Brain edema after experimental intracerebral hemorrhage: role of hemoglobin degradation products

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Object. The mechanisms involved in brain edema formation following intracerebral hemorrhage (ICH) have not been fully elucidated. The authors have found that red blood cell lysis plays an important role in edema development after ICH. In the present study, they sought to determine whether degradation products of hemoglobin cause brain edema.

Methods. Hemoglobin, hemin, bilirubin, or FeCl₂ were infused with stereotactic guidance into the right basal ganglia of Sprague–Dawley rats. The animals were killed 24 hours later to determine brain water and ion contents. Western blot analysis and immunohistochemistry were applied for heme oxygenase-1 (HO-1) measurement. The effects of an HO inhibitor, tin-protoporphyrin (SnPP), and the iron chelator deferoxamine, on hemoglobin-induced brain edema were also examined.

Intracerebral infusion of hemoglobin, hemin, bilirubin, or FeCl₂ caused an increase in brain water content at 24 hours. The HO-1 was upregulated after hemoglobin infusion and HO inhibition by SnPP-attenuated hemoglobin-induced edema. Brain edema induced by hemoglobin was also attenuated by the intraperitoneal injection of 500 mg/kg deferoxamine.

Conclusions. Hemoglobin causes brain edema, at least in part, through its degradation products. Limiting hemoglobin degradation coupled with the use of iron chelators may be a novel therapeutic approach to limit brain edema after ICH.

KEY WORDS • hemoglobin • iron • bilirubin • cerebral hemorrhage • brain edema • rat

In the United States, 15% of deaths due to cerebrovascular disease result from spontaneous ICH. Many patients with an intracerebral hematoma deteriorate progressively because of secondary brain edema formation. A better understanding of the pathophysiology of edema development after ICH may lead to better clinical management of these patients.

Recently, new details of the pathophysiological mechanisms involved in edema formation after ICH have been reported. These include clot retraction in the first several hours, the coagulation cascade and thrombin formation in the 1st day, and RBC lysis and hemoglobin neurotoxicity a few days later. Because hemoglobin-induced edema formation is triggered a few days after ICH, therapy targeted at hemoglobin and its degradation products may offer a new approach to therapy for ICH.

Hemoglobin breakdown products may have a key role in the formation of brain edema. Metabolism of hemoglobin by HO after RBC lysis results in the release of iron, carbon monoxide, and biliverdin. Subsequently, biliverdin reductase catalyzes the conversion of biliverdin to bilirubin.

Both enzymes are found in the brain, and inhibition of HO is associated with attenuation of brain edema after ICH.

Intracortical injection of iron, a potent catalyst for lipid peroxidation, causes focal epileptiform discharges and brain edema. Hemoglobin induces neuronal death in vitro, a process that is blocked by deferoxamine, an iron chelator. In addition, bilirubin is toxic to brain tissue, although it is considered to be an antioxidant at low concentrations.

In this study, we examined the effects of hemoglobin degradation products on the formation of brain edema. We also studied the effects of an HO inhibitor, SnPP, and the iron chelator deferoxamine, on hemoglobin-induced brain edema. Upregulation of HO-1 protein was examined using Western blot analysis and immunohistochemistry.

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 72 male Sprague–Dawley rats weighing 300 to 400 g were used in this study. The animals were anesthetized with 40 mg/kg intraperitoneally administered pentobarbital. The right femoral artery was catheterized for continuous blood pressure monitoring and
Part we examined HO-1 expression in the brain after hemoglobin intraperitoneally, on hemoglobin-induced brain edema. In the fourth part we studied the effects of the iron chelator deferoxamine, injected intraperitoneally, on hemoglobin-induced brain edema was investigated in this part. In the third part of hemoglobin. The effect of the HO inhibitor SnPP on hemoglobin-degradation products (hemin, bilirubin, and iron) on brain edema formation after an intracerebral infusion was evaluated by intraperitoneal infusion of hemoglobin and its breakdown products. These four groups received an infusion of 30 μl of saline, bovine hemoglobin (300 mg/ml in saline), hemin (12 mg/ml in saline), bilirubin (12 mg/ml in saline), or FeCl2 (10 mM in saline). The concentrations of injected hemoglobin breakdown products were the same as the concentrations found in rat RBCs. The animals were anesthetized again and decapitated 24 hours after infusion. Brain water, sodium, and potassium contents were measured.

Part 1. Five groups of five animals were used in the first part of the study. Each rat received a 30-μl infusion of normal saline, bovine hemoglobin (300 mg/ml in saline), hemin (12 mg/ml in saline), bilirubin (12 mg/ml in saline), or FeCl2 (10 mM in saline). The concentrations of injected hemoglobin breakdown products were the same as the concentrations found in rat RBCs. The animals were anesthetized again and decapitated 24 hours after infusion. Brain water, sodium, and potassium contents were measured.

Part 2. Four groups of five rats each were used in this part. These four groups received an infusion of 30 μl of saline, bovine hemoglobin (300 mg/ml in saline), hemin (12 mg/ml in saline), bilirubin (12 mg/ml in saline), or FeCl2 (10 mM in saline). The concentrations of injected hemoglobin breakdown products were the same as the concentrations found in rat RBCs. The animals were anesthetized again and decapitated 24 hours after infusion. Brain water, sodium, and potassium contents were measured.

Part 3. Three groups of five rats each were studied in this part. All the rats received a 30-μl infusion of hemoglobin, followed immediately by intraperitoneal infusion of 1 ml saline, 50 mg/kg deferoxamine, or 500 mg/kg deferoxamine (both dissolved in 1 ml saline). The animals were decapitated 24 hours later to determine brain water and ion contents.

Part 4. Two groups of six rats each either underwent a sham operation or received a 30-μl hemoglobin infusion. The rats were killed 24 hours later for Western blot analysis (three rats per group) or immunohistochemical studies (three rats per group).

### Brain Water and Sodium Contents

Animals were anesthetized and decapitated as described in our previous studies. The brains were removed, and a 3-mm thick coronal brain slice was cut 4 mm from the frontal pole. This section of brain was divided into its two hemispheres along the midline, and the cortex was separated from the basal ganglia bilaterally. The cerebellum was also detached to serve as control tissue. The brain samples were immediately weighed on an electronic analytical balance to obtain the wet weight. The samples were then dried in a gravity oven at 100°C for 24 hours to obtain the dry weight. Water contents were expressed as a percentage of wet weight; the formula for calculation was 

$$\frac{WW - DW}{WW} \times 100\%$$

where WW is the wet weight and DW is the dry weight. The dehydrated samples were digested in 1 ml of 1 M nitric acid for 1 week, and the sodium content of this solution was measured with an automatic flame photometer. Ion content was expressed in milliequivalents per kilogram of dehydrated brain tissue (mEq/kg dry wt).

### Use of SnPP

The SnPP was dissolved in normal saline with 1 M NaOH to obtain solutions at pH 8, and subsequently buffered with 6 M HCl to pH 7.4 for final injection solutions. The solutions (100 mM) were protected from light and freshly prepared just before use.

### Western Blot Analysis

Animals were reanesthetized and underwent transcardiac perfusion with saline. Brain tissues were sampled as described in Brain Water and Sodium Contents. The rats were decapitated and a coronal brain slice was cut as described for measurements of water content. The brain tissues were immersed in 0.5 ml Western blot sample buffer and were sonicated for 10 seconds. Twenty microliters of the sample solution was taken for the protein assay. Western blot analysis was performed as described previously. Briefly, 50 μg of protein was run on 10% polyacrylamide gels with a 4% stacking gel. The protein was transferred to a hybrid-C pure nitrocellulose membrane. The membranes were probed with a 1:1000 dilution of the polyclonal rabbit anti–rat HO-1 primary antibody. The membranes were immunoprobed again with a 1:2000 dilution of the secondary antibody (peroxidase-conjugated goat anti–rabbit immunoglobulin G). Finally, the antigen-antibody complexes were visualized with a chemiluminescence system and exposed to Kodak film. The relative densities of HO-1 protein bands were analyzed with a public-domain imaging software program.

### Immunohistochemical Studies

The rats were reanesthetized with 60 mg/kg intraperitoneally administered pentobarbital and perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). The brains were removed and kept in 4% paraformaldehyde for 6 hours, then immersed in 25% sucrose for 3 to 4 days at 4°C. The brains were embedded in optimal cutting temperature compound and 18-μm-thick sections were made with a cryostat. The sections were incubated according to the avidin–biotin complex method. The primary antibody was rabbit anti–rat HO-1 (1:800 dilution). Normal rabbit immunoglobulin G was used as a negative control.
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Statistical Analysis

All data in this study are presented as the means ± SD. Data from different animal groups were analyzed using analysis of variance with a Scheffé F-test or Student t-test. The differences were considered significant at probability values less than 0.05.

Sources of Supplies and Equipment

The Sprague–Dawley rats were purchased from Charles River Laboratories, Portage, MI. The stereotactic head frame was manufactured by Kopf Instruments, Tujunga, CA. The bovine hemoglobin was obtained from ICN Biomedicals, Inc., Aurora, OH. The hemin, bilirubin, and FeCl2 were supplied by Sigma Chemical Co., St. Louis, MO. The SnPP was purchased from Porphyrin Products, Inc., Logan, UT.

The electronic analytical balance (model AE 100) was acquired from Mettler Instrument Co., Hightstown, NJ. The automatic flame photometer (model IL943) was purchased from Instrumentation Laboratory, Inc., Lexington, MA. The protein assay kit was obtained from BioRad, Hercules, CA. The stereotactic head frame was manufactured by Kopf Instruments, Tujunga, CA. The hybrid-C nitrocellulose membrane and the ECL chemiluminescence system were supplied by Amersham International, Buckinghamshire, UK. The Kodak X-OMAT film was manufactured by Eastman Kodak, Rochester, NY. The NIH Image software (version 1.61) was obtained from the NIH, Bethesda, MD. The primary antibody (rabbit anti–rat HO-1) was acquired from StressGen, Victoria, BC, Canada. The optimal cutting temperature compound was obtained from Sakura Finetek U.S.A. Inc., Torrance, CA.

Results

Physiological parameters in all animal groups were recorded during intracerebral infusions. All physiological variables, including mean arterial blood pressure, blood pH, blood gases, hematocrit, and blood glucose, were controlled within normal ranges (Table 1).

At 24 hours after the intracerebral infusion of 30 μl of hemoglobin, brain swelling was found in the ipsilateral hemisphere, along with a midline shift (Fig. 1). Gross inspection of the brain revealed that most rats (four of five) in the hemoglobin infusion group developed transtentorial herniation. Herniation was not found in any animals in the control group, however.

Brain water content was increased in the ipsilateral basal ganglia 24 hours after intracerebral infusions of hemoglobin, hemin, bilirubin, and FeCl2, (82.2 ± 1.3%, 82.8 ± 2%, 81.8 ± 2.4%, and 83.5 ± 2.1%, respectively, compared with 77.9 ± 0.2% in the saline control; p < 0.01; Fig. 2A). Edema formation after hemoglobin, hemin, bilirubin, or FeCl2 infusions was associated with an accumulation of sodium ions (Fig. 2B).

Intracerebral coinjection of SnPP (90 nM) with hemoglobin attenuated hemoglobin-induced brain edema in the ipsilateral basal ganglia (80.2 ± 0.8% compared with 82.4 ± 1% in the hemoglobin group, p < 0.01) and cortex (80.5 ± 0.8% compared with 82.3 ± 1.6% in the hemoglobin group, p < 0.05) at 24 hours. We found that SnPP had no effect on brain water content in the contralateral hemisphere and cerebellum (Fig. 3).

Hemoglobin-induced brain edema was reduced in the ipsilateral basal ganglia (80.5 ± 1% compared with 82.4 ± 1%, p < 0.01) and cortex (80 ± 0.2% compared with 82.3 ± 1.3% in the control) by a large dose (500 mg/kg) of deferoxamine injected intraperitoneally. A low dose (50 mg/kg) of deferoxamine, however, had no significant effect on brain edema. The contralateral and cerebellar water contents were not affected by either deferoxamine dosage (Fig. 4).

In the normal brain, HO-1 protein was undetectable by Western blot analysis (data not shown). After an infusion of hemoglobin, however, HO-1 protein was upregulated in the ipsilateral basal ganglia 24 hours (3575 ± 850 pixels compared with 829 ± 369 pixels in saline control, p < 0.01; Fig. 5). Immunohistochemical studies also detected numerous HO-1 positive cells in the ipsilateral hemisphere 24 hours after hemoglobin infusion, whereas few HO-1 positive cells were found in the control group and in the contralateral hemisphere (Fig. 6).

Discussion

In this study we demonstrate that an intracerebral infusion of hemoglobin and its degradation products hemin, iron, and bilirubin, cause the formation of brain edema within 24 hours. Hemoglobin itself induces HO-1 upregulation in the brain, and HO inhibition by SnPP reduces hemoglobin-induced brain edema. In addition, an intraperi-
Toneal injection of a large dose of the iron chelator deferoxamine attenuates brain edema induced by hemoglobin. These results indicate that hemoglobin causes brain injury both by itself and through its breakdown products.

In a previous study it was demonstrated that an intracerebral infusion of lysed RBCs results in marked brain edema formation. This edema formation appears to be mediated by hemoglobin, because an intracerebral infusion of rat hemoglobin at concentrations found in erythrocytes also results in marked increases in brain water content. The osmolality of the infusate apparently does not contribute to the increases of brain water content, because no brain edema was found 2 hours after hemoglobin infusion. Osmotic equilibration occurs within approximately 30 minutes in the rat (Xi, et al., unpublished data).

Other studies indicate that hemoglobin has other deleterious effects on the brain. For example, an intracortical hemoglobin injection in rats produces chronic focal spike activity and gliosis. Hemoglobin inhibits Na+/K+ adenosine triphosphate activity, activates lipid peroxidation, exacerbates excitotoxic injury in cortical cell culture, and induces depolarization in hippocampal CA1 neurons. Koenig and Meyerhoff found that hemoglobin, at a concentration of 25 nM, induces the death of 50% of forebrain neurons in culture within 8 hours and 72% within 24 hours. A recent study in humans indicates that delayed brain edema after ICH is associated with a significant midline shift. We believe that this delayed edema (in the 2nd or 3rd weeks after onset of ICH in humans) is probably due to erythrocyte lysis and hemoglobin-induced brain damage. Oxyhemoglobin is a spasmogen that has been implicated in cerebral vasospasm after subarachnoid hemorrhage. In terms of ICH, an intraparenchymal infusion of lysed RBCs (oxyhemoglobin) failed to reduce cerebral blood flow in rats. In addition, ICH did not cause marked reductions of cerebral blood flow in animals. Finally, an intracerebral infusion of methemoglobin mimics the effects of lysed erythrocytes on edema formation.

In this study we have shown that intracerebral injection of hemin induces edema formation, indicating that this part of the hemoglobin molecule can cause brain injury. In addition, our study indicates that heme breakdown products that appear as the clot lyases play a major role in the formation of brain edema. Heme from hemoglobin is degraded by HO in the brain into iron, carbon monoxide, and biliverdin; the latter is then converted to bilirubin by biliverdin reduc-

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**Fig. 3.** Bar graph showing brain water contents in rats 24 hours after infusion of 30 μl of saline, vehicle, hemoglobin (Hb), or hemoglobin and SnPP. Values are expressed as the means ± SD. *p < 0.01 compared with saline or vehicle; **p < 0.05 compared with hemoglobin and SnPP; #p < 0.01 compared with the other groups.

**Fig. 4.** Bar graph showing brain water content in rats 24 hours after infusion of 30 μl of hemoglobin (Hb). The animals received intraperitoneal injections of either 1 ml saline (control) or 50 mg/kg or 500 mg/kg deferoxamine ([DFX] in 1 ml saline) right after intracerebral infusion of hemoglobin. Values are expressed as the means ± SD. #p < 0.01 compared with the saline group.

**Fig. 5.** Upper: The HO-1 levels were semiquantitated using Western blot analysis 24 hours after either saline control (lanes 1 to 3) or intracerebral infusion of hemoglobin (lanes 4 to 6). Lower: Bar graph depicting the results of Western blot analysis. Values are expressed as the means ± SD. #p < 0.01 compared with control.
Data from in vitro studies have shown that brief exposures (1–2 hours) of neurons to hemoglobin are not toxic, but their exposure to hemoglobin for 24 hours or more causes neuronal death. This delayed effect of hemoglobin may indicate that hemoglobin-induced brain injury results from its breakdown products.

Our study shows that an intracerebral infusion of iron causes brain edema, and that deferoxamine reduces hemoglobin-induced edema, indicating that iron plays an important role in edema formation after ICH. A cortical injection of iron causes focal epileptiform paroxysmal discharges. Iron and lipid peroxidation also have an important role in hemoglobin-induced brain injury. For example, a subpial injection of FeCl2 induces edema and lipid peroxidation in the brain. Iron can also stimulate the formation of free radicals, leading to neuronal damage; it is known that ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron react with lipid hydroperoxides to produce free radicals. Deferoxamine, an iron chelator, can cross the blood–brain barrier, and data from in vitro studies have shown that this substance reduces hemoglobin-induced brain Na+/K+ adenosine triphosphate inhibition and neuronal toxicity. Furthermore, antioxidants block neuronal toxicity induced by hemoglobin and iron.

Bilirubin is toxic to the brain although it is considered to be an antioxidant. Fifty years ago, Jackson injected bilirubin into the cisterna magna of dogs, causing severe inflammatory reactions. In more recent studies it has been demonstrated that bilirubin exerts many pernicious effects that cause brain injury, including inhibition of phosphorylation of the synaptic vesicle-associated protein synapsin I, disturbance in high-energy phosphate levels, reduction in mitochondrial activity, and disturbances in DNA synthesis, protein synthesis, and ion transport. Amit and Brenner found that bilirubin is toxic to neurons and astrocytes in vitro. Our results also indicate that bilirubin contributes to brain edema formation after ICH. It should be noted, however, that micromolar concentrations of bilirubin may also act as an antioxidant. Interruption of HO activity, which reduces bilirubin and free iron production, has provided a protective effect against hemorrhagic, ischemic, and traumatic brain injury.

Heme oxygenase consists of three enzymes, HO-1, HO-2, and HO-3. These forms have the following characteristics: HO-1, which has also been called heat shock protein 32, is induced by a variety of stimuli; HO-2, the constitutive isoform of HO, is localized mainly in the brain and testes; and HO-3 protein is a poor heme catalyst and is also found in the brain. Recent studies indicate that HO-1 upregulation increases free redox active iron production, which may cause cell injury, although other studies indicate that HO-1 plays an important role in cytoprotection against oxidative injury.

Our results demonstrate that hemoglobin upregulates HO-1 protein levels in the brain and that inhibition of HO by SnPP reduces hemoglobin-induced brain edema. From these results we infer that upregulation of HO-1 in a heme-rich environment that contains a high level of HO-2, which occurs in the perihematomal zone, might cause excess free iron and bilirubin accumulation, resulting in brain injury. This finding is supported by Suttner and Dennery, who found that HO-1 overexpression and reactive iron accumulation are associated with oxygen cytotoxicity. In addition, Lamb, et al., found that hemoglobin stimulates lipid peroxidation in microsomal and cellular systems and that peroxidation is reduced by HO inhibitors and iron chelators. When the concentration of heme products is not significantly increased, such as in cerebral ischemia, however, HO produces a low concentration of bilirubin and is neuroprotective.

Carbon monoxide is another product generated in the course of heme degradation by HO activity. Carbon monoxide is a free radical that causes brain injury that is analogous to nitric oxide–mediated damage.

Conclusions

After ICH, hemoglobin, the main product of lysed RBCs, plays a major role in delayed brain edema formation through its degradation products. Limiting hemoglobin degradation by HO inhibition and using iron chelators may be useful treatments for ICH.

References


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