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EFFECTS OF N^G-METHYL-L-ARGININE, N^G-NITRO-L-ARGININE, AND AMINOGUANIDINE ON CONSTITUTIVE AND INDUCIBLE NITRIC OXIDE SYNTHASE IN RAT AORTA

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Summary. A new selective inhibitor of the inducible nitric oxide synthase in the treatment of pathogenesis characterized by overproduction of nitric oxide may be useful. Therefore, we have examined the effects of two L-arginine analogues, N^G-methyl-L-arginine (L-NMA) and N^G-nitro-L-arginine (L-NNA), and aminoguanidine (AG) on the constitutive and inducible nitric oxide synthase in rat aorta. L-NNA induced greater contractions to phenylephrine than L-NMA whereas AG had no effect on dose-response curves to this α_1-agonist in rat aorta with endothelium. Relaxations to acetylcholine, adenosine triphosphate, and A 23187 were fully abolished by L-NNA, while L-NMA partially inhibited and AG did not affect the relaxations to these three vasodilators. L-NNA, L-NMA, and AG were equipotent in inhibiting the vascular hyporeactivity to phenylephrine induced by endotoxin in rat aortic rings with endothelium; however, the rate of onset of the maximum inhibitory effects of AG was slower than that obtained with L-NNA and L-NMA. L-arginine completely abolished the effects of AG, but only partially reversed the effects of L-NNA and L-NMA in LPS-treated rings. These results suggest that AG selectively inhibits inducible nitric oxide synthase, whereas L-NNA and L-NMA exert their effects on both the constitutive and inducible nitric oxide synthase.

Nitric oxide (NO·) is produced by the action of at least two different forms of NO· synthase (1). One type is activated by calcium/calmodulin and is constitutively present in endothelial cells and neurons (2,3). The second type is calcium/calmodulin independent and is only expressed after activation by endotoxin or cytokines. The activation of this inducible enzyme in smooth muscle cells (4-6) and in endothelial cells (7) of the vascular wall results in a delayed and prolonged release of a large amount of NO·. This overproduction of NO· leads to an inappropriate vasodilatation in endotoxin shock (8,9).

The analogues of L-arginine, N^G-nitro-L-arginine (L-NNA) or N^G-methyl-L-arginine (L-NMA) may be useful in treating patients with septic shock. However, the known inhibitors of the inducible enzyme can also exert their actions on the

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constitutive NO· synthase (NOS) present in endothelial cells, which may result in hypertensive side effects (10,11). Newer NOS inhibitors which are isoform specific may have less potential side effects.

Differences in the arginine binding sites of the constitutive and inducible forms of NOS are suggested by the potent inhibition of the constitutive enzyme by L-NNA, a weak inhibitor of inducible NOS (12). In contrast, L-NMA may have a selective ability to block basal but not agonist stimulated production of NO· in female rat aorta (13) and may be a powerful inhibitor of inducible NOS. It has been recently reported that aminoguanidine (AG) had no effect either on contractions evoked by phenylephrine or on relaxations evoked by acetylcholine in rat main pulmonary artery (14), but it reversed the vascular hyporeactivity induced by endotoxin in this vessel. Previous studies, however, had shown an increase in systemic arterial blood pressure after intravenous administration of high doses of AG as evidence of inhibition of vascular constitutive NOS in anesthetized rats (15).

In the present study we have examined the inhibitory effects of L-NMMA, L-NNA, and AG on the constitutive NOS (basal and agonist stimulated production of NO·) and on the inducible NOS (endotoxin induced production of NO·) in rat aorta with endothelium.

Materials and Methods

Drugs: Acetylcholine chloride, adenosine triphosphate, calcium ionophore (A23187), phenylephrine hydrochloride, endotoxin (lipopolysaccharides, Escherichia coli, 0111: B4), N^G-nitro-L-arginine, aminoguanidine bicarbonate and L-arginine were purchased from Sigma Chemical Co. (St. Louis, MO.); Dulbecco's modified Eagle's/Ham's F12 medium (DMEM/F12) was purchased from GIBCO BRL (Grand Island, NY). N^G-methyl-L-arginine monoacetate was synthesized by the general procedure of Corbin and Reporter (16). All drugs were prepared in distilled water.

Organ chamber studies: Aortic rings were prepared from male Wistar rats (250 g - 300 g) as described previously (17). The rings were suspended in 10 ml organ chambers under an optimal tension of 2.5 g - 3 g.The tension was recorded with an isometric force transducer connected to an input board in an IBM 386/30 mHz personal computer. Rings were maintained at 37°C in Krebs-Ringer solution containing (composition in mM) NaCl, 118.3; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; CaEDTA, 0.016; and glucose, 11.1 (control solution). The presence of endothelium was verified by addition of acetylcholine (10^{-6} M) in arteries contracted with phenylephrine (10^{-6} M). The preparations were then rinsed three times with warm control solution, rested 30 minutes and then incubated with either solvent, L-NNA,(L-NMA) or AG (10^{-4} M) (15, 30, or 60 minutes; with or without L-arginine 10^{-4} M) before a concentration-contraction curve to phenylephrine was obtained. In another set of experiments, after incubation with either solvent or inhibitors of NOS the preparations were contracted with phenylephrine (3×10^{-7} M to 1×10^{-6} M) and a cumulative dose-response curve was obtained for acetylcholine, adenosine triphosphate, or calcium ionophore (A 23187).

To induce NOS activity, aortic rings were incubated in 1 ml DMEM/F12 in the presence of 200 ng/ml of endotoxin (LPS) for 5 hours at 37°C in a cell culture incubator before suspension in the organ chambers.

Statistics: Results are expressed as means ± SEM. Statistical evaluation of the data was performed by Student's *t* test for paired or unpaired observations. P values less than 0.05 were considered significant.

Results

Basal release of NO. Phenylephrine (10^{-9} M to 10^{-5} M) evoked concentration-dependent contractions in aortic rings with endothelium. The addition of AG, L-NNA, or L-NMA (10^{-4} M) to the preparations for 1 hour had no effect on the basal tension. The contractions to phenylephrine were not affected in the presence of AG (Figure 1), but L-NMA and L-NNA shifted the dose-response curve to this α_1 agonist significantly to the left and significantly increased the maximal response from 2.06 ± 0.08 g to 2.26 ± 0.09 g and from 2.06 ± 0.08 g to 2.35 ± 0.15 g , respectively (Figure 1). Moreover, in the presence of L-NNA, contractions to phenylephrine were greater than those obtained in L-NMA-treated rings (Figure 1).

Agonist induced NO synthase activity. Following induction of a submaximal contraction with phenylephrine (3 X 10^{-7} M to 1 X 10^{-6} M), acetylcholine (ACH) (10^{-9} M to 10^{-5} M), adenosine triphosphate (ATP) (10^{-7} M to 10^{-4} M), and calcium ionophore (A 23187) (10^{-8} M to 10^{-6} M) induced concentration-dependent relaxations in aortic rings with endothelium. The maximal responses to phenylephrine were not significantly different for the three vasodilators in the presence or the absence of inhibitors of NOS (Data not shown).

Treatment of aortic rings with endothelium with L-NNA (10^{-4} M) for 1 hour resulted in a complete inhibition of relaxations to ACH (Figure 2A), A 23187

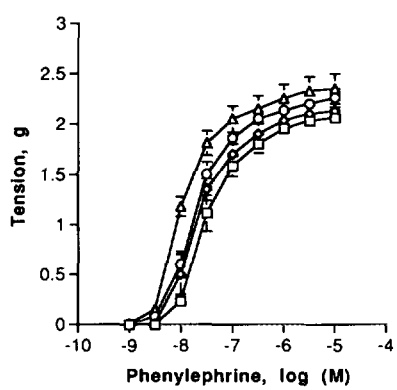

Figure 1. Effects of AG (◊), L-NNA (Δ), and L-NMA (O) (0.1 mM; 1hour) on the concentration-contraction curves evoked by phenylephrine (□) in rat aortic rings with endothelium. Results are presented as means ± SEM of four different experiments and are shown in absolute values.

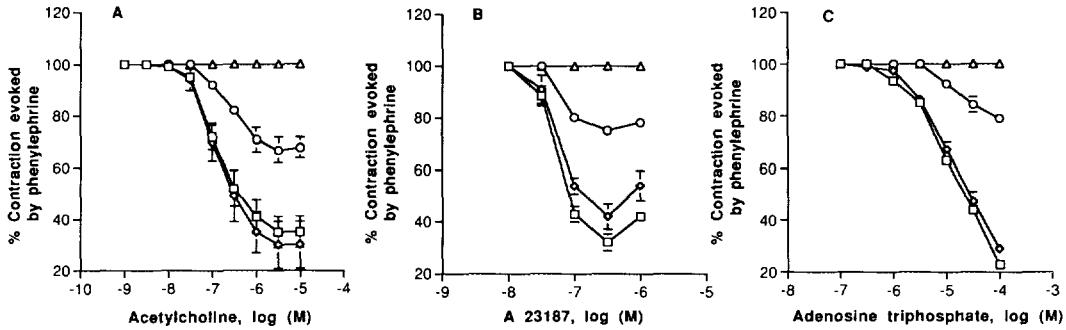

Figure 2. Effects of AG (◊), L-NNA (Δ), and L-NMA (O) (0.1 mM; 1hour) on the concentration-relaxation curves evoked by acetylcholine (□) (A); A 23187 (□) (B); and adenosine triphosphate (□) (C) in rat aortic rings with endothelium contracted with phenylephrine (3 X10^{-7} M to 1 X 10^{-6} M). Results are presented as means ± SEM of four-six different experiments and shown in absolute values.

(Figure 2B), and ATP (Figure 2C). Treatment with L-NMA (10^{-4} M) produced a significant but partial inhibition of relaxations to these three vasodilators (Figures 2A,B,C) . In contrast, AG (10^{-4} M) had no effect on relaxations induced by ACH (Figure 2A), A 23187 (Figure 2B), or ATP (Figure 2C).

LPS-induced NOS activity. The incubation of aortic rings with endothelium for 5 hours in culture medium containing 200 ng/ml LPS shifted the concentration-contraction curves to phenylephrine significantly to the right without significantly affecting the maximal response. L-NNA, L-NMA and AG did not affect the basal tension in LPS-treated rings with endothelium. Treatment of LPS-treated rings with AG for 15 minutes shifted the concentration-contraction curves to the α_1-agonist significantly to the right but had no effect on maximal contractions. Treatment for 30 minutes with this inhibitor induced a similar shift of dose-response curves to phenylephrine; however, this longer treatment significantly increased the maximal response from 1.92 ± 0.07 g to 2.25 ± 0.06 g (Figure 3A). When the treatment with AG was extended to 1 hour, the shift of the dose-response curve to phenylephrine, as well as the increase in the maximal contraction, were greater compared with the results obtained with 15, or 30 minutes of incubation (Figure 3A). Treatment for 15 minutes with L-NNA or L-NMA (10^{-4} M) significantly reversed the hyporeactivity to phenylephrine induced by LPS in rings with endothelium and significantly increased the maximal contractions from 1.92 ± 0.07 g to 2.25 ± 0.09 g with L-NMA and from 1.92 ± 0,07 g to 2.25 ± 0.06 g with L-NNA (Figures 3B and 3C, respectively). No additional inhibitory effects were obtained when the time of treatment was extended to 30 or 60 minutes (Figures 3B and 3C). After 1 hour incubation with AG, L-NMA or L-NNA contractions to phenylephrine were not significantly different in LPS-treated rings. The presence of L-arginine (10^{-4} M) shifted the concentration-contraction curves to phenylephrine significantly to the right and

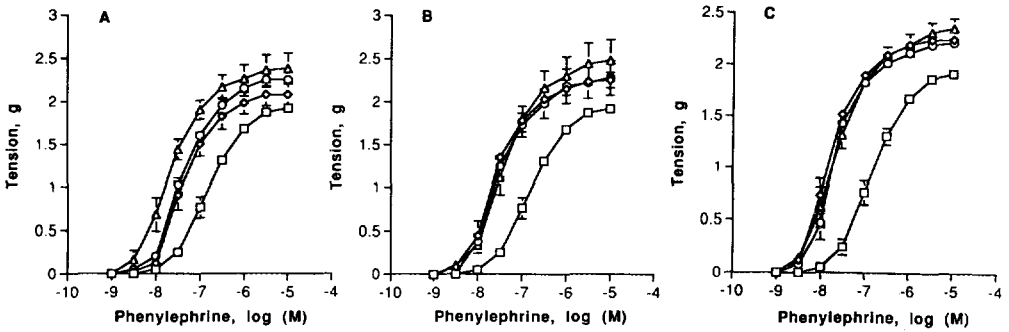

Figure 3. Effects of AG (A), L-NMA (B), and L-NNA (C) [0.1 mM; 15 min (◊); 30 min (O); 1 hour (Δ)] on the concentration-contraction curves evoked by phenylephrine (□) in rat aortic rings with endothelium which were preincubated for 5 hours in culture medium containing LPS (200 ng/ml). Results are presented as means ± SEM of four-five different experiments and are shown in absolute values.

significantly decreased the maximal response from 1.92 ± 0.07 g to 1.4 ± 0.13 g (Figure 4). The coincubation of L-arginine with AG for 1 hour abolished the inhibitory effects of AG on the vascular hyporeactivity to phenylephrine induced by LPS (Figure 4), whereas L-arginine only partially reversed the increased contractions to phenylephrine evoked by L-NNA or L-NMA in LPS-treated rings with endothelium (Figure 4).

Discussion

These results demonstrate that AG is equipotent to L-NMA and L-NNA as an inhibitor of endotoxin-induced NO· synthesis in the rat aorta. However, the lack of effect of AG on basal and agonist-stimulated NO· production suggests

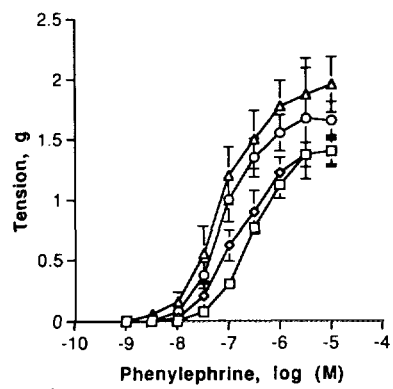

Figure 4. Effects of AG (◊), L-NMA (O), and L-NNA (Δ) (0.1 mM; 1 hour) on the concentration-contraction curves evoked by phenylephrine (□), in the presence of L-arginine (0.1 mM; 1 hour), in aortic rings with endothelium which were preincubated for 5 hours in culture medium containing LPS (200 ng/ml). Results are presented as means ± SEM of four different experiments and are shown in absolute values.

that this compound is a weak inhibitor of constitutive NOS compared with L-NMA and L-NNA. This suggests that AG is a selective inhibitor of NO· formation by the inducible isoform of NOS.

Rat aorta rings with endothelium are less sensitive to phenylephrine than rat aortic rings without endothelium. This difference is explained by the high basal production of NO· by endothelial cells. In our study, the observations that L-NMA and L-NNA increased contractions to phenylephrine and that AG did not affect contractions to this α_1-agonist suggest that L-NNA and L-NMA but not AG inhibit the basal release of NO· in rat aorta. These results are consistent with previous studies showing that AG was 15 times less potent than L-NMA on constitutive NOS in rat brain (18), was 40 times less potent in increasing mean arterial pressure in anesthetized rat (15), and had no effect on contractions to phenylephrine in rat pulmonary artery (14). The fact that L-NNA was more potent in inhibiting basal release of NO· is in agreement with previous results demonstrating that L-NNA was more potent than L-NMA in increasing contractions to phenylephrine in other vascular preparations (19). Likewise, the observations that: (1) L-NNA strongly inhibited the endothelium-dependent relaxations mediated by acetylcholine, adenosine triphosphate, and A 23187; (2) L-NMA showed a partial inhibition; and (3) AG did not prevent the relaxations to these vasodilators, further supporting the assumption that AG is a selective inhibitor of NO· production of the induced isoform of NOS. In comparison,
L-NNA AND L-NMA are more potent inhibitors of constitutive NOS. The rank order potency of these three inhibitors on basal NO· production (L-NNA > L-NMA >> AG) is similar to that on agonist stimulated NO· synthesis in the rat aorta.

The stimulation of rat aortas for several hours with endotoxin reduced their responsiveness to vasoconstrictors as previously reported (9). L-NNA and L-NMA were equipotent in reversing the vascular hyporeactivity to phenylephrine induced by endotoxin. The increase in contractions to phenylephrine with these two compounds was rapid in onset, since the maximal effect was already present after 15 minutes incubation, whereas the maximal inhibition of LPS-induced NO· synthesis by AG took longer than 30 minutes. This difference in the rate of onset of the inhibitory effects of AG is possibly due to variations in the uptake and/or mechanism(s) of action of this compound in cells of the arterial wall rather than by a difference of potency, since the three inhibitors were equipotent after 1 hour of incubation. These data are in line with previous results showing that analogues of L-arginine such as L-NMA are as potent as AG in reversing the LPS-induced vascular hyporeactivity in rat pulmonary artery (14) and in inhibiting the cytokine-induced NO· formation in a rodent insulinoma cell line (15). L-arginine potentiated the hypocontractility to

phenylephrine induced by LPS in rat aorta, indicating that its availability is rate limiting for induced NO· synthesis confirming previous reports (17). The fact that the increased contractions to phenylephrine induced by L-NMA and L-NNA were only partially reversed by L-arginine whereas those induced by AG were fully abolished by L-arginine are consistent with a mechanism of inhibition involving an arginine antagonist. A difference in affinity for the induced isoform of NOS among these three inhibitors or additional mechanisms may explain the partial reversion by L-arginine of the effects of L-NNA and L-NMA; a greater molar excess of L-arginine is required to reverse their effects fully. Taken together these results indicate that the three inhibitors exhibited a similar potency in inhibiting LPS-induced NO· production in rat aorta and that the rate of onset of the effects of AG was slower than that of L-NNA and L-NMA. Moreover, it is unlikely that a difference in the transport of AG into the cells can explain the lack of effects of this inhibitors on constitutive NOS since it effectively inhibited inducible NOS. Further experiments will be required to determine whether AG is a direct competitive inhibitor of the inducible NOS and/or it has effects on arginine transport, or utilization and consequently on the intracellular arginine concentration which limits the activity of inducible NOS. The latter hypothesis is suggested by the results showing a complete reversion of the effects of AG by L-arginine.

This study demonstrates the selectivity of AG for inhibition of NO· formation by the inducible isoform of NOS and confirms the difference in the arginine binding sites of the constitutive and inducible NOS. The selectivity of AG may decrease possible side effects that could occur as a result of inhibition of constitutive NOS formation. AG may be useful in the treatment of septic shock, however, its slow onset of action may limit its utility.

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