Carbon Monoxide–Activated Nrf2 Pathway Leads to Protection Against Permanent Focal Cerebral Ischemia

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Background and Purpose—Carbon monoxide (CO) is a gaseous second messenger produced when heme oxygenase enzymes catabolize heme. We have demonstrated that CO can be therapeutic in ischemia-reperfusion brain injury; however, it is unclear whether CO can also offer protection in permanent ischemic stroke or what mechanism(s) underlies the effect. Heme oxygenase-1 neuroprotection was shown to be regulated by Nrf2; therefore, we investigated whether CO might partially exert neuroprotection by modulating the Nrf2 pathway.

Methods—To evaluate the potential protective effects of CO, we exposed male wild-type and Nrf2-knockout mice to 250 ppm CO or control air for 18 hours immediately after permanent middle cerebral artery occlusion. Infarct volume and neurologic deficits were assessed on day 7. Molecular mechanisms of Nrf2 pathway activation by CO were also investigated.

Results—Mice exposed to CO after permanent ischemia had 29.6±12.6% less brain damage than did controls at 7 days, although amelioration in neurologic deficits did not reach significance. Additionally, 18-hour CO treatment led to Nrf2 dissociation from Keap1, nuclear translocation, increased binding activity of Nrf2 to heme oxygenase-1 antioxidant response elements, and elevated heme oxygenase-1 expression 6 to 48 hours after CO exposure. The CO neuroprotection was completely abolished in Nrf2-knockout mice.

Conclusions—Low-concentration CO represent a neuroprotective agent for combination treatment of ischemic stroke, and its beneficial effect would be at least partially mediated by activation of the Nrf2 pathway. (Stroke. 2011;42:2605-2610.)

Key Words: carboxyhemoglobin ■ heme oxygenase ■ mouse ■ neuroprotection ■ stroke

Low or near-physiologic doses of carbon monoxide (CO) have been shown to protect cells through potential anti-inflammatory, antiproliferative, or antiapoptotic effects; however, the exact cellular pathway(s) is still under investigation. CO is a soluble gas that is generated in cells almost exclusively through the degradation of heme by heme oxygenase (HO) enzymes. Heme oxygenase-1 (HO1) is known to be induced by the transcriptional factor Nrf2, which is considered to be a multiorgan protector and mediates neuroprotection by binding to antioxidant response elements (AREs) of antioxidant genes to increase their transcription. Normally, Nrf2 is bound to Keap1, largely localized in the cytoplasm. This quenching interaction maintains low basal expression of Nrf2-regulated genes. However, when cells are subjected to oxidative or xenobiotic stress, Nrf2 dissociates from Keap1, traverses to the nucleus, and activates the expression of phase II enzymes such as HO1, which is known to have some AREs. Exposure to CO produces protective effects similar to those of HO1 in many models, although it remains unclear in some cases whether the doses used represent physiologic or supraphysiologic levels. We have demonstrated that a low level of exogenous CO (250 ppm for 18 hours) can be neuroprotective against transient cerebral ischemia. Here, our goal was to determine whether CO is also protective in permanent focal cerebral ischemia, which is considered by some to have higher clinical relevance than transient cerebral ischemia. Furthermore, we investigated whether CO acts as a neuroprotective messenger in vivo via Nrf2 pathway activation.

Materials and Methods

Animals
First, all experiments were carried out in accordance with the National Institutes of Health guidelines and were approved by the institutional animal care and use committee. All randomly assigned mice were 8- to 10-week-old wild-type (WT) and Nrf2-knockout (Nrf2−/−) C57BL/6 mice.

CO Exposure
Mice were placed into a Plexiglas chamber at room temperature for exposure to air (control) or 250 ppm CO as monitored by a single gas
Permanent Middle Cerebral Artery Occlusion

Mice were first anesthetized with halothane (3% induction, 1.2% to 1.5% maintenance), and then a short vertical skin incision was made between the right eye and ear, a 2.0 mm burr hole was drilled, and the main trunk of the distal middle cerebral artery was occluded by bipolar coagulation. Interruption of blood flow at the occlusion site was confirmed by severance, and blood flow reduction was monitored by laser-Doppler flowmetry. Infarct volume was determined 7 days after permanent middle cerebral artery occlusion (pMCAO) by image analysis of five 2-mm-thick coronal sections stained with 1% 2,3,5-triphenyltetrazolium chloride and calculated as the percentage of the contralateral cortex, with correction for swelling.

Behavioral Tests

One experimenter held the mouse while another placed an adhesive tape (3 x 4 mm²) onto the plantar surface of each forepaw, and the contact time and removal time were recorded. The contact time represented the time taken for the animal to begin shaking or bringing the forepaw to its mouth. The removal time represented the time taken, once the tape had been sensed, for the animal to remove the tape. The mice were given a maximum of 2 minutes. Mice were tested before surgery and 7 days after surgery. The 28-point neurologic scoring system includes body symmetry, gait, climbing, circling behavior, front limb symmetry, compulsive circling, and whisker response. Each test was graded from 0 to 4, thus establishing a maximum deficit score of 28. For the gross locomotor test, mice were evaluated with the Home Cage Video Tracking System (MedAssociates) for 30 minutes at 7 days after CO or air exposure.

Electrophoresis Mobility Shift Assay for Nrf2

Nuclear protein was extracted by sucrose gradient centrifugation. Five micrograms of nuclear protein was incubated with 32P-labeled, double-stranded oligonucleotide probes containing ARE sequences of the HO1 promoter (5'-TGTGAATCACTGTTACCTT-3' and 3'-AAAATAAGCAGACAGTACCAA-5'); cold probes lacking 32P-label; and mutant probes containing mutant ARE sequences (5'-TTGTATAGCTGTCATGGTTT-3' and 3'-AAAATAAGCAGACAGTACCAA-5'). Positive-control nuclear protein was obtained from mouse neuronal cultures that were exposed to sulforaphane (an Nrf2 activator; 0.5 μmol/L).

Immunologic Analysis

The cytosolic protein was prepared as described previously. For immunoprecipitation, 15 μL of antibody (anti-Nrf2 or anti-Keap1, Santa Cruz) was added to 300 μg protein. The mixture was then incubated with glutathione-Sepharose 4B beads (GE Healthcare), and supernatant proteins were separated on gel, transferred to nitrocellulose membranes, and incubated with primary antibodies (anti-Keap1 or anti-Nrf2; 1:1000, Santa Cruz), anti-HO1 or anti-HO2 (1:1000, Stressgen Biotechnologies), anti-β-actin (1:3000, Sigma), anti-histone (1:8000, US Biological), and anti-MnSOD (1:5000; from T. Dawson, Johns Hopkins University). For immunohistochemistry, the frozen sections were blocked in normal goat serum and incubated with Nrf2 antibody and then with biotinylated secondary antibody (Vector). Immunoreactions were developed with the avidin-peroxidase–labeled biotin complex and visualized by the diaminobenzidine peroxidase substrate (Vector).

Statistical Analysis

Data were analyzed with Sigmasstat 3.0 software. All values are presented as mean±SD. For comparisons between 2 groups, a 2-tailed, unpaired Student t test was used. For comparisons among multiple groups, 1-way ANOVA with a Student-Newman-Keuls procedure was used. Statistical significance was set at P<0.05.

Results

Effect of Post-pMCAO CO Exposure on Infarct Size and Neurobehavioral Function

Eighteen hours of CO inhalation initiated immediately after ischemia significantly reduced infarct size, from 25.2±4.8% (air) to 16.4±3.7% (CO) (Figure 1A). Behavioral performances between air- and CO-treated mice did not reach significance when assessed with the neurologic deficit score and locomotor test (data not shown). However, in the tape-removal test, mice subjected to right-side pMCAO and exposed to air required significantly longer to contact (13.4±3.9 seconds) and remove (43.3±7.4 seconds) the tape from the left forepaw than did sham-operated mice (contact time, 7.9±3.7 seconds; removal time, 23.2±7.9 seconds). The mice exposed to CO had a trend toward improved ability to remove the tape compared with air-exposed mice (left).
A 75-kDa protein, corresponding to Keap1 that had bound to Nrf2, was observed in the brains of mice exposed to air, but its expression was significantly lower after 18-hour CO exposure (Figure 2A). Next, the lysates were precipitated with anti-Keap1 antibody and immunoblotted with anti-Nrf2 antibody. The 57-KDa protein corresponding to Nrf2 that had bound to Keap1 was highly detected in the brains of mice exposed to air, but its expression decreased with increasing time of CO exposure (Figure 2A). These results support the concept that CO promotes dissociation of Nrf2 from Keap1, the first step in Nrf2 nuclear translocation.

To further test whether CO could induce Nrf2 nuclear translocation, we measured Nrf2 protein in cytosolic and nuclear brain extracts. CO exposure did not significantly alter cytosolic Nrf2 levels. However, nuclear Nrf2 accumulation was significantly enhanced at 0 and 6 hours after 18-hour CO exposure and remained elevated for at least 6 hours after CO termination. Histone was used as a nuclear marker and as a protein loading control. Immunohistochemistry shows Nrf2 nuclear localization in the cortices of CO-exposed mice. In the air-treated group, most Nrf2 was cytoplasmic in cells with neuronal morphology. Nuclear staining of Nrf2 increased with increasing CO exposure time and remained high through 6 hours after CO exposure. The percentage of cells with nuclear Nrf2 staining was assessed in 6 nonoverlapping fields for each section (n=6 mice per group). O.D. indicates optical density. *P<0.05, **P<0.01.

CO Increases Nuclear Nrf2 Occupancy of AREs in the HO1 Promoter

Binding to the ARE by Nrf2 represents an indispensable process for transcriptional activation of ARE-responsive genes. Therefore, we measured Nrf2-ARE binding activity with Nrf2-specific, double-stranded oligonucleotide probes. We found that Nrf2-ARE was significantly increased at 0 and 6 hours after 18-hour CO exposure but had returned to nearly baseline by 24 hours (Figure 3A). Cultured neurons treated with the Nrf2 activator sulforaphane served as a positive control. To confirm that upregulation of HO1 expression was mediated by functional Nrf2, we used an excess concentration of cold probes to compete for radioactive complex formation and dominant-negative mutant probes to demonstrate Nrf2-ARE interactional specificity. Competition from cold probes completely inhibited Nrf2 binding with radioactive probes. In contrast, mutant probes failed to suppress binding activity (Figure 3B).

HO1 Induction After CO Exposure

HO1 has a large number of AREs within its promoter and is highly dependent on Nrf2 action. Therefore, we investigated the expression of HO1 and HO2 after CO exposure. As shown in Figure 4, HO1 expression gradually increased from 6 to 48 hours after 18-hour CO exposure. In contrast, CO had no effect on HO2 expression.

CO Is Not Protective in Nrf2−/− Mice

To further clarify the beneficial role of Nrf2 activation after CO treatment, we subjected Nrf2−/− and WT mice to pMCAO and exposure to 250 ppm CO or air for 18 hours and then measured infarct size and neurologic deficits at 7 days. Nrf2−/− mice had significantly larger infarct sizes and prolonged left-paw tape-removal times than did WT mice and did not show any improvements after CO exposure (Figure 5).
Additionally, CO did not induce HO1 expression in Nrf2−/− mice after CO exposure (see the online-only Data Supplement).

**Discussion**

The major findings are that 250 ppm CO exposure (1) significantly reduces infarct size but has no significant effect on behavioral deficits after permanent ischemia; (2) increases dissociation of Nrf2 from Keap1 and causes Nrf2 nuclear translocation in the brain; (3) increases the nuclear Nrf2 occupancy of AREs within the HO1 promoter; (4) induces time-dependent increases in HO1 expression; and (5) does not provide beneficial effects in Nrf2−/− mice. These data suggest that CO exposure after stroke can provide neurologic protection by activating the Nrf2 pathway.

Our previous study showed that 250 ppm CO exposure for 18 hours provided better neuroprotection than did other concentrations and durations (125 or 500 ppm for 12 or 18 hours). In addition, we found that 250-ppm CO treatment for 18 hours given immediately or after a 1-hour delay after ischemia provided significant protection against brain injury, as evidenced by decreased infarct volume. However, delaying CO exposure for 3 hours resulted in weaker protection (data not shown). Therefore, the therapeutic window for CO treatment in mice might be within 3 hours after ischemia. We chose to give CO immediately after pMCAO.

We first assessed neurologic deficit score and locomotor activity for behavioral evaluation. However, we did not find any significant difference between air- and CO-treated mice subjected to pMCAO on day 7 (data not shown). Nevertheless, according to Freret et al., the tape-removal test is more sensitive than other methods for detecting behavioral differences after pMCAO. We used this test and found that CO-exposed mice had a trend toward removing the tape from the left paw in less time than did air-exposed mice. A few factors that may have contributed to our detecting no significant effects on neurologic deficits include the following: (1) the injury in pMCAO is mainly confined to the cortex of the temporal lobe, but the primary somatosensory area is located in the postcentral gyrus of the parietal lobe; (2) the tape-removal test may have intrinsic limitations; (3) the limitations of behavioral testing in mice may be partly due to the high level of spontaneous recovery; and (4) CO may not be sufficiently robust per se to provide functional improvement with the given neurobehavioral tests. Taken together, we plan to consider more sensitive evaluation of neurologic deficits to reveal a benefit of CO treatment in future studies.

High CO concentrations can cause hypoxemia by competitive binding to oxygen-binding sites of hemoglobin to form carboxyhemoglobin (COHb). In humans, symptoms of CO poisoning begin to appear at 20% COHb, whereas death occurs between 50% and 80% COHb. In contrast, CO exposures associated with cytoprotection in rodents resulted in attained COHb levels within the 15% to 20% range. Based on our study, the blood COHb levels reached only 16.5±1.5% at 18 hours and returned to baseline relatively quickly after exposure. Whether compensation is possible, no changes in total hemoglobin levels were noted (data not shown). Therefore, the oxygen-carrying capacity of blood did not significantly change during CO exposure, although the possibility of subtle changes cannot be ignored.

Our data revealed that activation of the Nrf2 pathway might partially contribute to CO neuroprotection. Nrf2 has been considered to be a key regulator in cell survival mechanisms, and its activation induces expression of phase II enzymes, which attenuate tissue injury caused by oxidative stress. Dulak and colleagues reported that CO gas activates different kinases, such as phosphatidylinositol 3-kinase, protein kinase C, c-Jun NH2-terminal kinase, p38, and extracellular...
lular signal–regulated kinase, which lead to Nrf2 activation and downstream gene expression. Although much work has been conducted in regard to Nrf2 regulation, its exact activation remains unclear. It has been shown that CO-induced guanylate cyclase activation with cGMP production involves vasoregulation. However, it is unclear whether cGMP production is directly involved in Nrf2 activation by CO. Nrf2 gene function does not appear to be necessary for overall mouse development, growth, and fertility, and no developmental defects have been found in Nrf2-/- mice.10 Existing data have demonstrated that treatment with chemical activators of Nrf2, such as sulforaphane,11 can reduce cellular damage in WT mice but not in Nrf2-/- mice. In agreement with these findings, we found that exogenous CO induced Nrf2 activation, and its neuroprotection was abolished in Nrf2-/- mice.12 Our group and others have shown that Nrf2 activation may confer protection against stroke13 or oxidative stress–related neurodegenerative insults.14 Previous and current data position Nrf2 as a prime candidate for prevention of oxidative stress and subsequent neurotoxicity.

Nrf2 activation can coordinately upregulate expression of several antioxidative enzymes recognized to play important roles in combating oxidative stress, including HO1, superoxide dismutase, and glutathione S-transferase, as well as NADPH-regenerating enzymes. However, compared with other enzymes, the HO1 promoter is known to have a large number of ARE sequences to which Nrf2 can bind to induce its expression in a preferential manner.3 In addition, HO1 is more likely to exert a central role in neuroprotection because it is inducible to degrade free heme, and its metabolites, CO or biliverdin/bilirubin, can directly provide cytoprotection.5,14 It has been shown that HO1 may provide protection against excitotoxicity15 and cerebral ischemia.16 Therefore, we believe that HO1 could be a significant player, although other enzymes can also assist with CO neuroprotection. We further plan to use HO1-knockout mice or enzymatic inhibitors to further determine its unique contribution in CO protection, although we have found that Nrf2 activation caused by CO led to an increase in HO1 levels.

Recent findings have suggested the central role of reactive oxygen species induced by low levels of CO in CO-initiated preconditioning and protection by induction of antioxidant enzymes and protective signaling pathways.5 Eighteen hours of CO exposure may create such an oxidative preconditioning to induce Nrf2 activation for modulation of cerebral ischemia. Meanwhile, the formation of reactive oxygen species in response to low concentrations of CO may have both positive (signaling) and negative (damaging) effects, depending on the amount of reactive oxygen species formed and the cell type under investigation. Therefore, we cannot rule out the possibility that 18 hours of CO exposure might result in some factors that might contribute to ischemic injury, although no overall detectable toxicity was reported at the levels tested. Our data imply that controlled exposure to exogenous CO could be 1 means to harness the neuroprotective Nrf2 pathway for stroke treatment. The beneficial effects of CO are likely caused by a combination of factors. CO may also act as a vasodilator by modulating soluble guanylate cyclase17 or by activating calcium-activated potassium channels18 to induce vasorelaxation. Activation of the p38 mitogen-activated protein kinase signaling pathway modulated by CO has been implicated in anti-inflammatory19 and antiapoptotic20 effects. In addition, CO has been reported to have early thrombolytic effects after ischemia.21 Taken together, further investigation of the various mechanisms by which CO might be protective in permanent cerebral ischemia is warranted.

**Conclusions**

CO could be a neuroprotective agent in combination treatment for permanent ischemia, although it did not reach significance in attenuating several behavioral tests. As optimal delivery of CO is refined, the protective effects of CO could be extended to the treatment of ischemic stroke.

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Disclosures
None.

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Carbon monoxide-activated Nrf2 pathway leads to protection against permanent focal cerebral ischemia

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S1 Expression of HO1 in wildtype (WT) and Nrf2 knockout (Nrf2<sup>−/−</sup>) mice after carbon monoxide (CO) exposure. As shown in the figure, HO1 expression increased from 24 to 48 h after 18 h CO exposure in wildtype, but not in Nrf2-deleted mice.