Astrocyte Overexpression of Heme Oxygenase-1 Improves Outcome After Intracerebral Hemorrhage

Jing Chen-Roetling, PhD; Wei Song, MD, PhD; Hyman M. Schipper, MD, PhD; Christopher S. Regan; Raymond F. Regan, MD

Background and Purpose—Heme oxygenase-1 (HO-1) catalyzes the rate-limiting reaction of heme breakdown and may have both antioxidant and pro-oxidant effects. In previous studies, HO-1 overexpression protected astrocytes from heme-mediated injury in vitro. In the present study, we tested the hypothesis that selective astrocyte overexpression of HO-1 improves outcome after intracerebral hemorrhage.

Methods—Male and female transgenic mice overexpressing human HO-1 driven by the GFAP promoter (GFAP,HMOX1) and wild-type controls received striatal injections of autologous blood (25 μL). Blood–brain barrier disruption was assessed by Evans blue assay and striatal cell viability by methylthiazolyldiphenyl-tetrazolium bromide assay. Neurological deficits were quantified by digital analysis of spontaneous cage activity, adhesive removal, and elevated body swing tests.

Results—Mortality rate for wild-type mice was 34.8% and was similar for males and females; all GFAP,HMOX1 mice survived. Striatal Evans blue leakage at 24 hours was 23.4±3.2 ng in surviving wild-type mice, compared with 10.9±1.8 ng in transgenics. Perihematomal cell viability was reduced to 61±4% of contralateral at 3 days in wild-type mice, versus 80±4% in transgenics. Focal neurological deficits were significantly reduced and spontaneous cage activity was increased in GFAP,HMOX1 mice.

Conclusions—Selective HO-1 overexpression in astrocytes reduces mortality, blood–brain barrier disruption, perihematomal cell injury, and neurological deficits in an autologous blood injection intracerebral hemorrhage model. Genetic or pharmacological therapies that acutely increase astrocyte HO-1 may be beneficial after intracerebral hemorrhage. (Stroke. 2015;46:1093-1098. DOI: 10.1161/STROKEAHA.115.008686.)

Key Words: blood-brain barrier • heme oxygenase (decyclizing) • ischemic preconditioning • stroke

The heme oxygenases catalyze the breakdown of heme to carbon monoxide, ferrous iron, and biliverdin, which is subsequently reduced to bilirubin. The consequences of this reaction on outcome in hemorrhagic stroke models have been variable to date. Mice lacking heme oxygenase-1 (HO-1), the inducible isoform, sustained less injury and inflammation than their wild-type (WT) counterparts after collagenase-induced intracerebral hemorrhage (ICH). Unconditional knockout of HO-2, the predominant neuronal isoform, had the opposite effect, but it was somewhat protective in the blood injection ICH model. HO inhibitors, which have less usefulness in mechanistic studies because of their multiple off-target effects, have also been variably protective or deleterious.

These conflicting reports may be reconciled by consideration of the diverse biological activity of heme breakdown products and the selective vulnerability of neurons to iron. In cell culture, neuron loss after hemin or hemoglobin exposure is increased by HO-2 expression. This effect is mitigated by protein or low molecular weight iron chelators, resulting in net neuroprotection that may be mediated by the other reaction products. Astrocytes, which are relatively resistant to acute iron toxicity because of ferritin induction, are protected from hemin by both HO-1 and HO-2 in the absence of exogenous chelators. The effect of HO expression in any model may, therefore, depend on the cell population at greatest risk and the iron binding capacity of the cellular microenvironment.

These results suggest that nonselective targeting of HO expression or activity may not be an optimal therapeutic strategy. The efficacy of therapies that modulate HO in specific cell populations in vivo has not been defined. We have previously reported that increasing HO-1 expression in astrocytes was protective in a cell culture model of acute hemin toxicity. In the present study, we tested this concept in vivo, using transgenic mice expressing human HO-1 controlled by the GFAP promoter and an established ICH model.

Methods

Animals
Mice were transferred from the Schipper laboratory to Thomas Jefferson University where they were bred in a facility accredited
ICH Model
Mice were randomly selected from WT and GFAP.HMOX1 cohorts of similar age and were anesthetized with 2% isoflurane in oxygen delivered via a nose mask. Temperature was monitored with a rectal probe and maintained at 37±0.5°C with a heating lamp. The scalp was incised, and a burr hole was made at the following stereotactic coordinates relative to bregma: 2.5 mm lateral, 0.5 mm rostral. Blood obtained from a tail vein was loaded into a Hamilton syringe with 28 gauge needle, which was inserted 3.0 mm rostral. Blood–Brain Barrier Permeability Assay

Blood from a tail vein was collected for 5 μL of the solution obtained was extracted with 2 mL isopropanol. Absorbance (562 nm) of the solution obtained from each blood-injected striatum was normalized to that of the contralateral striatum to yield an index of cell viability. The close agreement of this method with perihematomal injury quantified by cell counts of striatal tissue sections has been established.2,18,20

Behavioral Outcome Measures
Functional outcome was assessed as previously described,21 via: (1) quantification of spontaneous cage activity in 1-hour videos, using Homecare Scan (CleverSys, Reston, VA); (2) adhesive removal test; and (3) elevated body swing test.

Immunoblotting and Immunostaining
The following antibodies were used: (1) anti-HO-1 (Enzo Life Sciences, Farmingdale, NY; Cat ADI-SPA-895) 1:4000 dilution for immunoblotting and 1:250 for immunostaining; (2) Anti-GFP (InVitrogen; Cat 130300) 1:1000 dilution; (3) anti-flag M2 antibody (Sigma-Aldrich; Cat F1804) 1:250 dilution. Methods have been detailed previously.18,20

HO-1 ELISA
Mouse and human HO-1 were quantified in injected and contralateral striata using ELISA kits marketed by Enzo Life Sciences (Cat. ADI-960-071 and ADI-EKS-800, respectively), following the manufacturer’s instructions.

Hemoglobin Assay
Blood (10 μL) collected from a tail vein was added to 5 μL of 3.5% sodium citrate to prevent clotting. Cells were lysed by passage through 3 freeze–thaw cycles. After centrifugation to remove debris, hemoglobin concentration was determined by the method of Winterbourne.22

Statistical Analyses
Mortality data were analyzed with Fisher’s exact test. Other data were analyzed with 1-way ANOVA and the Bonferroni multiple comparisons test.

Results
A total of 139 mice were used in these experiments (71 males and 68 females). The presence of the HMOX1 and tetracycline activator genes in transgenic mice was confirmed by genotyping (Figure 1A). Immunoblotting demonstrated a moderate increase in striatal HO-1 expression in GFAP, HMOX1 mice when compared with their WT counterparts (Figure 1B). Double immunofluorescence staining indicated that striatal HO-1 expression was predominantly localized to GFAP+ astrocytes in both WT and transgenic mice (Figure 2). Increased HO-1 immunoreactivity was apparent in the latter group, particularly in proximity to microvessels. Mean blood hemoglobin concentration was similar in WT and GFAP, HMOX1 mice (7.75±0.13 and 7.74±0.12 mmol/L heme, respectively).

Reduced Mortality in GFAP.HMOX1 Mice
Sixteen of 46 WT mice died within 24 hours of blood injection (Table), with similar mortality rates in males and females. No deaths were observed at later time points. All transgenic mice survived until completion of the experiment. Mortality reduction was significant for both males and females.
Astrocyte HO-1 Overexpression Reduces Blood–Brain Barrier Disruption

We have recently reported that systemic hemin therapy induces HO-1 overexpression in perivascular cells and reduces blood–brain barrier injury after experimental ICH. Because astrocyte HO-1 overexpression is particularly prominent in GFAP.HMOX1 mice adjacent to vessels (Figure 2), blood–brain barrier integrity was assessed 24 hours after striatal blood injection. Evans blue leakage into the striatal parenchyma at this time point in GFAP.HMOX1 mice was less than half of that in WT mice (Figure 3A).

Reduced Perihematomal Cell Loss in GFAP.HMOX1 Mice

Striatal blood injection resulted in loss of ≈ 40% of cells in the ipsilateral striata of WT mice, with minimal change between days 3 and 7 (Figure 3B). Striatal cell viability was significantly improved in transgenic mice (P<0.05–0.01 versus WT controls).

Effect of ICH on HO-1 Expression

Striatal HO-1 was quantified 7 hours after blood injection using ELISA kits specific for mouse or human HO-1. Consistent with previous observations, native mouse HO-1 expression

Table. Astrocyte HO-1 Overexpression Reduces Mortality After Intracerebral Hemorrhage

<table>
<thead>
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<th>WT Mortality, %</th>
<th>Tg HO-1 Mortality, %</th>
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<tr>
<td>Both</td>
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Mortality rates in WT and GFAP.HMOX1 mice (Tg HO-1) after striatal injection of 25-μL autologous blood. All deaths occurred within 24 hours. HO-1 indicates heme oxygenase-1; Tg, transgenic; and WT, wild-type.

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**Figure 1.** A, Polymerase chain reaction genotyping products demonstrating presence of Flag-HMOX1 (heme oxygenase-1 [HO-1]) and tTa genes in GFAP.HMOX1 mice. B, Immunoblots of striatal lysates of GFAP.HMOX1 transgenic (Tg) mice and wild-type (WT) controls, demonstrating increased HO-1 in transgenics; expression of transgene is confirmed by immunoreactivity to Flag peptide. Bar order correlates with lane order. *P<0.05 vs WT, 6 per condition.

**Figure 2.** Increased striatal heme oxygenase-1 (HO-1) expression in GFAP.HMOX1 (Tg) mice. Sections were immunostained with antibodies to HO-1 and GFAP. Increased HO-1 expression is apparent in GFAP.HMOX1 mice, particularly in perivascular astrocytes. WT indicates wild-type.

**Figure 3.** Astrocyte heme oxygenase-1 (HO-1) overexpression reduces blood–brain barrier breakdown and perihematomal cell death after intracerebral hemorrhage (ICH). A, Evans blue leakage into the striatum was quantified 24 hours after ICH, 7 to 8 per condition. B, Striatal cell viability was quantified 3 and 7 days by methythiazolyldiphenyl-tetrazolium bromide assay, 5 to 12 per condition. *P<0.05, **P<0.01 vs wild-type (WT).
in injected striata was increased ≈2.2-fold compared with contralateral in both WT and GFAP.HMOX1 mice (Figure 4). Human HO-1 was expressed at a higher level in transgenics and was similar in injected and contralateral striata.

**Effect of Astrocyte HO-1 Overexpression on Behavioral Outcome**

Spontaneous cage activity before striatal blood injection was similar in WT and GFAP.HMOX1 mice. It was decreased by ≈75% in WT mice 24 hours after injection, compared with ≈40% in transgenics (P<0.05; Figure 5). A similar benefit at this early time point only was observed in the adhesive removal test (P<0.001); surviving WT mice rapidly regained function. A persistent deficit was observed in the elevated body swing test in WT mice, and benefit was noted in transgenics at a later time point.

**Discussion**

This study provides the first evidence that selective overexpression of HO-1 in astrocytes is beneficial after ICH. The GFAP.HMOX1 transgene reduced mortality, an end point of obvious clinical relevance but an uncommon outcome measure in rodent ICH studies. Unequal mortality in WT and transgenic groups may have confounded the results of other planned outcome measures because the most severely injured mice in only the transgenic group survived for later testing. Although this bias would be predicted to diminish the observed benefit of astrocyte HO-1 overexpression, GFAP.HMOX1 mice nevertheless sustained significantly less blood–brain barrier injury, perihematomal cell loss, and sensorimotor deficits than surviving WT mice.

These observations are consistent with previous reports that peripheral hemin injection and ischemic preconditioning are protective in ICH models.21,24 Both of these therapies not only increase central nervous system (CNS) HO-1 expression but also have pleiotropic effects on signaling pathways and gene expression that limit their utility as mechanistic probes. The present findings provide more specific evidence that HO-1 overexpression per se is sufficient to manifest protection. It is noteworthy that Zeynalov et al25 observed that the benefit of ischemic preconditioning in a permanent middle cerebral artery occlusion model was lost in HO-1 knockout mice, consistent with an effect that also requires HO-1 expression.

![Figure 4](http://stroke.ahajournals.org/)

**Figure 4.** Striatal heme oxygenase-1 (HO-1) expression after intracerebral hemorrhage. Bars indicate mean mouse (A), human (B), and total (C) HO-1 in injected (Inj) and contralateral (Con) striata of wild-type (WT) and GFAP.HMOX1 (Tg) mice (±SEM, 6 per condition) 7 hours after striatal blood injection. ***P<0.001 vs contralateral expression (A) or corresponding WT condition (B and C).

![Figure 5](http://stroke.ahajournals.org/)

**Figure 5.** Astrocyte heme oxygenase-1 (HO-1) overexpression reduces neurological deficits. Bars represent mean values (±SEM; 5–7 per condition) at indicated days after striatal blood injection for (A) activity time (sum of seconds walking, feeding, vertical hanging, rearing and jumping during 1-hour recording); (B) percentage left swings; and (C) left minus right adhesive removal times.
Astrocytes play a key role in the development and maintenance of the blood–brain barrier in the rodent CNS. Experimental evidence suggests that HO-1 expression may be essential for optimizing this function in an oxidative environment. Alfieri et al have recently reported that preconditioning stimuli increase HO-1 expression primarily in perivascular astrocytes, associated with preservation of barrier function in a rat transient middle cerebral artery occlusion model. Consistent with this observation, Evans blue leakage into the brain parenchyma was decreased by about half in GFAP.HMOX1 mice, which overexpress HO-1 prominently in perivascular astrocytes. These results add to the growing body of evidence that HO-1 improves microvascular function after a wide variety of acute injuries, including cardiac, liver, and bowel ischemia-reperfusion, hemorrhagic shock, seizures, and sickle cell disease.

The protective effect of astrocyte HO-1 overexpression after ICH observed in 3 to 6-month-old GFAP.HMOX1 mice contrasts with the pathology that develops with aging in these transgenic animals. At 48 weeks, they exhibit behavioral disturbances characterized by hypokinesia and impaired prepulse inhibition, associated with glial iron deposition, oxidative mitochondrial injury, macroautophagy, and neuritic degeneration. The adverse effect of this transgene in older mice suggests that therapies producing a long-term increase in astrocyte HO-1 expression may be ineffective or poorly tolerated. It also highlights the need to test therapies that increase astrocyte HO-1 in older animals.

Striatal blood injection is usually a nonlethal event in rodents. In previous studies from this laboratory, mortality in Swiss-Webster or C57BL/6×129 mice has ranged from 0% to 34% in-hospital mortality in the United States. A moderate mortality rate facilitates its use as a primary outcome measure. Although most rodent studies are not designed to quantify the injury severity of clinical ICH, which also has a mortality rate of thrombin on glutamate release and glutamate receptor dysfunction. However, the effect of microglial HO-1 on hemorrhagic CNS injury is unknown, but previous results suggest that it may be deleterious. Wang and Doré reported that microglial activation and perihematomal free radical levels were reduced in conditional HO-1 knockout mice, consistent with a proinflammatory effect. This was associated with reduced mean lesion volume and early neurological deficits. The specific contribution of microglial HO-1 to ICH-related injury could be determined in future experiments using transgenic mice selectively overexpressing human HO-1 in the microglial compartment.

HO-1 is readily induced in cultured astrocytes, which are then robustly protected against heme-mediated injury. The translational potential of upregulating astrocyte HO-1 after ICH remains undefined, but initial reports suggest feasibility. Systemic therapy with the nonspecific inducers sulforaphane and hemin increased HO-1 in perivascular cells including astrocytes in naïve and injured rodent brains. This resulted in improvement in barrier function and neurobehavioral outcome, with clinically relevant time windows in traumatic brain injury and ICH models. The present results provide a rationale for the testing of genetic and pharmacological therapies that more specifically enhance HO-1 expression or activity in astrocytes after acute hemorrhagic injuries.

References


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