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Nonspecific blockade of vascular free radical signals by methylated arginine analogues

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Abstract

Methylated arginine analogues are often used as probes of the effect of nitric oxide; however, their specificity is unclear and seems to be frequently overestimated. This study analyzed the effects of N^G-methyl-L-arginine (L-NMMA) on the endothelium-dependent release of vascular superoxide radicals triggered by increased flow. Plasma ascorbyl radical signals measured by direct electron paramagnetic resonance spectroscopy in 25 rabbits increased by 3.8 ± 0.7 nmol/l *vs* baseline (28.7 ± 1.4 nmol/l, $P<0.001$) in response to papaverine-induced flow increases of 121 ± 12%. In contrast, after similar papaverine-induced flow increases simultaneously with L-NMMA infusions, ascorbyl levels were not significantly changed compared to baseline. Similar results were obtained in isolated rabbit aortas perfused *ex vivo* with the spin trap α-phenyl-N-*tert*-butylnitrone (N = 22). However, in both preparations, this complete blockade was not reversed by co-infusion of excess L-arginine and was also obtained by N-methyl-D-arginine, thus indicating that it is not related to nitric oxide synthase. L-arginine alone was ineffective, as previously demonstrated for N^G-methyl-L-arginine ester (L-NAME). *In vitro*, neither L-arginine nor its analogues scavenged superoxide radicals. This nonspecific activity of methylated arginine analogues underscores the need for careful controls in order to assess nitric oxide effects, particularly those related to interactions with active oxygen species.

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- N^G-methyl-D-arginine
- N^G-nitro-L-arginine methyl ester

Introduction

L-arginine analogues have been widely used as probes for the investigation of the effects of nitric oxide in a variety of *in vitro* and *in vivo* systems. Their established efficacy as nitric oxide synthase inhibitors has undoubtedly made a significant contribution to the understanding of nitric oxide biology (1,2) and may eventually have a clinical impact. Analogues modified at the terminal guanidine nitrogen, such as N^G-nitro-L-arginine methyl ester (L-NAME) or N^G-methyl-L-arginine (L-NMMA), were among the first nitric oxide synthase inhibitors described and are still the best known compounds of this class. However, the specificity of these substances - a basic condition for their use as pharmacological probes - has not yet been established. Although a variety of nonspe-

cific actions has been described (3-8), circumstantial evidence suggests that researchers in the area may significantly overestimate the specificity of L-arginine analogues. Recently, our laboratory reported data suggesting that increases in shear stress trigger the release of free radicals from the endothelium (9). These data were assessed by electron paramagnetic resonance (EPR) spectroscopic techniques, both *ex vivo* by measurement of radical adducts with spin traps and *in vivo* by direct measurements of plasma ascorbyl radical. In both cases, the increase in EPR signals was abolished by superoxide dismutase, suggesting that either the change in spin adduct or ascorbyl signals reflect the generation of superoxide radicals.

The purpose of the present study is to report the nonspecific blocking effect of methylated arginine analogues on flow-related free radical production observed with the use of the above EPR techniques and to call attention to other similar reported effects. These results may help clarify the interpretation of data on the putative biological roles of nitric oxide, particularly those related to its interaction with reactive oxygen species (10,11).

Material and Methods

Papaverine hydrochloride was obtained from the hospital pharmacy. Human recombinant CuZn-SOD was a kind gift of Dr. Pablo Valenzuela, Chiron Corp. (Emeryville, CA). All other compounds were from Sigma Chemical Co. (St. Louis, MO). L-NMMA and D-NMMA were used as acetate salts. L-NMMA acetate from Wellcome (the kind gift of Prof. Salvador Moncada) was also used. PBN (α-phenyl-N-*tert*-butylnitrone) was recrystallized from hexane.

In vivo ascorbyl radical measurements

The rabbit preparation used to assess plasma ascorbyl radical levels in response to iliac flow increases was described previously (9). Briefly, 25 male New Zealand white rabbits weighing 2.4 to 3.1 kg were anesthetized with sodium pentobarbital and instrumented with an electromagnetic flow probe at the origin of the right iliac artery. Rapid, transient and reproducible increases in blood flow were induced by bolus injections of papaverine (2 to 5 mg) delivered by a catheter advanced through the left femoral artery up to 0.5 to 1 cm above the iliac bifurcation. A short polyethylene catheter was advanced through a right femoral branch for blood collection at baseline, at maximal flow, and 15 min after papaverine injection. Systemic arterial pressure was continuously recorded from the left carotid artery. All rabbits were submitted to a first control papaverine bolus followed by a second one, simultaneously with a 3.8 ml/min infusion of 0.9% NaCl, L-arginine or its methylated analogues. The infusion was started 20 min after the first papaverine bolus at the initial rate of 1.0 ml/min; after 75% of the total volume was given, the second papaverine challenge was performed, and the infusion was terminated 1-2 min thereafter. Plasma ascorbyl levels were assessed by direct EPR spectroscopy as described (9).

Ex vivo aortic perfusion with PBN

The preparation of thoracic aortas from 27 other rabbits was also described previously (9). Briefly, rabbits were killed with pentobarbital and their thoracic aortas placed in a closed perfusion chamber (7.0 x 0.8 cm) connected on both sides to a closed tubing circuit with an open reservoir. Flow was provided in the physiological arterial direction by a roller pump without changing the perfusion pressure, even with increased flow. The system was primed with 6.0 ml of oxygenated Krebs-Henseleit solution at 37°C (9). The lipophilic spin-trap PBN (20 mM) was added immediately before starting arterial perfusion. In control experiments,

no L-arginine analogues were added; flow rate was started at 2 ml/min and maintained for 10 min. The first aortic fragment was then collected in liquid nitrogen. In the remainder of the vessel, the flow rate was increased to 12 ml/min for another 10 min and the fragment was then frozen. The same procedures were followed in other perfused aortas after the addition of methylated L-arginine analogues to the perfusate. Lipid extracts of arterial fragment homogenates were analyzed for PBN radical adducts, which were quantified by double integration of their EPR signals using TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) as a standard.

Superoxide radical production *in vitro* assay

The scavenger activity of arginine or its analogues was tested *in vitro* by the method of McCord and Fridovich (12) using xanthine/xanthine oxidase as the source of superoxide. Briefly, the reaction mixture contained 10 µM ferricytochrome c, 50 µM xanthine, and sufficient xanthine oxidase (about 6 nM) to produce a rate of reduction of ferricytochrome c at 550 nm of 0.030 absorbance units per min. Human recombinant SOD, with 16986 U/mg of protein, was used as standard for superoxide radical inhibition after the specified dilutions. L-NMMA, D-NMMA, L-NAME, or L-arginine was added to the system without SOD, each at 1.0 mM final concentration.

Statistical analysis

Data are reported as means ± SEM. Parameter changes *vs* baseline were analyzed by the paired *t*-test. Intergroup comparisons were tested by one-way ANOVA and the Student-Newman-Keuls multiple range test. The significance level was $P<0.05$. The Primer of Biostatistics computer program (by S.A. Glantz, McGraw Hill, 1992) was used.

Results

Effects of methylated L-arginine analogues on *in vivo* flow-dependent vascular free radical release

All pairwise control papaverine injections performed in each rabbit before the treatments with methylated L-arginine analogues were pooled to simplify data treatment (N = 25), since blood flow and ascorbyl radical changes were similar for all groups for this control condition. An intra-arterial bolus injection of papaverine (2 to 5 mg) caused an increase in right iliac flow of 121 ± 12% *vs* baseline levels of 16.0 ± 1.2 ml/min, which was accompanied by an increase in the ascorbyl radical levels of 3.8 ± 0.7 nmol/l *vs* baseline values of 28.7 ± 1.4 nmol/l for the control group. Baseline values for the other groups were as follows: L-NMMA (31.9 ± 1.7 nmol/l), D-NMMA (28.9 ± 2.2 nmol/l), L-arginine + L-NMMA (23.6 ± 1.8 nmol/l), and L-arginine (29.8 ± 2.8 nmol/l); none of these values was significantly different from the control group or each other. By 15 min after papaverine injection, ascorbyl radical levels had already returned toward baseline values, accompanying the normalization of blood flow. Figure 1 illustrates representative ascorbyl radical spectra and Figure 2 depicts the overall absolute change in plasma ascorbyl levels accompanying papaverine-induced flow changes during treatment with L-NMMA (10 mg/kg; N = 6), D-NMMA (10 mg/kg; N = 6), L-arginine + L-NMMA (100 mg/kg and 10 mg/kg, respectively; N = 5) and L-arginine (100 mg/kg; N = 3). L-NMMA, D-NMMA and L-arginine + L-NMMA did not induce significant changes in flow by themselves. L-arginine, at the dose of 100 mg/kg, significantly increased baseline flow from 21.3 ± 1.5 to 31.3 ± 2.8 ml/min ($P<0.02$). L-NMMA completely blocked flow-induced production of ascorbyl free radicals. This effect was shared with D-NMMA and was not reversed by excess

L-arginine (Figures 1 and 2).

Treatment with L-NMMA induced a sustained increase in arterial pressure from 108.8 ± 4.3 to 117.0 ± 3.1 mmHg ($P<0.05$), while D-NMMA and L-arginine did not induce significant changes. The co-infusion of L-arginine and L-NMMA completely prevented the increase in arterial pressure. The peak percent decrease in arterial pressure with papaverine after each of those treatments was similar to that observed before any treatment. None of these interventions induced significant changes in baseline ascorbyl radical levels, except for L-arginine, which caused a significant increase of 4.1 ± 0.9 nmol/l ($P<0.05$) in plasma ascorbyl levels from pretreatment values. L-lysine (100 mg/kg), a positively charged amino acid (as is L-arginine), induced no change in papaverine-induced blood flow or ascorbyl increases.

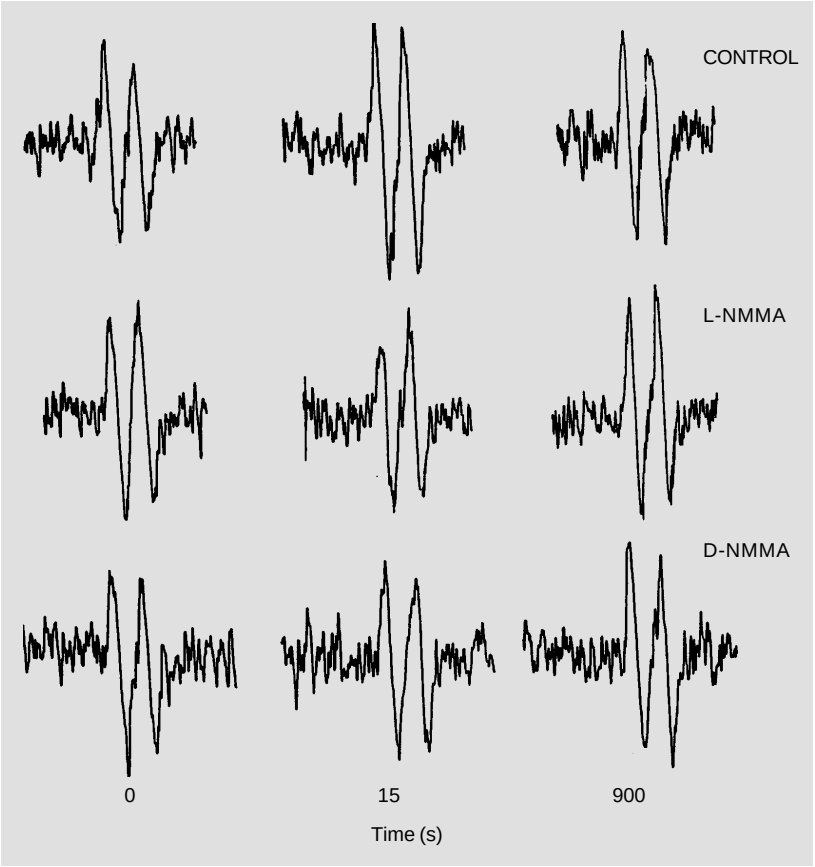

Figure 1 - Characteristic electron paramagnetic resonance spectra of arterial plasma ascorbyl radical (hyperfine splitting constant, a_H = 0.186 mT) and its change in response to a flow increase induced by intra-arterial papaverine (2 mg) in untreated control animals. The increase in ascorbyl signals was completely abolished in rabbits treated with L-NMMA and D-NMMA. Peak flow increases occurred at 15 s and returned to baseline levels by 120 s after papaverine injection. Data acquisition parameters were as follows: potency, 20 mW; frequency, 9.7 GHz; modulation amplitude, 0.1 mT; sweeping velocity 0.01 mT/s; time constant, 1000 ms.

Ex vivo aortic perfusion and detection of PBN radical adducts

The effects of each treatment with L-arginine analogues on the flow-induced change in PBN radical adduct yields are shown in Figure 3. In control experiments (N = 11) the changes in perfusion rate from 2 to 12 ml/min were accompanied by increases of PBN adduct levels from 3.2 ± 0.9 to 7.0 ± 1.5 pmol/mg ($P<0.005$). Both L-NMMA (0.1 mM; N = 4) and D-NMMA (0.1 mM; N = 4) completely inhibited free radical production, an effect not overcome by the coincubation of excess L-arginine with L-NMMA (1.0 mM and 0.1 mM, respectively; N = 3).

Effects of L-arginine and its analogues on superoxide radical production *in vitro*

The rate of ferricytochrome c reduction detected at 550 nm was 2.1 µM/min, with $\varepsilon_{550} = 15300\ M^{-1}\ cm^{-1}$. In our previous study with isolated rabbit aortas, the dose of SOD that effectively abolished the increase in PBN radical adduct signal was 100 µg/ml. This concentration completely inhibited superoxide production in the present assay. Further dilutions were then tested, so that at the final concentration of 2.5 fg/ml (thus, a dilution of $\approx 10^8$ times), SOD inhibited superoxide production by 32.8%. In contrast, L-NMMA, D-NMMA, L-NAME and L-arginine at 1.0 mM final concentration showed only negligible superoxide inhibition, with respective average values of 4.1, 7.1, 6.1 and 10.0%. Therefore, it is unlikely that methylated arginine analogues directly scavenged significant amounts of superoxide radicals in the present conditions.

Discussion

The present study reports the blocking effect of methylated arginine analogues on the release of free radicals triggered by increases in shear stress. This blocking effect was probably independent of nitric oxide synthase activity since it was not reversed by excess L-arginine and was shared by both the L- and D-isomers. Our previous work (9) showed that the flow-dependent increase in free radical signals was completely abolished by removal of the endothelium or by native superoxide dismutase and those signals were unchanged by catalase, indomethacin or L-NAME. Thus, the observed signal increases were probably due to superoxide radicals released extracellularly through an endothelium-dependent mechanism not involving cyclooxygenase or nitric oxide synthase activities. In addition, the reaction between nitric oxide and spin traps or ascorbate is quite unlikely to occur under those conditions (9,13,14), another argument against a role for nitric oxide in flow-triggered radical signals.

Several mechanisms might explain the present findings. It is unlikely that L-arginine analogues acted as nonspecific blockers of the flow transduction signaling cascade, since a number of previous studies showed that L-NMMA, even at a dose of 200 mg/kg, had no effect on flow-mediated vasodilation (15-17). It is also unlikely that those compounds directly scavenged superoxide radicals, since our experiments showed no *in vitro* scavenger activity of L-arginine or its analogues. Furthermore, our observations appear to be unrelated to the known differential effects of L-arginine analogues on superoxide generation from nitric oxide synthase. In this case, L-NAME as well as L-arginine, but not L-NMMA, completely blocked superoxide generation *in vitro* (18). In addition, in cultured endothelial cells exposed to low-density lipoproteins, such uncoupling of L-arginine uptake from nitric oxide synthesis was also prevented by L-NAME

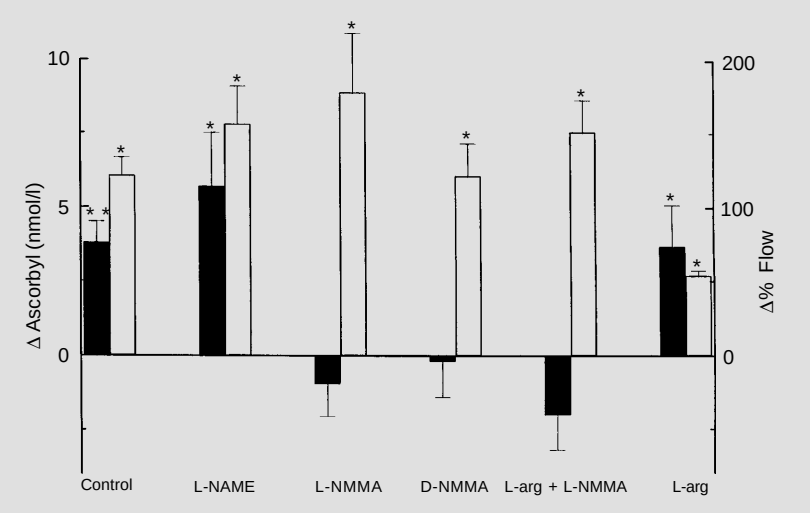

Figure 2 - The effects of several treatments on papaverine-induced increases in flow (open bars) and arterial plasma ascorbyl radical levels (closed bars). A pairwise control papaverine injection was performed in each rabbit before starting the treatments; however, since blood flow and ascorbyl radical changes were similar for all treatment groups for this control condition, the results were pooled for simplification (Control; N = 25). Neither L-NMMA (N = 6) nor D-NMMA (N = 6) altered papaverine-induced flow changes, but they both completely abolished the corresponding ascorbyl radical increase. Excess L-arginine coinfusion with L-NMMA (L-arg + L-NMMA; N = 5) did not overcome the blockade in ascorbyl increase. L-arginine (L-arg; N = 3) alone diminished papaverine-induced flow changes (P<0.05 *vs* other treatments), but did not impair ascorbyl radical increases. Data with L-NAME were previously published (9) and are shown only for comparison. **P<0.001, and *P<0.05 *vs* baseline, respectively (ANOVA and Student-Newman-Keuls multiple range test). Data are reported as means ± SEM.

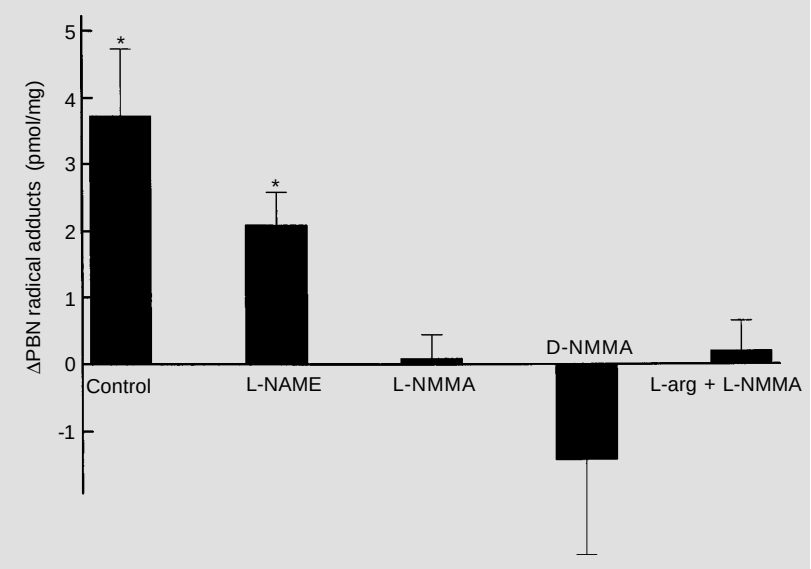

Figure 3 - Flow-related increases in α-phenyl-N-*tert*-butylnitrone (PBN) radical adduct yields in endothelialized aortas in the control (Control) condition and after treatment with L-NMMA (N = 4), D-NMMA (N = 4) and L-arginine + L-NMMA (N = 3). Data with L-NAME (N = 5) were previously published (9) and are shown only for comparison. Both L-NMMA and D-NMMA completely blocked radical generation. L-arginine did not reverse the blockade. *P<0.02 *vs* baseline. The SNK multiple range test showed that the control group was different from all groups, except for L-NAME. The D-NMMA group did not differ from the L-NMMA or L-arg + L-NMMA group. Data are reported as means ± SEM.

or L-arginine (19), in contrast to our data. The most likely mechanism for the arginine analogue effect involves their interaction with species derived from the flow-triggered radical (probably superoxide). These species are not exclusive blood or plasma constituents, since the blocking effect was observed *in vitro* as well as *in vivo*. One possibility is that catalytic metal complexes are involved in these processes. Peterson et al. (4) reported that L- and D-arginine analogues can complex with iron *in vitro* in such a way that NMMA and NAME exhibit different effects. Those nonspecific iron-chelating properties may inhibit cyclo- or lipoxygenases *in vivo* (20). In addition, L-NMMA was shown to attenuate copper-induced LDL oxidation *in vitro* (8). Interestingly, the metal chelators deferoxamine or 1,10 phenanthroline completely blocked flow-dependent free radical release *in vitro* or *in vivo* (data not shown), thus suggesting that redox active metals participate in this phenomenon, probably as secondary reactants.

The present findings add to other reports of nonspecific actions of L-arginine analogues. Some examples include the muscarinic receptor blocking effect of L-NAME (but not L-NMMA) (6), the decrease in L-arginine uptake by L-NMMA (but not L-NAME) (3) and antagonism of endothelium and nitric oxide-independent vasodilation due to amiloride or dibutyril cyclic AMP by L-NMMA (5). In pial arterioles, the vasoconstriction induced by L-NMMA or nitro-L-arginine may be due to cyclooxygenase-dependent superoxide production rather than nitric oxide synthase blockade (7). The different isoenzyme blocking profiles of methylated, as compared to nitrated, analogues are further consistent with other effects beyond nitric oxide synthase inhibition, although some peculiarities may be due to pharmacokinetic properties, e.g., the relative lipophilicity of L-NAME. In particular, L-NMMA exhibits a potent blocking effect of the macrophage isoenzyme, with characteristics of irreversibility (21), whereas nitro-L-arginine induces only a mild reversible blockade of the macrophage isoenzyme, but has a potent irreversible effect upon the brain isoenzyme (22). Finally, it should be noted that nitric oxide synthase is a mixed-function oxidase; thus, in some cases, documenting its enzymatic activity does not imply that the effect is mediated by nitric oxide (23). When nitric oxide levels were measured together with the arginine analogue effects, some significant unexpected discrepancies were reported (24).

Our data, as well as the observations discussed above, underscore the importance of adequate controls for the evaluation of the biological roles of nitric oxide. On the basis of these data, we propose that the use of L-arginine analogues as probes of nitric oxide synthase activity should be controlled by at least 3 of the 4 following procedures: 1) reversal of the observed analogue effects by excess L-arginine; 2) if L-arginine reverses the blockade, it should be proved that D-arginine does not reverse it; 3) observation of the effects of the D-analogue form; 4) observation of the effects of more than one blocking analogue. In addition, data should be preferably complemented with measurements of nitric oxide synthase activity or nitric oxide levels. In particular, our data document the role of procedures 3 and 4 (use of the D-analogues and use of more than one antagonist), which are simple, but rarely performed. These procedures should be particularly considered when evaluating the vascular interactions of nitric oxide with reactive oxygen species, or when assessing putative cytotoxic or cytostatic effects of nitric oxide.

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