The Indispensability of Heme Oxygenase-1 in Protecting against Acute Heme Protein-Induced Toxicity *in Vivo*

Karl A. Nath,* Jill J. Haggard,* Anthony J. Croatt,*
Joseph P. Grande,[†] Kenneth D. Poss,[‡] and
Jawed Alam[§]

From the Nephrology Research Unit* and the Department of Pathology,[†] Mayo Clinic/Foundation, Rochester, Minnesota; the Center for Cancer Research,[‡] Massachusetts Institute of Technology, Cambridge, Massachusetts; and the Department of Molecular Genetics,[§] Alton Ochsner Medical Foundation, New Orleans, Louisiana

Heme oxygenase (HO) is the rate limiting enzyme in the degradation of heme, and its isozyme, HO-1, may protect against tissue injury. One posited mechanism is the degradation of heme released from destabilized heme proteins. We demonstrate that HO-1 is a critical protectant against acute heme protein-induced toxicity *in vivo*. In the glycerol model of heme protein toxicity—one characterized by myolysis, hemolysis, and kidney damage—HO-1 is rapidly induced in the kidney of HO-1 +/+ mice as the latter sustain mild, reversible renal insufficiency without mortality. In stark contrast, after this insult, HO-1 -/- mice exhibit fulminant, irreversible renal failure and 100% mortality; HO-1 -/- mice do not express HO-1, and evince an eightfold increment in kidney heme content as compared to HO-1 +/+ mice. We also demonstrate directly the critical dependency on HO-1 in protecting against a specific heme protein, namely, hemoglobin: doses of hemoglobin which exert no nephrotoxicity or mortality in HO-1 +/+ mice, however, precipitate rapidly developing, acute renal failure and marked mortality in HO-1 -/- mice. We conclude that the induction of HO-1 is an indispensable response in protecting against acute heme protein toxicity *in vivo*. (Am J Pathol 2000, 156:1527–1535)

Heme oxygenase (HO) is the rate limiting enzyme in the degradation of heme.^{1–3} HO facilitates the opening of the heme ring and its conversion to biliverdin; during such conversion, iron is released from the heme prosthetic group and carbon monoxide is produced; subsequently, biliverdin is reduced to bilirubin by biliverdin reductase.^{1–3} HO-1 is the isozyme that is induced by heme, oxidants, cytokines, glycated albumin, and other stressors, and the induction of this isozyme is associated with

a protective response in some of these states.^{1–9} HO-2 is the constitutive isozyme that may be involved in the regulation of vascular tone and neural function effected, in part, through the cellular effects of carbon monoxide.^{10,11}

One posited, but primarily unsubstantiated, mechanism invoked for the cytoprotective effects of HO-1 in the setting of tissue injury centers on the degradation of heme released from intracellular heme proteins that are destabilized *pari passu* as cells are injured.^{1–3} The heme prosthetic group is widespread in cells as it is found in proteins that store or carry oxygen, cytochromes, and numerous enzymes involved in diverse aspects of cellular metabolism. Destabilization of heme proteins in injured cells may lead to the disengagement of heme from its linked protein moiety. The linkage of heme with a given protein moiety not only enables the functional activity of the specific heme protein, but also restrains free heme from exerting injurious effects. A large body of literature confirms the cytotoxicity of free heme,^{12–15} and indeed, there are clinical observations attesting to the marked nephrotoxicity of heme when the latter—used to induce remission in patients with acute intermittent porphyria—is inadvertently administered in inordinate doses.¹⁶ Heme is a lipophilic prooxidant that can impair lipid bilayers and organelles such as mitochondria and nuclei; heme can also destabilize the cytoskeleton; finally, heme can impair a number of enzymes.^{12–17} In injured cells, the recruitment of HO-1 may degrade heme released from intracellular heme proteins, thereby safeguarding against this mechanism of cytotoxicity.

Besides the removal of heme, the induction of HO-1 procures potentially cytoprotectant molecules such as ferritin, bilirubin, and carbon monoxide. Ferritin provides a storage site for iron,¹⁸ whereas bilirubin is a metabolite with recognized antioxidant properties.^{19,20} Carbon monoxide is a critical intracellular signaling molecule in neural tissue;¹⁰ additionally, carbon monoxide possesses vasodilatory, anti-inflammatory, and cytoprotectant properties.^{10,11,21,22} Thus, the salutary effects afforded by the induction of HO-1 may reside in the recruitment of bio-

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Address reprint requests to Dr. Karl Nath, Mayo Clinic, 200 First St., SW, 542 Guggenheim Bldg., Rochester, MN 55905. E-mail: nath.karl@mayo.edu.

chemically diverse cytoprotective substances, in addition to the reduction in an intracellular toxicant.

Much of the literature demonstrating a protective effect of induced HO-1, however, relies on pharmacological approaches which may exert effects besides those involving HO-1;^{23–25} the availability of genetically engineered mice deficient in HO-1 provides an opportunity to examine the protective effects of induced HO-1 without the confounding actions of such pharmacological manipulations.^{6,8} Moreover, very few studies have examined whether the potential cytoprotective effects of HO-1 are derived from restraining the tissue buildup of heme; and these studies do not include heme protein-induced toxicity. We thus examined the requirement for HO-1 in protecting against heme proteins—a possible intracellular toxicant in injured tissue, and one common to insults in which HO-1 is induced—using mice deficient in HO-1 (HO-1 $-/-$) or wild-type mice (HO-1 $+/+$). We used a long-established, *in vivo* model of heme protein-induced tissue injury, the glycerol model, in which we examined the relative sensitivity of these mice to such injury. The intramuscular injection of hypertonic glycerol induces myolysis and hemolysis thereby exposing tissues, especially the kidney, to large amounts of myoglobin and hemoglobin;^{26,27} acute renal failure dominates this disease model and the analogous clinical syndromes, such as those caused by crush injuries and a host of nontraumatic and medical disorders.²⁷ Using this model of heme protein-induced renal injury, as well as the direct examination of the nephrotoxicity of a specific heme protein (hemoglobin), we provide compelling evidence for the indispensability of HO-1 in protecting against heme protein-mediated injury *in vivo*.

Materials and Methods

HO-1 $-/-$ and HO-1 $+/+$ Mice

Homozygous HO-1 null mutants were generated by targeted disruption of the HO-1 gene as described by Poss and Tonegawa.²⁸ Colonies of mice were maintained by breeding HO-1 $-/-$ males with HO-1 $+/-$ females. Offspring were genotyped at the time of weaning by using polymerase chain reaction to amplify the wild-type and mutant alleles of genomic DNA obtained from tail samples. HO-1 $+/+$ mice (wild type) were used as controls. The characteristics of HO-1 $-/-$ mice, described previously,²⁸ include slower rate of somatic growth as compared to HO-1 $+/+$ mice; and thus to ensure that HO-1 $+/+$ and HO-1 $-/-$ mice received comparably severe glycerol-induced exposure to heme proteins, HO-1 $-/-$ mice were matched in body weight to HO-1 $+/+$ mice, the mean age of the HO-1 $-/-$ mice being 30 weeks whereas the mean age of the HO-1 $+/+$ mice was 20 weeks. Groups of HO-1 $+/+$ and HO-1 $-/-$ mice comprised similar numbers of male and female mice. Such groups were used in the study of the relative effects of the glycerol model of heme protein toxicity and the relative effects of a specific heme protein, namely, hemoglobin.

In additional studies of the nephrotoxic actions of hemoglobin in HO-1 $+/+$ and HO-1 $-/-$ mice, we examined the effects of hemoglobin in HO-1 $+/+$ and HO-1 $-/-$ mice that were much younger (age, 7 to 10 weeks) than those previously used (age, 20 to 30 weeks). In these additional studies using HO-1 $+/+$ and HO-1 $-/-$ mice in the age range 7 to 10 weeks, mean body weights of these mice were not significantly different (21.5 ± 1.5 g versus 22.7 ± 1.7 g, respectively; $P = \text{ns}$); the toxicity of a specific heme protein was thus further studied in younger mice similar in age and body weight.

The Glycerol Model of Heme Protein-Induced Injury

HO-1 $+/+$ and HO-1 $-/-$ mice, deprived of water overnight for 16 hours but allowed free access to rodent chow, were anesthetized with ether, and then injected with 50% glycerol in water, 7.5 ml/kg, half of the dose injected into each anterior thigh muscle.^{26,29} In initial studies, the renal effects of intramuscular injections of 50% glycerol at two doses, 7.5 ml/kg and 10 ml/kg, were examined in C57BL/6 mice.

Hemoglobin-Induced Acute Renal Failure

HO-1 $+/+$ and HO-1 $-/-$ mice were deprived of water for 16 hours but allowed free access to rodent chow. Mice were given two tail vein injections of mouse hemoglobin (Sigma Chemical Co., St. Louis, MO), each consisting of 90 mg/100 g mouse hemoglobin, and administered 1 hour apart.

Plasma Creatinine

Renal function was assessed by plasma creatinine concentrations; the latter were determined on plasma derived from tail vein blood samples and using a Beckman Creatinine Analyzer II (Beckman Instruments, Inc., Fullerton, CA).²⁹

Heme Content and Plasma Hemoglobin Concentrations

Plasma hemoglobin concentrations were determined by the method of Winterbourn.³⁰ Heme content of whole kidney homogenates was determined by the pyridine hemochromogen method.^{31,32}

Lactate Dehydrogenase and Creatine Kinase Activities

Plasma lactate dehydrogenase (LDH) activity was measured by determining the rate of formation of NADH.¹⁷ Plasma creatine kinase activity was measured by a colorimetric method using a Sigma Diagnostics Creatine Phosphokinase kit (Sigma Chemical Co.).

Figure 1. Effect of hypertonic glycerol (50%), 7.5 and 10 ml/kg, in dehydrated C57BL/6 mice on HO-1 mRNA expression, determined 6 hours after glycerol. The individual and mean standardized densitometric readings are provided below the Northern analysis.

Northern Analysis

RNA from kidneys was extracted using the Trizol method (Life Technologies, Inc., Gaithersburg, MD). Ten μ g of total RNA from each sample were separated on an agarose gel and transferred to a nylon membrane. Membranes were hybridized overnight with a 32 P-labeled mouse HO-1 cDNA probe. Autoradiograms were standardized, as previously described,³³ 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.

Western Analysis and Immunohistochemical Staining

Western analysis was performed using a polyclonal HO-1 antibody (SPA-895, Stressgen, Victoria, BC, Canada).³³ For immunohistochemical localization, tissue sections were fixed in 10% formalin and embedded in paraffin. Staining for HO-1 was performed using a polyclonal antibody (SPA-895, Stressgen) as the primary antibody, a horseradish peroxidase conjugated secondary antibody (R14745, Transduction Laboratories, Lexington, KY) and diaminobenzidine as substrate for localization.

Statistics

Results are expressed as means $\pm$ SEM, and are considered statistically significant for $P < 0.05$. For comparison between unpaired groups, the Student's *t*-test or the Mann-Whitney test was used as appropriate.

Results

Because the glycerol model in the mouse is not characterized as it is in the rat, we first examined the renal response to this insult in C57BL/6 mice. As in the rat, markers of renal injury (creatinine), muscle injury (creatinine kinase), and red cell injury (hemoglobin), are all reversibly elevated in mice subjected to heme protein-induced renal injury (see below); and, as shown in Figure 1, the kidney mounts a robust response consisting of the induction of HO-1 mRNA.

We proceeded to impose the glycerol model in HO-1 +/+ and HO-1 -/- mice, and to determine the severity of renal injury. Before this, we assessed the severity of myolysis and hemolysis by plasma creatine kinase and

Figure 2. Alterations in plasma creatinine in HO-1 +/+ mice (open columns) and HO-1 -/- mice (solid columns) after glycerol. Basal plasma creatinine was determined and mice were then dehydrated overnight. Hypertonic glycerol (50%) was injected (7.5 ml/kg) after which plasma creatinine was measured on sequential days. Each group consisted of eight mice before glycerol. *, $P < 0.05$ between HO-1 +/+ and HO-1 -/- mice on the same day.

plasma hemoglobin, respectively. Injection of hypertonic glycerol elevated plasma creatine kinase concentrations in HO-1 +/+ and HO-1 -/- mice to values that were not significantly different at 6 hours (257 ± 119 versus 82 ± 26 Sigma Units, $P = \text{ns}$, $n = 8$ in each group) and 24 hours (8 ± 4 versus 20 ± 7 Sigma Units, $P = \text{ns}$, $n = 6$ and $n = 5$, respectively); no significant difference was observed in this index before the administration of glycerol (13 ± 7 versus 4 ± 2 Sigma Units, $P = \text{ns}$, $n = 6$ in each group) in HO-1 +/+ and HO-1 -/- mice. Plasma hemoglobin levels in HO-1 +/+ and HO-1 -/- mice at 6 hours (75 ± 15 versus 142 ± 39 μ mol/L, $P = \text{ns}$, $n = 8$ in each group) and 24 hours (28 ± 5 versus 46 ± 17 μ mol/L, $P = \text{ns}$, $n = 6$ and $n = 5$, respectively) were not significantly different; nor were there significant differences in this index before glycerol (17 ± 1 versus 25 ± 4 μ mol/L, $P = \text{ns}$, $n = 6$ in each group). Thus, HO-1 +/+ and HO-1 -/- mice exhibit comparable degrees of muscle injury and hemolysis.

After glycerol, however, alterations in renal function were fundamentally different in HO-1 +/+ and HO-1 -/- mice (Figure 2). Although plasma creatinines were identical before the administration of glycerol, the plasma creatinine was markedly increased in HO-1 -/- mice by days 2 and 3 after the administration of hypertonic glycerol, findings that demonstrate greater deterioration in renal function in HO-1 -/- mice (Figure 2).

Worsened renal histological injury accompanied this greater impairment in renal function, as demonstrated by widespread necrosis of tubular epithelial cells and tubular cast formation in the kidney in HO-1 -/- mice on day 2 after glycerol (Figure 3, right panel); such histological features were uncommon in HO-1 +/+ mice in that only isolated tubules showed evidence of such tubular necrosis (Figure 3, left panel); no gross renal histological abnormalities were present in either group not subjected to

Figure 3. Photomicrographs of kidney sections 2 days after administration of glycerol to HO-1 $+/+$ and HO-1 $-/-$ mice. HO-1 $-/-$ mice subjected to glycerol (**right panel**) demonstrate diffuse and extensive tubular epithelial cell necrosis and tubular cast formation; such changes are much less prominent in HO-1 $+/+$ mice subjected to glycerol (**left panel**). Original magnification, $\times 200$.

glycerol (data not shown). In these mice in which renal histological studies were undertaken, worsened renal function was again observed in the HO-1 $-/-$ mice as compared to HO-1 $+/+$ mice subjected to glycerol: serum creatinine measurements on day 2 after glycerol were 0.9 ± 0.3 versus 2.7 ± 0.1 mg/dl, $P < 0.005$, $n = 6$ in each group.

Accompanying these differences in kidney function was biochemical evidence of marked cellular injury, as reflected by plasma levels of LDH (Figure 4a). LDH is a ubiquitous cytosolic enzyme, and its elevation in plasma

indicates cellular injury. Plasma LDH profiles in HO-1 $+/+$ and HO-1 $-/-$ mice were different: in HO-1 $+/+$ mice, plasma LDH levels acutely rose by 6 hours but normalized by 48 hours; in contrast, HO-1 $-/-$ mice demonstrate a progressive and sustained rise in LDH, and by 48 hours, these levels were increased 30-fold greater than levels in HO-1 $+/+$ mice, indicating massive tissue injury. Interestingly, HO-1 $-/-$ mice evinced minimally elevated LDH levels even in the basal state, perhaps reflecting the endogenous cytoprotective effects of HO-1 in unstressed mice.

Figure 4. a: Alterations in plasma lactate dehydrogenase (LDH) in HO-1 $+/+$ (open column) and HO-1 $-/-$ mice (solid column). Mice were subjected to glycerol as described in Figure 2, and plasma LDH was measured sequentially. *, $P < 0.05$ between HO-1 $+/+$ and HO-1 $-/-$ mice on the same day. **b:** Percent survival in HO-1 $+/+$ (open square) and HO-1 $-/-$ mice (solid square) subjected to glycerol-induced heme protein injury. Mice were subjected to glycerol as described in Figure 2, and these survival data are derived from studies summarized in Figure 2. Each group consisted of eight mice before the administration of glycerol.

Figure 5. **a:** Northern analysis for HO-1 expression in kidneys in HO-1 +/+ and HO-1 -/- mice, before (basal) and 6 hours after glycerol (7.5 ml/kg). Each lane represents RNA extracted from a single kidney of an individual mouse, and in each group, three mice were studied. **b:** Western analysis for HO-1 expression in kidneys of HO-1 +/+ and HO-1 -/- mice, before (basal) and 6 hours after the administration of hypertonic glycerol (7.5 ml/kg). Each lane represents a single kidney of an individual mouse, and in each group, two mice were studied. **c:** Increment in kidney heme content above basal values in whole kidney homogenates in HO-1 +/+ and HO-1 -/- mice 6 hours after the administration of hypertonic glycerol (7.5 ml/kg). Each group consisted of eight mice. *, $P < 0.05$ between HO-1 +/+ and HO-1 -/- mice.

The marked cellular injury in HO-1 -/- mice, in conjunction with more severe renal failure, after exposure to heme proteins, increased mortality in the HO-1 -/- mice; by 6 days, seven out of the eight animals in the HO-1 -/- group were dead, and by 15 days, all HO-1 -/- mice were dead; no deaths occurred in the HO-1 +/+ mice in response to the same dose of glycerol (Figure 4b).

The induction of myolysis and hemolysis by hypertonic glycerol promptly and prominently induced HO-1 mRNA and protein in the kidney in HO-1 +/+ mice; no such induction occurred in the HO-1 -/- mice (Figure 5, a and b). The lack of induction of HO-1 in the HO-1 -/- mice enhanced the accumulation of heme which occurs in this model: the increment in kidney heme content in HO-1

-/- mice was increased some eightfold above that which occurred in HO-1 +/+ mice (Figure 5c). The site of induction of HO-1 protein in the kidney in HO-1 +/+ mice after the administration of glycerol localized, primarily, to the proximal tubule (Figure 6).

To determine whether similar sensitivities would occur in HO-1 -/- mice when heme proteins are directly administered, we developed a model of heme protein-induced toxicity in which mouse hemoglobin was administered intravenously. In HO-1 +/+ mice such administration of mouse hemoglobin was associated with no elevation in plasma creatinine and no mortality (Figure 7, a and b). In stark contrast, hemoglobin so administered to HO-1 -/- mice led to acute renal failure and 75% mortality (Figure 7,

Figure 6. Immunoperoxidase staining for HO-1 in HO-1 +/+ mice, before and 6 hours after glycerol (7.5 ml/kg). In the basal state the kidneys of HO-1 +/+ mice do not express HO-1 (**left panel**). In response to glycerol prominent induction of HO-1 was observed in the proximal tubules of HO-1 +/+ mice (**right panel**). There was no evidence of HO-1 staining in HO-1 -/- mice either in the basal state or 6 hours after glycerol (data not shown). Original magnification, $\times 400$.

Figure 7. a: Alterations in kidney function as assessed by daily plasma creatinine in HO-1 +/+ mice (open column) and HO-1 -/- mice (solid column) after infusions of mouse hemoglobin. Each group consisted of eight mice before the administration of hemoglobin. *, $P < 0.05$ between HO-1 +/+ and HO-1 -/- mice on the same day. **b:** Percent survival in HO-1 +/+ (open square) and HO-1 -/- mice (solid square) after infusions of mouse hemoglobin as described in **a**. Each group consisted of eight mice before the administration of hemoglobin.

a and b). Thus, heme proteins per se are uniquely toxic in mice rendered deficient in HO-1.

After we established the remarkable sensitivity of HO-1 -/- mice to specific heme proteins, we proceeded to examine this effect in mice that were younger (7 to 10 weeks) than those previously used (20 to 30 weeks). In this age range of 7 to 10 weeks, HO-1 -/- mice exhibit body weights that are comparable to HO-1 +/+ mice, and do not display inflammatory and other histological changes that subsequently emerge in much older mice.²⁸ Serum creatinine was not different in these mice before the administration of hemoglobin (Figure 8); however, in response to hemoglobin (180 mg/100 g, as administered previously), fulminant nephrotoxicity occurred in the HO-1 -/- mice in that the serum creatinine was

some ninefold higher one day after hemoglobin (Figure 8). Such renal dysfunction was accompanied by widespread necrosis of tubular epithelial cells and tubular cast formation in the kidney in HO-1 -/- mice (Figure 9, right panel); histologically, the kidney appeared normal in HO-1 +/+ mice one day after hemoglobin (Figure 9, left panel). Thus, in HO-1 -/- mice at a relatively young age at which phenotypic changes pertaining to body weight and other parameters do not emerge, the vulnerability of the kidney to heme proteins is again present.

Discussion

The vulnerability of HO-1 -/- mice to challenges of heme proteins—administered either as a *bona fide*, *in vivo* disease model based on the injection of glycerol, or the direct infusion of hemoglobin—demonstrates the indispensability of HO-1 in protecting tissues exposed to heme proteins. This view was raised previously by studies that used pharmacological approaches in manipulating expression of HO activity.²⁹ However, such manipulations, as is increasingly recognized, may exert effects beyond those that involve the HO system; for example, pharmacological inhibitors of HO activity may affect nitric oxide synthase and guanylate cyclase activities, may exert prooxidant effects, or may possess other nonspecific actions;^{23–25} conversely, agents such as hemoglobin, used in the induction of HO, clearly exert a multitude of effects on other systems. Transgenic overexpression of HO-1 in endothelial cells *in vitro* has been shown to protect against heme proteins;³⁴ however, in contrast, studies of heme protein-induced or oxidant-induced injury to renal epithelial cells, and which have used tin protoporphyrin, have argued that the induction of HO-1 is cytotoxic in these circumstances.^{26,35} Although some of these results may be accounted for by actions of com-

Figure 8. Alteration in kidney function in younger mice (age, 7 to 10 weeks) as assessed by plasma creatinine in HO-1 +/+ mice (open column) and HO-1 -/- mice (solid column) after infusion of mouse hemoglobin ($n = 5$ and $n = 6$ in the HO-1 +/+ and HO-1 -/- group, respectively). *, $P < 0.05$ between HO-1 +/+ and HO-1 -/- mice on the same day.

Figure 9. Photomicrographs of histological sections from the kidney in HO-1 +/+ and HO-1 -/- mice 1 day after infusion of mouse hemoglobin as described in Figure 8. HO-1 -/- mice subjected to hemoglobin (**right panel**) demonstrated diffuse and extensive tubular epithelial cell necrosis; histologically, the kidney in the HO-1 +/+ mice appeared normal after hemoglobin (**left panel**).

petitive inhibitors other than those which involve the HO system, these findings also underscore the differing functional effects observed in the *in vivo* and *in vitro* settings in which tissue toxicity is examined. The present studies aid in resolving these issues by clearly demonstrating the necessity for HO-1 in protecting against heme protein toxicity *in vivo*.

We examined the sensitivity of HO-1 -/- mice to heme protein toxicity at multiple levels. Our initial studies used the glycerol model because this is a long-established and conventional method of studying the toxicity of heme proteins *in vivo*, especially with regards to their nephrotoxicity.²⁷ From these studies we proceeded to question whether the sensitivity of HO-1 -/- mice to glycerol-induced, heme protein-instigated, injury would also occur in response to heme proteins per se; and indeed, a specific heme protein, hemoglobin, proved markedly injurious to HO-1 -/- mice when administered in dosages that were innocuous in HO-1 +/+ mice. Having confirmed that this sensitivity of HO-1 -/- mice occurs in a model of heme protein toxicity (glycerol) and in response to a particular heme protein (hemoglobin), we then questioned whether such effects would also occur in younger HO-1 -/- mice. The rationale for the use of much younger animals was to address any concerns related to differences in body weight that emerge as these mice age; additionally, such younger mice are free from the mild inflammatory changes which evolve in older HO-1 -/- mice.²⁸ To this end, we thus used sufficiently young mice such that the HO-1 +/+ and HO-1 -/- groups were matched in body weight and age, and before the emergence of the previously described mild inflammatory changes.²⁸ Once again, dosages of hemoglobin that exerted no effect on the kidney (as assessed by plasma creatinine and histology) in HO-1 +/+ mice, induced

severe functional and structural damage to the kidney in HO-1 -/- mice.

That HO-1 -/- mice are compromised in their capacity to metabolize heme proteins is germane to the view that HO-1 is required in iron recycling.²⁸ In these previous studies which examined HO-1 -/- mice in the chronic, unstressed state, increased tissue deposition of iron was noted,²⁸ and it was suggested that HO-1 is involved in the homeostasis of iron as HO-1 serves to expel iron from cellular stores.²⁸ However, neither did this latter study, nor any other available study, address whether the deficiency of HO-1 in mice would render them acutely vulnerable *in vivo* to increased amounts of heme, the substrate that readily induces HO-1 and incriminated as an endogenous toxicant released from intracellular heme proteins in states of oxidant stress. This question is relevant not only from the pathobiology of oxidant injury but also from the clinical standpoint because tissues are exposed to large amounts of heme proteins in diseases characterized by myolysis and hemolysis.²⁷ Moreover, a single clinical case report has just appeared describing the deficiency of HO-1 in an infant; although this clinical observation demonstrates susceptibility of cells derived from this patient to oxidative stress *in vitro*, the importance of degradation of heme per se in such toxicity was unresolved.³⁶ Our data demonstrate that, in settings in which the absence of HO-1 renders organs such as the kidney exquisitely sensitive to heme proteins, marked elevations in tissue heme content occur.

Our studies, uncovering as they do, the toxicity of heme in the kidney and other organs, underscores the duality of heme: heme, linked as a prosthetic group to a number of proteins, provides diverse and indispensable cellular functions; and yet, in inordinate amounts, free

heme is markedly injurious to tissues. Such a mechanism of tissue injury may be relevant to the recently recognized sensitivity of HO-1 $-/-$ mice to endotoxin;³⁷ endotoxin is known to destabilize heme proteins and increase cellular heme content.³⁸ In such circumstances, the absence of HO-1 would render tissues particularly sensitive to the effects of endotoxin.

Previous studies demonstrate that the induction of HO-1 in states of heme protein-mediated injury is accompanied by increased ferritin synthesis;^{18,29} such increased amounts of ferritin, through the iron-binding and possibly other effects of this protein, may represent an added protectant against heme protein-mediated injury. Studies of ferritin synthesis in settings in which HO-1 is absent, as occurs in the HO-1 $-/-$ mouse, would be of interest. This HO-1 $-/-$ mouse model also provides an opportunity to test whether the deficiency of HO-1 renders tissues sensitive to the chronic inflammatory effects of heme proteins; additionally, it is of interest whether the deficiency of HO-1 renders tissues sensitive to oxidant and other forms of acute injury that are not overtly dependent on heme proteins.

Finally, the present studies contribute to the steadily accruing inventory on phenotypic patterns observed in knockout mice exhibiting the deletions of specific genes.³⁹ Interestingly, an increasing number of knockout models are described in which the anticipated phenotype is not observed, an occurrence that perhaps reflects an underlying redundancy such that the loss of a given gene may be compensated for by the recruitment of other genes. Additionally, phenotypic changes may be observed that are not anticipated from such genetic deletion or may be at odds with the presumed functional role of a given gene. Neither of these considerations—redundancy that denies the appearance of a predicted phenotype nor perplexity in accounting for the observed phenotype—pertains to the present findings in this knockout model: the genetic deletion of HO-1 renders tissue exquisitely vulnerable to the damaging effects of heme proteins, thereby attesting to the indispensability of HO-1 in protecting tissues so exposed, and the failure of other remaining antioxidant systems to compensate for the loss of HO-1 in this inimical circumstance.

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