Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 318 (2004) 88–94
BBRC
www.elsevier.com/locate/ybbrc

Heme oxygenase-2 gene deletion increases astrocyte vulnerability to hemin

Jing Chen and Raymond F. Regan*

Department of Emergency Medicine, Thomas Jefferson University Hospital, 1020 Sansom Street, 239 Thompson Building, Philadelphia, PA 19107, USA

Received 30 March 2004

Abstract

In a prior study, we observed that heme oxygenase-2 gene deletion protected murine cortical neurons from heme-mediated injury. In the course of these studies, constitutive HO-2 expression was observed in astrocyte cultures. The present study tested the hypothesis that astrocytes lacking the HO-2 gene would be less vulnerable to heme. Contrary to this hypothesis, gene deletion resulted in a 50–75% increase in cell death after 6 h exposure to 30 or 60 μM hemin, as measured by LDH release. A similar effect was observed when cell viability was assessed with the MTT assay. HO-2 gene deletion did not alter cellular expression of HO-1. The increased sensitivity of knockout astrocytes to hemin was reversed by increasing HO-1 expression by adenoviral gene transfer. These results suggest that heme oxygenase protects astrocytes from heme-mediated oxidative injury and highlight the disparate effect of HO in neurons and astrocytes.

Keywords: Free radical; Hemoglobin; Brain hemorrhage; Iron; Mouse; Oxidative stress; Stroke

The rate-limiting reaction in the metabolism of heme to bilirubin, iron, and carbon monoxide is catalyzed by the heme oxygenases (HO). Three isoforms have been identified in mammalian cells, which are encoded by separate genes. HO-1 is inducible, and in health is expressed predominantly by reticuloendothelial cells [1]; it is upregulated in most tissues by hemolysis and numerous other toxic perturbations to eliminate potentially toxic heme [2–4]. HO-2 is constitutively expressed, predominantly in brain, testis, and endothelium [5,6]. HO-3 is not yet well characterized, but appears to have lower activity than the other isoforms [7]. The redundancy and widespread distribution of the heme oxygenases are consistent with the diverse functions hypothesized for these enzymes. To date, experimental studies have suggested that HO mediates or modulates neurotransmission [8], inflammation [9], vasomotor tone [10], and antioxidant defense [11,12], in addition its originally described role in hemoglobin catabolism [13].

HO activity is relatively high in the CNS, and in health is due predominantly to the constitutive expression of HO-2 [14]. Little is known of the availability of its preferred substrate under normal conditions, but given the toxicity of free heme it is likely to be tightly regulated. This homeostasis is markedly disrupted by CNS hemorrhage. Since each hemoglobin molecule has four heme groups, the total heme content of blood is 8–10 mM. Extravascular erythrocytes lyse in the CNS via a complement-mediated mechanism [15]. The subsequent autoxidation of hemoglobin to methemoglobin decreases the affinity of the molecule for its heme moieties, resulting in its release [16]. In experimental hematomas, the concentration of free hemin, the oxidized form of heme, has been determined to be in the high micromolar range [17]. Growing evidence suggests that hemin toxicity contributes to injury to tissue adjacent to a hematoma [18,19].

Although the preponderance of evidence suggests that HO protects CNS cells from oxidative injury, disparate results have been reported in models that are relevant to CNS hemorrhage. Both Wagner et al. [20] and Huang et al. [18] have observed that nonselective HO inhibitors attenuated edema formation and hematoma volume in rodent and pig models of intracerebral hemorrhage. Since these agents may have nonspecific

* Corresponding author. Fax: 1-215-923-6225.
E-mail address: raymond.regan@jefferson.edu (R.F. Regan).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.03.187

effects that complicate the interpretation of experimental results [21], we have tested the effect of targeted deletion of the HO-2 gene on heme-mediated oxidative injury to CNS cells. In recent studies, we have observed that neurons derived from mutant mice were resistant to hemoglobin, but were more vulnerable to non-heme-mediated oxidative injury [22]. Since iron chelation was also protective, the toxic product of HO-2 activity in this model was likely iron. Although HO-2 expression is considered to be predominantly neuronal [6], in the course of these studies we also observed its expression in pure astrocyte cultures, in agreement with a prior report [23]. The effect of HO on heme-mediated injury may differ in neurons and astrocytes, since the latter rapidly increase production of ferritin after hemoglobin or hemin exposure and so may be better able to sequester iron [24]. The present study was therefore designed to test the effect of HO-2 gene deletion on heme-mediated injury to astrocytes.

Materials and methods

HO-2 knockout mice. All mice were obtained from our breeding colony and kept under standard conditions with a 12 h light/dark cycle, and allowed food and water ad libitum. HO-2 deficient mice with a C57BL/6 X 129/Sv background were generated by targeted disruption of the HO-2 gene as described elsewhere [25]. All breeding mice were the product of heterozygous matings. Animal care and treatments were in accordance with guidelines as described in "Principles of Laboratory Animal Care" (NIH publication No. 80-23, revised 1996) and approved by the Institutional Animal Care and Use Committee. Genotype was determined by polymerase chain reaction (PCR) using genomic DNA isolated from tail clippings, using previously published primers [22].

Culture preparation and hemin exposure. Cortical astrocyte cultures were prepared from 1 to 3 day postnatal wild type or HO-2 knockout mice. Cerebral cortices were dissected out and incubated for 50 min in 0.09% trypsin. After dissociation by trituration, the cell suspension was diluted in plating medium containing Eagle's minimal essential medium (MEM), 10% fetal bovine serum (Hyclone), 10% equine serum (Hyclone), 10 ng/ml epidermal growth factor (Sigma), 23 mM glucose, and 2 mM glutamine. Plating density was 1.0 dissociated hemisphere per 24-well plate. Cultures were incubated at 37 °C in a humidified 5% CO_2-containing atmosphere. Two-thirds of the medium was changed at 5 days in vitro (DIV) with medium containing MEM, 23 mM glucose, 2 mM glutamine, and 10% equine serum. Cultures were then fed twice weekly thereafter. The day prior to experiments, cultures were fed with MEM containing 10 mM glucose (MEM10), without serum.

Experiments were conducted after DIV 21. By this time, a confluent monolayer of cells is present, and over 95% are immunoreactive to antibodies raised against glial fibrillary acidic protein. Cultures were exposed to hemin in serum-free MEM10. Hemin was prepared as a 1 mM stock solution that was stored frozen until used. It was added directly to MEM10 at defined concentrations.

At the end of the exposure period, all cultures were examined under phase contrast microscopy. Cell injury was then quantified using two established methods: the MTT assay and LDH release assay. MTT assay is based on the reduction of the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan by metabolically active cells. At defined time points, cultures were exposed to a solution of MTT (0.025% in MEM10) for 10 min at 37 °C. An indigo-blue formazan precipitate was then visible in the monolayer. After aspiration of medium, it was extracted from cells with 250 μl isopropyl alcohol containing 0.04 N HCl. Absorbance of the resulting solution was measured in a microplate reader (Molecular Devices) at 562 nm with a reference wavelength of 650 nm. MTT reduction associated with 100% cell viability was determined from sister cultures subjected to medium exchanges only; data were normalized to this control.

Cell death was also assessed by assaying LDH activity in the culture medium at the end of the hemin exposure period, as previously described [26]. LDH activity associated with 100% cell death was determined from sister cultures treated with 0.3% Triton X-100 for 1 h; this treatment lyses all cells.

Immunoblotting. Cells were harvested using cell lysis buffer as described previously [22]. Proteins (15 μg total) were separated on a 15% polyacrylamide gel and transferred onto a polyvinylidene difluoride Immobilon-P transfer membrane filter (Millipore) using a semidry transfer apparatus (Bio-Rad). Nonspecific sites were blocked by incubating with 5% nonfat dried milk powder in buffer containing 20 mM Tris, 500 mM NaCl, and 0.1% Tween 20 (pH 7.5) for 1 h at 37 °C. Membranes were incubated at 4 °C overnight with a 1:1000 dilution of rabbit anti-HO-1 or anti-HO-2 (Stressgen Biotechnologies, Victoria, BC, Canada), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG at 1:2000 dilution at 37 °C for 1 h. Immunoreactive proteins were visualized using Super Signal West Femto Reagent (Pierce) and Kodak ImageStation 400.

Adenoviral propagation and infection of astrocytes. An adenovirus containing the coding sequence of the human HO-1 gene (Adv-hHO-1) was prepared as previously described [27]. A control virus that lacked the HO-1 gene but that was otherwise identical (Adv-null) was separately prepared. Viruses were propagated in HEK 293 cells, and titer was quantified by cytopathic effect assay. Astrocyte cultures were incubated with virus for 24 h in MEM10 containing 3.3% equine serum.

Data analyses. Data are shown as means ± SEM. Each experiment was carried out in triplicate from two to three dissections. For statistical analyses, one-way analysis of variance (ANOVA) was followed by the Bonferroni multiple comparison test.

Results

Effect of HO-2 gene deletion on hemin toxicity

Consistent with prior observations [24], continuous incubation of wild type astrocyte cultures with hemin for 6 h produced cell death, with release of approximately one-third of culture LDH after exposure to 30–60 μM (Fig. 1). In cultures prepared from HO-2 knockout mice and treated concomitantly with the same regimen, a significantly larger percentage of culture LDH was released. Inspection of cultures using phase-contrast microscopy was consistent with this observation (Fig. 2).

In order to assess cell injury by measuring metabolic activity, other cultures were treated with MTT at the end of the hemin exposure period. The formazan signal produced by knockout cultures after exposure to hemin for 4–8 h was consistently less than that produced by wild type cultures (Fig. 3), indicating decreased cell viability.

Effect of HO-2 gene deletion on HO-1 expression

In a prior study, HO-1 expression was observed to be increased in lung tissue from HO-2 knockout mice [12].

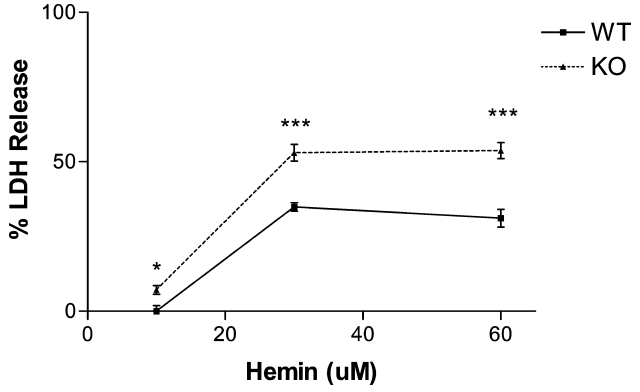

Fig. 1. HO-2 gene deletion increases astrocyte vulnerability to hemin. Cultures ($n = 10$–12/condition) were treated with indicated concentrations of hemin for 6 h. LDH activity (mean ± SEM) was then determined in the culture medium, and was scaled to that in sister cultures treated with 0.3% Triton X-100 for 1 h (= 100), which produces 100% cell lysis. $^{*}P < 0.05$; $^{***}P < 0.001$ versus wild type astrocytes treated with same concentration of hemin, Bonferroni multiple comparisons test.

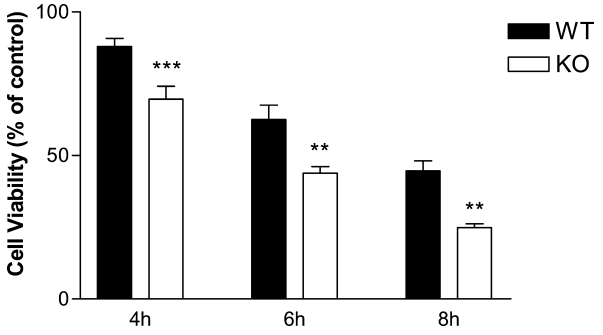

Fig. 3. Decreased cell viability in HO-2 knockout astrocytes after hemin exposure, quantified by MTT assay. Cultures ($n = 8$–12/condition) were treated with 30 μM hemin for indicated interval. MTT reduction is scaled to that in sister cultures that were subjected to media changes only (= 100). $^{**}P < 0.01$, $^{***}P < 0.001$ versus mean in wild type astrocytes treated with same concentration of hemin, Bonferroni multiple comparisons test.

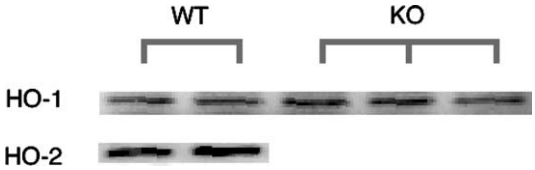

Fig. 4. Immunoblots of lysates from wild type and HO-2 knockout cultures, stained with polyclonal antibodies to HO-1 or HO-2. Relative density of HO-1 expression in knockout cultures 1.1 ± 0.04 versus 1.0 ± 0.098 for wild type cultures (means ± SEM, $n = 3$ each).

In order to determine if this compensatory effect also occurred in murine astrocytes, HO-1 expression was investigated by Western blot analysis. No difference in HO-1 expression was observed in lysates from wild type and knockout cultures (Fig. 4).

Effect of HO-1 gene transfer on vulnerability of knockout cultures to hemin

We hypothesized that the vulnerability of astrocytes in knockout cultures could be attenuated by increasing HO expression prior to hemin exposure. We therefore pretreated cells with an adenoviral vector that encodes the human HO-1 gene (adv-hHO-1) [27]. An increase in HO-1 expression was observed when cultures were treated with 10–100 MOI (multiplicity of infection) of this virus (Fig. 5). No increase was detected when cultures were treated with a control virus that was identical to adv-hHO-1 except that it lacked the HO-1 gene (adv-null).

Consistent with prior results, treatment with 30 μM hemin for 6 h resulted in death of about half of astrocytes that had not been pretreated with any virus. Most of this injury was prevented in cultures that had been

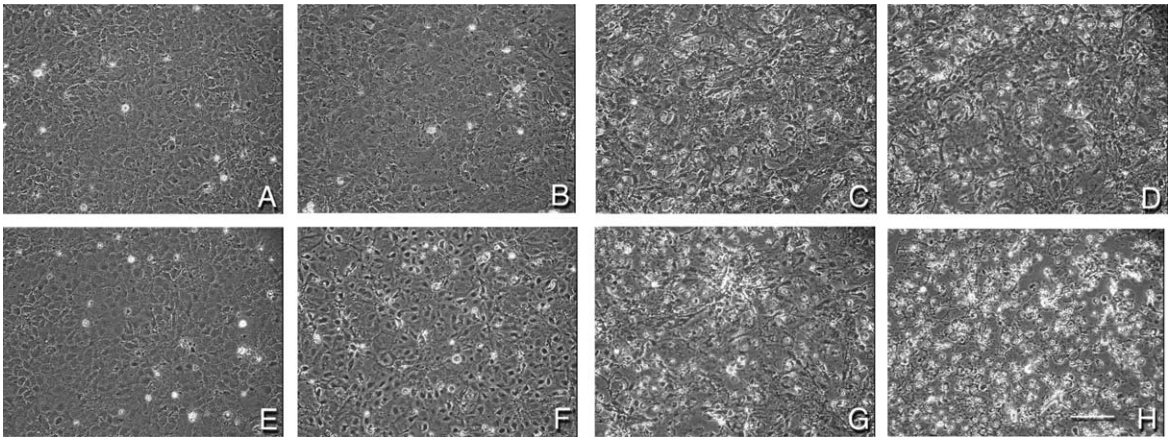

Fig. 2. Morphology of knockout and wild type cultures after hemin treatment. Phase-contrast photomicrographs of wild type (A–D) and HO-2 knockout cultures (E–H) treated for 6 h with the following hemin concentrations: (A,E), 0 μM; (B,F), 10 μM; (C,G), 30 μM; and (D,H), 60 μM. Scale bar = 100 μm.

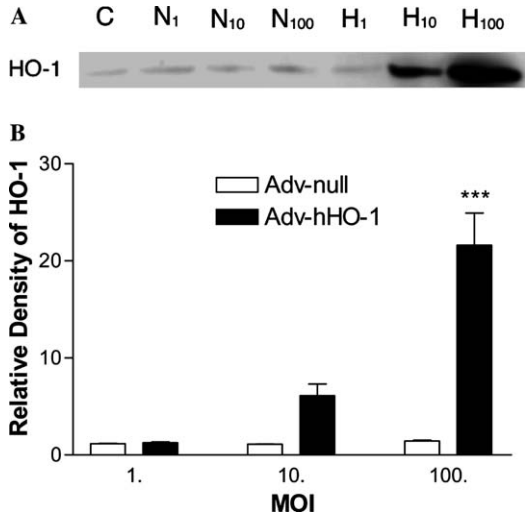

Fig. 5. Adenoviral transfer of the human HO-1 gene increases HO-1 expression in HO-2 knockout astrocytes. Cultures were treated with indicated doses (multiplicity of infection) of Adv-hHO-1 (H) or control adenovirus (Adv-null, N) for 24 h. Density values are expressed as means ± SEM of three experiments and are scaled to that in controls exposed to culture medium only (= 1). Equal loading of the experimental samples was verified with Coomassie blue.

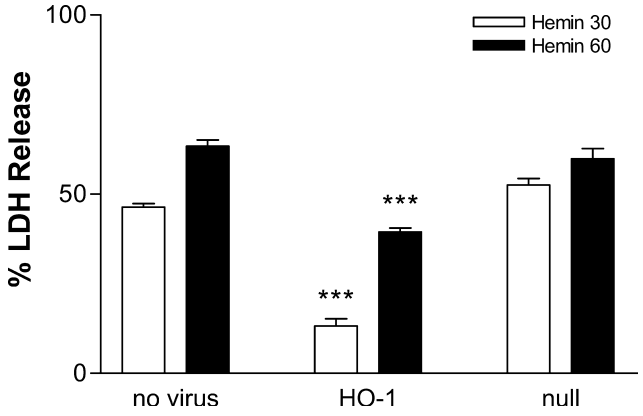

Fig. 6. HO-1 gene transfer reduces HO-2 knockout astrocyte death after hemin exposure. Cultures were treated with 100 MOI of either Adv-hHO-1 or Adv-null; additional controls were sister cultures incubated in culture medium only (no virus). After 24 h, the cultures were treated with 30 or 60 μM hemin for 6 h. $^{***}P < 0.001$ versus cultures treated with hemin without virus pretreatment.

pretreated with 100 MOI of adv-hHO-1 (Figs. 6 and 7); pretreatment with adv-null had no effect. Significant but moderate cytoprotection was also observed when the hemin concentration was increased to 60 μM.

Effect of HO-2 gene deletion on iron toxicity

In a prior study, we observed that HO-2 gene deletion increased the vulnerability of neurons to iron, while decreasing their vulnerability to heme-mediated injury. In contrast to neurons, wild type astrocytes are resistant to iron (unpublished observations). However, in order to determine if HO-2 gene deletion increased iron vulnerability, cultures were exposed to ferrous sulfate at the same concentrations that are highly toxic to neurons (Table 1). No cell injury was observed.

Discussion

In a prior study, we observed that heme oxygenase-2 gene deletion protected murine cortical neurons from heme-mediated oxidative injury [22]. The present study was designed to test the hypothesis that the same effect would be observed in cultured astrocytes. Contrary to this hypothesis, cultured astrocytes from mutant mice were actually more vulnerable to hemin. These divergent results suggest that HO alters the vulnerability of CNS cells to heme in a manner that depends on cell type. The effect of HO-2 gene deletion in astrocytes was less pronounced than in neurons [22], perhaps due to the protective effect of rapid HO-1 induction in astrocytes exposed to hemin [28].

Heme oxygenase catalyzes the conversion of hemin, a lipid soluble oxidant, to products that are biologically active. Its net effect on cell injury after hemin exposure is likely a complex function of the rate of formation of breakdown products and their beneficial or toxic effects. Bilirubin is an antioxidant that can protect CNS cells from oxidative stress [29]. However, at high concentrations it is toxic, and in the CNS it mediates the neonatal neurodegeneration of kernicterus; astrocytes are relatively resistant to this injury [30]. Free iron is an oxidant that initiates lipid peroxidation chain reactions via the Fenton reaction, or by decomposing membrane lipid peroxides to alkoxy- or peroxy radicals [31]. However, by inducing ferritin synthesis, it may indirectly protect some cell types from oxidative injury [24,32]. Carbon monoxide at physiologic concentrations serves as a gaseous neurotransmitter [8]; however, it also binds nonspecifically to hemoproteins including cytochrome C-oxidase [33]. Inhibition of the latter may contribute to the pathogenesis of exogenous carbon monoxide poisoning [34].

Hemin accumulates in cell membranes [35], where it is a very effective catalyst of lipid peroxidation chain reactions [36]. Its iron is essential for this effect, since iron-free protoporphyrin IX is nontoxic, while lipid-soluble iron chelators markedly reduce its toxicity [26]. Although astrocytes were vulnerable to low micromolar concentrations of hemin in this study, no injury was observed when they were exposed to identical concentrations of ferrous sulfate. The benefit of heme oxygenase to astrocytes exposed to hemin may be due at least in part to conversion of its iron to a water-soluble form. Ferritin synthesis induced by high cytoplasmic iron levels would then allow its sequestration, reducing its participation in free radical reactions [37].

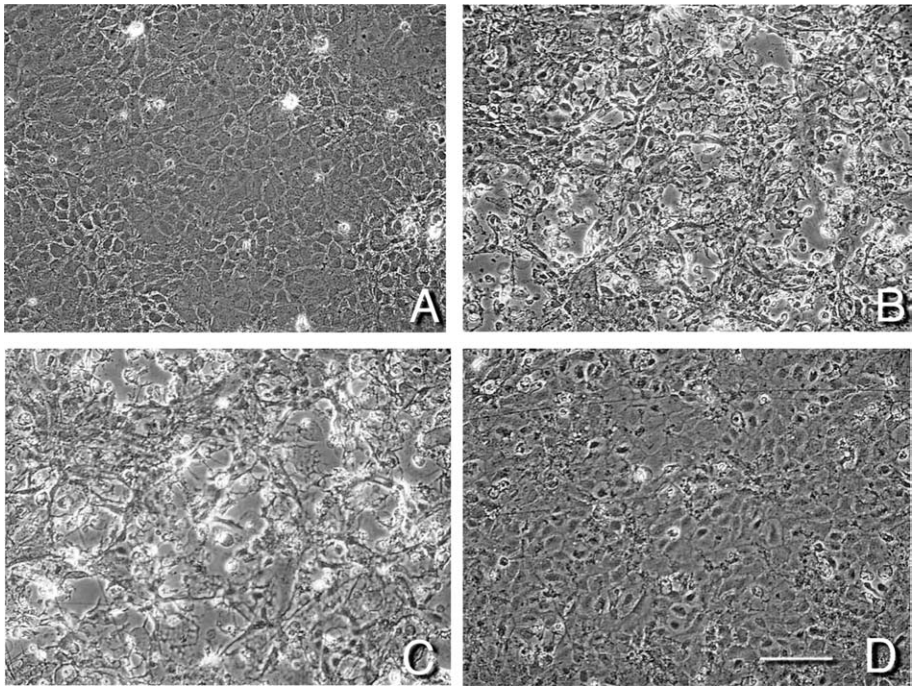

Fig. 7. Effect of HO-1 gene transfer on vulnerability of HO-2 knockout astrocytes to hemin. Phase-contrast photomicrographs of cultures treated as follows: (A) control culture subjected to medium changes only; (B) treated with hemin 30 μM for 6 h, without virus pretreatment; (C) pretreated with empty adenovirus (Adv-null) 100 MOI for 24 h and then treated with hemin as in B; and (D) treated with 100 MOI of adenovirus encoding human HO-1 gene and then treated with hemin. Scale bar = 100 μm.

Table 1
Astrocytes are not injured by ferrous sulfate

	Means ± SEM (WT)	Means ± SEM (KO)
Fe10	99.65 ± 3.79	104.05 ± 4.49
Fe30	98.74 ± 5.51	108.98 ± 4.80
Fe60	98.14 ± 4.96	109.31 ± 4.48
Medium	100.00 ± 6.10	100.00 ± 2.14

Cell viability as measured by MTT assay in cultures treated with indicated concentrations (μM) of ferrous sulfate for 6 h, scaled to that in sister cultures exposed to culture medium only (= 100).

In contrast to astrocytes, neurons are injured by exposure to low concentrations of inorganic iron, with an EC_{50} in primary cortical cultures of approximately 10 μM [22]. The disparate effect of HO-2 gene deletion in neurons and astrocytes may be explained by the inability of cultured neurons to tolerate these low iron concentrations. The molecular basis for this phenomenon has not been precisely defined and is likely multifactorial. Compared with astrocytes, neurons have considerably lower glutathione levels and so are more vulnerable to oxidative injury in general [38]. They are also more dependent on membrane glutamate transporters and cation pumps for termination of depolarization and maintenance of ionic gradients; these proteins are highly vulnerable to iron-mediated oxidation [39–41]. In addition, although neurons constitutively express ferritin, it is rich in H-subunits, resulting in a lower iron binding capacity and a tendency to aggregate and precipitate with iron loading [42,43].

Besides catalyzing heme breakdown, HO-2 is a hemoprotein that may bind two heme moieties at heme regulatory motifs [44]. It is therefore possible that the cytoprotection provided by HO-2 in this model is due to heme chelation, in a manner analogous to the protective effect of hemopexin [45]. Although a contribution by this mechanism cannot be excluded, it is noteworthy that the increased injury in HO-2 knockout cells was reversed by adenoviral transfer of the HO-1 gene and subsequent increase in HO-1 expression. Unlike HO-2, HO-1 lacks heme regulatory motifs and does not bind additional heme moieties [46].

Since heme oxygenase has opposite effects on heme-mediated injury in neurons and astrocytes, nonselective inhibition or induction of these enzymes may have limited therapeutic potential after CNS hemorrhage. HO inhibitors, e.g., may decrease neuronal injury but may worsen injury to astrocytes. Optimal treatment may require a strategy that increases astrocyte HO expression, while decreasing it or selectively inhibiting it in neurons. Although HO-2 is expressed in these astrocyte cultures, it has predominantly been observed in neurons in vivo [47], while HO-1 is induced in glial cells in response to hemorrhage, ischemia, and trauma [48,49]. A selective HO-2 inhibitor may be effective, but at the present time does not exist, and the similarity of HO-1

and HO-2 at their catalytic sites suggests that its development may be difficult [50]. Transfer of HO genes in sense or antisense orientation under the control of a cell type-specific promoter may permit increased expression in glial cells while decreasing it in neurons. CNS hemorrhage may be quite amenable to such genetic approaches, since growing evidence suggests that partial hematoma aspiration may have therapeutic benefit [51]. During this stereotactic procedure, injection of viral vectors encoding an HO isoform or other genes may attenuate heme-mediated injury to adjacent tissue. Further investigation of methods to optimize HO expression after CNS hemorrhage seems warranted.

Acknowledgments

This study was supported by a grant from the National Institutes of Health (1RO1NS042273). We thank Dr. Frank Sharp for providing HO-2 knockout mice that were used to establish our colony. We also thank Dr. Lee-Young Chau for providing adenoviral HO-1 vector and controls.

References

[1] N.G. Abraham, G.S. Drummond, J.D. Lutton, A. Kappas, The biological significance and physiological role of heme oxygenase, Cell. Physiol. Biochem. 6 (1996) 129–168.
[2] P.G. Matz, P.R. Weinstein, F.R. Sharp, Heme oxygenase-1 and heat shock protein-70 induction in glia and neurons throughout rat brain after experimental intracerebral hemorrhage, Neurosurgery 40 (1997) 152–160.
[3] A.E.M. Mautes, D.H. Kim, F.R. Sharp, S. Panter, M. Sato, N. Maida, M. Bergeron, K. Guenther, L.J. Noble, Induction of heme oxygenase-1 (HO-1) in the contused spinal cord of the rat, Brain Res. 795 (1998) 17–24.
[4] A.Z. Fernandez, F. Lopez, A. Tablante, E. Romano, E. Hurt-Camejo, G. Camejo, R. Apitz-Castro, Intravascular hemolysis increases atherogenicity of diet-induced hypercholesterolemia in rabbits in spite of heme oxygenase-1 gene and protein induction, Atherosclerosis 158 (2001) 103–111.
[5] G.M. Trakshel, R.K. Kutty, M.D. Maines, Purification and characterization of the major constitutive form of testicular heme oxygenase. The noninducible isoform, J. Biol. Chem. 261 (1986) 11131–11137.
[6] J.F. Ewing, M.D. Maines, In situ hybridization and immunohistochemical localization of heme oxygenase-2 mRNA and protein in normal rat brain: differential distribution of isozyme 1 and 2, Mol. Cell. Neurosci. 3 (1992) 559–570.
[7] W.K. McCoubrey, T.J. Huang, M.D. Maines, Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3, Eur. J. Biochem. 247 (1997) 725–732.
[8] R. Zakhary, K.D. Poss, S.R. Jaffrey, C.D. Ferris, S. Tonegawa, S.H. Snyder, Targeted gene deletion of heme oxygenase 2 reveals neural role for carbon monoxide, Proc. Natl. Acad. Sci. USA 94 (1997) 14848–14853.
[9] F.A. Wagener, A. Eggert, O.C. Boerman, W.J. Oyen, A. Verhofstad, N.G. Abraham, G. Adema, Y. van Kooyk, T. de Witte, C.G. Figdor, Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase, Blood 98 (2001) 1802–1811.
[10] R. Zakhary, S.P. Gaine, J.L. Dinerman, M. Ruat, N.A. Flavahan, S.H. Snyder, Heme oxygenase 2: endothelial and neuronal localization and role in endothelium-dependent relaxation, Proc. Natl. Acad. Sci. USA 93 (1996) 795–798.
[11] L.A. Applegate, P. Luscher, R.M. Tyrrell, Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells, Cancer Res. 51 (1991) 974–978.
[12] P.A. Dennery, D.R. Spitz, G. Yang, A. Tatarov, C.S. Lee, M.L. Shegog, K.D. Poss, Oxygen toxicity and iron accumulation in the lungs of mice lacking heme oxygenase-2, J. Clin. Invest. 101 (1998) 1001–1011.
[13] R. Tenhunen, H.S. Marver, R. Schmid, The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin, J. Lab. Clin. Med. 75 (1970) 410–421.
[14] G.M. Trakshel, R.K. Kutty, M.D. Maines, Resolution of rat brain heme oxygenase activity: absence of detectable amount of the inducible form (HO-1), Arch. Biochem. Biophys. 260 (1988) 732–739.
[15] Y. Hua, G. Xi, R.F. Keep, J.T. Hoff, Complement activation in the brain after experimental intracerebral hemorrhage, J. Neurosurg. 92 (2000) 1016–1022.
[16] D. Chiu, B. Lubin, Oxidative hemoglobin denaturation and RBC destruction: the effect of heme on red cell membranes, Sem. Hematol. 26 (1989) 128–135.
[17] P.B. Letarte, K. Lieberman, K. Nagatani, R.A. Haworth, G.B. Odell, T.A. Duff, Hemin: levels in experimental subarachnoid hematoma and effects on dissociated vascular smooth muscle cells, J. Neurosurg. 79 (1993) 252–255.
[18] F.P. Huang, G. Xi, R.F. Keep, Y. Hua, A. Nemoianu, J.T. Hoff, Brain edema after experimental intracerebral hemorrhage: role of hemoglobin degradation products, J. Neurosurg. 96 (2002) 287–293.
[19] J. Wu, Y. Hua, R.F. Keep, T. Nakemura, J.T. Hoff, G. Xi, Iron and iron-handling proteins in the brain after intracerebral hemorrhage, Stroke 34 (2003) 2964–2969.
[20] K.R. Wagner, Y. Hua, G.M. de Courten-Myers, J.P. Broderick, R.N. Nishimura, S.Y. Lu, B.E. Dwyer, Tin-mesoporphyrin, a potent heme oxygenase inhibitor, for treatment of intracerebral hemorrhage: in vivo and in vitro studies, Cell. Mol. Biol. 46 (2000) 597–608.
[21] L. Grundemar, L. Ny, Pitfalls using metalloporphyrins in carbon monoxide research, Trends Pharmacol. Sci. 18 (1997).
[22] B. Rogers, V. Yakopson, Z.P. Teng, Y. Guo, R.F. Regan, Heme oxygenase-2 knockout neurons are less vulnerable to hemoglobin toxicity, Free Rad. Biol. Med. 35 (2003) 872–881.
[23] G. Scapagnini, V. D'Agata, V. Calabrese, A. Pascale, C. Colombrita, D. Alkon, S. Cavallaro, Gene expression profiles of heme oxygenase isoforms in the rat brain, Brain Res. 954 (2002) 51–59.
[24] R.F. Regan, N. Kumar, F. Gao, Y.P. Guo, Ferritin induction protects cortical astrocytes from heme-mediated oxidative injury, Neuroscience 113 (2002) 985–994.
[25] K.D. Poss, M.J. Thomas, A.K. Ebralidze, T.J. O'Dell, S. Tonegawa, Hippocampal long-term potentiation is normal in heme oxygenase-2 mutant mice, Neuron 15 (1995) 867–873.
[26] R.F. Regan, Y. Wang, X. Ma, A. Chong, Y. Guo, Activation of extracellular signal-regulated kinases potentiates hemin toxicity in astrocyte cultures, J. Neurochem. 79 (2001) 545–555.
[27] S.H. Juan, T.S. Lee, K.W. Tseng, J.Y. Liou, S.K. Shyue, K.K. Wu, L.Y. Chau, Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development of atherosclerosis in apolipoprotein E-deficient mice, Circulation 104 (2001) 1519–1525.
[28] R.F. Regan, Y.P. Guo, N. Kumar, Heme oxygenase-1 induction protects murine cortical astrocytes from hemoglobin toxicity, Neurosci. Lett. 282 (2000) 1–4.
[29] R. Stocker, Y. Yamamoto, A.F. McDonagh, A.N. Glazer, B.N. Ames, Bilirubin is an antioxidant of possible physiologic significance, Science 235 (1987).

[30] L. Chuniaud, M. Dessante, F. Chantoux, J.P. Blondeau, J. Francon, F. Trivin, Cytotoxicity of bilirubin for human fibroblasts and rat astrocytes in culture. Effect of the ratio of bilirubin to serum albumin, Clin. Chim. Acta 256 (1996) 103–114.
[31] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, third ed., Oxford University Press, Oxford, 1999.
[32] G. Balla, H.S. Jacob, J. Balla, M. Rosenberg, K. Nath, F. Apple, J.W. Eaton, G.M. Vercellotti, Ferritin: A cytoprotective strategem of endothelium, J. Biol. Chem. 267 (1992) 18148–18153.
[33] O. Miro, J. Casademont, A. Urbanomarquez, F. Cardellach, Mitochondrial cytochrome *C* oxidase inhibition during carbon monoxide poisoning, Pharmacol. Toxicol. 82 (1998) 199–202.
[34] L.J. Young, W.S. Caughey, Pathobiochemistry of CO poisoning, FEBS Lett. 272 (1990) 1–6.
[35] N. Shaklai, Y. Shviro, E. Rabizadeh, I. Kirschner-Zilber, Accumulation and drainage of hemin in the red cell membrane, Biochim. Biophys. Acta 821 (1985) 355–366.
[36] J.J. Van den Berg, F.A. Kuypers, J.H. Qju, D. Chiu, B. Lubin, B. Roelofsen, J.A. Op den Kamp, The use of *cis*-parinaric acid to determine lipid peroxidation in human erythrocyte membranes:comparison of normal and sickle erythrocyte membranes, Biochem. Biophys. Acta 944 (1988) 29–39.
[37] P.M. Harrison, P. Arosio, The ferritins: molecular properties, iron storage function and cellular regulation, Biochim. Biophys. Acta 1275 (1996) 161–203.
[38] S.P. Raps, J.C.K. Lai, L. Hertz, A.J.L. Cooper, Glutathione is present in high concentrations in cultured astrocytes but not in cultured neurons, Brain Res. 493 (1989) 398–401.
[39] D.K. Anderson, E.D. Means, Lipid peroxidation in spinal cord: $FeCl_2$ induction and protection with antioxidants, Neurochem. Pathol. 1 (1983) 249–264.
[40] L. Leclerc, M. Marden, C. Poyart, Inhibition of erythrocyte (Ca^{2+} + Mg^{2+})-ATPase by nonheme iron, Biochim. Biophys. Acta 1062 (1991) 35–38.
[41] A. Volterra, D. Trotti, C. Tromba, S. Floridi, G. Racagni, Glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes, J. Neurosci. 14 (1994) 2924–2932.
[42] P. Santambrogio, S. Levi, A. Cozzi, E. Rovida, A. Albertini, P. Arosio, Production and characterization of recombinant heteropolymers of human ferritin H and L chains, J. Biol. Chem. 268 (1993) 12744–12747.
[43] P. Cheepsunthorn, C. Palmer, J.R. Connor, Cellular distribution of ferritin subunits in postnatal rat brain, J. Comp. Neurol. 400 (1998) 73–86.
[44] W.K. McCoubrey, T.J. Huang, M.D. Maines, Heme oxygenase-2 is a hemoprotein and binds heme through heme regulatory motifs that are not involved in heme catalysis, J. Biol. Chem. 272 (1997) 12568–12574.
[45] J.M.C. Gutteridge, A. Smith, Antioxidant protection by haemopexin of haem-stimulated lipid peroxidation, Biochem. J. 256 (1988) 861–865.
[46] Y. Ding, W.K. McCoubrey, M.D. Maines, Interaction of heme oxygenase-2 with nitric oxide donors—Is the oxygenase an intracellular "sink" for NO, Eur. J. Biochem. 264 (1999) 854–861.
[47] S.R. Vincent, S. Das, M.D. Maines, Brain heme oxygenase isoenzymes and nitric oxide synthase are co-localized in select neurons, Neuroscience 63 (1994) 223–231.
[48] K. Fukuda, S.S. Panter, F.R. Sharp, L.J. Noble, Induction of heme oxygenase-1 (HO-1) after traumatic brain injury in the rat, Neurosci. Lett. 199 (1995) 127–130.
[49] T. Nimura, P.R. Weinstein, S.M. Massa, S. Panter, F.R. Sharp, Heme oxygenase-1 (HO-1) protein induction in rat brain following focal ischemia, Brain Res. Mol. Brain Res. 37 (1996) 201–208.
[50] M.O. Rotenberg, M.D. Maines, Isolation, characterization, expression in *Escherichia coli* of a cDNA encoding rat heme oxygenase-2, J. Biol. Chem. 265 (1990) 7501–7506.
[51] G. Xi, R.F. Keep, J.T. Hoff, Pathophysiology of brain edema formation, Neurosur. Clin. N Am. 13 (2002) 371–383.