Mechanisms of Carbon Monoxide Attenuation of Tubuloglomerular Feedback

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Abstract—Carbon monoxide (CO) is a physiological messenger with diverse functions in the kidney, including controlling afferent arteriole tone both directly and via tubuloglomerular feedback (TGF). We have reported that CO attenuates TGF, but the mechanisms underlying this effect remain unknown. We hypothesized that CO, acting via cGMP, cGMP-dependent protein kinase, and cGMP-stimulated phosphodiesterase 2, reduces cAMP in the macula densa, leading to TGF attenuation. In vitro, microdissected rabbit afferent arterioles and their attached macula densa were simultaneously perfused. TGF was measured as the decrease in afferent arteriole diameter elicited by switching macula densa NaCl from 10 to 80 mmol/L. Adding a CO-releasing molecule (CORM-3, 5×10⁻⁵ mol/L) to the macula densa blunted TGF from 3.3±0.3 to 2.0±0.3 μm (P<0.001). The guanylate cyclase inhibitor LY-83583 (10⁻⁶ mol/L) enhanced TGF (5.8±0.6 μm; P<0.001 versus control) and prevented the effect of CORM-3 on TGF (LY-83583+CORM-3, 5.5±0.3 μm). Similarly, the cGMP-dependent protein kinase inhibitor KT-5823 (2×10⁻⁶ mol/L) enhanced TGF and prevented the effect of CORM-3 on TGF (KT-5823, 6.0±0.7 μm; KT-5823+CORM-3, 5.9±0.8 μm). However, the phosphodiesterase 2 inhibitor BAY-60-7550 (10⁻⁶ mol/L) did not prevent the effect of CORM-3 on TGF (BAY-60-7550, 4.07±0.31 μm; BAY-60-7550+CORM-3, 1.84±0.31 μm; P<0.001). Finally, the degradation-resistant cAMP analog dibutyryl-cAMP (10⁻⁴ mol/L) prevented the attenuation of TGF by CORM-3 (dibutyryl-cAMP, 4.6±0.5 μm; dibutyryl-cAMP+CORM-3, 5.0±0.6 μm). We conclude that CO attenuates TGF by reducing cAMP via a cGMP-dependent pathway mediated by cGMP-dependent protein kinase rather than phosphodiesterase 2. Our results will lead to a better understanding of the mechanisms that control the renal microcirculation. (Hypertension. 2012;59:1139-1144.)

Key Words: cGMP ▪ afferent arteriole ▪ TGF ▪ macula densa ▪ cAMP

The regulation of the renal microcirculation is influenced by many factors, including circulating hormones, sympathetic nervous system, and intrinsic autoregulatory mechanisms, most of which converge on the afferent arteriole (Af-Art), the main regulator of renal blood flow. Among the autoregulatory mechanisms, tubuloglomerular feedback (TGF) is a well-established feedback mechanism, initiated by an increase in NaCl reabsorption via the Na-K-2Cl cotransporter type 2 (NKCC2) at the macula densa. The macula densa, which is in close contact with the Af-Art, then releases ATP, which is hydrolyzed to adenosine and causes Af-Art constriction. Understanding the mechanisms of TGF regulation is essential to better understand the regulation of renal vascular resistance, glomerular filtration rate, and renal function.

Carbon monoxide (CO) is a low molecular weight gas that shares similar properties with another low molecular weight gas, namely NO, CO, like NO, is generated under physiological conditions. The synthetic enzymes that produce CO, heme oxygenase (HO) 1 and HO-2, are widely expressed in the kidney, both in vascular and tubular structures; thus, CO is endogenously produced in the kidney. Furthermore, we have shown recently that both HO isoforms are expressed in the macula densa. Both NO and CO exert important roles in the regulation of vascular tone and blood pressure, and we have reported recently that CO in the macula densa, just like NO, attenuates TGF in vitro and in vivo. However, to our knowledge, there is no information addressing the mechanism of action of CO in the macula densa, and, in fact, little is known about the signaling downstream from CO in renal epithelial cells. In the vasculature, the effects of CO are mainly mediated by activation of soluble guanylate cyclase and increases in cGMP. It is reasonable to postulate that cGMP is involved in the attenuation of TGF by CO, because we have shown previously that, in the macula densa, cGMP attenuates TGF.

The primary detection mechanism of TGF appears to be uptake of NaCl by means of the NKCC2, located in the apical membrane of macula densa cells. It is well known that NaCl absorption by the thick ascending limb can be increased by various hormones and autacoids that increase cAMP produc-
tion.3 cAMP then stimulates exocytic insertion of NKCC2 in the apical membrane via cAMP-dependent protein kinase9 and NKCC2-mediated NaCl transport.10 Similarly, it may be that, in the macula densa, cAMP production influences TGF by acting on NKCC2. Furthermore, it may be that cGMP acts to decrease cAMP, either via cGMP-dependent protein kinase (PKG), as shown in the cortical collecting duct,13 or via cGMP-stimulated (type 2) cyclic nucleotide phosphodiesterase (PDE2), as shown in the thick ascending limb.12 However, whether cAMP enhances TGF and whether it is involved in the mechanism by which CO attenuates TGF remain unknown.

Here we hypothesized that CO in the macula densa attenuates TGF by reducing cAMP via activation of PKG and PDE2. To address this hypothesis, we studied the effect of CO on TGF before and during inhibition of soluble guanylate cyclase, PKG, and PDE2 and in the absence and presence of a cell-permeant stable cAMP analog in the macula densa. For this we used a technique developed by us consisting of simultaneous perfusion of a microdissected Af-Art and its attached macula densa. This preparation has the advantage that it allows us to control pressure in the Af-Art and luminal fluid composition at the macula densa, as well as to obtain real-time images of the Af-Art, without the confounding effects of hemodynamic or hormonal influences.

**Methods**

New Zealand white rabbits weighing 1.5 to 2.0 kg (Covance, Covance, Battle Creek, MI) were given standard chow (Harlan Laboratories, Indianapolis, IN) and tap water ad libitum. Rabbits were anesthetized with ketamine (50 mg/kg, IM), xylazine (10 mg/kg, IM), and pentobarbital (25 mg/kg, IV). Kidneys were quickly harvested, sliced along their corticomedullary axis, and cooled to 4°C. All of the protocols were approved by Henry Ford Health System Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Af-Arts with the macula densa attached were microdissected and microprepurified, as described previously.13 Briefly, a single superficial Af-Art was isolated from each rabbit with its intact glomerulus and adherent tubular segments consisting of the terminal portion of the thick ascending limb, macula densa, and early distal tubule. Microdissection was performed by using fine forceps, at 4°C to 6°C and accomplished within an hour of harvesting the kidney. Samples were transferred to a 37°C chamber mounted on an inverted microscope where both the Af-Art and the end of either the distal tubule or thick ascending limb were cannulated with an array of concentric glass pipettes, as described previously.13 This system allows us to simultaneously perfuse the Af-Art and macula densa and to introduce chemicals by exchanging the perfusion solution in a few seconds, while keeping the holding and perfusion pipettes in place. It also keeps a constant pressure on the Af-Art perfusion (maintained at 60 mm Hg) and a constant flow at the macula densa (maintained at 20 nL/min), which is within the range of physiological flow rates.14 Another advantage of this system is that it makes it highly unlikely for any drugs present in the effluent of the tubular perfusate to diffuse through the bath and act directly in the Af-Art, because the exchange rate of bath perfusate is 1 mL/min; thus, effluents from the macula densa are immediately diluted 50000 times and washed out before they can act on other structures.15

**Experimental Protocols**

A 30-minute equilibration period was allowed before taking any measurements. Protocols consisted of 3 to 4 consecutive TGF responses induced by switching macula densa NaCl from 10 to 80 mmol/L. The first TGF response was used as a control, whereas subsequent TGF responses were induced in the presence of various pharmacological probes administered in the macula densa perfusate. Images of the Af-Art were acquired 5 minutes after the introduction of drugs at 5-seconds intervals, and 3 individual measurements of Af-Art diameter were taken at the site of maximum constriction and ±5 μm around it. The following experimental groups were studied: time controls (1a) 4 consecutive TGF responses with no drugs added; (1b) first TGF, control; and second and third TGF, CORM-3 (5×10−5 mol/L); (2) role of soluble guanylate cyclase in the attenuation of TGF by CO: first TGF, control; second TGF, CORM-3; third TGF, LY-83583 (10−6 mol/L); and fourth TGF, CORM-3+LY-83583; (3) role of PKG in the attenuation of TGF by CO: first TGF, control; second TGF, CORM-3 (5×10−5 mol/L); third TGF, KT-5823 (2×10−5 mol/L); and fourth TGF, CORM-3+KT-5823. (4) Role of PDE2 in the attenuation of TGF by CO: first TGF, control; second TGF, CORM-3 (5×10−5 mol/L); third TGF, BAY-60-7550 (10−6 mol/L); and fourth TGF, CORM-3+BAY-60-7550. (5) Role of cAMP in the attenuation of TGF by CO: first TGF, control; second TGF, CORM-3 (5×10−5 mol/L); third TGF, dibutyryl-cAMP (db-cAMP; 10−3 mol/L); and fourth TGF, CORM-3+db-cAMP.

**Chemicals and Solutions**

Ru(CO)3Cl, known as CORM-3, a CO-releasing molecule (synthesized by J.R. Fall, Dallas, TX), was used as a CO donor. This was freshly prepared before the experiments by dissolving the compound in distilled water. LY-83583, a soluble guanylate cyclase inhibitor, was purchased from Enzo Life Sciences (Farmingdale, NY). KT-5823, an inhibitor of PKG, and BAY-60-7550, a PDE2 inhibitor, were purchased from Cayman Chemical (Ann Arbor, MI). Db-cAMP, a cell-permeable analog of cAMP, was purchased from Tocris Bioscience (Minneapolis, MN).

**Statistics**

Paired t tests were used to compare the TGF responses, defined as the decrease in Af-Art diameter induced by switching macula densa NaCl from 10 to 80 mmol/L. Hochberg step-up procedure was used to adjust the P values for multiple comparisons. Values are expressed as mean ± SEM, and a P<0.05 was considered significant.

**Results**

**Time Controls**

We first tested whether TGF responses are stable and reproducible with time. Four consecutive TGF responses were induced by increasing the macula densa perfusate NaCl from 10 to 80 mmol/L. The first TGF response decreased Af-Art diameter by 2.9±0.2 μm (from 17.1±1.7 to 14.2±1.6 μm; n=6), and subsequent TGF responses were 3.2±0.1, 2.9±0.1, and 3.2±0.2 μm (Figure 1A). Thus, all 4 consecutive TGF responses were not significantly different, indicating reproducibility. We also tested the effect of the CO-releasing molecule CORM-3 (5×10−5 mol/L) when repeatedly added to the macula densa perfusate. The first (control) TGF response decreased Af-Art diameter by 3.7±0.2 μm (from 14.9±1.1 to 11.1±1.2 μm; n=6). The second and third TGF responses (in the presence of CORM-3) were attenuated to 1.5±0.3 μm (P<0.01 versus control) and 1.4±0.1 μm (P<0.001 versus control), respectively (Figure 1B). The 2 TGF responses performed in the presence of CORM-3 were not different from each other. These data suggest that CO attenuates TGF and that the effect of CORM-3 on TGF is reproducible over time, that is, it does not experience tachyphylaxis.
Role of Soluble Guanylate Cyclase in the Attenuation of TGF by CO

We then tested whether activation of the soluble guanylate cyclase/cGMP cascade is involved in the attenuation of TGF by CO. Control TGF decreased Af-Art diameter by 3.3±0.3 μm (from 19.2±0.6 to 15.9±0.7 μm; n=6). The CO-releasing molecule CORM-3 (5×10⁻⁵ mol/L) added to the macula densa perfusate attenuated TGF to 2.0±0.3 μm (P<0.001 versus control). The soluble guanylate cyclase inhibitor LY-83583 (10⁻⁵ mol/L) added to the macula densa perfusate enhanced TGF to 5.8±0.6 μm (P<0.001 versus control). CORM-3 failed to attenuate TGF in the presence of LY-83583, because TGF in the presence of both drugs was 5.5±0.3 μm (P value not significant versus LY-83583 alone; Figure 2). These data suggest that soluble guanylate cyclase inhibition potentiates TGF and that the attenuation of TGF by CO is mediated by activation of the soluble guanylate cyclase/cGMP system.

Role of PKG in the Attenuation of TGF by CO

Most of the effects of cGMP in the nephron are mediated by activation of either PKG⁷,¹⁵ or PDE2.¹⁶ We first tested whether PKG is involved in the attenuation of TGF by CO. Control TGF decreased Af-Art diameter by 3.2±0.3 μm (from 15.8±0.6 to 12.7±0.5 μm; n=6). CORM-3 added to the macula densa perfusate attenuated TGF to 1.7±0.2 μm (P<0.001 versus control). The PKG inhibitor KT-5823 (2×10⁻⁵ mol/L) added to the macula densa perfusate enhanced TGF to 6.0±0.7 μm (P<0.01 versus control). CORM-3 failed to attenuate TGF in the presence of KT-5823, because TGF in the presence of both drugs was 5.9±0.8 μm (P value not significant versus KT-5823 alone; Figure 3). These data suggest that the attenuation of TGF by CO is mediated by PKG.

Role of PDE2 in the Attenuation of TGF by CO

We then tested whether PDE2 is involved in the attenuation of TGF by CO. Control TGF decreased Af-Art diameter by
Role of cAMP in the Attenuation of TGF by CO

Finally, we investigated the role of cAMP in TGF and in the attenuation of TGF by CO. Control TGF decreased Af-Art diameter by 3.3±0.3 μm (from 15.4±1 to 12.1±1 μm; n=7). CORM-3 added to the macula densa perfusate attenuated TGF to 1.5±0.1 μm (P<0.001 versus control). The PDE-resistant, cell permeant cAMP analog db-cAMP (10⁻³ mol/L) added to the macula densa perfusate enhanced TGF to 4.6±0.5 μm (P<0.05 versus control). CORM-3 failed to attenuate TGF in the presence of db-cAMP, because TGF in the presence of both drugs was 5.0±0.6 μm (P<0.05 versus db-cAMP alone; Figure 5). These data suggest that cAMP potentiates TGF and that the attenuation of TGF by CO is mediated by cAMP.

Discussion

We report here that a CO donor attenuates TGF and that blocking either soluble guanylate cyclase or PKG, but not PDE2, can block this effect. Furthermore, preventing a decrease in cAMP by loading the macula densa with a cAMP analog can also block the attenuation of TGF by CO. These data support our hypothesis that CO in the macula densa attenuates TGF by reducing cAMP via cGMP/PKG pathway.

We reported previously that the HO system attenuates TGF, because inhibiting tubular HOs with stannous mesoporphyrin potentiated TGF, and adding exogenous CO to the macula densa perfusate attenuated TGF. Here we report on the mechanisms underlying the attenuation of TGF by CO. The signaling of CO in renal tubular epithelial cells has not been extensively studied. The soluble guanylyl cyclase/cGMP pathway mediates the protective effect of CO against cisplatin-induced toxicity in a proximal tubule cell line but not the antioxidant effects of CO in a mouse thick ascending limb cell line. Our data clearly demonstrate that, when soluble guanylate cyclase in the macula densa is inhibited, the effect of CO on TGF is prevented. This indicates that most of the effect of CO is attributed to activation of the soluble guanylate cyclase/cGMP system.

Because CO has been shown to cause vasodilation when applied directly to the Af-Art, it could be argued that the attenuation of TGF that we observed is attributed to diffusion of CO from the macula densa perfusate to the Af-Art. As explained in the Methods section, this is highly unlikely because of the dilutional effect of the bath perfusion. Furthermore, our data suggest that this is not the case, because the attenuation of TGF by CO was completely blocked by adding the soluble guanylate cyclase inhibitor LY-83583 to the macula densa perfusate; and we have shown previously that LY-83583 in the macula densa perfusate does not diffuse to the vascular compartment, because it did not alter the vasodilator effect of acetylcholine on the Af-Art (NO/soluble guanylate cyclase/cGMP–dependent vasodilator). Taken together, these data indicate that CO, similar to NO, acts locally in the macula densa by increasing cGMP.

Because inhibiting guanylate cyclase blocks the effect of both NO and CO on the TGF response and because it has been reported that in the vasculature CO could induce the vasodilator effect of acetylcholine on the Af-Art, it could be argued that the attenuation of TGF by CO was completely blocked by adding the soluble guanylate cyclase inhibitor LY-83583 to the macula densa perfusate; and we have shown previously that LY-83583 in the macula densa perfusate does not diffuse to the vascular compartment, because it did not alter the vasodilator effect of acetylcholine on the Af-Art (NO/soluble guanylate cyclase/cGMP–dependent vasodilator). Taken together, these data indicate that CO, similar to NO, acts locally in the macula densa by increasing cGMP.

Because inhibiting guanylate cyclase blocks the effect of both NO and CO on the TGF response and because it has been reported that in the vasculature CO could induce the release of NO, it could be argued that CO is acting via NO. However, we have shown previously that CORM-3 attenuates TGF even in the presence of the NO synthase 1 inhibitor 7-nitroindazole and that inhibiting endogenous CO synthesis with the HO inhibitor stannous mesoporphyrin
potentiates TGF even in the presence of the NO synthase inhibitor N^6-nitro-L-arginine methyl ester.\(^5\)

In the nephron, the effects of cGMP are mediated mainly by activation of PKG\(^7,15\) or PDE2\(^16\) and PDE2. PKGs are serine/threonine kinases encoded by 2 genes. PKG I is located predominantly in the cytoplasm, and PKG II is anchored to the plasma membrane by N-terminal myristoylation.\(^22\) We have shown previously that in the macula densa cGMP attenuates TGF, whereas blocking PKG potentiates TGF.\(^7\) To test whether PKG is involved in CO-induced attenuation of TGF, we used KT-5823, an inhibitor of PKG. We found that inhibition of PKG prevented the attenuation of TGF by CORM-3. To our knowledge, this is the first study to demonstrate that the attenuation of TGF by CO in the macula densa is a PKG-mediated process.

PDEs are a family of enzymes that hydrolyze cGMP and cAMP.\(^23\) PDE2 is unique in being markedly stimulated by cGMP to degrade cAMP.\(^24\) It has been shown that, in the thick ascending limb, cGMP activates PDE2, which, in turn, reduces cAMP and NKCC2-dependent Na entry.\(^16\) To test whether PDE2 is also involved in the attenuation of TGF by CO, we added the PDE2 inhibitor BAY-60-7550 to the macula densa perfusate. We found that PDE2 inhibition did not prevent the attenuation of TGF by CORM-3. Taken together, these data indicate that CO in macula densa attenuates TGF via a cGMP-dependent pathway mediated by PKG rather than PDE2.

Various factors that stimulate cAMP, such as arginine vasopressin, parathyroid hormone, and \(\beta\)-adrenergic agonists, are known to stimulate NaCl absorption in the thick ascending limb.\(^8\) Furthermore, cAMP is well known to increase NKCC2-dependent apical NaCl entry.\(^16,25-27\) likely by stimulating cAMP-dependent protein kinase, which phosphorylates NKCC2\(^28\) and increases exocytic insertion of NKCC2 in the apical membrane.\(^9\) If also true for the macula densa, this effect would be expected to potentiate TGF. Thus, cAMP and cGMP appear to have opposing actions on TGF. Moreover, cGMP, acting via PKG, inhibits adenyl cyclase in the collecting duct, and this cascade results in a decrease in intracellular cAMP.\(^11\) To test whether the attenuation of TGF by CO is mediated by a decrease in cAMP, we loaded the macula densa with a stable cAMP analog, db-cAMP. We found that db-cAMP enhanced TGF and prevented the attenuation of TGF by CORM-3. These data demonstrate that intracellular cAMP modulates TGF and that decreases in cAMP participate in the attenuation of TGF by CO.

The treatments that prevented the attenuation of TGF by exogenous CO in our study, namely, inhibition of guanylate cyclase or PKG and db-cAMP loading, also potentiated TGF when used by themselves. This may be because of blockade of endogenous CO. Our previous studies suggest that CO is endogenously produced in the macula densa, as we showed that both isoforms of HO are expressed in the macula densa and that blocking CO production with an HO inhibitor potentiates TGF.\(^3,5\) In addition, because NO shares at least part of the same signaling pathway with CO, it is possible that the potentiation of TGF seen with inhibitors of guanylate cyclase and PKG was partly attributed to inhibition of NO signaling.

Our finding that db-cAMP potentiates TGF is in apparent contradiction with 2 previous in vivo micropuncture studies showing that db-cAMP inhibited TGF.\(^29,30\) However, a direct vasodilatory effect of db-cAMP attributed to diffusion to the Af-Art cannot be excluded in in vivo studies, particularly because they only observed inhibition of TGF at high concentrations of db-cAMP, 10 times higher than the one that we used. Also in 1 study, perfusion was applied to the late proximal tubule, thus going through the thick ascending limb before reaching the macula densa, and because cAMP stimulates Na reabsorption in the thick ascending limb, it is likely that Na delivery to the macula densa was decreased, thus counteracting the direct effect of cAMP on TGF.

The HO system as a whole is antioxidant, because it transforms a pro-oxidant compound (free heme) into an antioxidant compound (biliverdin/bilirubin),\(^1\) and we have shown that biliverdin attenuates TGF by acting as an antioxidant.\(^3\) However, the effect of CO on oxidative stress is less clear. It has been reported that CO increases the generation of \(O_2^-\) from the mitochondria\(^31\) but inhibits the generation of \(O_2^-\) from NADPH oxidase 1.\(^32\) Current evidence suggests that the attenuation of TGF by CO is not attributed to an antioxidant effect. This assertion is based on 2 previously published experiments: the attenuation of TGF by Tempol was completely absent in the presence of the NO synthase inhibitor 7-nitroindazole\(^33\); that is, it was NO dependent; and the attenuation of TGF by CO is not affected whatsoever by 7-nitroindazole; that is, it is NO independent. Furthermore, NADPH oxidase 1, the enzyme associated with an antioxidant effect of CO, is not expressed in the macula densa.\(^34\)

**Perspectives**

In summary, we found that CO in the macula densa attenuates TGF by reducing cAMP via a cGMP-dependent pathway mediated by PKG rather than PDE2. Taken together with our previous studies,\(^3,5\) these data show that CO acts similarly, although independently from, NO. Thus, it is possible that, like NO, CO by inhibiting TGF favors Af-Art dilation, leading to increases in renal blood flow, glomerular filtration rate, and Na excretion. Considerable evidence indicates that CO is antihypertensive and natriuretic;\(^35\) our experiments suggest that part of those actions may be attributed to attenuation of TGF. Furthermore, because oxidative stress and angiotensin II are known inducers of HO-1\(^36\) but decrease NO in the macula densa,\(^33,37\) it is possible that the relative contribution of CO to TGF attenuation becomes greater in conditions with increased angiotensin II and/or oxidative stress.

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**Disclosures**

None.

**References**

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