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Ironing-Out Mechanisms of Neuronal Injury under Hypoxic–Ischemic Conditions and Potential Role of Iron Chelators as Neuroprotective Agents

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ABSTRACT

Iron is the most abundant transition metal in the brain, where it functions as an important cofactor in a host of vital metabolic processes and plays an absolutely essential role in cell viability. Free iron is also very toxic when present in high concentrations, thus placing this essential metal at the core of neurotoxic injury in a number of neurological disorders. The pivotal role of iron in cellular homeostasis, including its latent toxicity, necessitates a tight regulation of iron metabolism. Oxygen and iron appear to play an important role in iron homeostasis. They appear to exert their homeostatic role by modulating the proteins involved in a complex interplay between iron sensing, transport, and storage. These key regulatory proteins include ferritin (intracellular storage), transferrin (extracellular transport), transferrin receptor, and iron regulatory protein (sensor of intracellular iron concentration). The interplay of iron and oxygen is most intriguing in the setting of stroke, where hypoxia and free iron appear to interact in causing the subsequent neuronal death. Antiox. Redox Signal. 2, 421–435.

INTRODUCTION

THE STAGE FOR OUR CURRENT INTEREST in iron research was set long before we had any histochemical demonstration of iron as an integral component of biological systems. Indeed, iron therapeutics were used for various physical ailments in ancient China, 4,000 years ago. (Beard *et al.*, 1993). The first quantitative documentation of whole brain iron dates back to the late 19th century, when Zaleski (1886) completed a quantitative analysis of iron in several organs from a single patient with idiopathic thrombocytopenic purpura (ITP) (Koeppen, 1995). Zaleski made several important pioneering observations: (i) brain iron is presently largely in ferric (Fe^{3+}) form; (ii) tissue iron exists bound to organic substances; and (iii) gray matter has a much higher iron concentration than white matter. In 1922, Spatz published the most frequently cited article on brain iron; his 128-page report contains detailed discussions on methods of iron histochemistry and established the extrapyramidal motor centers as iron rich hubs of the brain. In 1980, Nguyen-Legros (1980) significantly enhanced Perls's traditional iron reaction to detect iron in individual components of the central nervous system (CNS), including axons and myelin sheaths (see Koeppen, 1995, for detailed review of the authors mentioned). More powerful tools for elucidating the localization and function of iron have now become available, and our understanding of this ubiquitous

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and physiologically indispensable transitional metal has come a long way since Zaleski's original observations of brain iron in the patient with ITP. Many questions, however, remain unanswered.

Indeed, studies of the mechanisms of neurodegeneration over the past several decades have highlighted a fundamental paradox of brain iron. Despite being absolutely essential for cell viability, free iron is very toxic when present in high concentrations in or around cells. By engaging in redox cycles with cellular molecules that act in concert as electron donors, ferrous (Fe^{2+}) and, to a lesser extent, ferric (Fe^{3+}) ions catalyze free radical-generating reactions, placing this essential transitional metal at the core of neurotoxic injury in a host of neurological disorders.

IRON IN THE BRAIN

Iron is the most plentiful transition metal in the brain. It not only functions as a cofactor for many heme and non-heme enzymes involved in cellular energy metabolism, but it also plays an essential role in many other metabolic processes including DNA, RNA, and protein synthesis, the formation of myelin and the development of dendritic trees (Connor and Fine 1986; Youdim *et al.*, 1991; Klausner *et al.*, 1993; Gerlach *et al.*, 1994). In the brain, iron is most abundant in the basal ganglia (at a concentration being equivalent to that in the liver, the third most abundant source of iron in the body (Beard *et al.*, 1993a,b). Within the basal ganglia, the globus pallidus has the highest concentration of iron, followed by the zona reticulata of the substantia nigra, red nucleus, and putamen (Hill *et al.*, 1985; Morris *et al.*, 1992b; Hallgren and Sourander, 1958). White and gray matter are equally enriched with iron within the basal ganglia. Attempts to link the accumulation of iron in the basal ganglia to the γ-aminobutyric acid (GABA) and dopaminergic neurotransmitter systems have been controversial (Hill *et al.*, 1985; Morris *et al.*, 1992a; Hallgren and Sourander, 1958; Hu and Connor, 1996).

The cerebral hemispheres have much less stainable iron as compared to the basal ganglia. Here, the white matter, where staining occurs in patches of cells, is relatively enriched compared to the cortical gray matter. Iron is predominantly stored within oligodendrocytes, cells of the CNS that produce and maintain myelin. Indeed, iron-containing oligodendrocytes are found in satellite positions near large neurons where they regulate iron availability to neurons. These oligodendrocytes are also found associated with blood vessels (perivascular cells) where they monitor transport of iron across the blood–brain barrier (BBB). Neuronal iron staining is characteristically different from staining seen in oligodendrocytes. Neurons (pyramidal neurons of the cortex and the hippocampus) have small punctata of iron reaction in their somata that, interestingly, increase in size with age. Tanycytes that line the third ventricle also show high iron concentrations, indicating that they may be involved in iron transport between the cerebrospinal fluid and brain (see Connor, 1994).

The iron transport protein, transferrin (Tf), is also predominantly found in the oligodendrocytes, but additional locations include tanycytes, endothelial cells, choroid plexus, epithelial cells, and ependymal cells (Connor, 1994). It is notable that iron and Tf are not distributed identically in the brain because Tf is not the main storage form of iron. Ferritin (Fr), a multimeric protein that binds up to 4,500 atoms, is the major storage form of iron. Indeed, there is 10 times more ferritin than transferrin in the cerebral cortex. About one-third of brain iron is stored in ferritin in the ferric form (Fe^{3+}) (Hallgren and Sourander, 1958), which is also predominantly distributed in glial cells, particularly in microglia and oligodendrocytes (Morris *et al.*, 1992a). It is evident that oligodendrocytes play an important role in maintaining iron homeostasis in the brain. However, the presence of ferritin heavy chains (see below) in some neurons suggests that these cells may also be capable of rapid iron uptake (Connor, 1994). This is further supported with evidence of increased intraneuronal iron staining associated with aging (Beard *et al.*, 1993a,b). With increasing age related iron load, astrocytes and microglia develop an increased role in iron regulation.

IRON METABOLISM

The pivotal role of iron in cellular homeostasis, including latent toxicity of iron, necessitates a tight regulation of iron metabolism. This highly regulated process involves a complex interplay between specific iron sensors, transporter proteins, and storage proteins. These key regulatory proteins include ferritin (intracellular storage), transferrin (extracellular transport), transferrin receptor, and iron regulatory protein (sensors of intracellular iron concentration).

Intracellularly, Fe^{3+} is sequestered by the iron storage protein Fr. Fr sequesters Fe^{3+} an an inorganic complex (ferrihydrite) inside a protein shell composed of 24 subunits. Mammalian Fr are copolymers composed of two chains, heavy (H-) and light (L-), each with a molecular weight of 20,000. L-rich Fr of liver and spleen have been associated with longer-term iron storage and H-rich Fr of heart and brain are associated with with rapid iron sequestration and detoxification. The catalysis of oxidation is clearly associated with the presence of the H-chain "ferroxidase center" (Arosio *et al.*, 1994). The ferroxidase center promotes the aerobic oxidation of ferrous ion (Fe^{2+}) back to the more inert ferric ion (Fe^{3+}) (Arosio *et al.*, 1994). These two chains assemble in various proportions to produce tissue-specific isoferritin populations, which have different functional properties depending on the specific needs of the cells. The H-chain has a fast rate of iron uptake, incorporates 100–300 Fe atoms/molecule, and takes up iron even at pH <6.0, when spontaneous iron oxidation is minimized. The L-chain also incorporates iron rapidly and has a higher efficiency of iron mineralization than the H-chain, which relates to the permeability of the protein coat to iron (see Arosio, 1994 for details). The H- and L-chains of ferritin have a cooperative role in the uptake and release of iron. First, the H-chain catalyzes oxidation of Fe^{2+} into Fe^{3+}. Once iron oxidation is catalyzed, the L-chain converts the newly oxidized iron into iron-core nuclei (iron nucleation) (Beaumont *et al.*, 1994).

There are two principal mechanisms of iron oxidation inside the ferritin core (see Chasteen *et al.*, 1994 for details): (i) protein catalysis pathway when small increments of Fe^{2+} [<50/protein] are introduced:

$$2\ Fe^{2+} + O_2 = 4H_2O \rightarrow 2FeOOH\ (core) + H_2O_2 + 4H^+$$

In this pathway superoxide does not appear to be a product of dioxygen reduction, nor is it an effective oxidant of Fe in apoferritin compared with O_2

(ii) When higher increments of Fe^{2+} [>200/protein] are introduced, the protein catalysis pathway is kinetically saturated and oxidation of excess Fe^{2+} occurs on the developing mineral surface of the core:

$$4\ Fe^{2+} = O_2 + 6\ H_2O \rightarrow 4\ FeOOH\ core + 8\ H^+$$

(Note that under conditions of excess iron H_2O_2 is not generated; this will be of relevance when we review regulation of iron metabolism.)

Extracellularly, the iron transport relies on Tf, which tightly binds iron in Fe^{3+} form. Each molecule of Tf binds two ferric ions. In the physiological pH range, iron is insoluble in the ferric state and has a propensity to catalyze noxious oxidative reactions. Both problems are overcome by Tf, which binds Fe^{3+} reversibly, but with sufficient affinity to resist hydrolysis and to hold it in a ferric state at an adequately low reduction potential to keep it from participating in redox reactions hazardous to the cell (Aisen, 1994).

At the cell surface, iron-laden Tf interacts with the Tf receptor (TfR). The receptor is a disulfide-linked homodimer of a 95,000-kDa glycosylated subunit. The complex is then internalized by receptor-mediated endocytosis and clathrin-coated vesicles. An ATP-dependent protein pump acidifies the internal milieu of the endosome. At pH near 5.6, iron dissociates from Tf, and after reduction to the ferrous state (Fe^{2+}) is transported through the endosomal membrane into the cytoplasm (see Aisen, 1994 for details). There is evidence that this function is served by the "natural resistance-

associated macrophage protein 2" (Nramp 2) also known as the DCT1 (divalent cation transporter 1) (Fleming *et al.*, 1998). The Tf-bound receptor is returned to the cell surface, where at pH 7.6 the TfR is once again released into the extracellular compartment.

Iron-mediated iron regulation

Iron-dependent cellular iron homeostasis is regulated, in part, post-transcriptionally by modulating mRNA stability of iron regulatory proteins. The cooperative interaction of *trans*-acting proteins, iron regulatory proteins (IRP), with *cis*-acting RNA motifs, termed iron-responsive elements (IRE), coordinate the differential expression of specific regulatory proteins (Weiss *et al.*, 1994a). The IRE consists of a short untranslated 30-nucleotide sequence that forms a stable stem–loop binding site for the IRP (Hentze *et al.*, 1987, 1996). Fr, TfR, and erythroid 5-aminolevulinate synthase (e-ALAS), all contain IREs. These motifs are present at the 5′ untranslated regions of Fr and e-ALAS, where IRP binding represses mRNA translations or the 3′ untranslated region of the TfR mRNA, and where IRP binding leads to mRNA stabilization (Klausner *et al.*, 1993; Hentze *et al.*, 1996).

IRP1, and a second family member, IRP2, are believed to function as the sensors of intracellular iron level via their iron-sulfur clusters. IRP-1 (also reported as IBP, FRP, and IRe-BP), which has been purified from a variety of mammalian tissues and cells, thus appearing to have been conserved during evolution, has a molecular weight of 98 kD and maps to chromosome 9 (Walden *et al.*, 1989; Yu *et al.*, 1992; Hu and Connor, 1996). IRP1 is a dual-function protein. When the cytosolic low-molecular-weight iron pool is adequate, IRP-1 is converted from an RNA binding protein to its [4Fe-4S] cytosolic aconitase form (Kaptain *et al.*, 1991). The mitochondrial aconitase (m-aconitase) is involved in the isomerization of citrate to isocitrate so that the six-carbon unit can undergo oxidative decarboxylation in the Krebs cycle. The metabolic role of cytosolic aconitase (c-aconitase), however, is not clear. IRP2 (also reported as IRE-BP B2 and IRF_B), which has been purified from rat liver and cloned from human and rat cDNA libraries, has a molecular weight of 104 kD with an amino acid sequence 57% identical and 75% similar to that of IRP1 and maps to chromosome 15 (Guo *et al.*, 1994, 1995a,b; Hu and Connor, 1996).

There are several functional distinctions between IRP1 and IRP2. First, iron regulates IRP1 and IRP2 via different mechanisms. Iron regulates IRP1 by modulating its RNA-binding affinity, whereas IRP2 is regulated by iron-mediated proteolysis (Samaniego *et al.*, 1994; Guo *et al.*, 1994, 1995b; Henderson and Kuhn, 1995). Second, the IRP1 binds and regulates the stability of many more genes than IRP2, with high-affinity IRP2 binding restricted to ferritin IREs (Ke *et al.*, 1998). Third, unlike IRP1, IRP2 is not modulated by nitric oxide (Phillips *et al.*, 1996a). Fourth, the 4Fe-4S cluster of IRP2 is different from IRP1, suggesting that they may sense iron by different mechanisms (Phillips *et al.*, 1996b). Finally, oxygen appears to modulate these proteins differentially with hypoxic decrease in IRP1-RNA binding activity and increase in IRP2-RNA binding activity (Hanson *et al.*, 1999).

Under iron deprivation states, IRP-RNA binding is activated and specifically binds to the IREs resulting in repression of ferritin and e-ALAS mRNA translation and increased stability of TfR mRNA. (Klausner *et al.*, 1993; Hentz and Kuhn, 1996). The decreased expression of ferritin results in increased mobilization of iron from intracellular stores, while the increased TfR expression results in increased internalization of iron-bound extracellular transferrin; both of these mechanisms increase the intracellular pool of available iron.

Conversely, increased intracellular iron concentrations cause inactivation of IRP, resulting in enhanced ferritin and e-ALAS translation and increased degradation of TfR mRNA. The enzyme ALAS, which catalyzes the synthesis of the first intermediate in heme biosynthetic pathways, is also involved in iron traffic between the cytosol and mitochondria. When intracellular iron is elevated, IRP-increased expression of ALAS shuttles iron out of the cytoplasm and into the mitochondria. Conversely, under conditions of iron deprivation, this enzyme is downregulated and cytosolic iron remains in the cytosol (see Fig. 1a,b). Although this process is of clear significance in differentiated erythroid tissue leading to regu-

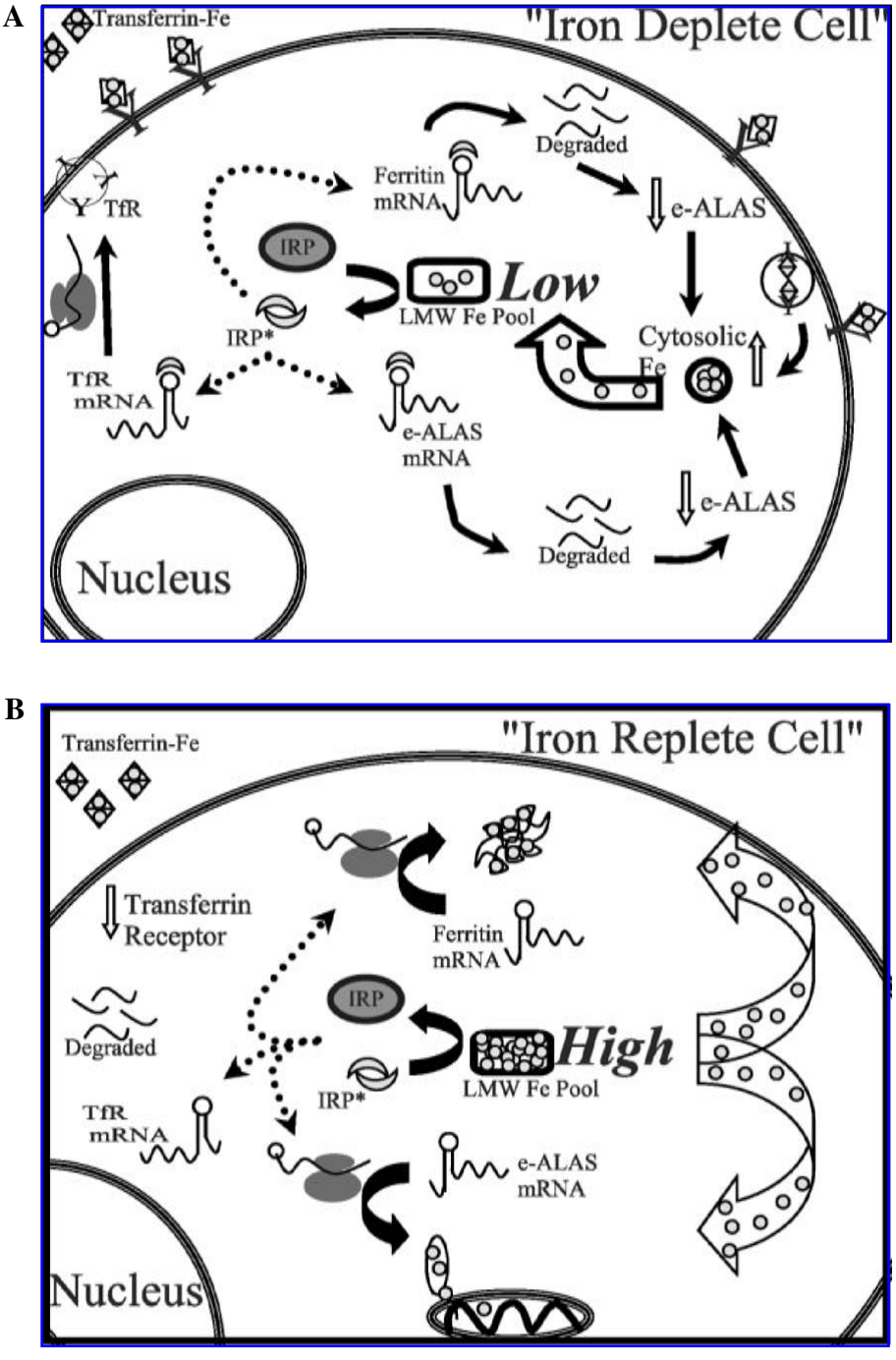

FIG. 1. **A. Iron Deplete Cell.** In this cell the LMW Fe Pool is low. This leads to the activation of IRPs. The activated IRPs bind to IREs in the 5′ end of ferritin and e-ALAS, resulting in destabilization of these two mRNAs and decreased Ferritin and e-ALAS protein synthesis. Decreased ferritin results in decreased intracellular iron sequestration and increased free iron for the LMW Fe pool. e-ALAS protein is also decreased, resulting in decreased shuttle of iron out of the cytoplasm into the mitochondria. Again, resulting in increased cytoplasmic iron for the LMW Fe pool. Finally, IRPs bind to the IREs in the 3′ end of the transferrin mRNA, resulting in increased mRNA stabilization and protein synthesis. The synthesized transferrin receptor is shuttled to the cell surface, where it binds extracellular iron laden transferrin and transports it intracellularly. This iron is also available to the LMW Fe pool. **B. Iron Replete Cell.** In this cell the LMW Fe pool is high. This leads to inactivation of IRPs. As a result there is no interaction between IRPs and mRNA IREs. Ferritin and e-ALAS mRNAs are not destabilized and these two mRNAs are translated. Ferritin and e-ALAs protein concentrations increase. Increased ferritin results in increased intracellular iron sequestration and decreased free iron for the LMW Fe pool. Increased e-ALAS protein results in increased shuttle of iron out of the cytoplasm into the mitochondria, again, resulting in decreased cytoplasmic iron for the LMW Fe pool. Conversely, without IRP binding the transferrin mRNA is destabilized, resulting in decreased protein synthesis. This leads to decreased internalization of extracellular iron.

lation of erythroid protoporphyrin biosynthesis, it also has potential implications for iron regulation in other systems not involved in heme biosynthetic pathways. In such systems, ALAS would mainly function to shuttle iron between the mitochondria and the cytosol, depending on the relative iron concentrations and the basic metabolic needs of the cell.

Hypoxia-mediated iron regulation

Cellular hypoxia is an important component of several pathophysiological conditions, including those related to ischemia. One of the adaptive responses employed by hypoxic mammalian cells is the regulated and specific alteration in gene expression imposed by hypoxia-induced transcriptional activation. Hypoxia-inducible factor-1 (HIF-1) is the most thoroughly characterized hypoxia-activated transcriptional regulator of gene expression. HIF-1 is a heterodimeric transcription factor expressed in all tissues in response to hypoxia (Maxwell *et al.*, 1993; Wang and Semenza, 1993a,b). It is composed of a 120-kD α-subunit and a 90- to 94-kD β-subunit (Semenza and Wang, 1992). Each subunit contains a PAS (PER-ARNT-SIM) domain that is required for heterodimerization of HIF-1α and HIF-1β subunits (Wang and Semenza, 1993a,b). Although there is significant basal expression of HIF-1β under normoxic conditions in most cell types, HIF-1α levels are low due to its rapid degradation (Semanza, 1998). The link between hypoxia and iron has been strengthened with the evidence of hypoxic regulation of proteins that regulate iron homeostasis.

Indeed, all the major genes of iron metabolism respond to hypoxia. First, it has been shown that hypoxia or $CoCl_2$ (which mimics hypoxia), results in a three-fold increase in TfR mRNA, despite a decrease of IRP1 activity (Tacchini *et al.*, 1999). Demonstration of a HIF-1 binding site in the TfR promoter suggests that the observed increase in TfR mRNA results from hypoxia-induced stabilization of HIF-1 and increased transcription of the TfR gene rather than a change in TfR mRNA stability (Tacchini *et al.*, 1999). Second, serum transferrin (Tf) levels increase in animals exposed to hypoxia (Simpson, 1996) and hypoxia increases Tf gene expression in hepatoma cells (Rolfs *et al.*, 1997). Of note, Tf is also a member of the HIF-1-regulated gene family (Bolann and Ulvik, 1987). Finally, as noted above, the activities of the RNA-binding proteins, IRP1 and IRP2 are also regulated by hypoxia. Whereas hypoxia exposure decreases IRP1-RNA binding activity, IRP2-RNA binding activity increases with hypoxia. The hypoxic increase in IRP2-RNA binding results from increased IRP2 protein levels (Hanson *et al.*, 1999). Unlike IRP1, IRP2 does not have a detectable aconitase activity. Hypoxia stabilizes the [4Fe-4S] aconitase active form of IRP1 at the expense of its RNA binding activity (Hanson and Liebold, 1998). Indeed, it appears that, with respect to hypoxic regulation of iron metabolism, IRP2 plays a more critical role than IRP1 (Hanson *et al.*, 1999) (see Fig. 2a,b).

How would hypoxia regulate iron metabolism? One proposed mechanism of hypoxic regulation of iron metabolism is via hydrogen peroxide (H_2O_2), possibly from a heme-containing oxygen sensor that acts as an IRP2 degradation signal (Hanson *et al.*, 1999). A decrease in peroxide output from such a sensor during hypoxia would thus act to stabilize IRP2. The use of oxygen-derived free radicals (in particular H_2O_2) in the regulation of hypoxic responses appears to be a general mechanism for regulating the stability of proteins that mediate hypoxic adaptation. For example, hypoxia-induced stabilization of HIF-1α protein is blocked in the presence of H_2O_2 (Fandrey *et al.*, 1994), suggesting that hypoxia-induced changes in the level of this reactive oxygen species may be involved in HIF-1 activation (Huang *et al.*, 1997; Hanson *et al.*, 1999). HIF-1α subunits are targeted for rapid degradation in normoxic cells by a proteosomal mechanism operating on an internal oxygen-dependent degradation domain (ODD) (Huang *et al.*, 1998). Under hypoxic conditions, IRP2 stability would depend on the relative cytosolic concentrations of both Fe^{2+} and H_2O_2. That is, the Fe^{2+} concentration may be high enough to inactivate the RNA binding activity of IRP1 and convert it to its aconitase form, but because of the simultaneous decrease in H_2O_2 (remember that also under conditions of Fe excess, H_2O_2 is not generated), the concentration may not be sufficient to signal the degradation of IRP2.

Data suggest that pVHL (Von Hippel-Lindau tumor suppressor gene product) might be re-

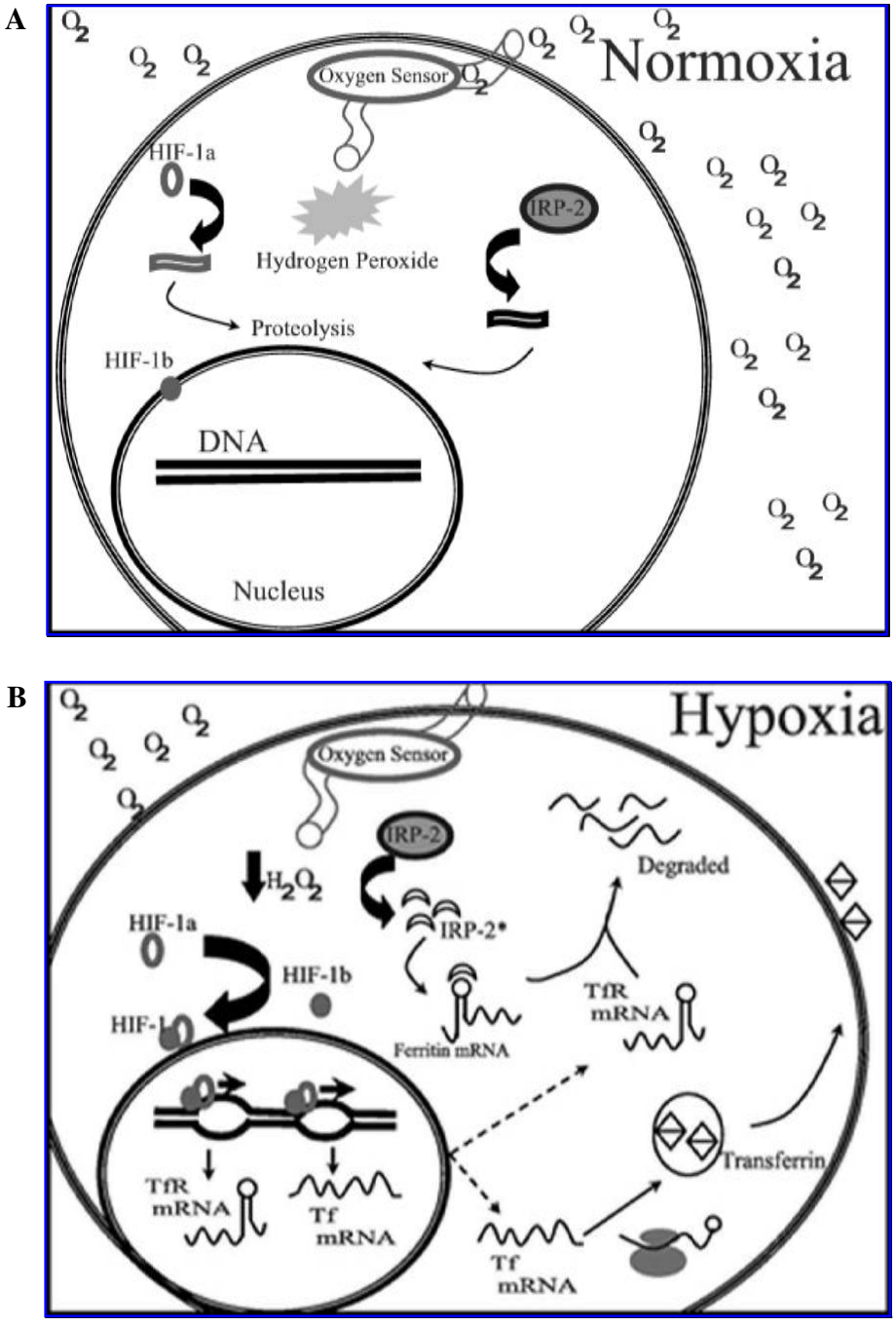

FIG. 2. **A. Normoxic Cell.** This cell is well oxygenated, and the oxygen sensor is saturated. This may lead to generation of H_2O_2, which is known to facilitate degradation of HIF-1α and IRP-2. **B. Hypoxic Cell.** This cell is hypoxic as signaled by the oxygen sensor, again possibly by a decrease in H_2O_2 concentration. HIF-1α is stabilized and after heterodimerizing with HIF-1β, HIF-1 translocates into the nucleus, where it binds to the hypoxia response element (HRE) upstream of a multitude of genes, including TfR and Tf. This leads to increased transcription of TfR and Tf mRNAs. The Tf mRNA is translated and transferrin I transported extracellularly, where is can bind available iron and remain poised to re-enter the cell via the receptor when the cell is ready. It is not clear if Tf plays an alternative role intracellularly. The TfR mRNA, however, is not stable without activated IRP and is subsequently degraded. Like HIF-1α, IRP2 is also stabilized and activated. IRP2 is specific to ferritin and binds to the 5′ end of the ferritin mRNA. As in the case of the "Iron Deplete" cell, this results in destability of the ferritin mRNA and decreased ferritin synthesis.

quired for oxygen-dependent HIF-1 degradation. The ability of pVHL to degrade HIF-1 appears to be dependent on iron. Treatment with iron chelators prevented the association of pVHL with HIF-1, suggesting that iron may be necessary for the interaction of pVHL with HIF-1 during immunoprecipitation studies (Maxwell *et al.*, 1999).

Leibold and co-workers (Hanson *et al.*, 1999) have suggested four provocative parallels between hypoxic regulation of HIF-1α and IRP2: (i) Both proteins accumulate during hypoxia by post-translational mechanisms involving increased protein stability; (ii) $CoCl_2$ stimulates the accumulation of both IP2 and HIF-1α, perhaps by inactivating a heme-containing O_2 sensor; (iii) iron chelators elevated the protein levels of both IRP2 and HIF-1α; (iv) the activation of IRP2 and HIF-1α by hypoxia and $CoCl_2$ occurs in all cell types, suggesting a global mechanism.

Clearly, the similarities between iron and oxygen metabolism suggest that iron and oxygen regulate overlapping cellular activities. Why would such apparently different stimuli elicit these common downstream pathways? Both iron depletion and hypoxia ultimately compromise cellular ATP generation by curtailing oxidative phosphorylation. In the iron-depleted cell, oxidative phosphorylation is arrested because this process depends on many enzymes containing iron–sulfur clusters. In the hypoxic cell oxidative phosphorylation is arrested due to substrate (oxygen) deficiency. It appears that the common cellular responses to iron depletion and oxygen depletion may be ways for a cell to compensate for its ATP-deprived status. In the case of iron depletion, the cell compensates in two ways. First, to restore the free iron available for essential cellular processes, the cell tries to increase its iron uptake and decrease its iron storage. Second, while the intracellular iron is being replenished, the cell tries to find other means of generating ATP. To this end, iron-depleted cells upregulate glycolytic enzymes and glucose transporters via a HIF-1-dependent pathway. Similarly, in the case of hypoxia, the cell compensates for ATP-depletion by increasing glycolysis. Here, the hypoxic stimulus causes the stabilization of HIF-1α, ultimately resulting in transcriptional upregulation of glycolytic enzymes and glucose transporters. Augmenting ATP generation may be one important mechanism by which iron chelators could prevent neuronal injury during hypoxic-ischemic insults.

IRON IN STROKE

Cerebral ischemia produces inadequate delivery of oxygen and substrate to brain tissue, resulting in energy failure and a complex cascade of events that may quickly lead to cell death. One of these events involves the liberation of iron from its cytoplasmic storage protein, ferritin, via reduction to the ferrous form. Iron-bound ferritin is reduced to its ferrous form and released into the cytoplasm where it can participate in deleterious redox reactions. In the context of ischemia, reduction, and release of stored iron is stimulated by acidosis (Qi *et al.*, 1995, Dorrepaal *et al.*, 1996), reactive oxygen species (*e.g.*, superoxide) (Biemond *et al.*, 1984; Bolann and Ulvik, 1987), and reactive nitrogen species (*e.g.*, nitric oxide) (Reif *et al.*, 1990). Moreover, ischemia-induced proteolysis also releases iron from storage proteins (Rothman, 1992). Finally, the enzyme heme oxygenase can liberate nonprotein-bound iron by catabolizing heme. Of note, when heme-containing iron gains access to the extracellular space (as a result of cell lysis), it is taken up by viable cells, a process that is enhanced by elevated intracellular calcium as well as, paradoxically, by increased intracellular iron (Kaplan *et al.*, 1991). Once liberated, ionic iron is then believed to catalyze the production of oxygen-derived free radicals by Fenton chemistry.

Experimental studies have shown that substantial amounts of chelatable iron are released from storage during hypoxia and ischemia (Bralet *et al.*, 1992). This chelatable iron has been attributed to the pools of non-protein-bound iron (also referred to as low-molecular-weight, LMW, iron), which is generated during ischemic insult. The levels of total and LWM iron increase in a region-specific manner during normoglycemic ischemia. Elevated LMW iron has been observed in ischemic cortical and subcortical brain regions, most prominently in the cerebellum, midbrain, hippocampus, and cortical gray matter.

Immunohistochemical examination of ischemic tissue in animal models of stroke, has

established temporal alterations in the distribution of iron metabolism-related proteins. In a four-vessel occlusion model of prolonged ischemia in the rat, iron deposits accompanied by enhanced Fr (intracellular iron storage) levels were demonstrated in the CA1 region at chronic stages (weeks 4–24) (Kondo *et al.*, 1995). Temporal alterations in iron staining during acute phases of ischemia were also evaluated in a bilateral occlusion model (Ishimaru *et al.*, 1996). In these studies there was an accumulation of Tf (extracellular iron transport) immunoreactivities that appeared in atrophied pyramidal neurons on day 4. Interestingly, this was associated with a slight decrease in Tf staining in the oligodendrocytes, which was subsequently restored on day 14. Prior to ischemia, all the neurons and glia were Fr positive, but on day 4 post ischemia, neurons lost their Fr staining. On day 7, Fr-positive glial cells became localized in the pyramidal layer and persisted to day 14. These observations suggest that 4 days after ischemia there is an increase of intraneuronal iron release, as revealed by the decreased Fr and increased Tf staining. With increasing neuronal death and subsequent cell lysis, there is an extracellular accumulation of iron, leading to increased uptake by the glial cells, thus explaining the increased Fr staining on day 7 in the reactive astrocytes and on day 4 in active phagocytes. In other studies, increased iron staining was first detected within the cytoplasm of neurons with pyknotic nuclei at 4 hr of recovery. Staining increased rapidly over the first 24 hr in regions of ischemic injury. By 7 days recovery, reactive glia and cortical blood vessels were also stained. Increased staining in gray matter persisted at 3 weeks of recovery, whereas myelinated, white matter tracks had fewer iron-positive cells compared to normal. (Plamer *et al.*, 1999).

After asphyxia in newborn infants and experimental animals, there is an increase of free iron in the plasma (van Bel *et al.*, 1994; Dorrepaal *et al.*, 1996; Shadid *et al.*, 1998). Leakage of plasma free iron into brain across a damaged BBB could occur and would be particularly damaging. In this scenario, iron would be directly taken up by cells in a manner that is independent of Tf, and the "free" iron would be quite redox active (Kaplan *et al.*, 1991). Such leakage of iron into the brain from the plasma would be unlikely to occur in focal insults. Interestingly, it has also been demonstrated that serum Fr levels are significantly higher in patients with a poor outcome in the setting of acute stroke and that they correlate with the degree of worsening of the neurological deficit (Davalos *et al.*, 1994). Thus while changes are observed in free and bound iron, both intracellularly and extracellularly, these observations do not constitute direct evidence for iron-mediated injury. It is possible that iron accumulation is just an epiphenomenona; an inevitable consequence of cellular death and proteolysis.

Mechanisms of iron-mediated injury during ischemia

1. Acidosis can enhance iron delocalization from ferritin and transferrin (Aisen, 1994; Li and Siesjo, 1997; Lipscome *et al.*, 1998). Acidosis shifts the equilibrium to the left, thus displacing iron from the HCO_3-TF complex.

$$Fe^{3+} + HCO_3^- + H_3TFn \Leftrightarrow FeTFnHCO_3^- + 3H^+$$

This is further enhanced because HCO_3^- is attacked by hydrogen ions during ischemia to yield CO_2 and H_2O. It has been proposed that lactic acidosis (as in hyperglycemic ischemia) increases iron dissociation from carrier proteins that use carbonate binding, whereas carbonic acidosis (from tissue CO_2) acts to stabilize bicarbonate ion and carbonate-bound iron (Rehncrona *et al.*, 1989). Increased total or free iron, however, has not been uniformly observed in animals treated with hyperglycemic ischemia (Lispcome *et al.*, 1998).

How could free iron liberated by acidosis then catalyze the generation of free radicals? It is widely held that free iron is a critical catalyst in converting hydrogen peroxide to hydroxyl radicals in a sequence of reactions known as the Haber-Weiss Reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+}OH + OH^- \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$$

Ferryl ions and $^{\cdot}OH$ are potent initiators of lipid peroxidation. That these reactions actually occur *in situ* has been supported by *in vitro* and *in vivo* experiments that have demonstrated that

acidosis can enhance the production of free radicals, as demonstrated by increased lipid peroxidation in hyperglycemic ischemia or more extensive damage when the pH (extracellular) was reduced to 6.6. Interestingly, cell damage was less extensive in the absence of oxygen, suggesting that free radicals play an important role in hyperglycemia-related damage only when some oxygen is supplied during ischemia or when it is resupplied during recirculation. Several studies have established that iron-catalyzed lipid peroxidation may be important in the pathophysiology of ischemic brain injury (see Hall, 1997 for detailed review).

2. Iron can catalyze tyrosine nitration by peroxynitrite. Iron catalysis of tyrosine nitration by peroxynitrite may also be an important mechanism and does not depend on the prior reduction of iron to the ferrous state (Beckman *et al.*, 1992). Peroxynitrite ($ONOO^-$) results from the interaction between $N^{\cdot}O$ and $^{\cdot}O_2^-$. In addition to direct damage caused by peroxynitrite, it can also protonate and decompose to $^{\cdot}OH$, leading to further injury. These steps are summarized in the following reactions.

$$N^{\cdot}O + {}^{\cdot}O_2^- \rightarrow ONOO^-$$
$$ONOO^- + H^+ \rightarrow ONOOH$$
$$ONOOH \rightarrow {}^{\cdot}OH + NO_2$$

Many biological effects of NO can be explained by its interaction with iron. It has been demonstrated that the loss of activity of the critical Krebs cycle enzyme aconitase in activated macrophages is a result of the formation of iron–nitrosyl complexes induced by the synthesis of NO, which interacts with the iron–sulfur clusters of the enzyme. It has also been shown that ferritin iron is also a target of NO, and that the activation of soluble guanyl cyclase by NO appears to be achieved by nitrosylation of the heme iron of the enzyme (see Weiss *et al.*, 1994a,b for detailed review).

Iron also regulates NO synthase (NOS) gene expression (Weiss *et al.*, 1994a,b). Increased intracellular iron levels result in a decrease of NOS activity, whereas depletion of intracellular iron strongly enhances the enzyme activity in interferon-γ/lipopolysaccharide (IFN-γ/LPS)-stimulated cells. Altered NOS mRNA levels rather than direct interference of Fe^{3+} and desferrioxamine with the NOS protein cause these differences in enzyme activity. Regulatory effects of Fe^{3+} and desferrioxamine are primarily caused by influencing nuclear gene transcription for inducible NOS. (iNOS) (Weiss *et al.*, 1994a,b).

3. Iron can catalyze free radical damage to DNA. It has also been speculated that iron-catalyzed free radical damage to DNA is responsible for some manifestations of ischemic cell injury (Krause *et al.*, 1988). Breaks in DNA are concentration-dependent and only occur with very high intracellular iron concentrations.

These mechanisms are reviewed in detail by Li and Siesjo (1997).

IRON CHELATORS

The significance of "free" iron in the pathology of ischemic injury is largely dependent upon whether pharmacological treatment by iron chelators can exert beneficial effects in experimental models of ischemia and oxidative stress (Kompala *et al.*, 1986; Cerchiari *et al.*, 1987; Ikeda *et al.*, 1989; Panter *et al.*, 1992; Rosenthal *et al.*, 1992; Rothman 1992; Oubidar *et al.*, 1994; Zaman *et al.*, 1999). It has been proposed that a small "chelatable" or "transit" iron pool exists in the cytosol, and that this pool is bound to low-molecluar-weight chelates such as nucleotides, organic acids, glycine, and cysteine (Bakkeren *et al.*, 1985; Mulligan *et al.*, 1986; Weaver and Pollack, 1989). Several studies have shown an increase in this LMW iron pool in the cerebral cortex under ischemic conditions (Krause *et al.*, 1985; Nayini *et al.*, 1985; Komara *et al.*, 1986; Bralet *et al.*, 1992). The level of LMW iron isolated from rat cerebral cortex averaged 0.2 μg/g, an amount equivalent to 1% of total tissue iron (21 μg/g; Bralet *et al.*, 1992); this amount was significantly enhanced with ischemia-associated acidosis (Oubidar *et al.*, 1994). The increase in LMW iron presumably reflects iron mobilization from ferritin, a process that requires reduction of ferric ions by reductants (*e.g.*, reduced flavins, ascorbate, cysteine, or superoxid anion), or alternatively proteolytic degradation of ferritin (Thomas and Aust, 1986; Thomas *et al.*, 1986; Monteiro *et al.*, 1989). In addition to chelating LMW iron, iron chelators can also mobilize iron from ferritin (Crichton *et al.*, 1980). A number of studies have shown that deferroxamine (DFO),

a high affinity chelator of Fe^{3+}, alone (Badylak and Babbas, 1986; Kompala *et al.*, 1986; Ikeda *et al.*, 1989) or in combination with superoxide dismutase (SOD) (Cerchiari *et al.*, 1987) improved survival and physiological function in various models of cerebral ischemia. By contrast, other studies failed to demonstrate cerebral protection by iron chelators (Cerchiari *et al.*, 1987; Fleischer *et al.*, 1987).

The efficacy of iron chelators depends on their ability to penetrate to subcellular compartments and cellular membranes where iron-dependent free radicals are generated. An untested assumption is that DFO is able to penetrate the BBB and the plasma membrane of neurons *in vivo*. Indeed, some studies have reported that DFO is unable to penetrate cells by passive diffusion (Rice-Evans *et al.*, 1989; Lloyd *et al.*, 1991). In addition, DFO has a short plasma half-life and its brain penetration seems to be limited. Indeed, after intravenous (i.v.) injection of 300 mg/kg in nephrectomized dogs, only traces of DFO were found in the cerebral spinal fluid (CSF) (Peters *et al.*, 1966). Plasma half-lives of DFO have been prolonged by coupling this chelator to polymeric carbohydrates such as dextran (Panter *et al.*, 1992) and hydroxyethyl starch (Rosenthal *et al.*, 1992). These DFO preparations have been found to improve clinical outcomes following head injury or cardiac arrest (Panter *et al.*, 1992; Rosenthal *et al.*, 1992). Because such polymers are unlikely to enter the brain, their efficacy supports an interavascular site of action. However, ischemia-induced alteration of the BBB may facilitate penetration of HES-DFO into the CNS, especially in models characterized by an abrupt and massive breakdown of the BBB such as traumatic brain injury. While the aformentioned lines of investigation support the salutary effects of DFO in cerebral injury, possibly through its ability to chelate redox active iron, several other potential protective models of action have been proposed.

Mechanisms of protection by deferroxamine

1. DFO inhibits iron-dependent free radical reactions. Free redox active iron is believed to be involved in mediating ischemic tissue injury by a variety of mechanisms, as already discussed. The Fe^{3+}-DFO complex (ferroxamine), unlike ferritin-bound Fe^{3+}, cannot by reduced at a significant rate to the Fe^{2+} form by free radicals, thus rendering DFO-bound Fe^{3+} unavailable to exacerbate oxidative damage. There are at least two problems with this putative mechanism. First, concentrations of DFO used to achieve protection in these studies far exceeds that required for chelation of "free iron." The concentration of "free iron" available in biological fluids, even under conditions of intense oxidant stress, are rarely greater than 5 μM; given the 1:1 stoichiometry of DFO to iron, concentrations of <10 μM of DFO should be adequate to chelate all the free iron. In human and animal studies with ischemia, the ranges of DFO used vary between 10 and 80 mg/kg, corresponding to plasma concentrations of 20–320 μM. Second, application of ferroxamine (preloaded DFO with Fe^{3+}) in some, but not all studies, was demonstrated to achieve the same level of protection as unloaded DFO, seriously questioning the role of iron chelation in the protective effects of DFO. Moreover, DFO is not absolutely specific as a chelator of Fe^{3+}, although the stability constant for formation of the Fe^{2+} complex is several orders of magnitude greater than that for other metal ions (Al, Cu, Zn).

2. DFO scavenges free radicals. Free radicals are well-known mediators of tissue injury not only in ischemia, but in a multitude of other disorders. Free radicals are generated more rapidly in the presence of iron and serve as one mechanism of iron-mediated injury. In addition to its iron-chelating properties, DFO has intrinsic radical-scavenging properties *in vitro* via its hydrogen-donating free hydroxamate groups (Halliwell, 1989, Nelson *et al.*, 1992). Through this scheme, DFO prevents injury by scavenging radicals rather than preventing their formation. DFO reacts with $\cdot OH$ at a diffusion-limited rate, as does iron-saturated DFO (ferrioxamine).

In addition to hydroxyl radicals, DFO also reacts with superoxide (O_2^-). However, the overall rate of DFO's reaction with superoxide (O_2^-) at pH 7.4 is much slower than the overall rate constant for enzymatic dismutation of O_2^- at that pH. This pathway would therefore require a much higher DFO concentration (mM) to proceed, as it is in competition with enzymatic dismutation of O_2^- by SOD and the

nonenzymatic reaction of O_2^- with other biological molecules, such as SOD and ascorbate.

3. DFO slows formation of hypohalous acids. DFO can be oxidized by peroxidase enzymes in the presence of peroxide to yield a mixture of nitroxide radicals. DFO can protect tissues by competing with halide ions for peroxidase enzymes and thereby can slow the formation of hypohalous acids. Halide ions are known to contribute to extensive tissue injury at sites of excessive phagocyte activation. The ability of DFO to act as a substrate for phagocyte-derived peroxidases seems to only be significant at concentrations greater than 100 μM.

4. DFO affects eicosanoid metabolism. Several groups have claimed that DFO can affect eicosanoid metabolism, but the reports are confusing. Although there is data demonstrating inhibition of prostacyclin and 12-lipoxygenase activity, there is also data that demonstrates an increase in prostaglandin synthesis.

5. DFO inhibits cell cycle transition. Proliferating cells have an essential requirement for iron, and iron chelators such as DFO block DNA synthesis and halt the cell cycle before the G_1/S boundary which is defined as the "safe point" in the cell cycle and associated with protection from trophic withdrawal-induced cell death (Farinelli and Greene, 1996). One potential mechanism of this protection is that injured postmitotic neurons undergo apoptosis resulting from an inappropriate attempt to re-enter the cell cycle (see Wang and Semenza, 1995, for detailed discussion). DFO at therapeutically relevant concentrations of 20–100 μM can inhibit cell proliferation *in vitro* and *in vivo*, thus providing protection.

6. DFO activates HIF-1 gene expression. DFO has been shown to stabilize HIF-1α and induce compensatory upregulation of a number of genes involved in energy metabolism, angiogenesis, and neuroprotection in response to oxygen-glucose deprivation (Semenza, 1989a,b; Semenza and Wang, 1992; Wang and Semenza, 1995). One of the established HIF-1-regulated genes is heme oxygenase-1. Heme oxygenase-1 and -2 are heme-degrading enzymes that generate ferrous ion and carbon monoxide as well as bile pigments biliverdin, and bilirubin, both of which are considered to be potent antioxidants (Maines *et al.*, 1986; Stocker *et al.*, 1987; Trakshel *et al.*, 1986; Marks *et al.*, 1991; Verma *et al.*, 1993; Liesuy and Tomaro, 1994). Interestingly, concomitant with induction of HO-1, the iron-binding protein ferritin is also up-regulated (most likely via iron-regulated ferritin expression), providing partial cytoprotection against iron-induced free radical damage (Eisenstein *et al.*, 1991; Vile and Tyrrell, 1993; Balla *et al.*, 1995). Data regarding the neuroprotective role of bilirubin has been controversial. While nanomolar concentration of bilirubin have been shown to be neurotoxic to primary astrocyte cultures (Amit and Brenner, 1993), it has also been reported that micromolar concentrations of bilirubin can scavenge lipid-peroxyl radicals even more effectively than α-tocopherol (Stocker *et al.*, 1987). A protective role for HO-1 has been supported by transgenic mouse studies. Mice overexpressing HO-1 are more resistant to cerebral ischemia-induced cell loss (Maines *et al.*, 1998; Panahian *et al.*, 1999). In addition to HO-1, other HIF-1-regulated genes that may be involved in ischemic protection include tyrosine hydroxylase (increased rate of breathing), VEGF (increased angiogenesis), erythropoietin (erythropoiesis), glycolytic enzymes, and glucose transporter (facilitate switch from aerobic to anaerobic energy metabolism). The proteins encoded by these genes function to maintain cellular oxygen homeostasis and energy balance under ischemic conditions.

Which of these mechanisms is operative most likely depends on the nature, duration, and locale of injury-inducing stimulus. However, it is clear from this discussion that one cannot reflexively use the protection by iron chelators as evidence that cell damage is occurring as the result of products of the Haber-Weiss reaction.

CONCLUSION

It is clear that we have come a long way from the primary observations of Zaleski in 1886 documenting the presence of iron in brain. We are now able to localize the distribution of iron in various pathological states, not only histopathologically but also radiographically with remarkable detail. We have a meticulous understanding of the elegant regulation of iron metabolism at the cellular level and the array

of regulatory proteins that are involved, and how this regulation is altered in hypoxic and ischemic states. While this understanding has paved the way for the clinical application of iron chelators, including DFO, to prevent stroke induced neuronal injury and loss, we are still faced with many unanswered questions.

ABBREVIATIONS

AL, Aluminum; BBB, blood brain barrier; CNS, central nervous system; CSF, cerebral spinal fluid; Cu, copper; DCTI, divalent cation transporter; DFO, ferroxamine; e-ALAS, erythroid 5-aminolevulinate synthase; Fr, ferritin; GABA, γ-aminobutyric acid; HIF-1α, heterodineric transcription factor in response to hyperoxia; HO-1, heneoxygenase-1; iNOS, inducible nitric oxide synthase; IRE, iron response elements; IRP, iron regulating protein; ITP, idopathic thrombocytopenic purpura; LMW, low molecular weight; NO, nitric oxide; Nramp2, natural resistance-associated macrophage protein 2; $ONOO^-$, peroxynitrite; pVHL, Von Hippel-Lindau tumor suppressor gene product; Tf, transferrin; TfR, transferrin receptor; Zn, zinc.

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