

Iron homeostasis is maintained in the brain, but not the liver, following mild hypoxia

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Alterations in iron metabolism or oxidative damage in response to hypoxic incidents have been examined following re-oxygenation of the hypoxic tissue. To understand the consequences of decreased tissue oxygen on iron load, metal-catalyzed redox activity and oxidative modifications in isolation from re-oxygenation, the present study exposed mice to either normoxia, or mild hypoxia (380 Torr; ~10% normobaric oxygen) where the tissue was not allowed to re-oxygenate prior to examination. Brain, liver and skeletal muscle were examined for Fe³⁺ load, metal-catalyzed redox activity and oxidative modifications to proteins (N^ε-(carboxymethyl)lysine), lipids (4-hydroxynonenal pyrrole) and nucleic acids (8-hydroxyguanosine). Hypoxia induced a 43% increase in the iron content of the liver ($P < 0.001$) as determined by ICP-MS and a 3.8-fold increase in Fe³⁺ load ($P < 0.001$) as determined by Perl's stain. There was a corresponding 2-fold increase in metal-catalyzed redox activity ($P < 0.01$) in the liver, but no change in the expression of oxidative markers. In contrast, non-significant increases in Fe³⁺ and metal-catalyzed redox activity were observed in the cerebral cortex, and molecular and granular layers of the hippocampus and cerebellum. Interestingly, hypoxia significantly decreased oxidative modifications to proteins and lipids, but not nucleic acids in most brain regions examined. In addition, hypoxia did not alter the Fe content of skeletal muscle, or the contents of Zn, Cu, Ni or Mn in liver, skeletal muscle, cerebral cortex or hippocampus. Together, these results indicate that there is a tighter regulation of iron metabolism in the brain than the liver, which limits the redistribution of Fe³⁺ following hypoxia.

Keywords: Iron homeostasis, brain, liver, skeletal muscle, mild hypoxia, oxidative damage, carboxymethyl lysine, 4-hydroxynonenal pyrrole, 8-hydroxyguanosine, edox activity

INTRODUCTION

During hypoxic-ischemic incidents, oxygen supply to tissue is decreased. Subsequent reperfusion of the tissue

results in a cascade of damage, involving the release of excitatory amino acids, disruption of calcium homeostasis and increased production of free radicals. This cascade leads to oxidative stress and cellular degeneration, yet the mechanisms leading to the marked oxidative damage are not understood. All tissues in the body are susceptible to damage induced by hypoxic-ischemic incidents, yet the brain is thought to be particularly vulnerable due to its high rate of oxygen consumption, large amount of unsaturated fatty acids in lipid membranes, high iron content, and relatively low antioxidant defenses.¹ These factors facilitate the peroxidation of lipids in the brain, as well as the oxidation of nucleic acids and proteins, which can lead to pronounced oxidative damage.

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Alterations in metal ion metabolism promote oxidative stress and cellular degeneration and may underlie the pathology in many neurodegenerative conditions.² Furthermore, iron chelators, such as deferoxamine, can protect numerous tissues against hypoxic-ischemic incidents, including brain and liver,^{3,4} suggesting an important role for iron in the oxidative damage caused by hypoxia-ischemia. However, under normal circumstances, the blood-brain barrier tightly regulates the transport of iron into or out of the brain,⁵ which helps to protect the brain from sudden alterations in systemic iron metabolism.

Alterations in iron metabolism or oxidative damage in response to hypoxic incidents have been examined following re-oxygenation of hypoxic tissue. In order to understand the consequences of decreased oxygen to tissues fully, it is necessary to consider the effects of hypoxia in isolation from re-oxygenation, since oxygenation will enhance oxidative damage, particularly if there is a prior shift in iron metabolism following hypoxia. In the present study, we have used an *in vivo* model of mild hypoxia to examine iron load and redox activity following exposure to hypoxia, without allowing tissue re-oxygenation prior to examination. Since the brain is generally well protected against systemic changes in iron metabolism by the blood-brain barrier, we have compared the effects of hypoxia on the brain to that in the liver. We also have examined oxidative modifications to proteins, lipids and nucleic acids to determine whether the brain is more susceptible to hypoxia-induced oxidative stress. We find that mild hypoxia alters iron metabolism and metal-catalyzed redox activity in the liver, but not in the brain, and that hypoxia does not increase oxidative modifications in the liver, yet decreases them in the brain.

MATERIALS AND METHODS

Animals and treatment

C57BL/6J mice aged 3 months ($n = 12$; 6 female, 6 male) were purchased from The Jackson Laboratory and housed in micro-isolator cages in the Animal Resource Center at Case Western Reserve University. Animal experimentation was approved by the Animal Care and Use Committee of Case Western Reserve University. Mice received standard rodent pellets and water *ad libitum* and were maintained on a 12 h:12 h light:dark cycle. Hypoxia was induced using a hypobaric chamber set at 380 Torr (0.5 atm), which is equivalent to 10% normobaric oxygen. Three female and three male mice were placed in the hypobaric chamber for 3 days, which was opened for 15–30 min each day for cage cleaning and food and water replenishment. The other six mice (3

female and 3 male) were kept under normal atmospheric conditions to act as normoxic controls. At the end of the experiment, mice were administered sodium pentobarbital and transcardially perfused with phosphate-buffered saline prior to collection of tissues. One liver lobe, skeletal muscle from the hind limb and one brain hemisphere from each animal were drop-fixed in methacarn for 2 days at 4°C and then transferred to 50% ethanol for histochemical analyses. In addition, one liver lobe, skeletal muscle from the hind limb and the other brain hemisphere were stored at –80°C for metal ion analyses. Prior to the start of the experiment, each mouse was numerically coded and only after the final collection of data were the results decoded.

Inductively coupled plasma-mass spectrometry (ICP-MS)

Liver, skeletal muscle and the cortical and hippocampal regions from one brain hemisphere were weighed and then dissolved in 1 ml of 70% metal grade nitric acid (Fisher Scientific, Pittsburgh, PA, USA) and incubated at 80°C in a water bath until the solution was clear. After cooling to room temperature, ~150 µl of 30% H₂O₂ was added; after the effervescence ceased, the samples were incubated at 70°C for 15 min and then cooled to room temperature. The final volume was brought to 10 ml with 1% metal-grade nitric acid. Samples were then analyzed for Fe, Zn, Cu, Ni and Mn content using inductively coupled plasma-mass spectrometry (ICP-MS; VG PlasmaQuad PQ2 Turbo Plus ICP-MS) at the Soil & Plant Analysis Laboratory, University of Wisconsin-Madison. Multi-element calibration standard solutions were prepared from single- and multi-element primary and/or in-house working standard solutions. Rhodium (Rh) was used as an internal reference standard. The metal ion contents of the buffer solutions were subtracted from the samples and the results expressed as microgram of metal ion per gram wet weight of tissue. Containers (bottles, vials, etc.) were soaked in 10% nitric acid overnight and rinsed with de-ionized water several times before use.

Histochemical analyses

Since the liver is relatively homogenous with a fairly consistent cyto-architecture, the liver sections were obtained from the middle of the lobe of liver and the regions of each section that were analyzed were randomly chosen, with all regions containing both hepatocytes and blood vessels. The brain cyto-architecture is not homogenous; therefore, distinct regions were chosen for analysis according to the Paxinos and Franklin mouse brain atlas.¹⁰ The brain was cut into sagittal

sections (Lat \pm 0.6–1.0 mm) to allow simultaneous viewing through numerous brain regions, defined as follows. Cerebral cortex (Ctx): three regions of grey matter in the parietal and frontal lobes (AP -3.0 mm to $+1.0$ mm). Hippocampus was divided into two regions based on the presence or absence of neuronal cell bodies: (i) hippocampal granular layer (Hipp. gran. layer) included the neuronal cell body layers of CA1, CA2 and the dentate gyrus; and (ii) hippocampal molecular layer (Hipp. mol. layer) included the oriens, stratum radiatum, and lacunosum moleculare layers and the molecular layer of the dentate gyrus. Cerebellum was analyzed in two regions: (i) the granular layer, made up primarily of granule cells, and including the outer layer of Purkinje cells; and (ii) the molecular layer, which contains relatively few neuronal cell bodies and is the main site of synaptic interactions between cerebellar interneurons and Purkinje cells. The granular and molecular layers were analyzed within the same cerebellar lobes, with regions selected from three lobes in both dorsal and ventral regions of the cerebellum.

Tissues were paraffin-embedded, sectioned at $7\ \mu\text{m}$ on a microtome, and collected onto charged microscope slides. The brain was cut into sagittal sections to allow simultaneous viewing through numerous brain regions. Prior to staining, sections were deparaffinized in xylene and rehydrated in descending ethanol concentrations to Tris-buffered saline (TBS; 50 mM Tris-HCl and 150 mM NaCl, pH 7.6). Serial sections were analyzed for ferric (Fe^{3+}) iron, redox activity, and oxidized proteins, lipids and nucleic acids.

Using a Zeiss Axiophot microscope (x20 objective; total area $1\ \text{mm}^2$) with a Zeiss Axiocam digital camera and Zeiss Image Analysis system (KS300), digital photomicrographs were taken and the intensity of the stain was determined. The light settings were kept constant between sections to ensure comparable analysis. The intensity of staining for each field was corrected for the background by subtracting the intensity of staining of an adjacent negative control section. For each mouse, on a single slide, three areas per region of analysis were quantitated and the values were averaged. Values were obtained from six normoxic mice and six hypoxic mice, and are expressed as mean \pm SEM. Statistical analysis was performed using one-way ANOVA and *post hoc* 2-tailed *t*-tests, with $\alpha = 0.05$.

Perl's stain

The presence of Fe^{3+} in tissue sections was detected using a modified Perl's stain.⁶ Briefly, sections were incubated in Perl's reagent, 7% potassium ferrocyanide in 3% HCl, for 2 h at 37°C , and enhanced by incubation in 0.75 mg/ml 3,3'-diaminobenzidine (DAB) and 0.015% H_2O_2 for 5 min. The Perl's reagent was omitted for negative control sections.

Metal-catalyzed redox activity

To determine the metal-catalyzed redox activity within tissues,⁷ sections were incubated with 0.75 mg/ml DAB and 3% H_2O_2 in Tris buffer (TB; 50 mM Tris-HCl, pH 7.6) for 45 min. Negative control sections received only TB. To verify that the staining observed was due to metal-catalyzed redox activity and not endogenous peroxidase activity, sections were pre-treated with 0.1 M deferoxamine (DFX) or 0.1 M diethylenetriaminepentaacetic acid (DTPA) for 19 h to chelate metal ions, and then the redox activity staining was performed.

Immunohistochemistry

Prior to immunohistochemical staining, endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 in methanol. To prevent non-specific binding, sections were blocked with 10% normal goat serum (NGS) in TBS for 30 min. Oxidative modifications were detected using antiserum to N^ϵ -(carboxymethyl)lysine (CML; 1:250),⁸ the lipid peroxidation adduct 4-hydroxynonenal pyrrole (HNE; Alexis, 1:250), and 8-hydroxyguanosine (8-OHG; Trevigen, 1:100). For 8-OHG staining, sections were pre-treated with proteinase K for 30 min prior to blocking. The primary antibodies were omitted for negative control sections. Immunostaining was developed by the peroxidase-antiperoxidase procedure⁹ using DAB (Dako, Glostrup, Denmark) as the co-substrate.

RESULTS

To compare the affect of hypoxia on metal ion metabolism in brain, liver and skeletal muscle, samples were analyzed for Fe, Zn, Cu, Ni and Mn content by ICP-MS. There was a significant 43% increase in the Fe content of the liver of hypoxic animals compared to normoxic animals, but there was no change in the Fe content of skeletal muscle, cerebral cortex or hippocampus (Fig. 1). The Fe content of liver in normoxic mice was 5–7-fold higher than skeletal muscle, cerebral cortex or hippocampus. Hypoxia did not induce any significant differences in the contents of Zn, Cu, Ni or Mn in any tissue examined (Fig. 1). The Zn content of liver in normoxic mice was approximately 3-fold higher than skeletal muscle, cerebral cortex or hippocampus. The Cu content of liver in normoxic mice was 7-fold higher than skeletal muscle and 2-fold higher than cerebral cortex or hippocampus. A similar trend was observed for Mn content, with the Mn content of liver in normoxic mice being approximately 15-fold higher than skeletal muscle and 4-fold higher than cerebral cortex or hippocampus. In contrast, the Ni content of the hippocampus of normoxic mice was approximately 2.5-fold higher than liver, skeletal muscle or cerebral cortex.

The hypoxia-induced increase in the Fe content of liver was confirmed using the Perl's stain (Fig. 2A).

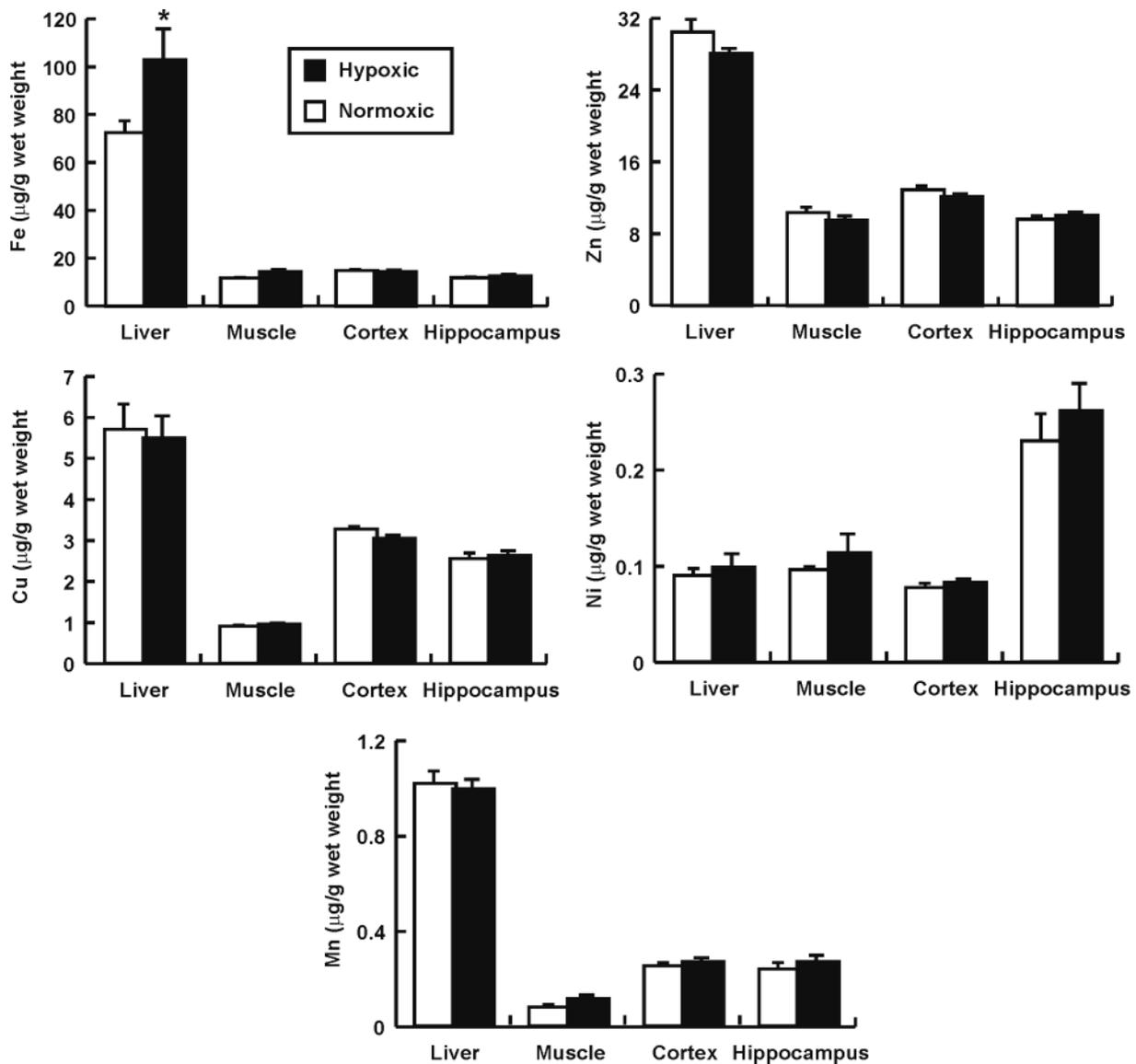


Fig. 1. Hypoxia induces iron accumulation in the liver. Liver, skeletal muscle, cerebral cortex and hippocampus were collected from normoxic and hypoxic mice and the contents of Fe, Zn, Cu, Ni and Mn were determined by ICP-MS. Results are expressed as $\mu\text{g/g}$ wet weight of tissue (mean \pm SEM, * $P < 0.001$, $n = 6$).

Exposure to mild hypoxia significantly increased the intensity of Fe^{3+} staining in the liver by 3.8-fold ($P = 0.001$; Figs 2A and 3A). The intensity of Fe^{3+} staining in the normoxic liver was 1.9–5.2-fold greater than the intensity of Fe^{3+} staining in the normoxic brain, depending on the brain region analyzed. Mild hypoxia increased the intensity of Fe^{3+} staining by 2.7-fold in the cerebral cortex, although this did not reach statistical significance ($P = 0.12$; Figs 2B and 3A). Similarly, in both the granular and molecular layers of the hippocampus, hypoxia tended to increase the intensity of Fe^{3+} staining, although this was not significantly different from normoxic mice ($P = 0.06$ and $P = 0.13$, respectively; Figs 2B and 3A). The intensity of Fe^{3+} staining in the granular and molec-

ular layers of the cerebellum was not altered by hypoxia, but it was lower than in the hippocampus ($P = 0.004$ and $P = 0.001$, respectively), but not the cerebral cortex ($P = 0.251$ and $P = 0.087$, respectively).

The metal-catalyzed redox activity within the tissue (see Figs 2 and 3B) was assessed by examining the ability of the tissue section to catalyze the H_2O_2 -dependent oxidation of DAB.⁷ Exposure to hypoxia increased the metal-catalyzed redox activity in the liver by 2-fold ($P = 0.007$; Figs 2A and 3B). In the cerebral cortex and the granular layer of the hippocampus, there was a small, but non-significant, increase in the metal-catalyzed redox activity of tissue following hypoxia ($P = 0.18$ and $P = 0.35$, respectively; Figs 2B and 3B). Pre-incubation

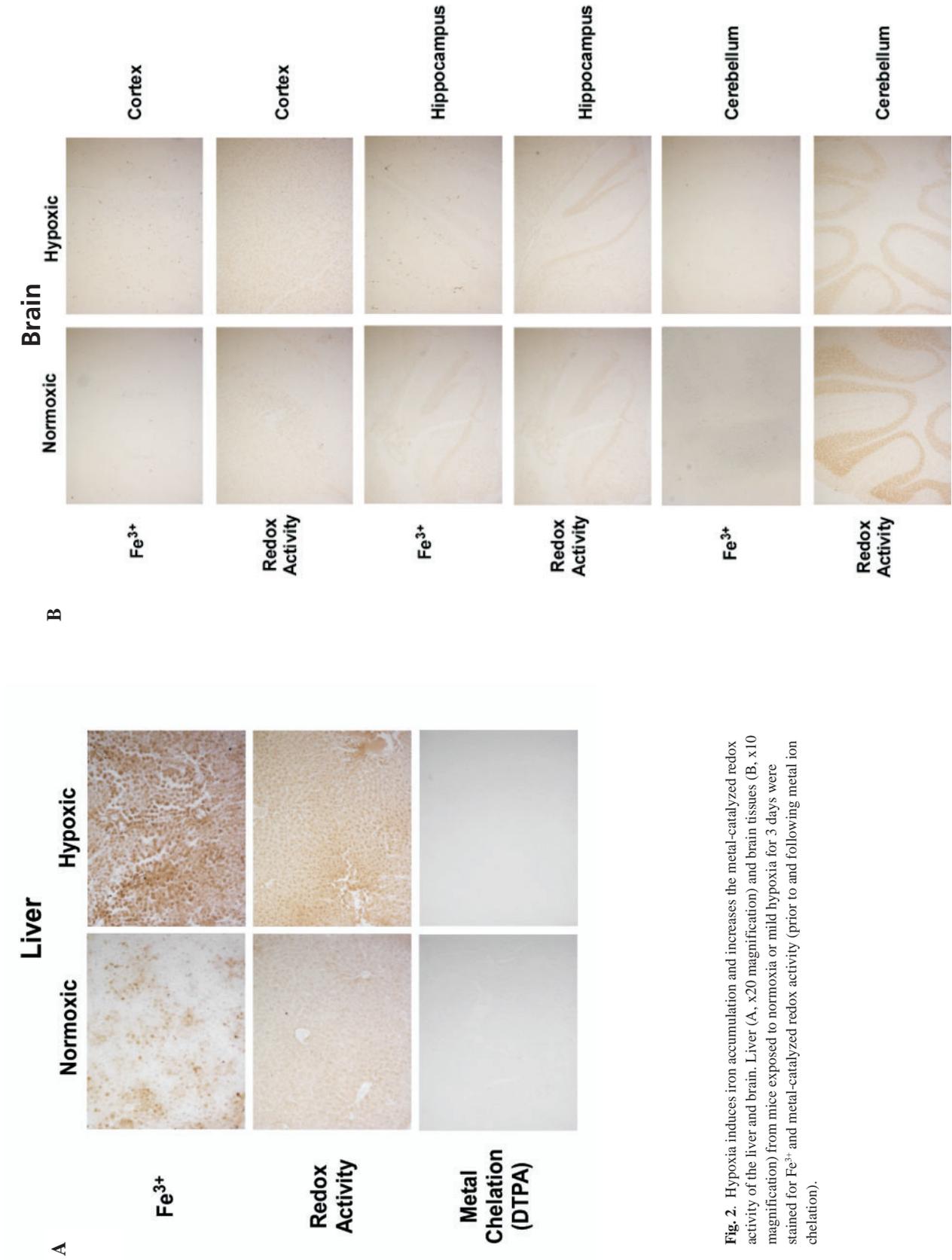


Fig. 2. Hypoxia induces iron accumulation and increases the metal-catalyzed redox activity of the liver and brain. Liver (A, x20 magnification) and brain tissues (B, x10 magnification) from mice exposed to normoxia or mild hypoxia for 3 days were stained for Fe³⁺ and metal-catalyzed redox activity (prior to and following metal ion chelation).

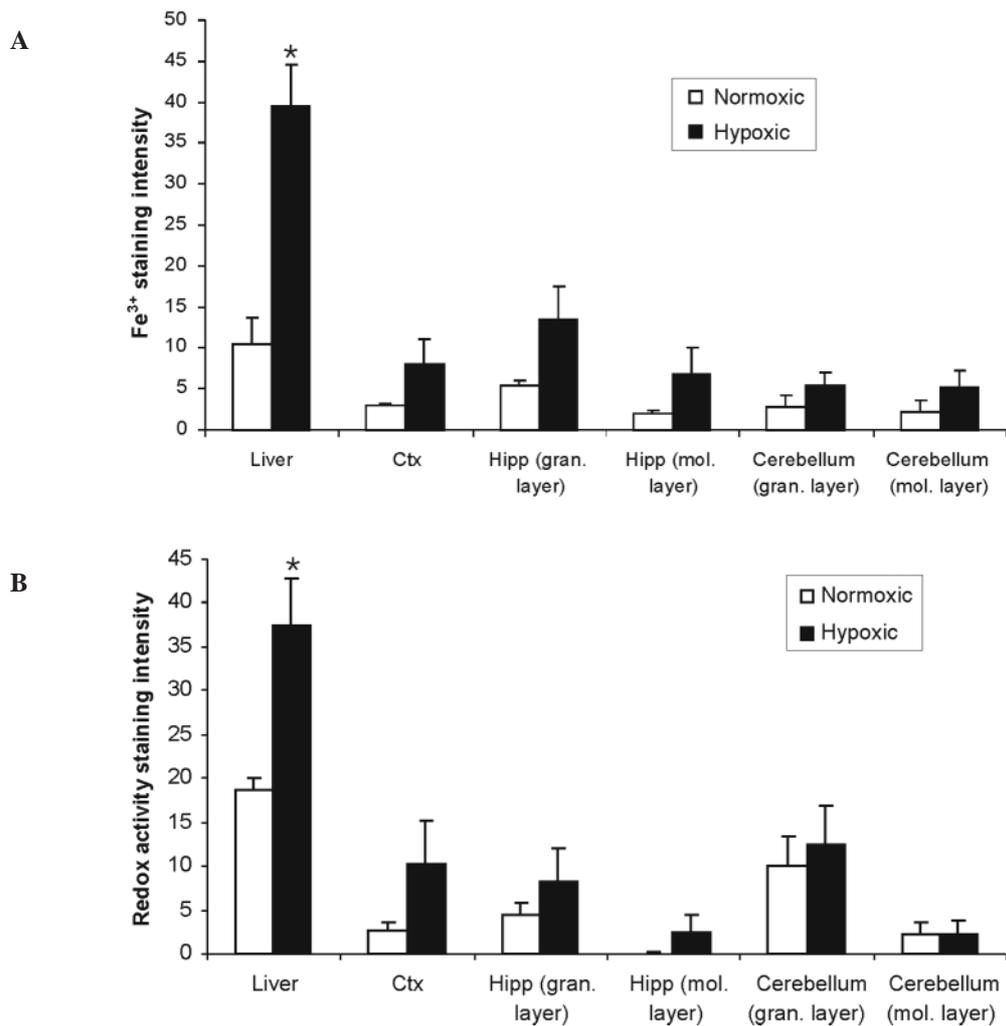


Fig. 3. Intensity of Fe³⁺ (A) and metal-catalyzed redox activity (B) staining in the liver and brains of mice exposed to normoxia or mild hypoxia for 3 days. * $P < 0.05$, compared to normoxic tissue.

of tissue sections (brain and liver) with DFX or DTPA completely abolished redox activity staining (Fig. 2A and data not shown), confirming that the staining was due to metal-catalyzed redox activity.

To determine whether there was a correlation between the intensity of Fe³⁺ staining and tissue metal-catalyzed redox activity, the data from all mice were combined (Fig. 4). Mice from both control and hypoxic groups were used since there was considerable variability in Fe³⁺ staining among mice. Regression analysis showed a positive correlation between Fe³⁺ intensity and tissue metal-catalyzed redox activity in the liver ($r^2 = 0.641$, $P = 0.0017$; Fig. 4A), indicating that the metal-catalyzed redox activity of the liver may be related to Fe³⁺ load. In contrast, there was no correlation observed between Fe³⁺ intensity and tissue metal-catalyzed redox activity in any brain region examined (Fig. 4B,C).

Markers of oxidative stress were examined immunohistochemically to detect oxidized proteins (CML), lipid

peroxidation products (HNE) and oxidized nucleic acids (8-OHG). The intensities of CML and HNE expression in the liver were lower than in any of the brain regions examined in this study ($P < 0.05$, paired samples t -tests; Fig. 5). Exposure to hypoxia did not alter the intensity of any of the oxidative stress markers in the liver. In the brain, however, hypoxia significantly decreased the intensity of HNE (Fig. 5B) staining in the cerebral cortex, the granular layers of the hippocampus and both the granular and molecular layers of the cerebellum by 59% ($P = 0.003$), 42% ($P = 0.028$), 68% ($P = 0.009$), and 66% ($P = 0.006$), respectively. However, the intensity of HNE staining was not significantly decreased in the molecular layers of the hippocampus ($P = 0.07$). The intensity of CML staining (Fig. 5A) was significantly decreased by hypoxia in both the granular and molecular layers of the hippocampus, and in the molecular layer of the cerebellum by 55% ($P = 0.024$), 62% ($P = 0.022$), and 56% ($P = 0.031$), respectively. However, the intensity of CML

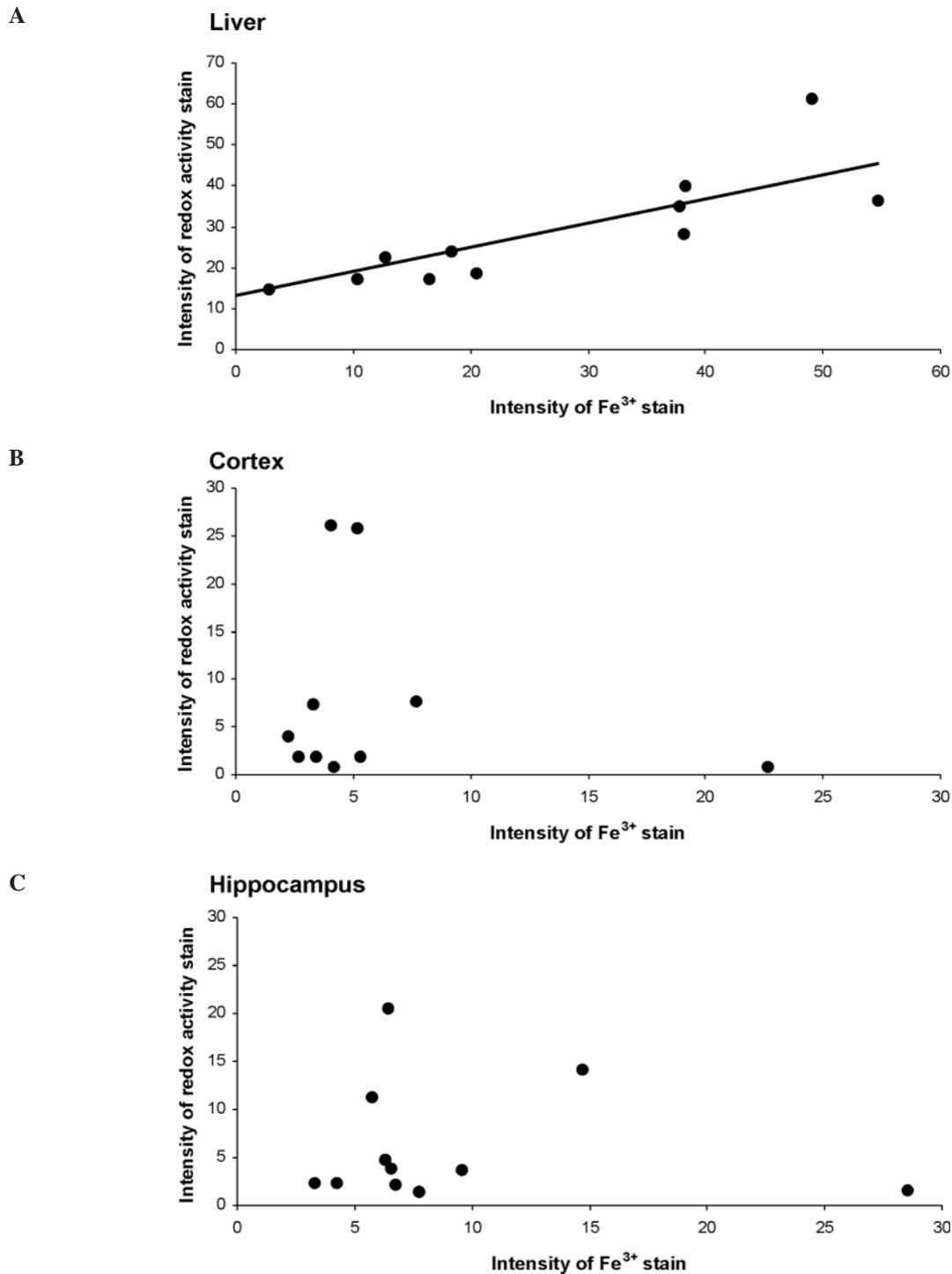


Fig. 4. Correlation between Fe^{3+} and tissue metal-catalyzed redox activity in the liver (A), cerebral cortex (B) and hippocampal granular layers (C). Data are pooled from all mice (both normoxic and hypoxic) combined. A significant correlation was observed in the liver, $r^2 = 0.641$ ($P < 0.05$).

staining was non-significantly decreased by hypoxia in the cerebral cortex ($P = 0.074$) and the granular layers of the cerebellum ($P = 0.059$). Hypoxia did not alter the expression of 8-OHG (Fig. 5C) in any of the brain regions examined.

DISCUSSION

Hypoxic-ischemic incidents are associated with oxidative damage to tissue that may be related to alterations in iron metabolism. While the blood-brain barrier partially

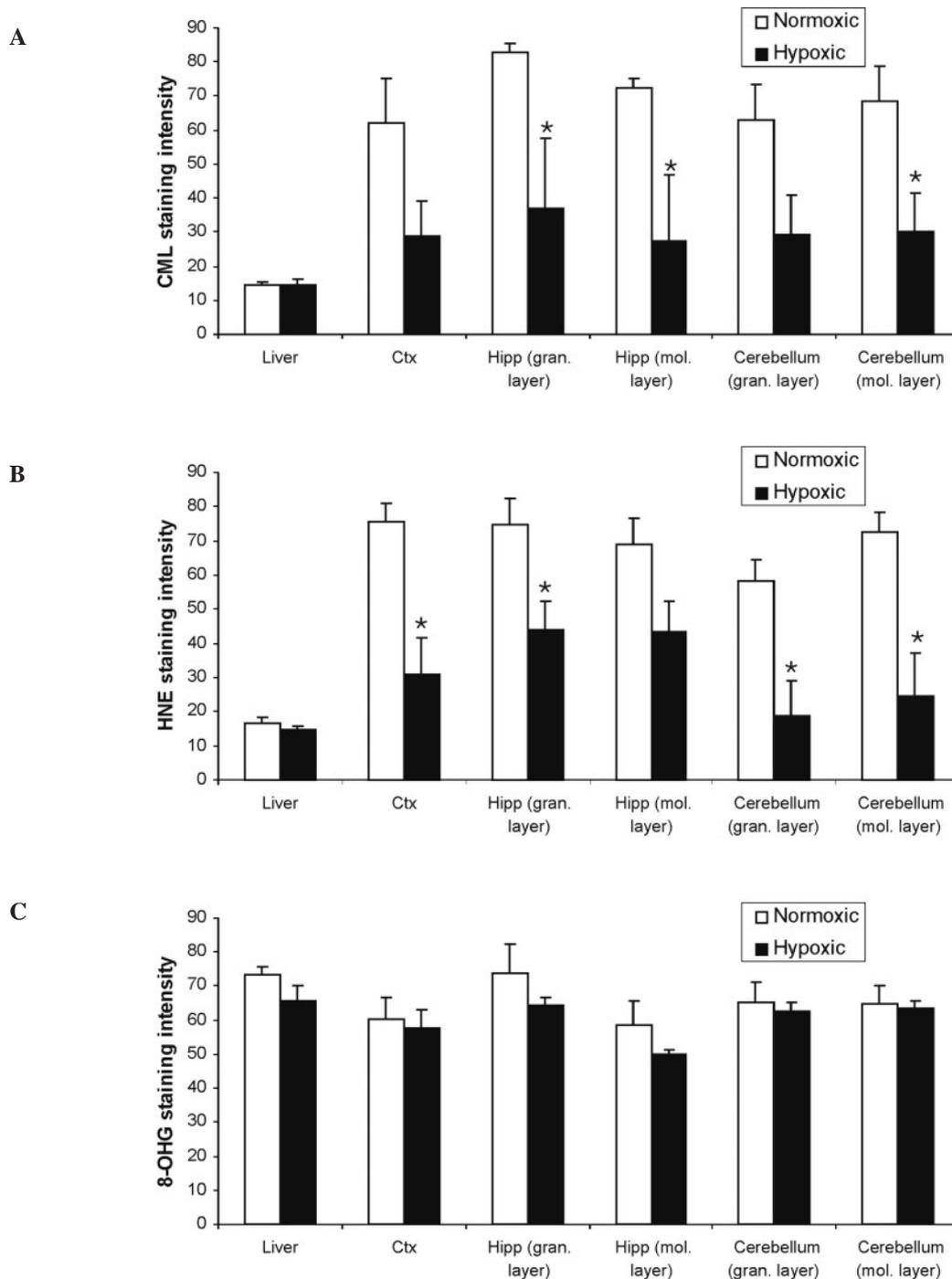


Fig. 5. Intensity of the oxidative stress markers CML (A), HNE (B), and 8-OHG (C) in the liver and brain following exposure to mild hypoxia. All measurements are relative within the oxidative marker measured. * $P < 0.05$, compared to normoxic tissue.

protects the brain from systemic fluctuations, it is particularly vulnerable to pro-oxidant stimuli. In this study, we have examined whether mild hypoxia induces changes in iron levels and metal-catalyzed redox activity in the liver and the brain, and the consequence of these changes on the expression of oxidative stress markers within the tissue. In addition to ICP-MS, the Perl's stain

was utilized to measure Fe^{3+} load since it allows for both the assessment of spatial and quantitative changes in iron metabolism.¹¹

When mice were chronically exposed to mild hypoxia, the Fe content of the liver increased substantially, supporting previous observations that hypoxia alters cellular iron metabolism. It is well established that hypoxia

increases intestinal iron absorption and the transfer of iron to the blood,¹²⁻¹⁵ presumably as a compensatory mechanism to improve blood oxygen-carrying capacity. Given the general need for a tight regulation of iron levels, the liver may remove some of this additional iron from the blood, thus increasing its own iron load. The increased Fe load in the liver (43%) as measured by ICP-MS is consistent with the finding that hypoxia increases the level of transferrin receptor in hepatocytes.¹⁶ Furthermore, the much greater intensity of Perl's staining (380%) in hypoxic liver may in fact be indicative of both an increase in Fe influx, as well as a redistribution of Fe away from ferritin since it is known that hypoxia decreases levels of ferritin.¹⁶ Fe released from the degradation of ferritin may be more available to react histochemically. Hypoxia has been shown to increase the iron content of liver in iron-supplemented mice,¹⁷ and the uptake of iron by more than 2-fold in cultures of myocardial cells from newborn rats.¹⁸ In contrast to the liver, the Fe³⁺ load in the brain was not significantly increased, suggesting that the brain may be more resistant to hypoxia-induced alterations in iron distribution. This could be related to the tight regulation of iron transport into or out of the brain by the blood-brain barrier.⁵ Alternatively, significant redistribution of brain iron might only occur following re-oxygenation since it has been reported that re-oxygenation increased the number of iron-containing microglial cells in the neonatal rat,¹⁹ and increased iron staining in neurons and blood vessels in a rat vessel-occlusion model.²⁰ Thus, mild hypoxia may not be sufficient to alter brain iron metabolism, while being sufficient to alter liver iron metabolism. While the brain is more sensitive to oxidative stress should there be a change in redox metal ion concentrations or distribution (Fig. 2), it is not more sensitive to changes in Fe concentration as indicated by our data. Thus the liver is more sensitive to hypoxia with regards to iron metabolism, although it appears to be less sensitive to oxidative stress (Fig. 5) than brain.

Hypoxia has been reported to promote the release of iron from intracellular stores in liver and kidney cells,²¹⁻²³ where it might form a pool of loosely bound Fe to low molecular weight molecules potentially available to promote redox reactions.²⁴ In the present study, we observed that hypoxia increased the metal-catalyzed redox activity within the liver. Since chelation of tissue metal ions with DFX or DTPA abolished the redox activity in liver tissue sections, we believe that we were assessing the metal-catalyzed redox activity of iron within these sections. This is further supported by a strong positive correlation between intensity of ferric iron staining and metal-catalyzed redox activity in the liver, regardless of whether the animal had been exposed to hypoxia or not. In contrast, mild hypoxia did not significantly increase the metal-catalyzed redox activity

within the brain, and there were no apparent correlations between iron load and metal-catalyzed redox activity in any of the brain regions examined. This further supports the idea that the brain and the liver have different homeostatic mechanisms regulating iron transport. However, it is possible that severe incidents of hypoxia may be sufficient to disrupt iron metabolism and subsequently promote an increased metal-catalyzed redox activity of affected brain tissue.

Oxidative markers against protein, lipids and nucleotides were used to assess the extent of damage to different macromolecules. Despite the increased iron load and metal-catalyzed redox activity of the liver, hypoxia did not change the expression of CML, HNE or 8-OHG. These findings support previous observations that hepatocytes exposed to hypoxia do not have corresponding lipid peroxidation.²¹ The lack of oxidative modifications may relate to a decreased rate of oxidative phosphorylation and generation of H₂O₂ in mitochondria as a result of decreased tissue oxygen due to the mild hypoxia. However, the increased iron load and metal-catalyzed redox activity of tissue illustrate the potential for the formation of reactive oxygen species following re-oxygenation. Thus, when hypoxic tissue is re-oxygenated, oxidative phosphorylation and H₂O₂ production will increase and oxidized iron could readily dismutate H₂O₂ leading to hydroxyl radical production and oxidative modifications. This scenario is supported by reports that HNE and 8-OHG expression are increased in the liver following ischemia-reperfusion injury.²⁵ A novel finding of this study was that mild hypoxia decreased the expression of CML and HNE in the brain, suggesting a reduced amount of oxidative modifications to proteins and lipids in the brain. Furthermore, the expression of 8-OHG in the brain after mild hypoxia was not altered, suggesting that oxidative modifications to nucleic acids in rat brain, as has been previously reported for both nuclear and mitochondrial DNA, requires severe hypoxia (only 4% oxygen).²⁶ Similar to that described above for the liver, ischemia-reperfusion also increased HNE and 8-OHG expression in rat brain.^{27,28}

CONCLUSIONS

Even under mild hypoxic conditions there is a major redistribution of tissue iron that could serve as a pro-oxidant source upon re-oxygenation, which may underlie the oxidative damage observed following reperfusion. The brain appears to be more resistant to hypoxia-induced changes in iron distribution and metal-catalyzed redox activity compared with the liver, suggesting that the blood-brain barrier may provide protection through the tight regulation of redox metal ion transport.

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