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Original Articles

Heme Oxygenase-2 Is Neuroprotective in Cerebral Ischemia

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Abstract

Heme oxygenase (HO) is believed to be a potent antioxidant enzyme in the nervous system; it degrades heme from heme-containing proteins, giving rise to carbon monoxide, iron, and biliverdin, which is rapidly reduced to bilirubin. The first identified isoform of the enzyme, HO1, is an inducible heat-shock protein expressed in high levels in peripheral organs and barely detectable under normal conditions in the brain, whereas HO2 is constitutive and most highly concentrated in the brain. Interestingly, although HO2 is constitutively expressed, its activity can be modulated by phosphorylation. We demonstrated that bilirubin, formed from HO2, is neuroprotectant, as neurotoxicity is augmented in neuronal cultures from mice with targeted deletion of HO2 ($HO2^{-/-}$) and reversed by low concentrations of bilirubin. We now show that neural damage following middle cerebral artery occlusion (MCAO) and reperfusion, a model of focal ischemia of vascular stroke, is substantially worsened in $HO2^{-/-}$ animals. By contrast, stroke damage is not significantly altered in $HO1^{-/-}$ mice, despite their greater debility. Neural damage following intracranial injections of *N*-methyl-D-aspartate (NMDA) is also accentuated in $HO2^{-/-}$ animals. These findings establish HO2 as an endogenous neuroprotective system in the brain whose pharmacologic manipulation may have therapeutic relevance.

Introduction

Heme oxygenase (HO) is the rate-limiting enzyme in heme degradation catalyzing cleavage of the heme ring to form ferrous iron, carbon monoxide (CO), and biliverdin. Biliverdin is rapidly reduced by biliverdin reductase to bilirubin (BR) so that in intact tissues biliverdin rarely accumulates and the physiologic product of HO is primarily BR. Two principal forms of HO have been distinguished and molecularly cloned (1). HO1 is an inducible enzyme, also designated as heat-shock protein-32, with new HO1 protein synthesis elicited by multiple stimulants, especially those associated with red blood cell damage, such as heme and other porphyrins. HO1 is con-

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centrated in tissues such as the spleen and liver, which degrade heme from aged red blood cells. By contrast, HO2 is constitutive and most concentrated in the brain and testes (2), accounting for the great majority of HO activity in the brain, where HO2 is localized to selective neuronal populations (1,3).

Of the three products of HO, CO has been most extensively studied, especially as a potential neurotransmitter in the brain and autonomic nervous system (1,3,4). Recent evidence implicates bilirubin as an antioxidant neuroprotectant (5,6). Neurotoxicity in brain cultures is exacerbated by HO inhibitors and in cultures from mice with targeted deletion of HO2 ($HO2^{-/-}$), whereas nanomolar concentrations of bilirubin are neuroprotective (5). BR elicits substantial antioxidant effects and is probably the most abundant endogenous antioxidant in mammalian tissues (6–8). Free radicals have been implicated as a causative agent in ischemic damage.

In the present study, we demonstrate that neural damage following middle cerebral artery occlusion (MCAO), a model of focal ischemia of vascular stroke, is substantially worsened in $HO2^{-/-}$ animals whereas stroke damage is not significantly affected in $HO1^{-/-}$ mice. These findings implicate HO2 as an endogenous neuroprotective system in the brain whose pharmacologic manipulation may have therapeutic relevance.

Materials and Methods

MCAO and Reperfusion

The study was conducted in accordance with National Institutes of Health (NIH) guidelines for the use of experimental animals, and protocols were approved by the Institutional Animal Care and Use Committee. Adult male mice (23–28 g) were obtained from Kenneth D. Poss and Susumu Tonegawa and have been described previously (4,9–11). The protocol used for MCAO was similar to one used before (12), with a few modifications. Briefly, under halothane anesthesia, a 6-0 monofilament was inserted into the internal carotid artery through the stump of the external carotid artery and advanced 6 mm past the origin of the pterygopalatine artery such that the blunted tip was positioned near the origin of the middle and anterior cerebral arteries. Because the posterior communicating artery was relatively large in these animals, a second monofilament was inserted via the external carotid artery and advanced up the internal carotid artery 4.5 mm past the pterygopalatine artery bifurcation to reduce collateral blood flow. Reperfusion was accomplished by withdrawal of the sutures and anesthesia was discontinued.

Using a different cohort of animals, the femoral artery was cannulated for measurement of arterial blood gases and blood pressure. Rectal temperature was maintained throughout the experiment with heating pads in all animals and no significant differences were observed among the different groups. The skull was thinned with a drill at 3 mm posterior and 5 mm lateral to the bregma, and a laser-Doppler flow probe was fixed in position. This region represents an area of dense ischemia. Red cell flux during ischemia and reperfusion was expressed as a percent of baseline.

In other cohorts of halothane-anesthetized mice, blood flow distribution was measured at 1 hr of ischemia by intravenous infusion of 4 μCi [^{14}C]iodoantipyrine (NEN Life Sciences, Boston, MA) over a 45-sec period. Arterial blood was sampled at 5-sec intervals to generate the time integral of the arterial concentration of the tracer. At 45 sec, mice were decapitated and brains quickly frozen. Autoradiographic images of 20-μm sections were digitized and the regional blood flow was calculated as described before (13).

Intrastriatal N-*methyl*-D-*aspartate (NMDA) Injection*

Mice were anesthetized and unilateral stereotaxic microinjections (0.3 μl over 3-min period) were performed using 0.1 M phosphate-buffered saline (PBS) with or without NMDA (Sigma) following the protocol described by Ayata et al. (14). Mice were killed by decapitation after 48 hr. Time-course and dose-response experiments were first performed to find the optimal conditions. Brains were frozen and 20-μm coronal cryostat sections were taken. The lesion area was measured by a computer-assisted system after Nissl staining.

Immunohistochemistry

Animals were perfused with 37°C Krebs/Henseleit buffer followed by 300 ml of 37°C 4% paraformaldehyde (pH 7.4). Brains were postfixed for 2 hr and cryoprotected. Free-floating brain sections (40 μm) were blocked with 4% normal goat serum for 1 hr in the presence of 0.2%

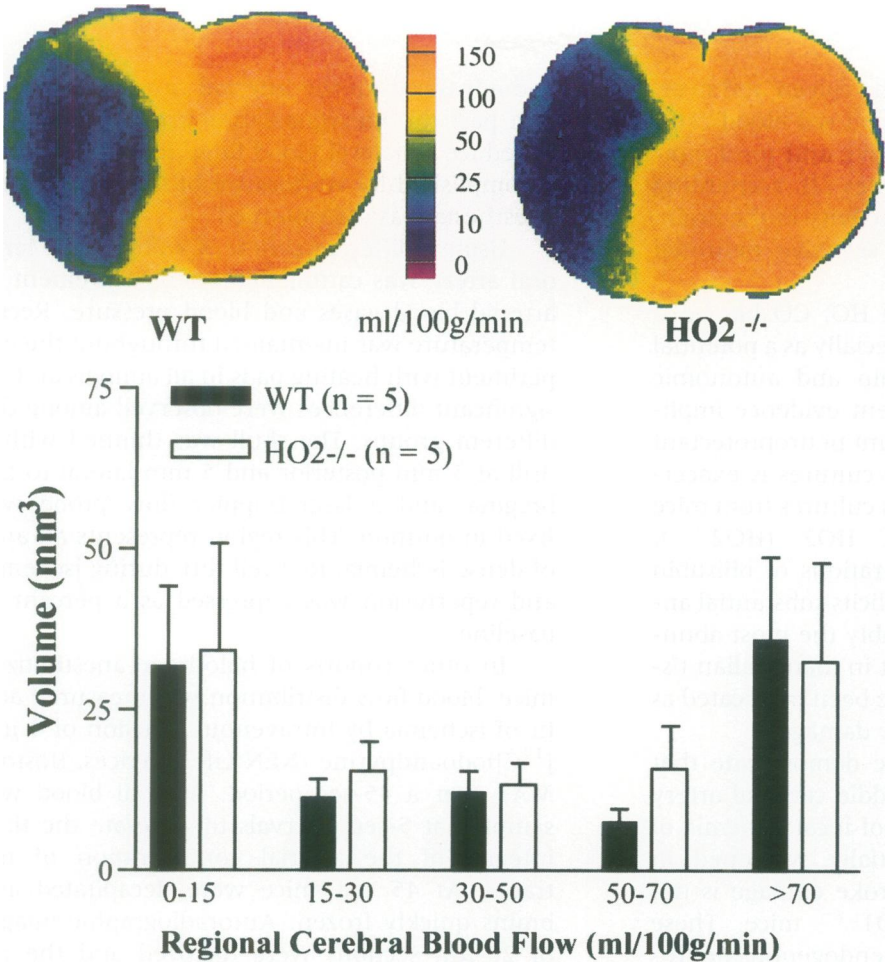

Fig. 1. Distribution of cerebral blood flow during ischemia in wild-type (WT) and HO2$^{-/-}$ mice. (Top) Digitized image of regional cerebral blood flow during focal ischemia in a WT and HO2$^{-/-}$ mouse determined by autoradiography of the iodoantipyrine tracer. (Bottom) Histogram depicting the relative volume of tissue in ischemic hemisphere partitioned into ranges of cerebral blood flow from WT (n = 5) and HO2$^{-/-}$ (n = 5) mice. There is no significant difference in blood flow distribution. Under similar conditions using laser-Doppler flowmetry measured over ischemic cortex, we observed a rapid decrease in red cell flux (approximately 85%) during the occlusion and a complete recovery to baseline after the filaments were withdrawn. No differences could be quantified between the WT and HO2$^{-/-}$ mice.

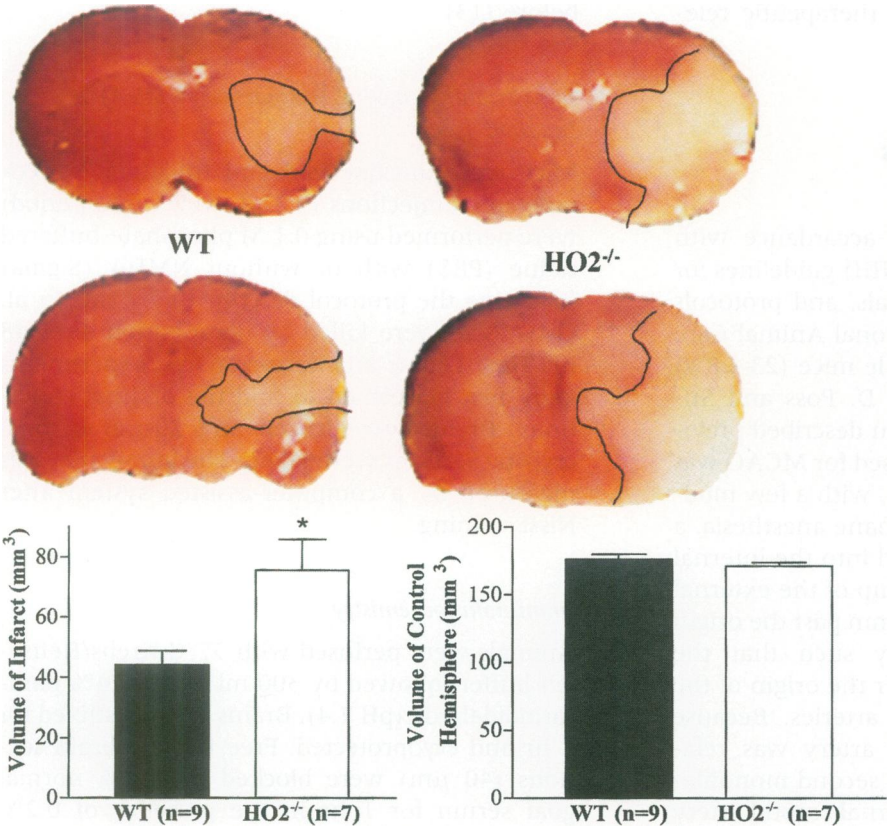

Fig. 2. Cerebral infarct volume is increased in HO2$^{-/-}$ but not wild-type (WT) mice following MCAO. Mice were submitted to 1 hr MCAO and 23 hr reperfusion. (Top) Representative coronal brain sections from WT and HO2$^{-/-}$ mice after MCA occlusion and reperfusion are shown. The infarct volume was determined by triphenyltetrazolium chloride staining. The white region representing the ischemic region was delineated and the surface area was measured. (Bottom) The infarct volume shown on the histogram was corrected for edema: the infarct represents 25 ± 3% of the hemisphere in WT (n = 9) and 44 ± 6% of the hemisphere in HO2$^{-/-}$ (n = 7) mice (*$p < 0.001$). No WT mice died and one HO2$^{-/-}$ mouse died.

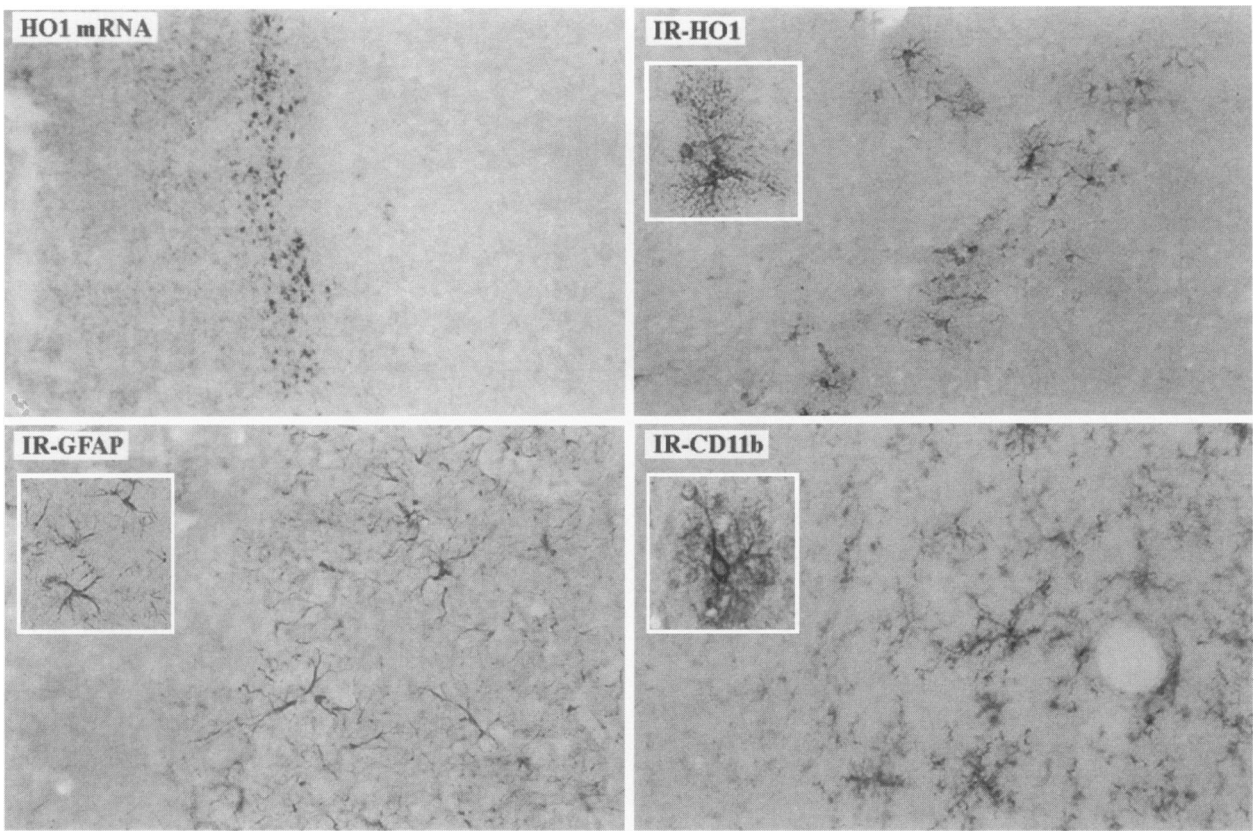

Fig. 3. HO1 induction following MCAO. Mouse brain sections were examined after 1 hr ischemia and 23 hr reperfusion using in situ hybridization (HO1 mRNA) and immunohistochemistry for immunoreactive HO1 (IR-HO1), glial (IR-GFAP; glial fibrillary acidic protein) and microglial (IR-CD11b) markers. HO1 mRNA and protein are induced in the border surrounding the infarct core. The induction appears to occur in reactive microglia.

Triton X-100 and incubated overnight at 4°C with the primary antiserum diluted in PBS containing 2% goat serum and 0.1% Triton X-100. Immunoreactivity was visualized with the Vectastain ABC Elite kit (Vector Laboratories, Inc., Burlingame, CA) using DAB as chromogen with or without nickel ammonium sulfate. The HO antibodies were obtained from Stressgen (Victoria, Canada). The other antibodies were markers for glia (GFAP; DAKO, Carpinteria, CA) and microglia (CD11b, Serotec, Raleigh, NC).

In Situ Hybridization

Probes used for in situ hybridization for HO1 and HO2 were 900 bp and 700 bp, respectively. These clones were in Bluescript and the sense and antisense were synthesized using T7 and T3 promotor. Brains were snap frozen and cut in 10-μm sections. The sections were permeabilized, blocked, and then hybridized overnight. Sections were developed using antidigoxygenin conjugated to phosphatase as described previously (15).

Results

Cerebral Blood Flow in Wild-type and HO2$^{-/-}$ Mice

MCAO resulted in stable decreases in cortical perfusion throughout the ischemic period as estimated by laser-Doppler flowmetry (Fig. 1, top). The percent reduction in perfusion during ischemia and recovery of blood flow during reperfusion were not different in wild-type (WT) and HO2$^{-/-}$ mice, which suggests a similarity of ischemic insult. Laser-Doppler flow measured over ischemic cortex indicated a rapid decrease in flow to approximately 15% of baseline after insertion of the two monofilaments. Flow was stable during ischemia and recovered completely to baseline after the filaments were withdrawn. There were no differences between WT and HO2$^{-/-}$ mice in the percent blood flow response

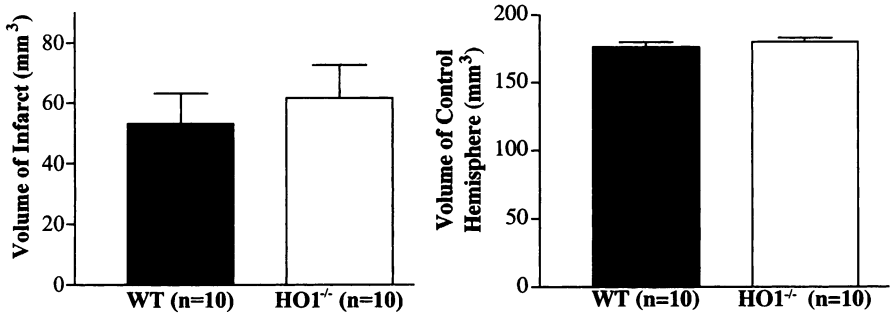

Fig. 4. Cerebral infarct volume is similar in $HO1^{-/-}$ and wild-type (WT) mice following MCAO. Mice were subjected to 1 hr MCAO and 23 hr reperfusion. The infarct volume shown on the histogram was corrected for edema: the infarct represented 31 ± 6% of the hemisphere in WT (n = 10) and 35 ± 7% of the hemisphere in $HO1^{-/-}$ (n = 10) mice. Two $HO1^{-/-}$ mice died, and no WT mice died.

or in mean arterial blood pressure. At 1 hr of ischemia, arterial pH (7.36 ± 0.002 vs. 7.34 ± 0.006), arterial P_{CO_2} (34 ± 2 vs. 34 ± 1 mmHg), and arterial P_{O_2} (138 ± 13 vs. 147 ± 12 mmHg) were similar in WT and $HO2^{-/-}$ groups.

To estimate absolute blood flow in separate cohorts, we examined intraischemic blood flow distribution as observed and quantified by [^{14}C]iodoantipyrine autoradiography (Fig. 1 bottom). The distribution of tissue volume recruited into low-flow zones within the ischemic hemisphere was similar in WT and $HO2^{-/-}$ groups. Mean blood flow in the nonischemic hemisphere was equivalent in WT (103 ± 4 ml/100 g/min; ±SE) and $HO2^{-/-}$ (104 ± 5 ml/100 g/min) mice.

Ischemic Volume Is Increased in $HO2^{-/-}$ Animals

The volume of infarcted brain tissue following 1 hr of ischemia and 23 hr of reperfusion was 71% greater in $HO2^{-/-}$ than in WT animals, whereas there was no difference in overall tissue volume between the two groups in the control hemisphere (Fig. 2).

HO1 Is Induced after MCAO and Reperfusion

Since HO1 is induced following many types of stress, one might anticipate alterations following MCAO. There are reports of increased HO1 protein by immunohistochemical staining (16–19) and HO1 mRNA by Northern blot analysis (17,19,20), but the exact cellular localization of the increase has been unclear. We observed a pronounced increase in HO1 staining (mRNA and protein) that was highly concentrated at the border of infarcted tissue (Fig. 3). The staining was localized to reactive microglia.

Ischemic Volume Is Similar in $HO1^{-/-}$ and WT Mice

The induction of HO1 after MCAO raises the possibility that it may mediate neurotoxic alterations. Accordingly, we evaluated ischemia in $HO1^{-/-}$ animals. We found no significant differences in infarct volume between WT and $HO1^{-/-}$ mice (Fig. 4).

NMDA Neurotoxicity Is Augmented in $HO2^{-/-}$ Mice

We sought to ascertain which mechanisms of stroke damage might account for the augmented stroke damage in $HO2^{-/-}$ animals. Neurotoxicity can be elicited by direct injections of NMDA into the brain. As reported previously (14), we observed substantial neural damage following NMDA injections into the cerebral cortex (Fig. 5). The extent of this damage was increased 3- to 4-fold in $HO2^{-/-}$ mice.

Discussion

The substantial increase in MCAO-associated neural damage in $HO2^{-/-}$ mice suggests that HO2 normally performs a neuroprotective role in the brain. However, it is conceivable that loss of HO2 causes nonspecific brain damage that worsens strokes. We think this is unlikely for the following reason. $HO2^{-/-}$ mice have been extensively characterized in behavioral studies as showing no defects in an extensive range of sensory and motor tasks, including measures of olfaction, motivation, exploratory behavior, motor coordination, strength, and balance (11), and long-term potentiation in brain slices is also normal (10). The only documented abnormalities have involved diminished penile ejaculation (11)

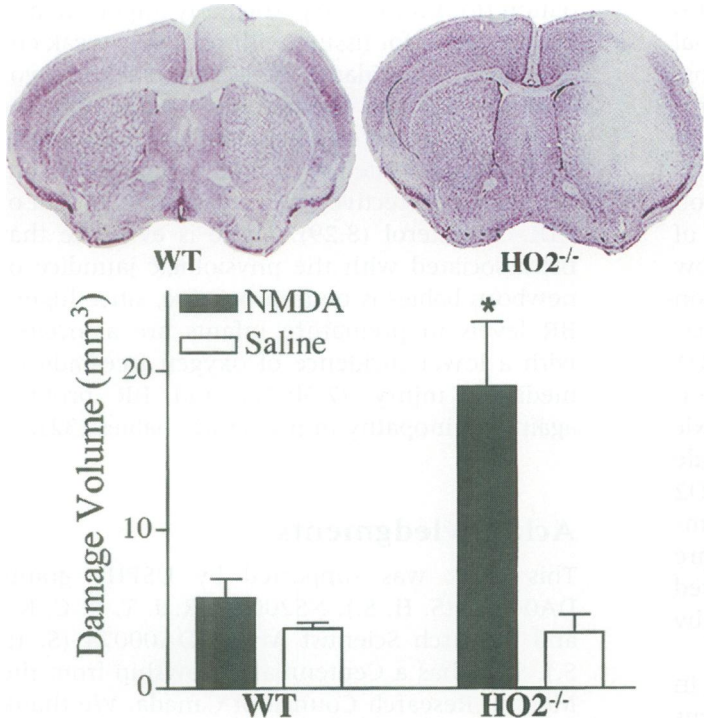

Fig. 5. NMDA damage is augmented in $HO2^{-/-}$ mice. (Top) Representative coronal brain sections Nissl stained after NMDA stereotaxic injections show the extent of damage in $HO2^{-/-}$ as compared to wild-type (WT) mice. The lesions were mostly localized to the striatum and to the adjacent cortex along with the needle track. (Bottom) The histogram depicts the relative volume of tissue damaged after saline or NMDA injection from WT and $HO2^{-/-}$ mice (n = 4 in each group).

and reduced nonadrenergic, noncholinergic neuronal transmission in the intestine (4). Gross locomotor behavior is normal, as is reproductive behavior and longevity. Sixteen-month-old $HO2^{-/-}$ animals (n = 15) performed the same as WT animals (n = 14) in a standard Morris water maze task (21): the distance swum (WT: 2208 ± 333; $HO2^{-/-}$: 2260 ± 203 cm) and the relative speed (WT: 24.4 ± 3.6; $HO2^{-/-}$: 25.0 ± 2.3 cm/sec) were comparable. By contrast, most $HO1^{-/-}$ mice die during embryonic life and those that survive usually die at an early adult age (9). Yet, we found no significant difference in MCAO neural damage in $HO1^{-/-}$ mice as compared to WT mice. This further emphasizes selectivity for HO2 in mediating neuroprotection and establishing that the greater stroke damage of $HO2^{-/-}$ mice is not related to general debility.

Stroke damage following blood vessel occlusion and reperfusion involves a variety of mechanisms. One component derives from the formation of oxygen free-radicals by hypoxic mitochondria. Hypoxia also triggers a massive release of glutamate, which causes neurotoxicity via stimulation of glutamate receptors, especially the NMDA subtype (14). We sought to ascertain which mechanisms of stroke damage might be reversed by HO activation and hence contribute to augmented stroke damage in $HO2^{-/-}$ animals. Neurotoxicity can be elicited by direct injections of NMDA into the brain and, as reported previously (14), we observed substantial neural damage following NMDA injections into the cerebral cortex (Fig. 3). The extent of this damage was increased 3- to 4-fold in $HO2^{-/-}$ mice. Thus, it appears that physiologic HO2 activity is neuroprotective against glutamate excitatoxicity that involves NMDA receptor activation.

What might account for the neuroprotective action of HO2? One of the enzyme's products, CO, is a putative neurotransmitter but it has not been directly linked to neuroprotective mechanisms (3,4). CO can dilate blood vessels, but there is no evidence that CO elaborated by HO2 in neurons regulates vascular reactivity. Moreover, the reduction in perfusion during the entire ischemic period and the volume of tissue subjected to ischemic levels of blood flow were the same in $HO2^{-/-}$ and WT mice. Thus the lack of hypothesized CO-mediated vasodilation during ischemia in $HO2^{-/-}$ mice does not account for the greater infarction volume in these animals. By contrast, there is direct evidence for a neuroprotective role for BR. In hippocampal and cerebral cortical cultures, the neurotoxic effects of hydrogen peroxide (5), hemin and the Alzheimer's $A\beta_{1-42}$ peptide (22) are substantially worsened by HO inhibitors and in cultures from $HO2^{-/-}$ mice. BR in nanomolar concentrations is neuroprotective against H_2O_2-induced toxicity

in these primary cultures. Phorbol esters also elicit neuroprotective actions in brain neuronal cultures by activating protein kinase C to phosphorylate and activate HO2 (5). Thus, it seems possible that diminished formation of BR in $HO2^{-/-}$ animals leads to greater neural stroke damage. HO participates in the efflux of iron from cells, as iron accumulates in tissues of $HO1^{-/-}$ mice while serum iron levels are low (9). Moreover, the physiologic egress of iron from fibroblasts is diminished in $HO1^{-/-}$ preparations and augmented by transfection of HO1 (23). Whether HO2 as well as HO1 regulates iron efflux is unclear. Ferrous iron is highly toxic following its interaction with hydrogen peroxide in the Fenton reaction, so that a loss of HO2 might lead to accentuated iron-mediated damage. It is noteworthy that brain levels of iron are similar in WT and the $HO2^{-/-}$ brains as observed by Prussian blue staining and quantified by atomic emission spectrometry (23).

Our findings may have clinical relevance in cerebral ischemia. Since HO2 deletion worsens stroke damage, activating the enzyme may be therapeutic. Drugs that augment HO2 activity would be predicted to be neuroprotective. Recently, we showed that activation of heme oxygenase activity is cytoprotective for cultured neurons, fibroblasts, and human embryonic kidney 293 cells (5,23). HO1 can be induced by a variety of agents, including several porphyrin derivatives (24). However, HO1 activation is not likely to be neuroprotective, as in our experiments, stroke damage was not worse in $HO1^{-/-}$ mice. Presumably, HO1 is not neuroprotective, because it is selectively localized to microglia, in contrast to HO2, which is exclusively neuronal in the brain. The one known class of substances that activates HO2 are phorbol esters that activate protein kinase C, which phosphorylates and activates HO2 (5). Phorbol esters are tumor promoters, which would preclude use for chronic therapy; however, acute treatment with such agents would be feasible. While most phorbol esters are lipophilic, water-soluble derivatives exist that could potentially be administered to stroke patients. Selectivity for protein kinase C activation might be feasible because of the numerous subtypes of the enzyme, some of which are specifically enriched in the brain (25), and activation of HO2 might occur to a greater extent with particular enzyme subtypes. Low doses of BR itself might also afford a therapeutic venue. BR is an effective antioxidant of peroxynitrite-mediated protein oxidation (26) and lipid peroxidation (6). BR may be particularly important as a cytoprotector for tissues with relatively weak endogenous antioxidant defenses such as the myocardium (27) and the nervous system (28). Interestingly, a decreased risk for coronary artery disease is associated with mildly elevated serum BR, with a protective effect comparable to that of HDL-cholesterol (8,29). There is evidence that BR associated with the physiologic jaundice of newborn babies is neuroprotective, since higher BR levels in premature infants are associated with a lower incidence of oxygen free-radical–mediated injury (7,30,31) and BR protects against retinopathy in premature babies (32).

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