Inhibition of Nitric Oxide Activity By Arginine Analogs in Human Renal Arteries

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**Background:** Plasma levels of endogenous guanidine compounds are increased in various pathologic conditions, including chronic renal failure. In the present study we tested the effects of some of these compounds on basal and stimulated nitric oxide activity in human renal arteries.

**Methods:** Rings from human renal arteries were obtained from 22 patients undergoing nephrectomy. The rings were suspended in organ baths for isometric recording of tension. We then studied the effects of N\textsubscript{G}-monomethyl-l-arginine (L-NMMA), N\textsubscript{G},N\textsubscript{G}-dimethyl-l-arginine (asymmetrical dimethylarginine [ADMA]), aminoguanidine (AG), and methylguanidine (MG) on artery rings under basal and stimulated conditions.

**Results:** In precontracted arteries, L-NMMA (1 μmol/L to 1 mmol/L) and ADMA (1 μmol/L to 3 mmol/L) caused concentration- and endothelium-dependent contractions (median effective concentrations [EC\textsubscript{50}] = 13.3 μmol/L and 17.5 μmol/L, respectively; E\textsubscript{max} = 15 ± 4% and 17 ± 4% of the response to 100 mmol/L KCl, respectively). Aminoguanidine (0.01 to 3 mmol/L) and MG (0.01 to 3 mmol/L) produced endothelium-independent contractions (E\textsubscript{max} = 9 ± 3% and 16 ± 2% of the response to 100 mmol/L KCl, respectively). L-arginine (1 mmol/L) but not d-arginine (1 mmol/L) prevented the contractions by L-NMMA and ADMA, but did not change contractions induced by AG and MG. In precontracted arteries, the relaxation to acetylcholine was decreased but not abolished by L-NMMA and ADMA. The remaining relaxation was reduced by charybdotoxin (0.1 μmol/L) and tetraethylammonium (1 mmol/L).

**Conclusions:** The results demonstrate that L-NMMA and ADMA reduce basal and stimulated nitric oxide activity in human renal arteries. An increase in the plasma concentrations of methylarginines associated with renal disease should be considered as a risk factor for endothelial dysfunction and abnormal vasomotor tone in human renal arteries. Am J Hypertens 2001;14:1142–1148 © 2001 American Journal of Hypertension, Ltd.

**Key Words:** Renal vascular resistance, nitric oxide, endothelium-derived hyperpolarizing factor.

Nitric oxide (NO) synthesized from l-arginine accounts for the powerful vasodilator effects of endothelium-derived relaxing factor\textsuperscript{1,2} and consequently plays a decisive role in determining vasomotor tone in several vascular beds, including the renal circulation.\textsuperscript{3–5} A number of guanidine substituted analogs of l-arginine are synthesized endogenously and can act as inhibitors of nitric oxide synthase,\textsuperscript{6,7} the enzyme responsible for the formation of NO from l-arginine. Of particular interest are N\textsubscript{G},N\textsubscript{G}-dimethyl-l-arginine (ADMA) and N\textsubscript{G}-monomethyl-l-arginine (L-NMMA). The plasma levels of both these l-arginine analogs are significantly increased in various pathologic conditions, including end-stage chronic renal failure,\textsuperscript{6,8} congestive heart failure,\textsuperscript{9} preeclampsia,\textsuperscript{10} peripheral arterial occlusive disease,\textsuperscript{11} and hypertension.\textsuperscript{12,13} Observations in the normal rat have shown that NO inhibition is associated with decreased renal plasma flow and increased renal vascular resistance, suggesting that NO exerts a tonic relaxing effect on the renal circulation.\textsuperscript{4,5,14} Recent experiments in the rat indicate that increased plasma levels of ADMA may play an important role in the appearance of hypertension in renal failure\textsuperscript{15} and in the pathogenesis of salt-sensitive hypertension.\textsuperscript{16} Furthermore, these compounds produced dose-dependent inhibition of nitrite production by macrophages (J774 cells)\textsuperscript{17} and reversed endothelium-dependent relaxation in human arterial grafts used for coronary bypass grafting.\textsuperscript{18} However, no attempt has been made to determine the direct effects of guanidine compounds on the vascular tone of human renal arteries or whether these
Methods
Sample Collection
Macroscopically normal segments of human renal arteries were obtained from 22 patients (12 men and 10 women, aged 42 to 72 years) undergoing nephrectomy for nonobstructive neoplasia. The study was approved by the ethics committee of our institution and informed consent was obtained from each patient. The arteries were immediately placed in chilled Krebs-Henseleit solution, and rings 3 mm long were cut for isometric recording of tension. The outside diameter of the rings was measured with an ocular micrometer with a Wild M8 zoom microscope (Heerbrugg, Switzerland) and ranged from 2 to 3 mm. In approximately 50% of the artery rings the endothelium was removed mechanically by inserting a roughened stainless-steel wire into the lumen and gently rolling the rings on wet filter paper.

Organ Bath Experiments
Two stainless-steel pins, 100 μm in diameter, were introduced through the arterial lumen. One pin was fixed to the organ bath wall and the other was connected to a force-displacement transducer (Grass FT03; Grass Instrument Division of Astromed, Inc., West Warwick, RI). Changes in isometric force were recorded on a Macintosh computer by use of Chart v 3.4/4 software and MacLab/8e data acquisition system (AD Instruments, Mountain View, CA). Each artery ring was set up in a 4-mL bath containing modified Krebs-Henseleit solution of the following millimole per liter composition: NaCl, 115; KCl, 4.6; MgCl₂·6H₂O, 1.2; CaCl₂, 2.5; NaHCO₃, 25; glucose 11.1, and disodium EDTA, 0.01. The solution was equilibrated with 95% O₂ and 5% CO₂ to give a pH of 7.3 to 7.4. Temperature was held at 37°C. The optimal resting tension was 3 g. The artery rings were allowed to attain a steady level of tension during a 2-h accommodation period before testing. Functional integrity of the endothelium was confirmed routinely by the presence of relaxation induced by acetylcholine (0.1 to 1 μmol/L) during the contraction obtained with norepinephrine (1 to 3 μmol/L).

All experiments were performed in the presence of 10 μmol/L indomethacin to inhibit prostanoid synthesis.

To study the contraction to guanidine compounds, concentration–response curves to L-NMMA (1 μmol/L to 3 mmol/L), ADMA (1 μmol/L to 3 mmol/L), AG (0.01 to 3 mmol/L), or MG (0.01 to 3 mmol/L) were determined in artery rings under resting tension. In another series of experiments concentration–response curves were determined in artery rings after evoking submaximal tone (approximately 0.8 g) with a threshold concentration (0.3 μmol/L) of norepinephrine. In a separate group of experiments the contractile effects of guanidine compounds were studied in arteries with norepinephrine-induced submaximal tone in the presence of L-arginine (1 μmol/L).

To study the effects of guanidine compounds on relaxation, vessels were precontracted with norepinephrine (1 μmol/L; approximately 2.5 g) and cumulative relaxation curves to either acetylcholine (0.003 to 10 μmol/L) or sodium nitroprusside (0.3 to 300 μmol/L) were constructed in the absence and in the presence of L-NMMA, ADMA, AG, or MG (all at 0.01 to 1 mmol/L). In a separate series of experiments the effects of guanidine compounds on acetylcholine-induced relaxation were studied in the presence of L-arginine (1 μmol/L).

In another series of experiments, the role of endothelium-derived hyperpolarizing factor (EDHF) in the acetylcholine-induced relaxation and the possible mediation by K⁺ channels were investigated. The relaxing effect of acetylcholine on intact vessel segments precontracted with norepinephrine and treated with L-NMMA (1 μmol/L) and indomethacin (10 μmol/L) was compared to the effect in segments that, in addition to L-NMMA and indomethacin, were also exposed to either 20 mmol/L potassium chloride (KCl), tetraethylammonium (TEA, 1 mmol/L), a nonselective inhibitor of K⁺ channels, 21 charybdoxin (0.1 μmol/L), an inhibitor of both large and intermediate conductance Ca²⁺-activated K⁺ channels, 22 iberiotoxin (0.1 μmol/L), an inhibitor of large conductance Ca²⁺-activated K⁺ channels, 23 or apamin (1 μmol/L), an inhibitor of small conductance Ca²⁺-activated K⁺ channels. 24 The inhibitors were applied to the organ bath 20 to 30 min before the concentration–response curves to acetylcholine were obtained.

Chemicals
The following drugs were used: acetylcholine chloride, norepinephrine hydrochloride, N⁶-n-monomethyl-L-arginine acetate, N⁶,N⁶-dimethylarginine hydrochloride, amino-guanidine hydrochloride, methylguanidine hydrochloride, l-arginine hydrochloride, indomethacin, sodium nitroprusside dihydrate, tetraethylammonium bromide, charybdoxin, apamin, iberiotoxin (Sigma Chemical Co., St. Louis, MO). Drugs were prepared and diluted in distilled water except for indomethacin, which was dissolved in absolute ethanol and sodium bicarbonate solution (150 mmol/L) and readjusted to pH 7.4 with hydrochloric acid before use. Stock solutions of the drugs were freshly prepared every day.
Data Analysis

All values are expressed as mean ± SEM. The contractile effects of L-NMMA, ADMA, AG, and MG were determined after evoking submaximal tone with norepinephrine (0.1 to 0.3 μmol/L). The change from the preexisting tension was expressed as a percentage of the response to KCl (100 mmol/L). Relaxation was expressed as a percentage of the norepinephrine-induced contraction.

EC₅₀ values (concentrations of agonist producing half-maximal contraction or relaxation) were determined from individual concentration–response curves by nonlinear regression analysis, and from these values the geometric means were calculated. The responses obtained in each subject were averaged to yield a single value. Therefore, all values are presented as the number of subjects. Differences between agonist- and antagonist-treated groups were assessed by two-way analysis of variance (ANOVA). Statistical significance was accepted at P < .05.

Results

Arteries under resting tension exposed to L-NMMA, ADMA, AG, and MG (1 μmol/L to 3 mmol/L) did not show significant changes in basal tension. In arteries with submaximal tone induced by a threshold concentration of norepinephrine (0.3 μmol/L; tension 0.8 g), L-NMMA (1 μmol/L to 3 mmol/L), and ADMA (1 μmol/L to 3 mmol/L) produced concentration-dependent increases in
tension in artery rings with endothelium but not in endothelium-denuded rings (Fig. 1). The EC\textsubscript{50} values for L-NMMA and ADMA were 13.3 μmol/L and 17.5 μmol/L, respectively (n = 6 for each compound). Aminoguanidine and MG augmented norepinephrine-induced tone at concentrations higher than 100 μmol/L; this response was endothelium independent. The EC\textsubscript{50} values were not determined for AG and MG as their concentration–response curves did not reach a plateau at concentrations up to 3 mmol/L (n = 6 for each compound). Previous addition of L-arginine (1 mmol/L), but not D-arginine (1 mmol/L) to arteries with norepinephrine-evoked submaximal tone, prevented the increase in tension induced by L-NMMA (n = 4) and ADMA (n = 4) but did not change contractions induced by AG (n = 4) and MG (n = 4) (Fig. 1).

Acetylcholine (0.003 to 10 μmol/L) caused endothelium-dependent relaxation (EC\textsubscript{50} = 47.1 nmol/L) in arterial rings contracted with norepinephrine (Fig. 2). The maximal relaxant response was 90.8 ± 2.9% in arteries with endothelium (n = 8) and 3.2 ± 1.1% in arteries without endothelium (n = 8). The relaxation induced by acetylcholine was inhibited in a concentration-dependent manner by L-NMMA (0.01 to 1 mmol/L) and ADMA (0.01 to 1 mmol/L) (Fig. 2). Maximal relaxations evoked by ace-

**FIG. 2.** Effects of L-NMMA, ADMA, AG, and MG on the relaxation of human renal arteries (n = 8) induced by acetylcholine. The inhibitory effects of L-NMMA and ADMA were completely prevented in the presence of L-arginine. Relaxation was tested in precontracted arteries with norepinephrine and it is expressed as a percentage of this contraction. Values are means ± SEM. Abbreviations as in Fig. 1.
tylcholine in the presence of L-NMMA (1 mmol/L, n = 5) and ADMA (1 mmol/L, n = 5) were 52.7 ± 7.7% and 46.4 ± 7.2%, respectively. The inhibitory effects of L-NMMA and ADMA on acetylcholine-induced relaxation were completely prevented in the presence of L-arginine (1 mmol/L). Neither AG (0.01 to 1 mmol/L, n = 4) nor MG (0.01 to 1 mmol/L, n = 4) had any effect on the relaxation induced by acetylcholine (Fig. 2). The remaining endothelium-dependent relaxation to acetylcholine, resistant to indomethacin and L-NMMA, was not affected by iberiotoxin (0.1 μmol/L, n = 5) or apamin (1 μmol/L, n = 5), whereas charybdotoxin (0.1 μmol/L, n = 5), TEA (1 mmol/L, n = 4), and KCl (20 mmol/L, n = 4) significantly reduced the relaxations (Fig. 3).

In endothelium-intact and in endothelium-denuded rings, sodium nitroprusside (0.3 to 300 nmol/L, n = 8) induced complete (100%) relaxation of precontracted artery rings, with an EC50 of 58.1 nmol/L. None of the guanidine compounds (100 μmol/L) modified the relaxation curves to sodium nitroprusside (n = 4 for each compound) (Fig. 4).

Discussion

The present study was designed to test the influence of various guanidine compounds on vascular tone and NO-dependent vasodilating function in human renal arteries. The basal (tone related) release of NO was determined indirectly by measuring the effects of L-NMMA and ADMA in precontracted artery rings. The NO synthesis can be inhibited by using L-NMMA and ADMA, competitive inhibitors of nitric oxide synthase.6,7 Therefore, differences in basal NO generation would be reflected in the level of contraction in response to L-NMMA or ADMA.25 The results demonstrate that L-NMMA and ADMA caused concentration-dependent contractions in arteries with norepinephrine-induced submaximal tone, but not in those under resting conditions. These contractile effects were endothelium dependent and were fully reversed by L-arginine, the substrate for the enzyme of NO synthesis. These findings indicate that L-NMMA and ADMA increase the tone of renal arteries by inhibiting the basal release of NO from the endothelium. The magnitude of the contractile effects suggests that NO production is particularly important in maintaining basal tone in the relatively large (2 to 3 mm) renal arteries used in this study.

The AG and MG produced endothelium-independent contractions and only at high concentrations. The L-arginine did not modify the contractions induced by AG and MG, thus indicating that this effect was not a consequence of inhibition of NO synthesis, but rather due to nonspecific interaction with the vascular smooth muscle. This finding
is not unexpected, as MG is structurally similar to AG, a compound reported to have a weak inhibitory effect on NO production by the vascular constitutive isoform of NO synthase. Non-specific contractions induced by high concentrations of AG and MG have previously been shown in human saphenous vein.

We also examined the effects of guanidine compounds on the relaxation induced by acetylcholine, which releases endothelium-derived relaxing factor, and by sodium nitroprusside, an exogenous NO donor. We observed that the relaxation induced by acetylcholine was significantly decreased by L-NMMA and ADMA. Because the relaxation to sodium nitroprusside was not impaired, the attenuated relaxation to acetylcholine appears to be a consequence of a decreased synthesis or release of endothelial NO. In contrast, AG and MG had no effect on the relaxation induced by acetylcholine and sodium nitroprusside, thus suggesting that these compounds do not affect the synthesis of NO.

The relaxant response to acetylcholine was substantially decreased but not abolished by L-NMMA and ADMA. This remaining dilatation may result from the action of acetylcholine on EDHF. Although the identity of this non-NO, nonprostanoid, endothelium-derived hyperpolarizing factor remains unknown, in vitro studies have shown that this factor causes hyperpolarization that has been attributed to an increase in K⁺ conductance of the smooth muscle cell membrane. Our results show that KCl, TEA, and charybdotoxin inhibited EDHF-induced relaxation, whereas iberiotoxin or apamin did not modify EDHF-induced relaxation. The results suggest that intermediate Ca²⁺-activated K⁺ channels may be involved in the acetylcholine relaxation in human renal arteries.

Our observations also indicate that exogenous L-arginine may compete with the NO synthase inhibitors to restore NO synthesis and endothelium-derived relaxation. It is well known that dietary L-arginine supplementation enhances the synthesis of endothelium-derived NO and restores endothelial vasodilator function in experimental models of kidney disease and in human renal failure.

Thus, it is possible that the beneficial effect of L-arginine could be due to reversal of the action of the competitive inhibition by ADMA and L-NMMA. However, the proof that L-arginine may overcome the decreased renal blood flow induced by high plasma levels of guanidine compounds is still lacking. The results of the present study indicate that relatively large human renal arteries are very sensitive to acute inhibition of NO synthesis. In this regard, our findings agree with the marked vasoconstrictor effects of NO synthase inhibitors on the renal microcirculation of the rat.

Nitric oxide in the renal vascular bed appears to control renal blood flow and glomerular capillary pressure. Aiello et al. found that renal synthesis of NO in rats with chronic renal failure is considerably lower than normal. Moreover, it has been shown that the net production of cyclic guanosine monophosphate in the aortic strips from rats with experimental renal failure is significantly decreased, which would reflect the reduced NO biosynthesis and would be brought about by the accumulation of ADMA in endothelial cells. Because endothelial cells are the main source of NO in all vascular beds, it could be assumed that the decreased NO synthesis by endothelial cells from the increased concentrations of ADMA and L-NMMA may contribute to increased renal vascular resistance.

In conclusion, this study demonstrates that L-NMMA and ADMA reduce basal and stimulated release of NO from the endothelium of human renal arteries. Inhibition of NO synthase by accumulation of methylarginines can lead to significant reductions of the arterial lumen or blood flow of the kidney. Impairment of NO formation in the vessel wall will predispose to vasoconstriction and favor platelet adhesion and aggregation, with the consequent release of vasoconstrictor substances that may exacerbate vasospasm. Thus, the results of the present experiments support the hypothesis that guanidine compounds should...
be considered as a risk factor for endothelial dysfunction and abnormal blood flow of human renal arteries. The study also suggests that a large part of acetylcholine relaxation is mainly dependent on a non-NO, nonprostanoid endothelium-dependent hyperpolarizing factor.

References


