Time-dependent action of carbon monoxide on the newborn cerebrovascular circulation

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Knecht KR, Milam S, Wilkinson DA, Fedinec AL, Leffler CW. Time-dependent action of carbon monoxide on the newborn cerebrovascular circulation. Am J Physiol Heart Circ Physiol 299: H70–H75, 2010. First published April 30, 2010; doi:10.1152/ajpheart.00258.2010.—Carbon monoxide (CO) causes cerebral arteriolar dilation in newborn pigs by the activation of large-conductance Ca²⁺-activated K⁺ channels. In adult rat cerebral and skeletal muscle arterioles, CO has been reported to produce constriction caused by the inhibition of nitric oxide (NO) synthase (NOS). We hypothesized that, in contrast to dilation to acute CO, more prolonged exposure of newborn cerebral arterioles to elevated CO produces constriction by reducing NO. In piglets with closed cranial windows, pial arteriolar responses to isoproterenol (10⁻⁶ M), sodium nitroprusside (SNP; 10⁻⁷ and 3 × 10⁻⁷ M), and l-arginine ethyl ester (l-Arg; 10⁻⁵ and 10⁻⁴ M) were determined before and after 2 h of treatment with CO. CO (10⁻⁷ M) caused transient dilation and had no further effects. CO (2 × 10⁻⁷ and 10⁻⁶ M) initially caused vasodilation, but over the 2-h exposure, pial arterioles constricted and removal of the CO caused dilation. Exposure to elevated CO (2 h) did not alter dilation to SNP or isoproterenol. Conversely, the NOS substrate l-Arg caused dilation before CO that was progressively lost over 90 min of elevated CO. If NO was held constant, CO caused dilation that was sustained for 2 h. We conclude that in neonates, cerebral arteriole responses to CO are biphasic: dilation to acute elevation with subsequent constriction from NOS inhibition after more prolonged exposure. As a result, short episodic production of CO allows function as a dilator gasotransmitter, whereas prolonged elevation can reduce NO to elevate cerebrovascular tone. The interaction between heme oxygenase/CO and NOS/NO could form a negative feedback system in the control of cerebral vascular tone.

nitric oxide; neonate

CARBON MONOXIDE (CO) is produced, along with biliverdin and iron, via the catabolism of heme by heme oxygenase (HO) (17). The constitutive isoform, HO-2, is highly expressed in astrocytes and the endothelium in the neonatal brain (15, 16, 20). CO functions as a gasotransmitter, causing dose-dependent vasodilation of cerebral arterioles in newborn pigs and adult rats (6, 16). Acute CO dilates cerebral arterioles through the activation of Ca²⁺-activated K⁺ (KCa) channels (26).

The effect of CO could be age-related, species-, and/or exposure time-dependent because in the adult rat skeletal muscle vasculature, prolonged exposure to CO causes vasoconstriction by the inhibition of nitric oxide (NO) synthase (NOS) (9). In adult rats, CO derived from HO-2 appears to tonically regulate cerebral vasodilation by decreasing NO production (8).

The actions of CO and NO on the cerebral vasculature appear to be interrelated. NOS, isolated from the rat cerebral cortex, can be inhibited by CO (24), and CO has been shown to suppress NOS in rat renal arteries (21). In the newborn piglet cerebral circulation, CO causes dilation that can be blocked by treatment with inhibitors of NOS (1, 14). While it is clear the NOS/NO and HO/CO systems can interact, this interaction remains incompletely understood in either the adult or the neonatal cerebral circulation.

We hypothesize that CO has a biphasic action on the newborn cerebrovascular circulation. This biphasic action allows CO to have two distinct functions: first, as a signaling molecule during short episodic release that produces vasodilation mediated by KCa channels and, second, as a tonic vasoconstrictor influence by inhibition of NOS during prolonged elevation of CO.

METHODS

The University of Tennessee Health Science Center Animal Care and Use Committee approved all animal procedures.

Newborn pigs (1–5 days old, 1–3.5 kg) were anesthetized with ketamine hydrochloride (33 mg/kg im) and acepromazine (3.3 mg/kg im), and sedation was maintained with α-chloralose (50 mg/kg iv). Animals were intubated via tracheostomies and ventilated with air. Femoral veins were cannulated for anesthesia injection. Cannulated femoral arteries were used for continuous blood pressure monitoring and drawing samples for blood gas and pH analysis. Blood gases, pH, and body temperature were maintained within normal ranges.

Cranial windows in vivo. The scalp was retracted, and an opening 2 cm in diameter was created in the skull over the cerebral cortex. The dura was cut without touching the brain, and the cut edges were retracted over the bone so that the periarchnoid space was not exposed to bone or damaged membranes. A stainless steel and glass cranial window was fitted in the hole and cemented in place with dental acrylic. The windows had side needle ports so fluid under the window could be exchanged and test compounds administered topically. The space under the window was filled with artificial cerebrospinal fluid (aCSF) equilibrated with 6% CO₂ and 6% O₂, producing gases and pH within the normal range for CSF (pH ∼ 7.35 and Pco₂ and Pco₂ ∼ 43 mmHg). Pial vessels were observed through the window with a dissecting microscope. Arteriolar diameters were measured with a video micrometer coupled to a television camera mounted on the microscope and a video monitor. The CO concentration in the CSF was measured by GC-MS as previously described (4, 10, 12, 15).

Experimental design. l-Arginine ethyl ester (l-Arg) was dissolved in aCSF to the desired concentration. Increasing concentrations (10⁻⁵ and 10⁻⁴ M) were sequentially instilled under the cranial window in a volume sufficient to fill the space. Arteriolar diameters were measured and recorded during 5 min after each instillation. We used the ethyl ester rather than l-arginine HCl because we determined previously that, while both caused pial arteriolar dilations that were blocked by N⁵-methyl-l-arginine, the ethyl ester form was 10-fold more potent, suggesting better cellular penetration and/or trapping.
inside the cell by cleavage of the ethyl ester by intracellular esterases (3).

Isoproterenol at $10^{-6}$ M was used as a control, endothelium-independent vasodilator. Sodium nitroprusside (SNP; $10^{-7}$ and $3 \times 10^{-7}$ M) was used to determine if dilation to NO itself was altered.

After a washout with aCSF free of additional agents, CO dissolved in aCSF ($10^{-6}$, $2 \times 10^{-7}$, or $10^{-7}$ M or vehicle) was instilled under the window. Arteriolar diameters were recorded before instillation and at 10-min intervals for 2 h. The aCSF with the desired concentration of CO was replaced every 10 min for the full duration to maintain the desired CO level. In the continued presence of CO, responses to L-Arg were determined at 10, 20, 30, 40, 60, 90, and 120 min.

In another group of piglets, the effects of prolonged CO were determined when NO was held constant. To hold NO constant, NOS was inhibited by including N-nitro-L-arginine (L-NNA; $10^{-3}$ M) in the aCSF and NO was provided in the form of the NO-releasing molecule SNP ($3 \times 10^{-7}$ M), which we assume provides a relatively constant NO concentration because the dilation was sustained over the 10-min period between the installations of fresh aCSF. Dilation to L-Arg was measured before and in the presence of L-NNA, L-NNA plus SNP, and CO ($10^{-5}$ M). Pial arteriolar diameters were measured, and fresh aCSF with L-NNA, SNP, and CO was instilled under the cranial windows at 10-min intervals during the 2-h period as described above.

In another group, chromium mesoporphyrin (CrMP; $1.5 \times 10^{-6}$ M, 2 h) was included in the aCSF to inhibit HO. Measurements were made of CO as described above.

Comparisons among three or more populations were made using ANOVA for repeated measures and Bonferroni post hoc tests. Comparisons between two groups used paired t-tests.

RESULTS

Figure 1 shows the effect of time of exposure to CO on pial arteriolar diameters. Topical application of CO caused pial arteriolar dilation. However, as exposure of the arterioles to CO was prolonged, pial arteriolar diameter decreased with continuous exposure to both $2 \times 10^{-7}$ M (Fig. 1A) and $10^{-6}$ M (Fig. 1B). With prolonged exposure, pial arteriolar diameter fell significantly below the diameter before exogenous CO at 90 min and 2 h in the $2 \times 10^{-7}$ and $10^{-6}$ M groups, respectively. By 2 h of exposure, pial arteriolar diameter was markedly less than the pre-CO diameter with both concentrations of CO. Two-hour exposure to vehicle control (aCSF free of other agents) had no effect on pial arteriolar diameter (Fig. 1C). A lower concentration of CO ($10^{-7}$ M) caused transient dilation not seen in vehicle controls, which was not sustained for 10 min (pial arteriolar diameters were $63 \pm 6$, $75 \pm 7$ ($P < 0.05$ compared with control), and $67 \pm 8$ μm for control, maximum dilation, and 10 min, respectively, $n = 7$ piglets). At $10^{-7}$ M, CO did not cause constriction over 2 h (pial arteriolar diameters were $63 \pm 6$ and $65 \pm 7$ μm at control and 2 h, respectively).

After 2 h of exposure, CO was removed by washout with aCSF free of other substances. Removal of CO ($2 \times 10^{-7}$ and $10^{-6}$ M) after 2 h of exposure resulted in an increase in pial arteriolar diameter to a diameter similar to that seen before the application of CO (Fig. 2).

Figure 3 shows the change in pial arteriolar diameter caused by the topical application of the NOS substrate L-Arg before and after 2 h of treatment with CO. Before treatment with CO, L-Arg caused concentration-dependent dilation of pial arterioles. After 2 h of treatment with either $2 \times 10^{-7}$ or $10^{-6}$ M, L-Arg had no effect on pial arteriolar diameter. Exposure to vehicle control for 2 h had no effect of vasodilation in response to L-Arg.

To investigate the time course of CO-induced loss of dilation to L-Arg, dilations were measured after different durations of treatment with CO ($10^{-6}$ M; Table 1). Multiple concentrations of L-Arg were not used for measurement until 60 min because the falling diameter from the 10-min peak made the determination of the dilation to the lower concentrations of L-Arg uncertain. Instead, we used one concentration of L-Arg ($10^{-4}$ M) given before and 20, 30, and 40 min after the beginning of CO. Dilation to L-Arg was decreased at 20 min of CO ($66 \pm 5$ to $77 \pm 5$ μm before CO ($P < 0.05$) and $73 \pm 5$ to $78 \pm 5$ μm at 20 min of $10^{-6}$ M CO ($P < 0.05$), $n = 5$ piglets). At 30 min of CO exposure, the dilation to L-Arg was further reduced but still present ($71 \pm 5$ to $76 \pm 5$ μm ($P < 0.05$)), but by 40 min, no dilation to L-Arg was detected ($69 \pm 5$ to $68 \pm 5$ μm).
60 min of continuous application of CO, the effect of L-Arg was markedly diminished (Table 1). At 90 min, and as also shown in Fig. 3, at 120 min, L-Arg did not cause pial arteriolar dilation (Table 1).

The time-course experiments on CO inhibition of NOS were done as a single time of CO treatment per piglet. The reason is the discovery that maintaining L-Arg elevated during the 2 h of CO (10⁻⁶ M) treatment attenuated the CO-induced inhibition of NOS (Table 2). Furthermore, the apparent (P > 0.05) decrease in diameter upon prolonged treatment with CO was greatly reduced. The data shown in Table 2 are from piglets in which repeated L-Arg (10⁻⁶, 10⁻⁵, and 10⁻⁴ M) applications were begun at 30, 60, 90, and 120 min of CO elevation.

In contrast to L-Arg-induced dilation, dilation to SNP was unaltered by CO exposure (Table 3).

In addition to examining the ability of exogenous CO to inhibit NOS and cause constriction of pial arterioles, we reduced the CO concentration in the CSF by inhibiting HO [69 ± 10 nM CO before treatment and 24 ± 4 nM CO during treatment with CrMP (P < 0.05), 90–120 min, n = 18 samples before CrMP and 17 samples during CrMP from 6 piglets]. Two hours of treatment with CrMP had no effect on pial arteriolar diameter (57 ± 5 μm before CrMP treatment and
Table 1. Effect of CO (10^{-6} M) application duration on pial arteriolar diameter responses to l-Arg

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<th>CO Duration</th>
<th>l-Arg Concentration</th>
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<tr>
<td></td>
<td>10^{-6} M</td>
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<tr>
<td>0 min</td>
<td>67 ± 3</td>
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<td>60 min</td>
<td>61 ± 6</td>
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<td>90 min</td>
<td>63 ± 5</td>
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<td>120 min</td>
<td>59 ± 9</td>
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Values are means ± SE (diameters; in μm); n = 28 arterioles in 14 piglets [0 min, before carbon monoxide (CO) treatment], 10 arterioles in 5 piglets (60 min), 10 arterioles in 5 piglets (90 min), and 4 arterioles in 2 piglets (120 min). l-Arg, l-arginine ethyl ester. *P < 0.05 compared with no l-Arg.

Table 2. Effect of elevated l-Arg) during 2 h of CO (10^{-6} M) application on pial arteriolar diameter responses to l-Arg

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<th>CO Duration</th>
<th>l-Arg Concentration</th>
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<tbody>
<tr>
<td></td>
<td>0 M</td>
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<tr>
<td>0 min</td>
<td>66 ± 5</td>
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<tr>
<td>120 min</td>
<td>62 ± 7</td>
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Values are means ± SE (diameters; in μm); n = 6 arterioles in 3 piglets. *P < 0.05 compared with no l-Arg.

Table 3. Effect of 2 h of CO (10^{-6} M) application on pial arteriolar diameter responses to SNP

<table>
<thead>
<tr>
<th>CO Duration</th>
<th>SNP Concentration</th>
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<tbody>
<tr>
<td></td>
<td>0 M</td>
</tr>
<tr>
<td>0 min</td>
<td>66 ± 5</td>
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<tr>
<td>120 min of CO</td>
<td>59 ± 5</td>
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<tr>
<td>CO off</td>
<td>67 ± 5</td>
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Values are means ± SE (diameters; in μm); n = 4 piglets. SNP, sodium nitroprusside. *P < 0.05 compared with no SNP.

56 ± 5, 56 ± 6, 54 ± 6, and 54 ± 6 μm at 30, 60, 90, and 120 min of CrMP treatment, respectively) and did not increase dilation to l-Arg.

The next series of experiments was designed to clamp NO constant so that alterations of NOS activity could not contribute to the results. l-NNA blocked pial arteriolar dilation to l-Arg (Fig. 4). The addition of SNP (3 × 10^{-7} M) with l-NNA caused a small dilatation but did not restore dilation to l-Arg (Fig. 4). When l-NNA and SNP were in the aCSF, the addition of CO (10^{-6} M) caused similar dilation of pial arterioles as it did without l-NNA or SNP being present (Fig. 5). This dilation was sustained for the 2 h of treatment (Fig. 5). When the CO was removed, pial arterioles constricted to the diameters of 2 h as before (Fig. 5).

At 2 h of treatment with l-NNA, SNP, and CO, l-Arg did not affect pial arteriolar diameters (Fig. 4). After the removal of l-NNA, SNP, and CO and flush of the cranial window with fresh aCSF again at 10 and 20 min, dilation of pial arterioles to l-Arg was restored (Fig. 4).

Pial arteriolar vasodilation in response to topical isoproterenol (10^{-6} M) was unaffected by 2 h of CO exposure and removal of CO (64 ± 6 to 78 ± 12 μm before CO vs. 61 ± 7 to 79 ± 10 μm after removal of CO after 2 h of exposure to 10^{-6} M CO, respectively).

DISCUSSION

The new findings reported here include the following: 1) while acute (10 min) treatment with CO causes concentration-dependent vasodilation of pial arterioles, prolonged elevated CO exposure causes progressive constriction of pial arterioles over 1–2 h (Fig. 1); 2) the NOS substrate l-Arg causes dilation of pial arterioles that progressively declines to zero during 2 h of elevated CO treatment (Table 1); 3) prolonged CO does not alter dilation to NO itself (Table 3); 4) removal of CO after 2 h of treatment results in vasodilation rather than vasoconstriction (Fig. 2); 5) maintenance of elevated l-Arg attenuates the CO-induced loss of dilation to l-Arg and CO-induced vasoconstriction (Table 2), and 6) if NO is held constant, CO-induced dilation is sustained over 2 h (Fig. 5). These data are consistent with the hypothesis that prolonged exposure of newborn pig pial arterioles to elevated CO causes constriction by inhibition of NOS. Therefore, episodic elevation of CO in the brain can function as a dilatory gasotransmitter in the regulation of cerebrovascular circulation, whereas prolonged elevation could result in a vasoconstrictor influence that may be involved in the provision of vascular tone and cause constriction under circumstances where CO levels are chronically elevated.

Physiological and pathological conditions under which CO-induced inhibition of NOS becomes of functional significance in place of, or in addition to, CO activation of K_{Ca} channel activity that causes dilation (16) are not known. The basal concentration of CO in piglet cortical periarachnoid CSF is in the range of 2 × 10^{-6} M (20–80 nM) (present study and Refs. 4, 10, and 15). CO (10^{-7} M) caused transient dilation and did not result in vasodilatation over 2 h of exposure (see RESULTS). In addition, inhibition of HO, which decreased CO in the aCSF, had no effect on pial arteriolar diameters over 2 h and did not increase dilation to l-Arg (see RESULTS). These data overall suggest that CO inhibition of NOS is not involved in the regulation of neonatal cerebrovascular tone under basal conditions. However, 2 × 10^{-7} M CO, which is physiological but above the baseline, causes loss of the CO-induced dilation and constriction linearly over the 2-h period (Fig. 1). These data suggest that CO may contribute to the regulation of NOS activity under conditions where CO production is increased even modestly for an extended period.

Dilation to 10^{-6} M CO is lost abruptly, and a constant diameter is then maintained for a full hour before a decline of pial arteriolar diameter occurs between 1 and 2 h of exposure (Fig. 1). CO (10^{-6} M) is a pathophysiological level that has been measured in newborn CSF during seizures (4). A plausible explanation for the difference in time courses between 2 × 10^{-7} and 10^{-6} M CO is that the dilation caused by 10^{-6} M CO, which results from K_{Ca} channel activation (16), may be sufficient to counteract the loss of basal NO/cGMP. Constriction may occur when the NO becomes progressively insufficient to provide the necessary permissive function for CO-induced dilation (11, 13). This speculation is consistent with the prevention of the loss of dilation to 10^{-6} M CO by the provision of a constant NO concentration (Fig. 5).

As previously reported (14), acute treatment with CO caused dilation of cerebral arterioles (Fig. 1). However, with prolonged exposure, arteriolar diameter returned to baseline and then progressively decreased below the baseline diameter.
When CO was removed after 2 h of exposure, dilation of the arterioles occurred rather than constriction (Fig. 2). That there was vasodilation upon the removal of CO is consistent with CO acting as a vasoconstrictor on resistance arterioles with prolonged exposure. A constrictor action of CO on vascular smooth muscle has been previously reported. Ishikawa et al. (8) reported that prolonged CO has an inhibitory effect on NOS, which causes the constriction of cerebral arterioles in adult rats, but the effects of acute CO were not examined. In the adult rat skeletal muscle vasculature, prolonged exposure to CO causes endothelial NOS-dependent constriction (9). In the present experiments, inhibition of NOS by CO was indicated by the lack of dilation to l-Arg after prolonged exposure to elevated CO (Table 1). The production of NO is dependent on synthetic enzyme activity and the availability of the enzyme substrate. With l-Arg being provided, ample substrate was made available, indicating that the enzyme, NOS, had been inhibited. High levels of l-Arg, which increase NOS activity, attenuated CO-induced NOS inhibition (Table 2). Similar dilation to SNP occurred before, during, and after prolonged CO exposure (Table 3), indicating that the vascular response to NO was intact.

The interactions between HO/CO and NOS/NO in the newborn cerebrovascular circulation have the potential to form a negative feedback loop. Prolonged elevation of CO inhibits NOS (Table 1). Decreased NOS activity could produce vasoconstriction by 1) reduction of NO directly increasing vascular tone, 2) insufficient NO to permit CO to cause dilation (1, 11, 13), and/or 3) decreased CO by loss of NO stimulation of HO-2 (12). The final mechanism would potentially close a negative feedback loop as decreasing NO reduces CO, thereby removing the inhibition of NOS.

The balance between the NO and CO systems is different in neonatal and adult cerebrovascular circulations. The role of NO in cerebrovascular regulation is diminished in the newborn pig compared with the juvenile pig (25, 27). Responses of newborn and baby piglet pial arterioles in vivo to CO are greater than in older pigs and adult rats (6). Also, the permissive enabling actions of NO and prostacyclin that are necessary for CO-induced dilation in newborn pigs are not necessary in juvenile pigs or adult rats (6).

Overall, the data of the present report are consistent with the hypothesis that the action of CO on the newborn cerebral circulation is biphasic: dilation to short episodic elevations of CO but constriction to prolonged exposure to elevated CO. Such a biphasic effect would allow episodic CO elevation to be used as a gaseous mediator of dilation in response to acute stimuli (16) and also for CO to contribute to the regulation of tonic cerebrovascular tone by modulation of NOS activity. In addition, persistent high levels of CO due to elevation of HO-1 by conditions injurious to the brain, while beneficial in other aspects (19), may reduce NO and contribute to the constriction that can accompany brain injury.

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