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Inhibitory mechanism of N^G-nitro-L-arginine on acetylcholine-induced depressor responses in dogs

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Abstract

The significance of the blood pressure elevation caused by N^G-nitro-L-arginine (L-NNA) to inhibitory mechanism of the drug on depressor responses to acetylcholine in anesthetized dogs was investigated. L-NNA (50 mg kg^{-1}, i.v.) elevated blood pressure to a plateau of 30–50 mm Hg above baseline level and shifted the dose–response curve for acetylcholine-induced responses to the right by about 70-fold. Prevention by hydralazine (1 mg kg^{-1}, i.v.) of the blood pressure elevation over baseline level caused by L-NNA attenuated the inhibitory effect of L-NNA on the responses to acetylcholine. Intravenous neostigmine (30 μg kg^{-1} bolus followed by 15 μg kg^{-1} min^{-1}) attenuated the inhibitory effect of L-NNA. The magnitude of the rightward shift in the dose–response curve for carbachol-induced depressor responses was only 3-fold. These results suggest that the accelerated acetylcholine metabolism by blood pressure elevation contributes to a considerable degree to the inhibitory mechanism of L-NNA.

Keywords: Acetylcholine; Blood pressure; Cholinesterase; N^G-Nitro-L-arginine; (Dog)

1. Introduction

Nitric oxide (NO) is formed by the catalytic conversion of L-arginine by the enzyme NO synthase (Palmer et al., 1988; Sakuma et al., 1988). L-Arginine analogues, such as N^G-nitro-L-arginine (L-NNA) and N^G-monomethyl-L-arginine, inhibit NO production in numerous cell types (Palmer et al., 1988; Knowles et al., 1989; Fukuto et al., 1990; Ishii et al., 1990; Ishii et al., 1991). In most in vivo studies, systemic administration of these NO synthase inhibitors caused profound pressor responses (Aisaka et al., 1989a; Gardiner et al., 1990a; Wang and Pang, 1990; Vargas et al., 1991; Van Gelderen et al., 1991) and attenuated the depressor responses to endothelium-dependent vasodilators, such as acetylcholine and bradykinin (Aisaka et al., 1989b; Rees et al., 1989; Whittle et al., 1989; Gardiner et al., 1990b; Rees et al., 1990; Van Gelderen et al., 1991). On the basis of these results, many investigators consider that NO plays an important role in both the regulation of blood pressure and endothelium-dependent vasodilation.

Recently, we have demonstrated that, in anesthetized dogs, persistent elevations of blood pressure caused by infusions of various vasoconstrictors attenuated acetylcholine-induced depressor responses via acceleration of acetylcholine metabolism (Nakahara et al., 1996). Therefore, elevation of blood pressure caused by L-NNA might be of importance in inhibiting depressor responses to acetylcholine. Thus, the purpose of this study was to examine how prevention of L-NNA-induced pressor response affected the inhibitory effect of the drug on acetylcholine effect. The results showed that L-NNA attenuated acetylcholine-induced responses through enhanced metabolism of acetylcholine by cholinesterase, in addition to inhibition of endothelial NO production.

2. Materials and methods

2.1. Surgical preparations

Healthy mongrel dogs of either sex, weighing 7–15 kg, were anesthetized with an initial dose of sodium pentobarbital (30 mg kg^{-1}, i.v.). Supplemental doses of the anesthetic were applied through the right cephalic vein

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when necessary. A tracheotomy was performed to facilitate spontaneous ventilation. In order to eliminate the influence of parasympathetic nervous activity, dogs were subjected to bilateral vagotomy. Polyethylene catheters were inserted into both cephalic veins for the injection of drugs. The right femoral artery was cannulated for measurement of blood pressure, which was recorded on a polygraph system (model RM-6300, Nihon Kohden, Tokyo, Japan) via a pressure transducer (model TDN-R, Gould, Oxnard, CA, USA) and preamplifier (model AP-641G, Nihon Kohden). Heart rate was measured with a cardiotachometer (model AT-601G, Nihon Kohden) triggered by the blood pressure pulse. Throughout the experimental period, the dog's body temperature was maintained at 37 ± 0.5°C with a thermostated operation table (model SN-662, Shinano, Tokyo, Japan). At least 40 min were allowed for stabilization before beginning the experiments.

2.2. Experimental procedures

In the first series of experiments ($n = 6$), we examined the effect of L-NNA on the depressor responses to acetylcholine. We determined the dose–response relationship of acetylcholine before and 30 min after administration of L-NNA (50 mg kg^{-1}, i.v.). The dose of L-NNA was determined based on our preliminary data indicating that this dose produced the maximal blood pressure elevation (Nakahara et al., 1995).

In the second series of experiments ($n = 5$), we examined how prevention of pressor response to L-NNA affected the inhibitory effect of the drug on depressor response to acetylcholine. We used a nonspecific vasodilator, hydralazine, to prevent pressor response to L-NNA. After determining control depressor responses to acetylcholine, we injected hydralazine (1 mg kg^{-1}, i.v.). L-NNA was injected 15 min after treatment with hydralazine. We compared control responses with those obtained after L-NNA under treatment with hydralazine.

In the third series of experiments ($n = 5$), we examined whether the acceleration of acetylcholine metabolism contributes to the inhibitory effect of L-NNA on depressor responses to acetylcholine. We examined the effects of carbachol, which is highly resistant to hydrolysis by cholinesterase, and neostigmine, a cholinesterase inhibitor, on the inhibitory effect of L-NNA. In the neostigmine group, dogs were first treated with neostigmine at an initial bolus dose of 30 μg kg^{-1}, i.v., and, then, a maintenance dose of 15 μg kg^{-1} h^{-1}, i.v., was continuously injected by means of a syringe pump (model 1140-001, Harvard Apparatus, South Natick, MA, USA). This was the maximum dose, identified in preliminary experiments, beyond which hemodynamic instability ensued.

2.3. Enzyme activity in blood

The cholinesterase activity in whole blood was measured by the method of Israël and Lesbats (1985) with a slight modification. In brief, blood samples (about 10 ml each) were collected from heparinized dogs before and 30 min after the L-NNA treatment and placed in contact with acetylcholine (1 mM) for 15–90 s at 37°C. After terminating the acetylcholine hydrolysis with neostigmine (1 mM), the fluid fraction was separated from blood cells by centrifugation at $1500 \times g$ for 30 min and incubated for 10 min at 37°C with a reaction mixture of the following composition: phenol, 30 mM; 4-aminoantipyrine, 0.6 mM; choline oxidase, 2.0 U ml^{-1}; horseradish peroxidase, 50 U ml^{-1}; KH_2PO_4, 20 mM and Na_2HPO_4, 80 mM (pH 7.4). The resultant red-colored quinone derivative was quantified by measuring absorption at 505 nm with a spectrophotometer (model U-2000, Hitachi, Tokyo, Japan). The exact volume of plasma in each blood sample was determined with the addition of choline (1 mM) as an internal standard. We confirmed, in preliminary experiments, that choline was not taken up by blood cells to any appreciable extent when the concentration was 200 μM or higher.

2.4. Drugs

The drugs used were acetylcholine chloride, atropine sulfate, L-arginine hydrochloride, choline chloride, hydralazine hydrochloride, neostigmine bromide (Sigma, St. Louis, MO, USA), carbamylcholine chloride (carbachol), 4-aminoantipyrine, choline oxidase, horseradish peroxidase, phenol (Wako Pure Chemical, Osaka, Japan), and N^G-nitro-L-arginine (Aldrich Chemical, Milwaukee, WI, USA). All of these compounds were dissolved in and/or diluted with 0.9% NaCl solution.

2.5. Data analysis

Changes in depressor responses are presented as percent changes from the basal levels of blood pressure obtained just before the drug administrations. To evaluate the magnitude of shift of dose–response curve for acetylcholine-induced depressor responses, we graphically obtained ED_{30} values, which were defined as the dose required to decrease mean arterial pressure by 30% before administration of L-NNA, from dose–response curves. Similarly, we used ED_{20} values to evaluate the magnitude of shift of dose–response curve for carbachol-induced depressor responses. Statistical analyses were made by Student's paired *t*-test and the Tukey's multiple comparison test after one-way analysis of variance. Differences were considered statistically significant if the *P* value was less than 0.05. All values are presented as means ± S.E.M.

3. Results

Fig. 1 shows that L-NNA (50 mg kg^{-1}, i.v.) elevated mean arterial pressure from 126 ± 5 mm Hg to 168 ± 9 mm Hg (panel A) and attenuated depressor responses to

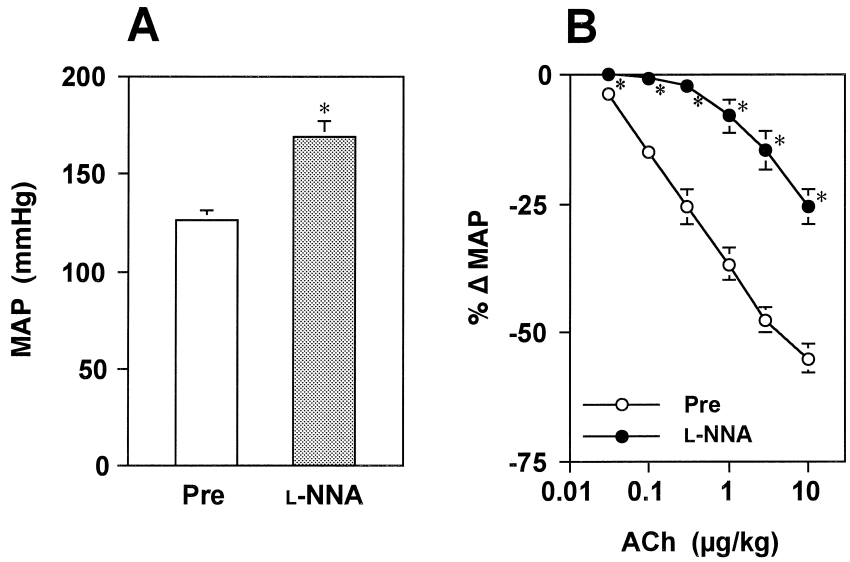

Fig. 1. Effects of N^G-nitro-L-arginine (L-NNA; 50 mg kg^{-1}, i.v.) on mean arterial pressure (MAP, A) and depressor responses to acetylcholine (ACh, B). Dose–response relationships for ACh-induced responses were obtained prior to (open circle) and 30 min after (closed circle) the administration of L-NNA. Percent changes in MAP were plotted against doses of ACh. Each column or point with a vertical bar represents mean ± S.E.M. of six experiments. *: $P < 0.05$ vs. corresponding pre-L-NNA injection (Pre) values.

acetylcholine (0.03–10 μg kg^{-1}, i.v.) (panel B). The magnitude of the rightward shift in the dose–response curve for acetylcholine-induced responses was about 70-fold. Pretreatment of dogs with L-arginine (500 mg kg^{-1}, i.v.) prevented the effects of L-NNA (data not shown).

Fig. 2 shows the effects of hydralazine (1 mg kg^{-1}, i.v.) on L-NNA-induced pressor responses and the inhibition by L-NNA of depressor responses to acetylcholine. Although hydralazine lowered mean arterial pressure 116 ± 8 mm Hg to 69 ± 6 mm Hg ($P < 0.05$), it kept mean arterial pressure after L-NNA close to the baseline (pre-hydralazine) level (panel A). After L-NNA under treatment with hydralazine, the shift in the dose–response curve for the depressor responses to acetylcholine was only 2- to 3-fold (panel B).

Fig. 3A shows the effect of L-NNA on depressor responses to carbachol (0.01–1 μg kg^{-1}, i.v.). Because depressor responses caused by the dose range of carbachol used in this study were antagonized by atropine (1 mg kg^{-1}, i.v.), it is apparent that carbachol lowers blood

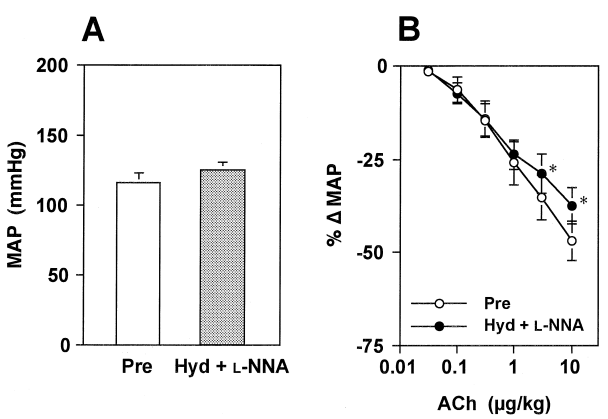

Fig. 2. Effects of N^G-nitro-L-arginine (L-NNA; 50 mg kg^{-1}, i.v.) on mean arterial pressure (MAP, A) and depressor responses to acetylcholine (ACh, B). Treatment with hydralazine (Hyd; 1 mg kg^{-1}, i.v.) was performed 15 min prior to L-NNA. Dose–response relationships for ACh-induced responses were obtained prior to (open circle) hydralazine and 30 min after (closed circle) the administration of L-NNA. Percent changes in MAP were plotted against doses of ACh. Each column or point with a vertical bar represents mean ± S.E.M. of five experiments. *: $P < 0.05$ vs. corresponding pre-Hyd injection (Pre) values.

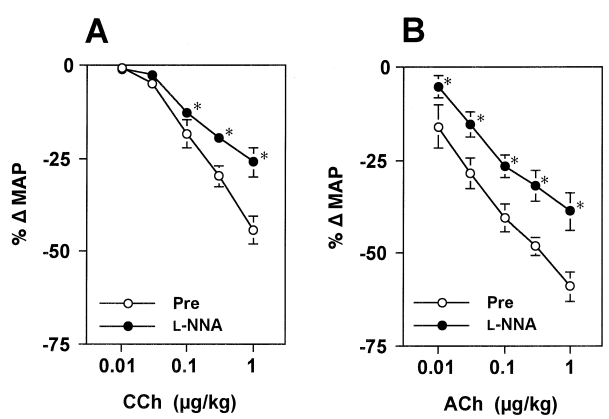

Fig. 3. Effects of N^G-nitro-L-arginine (L-NNA; 50 mg kg^{-1}, i.v.) on depressor responses to carbachol (CCh; A) and acetylcholine (ACh; B). In experiments performed to obtain data shown in panel B, dogs were treated with intravenous neostigmine (30 μg kg^{-1} bolus followed by 15 μg kg^{-1} h^{-1} infusion). Dose–response relationships for CCh- and ACh-induced responses were obtained prior to (open circle) and 30 min after (closed circle) the administration of L-NNA. Each point with a vertical bar represents mean ± S.E.M. of five experiments. *: $P < 0.05$ vs. corresponding pre-L-NNA (Pre) injection values.

pressure exclusively through stimulation of muscarinic cholinergic receptors, as in the case of acetylcholine. L-NNA shifted the dose–response curve for carbachol-induced depressor responses to the right by only 3-fold, whereas that for acetylcholine-induced responses was about 70-fold (compare Fig. 3A with Fig. 1B). Fig. 3B shows that intravenous neostigmine (30 $\mu g\ kg^{-1}$ bolus followed by 15 $\mu g\ kg^{-1}\ h^{-1}$ infusion) markedly diminished suppression by L-NNA of depressor responses to acetylcholine. Neostigmine slightly lowered mean arterial pressure from 123 ± 4 to 113 ± 4 mm Hg. However, the increases in blood pressure by L-NNA in neostigmine-treated dogs were the same as those observed in control dogs (43 ± 5 vs. 44 ± 7 mm Hg).

L-NNA (50 mg kg^{-1}, i.v.) did not change cholinesterase activity in blood (10.5 ± 0.8 vs. 10.3 ± 0.8 $\mu mol\ min^{-1}\ ml^{-1}$ blood, $n = 4$, $P > 0.05$).

4. Discussion

We demonstrated that L-NNA in dogs elevated blood pressure and markedly attenuated depressor responses to acetylcholine. The inhibitory effect of L-NNA on acetylcholine-induced responses was diminished by counteracting pressor response of L-NNA with hydralazine. These results suggest that elevation of blood pressure caused by L-NNA plays an important role in attenuating acetylcholine-induced depressor response.

In a previous study, we demonstrated a novel phenomenon that persistent elevations of blood pressure caused by infusions of various vasoconstrictors attenuated depressor responses to acetylcholine. One of the mechanisms underlying the phenomenon is acceleration of acetylcholine metabolism by elevation of blood pressure (Nakahara et al., 1996). In this study, the strength of the inhibitory effect of L-NNA on acetylcholine-induced responses was larger than that on carbachol-induced responses. Moreover, neostigmine decreased the inhibitory effect of L-NNA on acetylcholine-induced responses. Therefore, it is likely that elevation of blood pressure caused by L-NNA accelerates acetylcholine metabolism by cholinesterase, thereby attenuating depressor responses to acetylcholine.

Chowienczyk et al. (1993) reported interesting observations that N^G-monomethyl-L-arginine inhibited vasodilator responses to acetylcholine, but not to methacholine, in the human forearm vasculature. Based on these data, the authors proposed a hypothesis that methacholine acts predominantly through mechanisms other than the L-arginine-NO pathway; i.e., the effect of methacholine might be mediated by actions deep to the luminal surface of endothelium because susceptibility to cholinesterase is much lower than acetylcholine. Although they did not examine the effect of cholinesterase inhibition, our data is compatible with their results. The relative significance of metabolism of acetylcholine by cholinesterase in blood vessels in attenuating acetylcholine effect may be different between conditions under normal and NO synthase inhibition.

The magnitude of the shift by L-NNA in the dose–response curve for carbachol-induced responses was in good agreement with that for acetylcholine-induced responses under treatment with hydralazine (about 2- to 3-fold). In contrast, small (about 3-fold) difference remained even after neostigmine between the magnitudes of the L-NNA-induced rightward shifts in the dose–response curves for acetylcholine- and carbachol-induced responses. This seems to be attributable to insufficient inhibition of cholinesterase, because we could not use higher doses of the cholinesterase inhibitor to avoid the hemodynamic instability. Accordingly, to evaluate the role of NO for muscarinic receptors-mediated depressor responses in dogs, we should examine the effects of NO synthase inhibitors on carbachol- or acetylcholine-induced responses under counteraction of pressor responses to NO synthase inhibitors. The rightward shift by L-NNA of 2- to 3-fold in the dose–response curve for muscarinic receptor-mediated responses obtained in this study would reflect the extent of contribution of endothelial NO production to depressor responses induced by stimulation of muscarinic receptors.

The mechanism of accelerated acetylcholine metabolism by cholinesterase is unknown. Because intravenously administered acetylcholine is considered to be hydrolyzed by cholinesterase in the blood and the vascular wall, as a first step toward revealing the mechanism of enhanced acetylcholine metabolism, we compared activities of the enzyme in the whole blood before and after the L-NNA treatment. However, L-NNA did not increase enzyme activities. Further experiments, focused on the activities of cholinesterase located on the vascular wall in various tissues, should be conducted to elucidate the mechanism of this novel phenomenon.

In conclusion, acceleration of acetylcholine metabolism by cholinesterase observed after persistent elevation of blood pressure, rather than the inhibition of NO production in vascular endothelial cells, seems to play an important role in the inhibitory effect of L-NNA on acetylcholine-induced depressor responses in dogs.

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