Effects of Some Guanidino Compounds on Human Cerebral Arteries

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Background and Purpose—Accumulation of endogenous guanidino-substituted analogues of L-arginine in chronic renal failure might contribute to some of the vascular and neurological disorders of this pathology. We tested the hypothesis that in human cerebral arteries, some guanidino compounds may increase vascular tone, through nitric oxide (NO) synthase inhibition, and impair endothelium-dependent relaxation.

Methods—Rings of human middle cerebral artery were obtained during autopsy of 26 patients who had died 3 to 12 hours before. The rings were suspended in organ baths for isometric recording of tension. We then studied the responses to N\textsubscript{G}-monomethyl-L-arginine (L-NMMA), N\textsuperscript{G},N\textsuperscript{G}-dimethyl-L-arginine (asymmetrical dimethylarginine; ADMA), amino-guanidine (AG), and methylguanidine (MG).

Results—L-NMMA (10\textsuperscript{-6} to 3×10\textsuperscript{-4} mol/L) and ADMA (10\textsuperscript{-6} to 3×10\textsuperscript{-4} mol/L) caused concentration- and endotheli-um-dependent contractions (median effective concentrations [EC\textsubscript{50}] = 1.1×10\textsuperscript{-5} and 1.6×10\textsuperscript{-5} mol/L, respectively; E\textsubscript{max} = 35.5±7.9% and 43.9±5.9% of the response to 100 mmol/L KCl). AG (10\textsuperscript{-5} to 3×10\textsuperscript{-3} mol/L) and MG (10\textsuperscript{-5} to 3×10\textsuperscript{-3} mol/L) produced endothelium-independent contractions (E\textsubscript{max} = 44.3±8.8% and 45.7±5.8% of the response to 100 mmol/L KCl, respectively). L-Arginine (10\textsuperscript{-3} mol/L) prevented the contractions by L-NMMA and ADMA but did not change contractions induced by AG and MG. L-NMMA and ADMA inhibited endothelium-dependent relaxation induced by acetylcholine in a concentration-dependent manner; AG and MG were without effect.

Conclusions—The results suggest that the contractions induced by L-NMMA and ADMA are due to inhibition of endothelial NO synthase activity, whereas AG and MG do not affect the synthesis of NO. An increase in the plasma concentration of L-NMMA and ADMA associated with uremia is likely to represent a diminished release or effect of NO, and consequently, an increased cerebrovascular tone in uremic patients is highly conceivable. (Stroke. 1999;30:2206-2211.)

Key Words: cerebral arteries ■ endothelium ■ nitric oxide

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 cerebral circulation.\textsuperscript{3-7} The synthesis of NO can be specifically and competitively antagonized by arginine analogues such as N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA), N\textsuperscript{G},N\textsuperscript{G}-dimethyl-L-arginine (asymmetrical dimethylarginine; ADMA), amino-guanidine (AG), and methylguanidine (MG).

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Histochemical studies have demonstrated the presence of NO synthase immunoreactivity in the adventitia of rat and human cerebral arteries.\textsuperscript{9,10} Consistent with these observations, several reports have shown that NO from perivascular nerve endings mediates dilatation in the cerebral arteries through a nonadrenergic, noncholinergic mechanism,\textsuperscript{11-13} whereas NO of endothelial origin can modulate contractile responses of isolated human cerebral arteries to sympathetic stimulation.\textsuperscript{8}

Uremia is an established risk factor for cardiovascular disease and cerebrovascular accidents.\textsuperscript{14-16} Patients with end-stage chronic renal failure show an increase in plasma levels of N\textsuperscript{G}-N\textsuperscript{G}-asymmetrical dimethylarginine (ADMA), an endogenous inhibitor of NO synthesis, which might contribute to some of the vascular and neurological disorders of this pathology.\textsuperscript{17,18} Other guanidino-substituted analogues of arginine such as methylguanidine (MG) and L-NMMA also increase in renal failure and have been implicated as uremic toxins.\textsuperscript{17,19,20} Plasma levels of ADMA are also significantly elevated in a rat model of congestive heart failure,\textsuperscript{21} in patients with congestive heart failure,\textsuperscript{22} and in graded hemorrhagic shock in the pig.\textsuperscript{23} 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{24} and in the pathogenesis of salt-sensitive hypertension.\textsuperscript{25} Furthermore, these compounds produced dose-dependent inhibition of nitrite production by macrophages

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Stroke is available at http://www.strokeaha.org
(J774 cells) and reversed endothelium-dependent relaxation in human saphenous veins.\textsuperscript{26} Whether these compounds affect the responsiveness of human cerebral vessels remains to be determined. The purpose of the present study was to evaluate the potency and selectivity of ADMA, L-NMMA, MG, and aminoguanidine (AG) on endothelium-dependent and independent relaxation of human cerebral arteries. Because continuous release of NO from endothelial cells is an important determinant of the underlying smooth muscle tone in animals and humans,\textsuperscript{3,4,27–29} we also examined the ability of these compounds to inhibit basal NO release by measuring their effects on cerebrovascular tone.

Methods

Human cerebral arteries were obtained during autopsy of 26 patients (14 men and 12 women, aged 32 to 82 years) who had died 3 to 12 hours before. The cause of death varied: 12 patients had died of myocardial infarction, 10 were victims of thoracic trauma, and 4 had died of respiratory insufficiency. There was no relationship between age or cause of death and the ability of the vessels to develop tension in response to norepinephrine or KC or to relax in response to acetylcholine and sodium nitroprusside. The arteries were immediately placed in Krebs-Henseleit solution of the following composition (in mmol/L): NaCl 115, KCl 4.6, MgCl\textsubscript{2} \cdot 6H\textsubscript{2}O 1.2, CaCl\textsubscript{2} 2.5, NaHCO\textsubscript{3} 25, glucose 11.1, and disodium EDTA 0.01. Rings (4 mm long, 1 to 2 mm in outside diameter) were cut from branches of the middle cerebral artery. In ~50% of the artery rings, the endothelium was removed mechanically by inserting a roughened stainless steel wire into the lumen and gently rolling the vessel ring on wet filter paper.

Two stainless steel pins, 100 μm in diameter, were introduced through the arterial lumen. One pin was fixed to the organ bath wall while the other was connected to a force-displacement transducer (Grass FT03). Changes in isometric force were recorded on a Macintosh computer by use of CHART version 3.4/s software and a MacLab/8e data acquisition system (AD Instruments). Each artery ring was placed in a 4-mL bath filled with oxygenated (95% O\textsubscript{2}, 5% CO\textsubscript{2}) Krebs solution in the presence of indomethacin (10\textsuperscript{-7} M). To establish the resting tension for maximal force development, a series of preliminary experiments were performed on arteries of similar length and outer diameter that were exposed steadily until contractions were maximal. An applied resting tension of 2 g was found to be optimal. The vessels were allowed to attain a 2-hour accommodation period before testing. Functional integrity of the endothelium was confirmed by the absence of tension increases in artery rings with endothelium but not in rings without endothelium treated with L-arginine (10\textsuperscript{-4} mol/L), norepinephrine, and potassium chloride (100 mmol/L). Relaxation was expressed as a percentage of the change in tension from the preexisting tension as a percentage of the response to KC (100 mmol/L). Relaxation was expressed as a percentage of the norepinephrine-induced contraction.

To study contraction, concentration-response curves to L-NMMA (10\textsuperscript{-6} to 3 × 10\textsuperscript{-4} mol/L), ADMA (10\textsuperscript{-6} to 3 × 10\textsuperscript{-4} mol/L), AG (10\textsuperscript{-3} to 3 × 10\textsuperscript{-3} mol/L), or MG (10\textsuperscript{-5} to 3 × 10\textsuperscript{-5} mol/L) were determined in artery rings under resting tension. In another series of experiments, concentration-response curves were determined after evoking submaximal tone with 3 × 10\textsuperscript{-7} mol/L norepinephrine, and the change from the preexisting tone is expressed as a percentage of response to 100 mmol/L KC. Values are mean ± SEM.

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 (10\textsuperscript{-3} to 3 × 10\textsuperscript{-3} mol/L). The change from the preexisting tension was expressed as a percentage of the response to KC (100 mmol/L). Relaxation was expressed as a percentage of the norepinephrine-induced contraction.

EC\textsubscript{50} values (ie, 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. Median inhibitory concentration (IC\textsubscript{50}) values were expressed as concentrations of L-NMMA or ADMA that induced 50% inhibition of the relaxation induced by acetylcholine. The number of rings taken from each subject varied from 8 to twelve. The responses obtained in each subject were averaged to yield a single value. Therefore, all n values are presented as the number of subjects. Differences between agonist- and antagonist-treated groups were assessed by 2-way ANOVA. Statistical significance was accepted at P<0.05.

Results

There was no significant difference in the contractile response to 100 mmol/L KC between intact and denuded artery rings (2997 ± 127 versus 2728 ± 233 mg, P>0.05, n=10). Arteries exposed to L-NMMA, ADMA, AG, and MG (10\textsuperscript{-5} to 10\textsuperscript{-2} mol/L) did not show significant changes in resting tension. In the presence of a threshold concentration of norepinephrine (3 × 10\textsuperscript{-7} mol/L, tension ≈ 500 mg), L-NMMA (10\textsuperscript{-6} to 3 × 10\textsuperscript{-4} mol/L) and ADMA (10\textsuperscript{-6} to 3 × 10\textsuperscript{-4} mol/L) produced concentration-dependent increases in tension in artery rings with endothelium but not in endothelium-denuded rings (Figure 1). The EC\textsubscript{50} values for L-NMMA and ADMA were 1.1 × 10\textsuperscript{-5} and 1.6 × 10\textsuperscript{-5} mol/L, respectively (n=6 for each compound). AG and MG augmented

\begin{figure}
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\caption{Constrictions induced by N\textsuperscript{3}-monomethyl-L-arginine (L-NMMA, n=6) and N\textsuperscript{6},N\textsuperscript{6}-dimethyl-L-arginine (ADMA, n=6) on rings of human middle cerebral artery with and without endothelium and in rings with endothelium treated with L-arginine (10\textsuperscript{-5} mol/L, n=4). Constrictions were determined after evoking submaximal tone with 3 × 10\textsuperscript{-7} mol/L norepinephrine, and the change from the preexisting tone is expressed as a percentage of response to 100 mmol/L KC. Values are mean ± SEM.}
\end{figure}
norepinephrine-induced tone at concentrations >10^{-4} mol/L; this response was endothelium independent (Figure 2). The EC_{50} values were not determined for MG and AG, as their curves did not reach a plateau at concentrations up to 3×10^{-3} mol/L (n=6 for each compound). Previous addition of L-arginine (10^{-3} mol/L) prevented the increase in tension induced by L-NMMA (n=4) and ADMA (n=4) (Figure 1) but did not change contractions induced by AG (n=4) and MG (n=4) (Figure 2).

Acetylcholine (10^{-8} to 10^{-5} mol/L) caused endothelium-dependent relaxations (EC_{50}=1.0×10^{-7} mol/L) in arterial rings contracted with norepinephrine (Figure 3). The maximal relaxant response was 87.6±2.5% in arteries with endothelium (n=10) and 10.5±1.5% in arteries without endothelium (n=8). The relaxation induced by acetylcholine was inhibited in a concentration-dependent manner by L-NMMA (10^{-3} to 10^{-5} mol/L) and ADMA (10^{-5} to 10^{-3} mol/L), with IC_{50} values of 9.5×10^{-6} and 5.4×10^{-3} mol/L, respectively (Figure 3). Maximal relaxations evoked by acetylcholine in the presence of L-NMMA (10^{-3} mol/L, n=5) and ADMA (10^{-3} mol/L, n=5) were 41.1±11.1% and 43.8±8.4%, respectively. The inhibitory effects of L-NMMA and ADMA on acetylcholine-induced relaxation were completely prevented in the presence of L-arginine (10^{-3} mol/L). Neither MG (10^{-5} to 10^{-3} mol/L, n=4) nor AG (10^{-3} to 10^{-3} mol/L, n=4) had any effect on the relaxation induced by acetylcholine (Figure 4).

In endothelium-intact and endothelium-denuded rings, sodium nitroprusside (3×10^{-9} to 10^{-7} mol/L, n=8) induced complete (100%) relaxation of precontracted artery rings, with an EC_{50} of 5.1×10^{-9} mol/L. None of the guanidino compounds (10^{-4} mol/L) modified the relaxation curves to sodium nitroprusside (n=4 for each compound; results not shown).

**Discussion**

The study reported herein was designed to determine whether various guanidino compounds affect the tone of human cerebral arteries and inhibit endothelium-dependent relaxation. Our results demonstrate that L-NMMA and ADMA caused concentration-dependent contractions of cerebral arteries. The contractile effects were endothelium dependent and were reversed by L-arginine, the substrate for the enzyme for NO synthesis. These findings indicate that L-NMMA and ADMA increase the tone of cerebral 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 (1 to 2 mm) cerebral vessels used in this study. However, AG and MG produced endothelium-independent contractions and only at high concentrations. L-Arginine (10^{-3} mol/L) did not inhibit the contractions induced by AG and MG, thus indicating that the contractions were not a consequence of inhibition of NO synthesis but rather due to nonspecific interaction with the vascular smooth muscle. This finding is not unexpected, since 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.\textsuperscript{26,30,31} Nonspecific contractions induced by high concentrations of AG and MG have previously been shown in human saphenous vein.\textsuperscript{26}
We also examined the effects of guanidino compounds on the relaxation induced by acetylcholine, which releases endothelium-derived relaxing factor, and by sodium nitroprusside, which releases NO within the smooth muscle cells. We observed that the relaxation induced by acetylcholine was significantly decreased by L-NMMA and ADMA. Because the relaxation to sodium nitroprusside, an endothelium-independent vasodilator, was not impaired, the absence of 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 endothelial NO. These results are in contrast with those observed in isolated human saphenous veins in which high concentrations of MG (>10^{-3} mol/L) have been shown to reverse the endothelium-dependent relaxation induced by bradykinin or the endothelium-independent relaxation induced by sodium nitroprusside.26 Besides regional differences, the reversal of bradykinin-induced relaxation is most likely a consequence of functional antagonism resulting from the increased resting tension in precontracted rings with phenylephrine rather than inhibition of NO synthesis.26 Nevertheless, the concentrations of MG producing contractile effects in saphenous vein are much higher than those reported to occur in the plasma of patients with chronic renal failure (1 to 5×10^{-6} mol/L).19

Humans possess endogenous analogues of L-arginine, especially ADMA and L-NMMA, and the enzyme responsible for their synthesis is present in several tissues.32 However, there is controversy concerning absolute plasma values of ADMA and L-NMMA, largely due to the different analytical methods used. Plasma concentrations of ADMA in healthy volunteers are <10^{-6} mol/L17,33,34 and in uremic patients range from 1.0 to 8.7×10^{-6} mol/L.17,33 Concentrations of L-NMMA in healthy controls appear to be 10 times lower than that of ADMA,17,34 but are increased significantly (1.4×10^{-5} mol/L) in uremic patients.34 These values in uremic patients are within the range of concentrations tested in the present study. Plasma ADMA levels in normotensive and hypertensive rats (0.7×10^{-6} mol/L)25 are similar to those observed in healthy volunteers.

The vasodilator 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 endothelium-derived hyperpolarizing factor.35–37 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.38 In the rabbit middle cerebral artery, the 2 key endothelium-derived relaxing factors released by acetylcholine are NO and a prostanoid (presumably prostaglandin I₂).39,40 However, no evidence of prostanoid intervention was observed in postmortem human middle cerebral artery6 and in rat basilar artery,39 since acetylcholine-induced relaxation was unaffected by indomethacin, the inhibitor of cyclo-oxygenase.

A critical factor in the analysis of NO activity in postmortem human cerebral arteries is the possible time-dependent reduction of endothelial cell function. With regard to this, previous reports have shown that cerebral arteries obtained within 12 hours postmortem should be adequate for studies concerning smooth muscle contraction and endothelium-dependent and NO-induced relaxation.51–53

The question of whether high levels of guanidino compounds in biological fluids or brain tissue can influence the arterial lumen or blood flow of the brain under pathological conditions remains unknown. In the concentrations found in the plasma or urine of patients with chronic renal failure, it is possible that L-NMMA and ADMA would inhibit NO synthesis. On the other hand, AG and MG have no effect on the endothelium-dependent relaxation of human cerebral vessels. Inhibition of NO could play a role in the vasospasm that follows subarachnoid hemorrhage.44 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.45 Indeed, there are experimental observations showing that the levels of several guanidino compounds are markedly increased in human serum, cerebrospinal fluid, and various brain regions in uremic patients,18–46 An increase in guanidino compounds in uremia is likely to represent a diminished release or effect of NO, and consequently, a decrease of cerebral blood flow in uremic patients is highly conceivable. Indeed, a significant reduction in the middle cerebral artery and basilar blood flow velocity has been observed in uremic patients on dialysis.48,49 Thus, the results of the present experiments further support the hypothesis that guanidino compounds should be considered as possible uremic toxins that may play a primary role in the cerebrovascular and neurological disorders observed in uremia.

Acknowledgments

This work was supported by the Comisión Interministerial de Ciencia y Tecnología, Ministerio de Sanidad and Generalitat Valenciana.

References

There are a number of guanidino compounds, some of which are l-arginine analogues, that are synthesized endogenously and can act as inhibitors of nitric oxide synthase (NOS). Of particular interest are N\textsuperscript{G},N\textsuperscript{\alpha}dimethyl-l-arginine (asymmetrical dimethylarginine; ADMA) and N\textsuperscript{\alpha}monomethyl-l-arginine (L-NMMA). The circulating levels of both of these l-arginine analogues have been reported to be elevated in a variety of pathological conditions, including end-stage renal disease,

1. Preeclampsia,

2. Congestive heart failure,

3. and hypercholesterolemia. One possible manifestation of an increase...
in endogenous inhibitors of NOS is hypertension, a condition often associated with the pathologies listed above. Methyl-arginines may be produced in a variety of tissues and appear to be concentrated in the brain. The question therefore arises as to whether pathology-associated increases in circulating and cerebral levels of ADMA and L-NMMA are sufficient to impair NO-dependent cerebral vasodilating function. The only published work to date addressing this issue was performed in rats, and showed that ADMA, at concentrations that may be found in sera of uremic patients (10 μmol/L), was capable of constricting cerebral vessels in vivo and attenuating acetylcholine-induced vasodilation. The present study is the first to test the influence of endogenously generated guanidino compounds on vascular tone and NO-dependent vasodilating function in human cerebral arteries (middle cerebral artery rings obtained from cadavers). The authors found that both ADMA and L-NMMA, at 10 μmol/L and higher doses and with a similar potency, increased arterial tone and attenuated endothelium (acetylcholine)-dependent relaxation. The L-NMMA effect may not necessarily be of clinical relevance, insofar as endogenous levels of L-NMMA may only be one tenth of those measured for ADMA. Nevertheless, the ADMA findings do indicate that the levels achieved under a variety of pathological states, renal disease in particular, can have a marked influence on cerebrovascular tone. Moreover, when one combines these results with the reported reductions in circulating l-arginine levels in uremic patients, an even greater exacerbation of NO-dependent cerebral vasodilating function may result.

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*Stroke*. 1999;30:2206-2211
doi: 10.1161/01.STR.30.10.2206

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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