Neuroprotection by tempol in a model of iron-induced oxidative stress in acute ischemic stroke

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Iron is an essential nutritional element responsible for energy production and normal protein and mitochondrial enzyme function. Although iron plays a critical role in cell viability, free iron is toxic when present at high concentrations in and around cells (24). In the presence of oxygen, unbound iron generates hydroxyl radicals through the Fenton and Haber-Weiss reactions (11). Thus free iron released from cells under hypoxic conditions is capable of exerting its neurotoxic effects via the generation of hydroxyl radicals. Experimental and clinical data indicate an important role of iron in brain injury. Iron depletion or chelation reduces brain edema and metabolic failure in ischemia-reperfusion stroke models, and inhibition of iron-dependent lipid peroxidation attenuates neuronal necrosis (5). Therefore, antioxidants and ROS scavengers (such as desferoxamine, tempol, etc.) are being investigated as possible therapies for ischemic stroke (16). Tempol, a superoxide dismutase (SOD) mimetic, is highly cell permeable and crosses the blood-brain barrier (BBB) (21). We investigated the ability of tempol to reduce iron-induced cerebral ischemic injury. In addition, we investigated the role of ROS produced by the vasculature in iron-induced ischemic injury because ROS scavenging by nitric oxide donors exerts a neuroprotective effect in stroke (19), and ROS impair pial arteriolar dilatation in response to hypoxic-ischemic stress (8). We tested the hypothesis that iron-induced ROS generation in the cerebral vasculature adds to the oxidative stress during an ischemic episode after disruption of the blood-brain barrier.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (250–325 g) were purchased from Harlan (Indianapolis, IN). Rats were maintained on a 12:12-h light-dark cycle and were housed two per cage with access to food and water ad libitum. Four groups of rats were used for the middle cerebral artery occlusion (MCAO) experiments, and three groups of rats were used for the experiments involving the measurement of ROS. These studies complied with the protocols for animal use outlined by the American Physiological Society and were approved by the Institutional Animal Care and Use Committee.

Iron Treatment Protocols

In vivo. Iron was administered in the form of iron dextran (American Regent Lab). The iron-treated rats received a single injection of iron dextran intraperitoneally at a dose of 0.5 g/kg body wt 24 h before induction of MCAO. This dose has been shown to induce iron overload (27). Control rats received the same volume of 0.9% saline. Tempol (Sigma Chemical) was administered in the drinking water (1 mmol/l) (18, 22) for 3 days before the MCAO.

In vitro. Brain and carotid arteries (endothelium intact) were removed from naive rats and placed in physiological salt solution

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(PSS) (in mmol/l): 130 NaCl, 4.7 KCl, 1.18 KH₂PO₄, 1.17 MgSO₄·7H₂O, 14.9 NaHCO₃, 5.5 dextrose, 0.26 EDTA, and 1.6 CaCl₂. Arteries were cleaned free of adherent fat and connective tissue and were cut into 4-mm sections. The brain was sliced into 2-mm coronal sections. Iron(III) citrate (0.1, 1, and 5 μM) (FeC) (Sigma Chemical) was added to the tissues immediately before assessment of ROS generation (see below).

MCAO

Rats were anesthetized with pentobarbital sodium (50 mg/kg ip). The body temperature was maintained at 37°C during anesthesia. The MCAO was carried out according to the technique of Zea Longa et al. The common carotid artery was exposed by a midline incision, and the branches of the external carotid artery were cauterized. A 3–0 monofilament thread with a rounded tip was introduced into the external carotid artery in a retrograde fashion. This was advanced cranially into the internal carotid artery over a distance of exactly 19 mm, measured from the bifurcation of the common carotid artery. The thread was left in place, and the rats were allowed to recover. Postocclusion (24 h), the rats were anesthetized and decapitated, and the brains were carefully removed. The area of the infarction was quantified using 2,3,5-triphenyltetrazolium staining as described previously (9).

Lucigenin Assay

ROS production was measured by lucigenin assay as described previously (3). Lucigenin (5 μmol/l) was used for these studies; this concentration correlates well with electron spin resonance as a quan-
A quantitative measure of ROS production (15). The tissues were placed in PSS and allowed to equilibrate for 20 min at 37°C. Scintillation vials containing PSS with 5 /H9262 mol/l lucigenin were placed in a scintillation counter (Beckman LS6000IC), and after dark adaptation, background counts were recorded (in the case of the in vitro iron incubations, the FeC was also present in the vial at this time). Then the tissue was added to the vial, and the counts were recorded every minute for 20 min. The final five counts were averaged and expressed as counts per minute (cpm) above background per milligram of dried tissue.

**Iron Staining**

Rats were treated with iron dextran as described above. After 24 h the rats were euthanized, and the brain was removed and sliced into 2-mm coronal sections. The sections were fixed in 10% neutral buffered formalin before being paraffin embedded. The brain sections were then stained for iron (the iron staining was carried out at the pathology core at the Medical College of Georgia).

**Statistics**

Results were analyzed with the Student’s t-test (Bonferroni correction was applied when multiple analyses were performed). A P value < 0.05 was deemed to be statistically significant.

**RESULTS**

Iron-treated rats had significantly larger infarcts compared with saline-treated controls (51.83 ± 3.55 vs. 27.56 ± 3.28% HI iron treated vs. control, \( P = 0.01 \)) (Fig. 1). Pretreatment with tempol for 3 days before MCAO significantly reduced the infarct size in the iron-treated group (26.09 ± 9.57 vs. 51.38 ± 3.55% HI, iron + tempol vs. iron, \( P = 0.02 \)). Tempol administration to the control rats had no effect on infarct size (data not shown).

To directly demonstrate the role of iron-mediated ROS generation in ischemic injury, DHE staining of brains 30 min after MCAO in control and iron-treated rats was performed. Figure 2 shows representative confocal microscope images showing an increase in ROS production in the iron-treated rats compared with control. Treatment of the brain slices with PEG-SOD effectively scavenged the ROS generated in the iron-treated group, bringing it to control values.

To assess the ability of iron to generate ROS in the brain with an intact BBB, we measured ROS in the brain of rats that did not undergo an MCAO using the lucigenin assay. There was no difference in the ROS production from the brain of rats treated with iron compared with the untreated controls. Results are shown as cpm/mg of tissue (mean ± SE).

**Detection of Superoxide**

In situ superoxide generation was evaluated in brain 30 min after MCAO with the oxidative fluorescent dye dihydroethidine (DHE). Cryosections (15 μm) were incubated with DHE (2 μmol/l) in PBS, with or without polyethylene glycol (PEG)-conjugated SOD (250 U/ml), which was added 30 min before staining. Fluorescence images were obtained with a Bio-Rad MRC 1024 scanning confocal microscope. In each case, paired segments of control and iron-treated brain sections were analyzed in parallel with identical imaging parameters.
pretreated with iron for 24 h than from control rats. This suggests that with the BBB intact the exogenous iron may not significantly exacerbate oxidative stress. Similarly, there was no difference in the ROS generation from naive brain tissue incubated with FeC and control tissue (Fig. 3). However, in vitro incubation with ferric citrate (1 μM) did increase the ROS generation in the carotid arteries (Fig. 4) (17,801 ± 3,414 vs. 3,494 ± 365 cpm/mg of tissue, iron treated vs. control, P < 0.01). ROS generation in the arteries incubated with 0.1 μM FeC was not significantly different from untreated controls. ROS generation from arteries incubated with 5 μM FeC was similar to the response seen with 1 μM FeC (data not shown).

Brain sections from rats treated with iron dextran for 24 h and control rats were stained for iron. As shown in Fig. 5, minimal staining for iron was seen in the control sections (represented by blue color); however, in the iron-treated sections prominent staining was evident. This staining is primarily localized to the perivascular location and not in the brain parenchyma.

**DISCUSSION**

Ischemic injury involves complex interactions between multiple factors, including glutamate excitotoxicity, loss of membrane potential, and generation of ROS (4). However, without data concerning the neurotoxicity of iron in vivo, it has been difficult to delineate the contribution of iron-mediated oxidative stress in ischemic brain injury. Previous studies suggest that during ischemia a large amount of iron is released (24), and this iron can then participate in the generation of free radicals. Palmer et al. (17) have reported that, regardless of the source, there is an increase in the amount of iron available to promote oxidative stress in the neonatal rat brain following hypoxia-ischemia. Clinical studies by Davalos et al. (5) suggest a strong relationship between elevated iron stores and a poor prognosis during the early phase of ischemic stroke. This is consistent with our finding that administration of iron significantly exacerbates infarct size compared with control values after MCAO (Fig. 1). In addition, we have also shown using DHE staining that in animals subjected to MCAO, iron treatment significantly increases ROS production compared with control rats that underwent MCAO. Treatment of the tissue with PEG-SOD reversed the increased production of ROS in the iron-treated group, suggesting that superoxide is involved in the iron-induced ischemic injury (Fig. 2).

The in vitro incubations with FeC suggest that iron can increase ROS generation in the vasculature. By carrying out these studies in vitro we could assess the effects of iron alone without the confounding effects of flow and other circulating factors. While we did not measure ROS production from the carotid arteries of rats treated with iron, our results showing that iron treatment increases ROS generation in the brain after ischemia suggest that there is, indeed, increased ROS generation in the iron-treated rats. This, taken with the evidence that exogenous iron accumulates in the perivascular regions of the brain, lead us to suggest that during ischemia, when the BBB is breached, there is an increase in the entry of both iron and ROS into the brain from the vasculature. The ROS will exacerbate the ischemic damage, and the iron can then taken up by the surrounding cells, causing the propagation of the infarct. Other studies have shown using immunohistochemistry that there is release of iron stores from dying cells, which is taken up by the neighboring glial cells between day 4 and day 7 (13). However, our results suggest that that this mechanism is also of relevance in the early acute phase of stroke injury, i.e., in the first 24 h. The free iron that is taken up by the cells is redox active and responsible for the generation of hydroxyl radicals through the Haber-Weiss reaction. Hence, we propose that iron-mediated oxidative stress is a major factor in elevating the infarct size as pretreatment with tempol was able to reduce the iron-induced elevation in infarct size (Fig. 1), and PEG-SOD treatment decreased ROS production in the iron-treated group (Fig. 2). Tempol not only acts as a SOD mimetic; it also oxidizes Fe²⁺ ions, thus preventing the Fenton reaction and the generation of the hydroxyl radicals. Rak et al. (21) have reported the neuroprotective efficacy of intravenous administration of tempol in the rat model of transient ischemic stroke. However, we believe that this is the first study to show that tempol can reverse the ischemic damage exacerbated by elevated iron in the permanent MCAO model in the rat.

In our present study, although we administered iron dextran 24 h before the induction of ischemia, a pronounced effect on the infarct size was still observed. Hence, we speculate that the deleterious effect of iron manifests itself after the disruption of the BBB after the stroke. The mechanisms of brain iron

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Fig. 5. Representative brain sections with iron staining in animals with an intact blood-brain barrier treated with iron dextran for 24 h. Iron-stained areas are blue in color. Iron staining is localized in the perivascular region, and there is no staining in the brain parenchyma in the iron-treated group of animals. In the control animals, no iron staining is evident in the brain tissue. Results shown are representative of n = 3.
transport have not been clearly elucidated, and abnormally high levels of iron have been demonstrated in some regions of the brain in various neurodegenerative diseases, including Hallervorden-Spatz syndrome and Parkinson’s and Alzheimer’s disease. Under normal physiological conditions, brain cells obtain iron from transferrin (Tf). Most Fe$^{2+}$, after crossing the BBB, is oxidized to Fe$^{3+}$ by the ferroxidase activity of ceruloplasmnin, which then binds to Tf and is then acquired by the brain cells (20). Therefore, the transferrin pathway is the major mechanism for iron transport across the BBB (14). However, in case of stroke, free plasma iron could leak across the damaged BBB, and this iron would be taken up by cells in a Tf-independent manner (14). Thus excessive intracellular accumulation of iron induces oxidative stress and formation of ROS, triggering a pathological cascade of events that culminate in neuronal death. The notion that the BBB needs to be breached for iron to have its deleterious effects is supported by our findings that in vivo as well as in vitro exposure to low concentrations of iron with the intact BBB failed to induce significant oxidant stress (Fig. 3). Also, staining of brain sections for iron from animals not subjected to MCAO showed dense iron staining in the perivascular region of the iron-treated rats and almost none in the parenchyma (Fig. 5). The levels of iron in the brain postischemia were not measured. However, we did observe a marked increase in the ROS generation in the brain of iron-treated rats after ischemia compared with control rats after ischemia. The only difference between the two groups is the presence or absence of iron; therefore iron must be responsible for the increase in ROS generation. Whether the ROS in this case comes from the vasculature or from the brain tissue remains unclear, and further studies will be required to assess this.

Even though the neuronal source of ROS is extremely important, we believe that the role of the ROS generated in the cerebral vasculature is just as important. Pluta et al. (19) have shown the beneficial effects of restoring the cerebral circulation were enhanced by the administration of nitric oxide, which would scavenge the ROS. In our experimental model, we believe that the circulating iron can induce significant oxidative stress in the cerebral vasculature. This was evident from the fact that the iron-treated carotid arteries had significantly higher ROS production compared with untreated control vessels. We propose that the integrity of the BBB prevents the ROS from exerting their deleterious effects. However, with the disruption of the BBB during an ischemic stroke, iron and ROS produced in the cerebral microvasculature can gain access to the brain cells and exacerbate ischemic damage. Thus we believe that the cerebral vasculature plays an important role in maintaining the physiological homeostasis of cerebral function especially during pathological states.

Perspectives

Iron has been implicated in the pathogenesis of various neurological diseases such as Alzheimer’s, Parkinson’s, and stroke. In addition, ~10% of the American population carries the gene for hemochromatosis, and 1% suffer from iron overload (10). An increased absorption and deposition of iron in the various organs of the body characterize this disease. Studies by Davalos et al. (5) suggest that children with severe ischemic anoxic brain injury have extensive areas of iron deposition in the basal ganglia and periventricular white matter (6). Also, studies suggest the iron chelator desferoxamine is capable of reducing ischemic damage (23). Therefore, it would appear that iron is intricately involved in exacerbation of ischemic injury after hypoxia-ischemia. Our results support the hypothesis that iron-induced ROS generation in the vasculature has deleterious effects on the outcome of cerebral ischemia. Hence, understanding the role of iron-induced oxidative injury in stroke with a particular emphasis on elucidating the role of neuronal and vascular ROS may have significant therapeutic implications.

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