The Vasorelaxation of Cerebral Arteries by Carbon Monoxide

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Carbon monoxide (CO) is known to increase cerebral blood flow, but the effect of CO on the vascular tone of large cerebral arteries is uncertain. We tested whether CO affects cerebral artery tone by measuring tension generated by *ex vivo* segments of dog basilar artery upon exposure to CO. In cerebral artery segments contracted with either KCl or prostaglandin $F_{2\alpha}$, CO caused a concentration-related relaxation beginning with a concentration of 57 μM. Relaxation did not occur if CO was administered in the presence of bubbling carboxygen (95% O_2:5% CO_2), which reduces greater than 99% of CO from the solution. Furthermore, the CO-induced relaxation of cerebral artery segments was reduced in the presence of the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM)or the potassium channel blocker tetraethylammonium (TEA, 1 mM). Neither ODQ nor TEA completely eliminated the relaxation caused by CO and there was no additive effect if ODQ and TEA were administered together. These results suggest that cerebral arteries are directly relaxed by CO and that this relaxation depends upon the activation of guanylyl cyclase and the opening of potassium channels.

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Key words: carbon monoxide; cerebral artery; guanylyl cyclase; potassium channel

The study of how carbon monoxide (CO) affects the cerebral vasculature has developed along two lines: traditionally, CO has been considered a poison; however, more recently, CO has been considered as an endogenous regulator of cerebral vascular tone (as has been demonstrated with peripheral arteries [1]). Studies of CO intoxication have shown increased total cerebral blood flow (2–5), but none of these studies have examined the underlying mechanism thoroughly. CO is produced during the enzymatic breakdown of heme-containing proteins by heme oxygenases (reviewed in Ref. 6), and both the constitutive and inducible forms of heme oxygenase have been identified in the central nervous system and its vasculature (6–11). A recent study has suggested that CO produced by heme oxygenases increases cerebral blood flow during kainate-induced seizures (12). The interpretation of this study is limited because of the use of an antagonist of heme oxygenase (tin protoporphyrin) that also antagonized nitric oxide synthase and guanylyl cyclase (13). Certainly, future efforts will concentrate upon the role of endogenous CO in the regulation of cerebral blood flow.

Several mechanisms have been proposed to account for the increase in cerebral blood flow caused by CO. CO has been shown to dilate pial arterioles *in situ* (9), and it is also possible that CO increases cerebral blood flow by increasing cardiac output (14), although autoregulatory mechanisms should make this unlikely (15). Additionally, in light of CO's well-demonstrated ability to vasodilate large peripheral arteries (16–21), it initially was hypothesized that CO would dilate the cerebral arteries. Heretofore, in the only direct study of the effect of CO on the tone of large cerebral arteries, Brian *et al.* (22) concluded that CO did not reduce the tension generated by *ex vivo* rabbit and dog basilar and middle cerebral artery segments. However, in their experiments, the artery segments were exposed to CO in organ baths that were bubbled with carboxygen (95% oxygen: 5% carbon dioxide). Since the process of bubbling the organ bath with carboxygen sparges CO from the liquid (23), the amount of CO to which the cerebral artery segments were exposed may have been considerably less than what the investigators expected. Considering this, we reexamined the response of cerebral arteries to CO using an *ex vivo* preparation of dog basilar artery segments. Since CO treatment *in vivo* may cause cerebral artery dilation indirectly by affecting the partial pressure of oxygen in the blood (2) and/or brain tissue metabolic activity (24–27), we chose the *ex vivo* assay to avoid these confounding factors.

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Materials and Methods

Animal Handling and Cerebral Artery Harvesting. All procedures on animals were carried out under protocols approved by the Institutional Animal Care and Use Committee of the University of Chicago. Every effort was made to limit animal suffering, as well as to limit the number of animals used in these experiments.

Adult male mongrel dogs (15–30 kg body wt) were euthanized by exsanguination under general anesthesia with halothane. The brain was rapidly excised, and the basilar artery was gently removed and placed in Krebs-Henseleit buffer that was bubbled with 95% O_2/5% CO_2 (carboxygen). The buffer containing the artery was kept on ice until the artery could be prepared as described below.

Preparation of Cerebral Artery Segments and the Measurement of Isometric Tension. The basilar artery was cleaned of arachnoid tissue and any side branches were trimmed off. The artery was cut into 4-mm-long segments, and two hooks fashioned from stainless steel wires were run through the segment's lumen. Artery segments were individually hung in 5 ml of organ baths by fixing the lower hook to an immobile anchor at the bottom of the organ bath and attaching the upper hook to a strain gauge (Grass Model FT 03, Grass Instruments, Quincy, MA) by means of a 6-0 nylon thread. The strain gauge itself was mounted on an adjustable arm that could be raised or lowered to adjust the radial tension applied to the artery segment. Analog output from the strain gauge was connected through a preamplifier and an analog-digital converter to a computer equipped with software (Lakeshore Technologies, Chicago, IL) for the continuous recording of the tension measured by the strain gauge. Once placed in the organ bath, a resting tension of 0.75*g* was applied to the artery segment. This tension was selected based on length-tension curves generated in response to applications of 60 mM KCl and was a tension at which a maximal contraction was reached (28, 29) The organ baths were kept at 37°C by means of a circulating water heater.

The suspended artery segments were allowed to acclimate to the 0.75*g* resting tension over a period of 1 hr during which the tension was continuously readjusted to 0.75*g*. The buffer solution in the organ bath was changed every 20 min during the 1-hr acclimation period. After the artery segments had been acclimated to a stable baseline tension, the integrity of the segments' smooth muscle was assessed by measuring the contraction generated in response to 60 mM KCl (120 μL of 2.5 M KCl added to a 5-ml organ bath). In order to use an individual artery segment, it was necessary that it generated at least 0.5*g* of tension 2 min after exposure to 60 mM KCl; otherwise, the segment was considered nonviable and was discarded. In some experiments, 2 min after the second administration of KCl, sodium nitroprusside was added to the bath to a concentration of 100 μM. The relaxation of the KCl-contracted artery segments caused by nitroprusside was then measured; with this value, the relaxation caused by CO could be expressed as a ratio of "maximal" (e.g., nitroprusside-induced) relaxation to reduce the variability caused by the strength of the individual artery segments. At least 15 min was allowed following the KCl-nitroprusside treatment prior to experimental use during which the artery segments were washed with fresh Krebs-Henseleit buffer.

In most experiments, the flow of carboxygen into the organ bath was stopped before the beginning of the experiment. We have previously found that stopping the flow of carboxygen does not affect the resting cerebral artery tension, nor is the partial pressure of oxygen in the organ bath buffer reduced 30 min after the cessation of carboxygen bubbling. In preliminary experiments, the partial pressure of oxygen in the organ baths decreased from 580 ± 20 to 440 ± 20 and pH changed from 7.4 ± 0.1 to 7.6 ± 0.2 (n = 3). Contractions to KCl 60 mM changed from 1.3 ± 0.2 to 1.0 ± 0.3 g. Carboxygen bubbling was used in certain experiments with cerebral artery segments to demonstrate that it prevents the artery segments from responding to CO; these experiments are specifically noted in the Results section.

Preparation and Experimental Use of CO. The ability of CO to relax segments of cerebral artery was best measured by administering CO onto artery segments that had beforehand been contracted ("precontracted") (20). Precontraction of the artery segments was achieved by adding 60 mM KCl or 10 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to the organ bath, depending upon the experiment. These two compounds were chosen because of the different mechanisms by which they cause cerebral artery contraction. KCl acts to change the membrane potential, thereby directly depolarizing the artery's smooth muscle. $PGF_{2\alpha}$ acts through protein kinase C to release intracellular calcium stores (30, 31).

CO was administered onto cerebral artery segments by dissolving CO gas into Krebs-Henseleit buffer and then administering the solution into the organ bath. This was done as follows: pure CO gas (10 ml) was injected into a sealed glass vacuum tube containing Krebs-Henseleit buffer (5 ml). The CO-buffer mixture was vigorously shaken for 15 sec and stored at 4°C until use. Just prior to use, the tube was warmed to 37°C in a water bath and was again vigorously shaken. For determining the concentration-related effect of CO, serial 10-fold dilutions of the CO solution were made with Krebs-Henseleit buffer. CO-containing buffer was regularly supplemented with either 60 mM KCl or 10 μM $PGF_{2\alpha}$. This ensured that the precontraction caused by KCl or $PGF_{2\alpha}$ already present in the organ bath was not reduced by the administration of a diluting volume of CO solution.

Measurement of CO Concentration in Solution. CO concentration was measured by mixing 5 ml of 10g/dl purified human oxyhemoglobin A_0 (Hemosol, Inc., Toronto, Canada) with 1 ml of the CO-buffer mixture. One minute was allowed so that complete binding of CO by hemoglobin

occurred. The absorbance of CO-hemoglobin was then measured spectrophotometrically according to the method of Wolff and Bidlack (32), and the concentration of CO was calculated using an extinction coefficient of 58.7 nM^{-1} cm^{-1}. Using this technique, the concentration of CO in the mixture described above was 3.4 ± 0.4 mM in $n = 4$ samples. When 1 ml of this solution was added to an organ bath containing 5 ml of buffer, a concentration of 567 μM was obtained in the organ bath.

Drugs and Chemicals. Pure CO was purchased from Matheson Inc. (Joliet, IL). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one [ODQ] was purchased from Alexis Biochemicals (San Diego, CA). $PGF_{2\alpha}$, sodium nitroprusside, tetraethylammonium (TEA), and all inorganic ions were obtained from Sigma (St. Louis, MO). Human oxyhemoglobin A_0 was the generous gift of Hemosol Inc. and was supplied in 100% oxyhemoglobin form. The concentrations of all drugs and chemicals are reported as the concentration obtained after dilution into the organ bath solution.

Krebs-Henseleit buffer contained [in mM]: 113.7 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, and 10 glucose. KCl (60 mM) or $PGF_{2\alpha}$ (10 μM) was supplemented into the Krebs-Henseleit buffer when it was used to dissolve CO for reasons described previously.

Statistical Analysis. Data are presented as the means ± standard deviations. The concentration-related effect of a treatment was examined using one-way analysis of variance (ANOVA) with *post hoc* analysis performed by the Bonferroni method. Two individual treatment conditions were compared by unpaired Student *t* tests. Results of ANOVA or *t* tests are reported at the *P* level, with a $P < 0.05$ considered as statistically significant.

Results

Relaxation of Cerebral Artery by CO. Artery segments were precontracted with KCl so as to better observe any relaxant action of CO (20). The increase in artery tension after exposure to 60 mM KCl peaked within 0.5 to 1 min, and a fairly stable plateau was maintained for a few minutes thereafter. Once a stable plateau was reached, 1 ml of 3.4 mM CO-buffer mixture (supplemented with 60 mM KCl) was added to the organ bath producing a concentration of 567 μM CO in the organ bath. Compared against the addition of KCl-supplemented buffer without CO (Fig. 1, left tracing), the addition of buffer containing CO (right tracing) caused a rapid reduction in cerebral artery tension. In the aforementioned experiments CO was administered in the absence of carboxygen bubbling. Next, the effect of CO on cerebral artery tension in the presence of bubbling carboxygen was examined (Fig. 1, center tracing). Unlike the rapid reduction in tension caused by CO in the absence of carboxygen bubbling, there was only a minimal reduction in artery tension (compare with left tracing) at a concentration of 567 μM CO when it was administered in the presence of carboxygen bubbling.

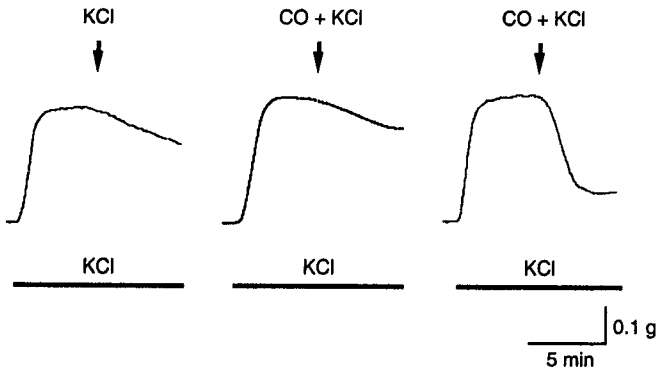

Figure 1. Representative tracings of CO-induced relaxation of dog basilar artery, and effect of bubbling with carboxygen. Krebs-Henseleit buffer (supplemented with 60 mM KCl) that was not mixed with CO was applied to basilar artery precontracted with 60 mM KCl (left tracing). This experiment was performed in the absence of carboxygen bubbling. Krebs-Henseleit buffer (supplemented with 60 mM KCl) containing CO was applied to precontracted artery segment in the presence (center tracing) or absence of carboxygen bubbling (right tracing). The final concentration of CO in the organ bath was 567 μM. These tracings are representative of $n = 4$ experiments conducted on cerebral artery segments taken from different dogs.

We also demonstrated that the relaxation caused by CO in KCl-precontracted cerebral artery segments was dependent upon the concentration of CO that was administered (Fig. 2). Serial 10-fold dilutions of the 3.4 mM CO buffer

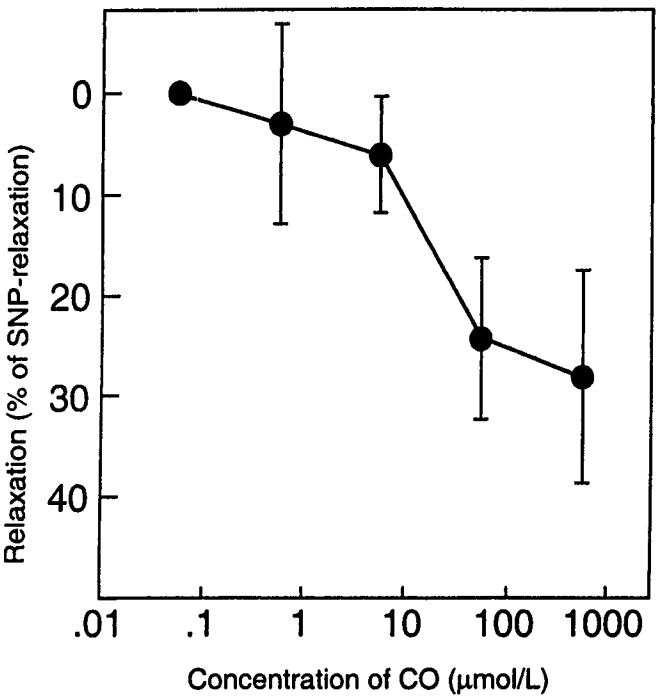

Figure 2. Concentration-related CO relaxation in dog basilar artery. In the absence of bubbling carboxygen, artery segments were precontracted with KCl. Two minutes after precontraction, CO was administered into the organ bath starting at a concentration of 0.57 μM and increasing in a logarithmic manner to 567 μM. Two minutes was allowed between administration of the CO and the recording of the tension measurement. The relaxation caused by CO is presented as the percentage of the relaxation induced by 100 μM sodium nitroprusside. $n = 7$ artery segments were examined. There was significant ($P < 0.05$) relaxation versus pre-CO tension at concentrations of 57 and 567 μM.

solution were made with Krebs-Henseleit buffer supplemented with 60 mM KCl. The dilutions of CO buffer were then administered in cumulative concentrations, allowing 2 min between administration of a concentration of CO and the measurement of cerebral artery tension. There was a concentration-related effect of CO on the tension of cerebral artery (n = 7 artery segments per concentration, one-way ANOVA, $P < 0.05$). *Post hoc* analysis indicated that CO-induced relaxation reached a maximum at 567 μM, although a significant relaxation was observed with as little as 57 μM CO. A lesser amount of CO (6 μM) had no effect on the tension generated by cerebral artery segments precontracted with KCl.

Effect of Carboxygen Bubbling on CO Concentration in Solution. In the previous section we demonstrated that cerebral artery segments are much less responsive to CO-induced relaxation when the CO is administered in the presence of bubbling carboxygen. We hypothesized that bubbling carboxygen sparges CO from the buffer solution (as demonstrated in Ref. 23) and thus reduced the effective concentration of CO in the organ bath. To test this hypothesis we measured the amount of CO in solution by spectrophotometric assessment of CO binding to oxyhemoglobin. CO binds rapidly and strongly to hemoglobin, and the resulting carboxyhemoglobin has a characteristic absorbance spectrum that readily distinguishes it from other forms of hemoglobin. Bubbling the CO buffer mixture with carboxygen in an organ bath for 2 min significantly decreased the concentration of CO from 567 ± 67 μM to 1.7 ± 0.1 μM (n = 4 samples, t test, $P < 0.01$). This represents on average a 99.7% reduction in CO content in the buffer solution. This was confirmed by spectrophotometry showing a loss of formation of carboxyhemoglobin when CO buffer was added to Krebs-Henseleit buffer that was bubbled with carboxygen (Fig. 3).

Effect of Antagonists of Guanylyl Cyclase and Potassium Channels on CO-Induced Relaxation of Cerebral Artery Tension. In peripheral artery CO has been shown to cause relaxation by means of guanylyl cyclase and potassium channels (16–21). We decided to examine the role of these factors in the response of cerebral artery to CO. Artery segments were precontracted in a pharmacological manner using $PGF_{2\alpha}$ instead of using KCl because $PGF_{2\alpha}$ acts via a specific receptor (33, 34), whereas KCl acts by directly depolarizing the cell membrane, and the addition of extracellular KCl changes the potassium electrochemical potential, which would complicate the study of potassium channel activity.

In order to determine the involvement of guanylyl cyclase in the relaxation caused by CO, artery segments were exposed to 10 μM ODQ and after a period of 5 min, the artery segments were precontracted with 10 μM $PGF_{2\alpha}$. The addition of ODQ caused a small contraction of the arterial segments, which was probably related to inhibition of vasodilator effect of nitric oxide. The magnitude of the contraction, however, was not sufficient to alter the contraction to subsequent addition of $PGF_{2\alpha}$. Two minutes after $PGF_{2\alpha}$ treatment, CO was added to the organ baths to a concentration of 567 μM. All solutions in this experiment contained 10 μM $PGF_{2\alpha}$ so that the amount of $PGF_{2\alpha}$ in the organ bath remained constant. In comparison with the effect of CO in the absence of ODQ, artery segments exhibited significantly smaller relaxations to CO in the presence of ODQ (Fig. 4). In the absence of ODQ, artery segments that had been precontracted with $PGF_{2\alpha}$ and then exposed to 567 μM CO were relaxed to 72% ± 10% of the precontracted tension (n = 6 artery segments); in the presence of ODQ, the tension was reduced by CO to 85% ± 6% of the precontraction level (n = 5 artery segments; t test, $P < 0.05$).

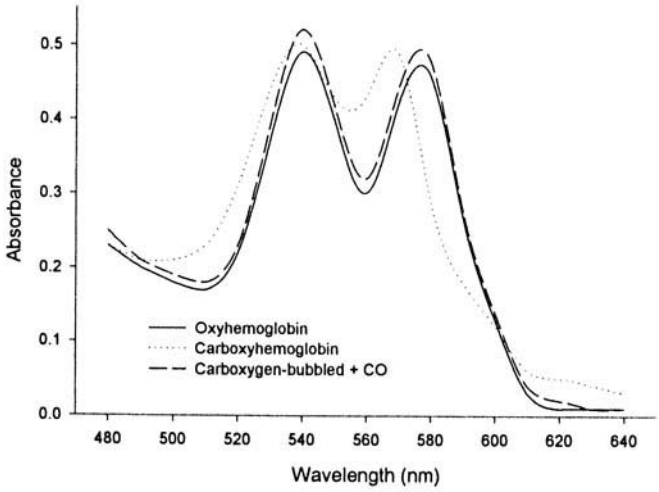

Figure 3. Representative spectrophotometric tracings of pure oxyhemoglobin before addition of any gases showing characteristic absorption maxima at 577 and 542 nm. Addition of oxyhemoglobin to a solution containing CO results in formation of carboxyhemoglobin with absorption maxima at 570 and 538.5 nm. If the CO buffer is bubbled with carboxygen, addition of oxyhemoglobin does not produce carboxyhemoglobin (carboxygen + CO), showing that CO is sparged from the solution by carboxygen.

The effect of the nonspecific potassium channel blocker TEA on the relaxation of cerebral artery segments by CO was also investigated. This experiment was performed as described above except that TEA was added to the organ baths to a concentration of 1 mM instead of ODQ. The addition of TEA caused a small contraction of the arterial segments, which was probably related to inhibition of vasodilator effect of potassium channels. The magnitude of the contraction, however, was not sufficient to alter the contraction to subsequent addition of $PGF_{2\alpha}$. Figure 4 shows that TEA partially inhibited the relaxation caused by 567 μM CO. In the presence of TEA, CO relaxed the artery segments to 86% ± 10% of the precontracted tension compared with a relaxation to 72% ± 10% of precontraction tension caused by CO administered in the absence of TEA (n = 5 artery segments; t test, $P < 0.05$).

Figure 4 also shows the combined effect of guanylyl cyclase inhibition and potassium channel blockade on CO-induced relaxation of cerebral artery segments. This experiment was similar to those described previously, except that both ODQ and TEA were added to the organ baths prior to

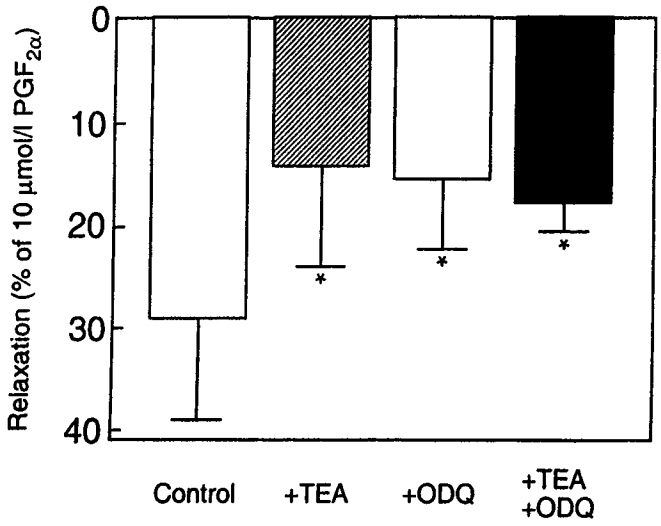

Figure 4. The effect of the potassium channel blocker TEA and the guanylyl cyclase inhibitor ODQ on CO-induced vasorelaxation. In the absence of bubbling carboxygen, artery segments were treated with ODQ (10 μM) and/or TEA (1 mM) and after a period of 5 min they were precontracted with 10 μM $PGF_{2\alpha}$. Mean contractions to $PGF_{2\alpha}$ were 1.1 ± 0.2 g (control), 1.0 ± 0.3 g (TEA), 1.1 ± 0.2 g (ODQ), and 1.0 ± 0.2 g (TEA + ODQ). After 2 min, CO buffer solution (supplemented with 10 μM $PGF_{2\alpha}$) was applied to achieve a concentration of 567 μM in the organ bath. This experiment was performed without either ODQ or TEA ("control"; n = 6 artery segments), or in the presence of TEA (1 mM; n = 5 artery segments), ODQ (10 μM; n = 5 artery segments), or both TEA and ODQ (n = 4 artery segments). Data are expressed as the percentage relaxation of the precontraction tension. An asterisk indicates significant ($P < 0.05$) reduction versus control group.

precontraction. Artery segments precontracted with 10 μM $PGF_{2\alpha}$ in the presence of both ODQ and TEA relaxed to 82% ± 3% of the precontraction tension after exposure to 567 μM CO (n = 4 artery segments). In comparison with the relaxation caused by CO in the absence of ODQ and TEA (e.g., 72% ± 10%), this was a significant inhibition of the effect of CO (t test, $P < 0.01$). However, when compared with the relaxation caused by CO in the presence of either ODQ or TEA, there was no additive inhibition of CO relaxation by co-administration of ODQ and TEA.

Discussion

The principle finding that we report here is that CO relaxed dog basilar artery segments in an *ex vivo* preparation. This was likely due to a direct action of CO on the vascular smooth muscle since, in our preparation, artery segments did not exhibit a functional endothelium (e.g., KCl-contracted artery segments did not relax in response to adenosine triphosphate 1–10 μM [29] or A23187 [35], data not shown). The relaxation caused by CO was concentration related, with significant reductions in artery tension occurring with concentrations of CO greater than 6 μM. A bath concentration of 567 μM CO produced about 30% of the relaxation observed in response to 100 μM sodium nitroprusside. This may relate to the observation that CO is less efficacious at stimulating guanylyl cyclase than is nitric oxide (36).

Although previous reports have hypothesized that CO acts as a cerebral artery vasodilator, this was not supported by the only direct examination of the effect of CO on cerebral artery tone. Using basilar and middle cerebral artery segments that had been precontracted with histamine, Brian *et al.* (22) were unable to demonstrate any CO-induced relaxation. The concentrations of CO that were reported to be present in the organ bath buffer in that study (up to 300 μM) are apparently in the range that caused artery relaxation in our experiments. However, in the study of Brian *et al.* (22), CO was administered into the organ baths while the baths were being bubbled with carboxygen. Our experiments, as well as those of others (23), showed that bubbling a solution with carboxygen reduces the CO concentration in the solution by two orders of magnitude, an effect that represents the relative insolubility of CO in solution when compared with that in oxygen and carbon dioxide gas. Brian *et al.*'s (22) experiments may have greatly overestimated the amount of CO to which the cerebral artery segments were exposed, and therefore, may have overlooked a relaxant effect of CO.

CO has been reported to relax extracranial vessels (i.e., porcine coronary artery and vein, rabbit aorta, and rat hepatic artery and portal vein) in a manner that involves activation of soluble guanylyl cyclase (16, 17, 19–21, 37). We found the relaxation of cerebral artery by CO was partially inhibited by antagonists of guanylyl cyclase and potassium channels. However, despite this similarity there may exist a significant difference between cerebral artery and peripheral artery in the mechanism by which CO causes relaxation. In rat tail artery, when both guanylyl cyclase and the large-conductance calcium-activated potassium channel (K_{Ca}) were antagonized, the effect on CO-induced relaxation was additive, indicating that the activation of the K_{Ca} potassium channel was not dependent upon the activation of guanylyl cyclase. The K_{Ca} channel is thought to be sensitive to cyclic guanosine monophosphate (38–41), however, there is also a direct interaction between CO and the K_{Ca} potassium channel (21) that could account for the observation in peripheral artery. In our experiments, simultaneous antagonism of guanylyl cyclase and potassium channels was not different than antagonism of either guanylyl cyclase or potassium channels alone, suggesting that these two downstream responses to CO are serially arranged.

In summary, we report here that CO caused a concentration-related vasorelaxation of cerebral artery. The relaxation of cerebral artery segments by CO was prevented if the CO was administered in the presence of bubbling carboxygen. Using a spectrophotometric assay for CO, we found that bubbling a solution with carboxygen reduced CO concentration by nearly 100-fold. Furthermore, we showed that CO-induced relaxation was sensitive to inhibitors of guanylyl cyclase (ODQ) (30, 42, 43) and potassium channels (TEA) (31). We conclude that CO causes relaxation of cerebral arteries by activation of guanylyl cyclase and the opening of potassium channels.

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