Characterization of a new double-filament model of focal cerebral ischemia in heme oxygenase-2-deficient mice

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Submitted 4 February 2003; accepted in final form 24 March 2003

Goto, Shozo, Kenji Sampei, Nabil J. Alkayed, Sylvain Doré, and Raymond C. Koehler. Characterization of a new double-filament model of focal cerebral ischemia in heme oxygenase-2-deficient mice. Am J Physiol Regul Integr Comp Physiol 285: R222–R230, 2003. First published March 27, 2003; 10.1152/ajpregu.00067.2003.—Variations in vascular anatomy in knockout mouse strains can influence infarct volume after middle cerebral artery (MCA) occlusion (MCAO). In wild-type (WT) and heme oxygenase-2 gene-deleted (HO2−/−) mice, infarcts were not reproducibly achieved with the standard intraluminal filament technique. The present study characterizes a double-filament model of MCAO, which was developed to produce consistent infarcts in both WT and HO2−/− mice. Diameters of most cerebral arteries were similar in WT and HO2−/− mice, although the posterior communicating artery size was variable. In halothane-anesthetized mice, two 6-0 monofilaments with blunted tips were inserted into the left internal carotid artery 6.0 and 4.5 mm past the pterygopalatine artery junction to reside distal and proximal to the origin of the MCA. The tissue “volume at risk” determined by brief dye perfusion in WT (59±2% of hemisphere; ±SE) was similar to HO2−/− (62±4%). The volume of tissue with cerebral blood flow <50 ml·min−1·100 g−1 was similar in WT (35±9%) and HO2−/− (36±11%) during MCAO and at 3 h of reperfusion (<2%). After 1 h MCAO, infarct volume was greater in HO2−/− (44±6%) than WT (25±3%). After increasing MCAO duration to 2 h, the difference between HO2−/− (47±4%) and WT (36±3%) diminished, but infarct volume remained substantially less than the volume at risk. Infusion of tin protoporphyrin IX, an HO inhibitor, during reperfusion after 1 h MCAO increased infarct volume in WT but not significantly in HO2−/− mice, although infarct volume remained less than the volume at risk. Thus greater infarct volume in HO2−/− mice is not attributable to a greater volume at risk, lower intracerebral blood flow, or poor reflow, but rather to a neuroprotective effect of HO2 activity. The double-filament model may be of use as an alternative in other murine knockout strains in which the standard filament model does not yield consistent infarcts.

cerebral blood flow; cerebral circulation; focal cerebral ischemia; heme oxygenase; mouse

Heme oxygenase (HO) converts pro-oxidant heme to biliverdin and carbon monoxide (CO), which can act as a neurotransmitter, vasodilator, and antiapoptotic agent. Biliverdin is rapidly converted to bilirubin, which can protect neurons from oxidative stress at low concentrations (6). Increasing the expression of the inducible isoforum of heme oxygenase, HO1, with a neuronal promoter in transgenic animals provides ischemic protection (22). However, basal expression of HO1 is normally extremely low in brain, and induction after ischemia is delayed 12–48 h with localization occurring primarily in microglia rather than neurons (5, 8, 13, 20). Thus this induction probably provides little immediate protection of neurons subjected to focal cerebral ischemia. However, neurons constitutively express heme oxygenase-2 (HO2), which confers resistance to oxidative stress (6). We have previously shown that infarct volume after 1 h of middle cerebral artery (MCA) occlusion (MCAO) was greater in mice with gene deletion of HO2 (HO2−/−) (5) and that inhibiting HO activity during reperfusion in wild-type (WT) mice also increased infarct volume (4). Together, these reports support the concept that endogenous HO activity in neurons is normally protecting neurons after 1 h MCAO.

In assessing the effect of a specific gene deletion in focal ischemic injury, it is important to determine if differences in vascular anatomy and cerebral blood flow (CBF) between WT and knockout animals might account for differences in infarct volume (16–19, 21, 28). When we started these studies with the standard intraluminal filament technique, the decrease in laser-Doppler flux (LDF) signal was modest, and infarcts were small and inconsistent in both WT and HO2−/− mice, even when larger filaments were tested. To overcome the apparent differences in cerebral vascular anatomy between these strains and C57BL/6 in which the filament technique ordinarily produces large and consistent infarcts, we developed a new procedure in which two filaments are inserted different distances into the same internal carotid artery of the mouse. In the present study, the characteristics of this double-filament technique are described in WT and HO2−/− mice. This model may be of use in other knockout strains of mice in which the standard single-filament technique is ineffective.

One approach used to normalize for variability in collateral blood flow during myocardial ischemia is to

First published March 27, 2003; 10.1152/ajpregu.00067.2003.
perfuse a dye during vascular occlusion to determine the “volume at risk” for ischemic injury. We used a similar approach in the brain after insertion of the two filaments. Using this double-filament technique, the first objective of the present investigation was to determine if there are differences between WT and HO2−/− mice 1) in the diameter of major cerebral arteries, 2) in the tissue volume at risk of injury determined by brief dye perfusion after placement of the filaments, 3) in the time course of cortical perfusion measured by LDF, and 4) in the distribution of CBF at each coronal level measured by indoantipyrine (IAP) autoradiography during MCAO and at 3 h of reperfusion where loss of CO generation in HO2−/− mice could conceivably result in poor intraischemic CBF or poor reflow. Analysis was performed at different coronal levels as well as on overall volume because variability in the size of the posterior communicating artery seen in different mouse strains may preferentially affect blood flow distribution in the posterior cortex (21). The second objective was to determine if increasing the duration of ischemia from the 1 h previously used (5) to 2 h results in sustained differences in infarct size between WT and HO2−/− mice, or if infarct volume approaches the volume at risk. Results with infusion of an HO inhibitor (4) were also analyzed in relation to the volume at risk to determine if increases in infarct volume approach the volume at risk.

METHODS

Focal cerebral ischemia. All procedures were approved by the Johns Hopkins University Animal Care and Use Committee. Wild-type (WT) and HO2−/− mice used for breeding were obtained from K. D. Poss and S. Tonegawa and have been described previously using embryonic stem cells derived from SV129 mice and C57BL/6 mouse blastocytes (23). Mice weighing between 20 and 28 g were anesthetized initially with 2% halothane and maintained on 1–1.5% halothane in O2-enriched air via a face mask. Rectal temperature was maintained at ~37°C during anesthesia with external heating. Focal ischemia was produced by a modification of the intraluminal filament technique. In preliminary experiments on WT and HO2−/− mice, insertion of either 6-0 or 5-0 monofilaments with blunted tips 6 mm into the internal carotid artery was found to produce only modest decreases in mono

filaments with blunted tips 6 mm into the internal carotid artery. Both WT and HO2−/− mice were obtained from K. D. Poss and S. Tonegawa and have been described previously using embryonic stem cells derived

mitochondria with 2% halothane and maintained on 1

flament technique. In preliminary experiments

albumin. Diameter of major arteries in the base of the brain

by heating and then coating the tip with cyanoacrylate glue. The left external carotid artery was ligated and cut. The common carotid artery was occluded with a silk suture. The first monofilament was inserted into the internal carotid artery through the stump of the external carotid artery and advanced 6.0 mm past the origin of the pterygopalatine artery. The blunted tip lay anterior to the origin of the MCA. The second monofilament was inserted via the external carotid artery and advanced into the internal carotid artery 4.5 mm past the pterygopalatine artery bifurcation. The filament tip lay just proximal to the origin of the MCA.

In survival protocols, the neck incision was closed, anesthesia was discontinued, and the mouse was allowed to awaken. To establish reperfusion, the mouse was briefly anesthetized with halothane, the suture around the common carotid artery was untied, and both monofilaments were withdrawn through the external carotid artery stump. The neck incision was sutured closed, and anesthesia was discontinued. Infarct volume was measured 24 h after induction of ischemia. The mice were killed by an overdose of halothane anesthesia. The brains were rapidly harvested, chilled, and cut into five coronal slabs. These 2-mm-thick sections were then incubated at 37°C in a 1% solution of 2,3,5-triphenyl-
tetrazolium chloride for 20 min followed by fixation in 10% buffered formalin. The surface area of viable regions stained red and of nonviable regions that remained pale were integrated on both the anterior and posterior surfaces of each section. The average of the anterior and posterior viable surface areas was multiplied by the section thickness and summed to obtain the volume of viable tissue. To correct for swelling of ischemic tissue, corrected infarct volume was calculated as: (volume of the contralateral hemisphere) – (volume of the ipsilateral hemisphere – measured infarct volume) (15). In one experiment, the volume at risk was compared with infarct volume after 2 h of ischemia in WT (n = 13) and HO2−/− (n = 12) mice and to infarct volume previously reported (5) after 1 h of ischemia in WT (n = 9) and HO2−/− (n = 7) mice. In another experiment, the volume at risk was compared with infarct volume previously reported (4) in groups receiving an intravenous infusion of vehicle (NaOH solvent titrated to pH of 7.6 with HCl) in WT (n = 11) and HO2−/− (n = 10) mice or tin protoporphyrin IX (SnPPix, 10 μmol·kg⁻¹·h⁻¹), an inhibitor of HO activity, in WT (n = 10) and HO2−/− (n = 10) mice for 23 h of reperfusion starting after 1 h of MCAO. This dose of SnPPix was found to inhibit HO activity in brain consistently without reducing nitric oxide synthase catalytic activity (4).

Temporal hemodynamics. To determine the time course of relative changes in perfusion and the extent of reperfusion in this model, perfusion was monitored continuously by laser-Doppler flowmetry in separate cohorts of five WT and five HO2−/− mice under halothane anesthesia. The femoral artery was catheterized for continuous monitoring of arterial pressure. The skull was thinned with a drill at 3 mm posterior and 5 mm lateral to the bregma, and a laser-Doppler probe (Moor Instruments) was fixed in position. This region represents an area of dense ischemia. Red blood cell flux was decreased by 90%, but reperfusion and reperfusion was expressed as a percentage of baseline.

Diameter of cerebral arteries. Five WT and five HO2−/− mice were anesthetized with halothane and were perfused transcardially at a rate of 6 ml/min with heparinized saline for 5 min and then with 10% buffered formalin for 4 min. For the final 10 s of perfusion, the mouse was perfused with a 1-ml saline solution containing 1.5% Evans blue and 1.25% albumin. Diameter of major arteries in the base of the brain was measured on a video microscopy system.

Volume at risk. After insertion of two 6-0 nylon monofilaments into the left internal carotid artery, seven WT and seven HO2−/− mice were perfused transcardially at a rate of 6 ml/min with the descending aorta clamped. Heparinized saline was perfused for 5 min followed by a 3-ml saline solution containing 2% Evans blue for 30 s. The whole brain was fixed in 10% buffered formalin for 24 h. The forebrain was sliced into 2-mm-thick coronal sections. Areas of distinct pallor and areas of light blue, which were in contrast to areas of dark blue, were considered to be areas at risk of infarction. These areas were digitally imaged and numerically integrated on both the anterior and posterior surfaces of each slice to obtain a volume at risk for each coronal slice.
**Intraischemic blood flow.** Ischemia was produced in five WT and five HO2/−/− mice for 60 min. After confirming neurological deficit, the mice were reanesthetized with halothane, and the femoral artery and vein were catheterized. At 60 min of ischemia, 4 μCi of [14C]IAA was infused intravenously at a constant rate of 108 μl/min for 45 s. Arterial blood was sampled at 5-s intervals for obtaining the arterial input function as described (24). The total volume of blood withdrawn was 100–160 ml. At 45 s of infusion, the anesthetized mouse was decapitated. The brain was quickly removed, frozen in 2-methylbutane on dry ice, and stored at −80°C. The brain was sliced into 20-μm-thick coronal sections on a cryostat, thaw mounted onto glass coverslips, and apposed to Kodak SB-5 film for 1 wk with 14C standards. Nine autoradiographic images at each of seven coronal levels (+2, +1, 0, −1, −2, −3, and −4 mm from the bregma) were digitized, and regional blood flow was calculated with image analysis software (Inquiry, Loats Associates). The number of pixels with blood flow in a particular range was summed and converted to volume of tissue. For each coronal level for each mouse, a histogram of volume of tissue with different ranges of CBF was constructed.

**Regional blood flow during reperfusion.** Ischemia was produced in four WT and four HO2/−/− mice for 60 min followed by 3 h of reperfusion. At 140 min of reperfusion, mice were reanesthetized with halothane, and a femoral artery and vein were catheterized. At 3 h of reperfusion, IAP was infused and regional CBF was measured as described above.

**Statistical analysis.** Data are expressed as means ± SE and were subjected to ANOVA and, where appropriate, the Newman-Keuls multiple range test at P < 0.05.

**RESULTS**

**Arterial diameter.** The diameter of the left posterior communicating artery was small in both WT and HO2/−/− mice (Fig. 1). Interestingly, the diameter of the right posterior communicating artery was larger than that of the left artery in WT mice but not in HO2/−/− mice in these cohorts. In the present model, the filaments are inserted on the left side. There were no other differences in the diameter of major arteries between WT and HO2/−/− mice.

**Volume at risk.** The blunted tip of the first inserted filament was anterior to the origin of the MCA (Fig. 2). The blunted tip of the second filament was just proximal to the origin of the MCA. These locations were observed consistently in all of the brains used for the volume-at-risk analysis. The volume at risk was determined from areas of pallor on coronal slabs after 30 s of perfusion with Evans blue dye (Fig. 3). The perfusion pressure was 86 ± 2 mmHg in WT and 89 ± 2 mmHg in HO2/−/− groups. The volume at risk was similar in WT and HO2/−/− mice, accounting for 59 and 62% of hemispheric volume, respectively (Fig. 4). The coefficient of variation (100 SD/mean) was similar in WT (10.5%) and HO2/−/− (9.3%) groups.

**Duration of MCAO.** Increasing the duration of MCAO from 1 to 2 h increased infarct volume in WT mice (Fig. 4). However, infarct volume was less than
the volume at risk. In HO2−/− mice, in contrast, infarct volume after 2 h MCAO was not different from 1 h MCAO, although infarct volume remained less than the volume at risk. Infarct volume in HO2−/− mice was greater than in WT mice after 1 or 2 h of ischemia, but the difference between WT and HO2−/− groups was reduced after 2 h ischemia.

**HO inhibitor.** Continuous intravenous infusion of the HO inhibitor SnPPIX (10 μmol·kg⁻¹·h⁻¹) starting at the onset of reperfusion after 1 h of MCAO resulted in increased infarction in WT (Fig. 5). Infarct volume remained less than the volume at risk. In HO2−/−, SnPPIX infusion increased infarction in the second anterior coronal section to values equivalent to the volume at risk. However, the overall infarct volume was not enhanced significantly by SnPPIX, and infarct volume remained less than the volume at risk in the more posterior sections. The infarct volume in the WT group receiving vehicle was less than the infarct volume of the other three groups.

**Cerebral hemodynamics.** Mean arterial blood pressure decreased slightly in both WT and HO2−/− groups maintained under halothane anesthesia throughout a 2-h period of MCAO (Fig. 6), but two-way ANOVA indicated no significant effect of mouse strain. The LDF measured over lateral parietal cortex decreased ~85% in both groups, and the percent decrease was not different between groups. The decreased flow remained stable during MCAO and recovered toward baseline values during early reperfusion.

**Fig. 2.** Photograph of base of fixed mouse brain after degradation of intravascular heme to show placement of 2 monofilaments proximal and distal to origin of middle cerebral artery from internal carotid artery.

**Fig. 3.** Examples of ventral view of whole brains (top) perfused for 30 s with Evans blue dye after placement of 3 monofilaments and of posterior view of coronal sections used for calculating the volume at risk in a WT and HO2−/− mouse.

**Fig. 4.** Bar graph of infarct volume after 1 h of focal ischemia in WT (n = 9) and HO2−/− (n = 7) mice and after 2 h of focal ischemia in WT (n = 13) and HO2−/− (n = 12) mice, and the volume at risk in WT (n = 7) and HO2−/− (n = 7) mice (mean ± SE). *P < 0.05 between 1-h and 2-h ischemic groups within the same strain; †P < 0.05 between corresponding values in WT and HO2−/− groups. Total hemispheric volume was equivalent in WT (170 ± 2 mm³) and HO2−/− (169 ± 2 mm³) mice.
At 60 min of MCAO, regional CBF was measured by IAP autoradiography at 1-mm incremental coronal levels from +2 mm through −4 mm relative to bregma. At each coronal level, the area of tissue with ranges of CBF in 10 ml·min⁻¹·100 g⁻¹ increments was measured (Fig. 7). Arterial P_{CO2} was 36 ± 3 mmHg in WT and 30 ± 3 mmHg in HO2⁻/⁻ groups at the time of CBF measurements. There were no differences in the intracranial tissue distribution of CBF between WT and HO2⁻/⁻ groups. Moreover, the volume of tissue with different degrees of intracranial blood flow was similar in WT and HO2⁻/⁻ groups at every coronal level, including posterior levels. In summing the volume from all coronal levels, 35 ± 9% of the hemisphere in WT and 36 ± 11% of the hemisphere in HO2⁻/⁻ had CBF <50 ml·min⁻¹·100 g⁻¹.

To determine if a delayed reperfusion deficit might contribute to greater infarction in HO2⁻/⁻ mice, regional CBF was also measured at 3 h of reperfusion after 1 h of MCAO. Mean arterial pressure was 84 ± 6 mmHg in WT and 79 ± 3 mmHg in HO2⁻/⁻ groups. Arterial P_{CO2} was 36 ± 2 mmHg in WT and 34 ± 2 mmHg in HO2⁻/⁻ groups. Total blood flow in the reperfused hemisphere (130 ± 7 ml·min⁻¹·100 g⁻¹) was similar to that in the contralateral hemisphere (141 ± 11 ml·min⁻¹·100 g⁻¹) of WT mice. These values were also similar to the total blood flow in the reperfused (140 ± 22 ml·min⁻¹·100 g⁻¹) and contralateral (144 ± 20 ml·min⁻¹·100 g⁻¹) hemispheres of HO2⁻/⁻ mice. The distribution of tissue volume with different ranges of CBF in the ipsilateral, reperfused hemisphere had a similar pattern in WT and HO2⁻/⁻ mice (Fig. 8). Moreover, the percentage of tissue with CBF of <50 ml·min⁻¹·100 g⁻¹ was only 2% in both groups and was only slightly greater than the 1.4% value in the contralateral hemisphere.

**DISCUSSION**

The present study describes the properties of a newly developed double-filament model of focal ischemia in WT and HO2⁻/⁻ mice in which the standard single-filament model failed to produce consistent infarction. The results demonstrate that nearly all cerebral arteries ipsilateral to the induced stroke have similar diameters in WT and HO2⁻/⁻ mice and that the volume at risk delineated by dye perfusion after double-filament placement was equivalent in WT and HO2⁻/⁻ mice. Moreover, the perfusion deficit evaluated temporally by LDF and spatially by IAP autoradiography was similar in WT and HO2⁻/⁻ strains. We conclude that differences in infarct volume between WT and HO2⁻/⁻ mice were not attributable to differences in the severity of the ischemic insult.
Others have indicated that variability in the single-filament technique among different strains of mice may be related to vascular anatomy (16), particularly variability in the posterior communicating artery (21, 28). We observed consistent diameter measurements of most of the major cerebral arteries, but the size of the posterior communicating artery was variable. This variability may have contributed to the inconsistent infarction seen with the use of a single filament in these mice. The lack of a difference in the CBF distribution in posterior coronal sections between WT and HO2−/− mice in the present study implies a similar overall MCA supply territory, although we cannot exclude possible differences in the precise location of watershed border regions between the MCA and other cerebral arteries (17). Volume-at-risk assessment is commonly used as a within-subject normalization procedure for analyzing infarct data in isolated hearts subjected to coronary artery occlusion, but it is rarely used in focal cerebral ischemia where animals are allowed to survive the surgery and where ischemia is often transient. We analyzed the volume at risk in separate cohorts not used for survival studies.

Differences in the MCA supply territory also need to be considered because this can differ among strains (17) and lead to differences in infarct volume (19). The equivalent volume at risk derived from dye perfusion in the WT and HO2−/− mice in the present study implies a similar overall MCA supply territory, although we cannot exclude possible differences in the precise location of watershed border regions between the MCA and other cerebral arteries (17). Volume-at-risk assessment is commonly used as a within-subject normalization procedure for analyzing infarct data in isolated hearts subjected to coronary artery occlusion, but it is rarely used in focal cerebral ischemia where animals are allowed to survive the surgery and where ischemia is often transient. We analyzed the volume at risk in separate cohorts not used for survival studies.

With the double-filament technique, the coefficient of variation was ~10% in these strains of mice. This variation is reasonably small and suggests that the use

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**Fig. 7.** Histograms of area of tissue (±SE) with different ranges of cerebral blood flow (CBF) at 7 individual coronal levels (A–G) in 5 WT and 5 HO2−/− mice at 60 min of focal ischemia. Coronal levels relative to bregma: +2 mm (A), +1 mm (B), bregma (C), −1 mm (D), −2 mm (E), −3 mm (F), −4 mm (G). H: area with CBF >70 ml·min⁻¹·100 g⁻¹ is plotted on a different scale for all coronal levels. The blood flow distribution was well matched between WT and HO2−/− mice.
of two filaments placed anteriorly and posteriorly to the origin of the MCA limits the contribution of variability in size of the posterior communicating artery or other arteries. Moreover, the coefficient of variation of the volume at risk was considerably smaller than the ~30% coefficient of variation of infarct volume. This comparison suggests that factors unrelated to perfusion significantly contribute to the biological variability of the model. Ideally, measurement of the volume at risk should be performed in the same mice that infarct volume is determined. However, perfusing dye at the time of harvesting the brain would require reinserting the filaments without the certitude of identical placement location. Moreover, with longer survival periods, vascular remodeling might occur. Therefore, normalizing infarct volume by the volume at risk within the same subject may not necessarily improve the statistical power in this model.

Evans blue dye is sometimes used to assess blood-brain barrier disruption. We do not believe that assessment of the volume at risk is confounded by the use of this dye because the duration of dye perfusion was relatively brief (30 s), whereas much longer circulatory times (~30 min) are generally used to detect extravasation (14). Moreover, the dye perfusion was performed within 20 min of filament insertion, whereas barrier disruption generally requires several hours of ischemia (2). Last, if extravasation were to occur, it would occur first in the ischemic core, but the dye did not reach the ischemic core in substantial amounts with the brief 30-s perfusion.

Increasing the duration of ischemia from 1 to 2 h in WT mice increased infarct volume from 25 to 36% of hemispheric volume, but infarct volume remained less than the volume at risk (~60% of hemisphere). Infarct volume in HO2−/− mice was also less than the volume at risk. The 85% reduction in LDF over the 2-h MCAO period in lateral parietal cortex indicates that the reduction in blood flow was severe and stable in the ischemic core. The issue of whether the flow reduction was equivalent in ischemic border regions was not distinguished by this probe location. However, the IAP autoradiographic data did not reveal any differences in the volume of tissue with low or intermediate levels of CBF. One limitation of the IAP technique is that some of the tracer may diffuse from high-flow regions to low-flow regions before freezing (12). However, this error should be similar in both the WT and HO2−/− mice. In terms of the volume of tissue with CBF <50 ml min−1 100 g−1, this volume of tissue is similar to the 36% infarct volume obtained after 2-h MCAO in WT mice. These results in the mouse are in general agreement with those in the rat where tissue with CBF <50 ml min−1 100 g−1 was destined to infarct (9, 11).

After removal of both filaments, the LDF signal rapidly returned to near-baseline levels. Not all studies using the single-filament technique in mice report com-
plete reperfusion measured by LDF (3). Because reperfusion deficits can be heterogeneous and may influence outcome, we chose to measure the spatial distribution of absolute blood flow by autoradiography. The 3-h time of reperfusion was chosen rather than a shorter time point to allow time for secondary energy failure, cell swelling, thrombus formation, and leukocyte adhesion to evolve and because LDF did not reveal a grossly abnormal deficit during the first hour of reperfusion. No evidence of hypoperfusion was seen at 3 h in the reperfused regions in either WT or HO2−/− groups, and the blood flow distribution was similar to that in the contralateral hemisphere. It is possible that ischemic durations >1 h would reveal reperfusion deficits. Nevertheless, these data indicate that a reperfusion deficit is unlikely to account for the greater infarct volume in HO2−/− after 1 h of MCAO.

Administration of the HO inhibitor SnPPiX increased infarct volume in WT mice, but infarct volume remained less than the volume at risk at each coronal level. In HO2−/− mice, SnPPiX increased infarction only in the second anterior section. In the remaining sections, infarct volume remained substantially less than the volume at risk, thereby indicating that the lack of effect of SnPPiX in these sections of HO2−/− mice was not attributable to inducing a maximum insult. The lack of an effect of SnPPiX on total infarct volume in HO2−/− mice implies that the primary effect of SnPPiX in WT mice is mediated by inhibition of HO2 activity rather than HO1 activity. The similar infarct volume previously observed in WT and HO1−/− mice (5) is consistent with this conclusion. Therefore, the neuronally constitutive isoform of HO (HO2) is primarily responsible for providing neuroprotection. Further, the observation that SnPPiX augmented injury when infused only during reperfusion implies that HO2 ordinarily acts to protect neurons after the ischemic insult. However, it is possible that differences in infarct volume between groups may be reduced if reperfusion was extended beyond 1 day.

The differences in infarct volume between WT and HO2−/− mice were greater after 1-h ischemia than after 2-h ischemia. Indeed, increasing the duration of ischemia from 1 to 2 h did not produce a significant increase in infarct volume in HO2−/− mice. These data indicate that constitutive HO2 provides neuroprotection primarily after relatively brief periods of transient focal cerebral ischemia. Apoptotic mechanisms of cell death may be more prominent after brief periods of ischemia (7). We reported that evoked apoptosis in cerebellar granule cells was enhanced in cultures from HO2−/− mice (4). After 1 h of MCAO, the number of cells with apoptotic bodies was also enhanced in HO2−/− mice (4). Thus constitutive HO2 may provide protection from 1 h of focal ischemia by limiting apoptotic mechanisms of cell death, whereas this neuroprotection may become overwhelmed by necrotic cell death mechanisms when ischemic duration is prolonged.

During ischemia and reperfusion, there is presumably an increase in the load of free heme as heme-containing proteins are degraded by the denaturing effects of oxidant and acidic damage and from active proteolysis. The mechanism of protection by HO2 may be mediated by removal of pro-oxidant heme, the substrate for HO, or by formation of CO and bilirubin. Our data indicate that CO-mediated vasodilation, which would result in enhanced perfusion during ischemia or improved reflow in WT mice, does not account for smaller infarcts in WT mice. The data do not exclude an antiapoptotic effect of CO as described in endothelial cells (25). Bilirubin in low concentrations is a potent antioxidant that can limit oxidative injury in endothelial cells and neurons (5, 26), possibly by being oxidized back to biliverdin by reactive oxygen species (1). However, neuroprotection is lost at high concentrations of bilirubin (5). Hence, the protection afforded by HO2 may depend on the enzymatic activity rate and the amount of available heme. Consistent with this concept, intracerebral injection of blood, which would be anticipated to provide a much larger heme load than ischemia, results in white matter edema that is attenuated rather than augmented by HO inhibition (10, 27). Therefore, the opposite effect of HO inhibition with MCAO vs. intracerebral injection of blood indicates that neuroprotection by HO may be selective for ischemic stroke.

In summary, we describe the use of a double-filament model of transient MCAO in a knockout strain of mice in which the standard single-filament technique was ineffective. The model produced a consistent volume at risk, reproducible and stable reductions in CBF, and homogeneous reperfusion. This model may be of use as an alternative in other knockout strains of mice where standard models of transient MCAO happen to be ineffective.

This work was supported by National Institutes of Health Grant NS-38084 (R. C. Koehler), by a grant-in-aid from the American Heart Association (S. Dore), and by an Atorvastatin Research Award (S. Dore) from Pfizer.

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