Integrative Physiology

Dual Pathways of Carbon Monoxide–Mediated Vasoregulation Modulation by Redox Mechanisms

Brian D. Lamon,* Frank F. Zhang,* Nitin Puri, Sergey V. Brodsky, Michael S. Goligorsky, Alberto Nasjletti

Rationale: Vascular tissues produce carbon monoxide (CO) via HO-dependent and HO-independent mechanisms; the former in tandem with biliverdin and iron and the latter as a lone product. CO has been shown to function as both a vasoconstrictor and vasodilator; however, factors that dictate the vasoregulatory phenotype of this gas are unknown.

Objective: We investigated whether CO-mediated vasoconstriction is mechanistically linked to enhanced reactive oxygen species production that masks vasodilatory pathways.

Methods and Results: Sprague–Dawley rat interlobar and interlobular arteries were examined in terms of superoxide (O₂⁻) generation and vascular reactivity in the absence and presence of antioxidants. Both authentic CO and the CO-releasing molecule (CORM)-3 constricted renal arteries and increased O₂⁻ production in a dose-dependent manner. The antioxidants tempol, ebselen, and deferoxamine inhibited CO-induced O₂⁻ production and converted CO from constrictor to dilator. CO-induced O₂⁻ generation was found to involve the activity of multiple oxidases including nitric oxide synthase, NADPH oxidase, xanthine oxidase, and complex IV of the mitochondrial electron chain. Furthermore, inhibition of these enzymes converted CO from constrictor to dilator. Similarly, biliverdin and bilirubin inhibited CO-induced O₂⁻ production and vasoconstriction, allowing for a vasodilatory response to CO to be expressed. CO-induced vasoconstriction was dependent on a non-thromboxane agonist of the thromboxane receptor, whereas vasodilatory mechanisms of CO relied on the activation of soluble guanylate cyclase and calcium-gated potassium channels.

Conclusions: CO-induced vasoconstriction involves the generation of reactive oxygen species, which, when negated, allows for the expression of vasodilatory pathways which are masked by the primary oxidative stress response to this gas. (Circ Res. 2009;105:775-783.)

Key Words: carbon monoxide ■ vascular reactivity ■ reactive oxygen species ■ oxidative stress ■ antioxidant

Vascular tissues generate carbon monoxide (CO) via heme oxygenase (HO)-dependent and HO-independent pathways. CO is liberated during heme metabolism by HO, with equimolar quantities of biliverdin and iron, whereas HO-independent sources of CO include oxidation of organic molecules and peroxidation of membrane lipids. Following seminal reports that described a vasorelaxant effect of CO in the hepatic microcirculation, evidence supporting a vasodilatory role of CO has accumulated. For example, pharmacological inhibition of HO product generation, presumably leading to a reduction in CO production, increases renal vascular resistance and constricts pressurized gracilis muscle arterioles. The functional role of HO in the vasculature, however, is not synonymous with the biological effects of CO because CO exerts pleiotropic actions in terms of vasoregulation, functioning as both a vasodilator and vasoconstrictor.

Exogenous CO was first shown to dilate rat coronary arteries and proposed to act in a manner similar to nitric oxide (NO); that is, via activation of soluble guanylate cyclase (sGC). The role of CO in regulating vascular tone has been proposed to be augmented under conditions in which NO bioavailability was reduced, a setting in which CO production was enhanced. CO has also been shown to hyperpolarize vascular smooth muscle via activation of calcium-activated potassium channels (KCa). Finally, CO was demonstrated to reduce constrictor responses to phenylephrine and 20-hydroxyeicosatetraenoic acid, while reducing the synthesis of the vasoconstrictor agent endothelin.

Although the majority of data support a prodilatory role for CO, evidence for the existence of vasoconstrictor effects of CO has also accumulated. CO binds sGC with 30 to 100 fold less potency than NO; thus, the ability of this gas to...
efficiently activate sGC in physiological settings has been in question. Furthermore, CO has been proposed to inhibit NO synthesis and/or actions, interfering with the expression of vasodilatory mechanisms mediated by NO. Additionally, both endogenous and exogenous CO were found to cause constriction of isolated rat gracilis muscle arterioles. To date, the mechanism of CO-induced vasoconstriction has not been elucidated.

Relevant to the apparent discrepancy as to whether CO functions as a vasodilator or vasoconstrictor are findings that vascular endothelial cells exposed to CO undergo oxidative stress. Because reactive oxygen species (ROS) have been implicated in pathways associated with direct vasoconstriction as well as impairment of vasodilation, we hypothesized that CO of vascular origin may promote vasoconstriction in a ROS-dependent manner. The present study was designed to investigate the vasoregulatory mechanisms associated with CO by examining the relationship between CO and ROS generation in rat renal arterial vessels.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Animals

Male Sprague–Dawley rats (250 to 300 g, Charles River, Wilmington, DE) were used in compliance with protocols approved by the Institutional Animal Care and Use Committee.

Assessment of Superoxide Production by Renal Vessels

Lucigenin chemiluminescence was used to measure superoxide (O$_2^-$) production by freshly isolated renal interlobar arteries as per a protocol modified from Omar et al. In an effort to further confirm levels of O$_2^-$ production by vessels, an intravital fluorescence microscopy detection technique was used. O$_2^-$ production by renal interlobar arteries was assessed by quantifying a fluorescent signal arising from oxidized dihydroethidium using a Nikon epifluorescence inverted microscope (Diaphot) outfitted with a SIT camera (Hamamatsu, Japan) as previously described.

Assessment of H$_2$O$_2$ and Peroxynitrite Generation by Renal Vessels

Freshly isolated renal interlobar arteries were exposed to authentic CO (1 μmol/L) for 1 hour at 37°C in gas-sealed vials. Supernatant was collected for measurement of H$_2$O$_2$ using Amplex red as previously described. As an index of peroxynitrite (ONOO$^-$) formation, vessels were homogenized in K$^+$ phosphate buffer, separated by SDS-PAGE, and probed with an anti-nitrotyrosine antibody (Millipore, 1:5000).

Detection of Isoprostane Production by Renal Vessels

Isoprostane production by renal interlobar arteries was assessed using the Cayman Chemical STAT-8-isoprostane EIA kit (CN-500431).

Assessment of Vascular Response to CO

Renal interlobar arteries were dissected into segments 1 to 2 mm in length and mounted on a pressure myograph (model CH/200/Q, Living System Instrumentation; Burlington, VT) as previously described. CO or the CO-releasing molecule (CORM)-3 was added to the superfusion buffer and changes in internal diameter were recorded. The response to CO was studied in vascular preparations pretreated and not pretreated with tempol, ebselen, deferoxamine, N$^\bullet$-nitro-l-arginine methyl ester (L-NAME), apocynin, allopurinol, rotenone, carbonyl cyanide m-chlorophenylhydroxylamine (CCCP), pe-gylated superoxide dismutase (SOD), pegylated catalase, polyethylene glycol monomethyl ether, biliverdin, bilirubin, uric acid, indomethacin, or a thromboxane receptor (TP) antagonist (SQ29548). Finally, the effect of CO in vessels pretreated with tempol was examined in the absence and presence of an inhibitor of sGC (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one [ODQ]) or the K$_c$ channel blockers tetraethylammonium (TEA) and ibetrixotoxin. Data are reported as internal diameter or change in internal diameter (in microns).

Effect CO on SOD and Catalase Activities

SOD and catalase activity was quantified in vascular homogenates from vessels treated with and without CO (1 μmol/L) at 37°C for 1 hour using commercial kits (Cayman Chemical 706002 and 707002, respectively).

Data Analysis

Data are expressed as means±SEM for the given number (n) of experiments. Results were analyzed by Student t test or by 2-way ANOVA and the Newman–Keuls post hoc test was performed. The null hypothesis was rejected at P<0.05.

Results

Effect of CO on Internal Diameter of Renal Arteries

Initial experiments were conducted to establish the vasoregulatory phenotype of CO in rat renal arteries under basal conditions. As shown in Figure 1A, exposure to authentic CO or the CO-releasing molecule CORM-3 reduced internal diameter of interlobular arteries in a dose-dependent manner. Comparatively, vasoconstrictor response to 0.1 and 1.0 μmol/L authentic CO correlated to 10 and 100 μmol/L CORM-3, respectively. These findings are consistent with gas chromatographic/mass spectrometric detection of CO release by CORM-3 in physiological buffers, in which ~1% of detectable CO recovery was observed (data not shown). Importantly, inactivated CORM-3, which does not actively release CO, had no effect on internal diameter of vessels.
Effect of CO on ROS Generation by Renal Arteries

As shown in Figure 1B, freshly dissected vessels generate $O_2^-$/H$_{2}O_2$ ex vivo. Exposure of vessels to CORM-3 led to a dose-dependent increase in lucigenin-detectable $O_2^-$ production that was 2-fold higher than baseline at 100 μmol/L CORM-3 (P<0.05). Dihydroethidium fluorescence, an indicator of $O_2^-$ production, was found to be stable in isolated renal arteries under basal conditions. Consistent with other detection methods, administration of CORM-3 led to a gradual increase in dihydroethidium fluorescence detection of $O_2^-$ production by interlobar arteries. Results are means±SEM. $^*P<0.05$ relative to i-CORM-3.

Effect of CO on ROS Generation by Renal Arteries

As shown in Figure 1B, freshly dissected vessels generate $O_2^-$ ex vivo. Exposure of vessels to CORM-3 led to a dose-dependent increase in lucigenin-detectable $O_2^-$ production that was ≈2-fold higher than baseline at 100 μmol/L CORM-3 (P<0.05). Dihydroethidium fluorescence, an indicator of $O_2^-$ production, was found to be stable in isolated renal arteries under basal conditions. Consistent with other detection methods, administration of CORM-3 led to a gradual increase in dihydroethidium-detectable $O_2^-$ production over a period of 1 to 3 minutes before stabilizing and remaining elevated during a plateau phase (Figure 1C). Importantly, inactivated CORM-3 was found to have no effect on vascular $O_2^-$ generation by either method (Figure 1B and 1C). Complementary studies confirmed CO was the bioactive molecule as authentic CO (1.0 μmol/L) increased $O_2^-$ generation by vessels from 62±3 to 99±9 cpm/μg protein (P<0.05), as measured by lucigenin chemiluminescence.

Although $O_2^-$ anion may be the initial ROS formed following exposure of renal arteries to CO, it is unclear whether other reactive intermediates are formed and/or propagate the vasoregulatory effects of CO. To this end, we examined the production of $H_2O_2$ and nitrotyrosine, the latter an indicator of ONOO$^-$ formation, in vessels exposed to CO. We observed a slight increase in $H_2O_2$ production that did not reach statistical significance, by renal interlobar arteries exposed to CO for 1 hour (3.54±1.1 versus 4.56±1.0 nmol/mg protein; n=6 and 7, respectively). Nitrotyrosine levels were undetectable under basal conditions and were unaltered following exposure to CO (data not shown).

Effect of Antioxidants on $O_2^-$ Production and Vascular Response to CO

To further investigate the impact of oxidative stress on the vasoregulatory actions of CO, antioxidants known to preferentially target various ROS were applied. Tempol (1 mmol/L), an SOD mimetic, prevented CORM-3-induced $O_2^-$ generation, as measured by the lucigenin chemiluminescence method. B, Effect of tempol (1 mmol/L), ebselen (1 μmol/L), deferoxamine (500 μmol/L), pegylated (peg) SOD (500 U/mL), peg-catalase (1000 U/mL), or empty peg (125 μg/mL) on vasostion of renal interlobular arteries to carbon monoxide. Results are means±SEM. $^*P<0.05$ relative to vehicle or baseline diameter.

Figure 1. A, Change in internal diameter of rat renal interlobular arteries in response to CORM-3 (1, 10, or 100 μmol/L) or CO (0.01, 0.1, and 1.0 μmol/L). $^*P<0.05$ relative to baseline. B, Measurement of $O_2^-$ production by renal interlobar arteries treated with increasing concentrations of inactivated CORM-3 (iCORM-3) or CORM-3, as measured by lucigenin chemiluminescence. C, Dihydroethidium fluorescence detection of $O_2^-$ production by interlobar arteries. Results are means±SEM. $^*P<0.05$ relative to i-CORM-3.

Figure 2. A, Assessment of tempol (1 mmol/L), ebselen (1 μmol/L), or deferoxamine (500 μmol/L) on CORM-3-induced $O_2^-$ generation, as measured by the lucigenin chemiluminescence method. B, Effect of tempol (1 mmol/L), ebselen (1 μmol/L), deferoxamine (500 μmol/L), pegylated (peg) SOD (500 U/mL), peg-catalase (1000 U/mL), or empty peg (125 μg/mL) on vasosigton of renal interlobular arteries to carbon monoxide. Results are means±SEM. $^*P<0.05$ relative to vehicle or baseline diameter.
converting the response to CO from constriction to dilation (Figure 2B). The capacity of ebselen and deferoxamine to convert CO from constrictor to dilator, however, may imply downstream ROS in these processes. Pretreatment of vessels with pegylated catalase (1000 U/mL) similarly converted the response of CO from constriction to dilation (Figure 2B). Polyethylene glycol monomethyl ether (125 µg/mL), used as a control for pegylated SOD and catalase, had no effect on CO-mediated vasoconstriction (Figure 2B). Collectively, these findings implicate O$_2^-$ and downstream intermediary reactive species in the implementation of the vasoregulatory actions of CO.

Effect of Inhibition of Vascular Oxidases on CO-Induced O$_2^-$ Production and Vasoactivity

To investigate the contribution of various oxidases to the implementation of CO-induced oxidative stress and vasoactivity, the effects of this gas were studied in the absence and presence of inhibitors of the major sources of O$_2^-$ in the vasculature: nitric oxide synthase (NOS), (NADPH)-oxidase, xanthine oxidase (XO), and the mitochondria. As shown in Figure 3, inhibition of NOS, NADPH oxidase, and XO with L-NAME (1 mmol/L), apocynin (100 µmol/L), and allopurinol (100 µmol/L), respectively, inhibited CORM-3–induced elevation in O$_2^-$ levels. Importantly, inhibition of NOS, NADPH oxidase, and XO also converted the response to CO from vasoconstriction to vasodilation (Figure 4).

The mitochondrial respiration chain has been reported to differentially generate O$_2^-$ depending on the specific activation or inhibition of particular oxidases. Rotenone (10 µmol/L), an inhibitor of complex I in the mitochondrial respiration chain did not alter O$_2^-$ production or internal diameter in response to CORM-3 and CO (Figures 3 and 4D), respectively. On the other hand, CCCP (5 µmol/L), an inhibitor of complex IV minimized ($P<0.05$) CORM-3–induced O$_2^-$ production by >70% (from 16±6.4 to 76.0±18.6 cpm/µg in controls and from 30±2.4 to 46±5.7 cpm/µg protein in vessels pretreated with CCCP) and converted CO from constrictor to dilator at 1000 nmol/L CO (Figure 4D).

Effect of Biliverdin and Bilirubin on O$_2^-$ Production and Vascular Response to CO

Consistent with previous reports demonstrating the antioxidant properties of biliverdin and bilirubin, 100 nmol/L either bile pigment inhibited the CORM-3–induced increase in O$_2^-$ production (Figure 5A). Consistent with our initial findings using other antioxidants, biliverdin and bilirubin converted the response of CO from vasoconstriction to vasodilation in a concentration-dependent manner (Figure 5B and 5C, respectively). Another endogenous antioxidant, uric acid (200 µmol/L) also converted the response of renal arteries to CO from vasoconstriction to vasodilation (internal diameters of $-3.0±0.0$ versus $4.8±0.9$...
Effect of Cyclooxygenase Inhibition and TP Receptor Antagonism on CO-Induced Vasoactivity

Pretreatment of renal interlobular arteries with indomethacin (1 μmol/L) did not alter the vasoconstrictor response to CO, suggesting cyclooxygenase metabolites do not contribute to vasoconstrictor mechanisms associated with this gas (Figure 6A). On the other hand, blockade of the TP receptor (SQ29548; 1 μmol/L) prevented (P<0.05) CO-induced vasoconstriction, implicating a non-thromboxane agonist of the TP receptor in mediating this response. Importantly, TP receptor blockade with SQ29548 had no effect on phenylephrine-induced (1 μmol/L) reduction in internal diameter of renal vessels (−25.1±15 versus −27.3±1.0 μm).

Oxidative stress promotes the formation of the vasoconstricting, nonenzymatic oxidation products of arachidonic acid known as isoprostanes.25 We found that incubation of freshly isolated renal interlobar arteries with CO (10 μmol/L) increased isoprostane formation from 201±47 to 393±60 pg/mg protein (P<0.05). Collectively, these observations suggest the contribution of an isoprostane to the vasoconstrictor actions of CO.

Effect of sGC Inhibition and KCa Channel Blockade on CO-Induced Vasoactivity

Pretreatment of renal interlobular arteries with tempol allows for the expression of a CO-induced vasodilatory response (Figure 6B). In this experimental setting, the dilatory action of CO was partially reduced (P<0.05) by ODQ (10 μmol/L) and completely prevented (P<0.05) by both TEA (1 mmol/L) and iberiotoxin (1 μmol/L). The combination of TEA and ODQ also prevented the vasodilatory actions of CO. In the absence of tempol, both ODQ and TEA sensitized (P<0.05) vessels to CO-induced vasoconstriction. ODQ enhanced CO-induced vasoconstriction from 5.0±0.6 to 7.3±0.6 μm and from 12.8±0.9 to 18.0±1.9 μm at 100 and 1000 nmol/L CO, respectively. TEA enhanced CO-induced vasoconstriction from 5.0±0.5 to 13.0±1.2 μm and from 12.8±0.9 to −21.5±0.5 μm, at 100 and 1000 nmol/L CO, respectively.
Effect of CO on SOD and Catalase Activities

We then investigated the capacity of CO to alter the enzymatic activities of 2 critical antioxidant enzymes. The activities of SOD and catalase were measured in homogenates from vessels exposed to CO (1 μmol/L) for 1 hour. CO did not significantly alter the activities of SOD (2.25 ± 0.57 versus 1.39 ± 0.04 U/mg protein in untreated and CO-treated, respectively) or catalase (22.4 ± 3.3 versus 27.5 ± 1.4 nmol H₂O₂ conversion/mg protein per minute in untreated and CO-treated, respectively).

Discussion

Vascular tissues generate CO which, depending on experimental conditions, can be implicated in mediating vasoconstriction as well as vasodilation.⁷,⁸,¹¹,¹⁵,¹⁶ We report here for the first time that both the vasoconstrictor and vasodilatory responses to CO are critically conditioned by redox mechanisms (Figure 7). The vasoconstrictor action is linked to increased oxidant activity which promotes formation of isoprostanates. The vasodilatory action is linked to mechanisms involving sGC and KCa channels, and requires conditions that offset the prooxidant activity of CO.

That CO and CORM-3 elicit constriction of isolated, pressurized, renal interlobar arteries is consistent with earlier reports that CO constricts pressurized gracilis muscle arterioles, an action attributed to inhibition of NO synthesis.¹⁵ The present study offers an alternative explanation to the vasoconstrictor action of CO that involves oxidative stress as a determinant for the generation of isoprostanates, which promote contraction of vascular smooth muscle and thus mediate the constrictor action of the gas. Consistent with early reports of prooxidant actions of CO in endothelial cells and brain, we found that both authentic CO and CORM-3 cause as increase of O₂⁻ levels in renal interlobar arteries.²⁻¹⁷,²⁴ This action of CO may entail activation of multiple oxidases, because the CO-induced elevation of vascular O₂⁻ levels was blunted or minimized in arterial vessels pretreated with L-NAME (NOS inhibitor), apocynin (NADPH oxidase assembly inhibitor), allopurinol (XO inhibitor), or CCCP (mitochondrial oxidase complex IV inhibitor). Relevant to this point, CO is capable of binding and inhibiting NOS.²⁷,²⁸ It is unclear, however, whether NOS inhibition by CO is accompanied by uncoupling of the enzyme with resultant generation of O₂⁻ as occurs in the presence of tetrahydrobiopterin deficiency. Cytochrome c oxidase, a constituent of mitochondrial oxidase complex IV, is also amenable to inhibition by CO with attendant generation of ROS, but the secondary activation of multiple oxidases by downstream intermediate prooxidant molecules such as H₂O₂, OH⁻ radical and ONOO⁻. These are volatile molecules that can rapidly cycle between species via pathways such as the dismutation of O₂⁻ to H₂O₂, the conversion of H₂O₂ and nitrite to ONOO⁻, the Fenton reaction–mediated generation of OH⁻ radical, or the combination of O₂⁻ and NO to form ONOO⁻.³¹ Previous studies offered evidence of feed-forward propagation of oxidative stress in the vasculature via H₂O₂-dependent activation of NADPH oxidases, XO, endothelial NOS uncoupling, and augmentation of intracellular iron.³² Despite a previous report that CO increased intracellular H₂O₂ production in the brain, the present observations that incubation of arterial vessels with CO failed to result in a significant augmentation of H₂O₂ or nitrotyrosine levels (an index of ONOO⁻) argues against the notion that H₂O₂ and ONOO⁻ are implicated in the propagation of the oxidative stress serving to sustain CO-induced elevation of vascular O₂⁻ levels.³³ However, the possibility that CO-induced oxidative stress is sustained by intermediate reacting molecules via activation of multiple oxidases fits well with our finding that pretreatment of arterial vessels with ebselen (a glutathione peroxidase mimetic that also scavenges ONOO⁻) or defereroxamine (a chelator of iron and other transition metals which limits OH⁻ radical generation) prevents CO from increasing O₂⁻. That defereroxamine blocked CO-induced increase in vascular O₂⁻ suggests that iron or other transition metals play a role in ROS propagation initiated by CO. Free iron can be deleterious to cells because of its participation in the Fenton reaction, which...
involves H₂O₂ and yields OH⁻ radical, a highly reactive oxidant toxic to biological molecules. That defereroxamine did not alter basal vascular levels of O₂⁻⁻ may be taken to indicate that under resting conditions metal-driven reactions promoting oxidative stress are nominal.

We have also given consideration to the possibility that CO-induced elevation of vascular O₂⁻⁻ levels results from an inhibitory action of the gas on antioxidant enzymes such as catalase and SOD. Catalase is a heme-containing enzyme which has been suggested to be a target for CO, leading to inhibition of its catalytic activity. This is not the case in our study, because treatment with CO did not alter catalase activity measured in freshly isolated arterial vessels acutely exposed to the gas. Treatment with CO was also without effect on the activity of SOD measured in isolated arterial vessels. Recently, CO was reported to inhibit cystathionine β synthase. Inhibition of this enzyme may overwhelm endogenous antioxidative defense mechanisms via excessive homocysteine accumulation and/or a reduction in intracellular glutathione.

Linking the increase in O₂⁻⁻ production to the vasoconstrictor actions of CO in renal arteries, we demonstrate that CO-induced vasoconstriction is converted to dilation by exogenous antioxidants and inhibition of intracellular sources of O₂⁻⁻. That a reduction in O₂⁻⁻ levels prevents CO-mediated constriction confirms a role for ROS in the constrictor response. However, the ability of antioxidants to convert the actions of exogenous CO from constrictor to dilator suggests that ROS may be simultaneously preventing the expression of vasodilatory pathways. In the present study, dilation to CO in the presence of antioxidants was found to be mediated by activation of sGC and K<sub>Ca</sub> channels, consistent with reports in other resistance vessels. Interestingly, sGC and K channels have been shown to be negatively regulated by ROS. BK<sub>Ca</sub> (big conductance K<sub>Ca</sub>) in rat cerebral arterial smooth muscle cells is reversibly inhibited by ONOO⁻, whereas ROS-mediated heme oxidation impairs sGC activation in blood vessels. Thus, antioxidant intervention may provide a dual impetus to both antagonize proconstrictor mechanisms, as well as to relieve inhibitory influences on vasodilator pathways (eg, K<sub>Ca</sub>, sGC) associated with oxidative stress.

The mechanism associated with CO-induced vasoconstriction, which appears to involve the generation of O₂⁻⁻ and potentially downstream ROS, has not been elucidated to date. ROS are known to lead to the generation of nonenzymatic metabolites of arachidonic acid known as isoprostanes that are capable of constricting vessels via activation of the TP receptor. As CO was found to enhance vascular isoprostane formation, we hypothesized that isoprostanes may be downstream mediators of CO-induced vasoconstriction. That a TP receptor antagonist, but not indomethacin, inhibited vasoconstriction to CO provides seminal evidence that isoprostane-mediated activation of the TP receptor mediates CO-induced vasoconstriction.

Paradoxically, we observe vasoconstriction in response to exogenous CO, yet previous work has demonstrated that a reduction in endogenous CO formation via the inhibition of HO activity similarly promotes vasoconstriction. These findings suggest that endogenously produced CO functions as a vasodilator, whereas exogenous CO functions as a vasoconstrictor. Because HO-mediated heme metabolism concurrently generates CO and endogenous antioxidants biliverdin/bilirubin, we hypothesize that cogeneration of biliverdin/bilirubin functions to neutralize prooxidant/-constrictor effects of endogenously formed CO. In fact, the prooxidant and pressor effects associated with angiotensin II– and DOCA salt–induced hypertension were reduced by elevated bilirubin levels.

Consistent with previous reports of bile pigments functioning as antioxidants, exogenous biliverdin and bilirubin inhibited O₂⁻⁻ production and vasoconstriction in response to CO. Concentrations of biliverdin and bilirubin used in the present study (10 to 1000 nmol/L) were consistent with previous work and believed to be in a physiological range, well below plasma concentrations (5 to 17 μmol/L). Ultimately, intracellular concentrations of biliverdin and bilirubin are contingent on lipid/water solubility, binding proteins, uptake/diffusion, and intracellular heme metabolism. Numerous mechanisms have been proposed regarding the antioxidant capacity of biliverdin and bilirubin. Perhaps the most impressive effects of bilirubin in terms of cellular protection is its ability to safeguard against lipid peroxidation. Plasma bilirubin may function as a chain-breaking antioxidant, acting on secondary oxidants (eg, ONOO⁻) involved in the propagation of ROS-mediated damage. Bilirubin was additionally shown to inhibit the activation process of NADPH oxidase, a major source of vascular O₂⁻⁻, and to inhibit protein kinase C activity–dependent ROS production. Furthermore, bilirubin may undergo a “recycling” process whereby biliverdin is converted to bilirubin via the enzyme biliverdin reductase, followed by bilirubin oxidation by ROS to biliverdin. It should also be noted that other endogenous antioxidants may provide an alternative to bile pigments, such as in a setting in which CO is formed independently of HO. For example, uric acid has been previously shown to suppress ROS production in response to angiotensin II, and we demonstrate herein the ability of this compound to convert the response of CO from constrictor to dilator. Collectively, these findings support the notion that the antioxidant capacity of endogenously formed compounds (biliverdin, bilirubin and uric acid) function to unmask dilatory mechanisms associated with CO.

It may be anticipated that acute administration of CO would elicit vasodilatation, because biliverdin/bilirubin would be present when redox balance is in equilibrium; however, the present studies were conducted in an isolated, non–blood perfused system. Plasma bilirubin has been shown to have a large capacity to combat oxidative stress; therefore, lack of this pigment may reduced the antioxidant capacity of the vessel wall and allow for CO to elicit vasoconstriction. Therefore, the effects of CO may be largely dependent on environmental redox balance or, in some cases, experimental condition. The debate as to whether CO of vascular origin functions as a vasodilator or vasoconstrictor has been fueled by conflicting reports in the literature. The role of HO in vasoregulation has oft focused on CO as the quintessential
bioactive product of heme metabolism; however, it may be prudent to consider that concurrent generation of biliverdin/bilirubin is critical in dictating the vasoregulatory phenotype of CO. As such, these considerations may be applicable to systems outside of the vasculature, a concept that is consistent with the work of other investigators demonstrating synergistic actions of CO and biliverdin.\textsuperscript{40} HO-independent sources of CO may also be physiologically relevant as CO formed as an isolated product likely increases ROS production, potentially leading to vasoconstriction. In conclusion, this study demonstrates, for the first time, that CO constricts renal arteries in a ROS-dependent manner that, when antagonized, allows for vasodilatory pathways associated with CO to become unmasked.

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Disclosures

None.

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**Methods: Online Supplement**

*Animals.* Rats were anesthetized with sodium pentobarbital (50mg/kg I.P.). Renal interlobar and interlobular arteries were dissected and placed in ice-cold gassed (95% O₂-5% CO₂) Krebs buffer and immediately used for ROS detection, assessment of vascular reactivity, or other analysis as described.

**Assessment of O₂⁻ Production by Renal Vessels.** The chemiluminescent signal was detected using a liquid scintillation counter (Beckman 6000TA; Long Island Scientific, Long Island, NY) with a single photomultiplier in out-of-coincidence mode. Dissected vessels were placed in oxygenated Krebs buffer and incubated for 30 m at 37°C. Scintillation vials containing 5-µmol/l lucigenin in 10-mmol/l HEPES-NaOH-buffered Krebs solution (pH 7.4) were prepared. Blank values were subtracted from those including samples, total protein was measured, and data was reported as counts per minute (cpm)/mg protein.

For measurements of superoxide production using fluorescence microscopy, vessels were loaded with 10-µmol/l DHE, incubated for 30 m at 37°C and illuminated at a wavelength of 490 nm in 30 s intervals using an automatic shatter (Lambda 10-2, Sutter Instruments) interfaced to MetaFluor software (Universal Imaging). MetaMorph software was used to analyze images collected at 620nm.

**Detection of Isoprostane Production by Renal Vessels.** Renal interlobar arteries (16 vessels/vial) were incubated at 37°C for 1 h in Krebs buffer containing 10⁻⁶mol/l phenylephrine with and without 10-µmol/l carbon monoxide or vehicle at 37°C for 1 h and snap frozen with 2.23µmol/l butylated hydroxytoulene to prevent lipid peroxidation and further generation of isoprostanes. Frozen samples were thawed and homogenized in 0.1M TRIS (pH 7.4) containing 1-mmol/l EDTA and 10-µmol/l indomethacin and
subsequently analyzed using the Cayman Chemical STAT-8-isoprostane EIA kit (CN-500431). Data were normalized to protein concentration and expressed as pg/mg protein.

**Effect CO on SOD and Catalase Activities.** Freshly dissected small renal and mesenteric resistance arteries from SD rats were incubated in gas sealed vials with and without CO (1-µmol/l) at 37°C for 1 hour. Incubation was terminated by flash freezing the vials and arteries were homogenized (20mM HEPES-70mM Sucrose-210mM Mannitol- 1mM EGTA buffer at pH 7.2 for SOD assay and 50mM K+ phosphate with 1mM EDTA buffer at pH 7.0 for catalase assay), centrifuged at 4°C (1500 x g for 5mins. for SOD assay and 10,000 x g for 15 mins for Catalase assay) and the supernatant was assayed for SOD and catalase activity using appropriate kits (Cayman Chemical 706002 and 707002, respectively).

**Chemicals and Reagents.** DHE was purchased from Molecular Probes (Eugene, OR), biliverdin and bilirubin from Frontier Scientific (Logan, UT) and ebselen from Alexis Biochemical, San Diego, CA. CORM-3 was a gift from Dr. John R. Falck from the University of Texas Southwestern Medical Center, Dallas, TX. Lucigenin, tempol, L-NAME, allopurinol, apocynin, deferoxamine, rotenone, pegylated SOD, pegylated catalase, ODQ, TEA, IBTX, indomethacin and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).