Effect of Vasospasm on Heme Oxygenases in a Rat Model of Subarachnoid Hemorrhage

Minoru Kuroki, MD; Kenji Kanamaru, MD, DMSc; Hidenori Suzuki, MD; Shiro Waga, MD, DMSc; Reiji Semba, MD, PhD

Background and Purpose—Subarachnoid hemorrhage (SAH)-induced heme oxygenase-1 (HO-1) in glia throughout the rat brain without affecting heme oxygenase-2 (HO-2). However, the relationship between cerebral vasospasm and the expression of heme oxygenases after SAH is thus far unknown. The purpose of the present study was to clarify the effect of vasospasm on the expression of heme oxygenases in a rat model of SAH.

Methods—Endothelin, hemolysate, hemolysate saturated with carbon monoxide (CO-hemolysate), and saline were injected into the cisterna magna of adult rats. Angiography was repeated before each injection and 15 and 60 minutes and 24 hours after each injection. Immunocytochemistry for HO-1, HO-2, and glial fibrillary acidic protein (GFAP) was performed 24 hours after the injection.

Results—A significant vasospasm occurred in the basilar artery after the injection of endothelin, hemolysate, and CO-hemolysate. The degree of vasospasm was most prominent 15 minutes after each injection. There was no significant difference in the degree of vasospasm among injections. The HO-1 was induced exclusively in the glial cells throughout the brain after injection of hemolysate and CO-hemolysate; however, it was not induced by endothelin and saline. In the dentate gyrus of the hippocampus and the molecular layer of the cerebellum, the HO-1-positive cells were also stained for GFAP, suggesting astrocytic glial cells. On the other hand, HO-2 immunoreactivity was abundant in neurons and was not affected by endothelin, hemolysate, CO-hemolysate, or saline.

Conclusions—It is suggested that heme per se, rather than ischemia induced by vasospasm, plays a pivotal role in the expression of HO-1 in this rat model. (Stroke. 1998;29:683-689.)

Key Words: subarachnoid hemorrhage ■ vasospasm ■ heme oxygenase ■ endothelins

Despite the fact that subarachnoid hemorrhage (SAH) causes vasospasm and induces stress proteins such as HSP70 and heme oxygenase-1 (HO-1),1–4 it is still unknown whether the stress proteins are caused by the vasospasm or the SAH itself. Free radical generation and lipid peroxidation may be very important for the occurrence of vasospasm after SAH.5–8 Heme oxygenase catalyzes the rate-limiting step in heme degradation, yielding iron, biliverdin, and carbon monoxide (CO); biliverdin is subsequently reduced enzymatically to bilirubin by biliverdin reductase.9 Both bilirubin and CO are thought to have important physiological functions, bilirubin as an antioxidant10 and CO as a messenger molecule.11

HO exists as two isozymes, namely, HO-1 and HO-2. HO-2 is the constitutive form, and under normal conditions it is by far the predominant isozyme in the brain, where it is expressed in the cerebellum, forebrain, brain stem, and diencephalon.12 HO-1 under normal conditions is detectable in selective neuronal populations of the hippocampus and in the α-motor neurons in the spinal cord, the red nucleus, and dorsal raphe.13 However, HO-1 is induced in response to stress inducers such as oxidative stress, cadmium, ultraviolet light, phorbol ester, and hypoxia.14 To date, direct demonstration of the relationship between vasospasm and heme oxygenase after SAH has not been researched. In the present study, we investigated the effects of vasospasm on heme oxygenases in a rat model of SAH.

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Materials and Methods

All protocols were evaluated and approved by the Animal Ethics Review Committee of the Mie University School of Medicine. The animals were cared for in accordance with the guideline for animal experiments at the Mie University School of Medicine.

Study Protocol

Male Sprague-Dawley rats weighing 350 to 500 g (SLC) were anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg), intubated with a 16- or 18-gauge elastic catheter, and allowed to breathe spontaneously. The rectal temperature was kept at approximately 37°C with an electric heating pad, and end-tidal CO2 was monitored (POET 601, Criticare System Inc). Anesthesia was maintained by repeated injections of chloral hydrate, if needed. After cannulation of both radial arteries with 22-gauge catheters, 0.3 mL autologous arterial blood was withdrawn, and the same volume of saline was injected in hemolysate and CO-saturated hemolysate (CO-hemolysate) groups. Both catheters were connected with a three-way stopcock to a pressure transducer (MK 12030 US, Baxter)
to measure blood pressure. Vertebral angiography was performed by manual injection of a contrast medium (0.3 mL Iomeron 400, Eisai) into both radial arteries simultaneously. The basilar artery diameter was determined at three different points: 0.5 mm above the junction of the vertebral arteries and halfway and just below the bifurcation of the posterior cerebral arteries. During the angiography, exposure factors were kept constant, and a radiopaque control standard was used for correction to constant magnification. An experienced person who was unaware of the treatment groups measured the diameter of the basilar artery three times with a calibrated optical micrometer (scale loupe No. 1983, PEAK).

After angiography, animals were placed in a head holder with the head at 30° of flexion. A midline skin incision was made from the middle of calvarium to the lower cervical spine. The occipital bone was cleared of muscular attachments by sharp dissection. Under a surgical microscope, the atlanto-occipital membrane was dissected and punctured for the injection of each material. The animals were randomly assigned into five groups: a sham-operation group (n = 6), an endothelin group into which 1.2 μg/kg endothelin (Sigma) was injected (n = 6), a saline group (n = 6), an endothelin group into which 1.2 × 10⁻¹⁰ mol/kg endothelin (Sigma) was injected (n = 7), a hemolysate group (n = 6), and a CO-hemolysate group (n = 6). After the injection of each material, angiography was repeated at 15 and 60 minutes. The catheters were then removed, and the wounds were closed. After 24 hours the animals were anesthetized again and a third angiography was performed in the same manner as described above. During the course of observation, the animals were allowed access to food and water ad libitum.

### Immunocytochemistry for Heme Oxygenases

The animals in the sham-operation (n = 3), saline (n = 3), endothelin (n = 6), hemolysate (n = 6), and CO-hemolysate (n = 6) groups were killed 24 hours after the operation. The rats were anesthetized with intraperitoneal chloral hydrate (600 mg/kg) and perfused with the use of a cardiac catheter with 200 mL normal saline followed by 500 mL 4% paraformaldehyde in 0.1 mol/L phosphate buffer saline (PBS, pH, 7.4). The brains were postfixed in 4% paraformaldehyde in 0.1 mol/L PBS containing 0.02% sodium azide. Tissue sections were washed twice in 0.1% gum arabic, the sections were developed with the silver enhancement kit (Amersham) for 20 minutes. Sections were then washed and incubated with rabbit polyclonal anti-GFAP for 24 hours at 48°C. Sagittal sections were cut into 50 mm thickness with a sliding microtome and placed in PBS containing 0.02% sodium azide. Tissue sections were washed twice with PBS for 5 minutes. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxide in PBS for 60 minutes, and nonspecific binding of antibodies was prevented by preincubation of sections in 10% normal goat serum. Sections were then incubated for 16 hours with antibodies against HO-1 (OSA 100, StressGen Biotechnologies) at a dilution of 1:1000 or against HO-2 by incubation with CO-hemolysate injection groups. Percentages indicate mean ± SD values in each group. pre indicates pretreatment; m, minutes after each treatment; and h, hours after each treatment. *P < 0.05, **P < 0.005.

and demonstrated no staining. After being washed in PBS, sections were incubated with biotinylated rabbit IgG antibodies (1:200) and freshly prepared ABC solution (1:50; Vectastain; Vector Laboratories), each for 1 hour.

Peroxidase activity was revealed by incubation with 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Chemical Institute) with 0.01% H₂O₂, and the sections were dehydrated, cleared, and mounted with Entellan (Merck).

### Immunofluorescent Double Labeling for Astrocyte

The immunogold–silver staining method for HO-1 followed by FITC double staining for GFAP was used. Sections were washed in PBS, coated with Logol's iodine solution for 10 minutes, and rinsed thoroughly. All traces of iodine were then removed with 2.5% sodium thiosulfite, and the sections were incubated with 1% skim milk for 10 minutes to block nonspecific background staining. Sections were incubated with antibodies against HO-1 for 16 hours, washed with PBS, and left in gold-labeled goat anti-rabbit IgG for 3 hours. After being washed with PBS and 0.2 mol/L phosphate buffer (pH 7.2) containing 0.1% gum arabic, the sections were developed with the silver enhancement kit (Amersham) for 20 minutes. Sections were then washed and incubated with rabbit polyclonal anti-GFAP for 24 hours at 48°C. Alternate control sections were incubated without anti-GFAP and demonstrated no staining. After being washed in PBS, the sections were incubated with FITC-conjugated goat anti-rabbit IgG antibody for 2 hours. Sections were placed on a coverslip in a mixture of glycerol/PBS (4:1), and fluorescence microscopy was performed with use of a microscope with a specific filter for FITC.

### Statistical Analysis

Data were expressed as mean ± SD. Comparisons within groups and intergroup comparisons were made with the use of ANOVA. The level of significance of all tests of comparison was P < 0.05.

### Selected Abbreviations and Acronyms

- CO-hemolysate: hemolysate saturated with carbon dioxide
- GFAP: glial fibrillary acidic protein
- HO-1: heme oxygenase-1
- HO-2: heme oxygenase-2
- SAH: subarachnoid hemorrhage

### Table: Physiological Parameters Before and After Injection

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>Mean Arterial Blood Pressure, mm Hg</th>
<th>Heart Rate, beats/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre 24 h</td>
<td>Pre 15 min</td>
<td>60 min</td>
</tr>
<tr>
<td>Saline (3)</td>
<td>383 ± 35</td>
<td>383 ± 35</td>
<td>98.7 ± 7.0</td>
</tr>
<tr>
<td>Endothelin (4)</td>
<td>441 ± 21</td>
<td>438 ± 23</td>
<td>94.7 ± 9.5</td>
</tr>
<tr>
<td>Hemolysate (6)</td>
<td>447 ± 25</td>
<td>446 ± 28</td>
<td>92.7 ± 6.8</td>
</tr>
<tr>
<td>CO-hemolysate (6)</td>
<td>445 ± 30</td>
<td>443 ± 40</td>
<td>94.3 ± 8.9</td>
</tr>
</tbody>
</table>

Pre indicates pretreatment; min, minutes after injection; h, hours after injection; and ND, not determined. Values are mean ± SD.
Results

Neurological Deficit and Physiological Parameters
No rats developed neurological deficits. Comparisons of mean values for body weight, mean arterial blood pressure, heart rate, and end-tidal CO₂ measured before and at 15 and 60 minutes and 24 hours after injection revealed there were no significant differences in the groups (Table 1).

Table 1. End-tidal CO₂, mm Hg

<table>
<thead>
<tr>
<th></th>
<th>pre</th>
<th>15 min</th>
<th>60 min</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>40.7±2.3</td>
<td>40.7±2.3</td>
<td>ND</td>
<td>41±2.3</td>
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<tr>
<td>39.0±1.8</td>
<td>38.8±1.4</td>
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<td>38.8±1.8</td>
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<tr>
<td>39.7±1.5</td>
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<td>37.2±1.8</td>
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<td>38.7±2.7</td>
<td>39.3±1.2</td>
<td></td>
</tr>
</tbody>
</table>

Angiographic Vasospasm
There was no significant vasospasm in the saline group. On the other hand, a significant vasospasm occurred in the basilar artery after intracisternal injections of endothelin, hemolysate, and CO-hemolysate (Fig 1). Vasospasm was most prominent at 15 minutes after each injection. At 60 minutes after injection, a significant vasospasm was noted in the hemolysate and CO-hemolysate groups. The basilar artery diameter returned to preinjection level after 24 hours.

Expression of HO-1
HO-1 immunoreactivity was detectable in the brains of sham-operated and saline-injected control rats (Fig 2A through 2C). HO-1-positive cells were noted in the hippocampal neurons, especially in the hilum of the dentate gyrus and hypothalamus. Occasionally, solitary HO-1 immunoreactive glial cells were observed in the striatum or cerebral cortex. In addition, the ependyma was slightly stained throughout the ventricles. After the injection of endothelin, HO-1 immuno-
reactivity was similar to that seen in the sham-operated and saline-injected groups (Fig 2E through 2G).

Marked induction of HO-1 immunoreactivity was noted throughout most regions of the brain following injection of hemolysate (Fig 3A through 3C) and CO-hemolysate (Fig 3E through 3G). A significant increase in the numbers of HO-1-positive cells occurred in most brain regions of the animals that received hemolysate and CO-hemolysate (Figs 2 and 3). The increased HO-1 immunoreactivity was localized in small cells diffusely distributed throughout the neocortex, hippocampus, and hindbrain (Fig 3).

Expression of HO-2
HO-2 immunoreactivity was noted in neurons throughout the brain. HO-2 expression in the hemolysate and CO-hemolysate groups was similar to that observed in the sham-operated and saline-injected animals (Figs 2D and 2H, 3D and 3H). There was no increase or decrease of HO-2 immunostaining detected in the animals in any of the groups in this study.

Double Labeling for HO-1 and GFAP
Double-labeling immunocytochemical studies revealed that HO-1 was induced primarily in GFAP immunoreactive astrocytes in the hippocampus (Fig 4A and 4B) and cerebellum (Fig 4C and 4D). In the cerebellum, HO-1 immunoreactivity was observed in the Bergmann glia cells, which extend long processes into the molecular layer (Fig 4C and 4D).

Discussion
The major findings of the present study were as follows: (1) significant vasospasm occurred in the basilar artery after injection of endothelin, hemolysate, and CO-hemolysate; (2) HO-1 was induced exclusively in glial cells throughout the brain after injection of hemolysate and CO-hemolysate but was not induced by endothelin or saline; and (3) no increase or decrease of HO-2 immunostaining was detected in animals in any of the groups.

This is the first study to demonstrate angiographic vasospasm and evaluate the effect of vasospasm on heme oxygenase
expression in a rat model. Endothelin, hemolysate, and CO-hemolysate all induced a significant vasospasm to the same degree. Reportedly, a 40% decrease of cerebral blood flow was demonstrated in the same rat model of SAH.14 It was inferred that if cerebral ischemia plays a major role in the expression of HO-1, induction of HO-1 would be observed in these three groups. However, HO-1 was not induced after the injection of endothelin in spite of vasospasm. It was demonstrated that injections of the protein BSA into the cisterna magna also did not induce HO-1.2 Hence, neither a nonspecific protein nor a compound that produces vasospasm induce HO-1. Therefore, neither a nonspecific protein nor a compound that produces vasospasm induce HO-1 throughout the brain. Therefore, cerebral ischemia after the injection of endothelin, hemolysate, and CO-hemolysate might not be a major causative factor for the expression of HO-1 in this model. Rather, heme per se might play a pivotal role in the expression of HO-1. The large amount of heme in blood is likely taken up into cells where it induces HO-1 and in turn is metabolized by HO-1.9 Hence, neither a nonspecific protein nor a compound that produces vasospasm induce HO-1.2 It was reported that oxyhemoglobin may be the causative factor of vasospasm in SAH. In the CO-hemolysate group, oxyhemoglobin was eliminated by saturating the hemolysate with CO gas. However, the hemolysate without oxyhemoglobin was as efficient as hemolysate with oxyhemoglobin in inducing acute vasospasm and HO-1. Therefore, oxyhemoglobin does not seem to be essential for the induction of acute vasospasm and HSP; CO-hemoglobin is as efficient as oxyhemoglobin. Because lipid peroxidation takes place in a subarachnoid clot,8 both nonenzymatic and enzymatic lipid peroxidation metabolites should be inducers of HO-1.15 HO-1 might be induced by the peroxidized lipid formed in the hemolysate. However, this model does not produce chronic cerebral vasospasm, and in this sense it has significant limitations in the study of the clinical entity of SAH. Thus, the expression of HO-1 in chronic vasospasm remains to be investigated.

It has been shown that SAH induces HO-1 in Bergmann glia in the molecular layer but not in cells in other layers, and there is diffuse induction in the glia in both cerebellar hemispheres and vermis.24 Hence it was postulated that the HO-1 induction in the cerebellum might reflect direct injury by blood products due to local release of large amounts of catalytically active iron and resulting oxidative injury.4 Since heme oxygenases are the rate-limiting step in the metabolism of heme to bilirubin,9 increases in their activity should increase the rate of degradation of heme, thus increasing the formation of bilirubin, which may in turn play a protective role as an antioxidant.10 Increased degradation of heme also increases the release of free iron from heme and allows the cell to begin the process of iron sequestration through ferritin.16 Degradation of heme produces what may be a physiological regulation of cGMP and plays a role in the regulation of cerebral blood flow through smooth muscle relaxation and vasodilatation.11,17 In rabbit and canine basilar arteries, however, it has been suggested that a direct effect of CO was not likely and that a reduction in oxygen delivery due to changes in the oxyhemoglobin dissociation curve may be responsible for increases in cerebral blood flow.18 The effects of CO on cerebral arterioles remains to be clarified.18 We are unaware of any previous demonstration of angio-graphic vasospasm after intracisternal injection of endothelin in rats.9 A number of points favor a role for endothelins as mediators of vasospasm, namely: (1) endothelins are extremely potent constrictors of human cerebral vessels20 and induce chronic vasospasm in dogs21; (2) endothelin A receptor antagonist FR139317 has been shown to reduce vasospasm in a two-hemorrhage model of SAH22; (3) production of endothelin is stimulated by various vasoactive substances present in the blood, including arginine vasopressin, angiotensin II, and thrombin19 (excessive local production of these substances is reported after SAH23,24); and (4) excessive concentrations of oxyhemoglobin may increase production of endothelin25,26 (significant increases of endothelin in the cerebrospinal fluid coincides with the appearance of clinically relevant vasospasm27).
In conclusion, intracisternal injection of endothelin did not induce HO-1. Heme per se, rather than ischemia induced by vasospasm, therefore plays a pivotal role in the expression of HO-1 in this rat model.

Acknowledgments
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References
pus, thalamus, hypothalamus, brain stem, and cerebellum. We have postulated that heme from hemoglobin is selectively taken up into microglia throughout the entire brain, where it induces the HO-1 gene by actions on a heme- or iron-sensitive element in the HO-1 promoter. These results suggest that the microglia have specialized systems for taking up the heme following subarachnoid hemorrhages and for metabolizing the heme to iron, biliverdin, and carbon monoxide.

An interesting finding not mentioned in the present study is that subarachnoid injections of lysed blood also produce focal regions of stress gene induction—including induction of HSP70 and HO-1. These focal regions vary in size and location and are produced by single injections of lysed blood but not by single injections of pure hemoglobin. We have speculated that these focal regions represent regions of ischemic injury produced by vasospasm resulting from the lysed blood injections. We propose that the focal regions of stress gene expression can be used to assess the efficacy of drugs that might be useful in the treatment of subarachnoid hemorrhage.

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