Published in final edited form as:

Neurotox Res. 2009 February ; 15(2): 133–137. doi:10.1007/s12640-009-9014-4.

Low Doses of Carbon Monoxide Protect Against Experimental Focal Brain Ischemia

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Abstract

Carbon monoxide (CO) is associated with central nervous system toxicity. However, evidence also indicates that CO can be protective, depending on its concentration. To determine if CO can be neuroprotective after ischemic brain injury, we subjected mice to transient middle cerebral artery occlusion and exposed them to different concentrations of CO. We found that in mice, low CO levels protected the brain from injury following 90-min transient focal ischemia and 48 h of reperfusion. When inhalation of 125 or 250 ppm CO began immediately at the onset of reperfusion, total hemispheric infarct volume was reduced by $32.1 \pm 8.9\%$ and $62.2 \pm 14.4\%$ respectively; with an extended therapeutic window of 1 to 3 h after ischemia, CO inhalation also attenuated infarct volume significantly. Furthermore, early CO exposure limited brain edema formation by $3.2 \pm 0.8\%$ (125 ppm) and $2.6 \pm 0.3\%$ (250 ppm). Finally, CO inhalation significantly improved neurological deficit scores at 48 h of survival time after ischemia. Transient elevation of carboxyhemoglobin levels returned rapidly to baseline when CO exposure was stopped. These findings suggest a potential application of CO to treat brain ischemic stroke.

Keywords

brain edema; gas; heme oxygenase; ischemic stroke; transient cerebral ischemia

INTRODUCTION

Exposure to high concentrations of carbon monoxide (CO) causes immediate elevation of carboxyhemoglobin (COHb) to toxic levels, triggering hypoxia. COHb-induced hypoxia leads in turn to neuronal cell death (Uemura *et al.*, 2001) and brain edema (Chu *et al.*, 2004). The lesions commonly found in CO-poisoned patients originate in specific brain regions rich in iron: the central white matter, globus pallidus, and hippocampus (Koehler and Traystman, 2002). However, physiologic concentrations of CO in the blood and basal levels of COHb are necessary for normal signaling pathways (Leffler *et al.*, 2006).

CO is released following the enzymatic degradation of heme by heme oxygenases (HO). It has been estimated that a minimum of 86% of endogenously generated CO is HO-dependent, while the remaining is formed from photo-oxidation, lipid peroxidation, and xenobiotic metabolism (Durante *et al.*, 2006). Using transgenic mice and HO inhibitors, we have previously shown that constitutively expressed HO2 is neuroprotective against stroke damage (Doré *et al.*,

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Conflict of Interest Statement: No conflict.

1999). In addition to HO-derived CO, CO-releasing molecules (Nakao *et al.*, 2003), and low concentrations of inhaled CO have been reported to protect against various insults through activation of anti-inflammatory, anti-apoptotic, and vasodilatory mechanisms (Otterbein, 2002; Leffler *et al.*, 2006).

Based on the protective properties of endogenous HO-generated CO, we tested the hypothesis that exogenous CO might provide protection against ischemic stroke. We designed an observational study of CO inhalation to describe the functional outcome of experimental transient focal brain ischemia.

MATERIALS AND METHODS

Animals

Experiments were carried out in accordance with the guidelines of the National Institutes of Health for the care and use of animals in research and were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University. All mice were 8–10-week-old male C57BL/6 (Charles River Laboratories, Wilmington, MA).

Transient Focal Cerebral Ischemia, Brain Edema, and Exposure to CO

Transient focal ischemia was induced by a 90-min occlusion of the middle cerebral artery (MCA) as described previously (Shah *et al.*, 2006). Successful occlusion was confirmed by an 87–90% reduction in cerebral blood flow (CBF), as measured by laser-Doppler flowmetry (Shah *et al.*, 2006) (see Table I). Body temperature was maintained at 36–37°C throughout the experiment. After reperfusion was begun and isoflurane anesthesia was discontinued, the animals were completely awake within 20–30 min. Then, neurological deficit was evaluated as follows: 0, normal motor function; 1, flexion of torso and of contralateral forelimb when held by the tail; 2, circling to the contralateral side but normal posture at rest; 3, leaning to the contralateral side at rest; 4, no spontaneous movement. Mice that had a neurological deficit scores (NDS) <2 at the time of reperfusion were excluded from the study. In one cohort, mice were exposed to normal air, 125 ppm, or 250 ppm CO at the onset of reperfusion. In additional cohorts, 250 ppm CO inhalation was initiated at 1 and 3 h after reperfusion. During CO exposure, mice were kept in a Plexiglas chamber (36 cm × 24 cm × 12 cm) at room temperature. The inflow in the chamber was adjusted to 1 L/min, and the outflow tube was secured to the Single Gas Analyzer CO91 (Universal Enterprises, Beaverton, OR), which allowed strict monitoring of CO levels in the chamber. After the 18-h exposure, mice were returned to their original cages. At 24 and 48 h of reperfusion, NDS testing was repeated. At 48 h of reperfusion, the mice were deeply anesthetized with 5% isoflurane, and the brains were processed for analysis of infarct volume or brain edema. Brains were harvested, sliced into 2-mm thick sections, and stained with 1% 2,3,5-triphenyltetrazolium chloride. Infarct volume was calculated as a percent of the contralateral hemisphere and corrected for swelling (Shah *et al.*, 2006). A separate cohort of animals was evaluated for focal ischemia-induced brain edema at 48 h of reperfusion. The ipsilateral and contralateral hemispheres were separated, weighed, dried at 100°C for 72 h, and then weighed again. The wet-to-dry ratio was calculated to determine the brain water content: $[(\text{Wet} - \text{Dry})/\text{Wet}] \times 100$.

Blood COHb Levels During Exposure to CO

Separate cohorts of mice were exposed to air or to 125 or 250 ppm CO to evaluate blood COHb levels at 1, 3, and 18 h during the exposure and soon after discontinuation of exposure, at 20 h. When gas flow was discontinued, or at the end of the experiment, mice were immediately anesthetized with 1.5% isoflurane, and 0.2 mL of venous blood was collected for COHb measurement using the OSM 3 Hemoximeter (Radiometer A/S, Copenhagen, Denmark).

Statistical Analysis

All values are expressed as mean $\pm$ standard error of the mean. Physiological variables, infarct volumes, brain water content, and COHb were compared to their corresponding controls by one-way ANOVA or Student's *t*-test. NDS is presented as median scores and interquartile ranges (25th and 75th percentiles) and analyzed by the non-parametric Kruskal-Wallis test. A *p*-value of <0.05 was considered to be significant.

RESULTS

CO inhalation initiated immediately upon reperfusion (0 h) attenuated ischemia-induced infarct volumes: a) in the cortex, from $55.5 \pm 4.3\%$ (airflow) to $37.1 \pm 4.1\%$ (125 ppm) and $19.2 \pm 7.3\%$ (250 ppm); b) in the caudate putamen (CP) complex, from $76.5 \pm 6.1\%$ to $67.2 \pm 5.4\%$ and $25.5 \pm 11.5\%$; and c) in the overall hemisphere, from $49.9 \pm 3.2\%$ to $33.9 \pm 4.4\%$ and $18.8 \pm 7.3\%$, respectively. CO (250 ppm) administered 1 and 3 h after initiation of reperfusion also significantly reduced infarct volumes, but less protection was observed after the 3-h delay. Infarct volumes at 1 h were $16.4 \pm 9.0\%$, $14.3 \pm 8.5\%$, and $15.4 \pm 7.2\%$ and at 3 h were $31.9 \pm 8.0\%$, $38.1 \pm 13.8\%$, and $33.4 \pm 7.5\%$ in the cortex, CP complex, and overall hemisphere, respectively (FIG. 1a–d).

The inhalation of 125 ppm CO initiated at 0 h and 250 ppm CO initiated at 0 or 1 h of reperfusion significantly improved NDS. CO exposure initiated 3 h after restoration of CBF did not improve NDS (Table I).

CO significantly reduced the edema formation in the ipsilateral hemisphere compared with that of the air-exposed mice ($83.3 \pm 0.7\%$) to $80.7 \pm 0.7\%$ (125 ppm) and $81.1 \pm 0.3\%$ (250 ppm) (FIG. 1e).

Exposure to 125 and 250 ppm CO increased blood COHb concentrations from $5.1 \pm 0.4\%$ (airflow) to $11.4 \pm 0.5\%$ (125 ppm) and $19.0 \pm 3.5\%$ (250 ppm), reaching a steady state at 3 h. Discontinuation of CO exposure returned COHb in the blood to physiologically normal levels within 2 h (FIG. 1f).

DISCUSSION

Based on reports of protective effects of CO (Otterbein, 2002; Koneru and Leffler, 2004), we sought to investigate the functional outcome of experimental brain ischemia in mice exposed to low CO concentrations. Although we observed mild, transient increases in COHb levels, mice exposed to 125 and 250 ppm CO following MCA occlusion developed significantly smaller cerebral lesions and less edema than those exposed to normal air. In addition, 250 ppm CO provided the best protection if the inhalation began at 0 or 1 h of reperfusion, as evidenced by decreased infarct volume, decreased edema, and improved neurological deficits. Delaying CO exposure after ischemia-reperfusion for 3 h resulted in weaker protection. Taken together, the data suggest that inhalation of CO may be considered as an alternative and/or adjuvant to current stroke therapies.

Knowledge of the ways in which CO affects various vascular and cellular signaling pathways could shed light on the mechanism(s) of protection against ischemic stroke. One possible mechanism by which CO can be protective is through vasodilatation. Because of its similarity in molecular structure to NO, CO is able to compete with NO for binding to heme-containing proteins. For example it might have similar actions on blood vessels/blood flow, cell death, free radical damage, etc. Similar to NO, CO can modulate soluble guanylyl cyclase and induce smooth muscle vasorelaxation. Furthermore, the binding of CO to heme molecules causes cerebral arterioles to dilate through increases of Ca^{2+} sparks, opening of Ca^{2+} -activated K^{+}

channels, and hyperpolarization of smooth muscle cells (Leffler *et al.*, 2006). This CO-induced vasodilatation would require NO and a basal level of cellular cyclic-GMP (Koneru and Leffler, 2004). The greater NO bioavailability in endothelial cells would lead to improved vasodilatory responses of cerebral arteries in post-ischemic conditions (Thom and Ischiropoulos, 1997). However, excessive release and slowed utilization of NO in ischemic conditions may cause formation of peroxynitrite (Thom and Ischiropoulos, 1997). Such phenomena (along with higher COHb) could potentially explain exacerbated ischemic brain injury in mice after inhalation of 500 ppm CO (data not shown).

Low CO doses have previously been reported to have anti-inflammatory effects (Otterbein, 2002). Exposure to CO has been shown to decrease infiltration of macrophages and neutrophils and suppress mobilization of the anti-inflammatory cytokine IL-10 (Otterbein, 2002). The potential protective effect of CO on ischemic reperfusion injury and brain edema could also result from a decrease in cytotoxic or vasogenic edema. CO has been reported to have early thrombolytic effects (Chen *et al.*, 2006) after ischemia that improve endothelial cell survival and likely help to preserve vascular integrity responsible for the development of vasogenic brain edema after reperfusion. Cytotoxic brain edema originates in cells undergoing stress and is reduced indirectly through suppression of inflammation, apoptosis, and free-radical formation on the first day of reperfusion.

Accidental exposure to high concentrations of CO renders severe consequences to the central nervous and cardiovascular systems that can be acute (hours to days) or delayed (weeks to months) (Weaver *et al.*, 2002). Case reports describe severe acute effects of CO poisoning that result in cell death, and brain edema formation (Uemura *et al.*, 2001; Chu *et al.*, 2004). Exposure to high concentrations of CO can produce blood concentrations of COHb greater than 30%, a level shown to cause neurotoxicity (Brunssen *et al.*, 2003). COHb blood levels exceeding 25% that result from accidental CO exposure cause delayed sequelae (Weaver *et al.*, 2002). On the other hand, COHb levels do not always correlate with delayed outcomes of CO exposure, and tolerance of the brain cells to CO poisoning and their ability to retain COHb have not been clearly established. In our study of controlled exposure to low CO concentrations, we describe acute outcomes only at 48 h that correspond with COHb concentrations of $11.4 \pm 0.5\%$ (125 ppm) and $19.0 \pm 3.5\%$ (250 ppm). Shorter CO exposure times (≤ 12 h) were not sufficient to provide significant neuroprotection against ischemic stroke, but 18 and 24 h of exposure resulted in similar neuroprotective effects (data not shown). Hence, we chose the 18-h exposure for all subsequent studies. Additional and ongoing studies are being performed to investigate the lowest effective dose and the minimal exposure time that can correlate with significant neuroprotection, such that side effects could also be minimized.

To our knowledge, this is the first report to demonstrate that controlled early post-ischemic exposure to CO concentrations of 125 and 250 ppm provides neuroprotection. We have also observed that naïve mice subjected to 250 and 500 ppm CO alone for 18 h had no detectable histological lesions at 30 h post-exposure, as demonstrated by postmortem 1% 2,3,5-triphenyltetrazolium chloride staining (unpublished observations); additionally, neither 125 nor 250 ppm CO exposure elevate brain water content after 30 h in naïve mice. These data indicate that low CO concentrations are unlikely to cause acute histologic damage of the brain or vascular tissues at 48 h. Although histologic evaluation of the CO-exposed animals is important to further document the potential harmful effects of CO on brain cells, we believe that at early time points, pathophysiological changes to the CNS and to blood gases and blood pressure are likely to occur only after high levels of CO exposure. However, potential delayed sequelae of CO exposure should be considered in long-term outcome studies. It is notable that in a clinical trial of 2-h controlled CO exposure up to 232 ppm produced no symptoms or changes in hemodynamics with COHb levels of 16–23% (Benignus *et al.*, 1987). Similarly, in our study spontaneous locomotor activity of mice exposed to 125 and 250 ppm CO was not

altered (data not shown) during or after exposure to CO for 18 h with corresponding COHb concentrations, and the increase in COHb appears to be transient.

One alternative explanation for the CO protection in an ischemia-reperfusion protocol is that when CO is given at an early time point, it might limit the surge in free-radical formation following reperfusion. In our study, CO exposure begun immediately at the onset of reperfusion and lasting for 18 h showed significant protection against ischemic-reperfusion injury, but a 3-h delay in CO exposure was less effective. Furthermore, exposure to 250 ppm CO that began immediately but with duration of only 3 h had no significant effect on infarct volume (data not shown). Determining the appropriate time course and duration for exposure in humans will be critical before CO can be used therapeutically.

CONCLUSIONS

We have shown that inhalation of 125 and 250 ppm CO is neuroprotective in the early stages of reperfusion after cerebral ischemia in mice. Anti-inflammatory, anti-apoptotic, vasodilatory, and early thrombolytic properties of CO could be involved in improved neuronal, glial, and endothelial cell survival in post-ischemic conditions. These properties of CO can be instrumental in efficiently restoring CBF in brain circulatory insufficiency and minimizing brain tissue damage. Further exploration of the pharmacodynamics, pharmacokinetics, and long-term effects of this gaseous agent is necessary to fully understand its role(s) in the physiology of the brain.

ACKNOWLEDGEMENTS

We especially thank Khodadad Namiranian, Kathleen K. Kibler, and all members of the Doré lab for assistance in this project.

Financial Support: This work was supported in part by grants from the National Institutes of Health NS046400 and the GEMI Fund (SD).

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FIGURE 1.

Inhalation of CO initiated at 0, 1, and 3 h of reperfusion after 90-min middle cerebral artery occlusion attenuated infarct volume in the: (a) cortex; (b) CP complex; and (c) overall hemisphere compared to air-flow-exposed mice. Air: 0 h, $n = 11$; 125 ppm CO: 0 h, $n = 10$; 250 ppm CO: 0 h, $n = 8$; 250 ppm CO: 1 h, $n = 6$; and 250 ppm CO: 3 h, $n = 10$. All values are mean $\pm$ SEM, * $p < 0.05$ and ** $p < 0.01$ vs. air flow control. (d) Representative images of 2,3,5-triphenyltetrazolium chloride-stained brain slices (anterior view) from air-flow-, 125 ppm CO-, and 250 ppm CO-exposed mice. (e) Inhalation of 125 and 250 ppm CO initiated at the onset of reperfusion limited brain edema formation in the ipsilateral hemisphere compared to air-flow-exposed mice. Brain water content of the contralateral hemisphere remained normal. Air, $n = 5$, 125 ppm CO, $n = 6$, and 250 ppm, $n = 6$. (f) Effect of CO on circulating carboxyhemoglobin (COHb). Exposure of mice to 125 and 250 ppm CO with a flow velocity of 1 L/min produced a steady-state blood COHb concentration of $11.4 \pm 0.5\%$ and $19.0 \pm 3.5\%$, respectively. COHb returned to normal levels within 2 h after discontinuation of CO gas exposure, and remained stable for up to 48 h after initiation of therapy. $n = 3 - 5$ per group; * $p < 0.05$ and ** $p < 0.05$ vs. air flow control.

Table 1

Cerebral Blood Flow and Neurological Deficit Scores

Parameter	Infarct Volume					Brain Water			
	Air Flow, 0 h	125 ppm, 0 h	250 ppm, 0 h	250 ppm, 1 h	250 ppm, 3 h	Air Flow, 0 h	125 ppm, 0 h	250 ppm, 0 h	250 ppm, 0 h
n	11	10	8	6	10	5	6	6	6
% decrease in	87.1 ± 1.3	87.1 ± 1.9	87.6 ± 1.6	87.8 ± 1.5	89.0 ± 1.6	90.4 ± 1.3	88.9 ± 1.4	89.2 ± 1.3	
CBF ^a									
NDS ^b									
Day 0	2 (2-2)	2 (2-2)	2 (2-2)	2 (2-2)	2 (2-2)	2 (2-2)	2 (2-2)	2 (2-2)	2 (2-2)
Day 1	2 (2-2)	1.5 (1-2)*	2 (1.5-2)	1 (1-1)*	2 (2-2)	2 (1.75-3)	2 (1-2)	1 (1-2)	1 (1-2)
Day 2	2 (1.25-2.75)	2 (1-2)*	1 (1-1.5)*	1 (1-1)*	2 (2-2)	3 (1.75-3)	1 (1-2)*	1 (1-1)*	1 (1-1)*
Mortality ^c	3/14	0/10	0/8	2/8	2/12	1/6	0/6	0/6	0/6

^a CBF, cerebral blood flow, values represent mean ± SEM

^b NDS, neurological deficit score, values represent median (25th–75th percentile)

^c Data are shown as the number of deaths/total number of animals.

* $p < 0.05$ vs. the corresponding air flow control.