Carbon monoxide induces vasodilation and nitric oxide release but suppresses endothelial NOS

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Carbon monoxide induces vasodilation and nitric oxide release but suppresses endothelial NOS. Am. J. Physiol. 277 (Renal Physiol. 46): F882–F889, 1999.—The vascular effects of carbon monoxide (CO) resemble those of nitric oxide (NO), but it is unknown whether the two messengers converge or exhibit reciprocal feedback regulation. These questions were examined in microdissected perfused renal resistance arteries (RRA) studied using NO-sensitive microelectrodes. Perfusion of RRA with buffers containing increasing concentrations of CO resulted in a biphasic release of NO. The NO response peaked at 100 nM CO and then declined to virtually zero at 10 µM. When a series of 50-s pulses of 100 nM CO were applied repeatedly (150-s interval), the amplitude of consecutive NO responses was diminished. NO release from RRA showed a dependence on L-arginine but not L-arginine, and the responses to CO were inhibited by pretreatment with N⁶-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthases (NOS). CO (100 nM) also suppressed NO release induced by 100 µM carbachol, a potent agonist for endothelial NOS (eNOS). RRA from rats in which endogenous CO production from inducible HO was elevated (cobalt chloride 12 h prior to study) also showed suppressed responses to carbachol. Furthermore, responses consistent with these findings were obtained in juxtamedullary afferent arterioles perfused in vitro, where the vasodilatory response to CO was biphasic and the response to acetylcholine was blunted. Collectively, these data suggest that the CO-induced NO release could be attributed to either stimulation of eNOS or to NO displacement from a cellular storage pool. To address this, direct in vitro measurements with an NO-selective electrode of NO production by recombinant eNOS revealed that CO dose-dependently inhibits NO synthesis. Together, the above data demonstrate that, whereas high levels of CO inhibit NOS activity and NO generation, lower concentrations of CO induce release of NO from a large intracellular pool and, therefore, may mimic the vascular effects of NO.

DURING THE LAST DECADE, nitric oxide (NO) has been established as one of the major synthetic products of the endothelial cell responsible for endothelium-derived vasorelaxing activity (10, 27, 28). It is synthesized in the process of L-arginine conversion to L-citrulline, a reaction catalyzed by a heme-containing member of the oxidoreductase family of enzymes, NO synthase (NOS)(28). In endothelial cells, endothelial NOS (eNOS) is basally expressed, and its resting activity accounts for the continuous low-level production of NO that participates in the maintenance of the basal vascular tone (9). It has been demonstrated that the heme moiety, which is essential for the assembly of functional homodimers and binding of L-arginine, can bind carbon monoxide (CO) (21). This latter molecule has recently been identified as a messenger in the central nervous system (40), and it is capable of activating guanylyl cyclase (4, 7, 15). In fact, CO has long been implicated in cGMP-induced vasorelaxation (7). CO is produced by heme oxygenase (HO), an enzyme that is constitutively expressed in the brain, liver, spleen, and some other tissues, or by its inducible isoform, which has a much broader, if not universal, expression (17, 18). Indeed, this inducible isoform, HO-1, has been considered an early stress-response factor upregulated by stimuli as diverse as ultraviolet irradiation, oxidant stress, and heat shock, among others (14).

Although the physiological effects of CO are somewhat similar to those of NO (Refs. 8, 18; and see DISCUSSION), it is not at all clear whether these two messenger systems have points of convergence resulting in feedback regulation of each other. There are, however, data demonstrating that NO may affect HO activity in rat brain and spleen (45). Furthermore, treatment of rat aortic smooth muscle cells with NO donors results in a dose-dependent induction of HO-1 (5). On the other hand, Ischiropoulos et al. (12) have demonstrated that CO poisoning results in a ninefold increase in NO production in rat brain, as detected by electron paramagnetic resonance spectroscopy. This contrasts with observations by other investigators (1) that CO inhibits the activity of NOS, thus preventing the increased synthesis of NO. It has recently been suggested that CO induces the release of preformed NO from intrinsic cellular binding sites, an effect that could reconcile these conflicting observations (34).

In the present study, an experimental system based on the use of highly sensitive and selective microelectrodes for the electrochemical detection of NO (38), as described below, allowed us to obtain the first direct evidence that low concentrations of CO induce the release of NO from isolated perfused renal resistance arteries (through a process that appears to require L-arginine metabolism). In contrast, the results also show that NO release is suppressed by higher CO concentrations and that exposure to exogenous CO or chronic elevation of endogenous CO production inter-
ferves with the ability of the muscarinic agonist carbachol to stimulate NO production. Furthermore, the results show that the vasodilatory action of CO in blood-perfused renal afferent arterioles in vitro reflects the above biphasic pattern of NO release rather than prevailing CO levels. Finally, data were obtained that demonstrate direct inhibition by high levels of CO of NO generation by recombinant endothelial NOS (eNOS) in vitro. We hypothesize that, although CO produces vasorelaxation, this is in large part the result of NO release from an intracellular pool, which masks an underlying, dose-dependent inhibition of eNOS by CO.

MATERIALS AND METHODS

In vitro monitoring of NO release from microdissected renal resistance arteries. Experiments were performed on intact microdissected renal resistance arteries. Sprague-Dawley rats (males weighing 200–250 g) were anesthetized with 110 mg/kg Inactin (Research Biochemicals, Natick, MA) and heparinized, and the left kidney was exposed via an abdominal incision. Renal arteries were catheterized, and kidneys were perfused with a pregassed Krebs-Ringer-bicarbonate solution containing 4% albumin. The kidneys were removed and dissected while perfused to expose the perihilar cortex, as described by Casellas and Moore (3). Tertiary branches of the renal artery (or primary arcuate arteries) were dissected free from the surrounding tissues, and all small branches were ligated. Arterial segments 300–400 µm in diameter and 2–3 mm long were transferred to a temperature-controlled chamber (37°C) and mounted on two glass cannulas (2 mm OD glass capillaries bent at ~45° and pulled to obtain a tip of approximately 200 µm OD). The proximal end of the vessel was tied to a double-channeled cannula used for perfusion and for continuous measurement of the luminal pressure. The distal end of the vessel was then tied to a collection cannula that drained through an adjustable flow resistor. The vessel was slightly stretched between the cannulas and perfused with the modified Krebs-Ringer-bicarbonate solution containing 0.1 mM L-arginine.

The NO concentration in the effluent was monitored with a porphyrin-electroplated, nafion-coated, carbon-fiber electrode (Bio-Logic Instruments; Grenoble, France; Ref. 38). The electrode was placed together with a Ag/AgCl reference electrode inside the distal cannula as close as possible to the tip. A silver auxiliary electrode was inserted into the bath. Measurements of NO oxidation at the electrode surface were made using differential pulse amperometry at 10 Hz with a holding potential of 0.65 V and a 0.05-V pulse with a computer-based electrochemical system (EMS-100; Bio-Logic Instruments, Grenoble, France). The electrode oxidation current was sampled at 10 Hz, and 20 sample-averaging was performed using EMS-100 software, resulting in mean values at 2-s intervals. Calibration of the electrode was performed before an experiment using dilutions of freshly prepared NO-saturated Krebs-Ringer solution.

Perfusion was done under conditions of slow constant flow and pressure. This was accomplished through the use of an outflow resistor. Under these conditions, the NO concentration in the effluent is linearly related to NO release from the vessel segment. All chemicals were applied to the perfusate and all measurements were performed at a luminal pressure of 100 mmHg.

At the beginning of each experiment, either 0.5 mM L-arginine or 0.1 mM carbachol was administered, and those vessels which did not show the characteristic prompt rise in NO release were discarded. CO was prepared as a saturated solution prior to each experiment and further diluted, as specified in RESULTS, to reach final concentrations in the perfusate of 0.01 to 10 µM.

Effects of induction of HO-1 and increased endogenous CO on agonist-stimulated NO production. In a separate series of experiments, Sprague-Dawley rats received subcutaneous injection of 25 µmol/100 g CoCl2. This dose has been shown to induce HO-1 in vivo (32). Twelve hours after the injection, the animals were killed, as detailed above, for the microdissection of renal resistance arteries. Control rats received a subcutaneous injection of isotonic saline.

Assessment of the hemodynamic effects of CO in blood-perfused renal afferent arterioles in vitro. These experiments utilized the in vitro juxtaglomerular (JG) nephron preparation (3). Briefly, rats were prepared as described above, except that the dissection was continued to expose superficial afferent arterioles on the perihilar cortex. Major arteries supplying the rest of the kidney were ligated. During dissection, the kidneys were perfused with a gassed Krebs-Ringer-bicarbonate (KRB) solution containing 4% albumin. During measurements, the kidneys were perfused with a blood solution prepared from donor rat blood. The red blood cells were separated, washed, and resuspended (~25% hematocrit) in KRB-6% albumin solution. The preparation was superfused with warmed (37°C) KRB solution with 1% albumin. In this study, all drugs were applied abuminally via the superfuse, except where indicated otherwise. The responses of JG mid-afferent arteriolar segments were assessed by measuring changes in lumen diameter via video microscopy as previously described (3).

Measurement of eNOS activity with NO-selective microelectrode in vitro. eNOS protein was purified from Escherichia coli that had been transformed with independent vectors for expression of eNOS and Gro ELS, as previously described (20). For each experiment, 25 pmol eNOS was used. To assess the effects of CO, eNOS activity was monitored with an NO-selective microelectrode in a stirred microcuvette at room temperature. The electrodes used were prepared as described above, although they were not placed inside of a collection cannula. In addition, these measurements were made using constant potential amperometry (0.7 V) using a highly sensitive potentiostat (InterMedical, Nagoya, Japan). The resulting signal was low-pass filtered at 0.5 Hz and sampled every 2 s. The NO electrode and reference were equilibrated in an intracellular buffer at room temperature with constant stirring until a stable baseline current was obtained. The composition of the buffer was 50 mM Tris·HCl, pH 7.4, 500 µM NADPH, 5 µM FAD, 5 µM flavin mononucleotide, 100 mM calmodulin, 0.01 mM CaCl2, and 20 µM L-arginine. To avoid interference by high levels of tetrahydrobiopterin (BH4), which is electroactive and unstable at physiological pH, 1 µM BH4 was added to the eNOS aliquots 12–24 h prior to the measurements. This permitted binding of the cofactor to the enzyme, which stabilizes the bound BH4, while much of the excess BH4 decays away. After obtaining a stable baseline, 25 pmol eNOS was pipetted into the cuvette, and the response was continuously recorded. For the determination of the effect of CO, different dilutions of CO-containing deionized water prepared from freshly prepared CO-saturated stock solution were added to the cuvette. When necessary, 0.2 to 2 mM Ni2+-monomethyl-L-arginine (L-NMMA) was added to the solution to verify the NO dependence of the recorded electrode current. At the completion of experiments, electrode function was tested with different dilutions of NO-saturated deionized water.
Statistical analyses were performed using paired or unpaired Student’s t-test or by repeated-measures ANOVA followed by a Dunnett’s multiple comparison test, with a $P < 0.05$ considered statistically significant. All values are presented as means ± SE.

**RESULTS**

Effects of CO on NO release from renal resistance arteries in vitro. Perfusion of microvessels with the Krebs-Ringer solution containing increasing concentrations of CO resulted in a biphasic release of NO detectable in the effluent (Fig. 1). The NO response peaked at 100 nM CO and then declined to virtually undetectable levels at 10 µM. As this is a sequential cumulative dose-response curve, the biphasic response could be due to several factors, including inhibition of eNOS at high CO levels and/or depletion of intracellular stores of NO after repeated exposure to CO. To explore this question, arterial segments were exposed to sequential pulses of 100 nM CO repeated at an interval of 150 s. As shown in Fig. 2, the second and third responses were significantly lower than the initial response. This blunting of NO release is consistent with CO-induced depletion of intracellular stores.

NO release from perfused vessels showed dependence on the presence of L-arginine but not D-arginine in the perfusate (Fig. 3), and the responses to CO were inhibited by the pretreatment with $N^G$-nitro-L-arginine methyl ester (L-NAME), an inhibitor of eNOS (Fig. 4). These data demonstrate that the release of NO in response to CO is dependent upon the enzymatic conversion of L-arginine to citrulline and NO. However, these data are also consistent with the possibility of NO release from an intracellular storage pool that is maintained in a dynamic steady state by NO generated by eNOS (see below).

The effects CO on stimulated NO release were then examined. The response to a potent agonist for eNOS, carbachol, was dramatically modified in the presence of CO, as shown in Fig. 5. Although 100 µM carbachol alone resulted in an immediate release of NO into the perfusate, this effect was virtually abolished in vessels perfused with 100 nM CO-containing solution. The observed suppression of NO release could not be attributed to the homologous desensitization of muscarinic receptors, as repeated infusion of carbachol alone, after a brief period of CO washout, elicited characteristic increases of NO release. Rather, the inability to further
raise NO release in the presence of CO may reflect some degree of eNOS inhibition by CO.

In vivo effects of cobalt chloride treatment to induce HO-1 on agonist induced NO release. To examine the effects of HO-1 induction and elevated production of endogenous CO on stimulated NO release, rats were injected with a potent inducer of HO-1, CoCl₂. This model has previously been established and broadly used to study the generalized induction of HO-1 (32). Twelve hours after the injection, renal resistance arteries were microprepared, and their responsiveness to carbachol was examined. As shown in Fig. 6, carbachol-induced NO release from vessel segments obtained from CoCl₂-treated rats was reduced significantly in comparison to the release from arterial segments from control rats.

Effect of CO in blood-perfused afferent arterioles in vitro. To determine whether the effects on NO release by CO in isolated renal resistance arteries are also evident in intact preglomerular vessels, blood-perfused J M afferent arterioles were exposed to increasing concentrations of CO in the superfusate solution. The results are shown in Fig. 7A. Significant vasodilation was seen after ~5 min exposure to 0.1 and 1.0 µM CO, whereas at 10 µM CO mean vessel diameter was not different from baseline. This pattern is similar to that seen for NO production in resistance artery segments, except that the CO concentration required for maximal dilation was ~10-fold higher. To determine whether this reflected uptake and binding by erythrocytes, 10 µM CO was also added to the blood solution at the end of each experiment (labeled 10+P in Fig. 7). This resulted in a modest, but significant vasoconstriction that was fully reversed after ~20 min exposure to CO-free solutions. Furthermore, addition of 10 µM CO to both superfusate and perfusate significantly reduced the vasodilatory response to abluminal application of 10 µM acetylcholine, which is consistent with the above findings in perfused resistance artery segments that CO blunts muscarinic eNOS stimulation.

In vitro experiments with the purified eNOS. This series of experiments was designed to address the question of whether CO-induced increases in NO release reflect enhanced de novo enzymatic production of NO, or does it reflect displacement by CO from intracellular stores that are maintained in dynamic equilibrium by NO production? To address this issue, in vitro studies were performed where the direct effect of CO on the activity of eNOS, the most likely isoform involved in...
the vasodilatory responses elicited from the luminal side of the vessels, was measured with an NO-selective porphyrinic microelectrode. As shown in Fig. 8A, addition of recombinant eNOS to the reaction buffer resulted in an immediate L-NMMA-inhibitable release of NO. These data validate the used preparations of recombinant eNOS and the technique of direct measurement of eNOS output (in preliminary experiments, the effect of CO on recombinant eNOS was also validated using [14C]arginine; data not shown). Application of CO diluted in the reaction buffer produced a dose-dependent suppression of NO generation, as shown in Fig. 8B (note that CO per se did not affect electrode current; Fig. 8C). CO did not interfere with NO directly, since the addition of NO-containing buffer to the CO-containing solution resulted in a typical rise in electrode oxidation current (Fig. 8D). These experiments directly demonstrate that CO inhibits eNOS activity within the physiological concentration range. Consequently, these results imply that the major source of CO-induced NO release is likely to be a CO-displaceable intracellular NO storage pool.

**DISCUSSION**

The data presented herein describe the vasodilatory and NO-releasing properties of CO, as detected using video microscopy of isolated perfused rat afferent arterioles and an NO-selective microelectrode to monitor NO release from renal resistance arteries. The data...
demonstrate that CO elicits NO release with a biphasic concentration dependence, where NO release is suppressed at high CO levels. Repeated exposure to brief pulses of CO results in an attenuation of the magnitude of the NO release, compared with the initial response. Furthermore, these NO responses depend on the presence of l-arginine in the perfusate and were inhibited by L-NAME. Pretreatment of vessels with CO reversibly blunted NO release induced by the muscarinic agonist carbachol. This inhibition of the response to carbachol was also seen in vessels from rats in which endogenous CO production was enhanced via induction of HO-1 with cobalt chloride. These effects of CO were further confirmed in blood-perfused J M afferent arterioles, where CO induced a biphasic pattern of vasodilation and blunted muscarinic vasorelaxation. Finally, direct measurement of NO production in a microassay with recombinant eNOS demonstrated that CO at concentrations 1.0 µM-10.0 µM invariably inhibits NO production by the enzyme.

The similarities between NO and CO are many (reviewed in Ref. 8). Both simple gases are produced endogenously by enzymes or enzymatic complexes possessing oxidoreductase activity, both activate guanylyl cyclase, although with different potencies, both can affect K channels in certain cell types, and both induce vasorelaxation (2, 7, 30, 43, 44). The distribution of guanylyl cyclase in the brain is colocalized with either NOS, or HO, or both, depending on the region (40). The endogenous production of CO can be substantial in some tissues. For example, in the esophageal sphincter, CO production reaches 1 nmol·mg protein⁻¹·h⁻¹, and CO participates in the relaxation of the circular smooth muscle (26).

The biological relevance of CO in vascular regulation has been established. Hypoxia, shear stress, and stretch all induce HO-1 and result in elevated CO production, especially by smooth muscle cells. The CO, in turn, stimulates cGMP production and produces a paracrine suppression in endothelial cells of mRNA for endothelin-1 and platelet-derived growth factor-B (19, 23, 24, 42). The physiological effects of CO vary from modulation of long-term synaptic plasticity in the hippocampus (46) to hypotension in rats inhaling the gas (12). The latter condition is associated with a marked elevation in NO and peroxynitrite levels accompanied by protein tyrosine nitrosylation (12). CO has been found to modulate hepatic sinusoidal perfusion by relaxing Ito cells (36). In the brain, CO increases blood flow in the cerebral vasculature, an effect that was found to be mediated via NO, and low concentrations of CO do not appear to suppress energy metabolism (22).

The data obtained in isolated perfused renal resistance arteries and afferent arterioles are consistent with the above findings, in that CO resulted in vasorelaxation and NO release into the perfusate. Moreover, the observed biphasic effect of CO on NO release from isolated perfused resistance arteries may have biological significance and may explain some previously contradictory findings. Low levels of CO, within the normal physiological range (41) may synergize with NO in eliciting vasorelaxation and modulating basal vascular tone, both by convergent actions on guanylyl cyclase activity and by CO-induced NO release from intracellular stores (34). This may be especially important in hypertensive states where HO-1 induction takes place and leads to a reduction in arterial pressure (13). Further evidence that low levels of CO may act synergistically with NO arises from studies by Scheele and coworkers (33), who utilized stopped-flow spectrophotometry to demonstrate that the kinetics of CO binding by neuronal NOS are such that binding at low CO concentrations is minimal under physiological conditions and in the presence of BH4 and arginine. Similar results were reported by Abu-Soud et al. (1) who demonstrated that, in the presence of l-arginine and BH4, the rate of CO binding to ferrous inducible NOS is considerably reduced.

In contrast, in several pathophysiological conditions, including septicemia, oxidant stress, and hemolysis, HO is induced and CO production is markedly enhanced. In these cases, the synergism between CO and NO may be lost, as high levels of CO will directly inhibit the generation of NO. This will lead to the depletion of cellular NO stores, as well as limit the responses to muscarinic agonists. Furthermore, higher concentrations of CO have been shown to induce oxidative stress and result in the production of peroxynitrite, which will also serve to limit the availability of NO (37). Such a decrease in NO levels in the endothelium would be accompanied by the loss of antithrombogenic activity of the endothelial layer and increased diapedesis of inflammatory cells (16, 29).

As discussed above, the source of the NO released in response to low levels of CO is most likely a preexisting intracellular heme-bound pool of NO, rather than a direct stimulation of eNOS. Indeed, the chemistry of the reaction of CO with the heme moiety of NOS argues against stimulation by CO. Furthermore, our direct measurements of eNOS activity show only inhibition by CO at relatively high levels (from 1.0 to 10 µM). This finding is consistent with those of McMillan et al. (21) and Fan et al. (6), who demonstrated that CO suppresses the activity of neuronal and inducible NOS. However, our studies also suggest that effects of CO on NO release are reversible within a relatively short interval. In this regard, the prediction of high-affinity binding of CO to the heme moiety has recently been questioned by Khartitonov et al. (15). These investigators demonstrated a high dissociation rate constant (28 s⁻¹) for CO binding to guanylyl cyclase. The authors provided additional evidence for the formation of a five-coordinate CO-guanylyl cyclase species that appears to be more active than the six-coordinate species. It is interesting to note that, if the NO released in response to CO represents NO displaced from a heme-bound intracellular pool, then this effect of CO is similar to that of long-wavelength ultraviolet light, which leads to the release of photo-induced relaxing factor, a substance identified as NO (7, 47).

The ability of CO and NO to stimulate guanylyl cyclase differs substantially (8, 35). Purified enzyme in
vitro was activated 130-fold by NO but only 4.4-fold by CO under similar conditions, and these findings have been confirmed in cell systems (11, 24). These data are in concert with the functional efficiency of CO-induced vasorelaxation found to be about one-thousandth that of NO (7). It appears, however, that an activator of soluble guanylyl cyclase, 3-(5-hydroxymethyl-2-furyl)-1-benzylindazole (YC-1), significantly enhances both CO- and NO-induced activation of the enzyme (reviewed in Ref. 8). Even if one assumes that an endogenous analog of YC-1 exists, the threshold concentration of CO is more than 100-fold higher than that necessary for NO stimulation of guanylyl cyclase. Our results in blood-perfused renal afferent arterioles are consistent with this concept, as the vasodilatory pattern mirrored the changes in NO release rather than the absolute level of CO present. Hence, in the context of vasorelaxation, CO has some paradoxical properties: its efficiency is low, and it leads to the reversible suppression of eNOS activity and production of superoxide, which further depletes free NO levels by generating peroxynitrite. Indeed, one wonders whether regulation of vascular tone is a primary function of CO, or whether it is just a secondary effect of another biologically meaningful action. Recent data on the accelerated motility of neutrophils exposed to CO (39) imply a potential role for CO in orchestrating the inflammatory response to cellular stress. If so, the associated CO-induced vasorelaxation observed by many investigators and confirmed here for the renal vasculature could represent one aspect of proinflammatory conditioning of vascular tissue.

In summary, the data obtained in renal preglomerular resistance arteries show that low levels of CO produce vasorelaxation and release NO, the source of which is most likely an intracellular pool of heme-bound NO that is normally maintained in a dynamic steady state by constitutive NO production by eNOS. At higher CO concentrations (above 1 µM), CO begins to inhibit eNOS activity, an effect that would eventually reduce NO release and is likely associated with the blunting of stimulated NO release. Although the biological significance of CO in regulating vascular tone under normal circumstances is unclear, CO-induced vasorelaxation may play an important role in mediating stress-induced inflammation.

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