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Differential Effects of Nitric Oxide Synthase Inhibitors in Rats

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We investigated the action of NOS inhibitors on NOS in rats. Both of nitric oxide synthase inhibitors, N^G-monomethyl-L-arginine (L-NMMA, 3 μM) or N^G-nitro-L-arginine methylester (L-NAME, 30 μM), augmented phenylephrine (PE, 10^{-7} M)-induced contraction which was inhibited by acetylcholine (ACh) in rat thoracic aorta. This augmentation by L-NAME or L-NMMA was attenuated with the treatment of NO precursor, arginine. ACh, however, decreased the augmentation induced by L-NMMA, but not by L-NAME. Superoxide dismutase (SOD, 50 u/ml) potentiated an inhibitory effect of ACh on the PE (10^{-7} M)-induced contraction. It has been known that platelet activating factor itself induces iNOS. Platelet activating factor (PAF, 10^{-7} M) inhibited PE (10^{-7} M)-induced contraction. Pretreatment with L-NMMA (30 mM) or L-NAME (30 mM) significantly blocked the inhibitory action of PAF on PE-induced contraction. L-NMMA (100 mM) or L-NAME (100 mM) reduced nerve conduction velocity (NCV) relevant to nNOS in rat sciatic nerve. ACh attenuated the reduction of NCV by L-NMMA-, but not by L-NAME-induced reduction of NCV. These results suggest that L-NMMA and/or L-NAME have different action on three types of NOS in rats.

Key Words: Nitric oxide, Nerve conduction velocity, N^G-monomethyl-L-arginine, N^G-nitro-L-arginine methylester, Superoxide anion, Platelet activating factor

INTRODUCTION

Nitric oxide (NO) is an unique endogenous substance involved in the regulation of a variety of physiological and pathological process (Moncada et al, 1991). NO has an important role in smooth muscle contractility, platelet reactivity, neurotransmission and cytotoxic action. Thus, the modulation of NO in these processes is a therapeutic consideration in controlling the physiology and pathology.

NO is synthesized from the amino acid L-arginine by an enzyme, the NO synthase (NOS). From the many researches, it has been shown that there are three types of NOS in biological system (Lancaster, 1992). Two of them are constitutive, cytosolic and calmodulin dependent NOS, eNOS and nNOS. The NO released by these enzymes in short time acts as vasorelaxation factor and neurotransmitter, respectively. The other enzyme is induced after activation of macrophages, endothelial cells and a number of other cells by cytokine. Once expressed, the induced NOS (iNOS) produces NO for a long time.

Inappropriate release of NO has been linked to the pathogenesis of a number of disease states including septic shock, neurodegeneration, stroke, inflammation, migraine, nociception and diabetes (Hobbs et al, 1999). Therefore, the regulation of NO in the diseases with the inhibition of NOS has been potentially beneficial. However, the nonselective blockade of NOS maybe has a possibility that it will interfere with the beneficial physiology of NO. For example, NO may have several protective actions such as inhibition of platelet aggregation and smooth muscle proliferation in atherosclerosis.

L-arginine hydrochloride, N^G-nitro-L-arginine methylester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA) are analogues of a substrate of NOS, L-

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arginine. Both of them have been used as competitive and nonselective NOS inhibitors. However, recent study reported that L-NMMA is able to act as a substrate but not inhibitor since it is metabolized to form L-arginine. Thus, our study is designed to investigate the inhibitory effects of L-NMMA and L-NAME on the NOS isoforms in rats.

METHODS

Materials

Acetylcholine bromide, L-arginine hydrochloride, N^G-nitro-L-arginine methylester (L-NAME), N^G-monomethyl-L-arginine (L-NMMA), superoxide dismutase, phenylephrine hydrochloride and catalase were obtained from Sigma. All drugs were dissolved and diluted in saline (0.9%).

PAF in chloroform was stored at below −20°C until use. The chloroform was evaporated under a stream of nitrogen gas, and Krebs solution containing 0.5% bovine serum albumin was added immediately before use, then the PAF was dissolved completely by sonication. Further dilution was made with Krebs solution containing 0.1% bovine serum albumin.

Organ bath experiments

Female Sprague-Dawley rats weighing 250~300 g were used. The thoracic aortae were rapidly removed, freed of adhering fats and connective tissues and cut into ring segments of 3~5 mm length with parallel razors. Two stainless wires were inserted into the lumen of the aortic ring. One wire was connected to a transducer and the other U-shaped wire was anchored to a glass holder. The holders were placed in a 15 ml organ bath at 37°C containing oxygenated (95% O_2+ 5% CO_2) Krebs solution of the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24 and glucose 11. The organ bath and holder were siliconed to prevent adsorption of PAF to surface.

The resting tension of the preparations was maintained at 1.0 g or 2.0 g, respectively, for the PAF group or the others, and the preparations were equilibrated for 60min before the start of the experiments.

For measurement of relaxation, the arteries were precontracted with phenylephrine and L-NMMA and L-NAME were pretreated 10 min before induction of contraction with phenylephrine. Isometric tension was measured by means of a force transducer (FT03, Grass Co., Quincy, MA) and recorded on a polygraph (79E, Grass Co., Quincy, MA).

Measurement of nerve conduction velocity

Animals were anesthetized by i.p. injection of 1.5 g/kg urethane. The body temperature was maintained at 37°C with a warming pad. Sciatic NCV was determined non-invasively by stimulating proximally at the sciatic notch and distally at the ankle via bipolar electrodes with supramaximal stimulation (stimulator S48, Grass, Quincy, MA, USA). The proximal and distal latencies of the compound muscle action potentials, recorded on a oscilloscope (OS3020, Goldstar, Seoul, Korea) via bipolar electrodes from the first interosseous muscle of the hind paw, were measured from the stimulus artifact to the onset of the negative M-wave deflection, subtracted and divided into the distance between the stimulating and recording electrodes giving a value for NCV in m/s.

RESULTS

Differentially inhibitory effects of NOS inhibitors, L-NMMA and L-NAME on vasorelaxation

Phenylephrine (10^{-7} M)-induced contraction (Fig. 1)

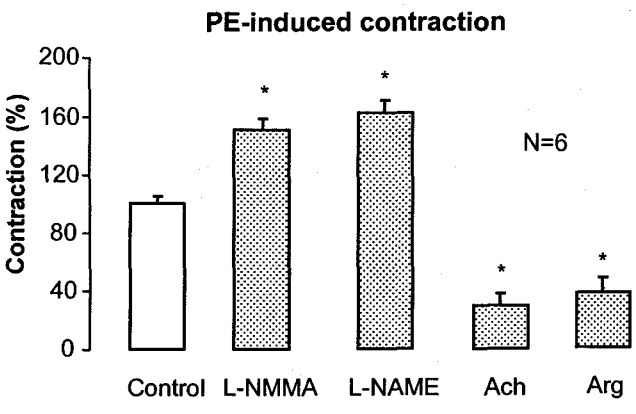

Fig. 1. The effects of agents on the PE-induced contraction in rat thoracic aorta. L-NMMA (30 μM) and L-NAME (30 μM) augmented, but acetylcholine (10^{-6} M) inhibited the PE (10^{-7} M)-induced contraction. Error bars are SEM. *means significant difference from the value obtained from control ($P<0.01$).

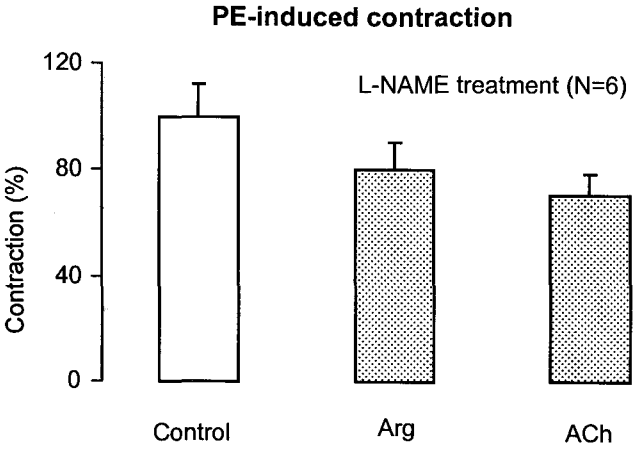

Fig. 2. The pretreatment of L-NAME (30 μM) inhibited the relaxation of Arg (10^{-2} M) and acetylcholine (10^{-6} M) on PE (10^{-7} M)-induced contraction in rat thoracic aorta. Error bars are SEM.

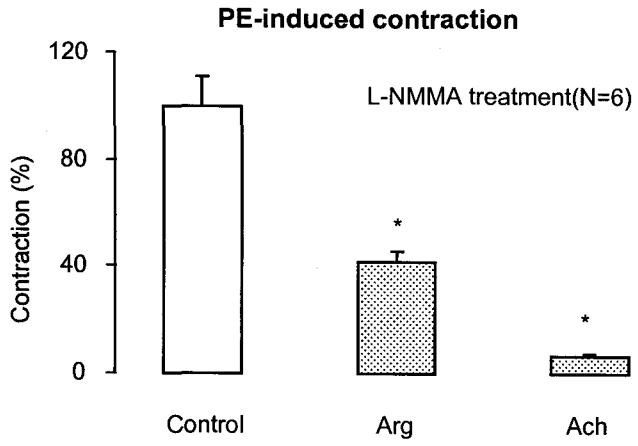

Fig. 3. The pretreatment of L-NMMA (30 μM) did not inhibit the relaxation of Arg (10^{-2} M) and acetylcholine (10^{-6} M) on PE (10^{-7} M)-induced contraction in rat thoracic aorta. Error bars are SEM. *means significant difference from the value obtained for control ($P<0.01$).

was potentiated by L-NMMA (30 mM) or L-NAME (30 mM) but attenuated by acetylcholine (10^{-6}). The pretreatment of L-NAME (30 mM) blocked the significant relaxation of rat aortic ring induced by L-arginine and acetylcholine (Fig. 2). However, with the pretreatment of L-NMMA, there was no change in L-arginine and acetylcholine induced relaxation (Fig. 3).

The exposure to superoxide dismutase (50 u/ml) following induction of phenylephrine (10^{-7} M)-induced contraction in rat thoracic aorta potentiated acetylcholine (10^{-6} M)-induced relaxation (Fig. 4).

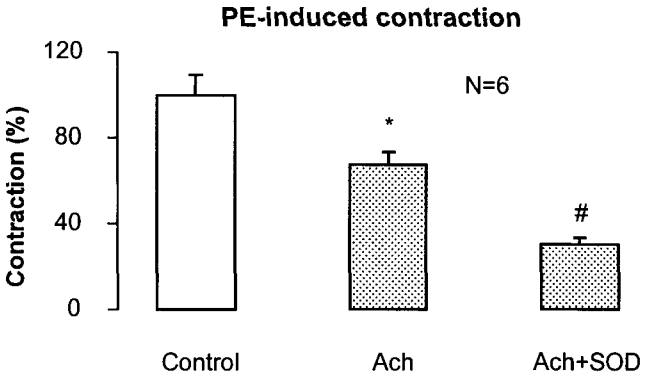

Fig. 4. Effect of superoxide dismutase on ACh-induced relaxation in rat thoracic aorta pretreated with phenylephrine. SOD enhanced the inhibition of phenylephrine-induced contraction by ACh. PE (10^{-7} M), acetylcholine (10^{-6} M) and SOD (50 u/ml) were used. *and #mean significant difference from the value obtained for control and acetylcholine, respectively ($P<0.01$).

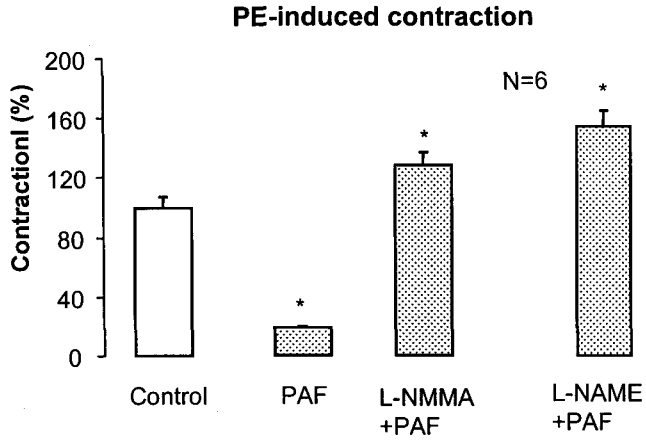

Fig. 5. Inhibition of platelet-activating factor-induced relaxation by L-NMMA or L-NAME in rat thoracic aorta. L-NMMA, L-NAME (30 μM), PE (10^{-7} M) and PAF (10^{-7} M) were used. Error bars are SEM. *means significant difference from the value obtained for control ($P<0.01$).

Effects of L-NMMA and L-NAME on the PAF-induced relaxation

PAF (10^{-7} M) caused a relaxation of PE (10^{-7} M)-induced contraction in rat thoracic aorta. Pretreatment with L-NMMA (30 μM) and L-NAME (30 μM) produced significant inhibition of the PAF-induced relaxation (Fig. 5).

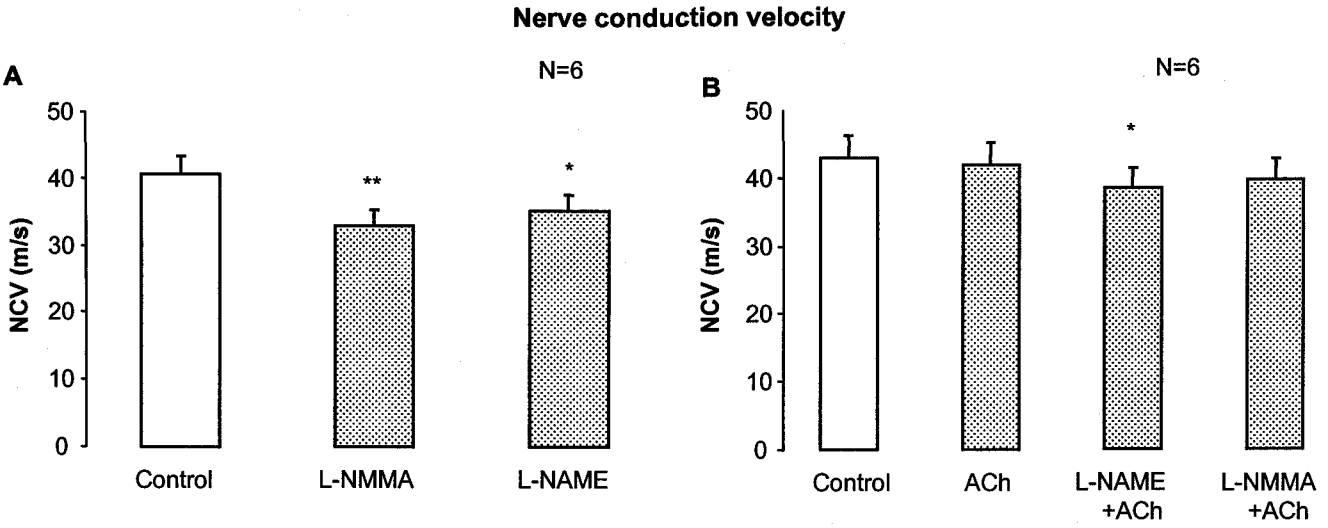

Fig. 6. Inhibitory effect of NOS inhibitors, L-NMMA and L-NAME, on nerve conduction velocity in rat sciatic nerve untreated or treated with ACh. A and B show the results in the nerve untreated or treated with acetylcholine, respectively. Control (0.9% saline), L-NMMA (100 μM), acetylcholine (100 μM) and L-NAME (100 μM) were used. Error bars are SEM. * and ** mean significant difference from the value obtained for control (*: $P<0.05$, **: $P<0.01$).

Effects of L-NMMA and L-NAME on the nerve conduction velocity

Nerve conduction velocity was measured in rat sciatic nerve using 6 ml chamber. Treatment with L-NMMA (100 μM) and L-NAME (100 μM) produced significant reduction of nerve conduction velocity (Fig. 6A). Acetylcholine (100 μM) had no significant effect by itself on the normal conductions. ACh (100 μM) restored the reduction of nerve conduction velocity induced by L-NAME but not L-NMMA (Fig. 6B).

DISCUSSION

The vascular endothelium is known to produce a powerful substance, endothelium-derived relaxing factor (EDRF), which dilates blood vessels and inhibits platelet aggregation (Furchgott et al, 1980; Azuma et al, 1986). There is increasing evidence that EDRF is nitric oxide or a closely related nitrosothiol (Palmer et al, 1988; Myers et al, 1990).

The nitric oxide synthase relevant to vasorelaxation is eNOS, constitutive cytochrome P450-type hemoprotein that catalyzes the formation of NO and citrulline from L-arginine in the presence of calmodulin and NADPH (Marletta et al, 1988). It was already known that NO released by eNOS activates guanylate cyclase, the key enzyme involved in smooth muscle relaxation (Anthony et al, 1995).

We have examined separately the effects of L-NMMA and L-NAME on basal and ACh-stimulated production of nitric oxide. L-NMMA selectively blocked basal but not ACh-stimulated production of NO. Based on these results, we also examined differential sensitivity of basal and ACh-stimulated NO activity in the superoxide anion induced destruction of NO, PAF-induced relaxation and nerve conduction velocity.

Two inhibitors of nitric oxide synthase, L-NAME and L-NMMA augmented phenylephrine-induced contraction in rat aorta in an L-arginine-reversible manner. This augmentation may occur as a consequence of inhibition of production of nitric oxide by eNOS. It is well established that exogenous superoxide dismutase potentiates activity of nitric oxide by eNOS in cascade bioassay systems (Frew et al, 1993). We found that SOD produced a potentiation of acetylcholine (10^{-6} M)-induced relaxation. Therefore, post-treatment with SOD seems to potentiate both basal and agonist-stimulated activity of nitric oxide by eNOS. Platelet activating factor (PAF) induces endothelium-dependent relaxation of arterial segments (Cervoni et al, 1983). The vasorelaxant effect of PAF is mediated by at least NO (Kamata et al, 1989). It is now known that PAF is involved in the lipopolysaccharide induc-

tion of calcium-independent NOS (iNOS), and that PAF itself induces this type of NOS (Szabo et al, 1993). Thus, there remains the possibility that PAF-induced vascular hyporeactivity is mediated by NO.

Endothelium-dependent relaxation of smooth muscle has been shown to be impaired in experimental diabetes. The mechanisms underlying this deficit are unclear but may involve depletion of NO or altered smooth muscle sensitivity to NO. Impaired local synthesis of NO may alter basal vascular contraction either by reduced activation of soluble guanylate cyclase in vascular smooth muscle or by decreased Na^+/K^+-ATPase potentially resulting in decreased endoneuronal blood flow (Stevens et al, 1995). Alternatively, although local endoneuronal NO depletion may directly contribute to decreased endoneurial blood flow, the potential of as an inhibitory neurotransmitter and its role in the maintenance of blood pressure via modulating sympathetic contraction suggest another possible mechanism. Thus in diabetes, if NO depletion at the level of the sympathetic ganglia is an early event, this may result in increased vasoconstricting sympathetic contraction to the nerve vasculature precipitating nerve ischaemia and acute nerve conduction velocity (NCV) slowing. Metabolic competition for NADPH by aldose reductase and NOS has been proposed as a potential mechanism by which these hypotheses are linked. Aldose reductase-mediated consumption of NADPH may not only directly impair the activity of NOS, but may also lead to increased levels of superoxide radicals. Support for metabolic competition between NOS and aldose reductase is provided by the fact that aldose reductase inhibitors not only restore osmolyte balance in the nerve but also improve nerve blood flow (Stevens et al, 1995; Stevens, 1995).

Furthermore, nitric oxide is involved in the neuropathy (Stevens et al, 1995). It seems that nNOS is involved in the etiology of the neuropathy. Inhibitor of nitric oxide synthase was found to slow nerve conduction velocity and reduce Na/K-ATPase activity in chronically treated rats by inhibiting the production of NO by nNOS. We measured nerve conduction velocity in normal rat using 6 ml chamber. L-NMMA and L-NAME were directly treated in exposed rat sciatic nerve. Nerve conduction velocity was reduced by treatment with L-NAME and L-NMMA in these conditions. We also found that L-NMMA-induced reduction was reversed by treatment with acetylcholine. But, treatment with acetylcholine did not significantly reverse L-NAME-induced reduction. We confirmed that basal production of nitric oxide was predominantly involved in rat sciatic nerve conduction velocity.

In conclusion, the L-NMMA and L-NAME inhibits all type of NOS isoforms. These results suggest that L-NMMA and/or L-NAME have different action on three types of NOS.

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REFERENCES

Anthony RB, Frederick WF, D. Lyn HW. NO, nitrosonium ions, nitro oxide ions, nitrosothiols and iron-nitrosyls in biology: a chemist's perspective. *TiPS* 16: 18−22, 1995

Azuma H, Ishikawa M, Sekizaki S. Endothelium-dependent inhibition of platelet aggregation. *Br J Pharmacol* 88: 411−415, 1986

Cervoni P, Herzlinger HE, Lai FM, Tanikella TK. Aortic vascular and arterial response to (±)-1-O-octadecyl-2-acetyl-glyceryl-3-phosphorylcholine. *Br J Pharmacol* 79: 97, 1983

Frew JD, Paisley K, Martin M. Selective inhibition of basal but not agonist-stimulated activity of nitric oxide in rat aorta by NG-monomethyl-L-arginine. *Br J Pharmacol* 110: 1003−1008, 1993

Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288: 373−376, 1980

Hobbs AJ, Higgs A, Moncada S. Inhibition of nitric oxide synthase as a potential therapeutic target. *Annu Rev Pharmacol Toxicol* 39: 191−220, 1999

Kamata K, Mori K, Shigenobu K, Kasuya Y. Endothelium-dependent vasodilator effects of platelet activating factor of rat resistance vessels. *Br J Pharmacol* 98: 1360, 1989

Lancaster JR Jr. Nitric oxide in cells. *Am Scientist* 80: 248−259, 1992

Marletta MA, Yoon PS, Iyengar R, Leaf CD, Wishnok JS. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry* 27: 8706−8711, 1988

Moncad S, Palmer RMJ, Higgs EA. Nitric oxide: phgysology, patholgy, and pharmacology. *Pharm Rev* 43: 109

−142, 1991

Myers PR, Minor RL Jr, Guerra R Jr, Bates JN, Harrison DG. Vasorelaxant properties of endothelium derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. *Nature* 345: 161−163, 1990

Palmer RMJ, Ashton DS, Moncada S. Vascular endothelial cells synthesis nitric oxide from L-arginine. *Nature* 333: 664−666, 1988

Stevens MJ. Nitric oxide as a potential bridge between the metabolic and vascular hypotheses of diabetic neuropathy. *Diabet Med* 12: 292−295, 1995

Stevens MJ, Feldman EL, Greene DA. The aetiology of diabetic neuropathy: the combined roles of metabolic and vascular defects. *Diabet Med* 12: 566−579, 1995

Szabo C, Wu CC, Mitchel JA, Gross SS. Thiemermann C. & Vane JR. Platelet-activating factor contributes to the induction of nitric oxide synthase by bacterial lipopolysaccharide. *Circ Res* 73: 991, 1993