of reactive oxygen species reduce structural and functional renal injury in this model [24, 25]. We provide the first demonstration in this model that heme oxygenase is induced. However, in contrast to effects observed in the cisplatin model, we found no evidence for a functional effect accruing from inhibition of heme oxygenase activity. These findings are instructive in that they underscore the heterogeneous effects resulting from the induction of heme oxygenase in diseased states. In settings such as gentamicin nephropathy wherein the induction of heme oxygenase leaves the kidney subjected to oxidative stress unaided, it is likely that the induction of heme oxygenase fails to fulfill the critical requirements of an effective antioxidant strategy [7], that is, the provision of adequate amounts of an appropriate scavenger either at cellular loci where oxidants are generated or at those vulnerable and vital cellular targets that suffer the brunt of oxidative attack. Thus in states of tissue injury wherein it is induced, heme oxygenase can be a protectant or in others a seemingly disinterested bystander [40]. Such divergent functional effects arising from the induction of heme oxygenase urge caution in inferring the functional significance of its induction in one given setting based on conclusions derived from another.

In conclusion, this study provides the first demonstration that a model of oxidant-induced toxic nephropathy and one not dependent on the delivery of heme proteins to the kidney—in cisplatin nephropathy—the induction of heme oxygenase affords protective effects. In this model increased amounts of endogenous heme is detected in the kidney, and such heme may contribute to the induction of heme oxygenase and the injury that ensues. Induction of heme oxygenase also occurs in another oxidant-driven, toxic nephropathy model, gentamicin nephropathy, but in this latter model, inhibiting its activity fails to exacerbate the course of renal insufficiency.

Acknowledgments

These studies were supported by NIH grants to Karl Nath (R01- DK 47060) and to Jawed Alam (R01- DK 43135). This work was presented in part at the American Society of Nephrology meeting in Orlando, Florida, October 1994 and was published in abstract form JASN 5:915, 1994.

Reprint requests to Karl A. Nath, M.D., Department of Medicine, Renal Division, Box 736 UMHC, University of Minnesota, 516 Delaware St SE, Minneapolis, Minnesota, USA

References

1. Tenhunen R, Marver HS, Schmid R: The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc Natl Acad Sci USA* 61:748–755, 1968
2. Maines MD: Heme oxygenase: Function, multiplicity, regulatory mechanisms and clinical application. *FASEB J* 2:2557–2568, 1988
3. Abraham NG, Lin JH, Schwartzman ML, Levere RD, Shibahara S: The physiological significance of heme oxygenase. *Int J Biochem* 20:543–558, 1988
4. Keyse S, Tyrrell R: Heme oxygenase is the major 32-kDA stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci USA* 86:99–103, 1989
5. Stocker R: Induction of haem oxygenase as a defense against oxidative stress. *Free Rad Res Commun* 9:101–112, 1990
6. Applegate LA, Luscher P, Tyrrell RM: Induction of heme oxygenase: A general response to oxidant stress in cultured mammalian cells. *Cancer Res* 51:974–978, 1991
7. Nath KA: The functional significance of the induction of heme oxygenase by oxidative stress. *J Lab Clin Med* 123:461–463, 1994
8. Hebbel RP, Eaton JW: Pathobiology of heme interaction with the erythrocyte membrane. *Semin Hematol* 26:136–149, 1989
9. 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
10. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN: Bilirubin is an antioxidant of possible physiologic significance. *Science* 235:1043–1046, 1987
11. Llesuy SF, Tomaro ML: Heme oxygenase and oxidative stress: Evidence of involvement of bilirubin as physiological protector against oxidative damage. *Biochim Biophys Acta* 1223:9–14, 1994
12. Zhang J, Piantadosi CA: Mitochondrial oxidative stress after carbon monoxide hypoxia in rat brain. *J Clin Invest* 90:1193–1199, 1992
13. Sugihara K, Nakano S, Koda M, Tanaka K, Fukuishi N, Gemba M: Stimulatory effect of cisplatin on production of lipid peroxidation in renal tissues. *Jpn J Pharmacol* 43:247–252, 1987
14. Hannemann J, Baumann K: Cisplatin-induced lipid peroxidation and decrease of gluconeogenesis in rat kidney cortex: Different effects of antioxidants and radical scavengers. *Toxicology* 51:119–132, 1988
15. Sadzuka Y, Shoji T, Takino Y: Mechanism of the increase in lipid peroxide induced by cisplatin in the kidneys of rats. *Toxicol Lett* 62:293–300, 1992

16. BRADY HR, ZEIDEL ML, KONE BC, GIEBISCH G, GULLANS SR: Differential actions of cisplatin on renal proximal tubule and inner medullary collecting duct cells. *J Pharmacol Exp Ther* 265:1421–1428, 1993
17. MASUDA H, TANAKA T, TAKAHAMA U: Cisplatin generates superoxide anion by interaction with DNA in a cell-free system. *Biochem Biophys Res Commun* 203:1175–1180, 1994
18. MCGINNESS JE, PROCTOR PH, DEMOPOULOS HB, HOKANSON JA, KIRKPATRICK DS: Amelioration of *cis*-platinum nephrotoxicity by orgotein (superoxide dismutase). *Physiol Chem Phys* 10:267–277, 1978
19. DOBYAN DC, BULL JM, STREBEL FR, SUNDERLAND BA, BULGER RE: Protective effects of O-(β-hydroxyethyl)-rutoside on *cis*-platinum-induced acute renal failure in the rat. *Lab Invest* 55:557–563, 1986
20. APPENROTH D, WINNEFELD K: Role of glutathione for cisplatin nephrotoxicity in young and adult rats. *Renal Fail* 15:135–139, 1993
21. KAMEYAMA Y, GEMBA M: The iron chelator, deferoxamine, prevents cisplatin-induced lipid peroxidation in rat kidney cortical slices. *Jpn J Pharmacol* 57:259–262, 1991
22. WALKER PD, SHAH SV: Gentamicin enhanced production of hydrogen peroxide by renal cortical mitochondria. *Am J Physiol* 253 (Cell Physiol 22):C495–C499, 1987
23. GUIDET BR, SHAH SV: *In vivo* generation of hydrogen peroxide by rat kidney cortex and glomeruli. *Am J Physiol* 256 (Renal Fluid Electrol Physiol 25):F158–F164, 1989
24. WALKER PD, SHAH SV: Evidence suggesting a role for hydroxyl radical in Gentamicin-induced acute renal failure in rats. *J Clin Invest* 81:334–341, 1988
25. NAKAJIMA T, HISHIDA A, KATO A: Mechanisms of protective effects of free radical scavengers on gentamicin-mediated nephropathy in rats. *Am J Physiol* 266 (Renal Fluid Electrol Physiol 35):F425–F431, 1994
26. NATH KA, SALAHUDEEN AK: Induction of renal growth and injury in the intact rat kidney by dietary deficiency of antioxidants. *J Clin Invest* 86:1179–1192, 1990
27. SALAHUDEEN AK, CLARK EC, NATH KA: Hydrogen peroxide-induced renal injury. A protective role for pyruvate *in vitro* and *in vivo*. *J Clin Invest* 88:1886–1893, 1991
28. ROSENBERG ME, CHMIELEWSKI D, HOSTETTER TH: Effect of dietary protein on rat renin and angiotensinogen gene expression. *J Clin Invest* 85:1144–1149, 1990
29. CORREA-ROTTER R, MARIASH CN, ROSENBERG ME: Loading and transfer control for Northern hybridization. *Biotechniques* 12:154–158, 1992
30. MAINES MD, TRAKSHEL GM, KUTTY RK: Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible. *J Biol Chem* 261:411–419, 1986
31. BALLA G, JACOB HS, BALLA J, ROSENBERG ME, NATH KA, APPLE F, EATON JW, VERCELLOTTI GM: Ferritin: A cytoprotective antioxidant stratagem of endothelium. *J Biol Chem* 267:18148–18153, 1992
32. PAUL KG, THEORELL H, AKESON A: The molar light absorption of pyridine ferroprotoporphyrin (Pyridine Haemochromogen). *Acta Chem Scand* 7:1284–1287, 1953
33. BERRY EA, TRUMPOWER BL: Simultaneous determination of hemes a, b, and c, from pyridine hemochrome spectra. *Anal Biochem* 161:1–15, 1987
34. ELLMAN GL: Tissue sulfhydryl groups. *Arch Biochem Biophys* 82:70–77, 1959
35. LAWRENCE RA, BURK RF: Glutathione peroxidase activity in selenium deficient rat liver. *Biochem Biophys Res Commun* 95:351–358, 1976
36. AEBI H: Catalase, in *Methods of Enzymatic Analysis* (vol 2), edited by HU BERGMEYER, New York, Academic Press, Inc., 1974, pp 673–684
37. NATH KA, PALLER M: Dietary Deficiency of antioxidants exacerbates ischemic injury in the rat kidney. *Kidney Int* 38:1109–1117, 1990
38. PALLER MS, NATH KA, ROSENBERG ME: Heme oxygenase is not expressed as a stress protein after renal ischemia. *J Lab Clin Med* 122:341–345, 1993
39. STERN A: Drug-induced oxidative denaturation in red blood cells. *Semin Hematol* 26:301–306, 1989
40. NATH KA, AGARWAL A, VOGT BA: Functional consequences of induction of heme oxygenase. *Contemporary Issues in Nephrology* (in press)
41. BRADY HR, KONE BC, STROMSKI ME, ZEIDEL ML, GIEBISCH G, GULLANS SR: Mitochondrial injury: An early event in cisplatin toxicity to renal proximal tubules. *Am J Physiol* 258 (Renal Fluid Electrol Physiol 27):F1181–F1187, 1990
42. GORDON JA, GATTONE VH II: Mitochondrial alterations in cisplatin-induced acute renal failure. *Am J Physiol* 250(Renal Fluid Electrol Physiol 19):F991–F998, 1986
43. MAINES MD: Differential effect of cis-platinum (cis-diamminedichloro-platinum) on regulation of liver and kidney haem and haemoprotein metabolism. Possible involvement of gamma-glutamyl-cycle enzymes. *Biochem J* 237:713–721, 1986
44. MARKS GS, BRIEN JF, NAKATSU K, MCLAUGHLIN BE: Does carbon monoxide have a physiological function? *Trends Pharmacol Sci* 12: 185–188, 1991
45. FURCHGOTT RF, JOTHIANANDAM D: Endothelial-dependent and independent vasodilatation involving cGMP: relaxation induced by nitric oxide, carbon monoxide and light. *Blood Vessels* 28:52–61, 1991
46. VERMA A, HIRSCH DJ, GLATT CE, RONNETT GV, SNYDER SH: Carbon monoxide: A putative neural messenger. *Science* 259:381–384, 1993
47. WANG J, ROUSSEAU DL, ABU-SOUD HM, STUEHR DJ: Heme coordination of NO in NO synthase. *Proc Natl Acad Sci USA* 91:10512–10516, 1994
48. TOLINS JP, PALMER RM, MONCADA S, RAIJ L: Role of endothelium-derived relaxing factor in regulation of renal hemodynamic responses. *Am J Physiol* 258 (Heart Circ Physiol 27):H655–H662, 1990
49. BACHMANN S, MUNDEL P: Nitric oxide in the kidney: Synthesis, localization, and function. *Am J Kidney Dis* 24:112–129, 1994
50. LUSCHER TF, BOCK HA: The endothelial L-arginine/nitric oxide pathway and the renal circulation. *Klin Wochenschrift* 69:603–609, 1991