Inhibition of 20-HETE Production Contributes to the Vascular Responses to Nitric Oxide

Magdalena Alonso-Galicia, Heather A Drummond, K Kishta Reddy, John R Falck, Richard J Roman

Abstract Nitric oxide (NO) inhibits a variety of heme-containing enzymes, including NO synthase and cytochrome P4501A and 2B1. The present study examined whether NO inhibits the production of 20-hydroxyeicosatetraenoic acid (20-HETE) by cytochrome P4504A enzymes and whether blockade of the production of this substance contributes to the vascular effects of NO. Sodium nitroprusside (SNP), 10⁻³, 10⁻⁴, and 10⁻⁵ mol/L, reduced the production of 20-HETE by renal microsomes incubated with arachidonic acid to 71±5%, 20±4%, and 1±2% of control, respectively (n=5). Similar results were obtained with the use of 1-propanamine, 3-(2-hydroxy-2-nitroso-1-propylhydrazono) (n=3). To determine whether inhibition of 20-HETE contributes to the vasodilatory effects of NO, the effects of dibromo-dodecenylmethylsulfinide (DDMS), a selective inhibitor of the formation of 20-HETE, on the response to SNP (10⁻⁷ to 10⁻¹ mol/L) were examined in rat renal arterioles preconstricted with phenylephrine (n=5). SNP increased vascular diameter in a concentration-dependent manner to 82±4%, 29±4%, and 4±2% of control, respectively (n=5). Similar results were obtained with the use of 6-(2-hydroxy-1-methyl-2-mtrhydroxamino)N-methyl (MAHMA NONOate) averaged only 20% of those seen during control. In other experiments, MAP increased by 32±4% and RBF fell to 56±3% of control after administration of N-nitro-L-arginine (L-NArg) (10 mg/kg IV). After DDMS (10 mg/kg, n=7 rats), the MAP and RVR responses to L-hexamethonium, 6-(2-hydroxy-1-methyl-2-nitrohydroxamino)N-methyl (MAHMA NONOate) averaged only 20% of those seen during control. In this regard, it should be noted that NO has recently been reported to inhibit NO synthase and P450 enzymes, and potentiates tubuloglomerular feedback responses. These results indicate that NO inhibits cytochrome P4504A enzymes and that inhibition of the production of 20-HETE contributes to the vasodilatory effects of NO (Hypertension. 1997;39[part 2]:320-325.)

Key Words • nitric oxide • vasculature • enzymes

Recent studies have indicated that the effects of many renal vasodilators are dependent on the release of NO from the endothelium. Blockade of NO synthesis increases arterial pressure, decreases RBF, and potentiates tubuloglomerular feedback responses. These results indicate that tonic release of NO plays an important modulatory role in the regulation of both renal and peripheral vascular tone. It is generally assumed that the vasodilatory effects of NO are mediated by cGMP secondary to stimulation of guanylyl cyclase. This conclusion is based on the observations that endothelium-dependent vasodilators and NO donors increase cGMP in vascular tissue and that methylene blue and other inhibitors of guanylyl cyclase in many vessels can eliminate the vasodilatory response. However, this generalized scheme for NO-induced vasodilation has been questioned recently because there are an increasing number of reports that NO donors and/or endothelium-dependent dilators can produce vasodilation and/or membrane hyperpolarization in some vessels in the presence of guanylyl cyclase inhibitors.

In the renal microcirculation, there have been no direct pharmacological or biochemical studies to examine the mechanisms by which NO or endothelium-dependent dilators alter renal vascular tone. It is not known whether the renal vasodilatory response to NO is dependent on the generation of cGMP or if a "cGMP-independent" signaling pathway also contributes to this response. In this regard, it should be noted that NO has recently been reported to inhibit NO synthase and P450 enzymes, and potentiates tubuloglomerular feedback responses. These results indicate that NO inhibits cytochrome P4504A enzymes and that inhibition of the production of 20-HETE contributes to the vasodilatory effects of NO. Therefore, the purpose of the present study was to determine whether NO inhibits enzymes of the P4504A family and whether blockade of the production of 20-HETE contributes to the effects of NO on renal vascular tone, both in vivo and in vitro.

Methods

Experiments were performed on 10- to 12-week-old male Sprague-Dawley rats purchased from Harlan Sprague Dawley Laboratories (Indianapolis, Ind). The rats were housed in an animal care facility at the Medical College of Wisconsin, which is approved by the American Association for the Accreditation of Laboratory Animal Care, and had free access to food and water.
Selected Abbreviations and Acronyms

AA = arachidonic acid  
DDMS = dibromo-dodecyl-methylsulphimide  
DHETEs = 2-hydroxy-6-oxo-6-oxodecanedioic acid  
EETs = 12-epoxyeicosatetraenoic acids  
20-HETE = 20-hydroxyeicosatetraenoic acid  
HPLC = high-performance liquid chromatography  
L-NARG = L-arginine  
MAHMA NONate = 1-hexamine, 6-(2-hydroxy-1-methyl-2-nitrosopyridinyl)-N-methyl  
MAP = mean arterial pressure  
NO = nitric oxide  
17-ODYA = 17-octadecynolic acid  
PAPA NONOate = 1-propanamine, 3-(2-hydroxy-2-nitroso-1-propylhydr zona)  
RBF = renal blood flow  
SNP = sodium nitroprusside

All protocols involving animals received approval by the Animal Care Committee of the Medical College of Wisconsin

Renal Metabolism of AA

Adult male rats were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). The kidneys were removed and placed in ice-cold 0.9% NaCl solution. The renal cortex was homogenized in a 10 mmol/L potassium phosphate buffer (pH 7.4) containing 250 mmol/L sucrose, 1 mmol/L EDTA, and 10 mmol/L magnesium chloride, and microsomes were prepared by differential centrifugation as we have previously described. A P4504A enzyme activity was assayed by incubating renal cortical microsomes (0.5 mg) for 30 minutes at 37°C with [14C]AA (0.1 μCi, 50 μmol/L, Amersham Corp) in 1 mL of a 100 mmol/L potassium phosphate buffer (pH 7.4) containing 5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L Na2HPO4, and an NADPH-regenerating system (10 mmol/L 1-succinate and 10 mmol/L NADPH, and an NADPH-reductase system) (system to control conditions and after addition of the various compounds to be tested). The reactions were terminated by acidification of pH 4 through the use of 0.1 mol/L formic acid and followed by extraction of the tissue and media with ethyl acetate. Metabolites were separated through the use of a 25 cm×2 mm ID (Supelco Inc) C18-reverse-phase HPLC column and a linear elution gradient ranging from acetone/water/acetic acid (50/50/0.1) to acetonitrile/acetic acid (100/0.1) over a 40-minute period. The radioactive products were monitored with the use of a radioactive flow detector (model 120, Radiomatic Instrument Co)

Protocol 1: Effects of NO Donors on AA Metabolism

Renal cortical microsomes from male rats were incubated with [14C]AA for 30 minutes in the presence of NADPH under control conditions and after addition of various concentrations of SNP (10−5, 10−4, and 10−3 mol/L) or PAPA NONoate (10−5, 10−4, and 10−3 mol/L) (Cayman Chemical Co), a non-cyanide-releasing NO donor, to the incubation. The reactions were terminated by acidification of pH 4 through the use of 0.1 mol/L formic acid and followed by a lipid extraction of the tissue and media. Metabolites were then separated by reverse-phase HPLC as described above.

Protocol 2: Role of 20-HETE In the Response to NO Donors in Isolated, Perfused Renal Arterioles

Experiments were performed on isolated, perfused renal arterioles (<100 μm) microdissected from the kidneys of adult male rats. Rats were anesthetized with sodium pentobarbital (50 mg/kg IP). The left kidney was removed and placed in ice-cold physiological saline solution (PSS) containing (in mmol/L) 119 NaCl, 4.7 KCl, 1.6 CaCl2, 1.2 MgSO4, 1.2 NaH2PO4, 12 NaHCO3, 10 glucose, and 0.03 EDTA, pH 7.4. The kidney was hemisedected, and small interlobular arterioles with 50- to 100-μm ID were removed by microdissection. The vessels were mounted on glass micropipettes in a water-jacketed perfusion chamber containing PSS that was equilibrated with a 95% O2−5% CO2 gas mixture and maintained at 37°C. Indomethacin (5 μmol/L, Sigma Chemical Co) and bacleralen (0.5 μmol/L, Biomol) were added to the bath to block the endogenous metabolism of AA through the cyclooxygenase and lipooxygenase pathways, as we have previously described. Vessels were secured to the pipettes with 10-0 silk suture, and side branches were tied off. The micropipette was connected to a pressurized reservoir to allow for control of intraluminal perfusion pressure, which was monitored with the use of a transducer (Cobe). After mounting, the vessels were stretched to the in vivo length with the use of an eyepiece micrometer, the outflow cannula was clamped off, and intraluminal pressure was set to 90 mm Hg during the experiment.

After a 30-minute equilibration period, the vessels were preconstricted with phenylephrine (1 μmol/L, Sigma Chemical Co), and a cumulative concentration-response curve for SNP (10−10 to 10−4 mol/L, Sigma Chemical Co) was obtained. Vascular diameters were measured 1 minute after the addition of SNP to the bath with the use of a video system composed of a stereomicroscope (Carl Zeiss, Inc.), a CCTV video camera (KP-130AU, Hitachi), a videocassette recorder (AG-7300, Panasonic), a video monitor (CVM-1271, Sony), and a video measuring system (VIA-100, Boeckeler Instrument Co). The bath solution was then exchanged for freshly prepared PSS containing 5 μmol/L indomethacin, 0.5 μmol/L bacleralen, and 25 μmol/L DDMS (n=5 rats) or vehicle (n=5 rats) 30 minutes after blockade of the production of 20-HETE by DDMS, an experimental cumulative concentration-response curve for SNP was obtained after again preconstricting the vessels with phenylephrine (1 μmol/L).

To determine whether the effects of DDMS were specific to NO donors and not caused by a generalized inhibitory action, we studied the effects of DDMS on the vasodilatory responses to the NO-independent dilators dibutyryl-cGMP and adenosine. In these studies, cumulative concentration-response curves for N2,2′-O-dibutyrylguanosine 3′,5′-cyclic monophosphate (dibutyryl-cGMP) (Sigma Chemical Co, n=3 vessels, 3 rats) and adenosine (Sigma Chemical Co; n=3 vessels, 3 rats) were obtained in vessels preconstricted with phenylephrine under control conditions and after 30 minutes of blockade of the production of 20-HETE by DDMS (25 μmol/L).

Finally, cumulative concentration-response curves for SNP also were obtained in phenylephrine-preconstricted renal microvessels under control conditions and after addition of 100 mmol/L of 20-HETE to the bath to determine whether preventing the fall in 20-HETE levels would block the vasodilatory response to SNP (n=6 vessels, 5 rats).

Protocol 3: Influence of 20-HETE on the Renal and Peripheral Vascular Responses to NO Donors

Adult male rats were anesthetized with ketamine (30 mg/kg) and thionitrousbarbital (Ineetan) (100 mg/kg) and maintained at 37°C. Cannulas were placed in a femoral artery and vein for the measurement of systemic blood pressure and intravenous infusions. The rats received an intravenous infusion of 0.9% NaCl solution containing 3% albumin at a rate of 1.2 mL/h throughout the experiment. After surgery and a 30-minute equilibration period, MAP and RBF (measured with an electromagnetic flowmeter) were recorded during a control period and in response to 2-minute intravenous infusion of the very short-acting NO donor MAHMA NONoate (Cayman Chemical Co) at doses of 1, 3, 5, and 10 nmol/min. This donor was selected because it does not accumulate in the systemic circulation and blood pressure and RBF rapidly return to control (within 15 seconds) after the infusion is stopped. Five minutes, however, was allowed between each dose to allow for full equilibration of the preparation before testing the effects of the next dose. After the control responses were recorded, a 2 mg IV bolus injection of DDMS (6 mg/kg) was given followed by a maintenance infusion at a rate of 1.2

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**Results**

**Effects of DDMS on the Renal Metabolism of AA**

The effects of various concentrations of DDMS on the metabolism of AA by rat renal cortical microsomes are presented in Fig 1. Under control conditions (Fig 1A), renal cortical microsomes produced 14-15-, 11,12-, and 8,9-DiHETEs (retention times, 7, 8, and 8.5 minutes, respectively) and 20-HETE (retention time, 10 minutes) when incubated with AA. Addition of DDMS (Fig 1B) selectively reduced the formation of 20-HETE. At concentrations of 10^{-6} and 10^{-5} mol/L, DDMS inhibited the endogenous production of 20-HETE by rat renal cortical microsomes to 81±4% and 18±9% of control values, respectively (n=3), but it had very little effect on the formation of EETs and DiHETEs (Fig 1C).

**Protocol 1: Effects of NO Donors on Renal Metabolism of AA**

The results of these experiments are summarized in Fig 2. Under control conditions, rat renal cortical microsomes produced 20-HETE, 14,15-, 11,12-, and 8,9-DiHETEs when incubated with AA (Fig 2A). Addition of 10^{-3} mol/L SNP to the incubation media completely blocked the metabolism of AA (Fig 2B). The effects of SNP on the renal metabolism of AA were concentration dependent. At concentrations of 10^{-5}, 10^{-4}, and 10^{-3} mol/L, SNP reduced the production of 20-HETE to 71±5%, 29±4%, and 42±2% of control (Fig 2C) and DiHETEs to 100±4%, 54±3%, and 10±3% of control.

**Protocol 4: Influence of 20-HETE on the Renal and Peripheral Vascular Responses to L-NArg**

Additional experiments were performed to test the effects of systemic administration of the 20-HETE inhibitor on the renal and peripheral vascular responses to MAHMA NONOate were once again assessed.

**Statistics**

Values are presented as mean±SEM. The significance of the differences in mean values within and between groups was examined with the use of ANOVA for repeated measures followed by Duncan’s multiple range test. A value of P<0.05 with a two-tailed test was considered significant.
Under control conditions, SNP (10⁻⁷ to 10⁻³ mol/L, n=6) increased the inner diameter of vascular 20-HETE levels produced by SNP. Concentration of 20-HETE was added to the bath to preclude the fall in vascular 20-HETE levels produced by SNP. Under control conditions, SNP (10⁻⁷ to 10⁻¹ mol/L, n=6) vessels, 5 rats) increased the inner diameter of these vessels by 64±4% of control. After fixing of vascular 20-HETE levels, the diameter of these vessels increased by only 24±4% of control. These results suggest that a fall in 20-HETE levels contributes significantly to the vasodilator response to NO donors.

To determine whether DDMS has any nonspecific inhibitory effects, the effects of this drug on the renal vasodilator responses to the NO-independent dilators adenosine and dbutryryl-cGMP were also examined. Under control conditions, adenosine (10⁻⁷ to 5 × 10⁻⁴ mol/L, n=3 vessels, 3 rats) increased the inner diameter of vessels preconstricted with phenylephrine by 68±10% of control. After blockade of the synthesis of 20-HETE with DDMS, adenosine increased vascular diameter by 79±9% of control. Similarly, dbutryryl-cGMP (10⁻⁷ to 10⁻⁴ mol/L, n=3 vessels, 3 rats) increased the diameter of vessels preconstricted with phenylephrine by 69±6% of control and by 61±10% of control after the synthesis of 20-HETE was blocked with DDMS.

Protocol 3: Role of 20-HETE in the Renal and Peripheral Vascular Responses to NO Donors

These experiments examined the effects of systemic administration of DDMS on the renal and peripheral vascular responses to NO donors in the rat in vivo. Control MAP averaged 115±5 mm Hg, RBF was 6.7±1 mL/min per gram of kidney weight, and RVR averaged 17±13 mL⁻¹ min⁻¹ g⁻¹ mm Hg⁻¹ (n=7 rats). Under these conditions, intravenous infusions of MAHMA NONOate at doses of 1, 3, 5, and 10 nmol/min reduced MAP to 99±3, 85±3, 75±6, and 68±6 mm Hg, respectively (Fig 4, top), and lowered RVR to 15±3%, 26±2%, 30±3%, and 34±4% of control values, respectively (Fig 4, bottom). After administration of DDMS (10 mg/kg), baseline MAP fell to 103±6 mm Hg and RVR was lowered to 15.3±1.5 mL⁻¹ min⁻¹ g⁻¹ mm Hg⁻¹ After DDMS pretreatment, the vasodilatory responses to NO donors were blocked.
Peripheral Vascular Responses to L-NArg NO synthase and P450 enzymes of the 1A and 2B19 and polarization of VSM in many vascular beds in the pres-vasodilators can produce vasodilation and/or hyper-

Discussion

A large number of studies suggest that tonic release of NO plays an important modulatory role in the regulation of both renal and peripheral vascular tone. It is generally believed that the vasodilatory effects of NO are mediated by cGMP secondary to the stimulation of guanylyl cyclase in vascular smooth muscle (VSM) cells and the production of cGMP. However, an increasing number of studies have indicated that NO donors or endothelium-dependent vasodilators can produce vasodilation and/or hyperpolarization of VSM in many vascular beds in the presence of guanylyl cyclase inhibitors. The mechanism of these cGMP-independent effects of NO remains to be determined.

In the present study, we explored the possibility that NO may inhibit the formation of 20-HETE and that this may contribute to the vasodilatory effects of NO. This hypothesis is based on the recent observations that NO inhibits NO synthase and P450 enzymes of the 1A and 2B19 and 3C10 families and from studies indicating that renal VSM cells metabolize AA through a P450-dependent pathway to 20-HETE and that this system plays a central role in the regulation of renal vascular tone. The present finding that two different NO donors (SNP and PAPA NONOate) blocked the formation of 20-HETE and P450 enzymes, with a dibromo modification on the terminal carbon designed to bind to the active site of the enzyme. In addition, the molecule has a methyl sulfimide modification on the C1 carbon to enhance its solubility in aqueous solutions and to increase the half-life by blocking oxidation of the molecule. The effects of DDMS on the metabolism of AA in the kidney were evaluated by incubating renal cortical microsomes with 14C-AA AA in the presence of NADPH. We demonstrated that DDMS at a concentration of 10 μmol/L, selectively inhibited the formation of EETs and DiHETEs at higher concentrations (100 μmol/L), however, DDMS, like 17-ODYA, did inhibit the formation of EETs and DiHETEs by renal cortical microsomes (data not shown). Thus, it appears that DDMS is a first-generation, selective inhibitor of 20-HETE production, with an =10-fold selectivity to inhibit renal ω-hydroxylation over epoxygenase activity.

In the present study, administration of DDMS at a concentration similar to that which inhibited the formation of 20-HETE by renal microsomes in vitro greatly attenuated the vasodilatory response to SNP in renal arterioles preconstricted with phenylephrine. We have also observed similar effects using 17-ODYA (data not shown). These observations support the hypothesis that inhibition of the formation of 20-HETE in renal arterioles contributes to the vasodilatory response to NO, however, the mechanism of this interaction remains to be established.

In previous studies, we have reported that 20-HETE is an endogenously formed constrictor of preglomerular renal and cerebral arteries that normally inhibits the opening of the large-conductance Ca2+-activated K+ channels in VSM. Inactivation of this channel results in membrane depolarization and vasoconstriction by increasing calcium influx through voltage-sensitive Ca2+ channels. When NO binds to P450 enzymes in VSM, it inhibits the endogenous production of 20-HETE. The subsequent fall in 20-HETE levels leads to activation of the large-conductance Ca2+-activated K+ channels and membrane hyperpolarization. Hyperpolarization of renal VSM cells would be expected to decrease calcium influx through voltage-sensitive Ca2+ channels and promote vasodilation. When the endogenous synthesis of 20-HETE is blocked with DDMS, or micellar levels of 20-HETE are fixed by exogenous addition of the compound, NO cannot lower 20-HETE levels and promote vasodilation by hyperpolarizing the membrane through activation of Ca2+-activated K+ channels. Thus, the response to NO donors is attenuated by 70%. The residual vasodilatory response to NO donors in vessels treated with DDMS or 20-HETE may represent the cGMP-dependent component of the vasodilatory response to NO. It is important to note that the vasodilatory response to NO-independent dilators adenosine and dibutyryl-cGMP in renal vessels was not significantly altered by DDMS. These results suggest that the inhibitory effects of DDMS on renal vasodilatory responses are specific to NO-dependent dilators.

Experiments were also performed to determine the possible role of 20-HETE in the renal and peripheral vasodilatory effects of NO donors and synthesize inhibitors in rats in vivo. In these experiments, the dose of DDMS was chosen on the basis of preliminary experiments indicating that infusion of DDMS (10 mg/kg) over a 1-hour period (either into the renal artery or intravenously) produced the same...
degree of renal vasodilation and blockade of the autoregulation of RBF as a dose of 17-ODYA, which reduces renal 20-HETE production by 70%. In addition, Brand-Scheber et al. have recently reported that a similar dose of DDMS effectively reduces the urinary excretion of 20-HETE, an index of renal 20-HETE production. We found that DDMS inhibited the renal vasodilatory response to graded infusions of an NO donor by ~80%. It also inhibited the rise in renal vascular resistance produced by a maximal vasoconstrictor dose of an inhibitor of NO synthesis by ~50%. These findings are consistent with the results of the in vitro studies on isolated renal arterioles and together suggest that inhibition of the production of 20-HETE may contribute to the vasodilatory effects of NO in the renal microcirculation.

It was also surprising that systemic administration of DDMS greatly attenuated the fall in blood pressure produced by intravenous infusion of MAHMA NONOate and the rise in systemic blood pressure produced by L-NArg. These observations suggest that NO may tonically inhibit the formation of 20-HETE in the rat in vivo in vascular beds other than the kidney and the brain, in which we have established a prominent role for this substance in the regulation of vascular tone. Clearly, more work is needed to map the vascular beds that produce 20-HETE and the relative contribution of 20-HETE and NO to the regulation of basal vascular tone.

In summary, the present results suggest that NO inhibits renal cytochrome P450 enzymes and that inhibition of the endogenous production of 20-HETE contributes to the effects of NO on renal and peripheral vascular tone.

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