Heme-oxygenase-mediated iron accumulation in the liver

Zia A. Khan, Yousef P. Barbin, Mark Cukiernik, Paul C. Adams, and Subrata Chakrabarti

Abstract: Heme oxygenase (HO) isozymes, HO-1 and HO-2, catalyze the conversion of heme to iron, carbon monoxide, and biliverdin. The present study was aimed at elucidating the role of the HO system in iron accumulation and oxidative stress in the liver. We have also studied the regulation of an iron exporter, ferroportin-1 (FPN-1), as an adaptive response mechanism to increased iron levels. Sprague-Dawley rats were treated with HO inducer hemin or HO inhibitor tin-protoporphyrin IX (SnPPIX) for 1 month. A portion of liver tissues was subjected to RT-PCR for HO-1, HO-2, and FPN-1 gene expression as well as an HO activity assay. Paraffin-embedded tissues were stained for iron with Prussian blue. Hepatic iron concentration was measured by High Resolution-Inductively Coupled Plasma-Mass Spectrometry. 8-hydroxy-2′-deoxyguanosine (8-OHdG) stain, a sensitive and specific marker of oxidative DNA damage, was performed to assess oxidative stress. Hemin treatment led to augmented HO expression and activity in association with increased iron accumulation and oxidative stress. FPN-1 expression was also found to be upregulated. SnPPIX treatment reduced HO activity, intracellular iron levels, and oxidative stress as compared to controls. Our data provides evidence of increased HO activity as an important pro-oxidant mechanism leading to iron accumulation in the liver.

Key words: Kupffer cells, hepatocytes, iron overload, ferroportin-1, oxidative stress.

Résumé : Les isoenzymes de l'hème oxygénase (HO), HO-1 et HO-2, catalysent la conversion de l'hème en fer, en monoxyde de carbone et en biliverdine. La présente étude a eu pour but de clarifier le rôle du système HO dans l'accumulation de fer et lors du stress oxydatif dans le foie. Nous avons aussi examiné la régulation d'un transporteur de fer, ferroportine-1 (FPN-1), en tant que mécanisme de réponse adaptative aux augmentations des taux de fer. Des rats Sprague-Dawley ont été traités avec l'inducteur de l'HO, hémine, ou l'inhibiteur de l'HO, Sn-protoporphyrine IX (SnPPIX), pendant 1 mois. Une portion de tissus hépatiques a été soumise à une analyse par RT-PCR, pour déterminer l'expression génique de HO-1, HO-2 et FPN-1, ainsi qu'à un dosage de l'activité de l'HO. Des tissus inclus en paraffine ont été colorés avec du bleu de Prusse pour visualiser le fer. La concentration hépatique en fer a été mesurée par spectrométrie de masse à plasma induit à haute résolution. La 8-hydroxy-2′-désoxyguanosine (8-OHdG), un marqueur sensible et spécifique des dommages oxydatifs de l'ADN, a été utilisée pour évaluer le stress oxydatif. Le traitement à l'hémine a provoqué une augmentation de l'expression et de l'activité de l'HO en association avec une augmentation de l'accumulation de fer et du stress oxydatif. L'expression de la FPN-1 a aussi été augmentée. Le traitement à la SnPPIX a réduit l'activité de l'HO, les taux intracellulaires de fer et le stress oxydatif comparativement aux valeurs témoins. Nos résultats montrent que l'augmentation de l'activité de l'HO constitue un mécanisme pro-oxydant important induisant une accumulation de fer dans le foie.

Mots clés : cellules Kupffer, hépatocytes, surcharge en fer, ferroportine-1, stress oxydatif, molécules.

[Traduit par la Rédaction]

Introduction

Iron is an essential metal for all living organisms. Unlike other transition metals, iron homeostasis is only maintained by regulating absorption. This suggests that the regulation of iron absorption is tightly controlled to prevent toxicity induced by iron overload. However, disorders of iron metabolism are quite prevalent. In situations where plasma iron exceeds the capacity of transferrin, parenchymal cells, including hepatocytes, accumulate iron (Andrews 2000). Hepatocytes are quite efficient in maintaining the equilibrium between intracellular iron and circulating iron (Andrews 2000; Cook et al. 1970). In contrast to parenchymal iron-loading in primary disorders, such as hemochromatosis,

Received 25 September 2003. Accepted 7 June 2004. Published on the NRC Research Press Web site at http://cjpp.nrc.ca on 27 August 2004.

Z.A. Khan, Y.P. Barbin, M. Cukiernik, P.C. Adams, and S. Chakrabarti.[1,2] Department of Pathology, Department of Microbiology and Immunology, University of Western Ontario, London, ON N6A 5C1, Canada.

[1]Corresponding author (e-mail: schakrab@uwo.ca).
[2]Present Address: Department of Pathology, DSB 4011, University of Western Ontario, London, ON N6A 5C1, Canada.

 doi: 10.1139/Y04-052

excess iron generated from the catabolism of erythrocytes is primarily deposited in reticuloendothelial cells (Andrews 2000). Reticuloendothelial cells have a very limited capacity to use circulating transferrin iron (Hershko 1977; Hershko et al. 1998). These cells primarily derive iron from the catabolism of hemoglobin, which is the major site of iron storage and use (Andrews 2000; Custer et al. 1982; Hershko 1977; Hershko et al. 1998).

Long-term blood transfusions, the parenteral route of iron overload, cause various complications in the absence of chelation therapy, including liver cirrhosis, growth failure, diabetes, and cardiomyopathy (Buja and Roberts 1971; Cohen 1990; Gabutti and Borgna-Pignatti 1994; Halliday and Searle 1996; Harmatz et al. 2000; Mckay et al. 1996; Pippard et al. 1982). It is believed that the unregulated absorption of iron in conditions such as β-thalassemia may increase total iron burden (Hershko et al. 1978; Hershko and Rachmilewitz 1979; Jean et al. 1970). With frequent blood transfusions, this burden significantly increases, leading to dysfunction of the heart, liver, and endocrine glands. Hepatic siderosis resulting from chronic red blood cell transfusions is a consequence of hemoglobin catabolism (Custer et al. 1982). One important regulator of heme homeostasis is the heme oxygenase (HO) system (Buelow et al. 2001, Maines 1997, Tenhunen et al. 1969). HO is an enzyme system that physiologically degrades heme to biliverdin, a bile pigment. Free iron and carbon monoxide are produced as byproducts of the HO-mediated catalysis of heme (Buelow et al. 2001; Maines 1997).

The HO system is localized to the endoplasmic reticulum of cells. This system is made up of three isozymes, HO-1, HO-2, and HO-3 (Maines 1997; McCoubrey et al. 1997). HO-3 is a recently discovered isozyme that exhibits poor heme catalyst activity. HO-2 is believed to be the constitutively expressed form of HO. However, recent evidence has indicated that HO-2 can be induced by various HO agonists, such as adrenal glucocorticoids, and by tissue damage, such as spinal cord injury (Goda et al. 1998; Maines 1997; Panahian and Maines 2001; Sharma et al. 2000). HO-1 is the inducible isozyme and is a minor constituent in the normal, unstimulated liver. However, in the induced liver, HO-1 activity is the predominant mechanism of heme degradation (Maines 1988). It is possible that with increased HO activity, pro-oxidant iron accumulates in hepatocytes and other cells residing in the liver tissue, which can lead to increased oxidative stress and liver dysfunction.

In this study, we evaluated the role of the HO system in the pathogenesis of iron accumulation and oxidative damage in the liver, which may be of significance in secondary iron-overload disorders. Long-term blood transfusions are associated with secondary iron overload and hepatic siderosis, which involves the increased catabolism of hemoglobin (Custer et al. 1982). Because the HO system is the primary regulator of hemoglobin catabolism, we sought to determine whether the alteration of HO activity could mediate increased iron accumulation and oxidative damage in the liver. In addition, we considered the regulation of ferroportin-1 (FPN-1; also known as MTP-1 or IREG-1) expression, an important iron exporter (Abboud and Haile 2000; McKie et al. 2000), to be a possible adaptive mechanism in the prevention of intracellular cytotoxic iron levels.

Methods and materials

Animals

Male Sprague–Dawley rats (Charles River Laboratories, St. Constant, Que.), weighing approximately 200–250 g, were randomly divided into 2 groups. One group was treated with hemin, an HO inducer (50 mg/(kg·day); s.c. administration; $n = 6$) (Abraham et al. 1996; Tulis et al. 2001). To determine whether there is a physiological/basal role for the HO system in the homeostasis of iron in the liver, the second group was treated with a potent competitive inhibitor of HO, tin-protoporphyrin IX (SnPPIX; 37.50 mg/(kg·day); i.p. administration; $n = 6$) (Tulis et al. 2001; Drummond and Kappas 1981). Each porphyrin (hemin and SnPPIX) was dissolved in alkaline solution (0.1 mol/L NaOH in the presence of dimethyl sulfoxide (DMSO)) and reconstituted with PBS (final pH 7.4). Age- and sex-matched animals were used as controls, and were given PBS/DMSO as vehicle.

After 1 month of treatment, rats were euthanized (1 day after last treatment), and liver tissue was obtained and divided into portions. A portion of liver tissue was snap frozen in liquid nitrogen for quantitative real-time reverse transcription (RT)-PCR. A portion was fixed in formalin and embedded in paraffin for histological analysis. In addition, separate portions of frozen tissue were used for iron measurement and assessment of HO activity.

All animals were cared for in accordance with *The Guiding Principles in the Care and Use of Animals*. The University of Western Ontario Council on Animal Care Committee formally approved all experimental protocols.

Iron and copper measurement in liver tissue

Iron and copper content of the liver was measured with Finnigan MAT Element High Resolution Inductively Coupled Plasma Mass Spectrometry (HR-ICP-MS) (ThermoQuest, San Jose, Calif.) in accordance with manufacturer's recommendations. Briefly, liver samples (0.5–10 mg) were heat-dried at 100 °C and weighed. Concentrated HNO_3 was added, and samples were heated again, at 103 °C for 15–30 min. Samples were diluted to 10 mL before iron and copper concentrations were measured. Metal concentrations were expressed as μg metal/g tissue.

Iron staining in liver tissue

Paraffin-embedded tissue was used for Perl's Prussian blue staining and localization of iron, using standard procedures (Bunting 1949). Briefly, tissue sections 4 μm thick were treated with HCl (5%) to liberate ferric ions. The samples were then treated with 5% potassium ferrocyanide to produce insoluble ferric ferrocyanide. The slides were counterstained with Neutral red.

RNA isolation and cDNA synthesis

Frozen tissue sections were used to isolate total RNA using TRIZOL™ (Invitrogen Canada, Burlington, Ont.), as described elsewhere (Evans et al. 2000). DNAse treatment was carried out to degrade any contaminating DNA in RNA samples (Invitrogen). RNA was quantified by measuring ultraviolet absorbance at 260 nm, and purity of samples was determined by calculating optical density at 260:280 nm. cDNA was synthesized using 3 μg of total RNA with oligo-

Table 1. Primer sequences and real-time RT-PCR temperature profiles.

Primer	PCR parameters*	
(5′→3′)	Phase	Temperature/time
HO-1	Denaturation	95 °C/0 s
(+) ACAACCCCACCAAGTTCAAA	Annealing	55 °C/5 s
(–) CCTCTGGCGAAGAAACTCTGA	Extension	72 °C/10 s
	Signal	83 °C/1 s
HO-2	Denaturation	95 °C/0 s
(+) ATGGCAGACCTTTCTGAGCTC	Annealing	59 °C/5 s
(–) CTTCATACTCAGGTCCAAGGC	Extension	72 °C/24 s
	Signal	85 °C/1 s
FPN-1	Denaturation	95 °C/0 s
(+) GACTGTATCACCACAGGATA	Annealing	49 °C/5 s
(–) CATGTATAAACCTAGAACGG	Extension	72 °C/13 s
	Signal	81 °C/1 s
β-Actin	Denaturation	95 °C/0 s
(+) CCTCTATGCCAACACAGTGC	Annealing	58 °C/5 s
(–) CATCGTACTCCTGCTTGCTG	Extension	72 °C/5 s
	Signal	83 °C/1 s

* Initial denaturation was 1 min. Ramp rate was 20 °C per second.

(dT) primers and Superscript-II™ MMLV-reverse transcriptase (Invitrogen).

Real-time quantitative RT-PCR

Real-time RT-PCR was carried out in a LightCycler™ (Roche Diagnostics Canada, Laval, Que.), using SYBR Green I detection platform. PCR reactions were performed in glass microcapillaries (Roche Diagnostics), in a total volume of 20 μL. The reaction mixture consisted of 2.5 μL of 10X PCR buffer, 1.25 μL of 5 mmol/L dNTP, 1.2 μL of 50 mmol/L $MgCl_2$, 1 μL of each forward and reverse 10 μmol/L primers (Table 1), 0.5 μL of 5 units/μL Platinum Taq polymerase, 0.75 μL of 10X SYBR Green I (Molecular Probes, Eugene, Ore.), 10.8 μL H_2O, and 1 μL cDNA template.

During PCR reactions, an additional step was incorporated after the extension phase to minimize signal interference from primer-dimers and nonspecific amplification products. This signal-acquisition phase was empirically determined by obtaining the melting temperature (T_m) of all products using a melting curve analysis (MCA). During MCA, the post-PCR products were allowed to anneal at 60 °C before raising the temperature to 0.10 °C/s. The signal-acquisition step was set to 2–3 °C below the T_m of specific product (Table 1). mRNA levels were quantified using the standard curve method. Standard curves were constructed using serially diluted standard template. All PCR reactions were subjected to MCA to determine their specificity of amplification. In addition, PCR products were subjected to gel electrophoresis (2% agarose) to determine the size of the amplified products. The data were normalized to β-actin to account for the differences in reverse transcription efficiencies and the amount of template in the reaction mixtures.

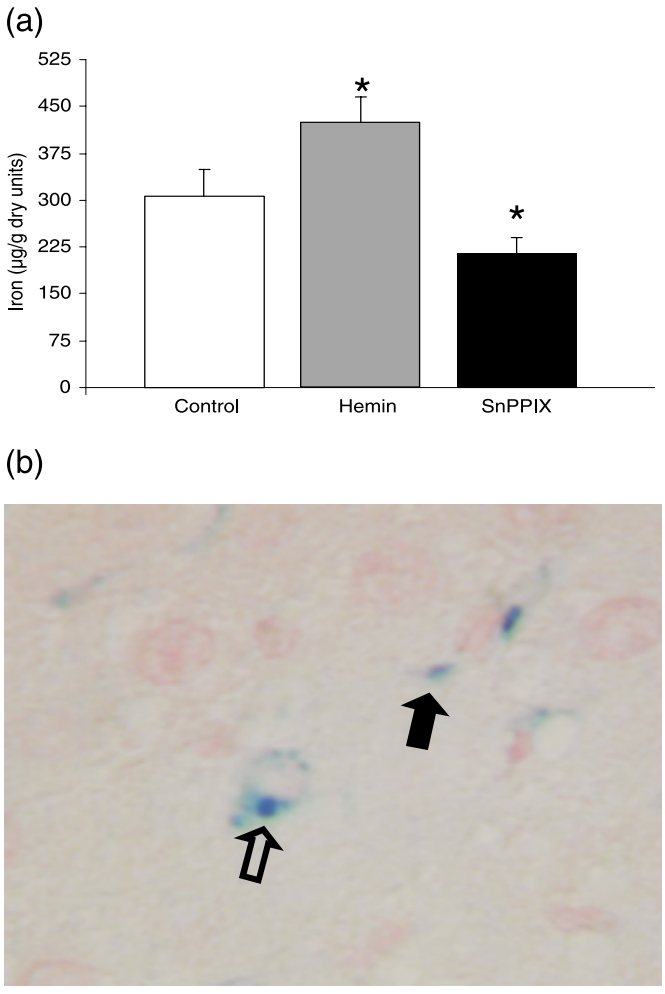
HO activity assay

Liver HO activity was measured as described elsewhere (Trakshel et al. 1986). Briefly, microsomal fraction was isolated from harvested liver tissue and added to a reaction mixture that contained 0.1 mol/L potassium phosphate buffer (pH 7.4). Mouse liver cytosol was also added to the reaction mixture as a source of biliverdin reductase. The reaction was initiated with the addition of 25 μmol/L of the HO inducer hemin and 0.4 mmol/L NADPH. The reactions were performed in a shaker bath at 37 °C for 30 min, and terminated by cooling the samples on ice. Concentration of bilirubin was calculated as the difference in absorbance at 470 and 530 nm, using the extinction coefficient of 40 $(mmol/L)^{-1} \cdot cm^{-2}$. HO activity was then expressed as pmol/(h·mg) protein. All reactions were performed in triplicate.

Immunohistochemistry

Paraffin-embedded liver tissue sections were stained for HO-1, HO-2, FPN-1, and 8-hydroxy-2′-deoxyguanosine (8-OHdG), using standard immunohistochemical techniques. Briefly, 5-μm sections were transferred to positively charged slides. Monoclonal anti-rat HO-1 antibody (1:250) (Stressgen Biotechnologies Corp., Victoria, B.C.), polyclonal anti-rat HO-2 antibody (1:200) (Stressgen Biotechnologies Corp.), anti-rabbit FPN-1 antibody (1:200) (ResGen, Invitrogen Corporation, Huntsville, Ala.), or monoclonal anti-mouse 8-OHdG (1:150) (Chemicon Lab, Temecula, Calif.) was used for staining. FPN-1 antibody recognizes the following sequence of FPN-1 proteins: CGKQLTSPKDTEPKPLEGTH. This antibody has been previously characterized and has been shown to be specific for FPN-1 (McKie et al. 2000). Secondary antibodies conjugated with horseradish peroxidase (BioRad Laboratories, Hercules, Calif.) were used to produce signals from the chemiluminescent substrate diaminobenzidine (Amersham Biosciences UK Limited, Buckingshire, U.K.). Negative controls were incubated with PBS without primary antibody. Specificity of the antibodies was confirmed by blocking tissue sections with 10% horse serum. The experiments were performed in triplicate and slides were read by two investigators who were unaware of the particular treatment. For 8-

Fig. 1. Iron accumulation in liver tissue showing (*a*) iron levels, as assessed by HR-ICP-MS, and (*b*) histochemical analysis of iron in liver, as assessed by Perl's Prussian blue staining of hemin treated rats. *, $p < 0.05$ vs. controls; $n = 6$/treatment; positive iron staining is observed as blue stain (open arrow, iron staining in a hepatocyte; solid arrow, iron staining in a Kupffer cell); magnification ×400.



OHdG staining, the number of positive cells in 10 random fields were counted and expressed as the percentage of total cells.

Statistical analysis

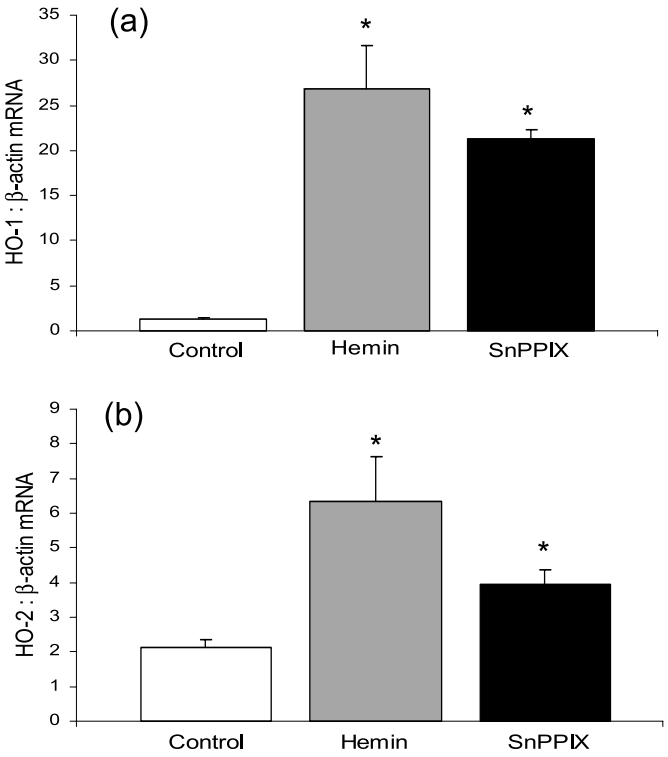
All values are given as mean ± SEM. Values were first analyzed with ANOVA, and then analysis between the 2 groups was conducted with the unpaired Student's *t* test. A *p* value of 0.05 or less was considered significant.

Results

Clinical monitoring of animals

Clinical monitoring was performed by measuring body weight gain, systolic blood pressure, and liver weight at the time of sacrifice. There was no significant difference among treatment groups in any of the parameters used for monitoring (data not shown).

Fig. 2. Real-time RT-PCR amplification of HO-1 and HO-2 mRNA from liver tissue, showing (*a*) quantification of HO-1, and (*b*) quantification of HO-2. HO-isozyme transcripts were expressed as a ratio of target mRNA to β-actin mRNA. *, $p < 0.05$ vs. controls; $n = 6$/treatment.



Iron accumulation in the liver

Rats treated with hemin had a higher concentration of liver iron than controls, as determined by HR-ICP-MS (Fig. 1*a*; $p < 0.05$). In contrast, rats treated with the inhibitor of HO activity (SnPPIX) had a significantly lower concentration of liver iron than controls (29% reduction vs. controls; $p < 0.05$). Our data indicate that basal HO activity is involved in the physiological homeostasis of liver iron, because SnPPIX treatment reduced iron to levels well below those in controls.

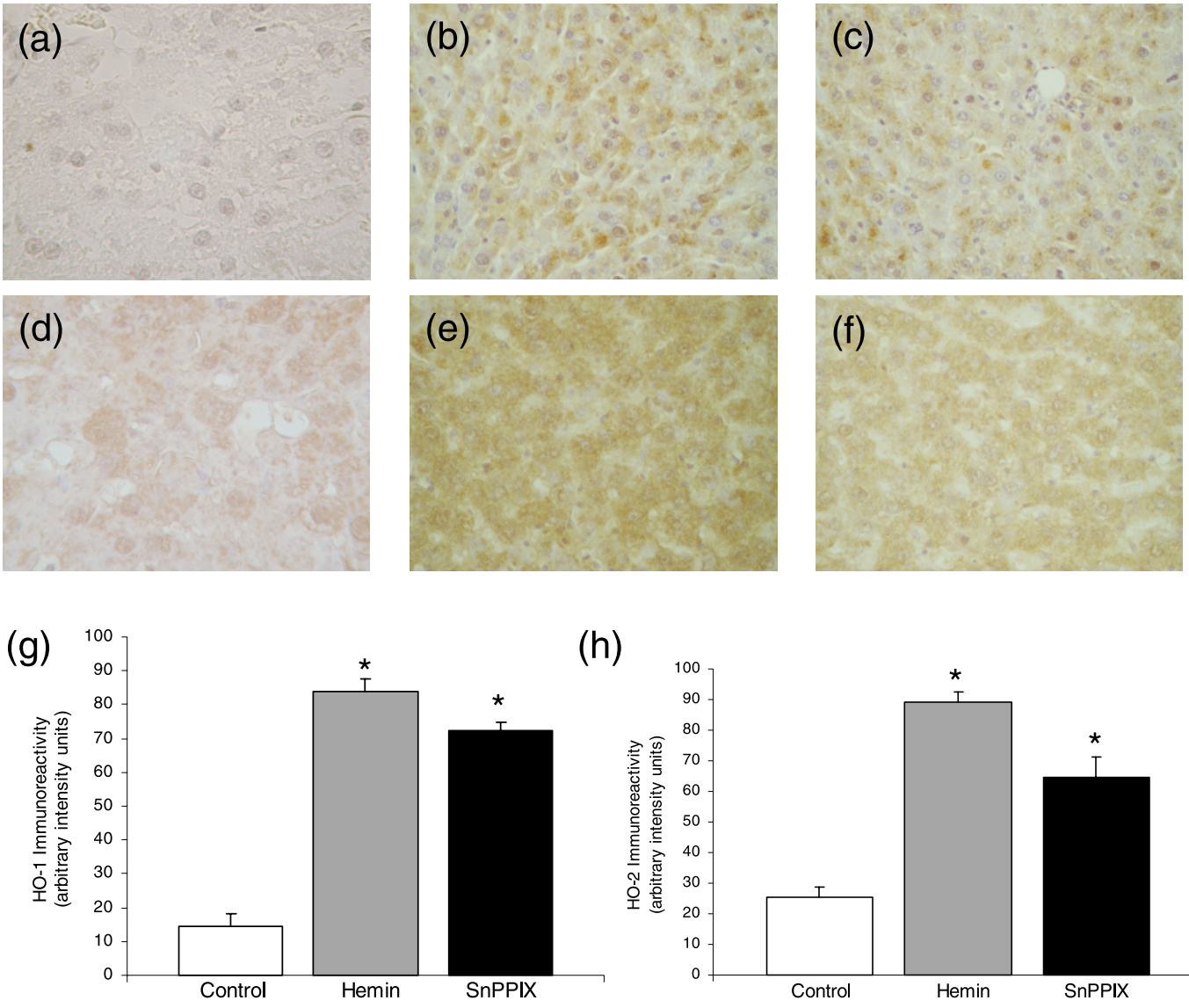
Histochemical analysis of iron in liver tissue

In secondary iron-overload situations, iron is primarily accumulated in reticuloendothelial cells. It is possible that iron is subsequently redistributed to hepatocytes and other peripheral tissues. We used Prussian blue stain to localize major sites of iron accumulation in liver tissue. Both Kupffer cells and hepatocytes in hemin-treated animals were strongly stained for iron (Fig. 1*b*). This finding supports the notion that iron is redistributed in various cell types within the liver.

HO isozyme mRNA and protein expression

We used a novel real-time RT-PCR-based assay to quantitate mRNA levels of HO-1 and HO-2. Our data show a significant increase in expression of both HO-1 and HO-2 mRNA levels in rats treated with hemin (Figs. 2*a* and 2*b*). Treatment with SnPPIX also resulted in the upregulation of HO-1 and HO-2 mRNA levels. These results are consistent

Fig. 3. Immunohistochemical analysis of HO-1 and HO-2 in liver tissue, showing (*a*) HO-1 in control rats, (*b*) HO-1 in hemin-treated rats, (*c*) HO-1 in SnPPIX-treated rats, (*d*) HO-2 in control rats, (*e*) HO-2 in hemin-treated rats, (*f*) HO-2 in SnPPIX-treated rats, (*g*) semi-quantitative analysis of HO-1 protein, and (*h*) semi-quantitative analysis of HO-2 protein. * $p < 0.05$ vs. controls; magnification ×400.



with previous studies that have demonstrated the ability of SnPPIX to upregulate mRNA and protein levels of the HO system while inhibiting activity of both preformed and newly synthesized HO (Sardana and Kappas 1987).

Paraffin-embedded liver tissue sections were analyzed immunohistochemically for HO-1 and HO-2 protein expression and localization. Hemin treatment increased HO-1 and HO-2 protein expression in both hepatocytes and Kupffer cells (Fig. 3). SnPPIX treatment also upregulated HO isozyme protein levels, which is consistent with mRNA results.

HO activity

Our next objective was to determine whether the alteration of HO mRNA and protein levels correlate with HO activity. Our data show that hemin treatment lead to a significant increase in HO activity in liver microsomal fractions (Fig. 4).

Fig. 4. HO activity assessed as the concentration of bilirubin produced from liver microsomal fractions, and expressed as bilirubin (BR) produced per hour per milligram of protein. *, $p < 0.05$ vs. controls; $n = 6$/treatment.

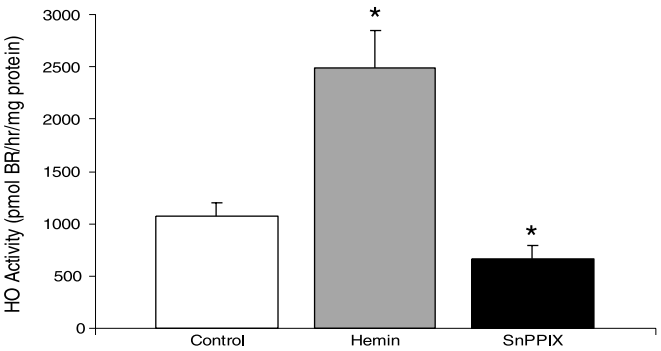
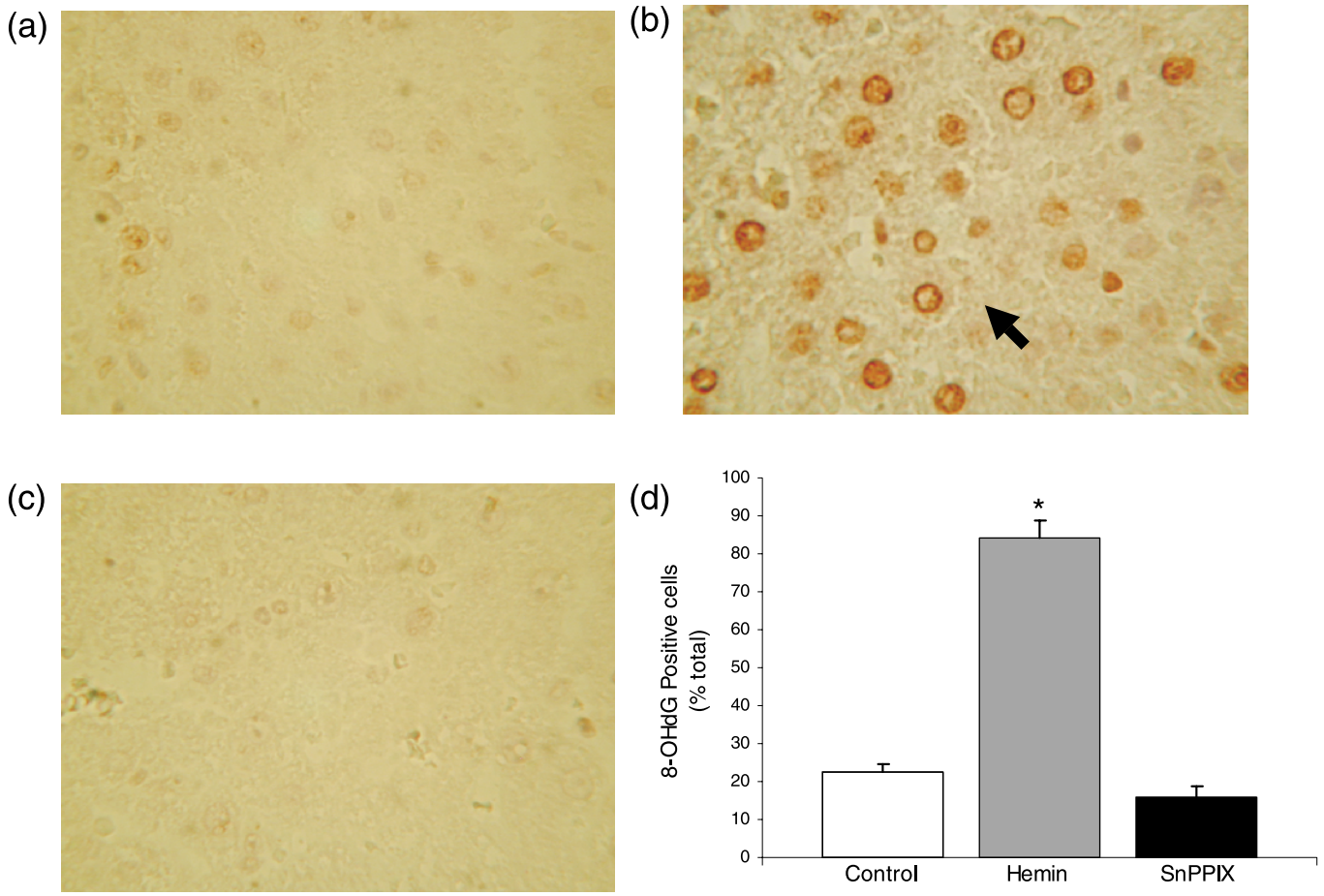


Fig. 5. 8-OHdG immunoreactivity in liver tissue of (*a*) control rats, (*b*) hemin-treated rats, and (*c*) SnPPIX-treated rats. (*d*) semi-quantitative analysis of 8-OHdG-positive cells (8-OHdG positivity is shown as brown nuclear stain (arrow)); magnification ×400. *, $p < 0.05$ vs. controls; $n = 6$/treatment.



In rats treated with SnPPIX, HO activity was 28% lower than it was in controls ($p < 0.05$). These results are consistent with iron concentration in the liver tissue.

HO-mediated increased oxidative stress in the liver

We used 8-OHdG as a marker of oxidative damage to the liver. Previous studies have shown 8-OHdG to be the most sensitive and specific marker of DNA damage induced by oxidative stress (Floyd et al. 1986). In addition, 8-OHdG staining has previously been used to detect oxidative DNA damage in chronic liver diseases (Kitada et al. 2001). Our data show significantly increased oxidative DNA damage to the liver tissue of hemin-treated rats (Fig. 5). HO inhibitor, SnPPIX, reduced the number of 8-OHdG-positive cells in liver tissue.

Copper levels in liver tissue

To determine whether iron accumulation in the liver was specifically the result of increased HO activity, we measured the concentration of copper, which is a transition metal that shares many metal transporters with iron. Our data indicate no significant changes in copper concentration in any of the animal groups (control, 9.78 ± 3.55; hemin, 11.05 ± 2.89; SnPPIX, 10.08 ± 7.6 µg/g tissue). Because copper is transported by the same metal transporters as iron, our data demonstrate that the accumulation of iron in liver tissue of hemin-treated rats is mediated by augmented HO activity.

FPN-1 expression

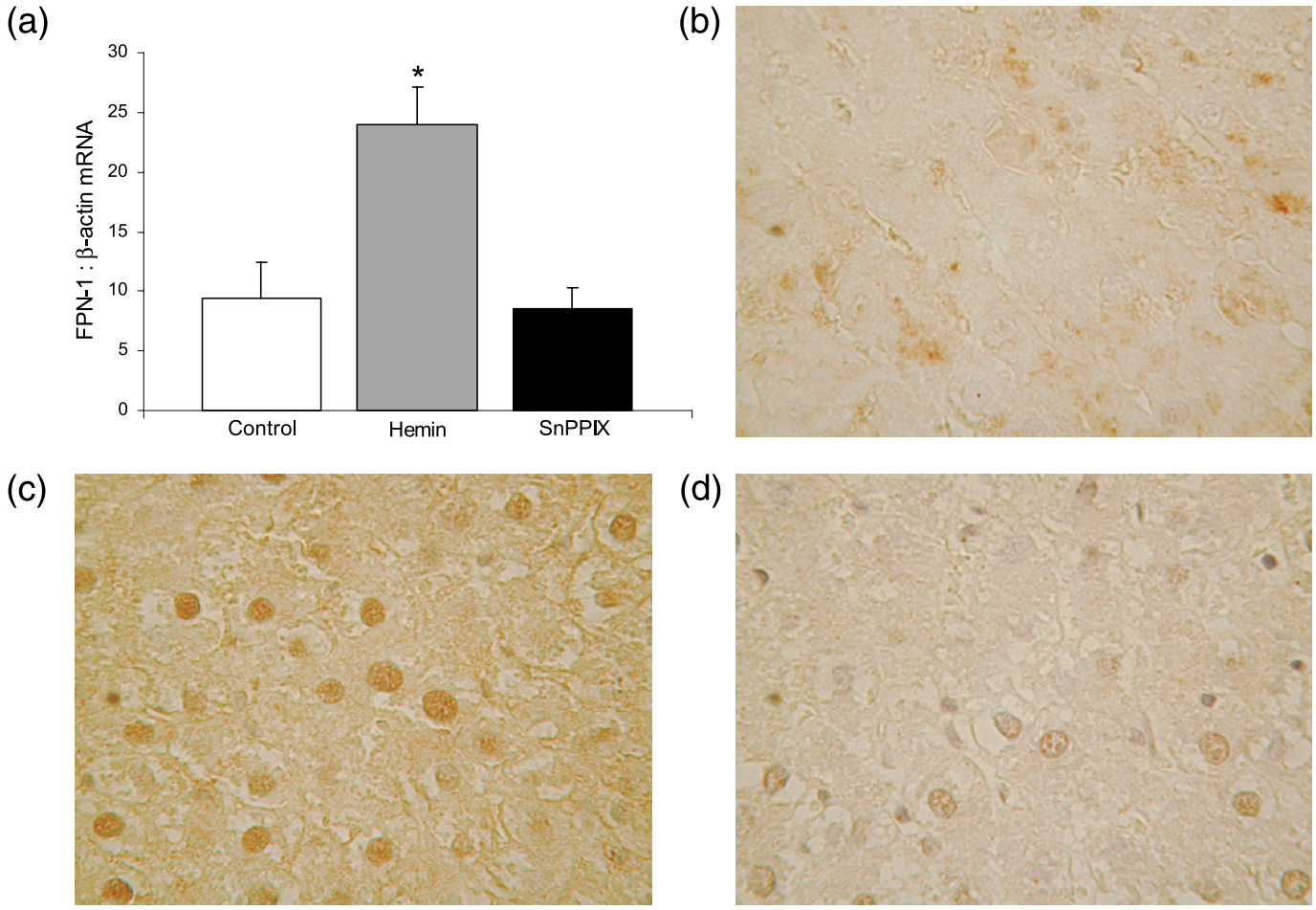
We evaluated whether the expression of FPN-1, an exclusive iron exporter, is altered in association with changes in iron levels in the liver. Our mRNA data indicate that FPN-1 expression parallels iron levels in the liver (Fig. 6*a*). Hemin treatment resulted in increased iron accumulation in the liver, with concurrent upregulation of FPN-1 mRNA levels (Fig. 6). SnPPIX treatment, on the other hand, was not associated with FPN-1 mRNA alteration (Fig. 6*a*).

Immunohistochemical analysis of FPN-1 revealed increased protein levels in liver tissue of hemin-treated rats (Figs. 6*b*–6*d*). FPN-1 staining shows increased levels of protein in both the cytoplasm and nucleus, with the increase being more prominent in the nucleus. SnPPIX treatment had no significant effect on FPN-1 protein expression.

Discussion

In this study, we showed that the HO enzyme system plays a mediating role in iron accumulation and oxidative stress in the liver. We demonstrated that the HO system is involved in physiological iron homeostasis, as selectively in-

Fig. 6. FPN-1 mRNA and protein expression in liver tissue, showing (*a*) real-time RT-PCR quantification of FPN-1 mRNA, and immunohistochemical analysis of FPN-1 in liver tissue of (*b*) control rats, (*c*) hemin-treated rats, and (*d*) SnPPIX-treated rats. FPN-1 transcripts were expressed as a ratio of target mRNA to β-actin mRNA (FPN positivity is shown as brown cytoplasmic and nuclear stain); magnification ×400. * p, < 0.05 vs. controls; n = 6/treatment.



hibiting HO activity, resulted in less iron in the liver. In addition, the increased activity of HO, brought about by the administration of a direct HO inducer, lead to increased intracellular iron levels, in both hepatocytes and Kupffer's cells, in association with increased oxidative DNA damage to liver tissue. Furthermore, FPN-1 expression was found to be upregulated in hemin-treated rats, suggesting a possible adaptive mechanism for exporting excess iron from the cells.

Evidence accumulated to date suggests that inducers regulate both transcriptional and translational activities of HO-1 (Buelow et al. 2001; Maines 1997). In contrast, information about the regulation of HO-2 is scarce. However, recent evidence indicates that HO-2 can be induced by tissue injury and by various agonists, such as adrenal glucocorticoids (Goda et al. 1998; Maines 1997; Panahian and Maines 2001; Sharma et al. 2000). Our data support the notion that both HO-1 and HO-2 can behave as inducible enzymes, at least in the induced liver. Our findings indicate that treatment with hemin, a well-characterized HO inducer, resulted in increased mRNA and protein expression, as well as increased activity of the HO system. This increase in activity coincided with an increase in intracellular iron content and oxidative stress. Furthermore, copper levels were not found to be significantly altered in hemin-treated rats. Because copper transport involves the same metal transporters as iron, our results support the notion that iron accumulation was mediated by the HO system rather than through any other iron intrusion mechanism. The HO system was also found to be important for the homeostasis of iron in the liver; rats treated with SnPPIX had lower levels of intracellular iron, and associated HO activity was reduced. A recent study of mice lacking HO-1 demonstrates a role for HO in iron homeostasis (Poss and Tonegawa 1997). These mice exhibited higher iron levels in both kidney and liver tissues. Because HO-2 was not measured at the mRNA, protein, or activity levels, it is possible that HO-2 expression and activity was the arbitrator of such iron accumulation. Our data demonstrate the upregulation of HO-2 at both the mRNA and protein levels, which could contribute to total HO activity.

Most studies that are focused on the HO system are devoted to 2 products of the enzymatic reaction: biliverdin and carbon monoxide. The other byproduct, redox-active iron, has not been thoroughly investigated. Iron is a potent cytotoxic element, by virtue of its ability to participate in the Fenton reaction, which gives rise to hydroxyl radicals. The consequence of such immediate iron release is not com-

pletely known. However, several in vivo studies have documented oxidant toxicity resulting from increased iron levels (Aust et al. 1985; Cadenas 1989). In addition to releasing iron from heme catabolism, the HO system may also increase oxidant toxicity by producing hydrogen peroxide as a byproduct (Noguchi et al. 1983). It is possible that cells have an adaptive mechanism to deal with excess intracellular iron. It has been suggested that increased intracellular iron from HO-mediated enzymatic reactions increase ferritin levels and result in the sequestration of iron (Bauer and Bauer 2002). However, patients with iron overload exhibit increased iron and oxidative stress, despite upregulated ferritin levels (Kassab-Chekir et al. 2003). It is possible that intracellular iron may overwhelm the ability of ferritin to sequester redox-active iron and produce oxidative stress. However, such a notion requires further investigation. Recently, a transporter responsible for the export of iron across biological membranes has been identified. This transporter, FPN-1, is expressed in villus cells of the small intestine and on the basolateral surface of polarized epithelial cells, where it participates in the basolateral transfer of iron (McKie et al. 2000). In addition, FPN-1 has been localized to both Kupffer cells and hepatocytes in the liver (Abboud and Haile 2000; Yang et al. 2002; Donovan et al. 2000). The role of FPN-1 in iron export is supported by the presence of an iron-response element, which suggests that upregulation of FPN-1 protein is rapid in the presence of elevated iron levels. A recent study, in which FPN-1 was experimentally expressed in *Xenopus* oocytes, has confirmed its role in iron release from intracellular compartments (McKie et al. 2000). Our data confirm the upregulation of this transporter in response to increased intracellular iron. It is possible that HO activity overwhelms the capacity of FPN-1 to export iron, which leads to accumulation and increased oxidative stress. However, such notions require further investigation. It has been suggested that the efficient release of heme iron to the extracellular space requires an iron exporter, because of the microsomal localization of the HO system (Bauer et al. 1998; Baranano et al. 2000; Immenschuh et al. 2003; Kikuchi and Yoshida 1983). Interestingly, FPN-1 has been shown to be intracellular in Kupffer cells, and membrane-associated in hepatocytes (Abboud and Haile 2000; Yang et al. 2002). The HO system may interact with FPN-1 in the process of heme-iron use.

In conclusion, we have demonstrated that the HO system is involved in the homeostasis of iron in the liver, and that it may be involved in the accumulation of iron and the increase in oxidative stress in the liver. This could be the major pathogenic mechanism of iron loading and tissue damage in secondary iron-overload disorders. The HO system may prove to be a target for therapy, in combination with iron chelators, for patients requiring frequent blood transfusions. However, such a notion requires further investigation because the inhibition of HO may also lead to increased levels of pro-oxidant heme in the liver.

Acknowledgements

The authors acknowledge grant support from the Canadian Liver Foundation and Lawson Health Research Institute.

References

Abboud, S., and Haile, D.J. 2000. A novel mammalian iron-regulated protein involved in intracellular iron metabolism. J. Biol. Chem. **275**: 19 906 – 19 912.

Abraham, N.G., Drummond, G.S., Lutton, J.D., and Kappas A. 1996. The biological significance and physiological role of heme oxygenase. Cell Physiol. Biochem. **6**: 129–168.

Andrews, N.C. 2000. Iron metabolism: iron deficiency and iron overload. Annu. Rev. Genomics Hum. Genet. **1**: 75–98.

Aust, S.D., Morehouse, S.A., and Thomas, C.E. 1985. Role of metals in oxygen radical reactions. Free Radic. Biol. Med. **1**: 3–25.

Baranano, D.E., Wolosker, H., Bae, B.I., Barrow, R.K., Snyder, S.H., and Ferris, C.D. 2000. A mammalian iron ATPase induced by iron. J. Biol. Chem. **275**: 15 166 – 15 173.

Bauer, M., and Bauer, I. 2002. Heme oxygenase-1: redox regulation and role in the hepatic response to oxidative stress. Antioxid. Redox. Signal. **4**: 749–758.

Bauer, I., Wanner, G.A., Rensing, H., Alte, C., Miescher, E.A., Wolf, B., Pannen, B.H., Clemens, M.G., and Bauer, M. 1998. Expression pattern of heme oxygenase isoenzymes 1 and 2 in normal and stress-exposed rat liver. Hepatology, **27**: 829–838.

Buelow, R., Tullius, S.G., and Volk, H.D. 2001. Protection of grafts by hemoxygenase-1 and its toxic product carbon monoxide. Am. J. Transplant. **1**: 313–315.

Buja, L.M., and Roberts, W.C. 1971. Iron in the heart. Etiology and clinical significance. Am. J. Med. **51**: 209–221.

Bunting, H. 1949. The histochemical detection of iron in tissue. Stain. Technol. **24**: 109–115.

Cadenas, E. 1989. Biochemistry of oxygen toxicity. Ann. Rev. Biochem. **58**: 79–110.

Cohen, A. 1990. Treatment of transfusional iron overload. Am. J. Pediatr. Hematol. Oncol. **12**: 4–8.

Cook, J.D., Marsaglia, G., Eschbach, J.W., Funk, D.D., and Finch, C.A. 1970. Ferrokinetics: a biologic model for plasma iron exchange in man. J. Clin. Invest. **49**: 197–205.

Custer, G., Balcerzak, S., and Rinehart, J. 1982. Human macrophage hemoglobin-iron metabolism *in vitro*. Am. J. Hematol. **13**: 23–36.

Donovan, A., Brownlie, A., Zhou, Y., Shepard, J., Pratt, S.J., Moynihan, J., Paw, B.H., Drejer, A., Barut, B., Zapata, A., Law, T.C., Brugnara, C., Lux, S.E., Pinkus, G.S., Pinkus, J.L., Kingsley,P.D., Palis, J., Fleming, M.D., Andrews, N.C., and Zon, L.I. et al. 2000. Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. Nature, **403**: 776–781.

Drummond, G.S., and Kappas, A. 1981. Prevention of neonatal hyperbilirubinemia by tin protoporphyrin IX, a potent competitive inhibitor of heme oxidation. Proc. Natl. Acad. Sci. U.S.A. **78**: 6466–6470.

Evans, T., Deng, D.X., Chen, S., and Chakrabarti, S. 2000. Endothelin receptor blockade prevents augmented extracellular matrix component mRNA expression and capillary basement membrane thickening in the retina of diabetic and galactose-fed rats. Diabetes, **49**: 662–666.

Floyd, R.A., Watson, J.J., Wong, P.K., Altmiller, D.H., and Rickard, R.C. 1986. Hydroxyl free radical adduct of deoxyguanosine: sensitive detection and mechanisms of formation. Free Radic. Res. Commun. **1**: 163–172.

Gabutti, V., and Borgna-Pignatti, C. 1994. Clinical manifestations and therapy of transfusional haemosiderosis. Bailliere's Clin. Haematol. **7**: 919–940.

Goda, N., Suzuki, K., Naito, M., Takeoka, S., Tsuchida, E., Ishimura, Y., Tamatani, T., and Suematsu, M. 1998. Distribution

of heme oxygenase isoforms in rat liver. Topographic basis for carbon monoxide-mediated microvascular relaxation. J. Clin. Invest. **101**: 604–612.

Halliday, J.W., and Searle, J. 1996. Hepatic iron deposition in human disease and animal models. Biometals, **9**: 205–209.

Harmatz, P., Butensky, E., Quirolo, K.R., Ferrell, L., Moyer, T., Golden, D., Neumayr, L., and Vichinsky, E. 2000. Severity of iron overload in patients with sickle cell disease receiving chronic red blood cell transfusion therapy. Blood, **96**: 76–79.

Hershko, C. 1977. Storage iron regulation. Prog. Hematol. **10**: 105–148.

Hershko, C., and Rachmilewitz, E.A. 1979. Mechanism of desferrioxamine-induced iron excretion in thalassaemia. Br. J. Haematol. **42**: 125–132.

Hershko, C., Grady, R.W., and Cerami, A. 1978. Mechanism of iron chelation in the hypertransfused rat: definition of two alternative pathways of iron mobilization. J. Lab. Clin. Med. **92**: 144–151.

Hershko, C., Link, G., and Cabantchik, I. 1998. Pathophysiology of iron overload. Ann. N.Y. Acad. Sci. **850**: 191–201.

Immenschuh, S., Baumgart-Vogt, E., Tan, M., Iwahara, S., Ramadori, G., and Fahimi, H.D. 2003. Differential cellular and subcellular localization of heme-binding protein 23/peroxiredoxin I and heme oxygenase-1 in rat liver. J. Histochem. Cytochem. **51**: 1621–1631.

Jean, G., Terzoli, S., Mauri, R., Borghetti, L., Di Palma, A., Piga, A., Magliano, M., Melevendi, M., and Cattaneo, M. 1970. Cirrhosis associated with multiple transfusions in thalassaemia. Arch. Dis. Child. **59**: 67–70.

Kassab-Chekir, A., Laradi, S., Ferchichi, S., Haj Khelil, A., Feki, M., Amri, F., Selmi, H., Bejaoui, M., and Miled, A. 2003. Oxidant, antioxidant status and metabolic data in patients with beta-thalassemia. Clin. Chim. Acta, **338**: 79–86.

Kikuchi, G., and Yoshida, T. 1983. Function and induction of the microsomal heme oxygenase. Mol. Cell Biochem. **53–54**: 163–183.

Kitada, T., Seki, S., Iwai, S., Yamada, T., Sakaguchi, H., and Wakasa, K. 2001. *In situ* detection of oxidative DNA damage, 8-hydroxydeoxyguanosine, in chronic human liver disease. J. Hepatol. **35**: 613–618.

Maines, M.D. 1988. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. FASEB J. **2**: 2557–2568.

Maines, M.D. 1997. The heme oxygenase system: a regulator of second messenger gases. Annu. Rev. Pharmacol. Toxicol. **37**: 517–554.

McCoubrey, W.K., Huang, T.J., and Maines, M.D. 1997. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. Eur. J. Biochem. **247**: 725–732.

Mckay, P.J., Murphy, J.A., Cameron, S., Burnett, A.K., Campbell, M., Tansey, P., Franklin, and I.M. 1996. Iron overload and liver dysfunction after allogeneic or autologous bone marrow transplantation. Bone Marrow Transplant. **17**: 63–66.

McKie, A.T., Marciani, P., Rolfs, A., Brennan, K., Wehr, K., Barrow, D., Miret, S., Bomford, A., Peters, T.J., Farzaneh, F., Hediger, M.A., Hentze, M.W., and Simpson, R.J. 2000. A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. Mol. Cell **5**: 299–309.

Noguchi, M., Yoshida, T., and Kikuchi, G. 1983. A stoichiometric study of heme degradation catalyzed by reconstituted heme oxygenase system with special consideration of the production of hydrogen peroxide during the reaction. J. Biochem. **93**: 1027–1036.

Panahian, N., and Maines, M.D. 2001. Site of injury-directed induction of heme oxygenase-1 and -2 in experimental spinal cord injury: Differential functions in neuronal defense mechanisms? J. Neurochem. **76**: 539–554.

Pippard, M.J., Callender, S.T., and Finch, C.A. 1982. Ferrioxamine excretion in iron-loaded man. Blood, **60**: 288–294.

Poss, K.D., and Tonegawa, S. 1997. Heme oxygenase 1 is required for mammalian iron reutilization. Proc. Natl. Acad. Sci. U.S.A. **94**: 10 919–10 924.

Sardana, M.K., and Kappas, A. 1987. Dual control mechanism for heme oxygenase: tin(IV)-protoporphyrin potently inhibits enzyme activity while markedly increasing content of enzyme protein in liver. Proc. Natl. Acad. Sci. U.S.A. **84**: 2464–2468.

Sharma, H.S., Alm, P., Sjoquist, P.O., and Westman, J. 2000. A new antioxidant compound H-290/51 attenuates upregulation of constitutive isoform of heme oxygenase (HO-2) following trauma to the rat spinal cord. Acta Neurochir. Suppl. **76**: 153–157.

Tenhunen, R., Marver, H.S., and Schmid, R. 1969. Microsomal heme oxygenase. Characterization of the enzyme. J. Biol. Chem. **244**: 6388–6394.

Trakshel, G.M., Kutty, R.K., and Maines, M.D. 1986. Purification and characterization of the major constitutive form of testicular heme oxygenase. The noninducible isoform. J. Biol. Chem. **261**: 11 131 – 11 137.

Tulis, D.A., Durante, W., Peyton, K.J., Evans, A.J., and Schafer AI. 2001. Heme oxygenase-1 attenuates vascular remodeling following balloon injury in rat carotid arteries. Atherosclerosis, **155**: 113–122.

Yang, F., Liu, X.B., Quinones, M., Melby, P.C., Ghio, A., and Haile, D.J. 2002. Regulation of reticuloendothelial iron transporter MTP1 (Slc11a3) by inflammation. J. Biol. Chem. **277**: 39 786 – 39 791.