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Hemoglobin and Iron-Evoked Oxidative Stress in the Brain: Protection by Bile Pigments, Manganese and *S*-Nitrosoglutathione

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In the present *in vitro* and *in vivo* study we investigated the pro-oxidant effects of hemoglobin, as well as the antioxidant effects of its metabolites, in the brain. Incubation of rat brain homogenates with hemoglobin (0–10 μM) but not hemin induced lipid peroxidation up to 24 h ($EC_{50} = 1.2$ μM). Hemoglobin's effects were similar to ferrous ion ($EC_{50} = 1.7$ μM) and were blocked by the chelating agent deferoxamine ($IC_{50} = 0.5$ μM) and a nitric oxide-releasing compound *S*-nitrosoglutathione ($IC_{50} = 40$ μM). However, metabolites of hemoglobin – biliverdin and bilirubin – inhibited brain lipid peroxidation induced by cell disruption and hemoglobin (biliverdin $IC_{50} = 12$–30 and bilirubin $IC_{50} = 75$–170 μM). Biliverdin's antioxidative effects in spontaneous and iron-evoked lipid peroxidation were further augmented by manganese (2 μM) since manganese is an antioxidative transition metal and conjugates with bile pigments. Intrastriatal infusion of hemoglobin (0–24 nmol) produced slight, but significant 20–22% decreases in striatal dopamine levels. Whereas, intrastriatal infusion of ferrous citrate (0–24 nmol) dose-dependently induced a greater 66% depletion of striatal dopamine which was preceded by an acute increase of lipid peroxidation. In conclusion, contrary to the *in vitro* results hemoglobin is far less neurotoxic than ferrous ions in the brain. It is speculated that hemoglobin may be partially detoxified by heme oxygenase and biliverdin reductase to its antioxidative metabolites in the brain. However, in head trauma and stroke, massive bleeding could significantly produce iron-mediated oxidative stress and neurodegeneration which could be minimized by endogenous antioxidants such as biliverdin, bilirubin, manganese and *S*-nitrosoglutathione.

Keywords: Hemoglobin, bile pigments/biliverdin/bilirubin, manganese, *S*-nitrosoglutathione/GSNO, brain lipid peroxidation, dopamine

INTRODUCTION

Previous *in vitro* studies have demonstrated that hemoglobin is a pro-oxidant and may be neurotoxic.[1–3] These pro-oxidative effects are explained by the release of iron-containing heme from hemoglobin, which converts hydrogen peroxide to cytotoxic hydroxyl radicals.[4] Under normal circumstances, plasma hemoglobin is not able to cross the blood–brain barrier. Nonetheless under pathophysiological conditions, such as

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subarachnoid hemorrhage, head trauma and stroke, lysed erythrocytes release large amounts of hemoglobin and iron inside the brain. Neurons and glial cells will therefore be exposed to toxic byproducts of hemoglobin, which could cause oxidant stress, delayed neurotoxicity and liquefaction of brain tissue. Recent *in vivo* studies indicate that ferrous ion is a potent pro-oxidative transition metal,[5,6] while manganese appears to be a potent antioxidant.[7–9] In addition, nitric oxide released from *S*-nitrosoglutathione (GSNO) inhibits iron-induced brain lipid peroxidation and nigral damage *in vivo*.[6] In this study, the effects of these atypical antioxidants on hemoglobin-induced brain lipid peroxidation were thoroughly examined.

Enzymatic metabolism of hemoglobin by heme oxygenase results in the release of iron and also the formation of biliverdin and carbon monoxide.[10] Subsequently, biliverdin reductase catalyzes the conversion of biliverdin to bilirubin. Because both enzymes are mainly localized in the liver, biliverdin and bilirubin become the major components of bile pigments. However, heme oxygenase and biliverdin reductase also exist in the brain.[11] Earlier research has focused on the putative antioxidant effects of bilirubin, which may scavenge *in vitro* superoxide anion,[12] hypochlorous acid[13] and peroxyl lipid radicals.[14] These putative antioxidative effects of bile pigments have not been thoroughly investigated in the brain. Therefore, in the present study, we also compared the effects of biliverdin and bilirubin on hemoglobin-induced oxidative stress in the brain.

MATERIALS AND METHODS

Animals The experiments were carried out with male Sprague–Dawley rats (Taconic Farms, Germantown, NY, USA; 250–350 g). Prior to and after the operation rats were housed three to a cage. Food pellets and tap water were provided to the rats *ad libitum*. The room temperature was kept at 20–22°C with a 12 h life circle and a relative humidity of 45–60%. The experimental protocol was approved by the Animal Care and Use Committee of the National Institute of Mental Health.

In vitro brain lipid peroxidation assay Rats were decapitated and cortical brain samples were dissected and stored at −70°C. Brain samples were homogenized in ice cold Ringer's buffer using an ultrasonic cell disrupter (Heat Systems Inc., Farmingdale, NY) and diluted to 50 mg/ml with ice cold Ringer's solution. As a model of head trauma brain homogenates were incubated at 37°C without adding hemoglobin or ferrous ion in a shaking waterbath for 2–24 h. In other experiments hemoglobin or ferrous ion was added to brain homogenates to mimic hemorrhage-induced brain injury. Drugs were added to homogenates at the beginning of incubation. Fluorescent end-products of brain lipid peroxidation, which consist of cross-linked products of primary amines with reactive aldehyde species derived from peroxidized polyunsaturated fatty acids, were determined using a microassay procedure as previously described by Mohanakumar *et al.*[5] modified from Dillard and Tappel.[15] These fluorescent products represent a reliable biological marker for lipid peroxidation. Briefly, after incubation, a 200 µl aliquot of brain homogenate was transferred to another tube containing 200 µl chloroform and 100 µl methanol. The mixture was vortexed and kept on ice for about 15 min. After centrifugation at 8000 rpm for 6 min 150 µl of the chloroform extract was transferred to another tube containing 50 µl methanol. The relative fluorescent intensities of the aldehyde products in 3.75 mg tissue extract (100 µl) were measured in a Perkin Elmer LS 50B spectrofluorometer (activation wavelength 356 nm and emission wavelength 426 nm). Where applied, NaOH (0–600 µM) was added to incubation mixture and served as sham controls for the experiments using biliverdin, bilirubin and hemin. The spectrofluorometer was calibrated with quinine sulphate. All samples were assayed in triplicate.

Intrastriatal infusion of drug Male rats were anesthetized with chloral hydrate (400 mg/kg, i.p.). Body temperature was recorded with a rectal probe and maintained at 37°C by a thermal blanket system (Harvard Apparatus). For microinfusion of ferrous citrate, hemoglobin, and vehicle to the caudate nucleus, rats were placed in a stereotaxic head holder with the mouth bar set at −3.5 mm. Microinjections were made via a 29g needle connected by plastic PE-10 tubing to a Hamilton syringe which was mounted on a syringe infusion pump. The bregma was used as rostral–caudal zero point. Injections into the caudate putamen were made unilaterally (right-sided) by placing the needle at a level of 0.2 mm posterior from the bregma, 3 mm laterally and 6 mm deep (according to the rat atlas of Paxinos and Watson[16]). Drugs (in 6 µl) were unilaterally infused at a rate of 0.2 µl/min into the right caudate nucleus. After infusion the wound on the skull was closed with either sutures or wound clips. The rats were kept warm under a heating lamp until they regained consciousness.

In vivo brain lipid peroxidation assay Rats were sacrificed by decapitation at 24 h, 3 days or 7 days after intrastriatal infusion of ferrous citrate, hemoglobin or vehicle. Their brains were removed and the caudate nucleus was dissected and removed from both sides. Fluorescent end-products of lipid peroxidation in the whole striatum were measured as previously described.[5]

Measurement of striatal dopamine levels Rats were sacrificed by decapitation at 7 days after intrastriatal infusion of ferrous citrate, hemoglobin or vehicle. The striatum of both sides was dissected and homogenized in twenty volumes of 0.1 N perchloric acid containing 10 mM EDTA. After mixing, the percholate extract was chilled on ice for at least 20 min. After centrifugation at 8000 rpm for 6 min the supernatant was transferred to another tube for HPLC-EC measurement of dopamine and its metabolites.[17]

Statistical Analysis Data were analyzed by one-way analysis-of-variance (ANOVA), followed by Bonferroni as *post hoc* test, or by a paired student *t*-test. The level of significance was set at 95% confidence limit. All data are expressed as the mean ± SEM.

Materials Ferrous ammonium sulphate, bovine hemoglobin, bovine hemin, deferoxamine mesylate, biliverdin dihydrochloride, bilirubin, manganese chloride, chloroform, methanol and chloral hydrate were purchased from Sigma Chemical Co, St. Louis, MO, USA; copper(II) chloride from Aldrich, Milwaukee, WI, USA; citric acid from Reagents Inc. Charlotte, NC, USA; GSNO from Calbiochem, La Jolla, CA, USA.

Compounds were freshly dissolved in Ringer's solution and kept on ice. Biliverdin, bilirubin and hemin (10 mM stock solution) were dissolved in 20 mM NaOH. For the *in vivo* experiments drugs were dissolved in sterilized Ringer's solution as passed through a 0.22 µm filter membrane (Millipore Products Division, Bedford, MA, USA). Ferrous citrate was prepared by mixing equimolar concentrations of ferrous ammonium sulphate and citric acid. Bovine hemoglobin was bought as lyophilized powder, and therefore might have been predominantly methemoglobin. A photo-degraded, nitric oxide-exhausted GSNO solution was obtained by leaving a freshly prepared solution under a 100 Watt light bulb for at least 48 h. This procedure causes a slow release of nitric oxide from GSNO, resulting in the formation of GSSG and nitrites or nitrates.[18]

RESULTS

In Vitro Results

Effects of iron, hemoglobin and its metabolites biliverdin and bilirubin on cell disruption-induced brain lipid peroxidation The process of disrupting brain tissue by sonication released iron and initiated the peroxidation of polyunsaturated fatty acids which lasted for more than 24 h. Incubation of brain homogenates at 37°C for 2–24 h significantly increased the formation of

peroxidized brain lipids assayed fluorimetrically. The cell disruption-induced lipid peroxidation was further augmented by adding ferrous ions ($EC_{50} = 1.7\,\mu M$) but not copper ions to the incubation mixture (Figure 1). Addition of hemoglobin (0–10 μM) significantly increased the peroxidation of brain lipids in a concentration-dependent manner ($EC_{50} = 1.2\,\mu M$, Figure 2A). However, the maximal effect of ferrous ion was higher than that of hemoglobin. The concentration-response curves of iron and hemoglobin iron complex on cell-disruption-induced brain lipid peroxidation were bell-shaped, since higher non-physiological concentrations of iron (> 20 μM) may interfere with the redox cycling of oxygen and iron complexes.

Hemoglobin has four iron-containing polypeptide side chains while hemin (0–100 μM) consists of only one iron-containing polypeptide chain, which caused no such pro-oxidative effects. In fact, hemin induced antioxidative effects in brain homogenates (Figure 2A). Similarly, incubation of brain homogenates for 3 h at 37°C with biliverdin (0–250 μM) or bilirubin (0–1000 μM) concentration-dependently decreased brain lipid peroxidation (Figure 2B). In brain tissue, biliverdin was a more potent antioxidant than bilirubin; the IC_{50} values for biliverdin and bilirubin were 30 and 170 μM, respectively.

Effects of biliverdin and bilirubin on the pro-oxidant effects of hemoglobin in brain homogenates Biliverdin (0–100 μM) concentration-dependently inhibited the tissue disruption-induced peroxidation of brain lipids without adding hemoglobin. In addition, biliverdin concentration-dependently inhibited lipid peroxidation stimulated by 1 μM hemoglobin (Figure 3A). A comparable effect was observed with bilirubin (0–1000 μM, Figure 3B). The IC_{50} values of biliverdin and bilirubin in suppressing the pro-oxidative effects of 1 μM hemoglobin were 12 and 75 μM, respectively.

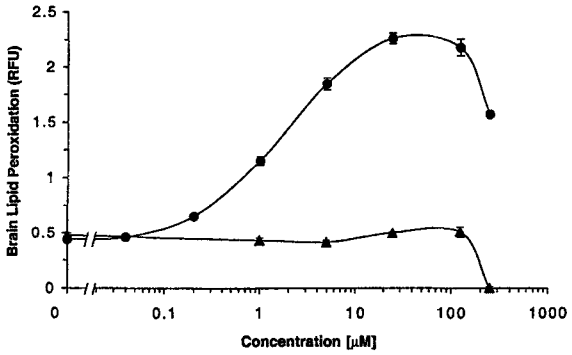

FIGURE 1 Effects of Fe^{2+} and Cu^{2+} metal ions on peroxidation of brain lipids *in vitro*. Fe^{2+} (●, 0–125 μM) and Cu^{2+} (▲, 0–250 μM) were added to 1 ml rat brain homogenates (50 mg cortical tissue/ml of Ringer's solution). After a 2 h incubation at 37°C fluorescent products of lipid peroxidation were extracted and measured (excitation/emission wavelength 356/426 nm). Data represent the mean ± SEM of relative fluorescence intensity units (RFU) in 3.75 mg brain tissue ($n = 3$).

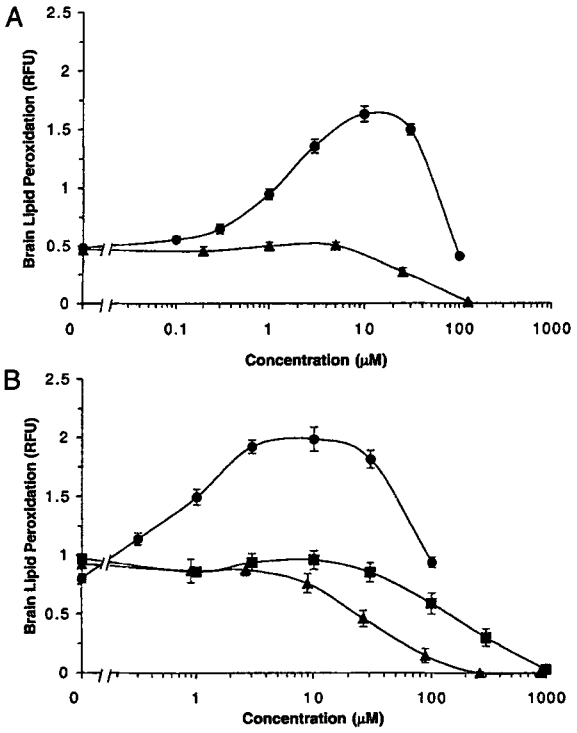

FIGURE 2 Effects of hemin and metabolites of hemoglobin on peroxidation of brain lipids *in vitro*. (A) Hemoglobin (●, 0–100 μM) and hemin (▲, 0–125 μM) were added to 1 ml rat brain homogenates. (B) Hemoglobin (●, 0–100 μM), biliverdin (▲, 0–250 μM), and bilirubin (■, 0–1000 μM) were added to 1 ml rat brain homogenates. Samples were incubated at 37°C for 2–3 h; and fluorescent end-products of lipid peroxidation were measured (excitation/emission wavelength 356/426 nm). Data represent the mean ± SEM of RFU in 3.75 mg brain tissue ($n = 3$–4).

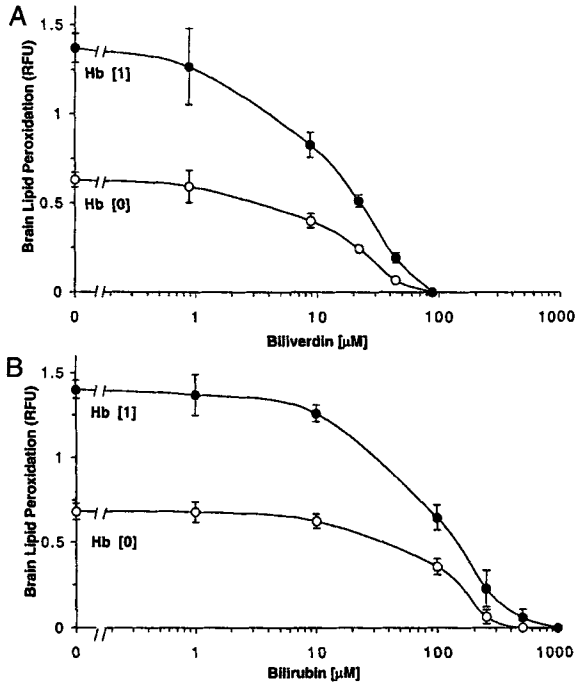

FIGURE 3 Antioxidative effects of (A) biliverdin (0–100 μM) and (B) bilirubin (0–1000 μM) on hemoglobin-induced brain lipid peroxidation *in vitro*. Hemoglobin-induced lipid peroxidation in rat brain homogenates (50 mg cortical tissue/ml of Ringer's solution) was measured by assaying fluorescent products of lipid peroxidation after incubation with either Ringer's solution (○, Hb[0]) or 1 μM hemoglobin (●, Hb[1]) at 37°C for 2 h. Data represent the mean ± SEM of accumulated fluorescent end-products of lipid peroxidation (RFU) in 3.75 mg brain tissue ($n = 3$–6).

Effects of deferoxamine on the pro-oxidant effects of hemoglobin in brain homogenates When the incubation period was increased from 2 to 24 h, a significant 5- and 2.6-fold increases in cell disruption- and hemoglobin-evoked brain lipid peroxidation was observed, respectively. The chelating agent deferoxamine (0.1–10 μM) blocked tissue disruption-induced brain lipid peroxidation in a concentration-dependent manner in both acute 2 h and chronic 24 h experiments (Figure 4). The pro-oxidative effects of 1 μM hemoglobin were also inhibited by deferoxamine for an experimental period up to 24 h.

Effects of GSNO on hemoglobin-induced brain lipid peroxidation GSNO blocked hemoglobin-induced lipid peroxidation in brain homogenates

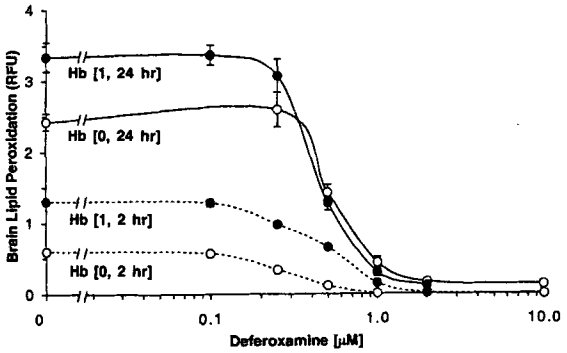

FIGURE 4 Effects of deferoxamine on hemoglobin-induced brain lipid peroxidation *in vitro*. Hemoglobin-induced lipid peroxidation in 1 ml rat brain homogenates was measured by assaying fluorescent products of lipid peroxidation after incubation with hemoglobin 0 μM (○) or 1 μM (●) at 37°C for 2 (---) and 24 (—) h. Deferoxamine (0–10 μM) was added to the incubation mixture at time zero. Data represent the mean ± SEM of accumulated fluorescent end-products of lipid peroxidation (RFU) in 3.75 mg brain tissue ($n = 5$–7).

in a concentration-dependent manner (IC_{50} = 40 μM; Figure 5A). Photo-degraded, nitric oxide-exhausted GSNO (20–100 μM) did not have any significant effect on the peroxidation of brain lipids enhanced by hemoglobin (Figure 5B).

Effects of biliverdin and manganese on iron-induced brain lipid peroxidation Manganese (Mn^{2+}) was recently shown to be a potent antioxidant in the brain with an IC_{50} value of 2 μM.[9] Indeed, Mn^{2+} (2 μM) suppressed cell disruption-induced brain lipid peroxidation for more than 50% (Figure 6). An almost complete inhibition of spontaneous brain lipid peroxidation was achieved when a mixture of biliverdin (30 μM) and manganese (2 μM) was added to the brain homogenate. Iron-induced brain lipid peroxidation was also suppressed by a combination of biliverdin and manganese.

In Vivo Results

Comparison of the pro-oxidative effects of hemoglobin and ferrous citrate in the striatum of the rat After intrastriatal micro-infusion of ferrous citrate (12 nmol) into the caudate nucleus, a significant and long-lasting increase of fluorescent end-products of lipid peroxidation was

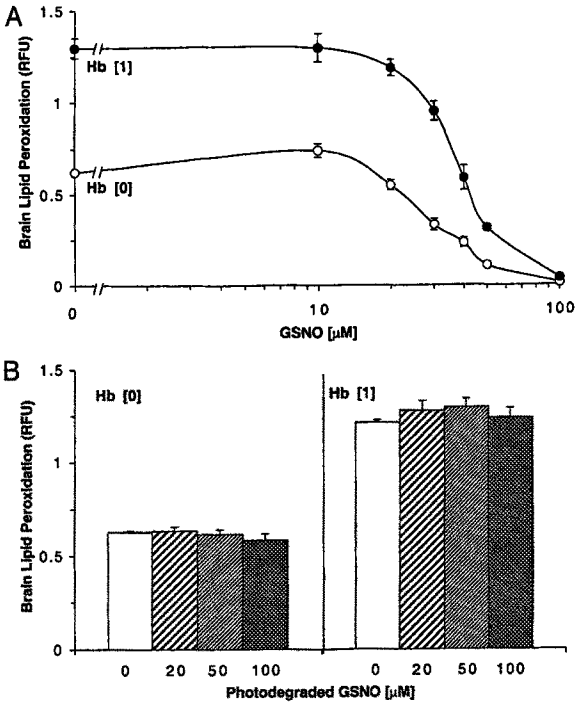

FIGURE 5 Effects of GSNO on hemoglobin-induced brain lipid peroxidation *in vitro*. (A) The effect of freshly prepared nitric oxide-releasing GSNO on lipid peroxidation of rat brain homogenates induced by hemoglobin (0 μM, ○; 1 μM, ●). (B) The effect of photodegraded, nitric oxide-exhausted GSNO on lipid peroxidation of rat brain homogenates induced by hemoglobin (0 and 1 μM). Lipid peroxidation as reflected by accumulated fluorescent products in 3.75 mg brain tissue was measured after incubation at 37°C for 2 h. Data represent the mean ± SEM of RFU ($n = 3$).

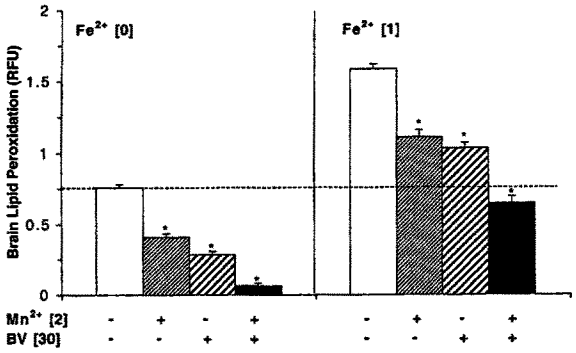

FIGURE 6 Effects of manganese (Mn^{2+}) and biliverdin (BV) on iron-induced brain lipid peroxidation *in vitro*. Manganese (2 μM) or biliverdin (30 μM) or a mixture of both was added to rat brain homogenates (50 mg cortical tissue/ml of Ringer's solution). Brain lipid peroxidation was induced by Fe^{2+} (0 and 1 μM) and reflected the accumulated fluorescent products in 3.75 mg brain tissue measured after incubation at 37°C for 2 h. Data represent the mean ± SEM of RFU ($n = 3$). *$P < 0.05$ significantly different from control.

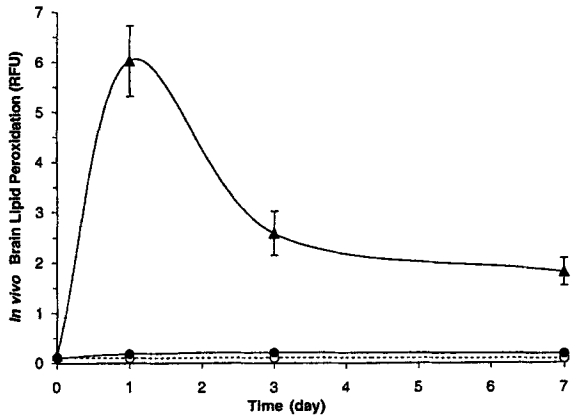

FIGURE 7 *In vivo* lipid peroxidation caused by intrastriatal infusion of hemoglobin and ferrous citrate. Hemoglobin (12 nmol, ●), ferrous citrate (12 nmol, ▲), or Ringer's solution (○) were infused into the right caudate nucleus of anesthetized rats on day 0. At different time points after treatment, rats were decapitated and the right striatum was dissected and processed for the measurement of fluorescent end-products of lipid peroxidation. RFU = relative fluorescence intensity units in whole right striatum. Data represent the mean ± SEM ($n = 4$–6).

observed in the ipsilateral caudate nucleus (Figure 7). Ferrous citrate increased lipid peroxidation and peaked at 24 h (30-fold). Thereafter lipid peroxidation slowly declined; a 15-fold increase was observed after 3 days and a 10-fold after 7 days. In accordance, tissue damage of the ipsilateral caudate nucleus was visually observed throughout the 7 days. Subsequently, ferrous citrate induced a marked 40% (12 nmol) and 66% (24 nmol) decrease in striatal dopamine levels. (Figure 8).

To our surprise, intrastriatal micro-infusion of hemoglobin (12 nmol) induced a small insignificant lipid peroxidation in the striatum *in vivo* (Figure 7). However, a brownish precipitation/ pigment was visually observed 24 h later at the injection site, whereas after 3 and 7 days the striatum appeared visually similar to the control striatum. Even though hemoglobin did not appear to have a significant effect on brain lipid

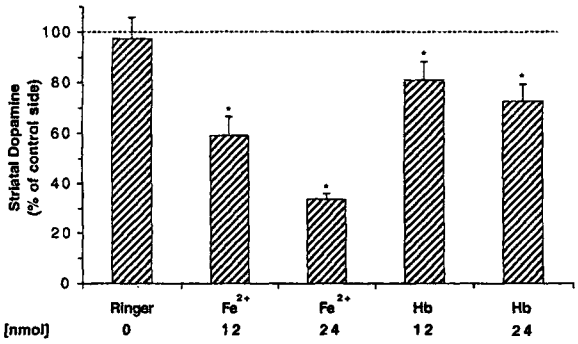

FIGURE 8 Effects of intrastriatal infusion of hemoglobin and ferrous citrate on dopamine content in the striatum. Striatal dopamine levels were assayed by HPLC procedure 7 days after the infusion of drugs (12 and 24 nmol) into the right caudate putamen of anesthetized rats. Each bar represents the mean ± SEM ($n = 5$–10) of dopamine levels as % of contralateral side. Control striatal dopamine level was 71 ± 2 pmol/mg. *$P < 0.05$ significantly different from contralateral side.

peroxidation, 12 nmol hemoglobin did deplete by 20% dopamine in the ipsilateral striatum as compared to the contralateral control side (Figure 8). It appeared that 12 nmol is near the maximal dose, since infusion of 24 nmol of hemoglobin significantly decreased striatal dopamine by approximately 22% ($P < 0.05$).

DISCUSSION

In the first part of this study we investigated the pro-oxidative effects of hemoglobin and the putative antioxidative properties of its metabolites, biliverdin and bilirubin on tissue disruption-induced peroxidation of brain lipids *in vitro*. The present results confirmed that iron moieties of hemoglobin can induce significant oxidative stress in brain homogenates ($EC_{50} = 1.2\,\mu M$), since it was blocked by deferoxamine. The present new results indicated that bile pigments (i.e. biliverdin and bilirubin) are potent antioxidants in brain preparations. These *in vitro* results suggest that biliverdin is approximately 5 times more potent than bilirubin in suppressing lipid peroxidation in brain samples. Moreover, hemoglobin- or iron-induced brain lipid peroxidation can be suppressed by putative endogenous antioxidants such as biliverdin, manganese, deferoxamine and GSNO. In addition, the pro-oxidative effects of hemoglobin were compared to those of ferrous citrate – a small molecular weight iron complex[5] on oxidative stress and dopamine depletion in the striatum *in vivo*. The present results suggest that hemoglobin metabolites are potent antioxidants, which could inhibit the propagation of lipid peroxidation in both the head trauma model (e.g. brain tissue disruption) and cerebral hemorrhage model (e.g. hemoglobin-induced).

Role of Iron in Hemoglobin-Mediated Brain Lipid Peroxidation *In Vitro*

Confirming previous *in vitro* studies,[19–21] the present results demonstrated that hemoglobin initiates lipid peroxidation in cortical brain homogenate with an EC_{50} value of approximately 1.2 μM (Figure 2A). The high redox potential produced by four iron-containing alpha and beta polypeptide chains in hemoglobin could contribute to its pro-oxidative properties since hemin (a single iron-containing polypeptide chain of hemoglobin) did not promote lipid peroxidation (Figure 2A). Based on the fact that brain lipid peroxidation induced by hemoglobin can be completely inhibited by the commonly used iron chelator deferoxamine ($IC_{50} = 0.5\,\mu M$, Figure 4), we and others[22] suggested that hemoglobin may release or expose at least one of its four heme irons for initiating oxidative stress in brain homogenates that contain high amounts of polyunsaturated fatty acids.

Ferrous ion produced a similar concentration-response curve as hemoglobin (0–10 μM) in brain homogenates. However, free ferrous ions evoked a higher maximal effect since it can easily undergo redox reaction in the presence of brain homogenates that contain citrate and ascorbate. Iron complex is capable of stimulating hydroxyl radical production via the Fenton reaction,

especially in the presence of citrate and ascorbate.[4,5,23] The formation of oxo-iron species can lead to stimulation of peroxidation of polyunsaturated fatty acids[24] which is blocked *in vivo* by nitric oxide[23,25] and *S*-nitrosothiols such as GSNO.[6] Despite the fact that copper can generate hydroxyl free radicals and cause diene formation in human low-density lipoproteins,[26] its expected pro-oxidative properties in the brain cannot be demonstrated in brain homogenates (Figure 1). Unexpectedly, the other transition metal manganese does not initiate brain lipid peroxidation at physiological pH and in fact it terminates the propagation of oxidative stress *in vitro*[26] and *in vivo*.[9] This new information infers that polyunsaturated fatty acids in brain homogenates are prone to oxidative stress generated by iron complexes including hemoglobin, but not copper or manganese.

The present finding that freshly prepared nitric oxide donor GSNO suppresses hemoglobin's pro-oxidative effects is very intriguing (Figure 5). This finding is consistent with a recent new report that GSNO is more potent than GSH in suppressing iron-induced free radicals generation and brain lipid peroxidation in both *in vitro* and *in vivo* preparations.[6] Sham control results were obtained in experiments using light-exposed GSNO preparations since they can no longer release nitric oxide, which is an atypical antioxidant. This result infers that nitric oxide released from freshly prepared GSNO may contribute to the observed GSNO protection against hemoglobin-mediated brain lipid peroxidation. It has been proposed that nitric oxide may scavenge lipid peroxyl radicals and hence terminate the lipid peroxidation chain reactions caused by iron complexes.[27,6] The present findings are also supported by prior reports that nitric oxide inhibits the linoleic acid peroxidation catalyzed by hemoglobin[28] and iron-induced oxidative brain damage *in vivo*.[23,25] Furthermore, Rauhala *et al.*[6] have recently shown that GSNO is a potent neuroprotectant *in vivo* by inhibiting iron-induced nigral injury and dopamine depletion. These putative antioxidative and neuroprotective properties of nitric oxide and GSNO have consistently been demonstrated in both *in vitro* and *in vivo* preparations supporting a new biological role of nitric oxide as a neuroprotective antioxidant in the brain.[29]

Antioxidative Properties of Manganese, Biliverdin and Bilirubin

Despite similar *in vitro* pro-oxidative properties of macromolecular hemoglobin and small molecular ferrous ions (Figures 1 and 2A), the present *in vivo* results, however, indicate that hemoglobin is far less potent than ferrous citrate in causing oxidative stress (Figure 7) and dopamine depletion in the striatum (Figure 8). One apparent explanation is that large molecular weight hemoglobin does not easily diffuse as the small molecular weight ferrous citrate inside the brain. Two other mechanisms may explain the loss of pro-oxidative potency of hemoglobin in the present *in vivo* study. (1) Iron released from the hemoglobin may be sequestered by astroglia and macrophages in the brain. This free iron can then be converted into ferritin for iron storage, which consequently diminishes its ability to induce oxidative stress and damage in the brain.[30,31] (2) The second mechanism possibly involves the presence of heme oxygenase and biliverdin reductase.[10,11,32] These two enzymes convert pro-oxidative hemoglobin into the antioxidants biliverdin and bilirubin and exist not only in the hepatic tissue but also in the brain tissue.[11,32] Although hemoglobin metabolites can act as an iron chelator[33] or a scavenger for hypochlorous acid,[13] earlier studies also suggest that they also scavenge lipid peroxyl radicals.[14] Unfortunately, in the present study we do not know how much biliverdin and bilirubin are produced from hemoglobin over time *in vivo*.

To the best of our knowledge, this is the first study to report antioxidant effects of biliverdin and bilirubin in brain tissue. Biliverdin is five times more potent than bilirubin in the

suppression on brain lipid peroxidation. Moreover, biliverdin's antioxidative potency is further augmented by manganese – an atypical antioxidative transition metal (Figure 6).[7–9,26] The daily intake of manganese is about 2–10 mg and most of the circulating manganese is bound to bile pigments.[34] Therefore, it can be speculated that the manganese complex of conjugated biliverdin with its potent antioxidative properties may have a physiological relevance in the protection of the hepatoenteric system, the cardiovascular system and the central nervous system against oxidant stress and associated damage.

It is postulated that there is a homeostasis in the brain between the pro-oxidative effects of hemoglobin and anti-oxidative effects of its metabolites such as biliverdin and bilirubin. Low amounts of hemoglobin may not induce severe brain lipid peroxidation because of the induction of heme oxygenase-1 in the brain.[35] However, in stroke and head trauma cases, exposure of concussed brain tissue to excessive amount of lysed extravascular red blood cells could lead to the generation of toxic byproducts of hemoglobin, such as iron complexes, reactive oxygen species and lipid radicals.[36] These hemoglobin-derived iron complexes and oxygen radicals may induce oxidative stress, vasospasm, neurotoxicity, neurodegeneration and ultimately liquefaction in the brain. Finally, the present new findings indicate that bilirubin, biliverdin manganese and GSNO may be endogenous antioxidants that could provide protection of neurons brain cells, and endothelial cells against the free radical-mediated damage caused by iron complexes such as hemoglobin and ferrous citrate.

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