Nitric Oxide Synthesis Inhibition Promotes Renal Production of Carbon Monoxide

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Abstract—We tested the hypothesis that the status of NO synthesis influences the renal heme-heme oxygenase system. Studies were conducted in untreated rats and rats treated with the NO synthesis inhibitor NG-nitro-L-arginine methyl ester for 2 days. Treated and untreated rats were contrasted in terms of renal expression of heme oxygenase-1 and -2, renal carbon monoxide (CO)-generating activity, and urinary CO concentration and excretion rate. Heme oxygenase-1 and -2 proteins were similarly expressed in the kidneys of untreated and treated rats. In contrast, the NADPH-dependent component of the CO-generating activity of renal homogenates incubated with heme (a measure of heme oxygenase activity) was higher (P<0.05) in kidneys from rats treated with the NO synthesis inhibitor relative to corresponding data in untreated rats (1015±95 versus 379±111 pmol CO/mg per hour). Similarly, relative to corresponding data in untreated rats, rats treated with the NO synthesis inhibitor displayed increased (P<0.05) urinary CO concentration (920±174 versus 2286±472 pmol/mL) and urinary CO excretion (4.7±0.4 versus 14.3±2.7 pmol/min). This study demonstrates that NO synthesis inhibition upregulates the urinary concentration and excretion rate of CO, and the HO-dependent generation of CO by renal homogenates, without affecting the expression of renal heme oxygenase isoforms. Our findings imply that endogenous NO is an inhibitory regulator of renal CO generation by HO.

Key Words: kidney ■ carbon monoxide ■ nitric oxide ■ heme oxygenase

Carbon monoxide (CO), a product of heme metabolism by heme oxygenase isoforms (HO)-1 and -2, has been linked to the regulation of arterial tone and/or reactivity. Nitric oxide (NO), a product of L-arginine metabolism by NO synthase isoforms (NOS), is a major contributor to mechanisms of vasodilation in several vascular beds. The heme-HO and the L-arginine-NOS pathways interact at multiple sites and influence each other’s level of activity and function. On one hand, ex vivo studies indicate that CO inhibits NOS activity and attenuates the expression of vasodilatory mechanisms mediated by NO. On the other hand, NO decreases the catalytic activity of HO, promotes HO-1 protein expression and cellular uptake of heme, and interferes with the ability of CO to stimulate large conductance Ca2+-activated K+ channels in vascular smooth muscle cells and produce vasodilation.

It is difficult to predict the impact of variations in NO synthesis on the activity of the heme-HO system, because NO downregulates the activity of constitutively-expressed HO-2 while upregulating HO-1 protein expression. Information on this point is relevant to the notion that the status of NO synthesis conditions the vasomotor response to HO inhibition in gracilis muscle arterioles and renal interlobular arteries ex vivo, and in the rat kidney and hind limb in vivo. For example, after NOS inhibition, an increase in HO product generation may help condition the associated intensification of the vasoconstrictor effect of HO inhibitors.

The present study was undertaken to determine whether treatment with an NOS inhibitor affects the activity of the renal heme-HO system. We contrasted untreated rats and rats undergoing treatment with a NOS inhibitor in terms of renal expression of HO-1 and HO-2, renal CO-generating activity, and urinary CO concentration and excretion.

Methods

Drugs and Solutions

Stannous mesoporphyrin (SnMP; Frontier Scientific), a nonselective HO inhibitor, was dissolved in 50-mmol/L NaCO3. All other drugs were from Sigma Chemical Co. S-nitroso-N-acetylpenicillamine (SNAP), a donor of NO, was dissolved in 0.1-mol/L potassium phosphate buffer pH 7.4; Ferriprotoporphyrin IX chloride (hemin) was dissolved in 0.1-mol/L NaOH and the pH was adjusted to 7.8 with 0.1-mol/L HCl. The NOS inhibitor N5-nitro-L-arginine methyl ester (L-NAME) was dissolved in tap water (0.72±0.00 and 0.23±0.02 mg/mL in short- and long-term treatment studies, respectively).

Experimental Protocols

Protocols were approved by the Institutional Animal Care and Use Committee. Studies were conducted on male Sprague-Dawley rats (Charles River, Wilmington, Mass; 300 to 325 g body weight) treated and not treated with L-NAME. L-NAME was provided in the

Received September 30, 2003; first decision October 27, 2003; revision accepted November 26, 2003.

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Hypertension is available at http://www.hypertensionaha.org

DOI: 10.1161/01.HYP.0000111721.97169.97
drinking water at a daily dose of 74.3±1.0 and 29.8±0.5 mg/kg in short- and long-term treatment studies lasting 2 days and 6 weeks, respectively.

On the last day of treatment, control and experimental animals anesthetized with thiobotubarbitral (50 mg/kg IP) and ketamine (30 mg/kg IM) were prepared for assessment of urinary CO. The rats were instrumented with polyethylene cannulas in the trachea (PE-205) to aid ventilation, the left femoral artery (PE-50) for measurement of blood pressure, the left femoral vein (PE-50) for administration of drugs and fluids (0.15-mol/L NaCl; 2.7 mL/h throughout the study), and the bladder (PE-60) for urine collection. After a 60-minute equilibration interval, 2 or 3 urine samples (100-μL each) for CO analysis were collected consecutively. Animals in the short-term L-NAME treatment protocol were subsequently injected with the HO inhibitor SnMP (40 μmol/kg IV) and, after a 45-minute interval, urine intervals were again collected as described above. In other animals not instrumented for urine collection, the kidneys were excised and frozen in liquid N2 for later assessment of HO isoform protein expression and CO-generating activity.

Assessment of HO Isoform Expression

Kidneys were homogenized in ice-cold 50-mmol/L Tris-HCl buffer, pH 7.4, containing 1% NP-40, 0.25% sodium deoxycholate, 1-mmol/L EDTA, and 10% protease inhibitor cocktail (Sigma Chemical Co). Homogenates were centrifuged (10 000 g for 30 minutes) and the supernatant was saved for protein assay and Western blot analysis in triplicate of HO-1, HO-2, and β-actin according to published procedures utilizing antibodies provided by Stresgen Biotechnologies. Immunocomplexed bands were visualized and quantified by densitometric analysis. Data are expressed as the HO isoform/β-actin ratio.

Assessment of CO-Generating Activity

Kidneys were homogenized in ice-cold 0.1-mol/L potassium phosphate buffer, pH 7.4, containing 1.37-mmol/L NaCl, 0.027-mmol/L KCl, 0.1-mmol/L butylated hydroxytoluene, and 10% protease inhibitor cocktail (Sigma Chemical Co). Subsequently, the homogenates were centrifuged (10 000 g for 30 minutes at 4°C), and the supernatant was assayed, and an aliquot (10 to 20 μL containing ~300-μg protein) was reacted with 40-μmol/L heme in the presence and absence of a NADPH-generating system consisting of 10-mmol/L MgCl2, 6.8-mmol/L glucose-6-phosphate, 3.3-U/mL glucose-6-phosphate dehydrogenase, and 2.6-mmol/L NADPH. When so noted, SNAP (0.01 to 1.0 mmol/L) was included in the incubation mixture to test the effect of NO on the CO-generating activity of homogenates prepared from kidneys of L-NAME-treated rats.

The reaction mixture (1 mL final volume) was incubated for 1 hour at 37°C in 2-mL amber vials capped with rubberized Teflon liners. Subsequently, the vials were placed on ice, an internal standard made of isotopically-labeled CO (13C18O, Sigma Chemical Co). Homogenates were centrifuged (10 000 g for 30 minutes) to aid ventilation, the left femoral artery (PE-50) for measurement of blood pressure, the left femoral vein (PE-50) for administration of drugs and fluids (0.15-mol/L NaCl; 2.7 mL/h throughout the study), and the bladder (PE-60) for urine collection. After a 60-minute equilibration interval, 2 or 3 urine samples (100-μL each) for CO analysis were collected consecutively. Animals in the short-term L-NAME treatment protocol were subsequently injected with the HO inhibitor SnMP (40 μmol/kg IV) and, after a 45-minute interval, urine intervals were again collected as described above. In other animals not instrumented for urine collection, the kidneys were excised and frozen in liquid N2 for later assessment of HO isoform protein expression and CO-generating activity.

Analysis of CO in Urine

CO was measured by gas chromatography-mass spectroscopy as previously described, in urine specimens collected into amber vials (2 mL), capped with rubberized Teflon liners perforated with one G-23 and one G-30 needle which, respectively, allow the specimens to flow into the vials under isobaric conditions. Immediately after completion of sample collection, the internal standard was added, the needles were removed, the perforations were sealed, and the samples were analyzed.

Results

Effects of Short-Term L-NAME Treatment on the Renal Heme-HO System

The mean arterial pressure of rats treated with L-NAME for 2 days exceeded that of untreated rats (140±3 versus 106±3 mm Hg, P<0.05). Shown in Figure 1, untreated (n=3) and L-NAME-treated (n=3) rats did not differ significantly from each other in terms of renal expression of HO-1 and HO-2 protein, relative to β-actin expression. Also shown in Figure 1, untreated (n=5) and L-NAME-treated (n=5) rats did not differ in terms of CO-generating activity of renal homogenates incubated with heme in the absence of NADPH. However, the CO-generating activity of renal homogenates incubated with heme in the presence of NADPH was 2.5-fold higher (P<0.05) in samples derived from L-NAME-treated rats than from the untreated controls. Consequently, the NADPH-dependent component of CO-generating activity, a measure of HO activity, was increased (P<0.05) 2.7-fold in kidneys from rats treated with the NOS inhibitor relative to untreated controls.
levels in control kidneys. Inclusion of the NO donor SNAP into incubation mixtures of heme and homogenates of kidney from L-NAME-treated animals (n=4) decreased (P<0.05) NADPH-dependent, but not NADPH-independent, generation of CO over the concentration range 100 to 1000 μmol/L (Figure 2). Thus, NO inhibits HO-catalyzed generation of CO by renal homogenates.

Figure 3 displays data on urinary CO concentration and excretion in untreated (n=5) and L-NAME treated (n=7) rats before and after administration of SnMP. Before treatment with SnMP, values of urinary CO concentration and excretion rate in untreated rats were exceeded (P<0.05) by corresponding values in rats treated with L-NAME for 2 days. The administration of the HO inhibitor SnMP acutely decreased (P<0.05) urinary CO concentration and excretion rate in rats with and without L-NAME treatment. Mean arterial pressure was not affected significantly by SnMP administration to untreated (106±3 versus 100±2 mm Hg) or L-NAME-treated rats (140±3 versus 133±3 mm Hg). Urine volume was similarly unaffected consistently by SnMP in untreated (8.8±4.0 versus 28.4±14.2 μL/min, P=0.2795) or L-NAME treated rats (7.7±1.8 versus 13.5±4.4 μL/min, P=0.2568).

Effects of Long-Term L-NAME Treatment on the Renal Heme-HO System

The mean arterial pressure of rats treated with L-NAME for 6 weeks (n=5) exceeded that of untreated rats (n=5, 144±5 versus 111±4 mm Hg, P<0.05). As shown in Figure 4, HO-1 and HO-2 proteins were similarly expressed in kidneys of untreated and L-NAME treated animals. In contrast, the values of urinary CO concentration and excretion in rats treated with the NOS inhibitor were clearly elevated (P<0.05) relative to corresponding values in untreated rats. Hence, renal HO isoform expression and urinary CO were similarly affected by short- and long-term treatment with L-NAME.

Discussion

Renal vascular and tubular structures express HO-2 constitutively; they also express HO-1, particularly in response to injurious conditions.16,19 HO-derived CO is found in the urine and renal interstitium of rats.18 HO-dependent generation of CO and other products is relevant to the regulation of renal function, because pharmacological inhibition of HO isoforms results in renal vasoconstriction and increased reactivity of the renal vasculature to constrictor agonists.3,5,20

The present study demonstrates for the first time that the status of NO synthesis influences the urinary concentration...
and excretion rate of CO in anesthetized rats. It also confirms that urinary CO concentration and excretion rate fall after treatment with SnMP, an indication that urinary CO arises from HO-catalyzed generation of the gas, presumably within the kidney. Accordingly, our finding that NO synthesis inhibition with l-NAME promotes urinary excretion of CO implies that endogenous NO is an inhibitory regulator of the renal heme-HO system.

According to our results, upregulation of urinary CO levels after NOS inhibition is not attributable to upregulation of HO isoforms, as the renal expression of HO-1 and HO-2 was not significantly affected by either short- or long-term treatment with l-NAME. Rather, our study suggests that elevation of urinary CO after NOS inhibition is related to an increase in the CO-generating activity of renal HO. In this regard, we found that the NADPH-dependent component, but not the NADPH-independent component, of CO-generating activity is increased in kidneys of l-NAME-treated rats relative to corresponding values in kidneys of untreated controls. As NADPH-dependent generation of CO is a measure of HO activity, our results suggest that NO synthesis inhibition with l-NAME upregulates the CO-generating activity of renal HO. Although it is a distinct possibility, it remains to be determined whether NOS inhibition also promotes CO production in tissues other than the kidney.

That NOS inhibition enhances HO-dependent generation of CO in the kidney implies that endogenous NO inhibits renal HO. In support of this notion, we found that the NO donor SNAP inhibits NADPH-dependent, but not NADPH-independent, generation of CO by renal homogenates derived from l-NAME-treated rats. This finding is in agreement with previous reports that exogenous NO inhibits HO activity. According to one study, the inhibitory effect of NO on HO activity is a consequence of heme nitrosylation which reduces the suitability of the modified porphyrin as a substrate for HO. According to another study, NO combines with the heme bound to the heme regulatory motif of HO-2 and selectively decreases the catalytic activity of HO-2. Our study offers no information on whether or not upregulation of renal CO-generating activity and urinary CO levels after NOS inhibition rely on upregulation of the activity of one or both renal HO isoforms.

Augmentation of renal CO production after NOS inhibition may have major functional consequences, because HO-derived CO is believed to decrease renal vascular reactivity to constrictor stimuli and promote renal vasodilation. For example, it is conceivable that upregulation of renal CO production during NOS inhibition brings about attenuation of the renal vasoconstriction known to be associated with diminished production of NO. This notion is in keeping with a recent report that the renal vasocsontriction ensuing after administration of the HO inhibitor SnMP is greatly magnified in rats pretreated with l-NAME. Consideration should also be given to the possibility that upregulation of renal CO production impacts on renal excretory functions, because HO inhibition was shown to promote diuresis and natriuresis due to decreased absorption of fluid and sodium in the loop of Henle. In the present study, however, urine volume was not consistently affected by SnMP in either untreated or l-NAME-treated rats.

In summary, the present study demonstrates that NOS inhibition with l-NAME elevates urinary CO levels and promotes urinary CO excretion without affecting the renal expression of either HO-1 or HO-2. After NOS inhibition, the augmentation of urinary CO concentration and excretion rate appears linked to an increase in the CO-generating activity of renal HO. These findings imply that endogenous NO exerts a tonic inhibitory influence on the heme-HO system of the kidney.

**Perspectives**

**Previous reports established that NOS inhibition enhances the functional relevance of the heme-HO system, facilitating CO-induced vasodilation and intensifying the vasoconstriction that accompanies inhibition of HO.** Such observations, in conjunction with the present finding that NO inhibition upregulates renal CO generation, are compelling reasons to consider the heme-HO system a potential player in the homeostatic response to disorders that feature deficient synthesis or bioavailability of NO. Indeed, there is evidence that the renal heme-HO system subserves a renoprotective role in angiotensin-dependent models of hypertension in which NO bioavailability is believed to be diminished.

**Acknowledgments**

This study was supported by the National Institutes of Health Grants HL18579 and HL34300. We thank Jennifer Brown for secretarial assistance.

**References**


