Iron and Iron-Handling Proteins in the Brain After Intracerebral Hemorrhage

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**Background and Purpose**—Evidence indicates that brain injury after intracerebral hemorrhage (ICH) is due in part to the release of iron from hemoglobin. Therefore, we examined whether such iron is cleared from the brain and the effects of ICH on proteins that may alter iron release or handling: brain heme oxygenase-1, transferrin, transferrin receptor, and ferritin.

**Methods**—Male Sprague-Dawley rats received an infusion of 100 μL autologous whole blood into the right basal ganglia and were killed 1, 3, 7, 14, or 28 days later. Enhanced Perl’s reaction was used for iron staining, and brain nonheme iron content was determined. Brain heme oxygenase-1, transferrin, transferrin receptor, and ferritin were examined by Western blot analysis and immunohistochemistry. Immunofluorescent double labeling was performed to identify which cell types express ferritin.

**Results**—ICH upregulated heme oxygenase-1 levels and resulted in iron overload in the brain. A marked increase in brain nonheme iron was not cleared within 4 weeks. Brain transferrin and transferrin receptor levels were also increased. In addition, an upregulation of ICH on ferritin was of very long duration.

**Conclusions**—The iron overload and upregulation of iron-handling proteins, including transferrin, transferrin receptor, and ferritin, in the brain after ICH suggest that iron could be a target for ICH therapy. (Stroke. 2003;34:2964-2969.)

**Key Words:** cerebral hemorrhage ■ ferritin ■ iron ■ receptors, transferrin ■ transferrin

Spontaneous intracerebral hemorrhage (ICH) is a common and often fatal stroke subtype. If the patient survives the ictus, the resulting hematoma within brain parenchyma triggers a series of events leading to secondary insults and severe neurological deficits. Although the hematoma in humans gradually resolves within several months, restoration of function is gradual and usually incomplete.

Animal studies have elucidated several mechanisms that result in brain injury, particularly brain edema formation, after ICH. These include thrombin, produced during clot formation, and hemoglobin and hemoglobin breakdown products, released as the erythrocytes within the hematoma start to lyse. Iron, a hemoglobin degradation product, plays a key role in neurodegeneration in many disease states, and an excess increase in brain iron can result in lipid peroxidation and free radical formation. Evidence suggests that iron and oxidative stress contribute to delayed edema formation after ICH.

Despite the potential for massive iron release into the brain as a result of hemoglobin degradation, there have been few studies on the fate of iron after ICH and on the temporal relationship between enzymes that may release and regulate iron. The key enzyme for heme degradation is heme oxygenase (HO). It cleaves heme to release carbon monoxide, iron, and biliverdin. HO-1 is upregulated in the brain after ICH.

HO inhibition reduces hemoglobin-induced brain edema. A number of proteins, including transferrin (Tf), Tf receptor (TfR), and ferritin, are involved in maintaining brain iron homeostasis. Tf, an 80-kDa protein, is a major iron distributor in the brain. Cellular uptake of Tf-bound iron is achieved by binding to the TfR. Brain Tf levels are altered in various neurodegenerative disorders. TfR is normally expressed only on brain parenchymal cells at low levels. Brain TfR can be altered under pathophysiological conditions. Decreases in brain TfR levels are associated with Alzheimer’s disease and other neurodegenerative diseases. Ferritin, an iron-storage protein, has been found in the brain. Ferritin has 2 subunits, the ferritin-heavy (H)-chain (21 kDa) and ferritin-light (L)-chain (19 kDa), and is found mainly in glial cells but not neurons.

The present study examines the temporal relationship among HO-1 upregulation, iron accumulation, Tf, TfR, and ferritin (both L chain and H chain) levels after ICH in the rat.

**Materials and Methods**

**Animal Preparation and Intracerebral Infusion**

The protocols for these animal studies were approved by the University of Michigan Committee on the Use and Care of Animals. A total of 95 adult male Sprague-Dawley rats (Charles River Laboratories) weighing 300 to 350 g were used in the study. The rats
were anesthetized with pentobarbital (40 mg/kg IP). The right femoral artery was catheterized for continuous blood pressure monitoring and blood sampling. The rats were placed in a stereotactic head frame (Körd Instruments), and a 1-mm cranial burr hole was drilled on the right coronal suture 4 mm lateral to midline. Then, 100 μL autologous whole blood was infused into the right caudate at a rate of 10 μL/min through a 26-gauge needle (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 4.0 mm lateral to the bregma). Control rats only received a needle insertion. Body temperature was maintained at 37.5°C with a feedback-controlled heating pad. Blood pH, PaO₂, PaCO₂, hematocrit, and glucose levels were monitored.

Experimental Groups
This study was performed in 3 parts. Rats received either a needle insertion or an intracerebral infusion of 100 μL autologous whole blood. The rats were killed 1, 3, 7, 14, or 28 days later. Part 1 examined HO-1, Tf, TfR, ferritin-L-chain, and ferritin-H-chain levels by Western blot analysis (n=3 to 4 at each time point). Part 2 investigated HO-1, Tf, TfR, ferritin, and ferritin-L-chain and ferritin-H-chain immunoreactivities by immunohistochemistry and immunofluorescent double labeling. Enhanced Perl’s reaction was used for iron staining (n=3 to 4 at each time point). Part 3 examined nonheme brain tissue iron contents (n=5 to 6 at each time point).

Nonheme Brain Tissue Iron Determination
Rats were killed at 1, 3, 7, 14, and 28 days after ICH. The brains were perfused with saline before decapitation. A coronal slice ~4 mm thick around the injection needle tract was cut, divided into ipsilateral and contralateral sides, and weighed. Nonheme brain tissue iron was determined according to the method described by Weinfeld et al. Briefly, the brain was homogenized, and then 1 mL of 8.5 mol/L HCl was added. Brain samples were hydrolyzed at 90°C for 60 minutes. After cooling, 2 mL of 20% trichloroacetic acid was added to precipitate proteins, and supernatant was collected after centrifuge. The supernatant was run through an acid-washed filter, and the precipitate was washed with 1 mL of 4.25 mol/L HCl plus 20% trichloroacetic acid (1:1). The supernatant was collected, and 4 mL of 1 mol/L sodium citrate was added. pH was regulated to 3.1, and the final volume was adjusted to 25 mL. Total nonheme iron content was assayed by a spectrophotometer with ferrozine as the color reagent.

Western Blot Analysis
Rats were anesthetized and killed at different time points for Western blot analysis. They underwent intracardiac perfusion with saline. The brains were removed, and a 3-mm-thick coronal brain slice was cut with a blade ~4 mm from the frontal pole. The ipsilateral and contralateral basal ganglia samples were dissected from the slice. Protein concentration was determined by Bio-Rad protein assay kit. Protein (50 μg) from each sample was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a Hybond-C pure nitrocellulose membrane (Amersham). Membranes were probed with a 1:2500 dilution of the primary antibody and a 1:2500 dilution of the secondary antibody. The primary antibodies were polyclonal rabbit anti-human TF (Dako; 1:2500 dilution), monoclonal mouse anti-human TIR (ZYMED Laboratories Inc; 1:2000 dilution), polyclonal rabbit anti-rat ferritin-L-chain (Dr J Connor, Pennsylvania State University; 1:2500 dilution), polyclonal rabbit anti-rat ferritin-H-chain (Dr J Connor; 1:2500 dilution), or polyclonal rabbit anti-rat HO-1 (StressGen; 1:2500 dilution). The antigen-antibody complexes were visualized with the ECL chemiluminescence system (Amersham) and exposed to Kodak X-OMAT film. The relative densities of bands were analyzed with NIH Image (version 1.61).

Immunohistochemistry
Immunohistochemistry was performed as previously described. Briefly, rats (n=3 to 4 each time point) were reanesthetized with pentobarbital (60 mg/kg IP), followed by intracardiac perfusion with 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline, pH 7.4. The brains were removed, kept in 4% paraformaldehyde for 6 hours, and then immersed in 25% sucrose for 3 to 4 days at 4°C. They were then placed in embedding compound and sectioned (18-μm slices) on a cryostat. Sections were incubated with avidin-biotin complex technique. Primary antibodies were polyclonal rabbit anti-rat HO-1 IgG (StressGene; 1:400 dilution), polyclonal rabbit anti-human Tf (Dako; 1:200 dilution), monoclonal mouse anti-human TIR (ZYMED Laboratories Inc; 1:200 dilution), polyclonal rabbit anti-human ferritin IgG (Daco; 1:400 dilution), rabbit anti-rat ferritin-L-chain IgG (1:400 dilution), and rabbit anti-rat ferritin-H-chain IgG (1:400 dilution). Normal rabbit IgG or mouse IgG was used as negative control.

Immunofluorescent Double Labeling
For immunofluorescent double labeling, the primary antibodies were rabbit anti-human ferritin IgG (Daco; 1:100 dilution), rabbit anti-rat ferritin-L-chain IgG, rabbit anti-rat ferritin-H-chain IgG, mouse anti-rat OX-42 (Serotec; 1:100 dilution), and mouse anti-GEAP (Chemicon International Inc; 1:100 dilution). Rhodamine-conjugated goat anti-rabbit antibody (Boehringer Mannheim Bio; 1:100 dilution) and fluorescein isothiocyanate (FITC)–labeled horse anti-mouse antibody (Vector; 1:100 dilution) were used as second antibodies. The double labeling was analyzed using a fluorescence microscope.

Enhanced Perl’s Reaction
Brain sections were incubated in Perl’s solution (1:1, 5% potassium ferrocyanide and 5% HCl) for 45 minutes, washed in distilled water, and incubated again in 0.5% diamine benzidine tetrahydrochloride with nickel for 60 minutes.

Statistical Analyses
All data in this study are presented as mean±SD. Data were analyzed either by analysis of variance with a Scheffé’s multiple comparisons test or by Student’s t test. Differences were considered significant at P<0.05.

Results
Physiological data were measured before intracerebral blood infusion. The mean values of blood pH, blood gases, mean arterial blood pressure, hematocrit, and blood glucose were controlled in normal ranges (PaO₂, 80 to 120 mm Hg; PaCO₂, 35 to 45 mm Hg; pH, 7.40 to 7.50; mean arterial blood pressure, 70 to 110 mm Hg; hematocrit, 35% to 45%; and blood glucose, 6 to 9 mmol/L). HO-1 immunoreactivities were very low in the normal cerebral hemispheres of the adult rat. However, HO-1 protein levels were increased markedly in the ipsilateral basal ganglia the first day after ICH (6153±678 versus 1048±166 pixels in the sham, P<0.01). HO-1–positive cells were found in the perihematomaal zone, and most of the HO-1–positive cells were microglia (Figure 1A and 1B). By Western blot analysis, the time course study of HO-1 showed that HO-1 was increased at day 1, peaked at day 3, and was still detectable at day 28 after ICH (Figure 1C).

The release of iron from the breakdown of hemoglobin occurred during intracerebral hematoma formation. By enhanced Perl’s reaction, iron-positive cells were found in the perihematomaal zone as early as the first day. Perl’s–positive cells were neurons the first day (Figure 2C) and glial cells several days later (Figure 2F and 2I). Lysis of erythrocytes resulted in a buildup in nonheme brain tissue iron starting at day 3 (56.1±9.8 versus 32.4±4.0 μg/g in the contralateral side; n=5; P<0.05) after ICH and reaching a plateau after
~7 days (94.3±31.4 versus 34.7±5.7 μg/g in the contralateral side; n=5; P<0.05). Brain nonheme iron remained at high levels for at least 28 days (Figure 2J).

Tf, through binding to its receptor, is involved in the transport of iron into cells. By Western blot analysis, both Tf and TfR protein levels were significantly increased in the ipsilateral basal ganglia after ICH (Figure 3). In the normal rat brain, Tf is present in oligodendrocytes, and TfR is expressed at high levels in endothelial cells. After ICH, we found that most Tf-positive cells were still oligodendrocytes, but many neuronlike TfR-positive cells were found in the perihematomal zone (data not shown).

Ferritin is a key iron-storage protein in the brain. We found that both ferritin-L-chain and ferritin-H-chain protein levels were increased after ICH. Western blot analysis showed that ferritin-L-chain protein levels in the perihematomal zone were low at day 1, increased significantly at day 3, and remained at high levels at day 28 (Figure 4A). Figure 4B shows the time course of ferritin-H-chain levels in the perihematomal zone, which also increased after 3 days and remained at high levels at least for 4 weeks. Compared with sham-operated rats, both ferritin-L-chain and ferritin-H-chain levels were significantly higher after ICH (ferritin-L-chain, a 40-fold increase; ferritin-H-chain, a 3-fold increase). By immunohistochemistry, a few ferritin–, ferritin-L-chain–, and ferritin-H-chain–positive cells were found in the normal brain and ipsilateral basal ganglia after needle insertion. The numbers of ferritin-positive cells were increased around the hematoma at the first day and were still at high levels 1 month later (Figure 5). Most ferritin-positive cells were glialike cells. Only a few ferritin-positive neurons were found in the first several days after ICH (data not shown). Double labeling demonstrated that most ferritin was either microglial cells or astrocytes (Figure 6).

Discussion

Although the mechanisms of brain injury after ICH are complex and not well understood, hemoglobin and its degradation products may play a key role.\textsuperscript{5,6,16} The present study demonstrates HO-1 induction, nonheme iron accumulation, and iron-handling protein upregulation in the brain after ICH. The temporal relationship between HO-1 (peaking at day 3) and nonheme iron (peaking at 1 to 2 weeks) indicated that an increase in perihematomal HO-1 content may accelerate heme degradation and iron overload in the brain. Accumulation of iron may then result in brain injury. However, the degree of brain injury may be limited by changes in iron-
handling proteins. In particular, an upregulation in brain ferritin level may be neuroprotective.

The prominent increase in nonheme iron is probably due to degradation of the hemoglobin. The remarkable induction of HO-1 in the ipsilateral basal ganglia was found as early as day 1 and became even higher at day 3. HO consists of 3 enzymes: HO-1, HO-2, and HO-3. In our present study, HO-1 protein levels were significantly increased (Figure 1) and iron-positive neurons were found around the clot 24 hours after ICH. Early expression of HO-1 may result from induction by plasma proteins and thrombin in particular. Turner et al. and our laboratory have reported that hemoglobin can upregulate HO-1 in the brain. We found that most HO-1-positive cells were microglial cells. This result is supported by our early studies and others reports. Matz et al. found that HO-1 expression is increased primarily in microglia cells in an ICH model. An increase in HO-1 content in the perihematoma zone may cause excess free iron accumulation, contributing to brain injury such as brain edema. Indeed, HO inhibition reduces brain edema in rats and pigs. However, it should be noted that overexpression of HO-1 protects neurons from oxidative injury.

Iron is essential for normal brain function, but iron overload may have devastating effects. After erythrocyte lysis, iron concentrations in the brain can reach very high levels, possibly contributing to acute brain edema formation (first week) and delayed brain atrophy (1 month later). To avoid the influence of the hemoglobin released from the hematoma, the present experiments used nonheme tissue iron to evaluate the natural time course of the brain iron accumulation after ICH. Our data showed a 3-fold increase in brain nonheme

![Figure 3. Western blot analysis showing Tf (A) and TfR (B) protein levels in the ipsilateral basal ganglia 3 days after sham operation and ICH. Values are mean±SD. n=3. #P<0.01 vs sham.](image)

![Figure 4. Western blot analysis showing time course of ferritin-L-chain (A) and ferritin-H-chain (B) protein levels in ipsilateral basal ganglia after ICH. Bar graphs show analysis of the Western blotting. Values are mean±SD. n=3. #P<0.01 vs day 1.](image)

![Figure 5. Ferritin immunoreactivities in ipsilateral basal ganglia 28 days after needle insertion (A) or ICH (B). Scale bar=20 μm.](image)
iron in the perihematomal zone after ICH in rats. The peak in nonheme iron accumulation 1 to 2 weeks after ICH was delayed compared with the rise in HO-1. This probably reflects the delayed lysis of erythrocytes after ICH.5

Iron overload can cause brain injury via many pathways such as lipid peroxidation and formation of free radicals.8 It is well known that iron reacts with lipid hydroperoxides to produce free radicals. Free radicals attack DNA and cause oxidative brain injury. Our previous study has shown oxidative brain injury after ICH.9 In addition, antioxidants block the neuronal toxicity induced by hemoglobin and iron.12

Deferoxamine, an iron chelator, reduces hemoglobin-induced brain Na+,K+–ATPase inhibition, brain edema, and neuronal toxicity.6,22

Ferritin, a naturally occurring iron chelator, is involved in maintaining brain iron homeostasis, and the brain can produce ferritin. Ferritin has 2 subunits: ferritin-H-chain, which is related to iron utilization, and ferritin-L-chain, which is associated with iron storage.12 Ferritin protein synthesis is regulated mostly posttranscriptionally by iron-mediated or non–iron-mediated induction. Hemoglobin degradation products such as iron and heme are strong ferritin inducers through iron regulatory protein. Our present data found that the upregulation of ferritin-H-chain and ferritin-L-chain paralleled the increase in nonheme brain tissue iron, suggesting that regulation of ferritin synthesis after ICH is mainly iron mediated. However, non–iron-mediated ferritin upregulation may also occur in the perihematomal zone. For example, tumor necrosis factor-α can induce ferritin synthesis, and a significant increase in tumor necrosis factor-α content is found 2 hours after ICH.24,25

In normal brain, ferritin is present in neurons and glial cells. In the present study, we found that most ferritin and its subunits in the perihematomal zone are glial cells (microglia and astrocytes). This result is supported by the study of Koeppen et al,19 who reported that most ferritin- and iron-positive cells around intracerebral hematoma were microglia. It should be noted that, under certain circumstances, brain iron accumulation may induce injury even after iron has been combined to ferritin. Thus, iron can be released when it is reduced into the ferrous form by superoxide.8

The extent to which iron-mediated toxicity can be prevented by the export of iron from the brain is still unclear. There seemed to be a slight decrease in nonheme iron 4 weeks after ICH, but this did not reach significance. Tf and TIR are involved in the transport of iron across biological membranes. In normal brain, TIR is expressed at very high levels in the blood-brain barrier, where it is involved in iron uptake into the brain. However, a recent report indicates that there is rapid efflux of Tf from brain to blood across the blood-brain barrier,26 suggesting that Tf and TIR could contribute to iron clearance when there is brain iron overload. The present study demonstrates that both Tf and TIR levels are markedly increased after ICH. However, Tf appeared to be present primarily in oligodendrocytes, as in the normal brain where it is an important factor for myelination.16 Some of the TIR upregulation appeared to be in perihematomal neurons where perhaps it is involved in the clearance of iron. The role of Tf and TIR upregulation after ICH needs further investigation.

In summary, iron overload and iron-handling protein upregulation in the brain after ICH suggest that iron could be a target for ICH therapy. Because most of erythrocytes start to lyse several days after ICH, the potentially delayed time window of such injury may facilitate treatment.

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