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Vascular Responses after Long-Term Inhibition of Nitric Oxide Synthesis in Newborn Dogs

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Summary

The effect of long-term inhibition of nitric oxide synthase on the relaxation and contraction ability of the thoracic aorta, carotid and pulmonary arteries was studied in the early postnatal period. Starting from the fifth day after birth, puppies were administered N^G-nitro-L-arginine methyl ester (L-NAME, 50 mg/kg/day subcutaneously) for 6 weeks. After this period, mean blood pressure increased from the control value of 94±14 mm Hg to 168±5 mm Hg ($P<0.01$) and the heart/body weight ratio from 6.22±0.25 to 8.23±0.45 ($P<0.01$). In control arterial rings precontracted by phenylephrine (10^{-5} mol/l), acetylcholine caused dose-dependent relaxations; the maximal values were reached in the range of 10^{-8} to 10^{-6} mol/l. In arteries from L-NAME treated puppies, acetylcholine also induced dose-dependent relaxations, the maximum values in the thoracic aorta (81.0±2.9 %) and carotid artery (87.2±6.9 %) were significantly reduced, not, however, in the pulmonary artery (76.4±7.8 %). Dose-response curves to acetylcholine in all the examined arteries from L-NAME-treated animals were shifted to the right indicating a decrease in sensitivity to acetylcholine. Neurogenic contractions, induced by electrical stimulation of adrenergic nerves, were not significantly altered in the thoracic aorta and carotid artery. However, in the pulmonary artery the contractions were greater at high frequency of stimulation. The findings that (i) submaximal doses of L-NAME attenuate acetylcholine-induced relaxation only slightly, and (ii) that it does not appreciably influence adrenergic contractions justify the hypothesis that the endothelium of vessels in newborn dogs is very probably endowed with a high content of nitric oxide synthase.

Key words

Newborn dogs – Long-term inhibition – Nitric oxide – Endothelium-dependent relaxation – Neurogenic contraction

Introduction

Long-lasting inhibition of nitric oxide (NO) synthase with L-arginine analogues causes systemic arterial hypertension. This so-called "nitric oxide-deficient hypertension" (Dananberg *et al.* 1993) in adult animals is usually accompanied by an attenuation of endothelium-dependent relaxation induced by dilator agents, and enhancement of the responses to constrictor stimuli (Manning *et al.* 1993, Holécyová *et al.* 1996, Török *et al.* 1995).

It has been shown that endothelium-dependent relaxation is already present in the perinatal period (in foetuses and newborns) in vessels of various species, such as pigs (Zellers and Vanhoutte 1991, Liu *et al.* 1992), lambs (Gao *et al.* 1995a,b), guinea-pigs (Thompson and Weiner 1993) and dogs (Török and Gerová 1994). The data suggest that endothelium derived nitric oxide already plays an important role in regulation of vascular tone in the prenatal and early postnatal period. Since several vessels still lack sympathetic innervation in this early period (Doležel *et al.* 1990), the role of NO in the regulation of vascular tone seems to be even more important. However, no data are available concerning the effect of long-term inhibition of NO production on the cardiovascular system during the early postnatal period.

Our aim was to study the effect of long-term nitric oxide synthase inhibition by N^G-nitro-L-arginine

methyl ester (L-NAME) on the blood pressure, heart weight and reactivity of arteries of newborn dogs.

Material and Methods

Our experiments were performed on 13 animals. The project was approved by the local Institutional Animal Committee. Starting from the day 5 after birth, five puppies were treated for 6 weeks with N^G-nitro-L-arginine methyl ester (L-NAME, 50 mg/kg/day subcutaneously); 8 puppies served as controls. After this period, the animals of both groups were anaesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). The carotid artery was prepared, cannulated and connected to a Statham pressure transducer.

After the blood pressure had been measured, the animals were sacrificed by bleeding from the carotid artery. Segments of carotid and external pulmonary arteries as well as the thoracic aorta were rapidly prepared and placed in an oxygenated (95 % O_2 + 5 % CO_2) physiological salt solution (PSS). The composition of the PSS in mmol/l was: NaCl 118, KCl 5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11, $CaNa_2$.EDTA 0.03, ascorbic acid 0.55. The pH of the solution was 7.3–7.4.

The arteries were cut into approximately 3–4 mm long rings with special care taken to preserve the endothelium, and the nerve fibres in the adventitia.

The arterial rings were vertically fixed between hooks in an incubation bath (20 ml capacity). The hook anchoring the upper end of the ring was connected to the level of a force-displacement transducer Sanborn FT 10. The resting tension was adjusted to 20 mN for the thoracic aorta and to 10 mN for the carotid and pulmonary arteries.

Before starting of the experiment, all vessel rings were allowed to equilibrate in the bathing media for 60 to 90 min, during which time the solution was replaced every 15 min.

Arterial rings were precontracted submaximally with phenylephrine (10^{-5} mol/l) before being exposed to a dilator agent acetylcholine (ACh). The increase in tension with phenylephrine was considered to be 100 % and the relaxation responses were calculated as a percentage of this contraction.

Transmural electrical stimulation of adrenergic nerves was accomplished with platinum electrodes situated on either side of and parallel to the rings. To obtain neurogenic contractile responses and to avoid direct stimulation of smooth muscles, the following stimulation parameters were used: square wave pulses 0.2 ms duration, supramaximal voltage, 1–64 Hz, time of stimulation 20 s. Using these parameters, stimulation caused tetrodotoxin-sensitive contractions of arteries that are abolished by the α_1-adrenoceptor antagonist prazosin (Tesfamariam *et al.* 1987, confirmed by our preliminary results).

To prevent the effect of catecholamines released from adrenergic terminals, the contraction-response curves to potassium chloride were determined in rings that were previously incubated with phentolamine (10^{-5} mol/l). In all experiments indomethacin (10^{-5} mol/l) was used to prevent the formation of vasoactive prostaglandins.

Drugs

The following drugs were used: phenylephrine, acetylcholine, indomethacin, sodium nitroprusside, N^G-nitro-L-arginine methyl ester (all from Sigma), and phentolamine methane sulphonate (Regitine, Ciba-Geigy). All drugs were dissolved in distilled water; indomethacin was first solubilized in 0.2 mol/l Na_2CO_3 and diluted with distilled water.

Statistical analysis

The results are expressed as means ± S.E.M. For the comparison of statistical difference between the groups, one-way analysis of variance (ANOVA) was used. Values were considered significant when $P<0.05$.

Table 1
Basic parameters of control and experimental group

	Mean arterial blood pressure (mm Hg)	Heart rate (beats/min)	Heart weight/ body weight (mg/g)	n
Control	95±14	205±16	6.22±0.25	8
L-NAME 50 mg/kg/day	168±5**	194±8	8.23±0.45**	5

*Values are means ± S.E.M. ** $p<0.01$ for L-NAME vs Control*

Results

General characteristics

The mean arterial blood pressure of puppies treated by L-NAME for 6 weeks was significantly higher (168±5 mm Hg) than the blood pressure of age-matched controls (95±14 mm Hg, $P<0.01$) (Table 1). The heart rate did not significantly differ in the two groups. The heart/body weight ratio increased from 6.22±0.25 to 8.23±0.45 ($P<0.01$) indicating clear-cut cardiac hypertrophy.

Endothelium-dependent receptor-mediated relaxation

Acetylcholine produced a concentration related relaxation of phenylephrine-precontracted thoracic aortas from control puppies. At the concentration $3x10^{-7}$ mol/l, ACh caused almost complete relaxation (Fig. 1A). In aortic rings from hypertensive puppies, ACh also induced concentration-related relaxation but its maximum was attenuated and averaged 81.0±2.9 %, $p<0.01$.

The carotid artery rings from control animals yielded concentration-dependent relaxation which was already completed at 10^{-8} mol/l of ACh. The carotid artery rings from puppies treated by L-NAME relaxed to ACh also readily and the maximum was significantly lower than in control rings, averaged 87.2±6.9 %, $p<0.05$ (Fig. 1B).

Figure 1C illustrates the acetylcholine-induced relaxation of the pulmonary artery in control and experimental animals. The relaxation of the pulmonary artery from L-NAME-treated animals was significantly attenuated up to ACh concentration 10^{-7} mol/l in comparison to control pulmonary rings. Maximum relaxation (76.4±7.8 %) did not significantly differ, however, from the control animals.

Dose-response curves to acetylcholine in all the examined arteries from L-NAME-treated animals were shifted to the right indicating a slight decrease in their sensitivity to acetylcholine.

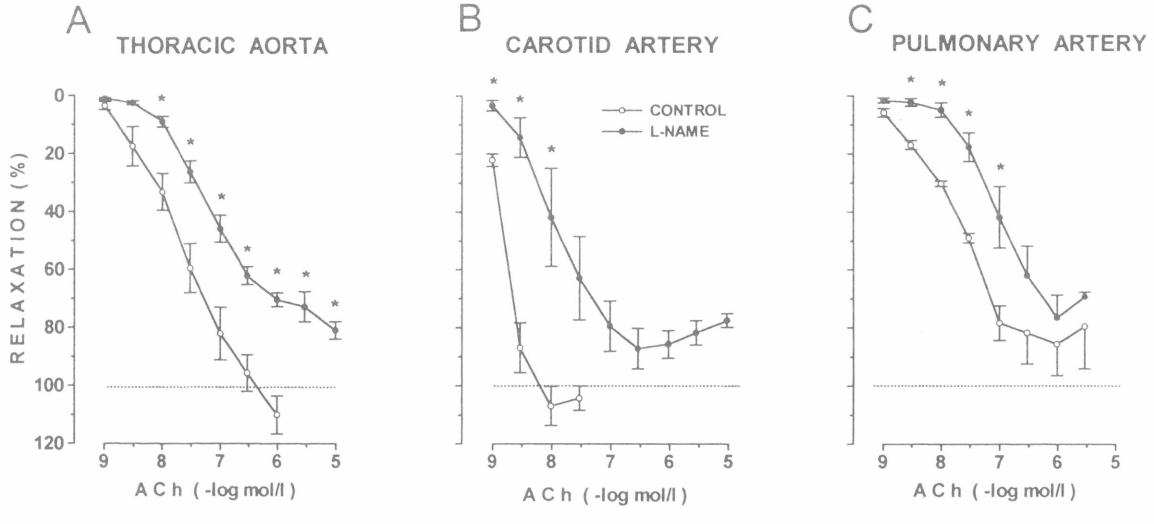

Fig. 1

*Cumulative concentration-response curves for acetylcholine in rings of thoracic aorta (A), carotid artery (B) and pulmonary artery (C) in control and L-NAME treated puppies. The acetylcholine-induced relaxations were obtained during contractions caused by phenylephrine (10^{-5} mol/l) and expressed as percentage of phenylephrine contraction (= 100 %). The symbols represent mean values ± S.E.M., n = 5–8, * $p<0.05$, L-NAME vs. control.*

Contraction induced by transmural nerve stimulation (TNS)

TNS produced a frequency-dependent increase in tension. In control aortic rings, the maximum tension was reached at 64 Hz and it represented approx. 35 % of the 100 mmol/l KCl-induced contraction. In hypertensive puppies, the frequency-response curve of aortic rings did not differ significantly from the controls (Fig. 2A).

The same pattern of neurogenic responses was also observed in carotid arteries (Fig. 2B).

It was necessary to use a higher frequency of stimulation in pulmonary artery of both control and hypertensive animals to obtain marked contractions. The TNS-induced contractions were greater in hypertensive animals at a higher frequency of stimulation (Fig. 2C).

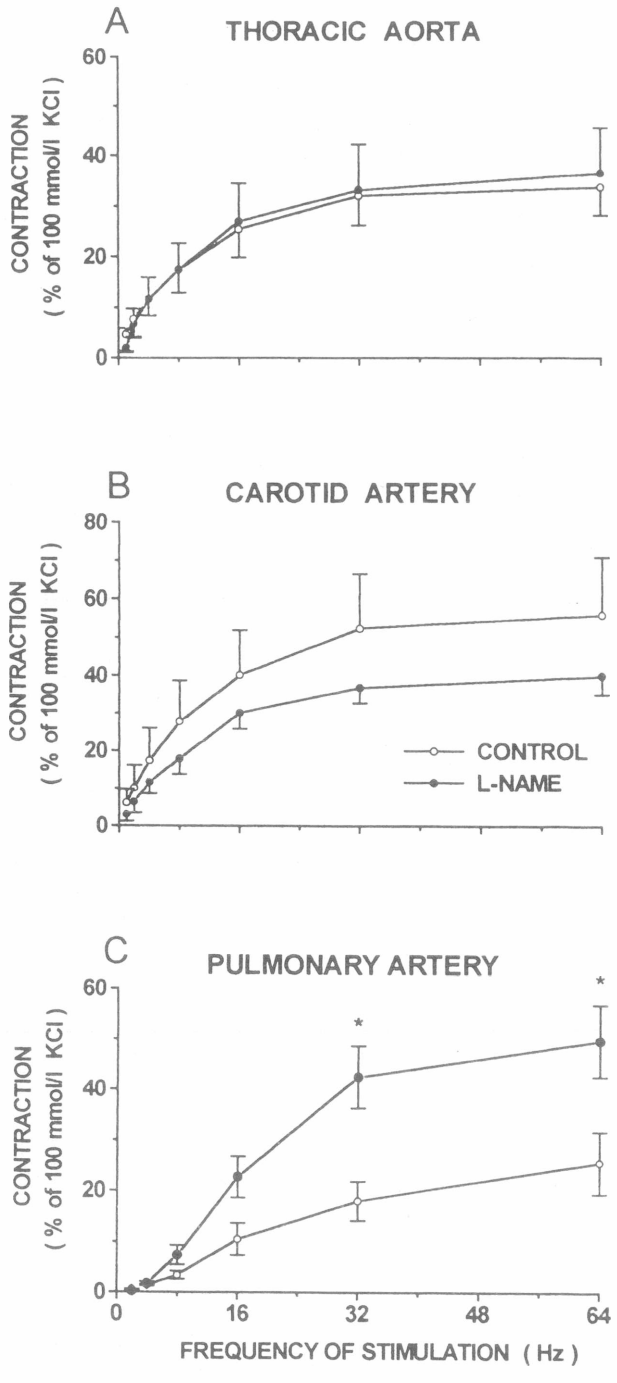

Fig. 2
*Contractile responses induced by electrical field stimulation of adrenergic nerves in rings of thoracic aorta (A), carotid artery (B) and pulmonary artery (C) in control and L-NAME treated puppies. Contractions are expressed as a percentage of 100 mmol/l KCl-induced contractions. The symbols represent mean values ± S.E.M., n = 5–8, * $p<0.05$, L-NAME vs. control.*

Receptor-independent contractions

Cumulative doses of KCl (10–100 mmol/l) induced contractions of the pulmonary artery. When normalized to maximal contraction (Fig. 3A) or expressed in absolute terms, as mN/mm^2 of tension developed (Fig. 3B), the concentration-response curves to KCl in control and L-NAME-treated puppies did not differ significantly.

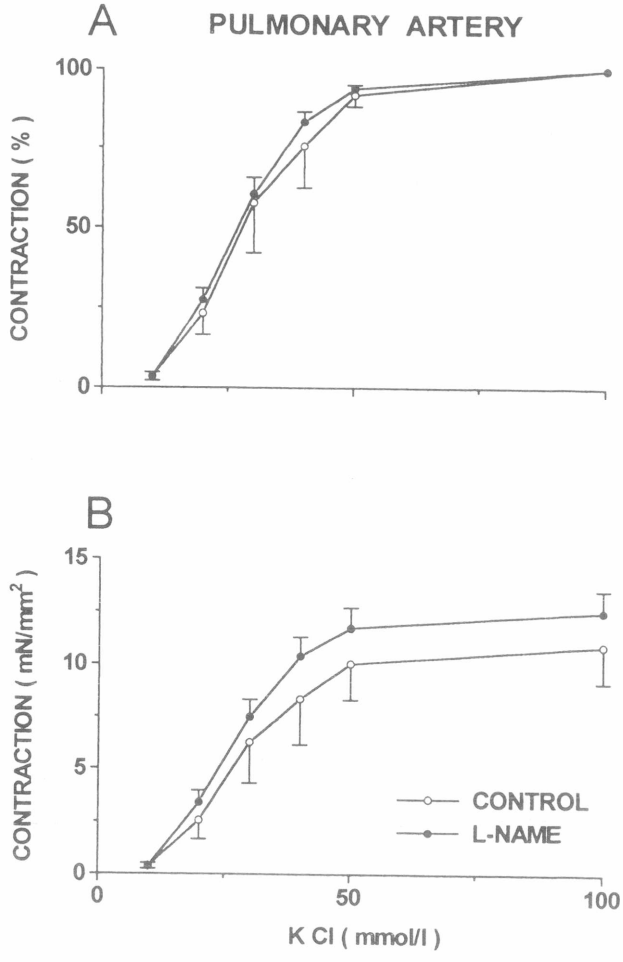

Fig. 3
Cumulative concentration-response curves to potassium chloride in pulmonary artery rings from control and L-NAME treated puppies. On the top (A), data are normalized to the maximal tension developed by 100 mmol/l KCl and expressed as a percentage of this maximal tension. At the bottom (B), contractions are expressed in N/mm^2 of tension developed to the agonist. The symbols represent mean values ± S.E.M., n = 5.

Discussion

The inhibition of nitric oxide synthesis in newborn dogs lasting 6 weeks increased the blood pressure and the heart/body weight ratio, thus indicating the presence of cardiac hypertrophy. These findings are in agreement with the data obtained in adult animals of different species with chronic inhibition of NO synthesis (Morton *et al.* 1993, Delacretaz *et al.* 1994, Török *et al.* 1995).

However, the main result of our study was finding that although the newborns received L-NAME for 6 weeks, the thoracic aorta, the carotid and pulmonary arteries still profoundly relaxed to acetylcholine and the resultant relaxation represented a 76–87 % of the tension induced by phenylephrine (Fig. 1).

Acetylcholine-induced relaxation resistant to long-term inhibition of NO synthase is not probably due to the release of vasodilator prostaglandins, since all experiments were performed in the presence of indomethacin, an inhibitor of cyclooxygenase. There are other possibilities to explain the marked residual acetylcholine-induced relaxation. The level of NO synthase could be so high in the endothelium of newborns that a submaximal dose of L-NAME still maintained a high level of NO production. Further support for the idea of a high content of NO synthase in newborns is provided by the fact that in control arteries of puppies 6 weeks of age complete relaxation pf arteries (thoracic aorta, carotid artery) was observed at lower concentrations of ACh than in the same vessels of adult animals of different species (Furchgott and Zawadzki, 1980, Török *et al.* 1993, 1995, Holécyová *et al.* 1996).

High endothelial NO production in foetal and early postnatal period was already demonstrated (Abman *et al.* 1990, Shaul *et al.* 1993). This explanation could be supported the findings of Arnal *et al.* (1994) on endothelial cells in culture. They found a sixfold higher content of NO synthase in growing endothelial cells compared with quiescent mature cells.

Another explanation might be considered, namely that endothelium-dependent relaxation is resistant to the long-lasting action of L-NAME, as was observed under similar conditions in other arteries (Zygmunt *et al.* 1994, Hatake *et al.* 1995). The relaxation of arteries induced by ACh is due, besides the NO mechanism, to a hyperpolarization of the smooth muscle membrane. An endothelium-hyperpolarizing factor (EDHF) released from endothelial cells (Chen *et al.* 1988), is also supposed to underlie, at least partially, this relaxation. Extensive relaxation in arteries of puppies could be explained by the involvement of EDHF in the relaxation resistant to the blockade of L-NAME.

Several studies have demonstrated that sympathetic nerve activity is at least shortly elevated after L-NAME administration (Matsuoka *et al.* 1994) suggesting that neurogenic mechanisms may also contribute to the elevation of arterial pressure. The function of the adrenergic innervation in arteries was therefore examined in both control and L-NAME treated puppies.

In our experiments, the neurogenic contractions of thoracic aorta and carotid arteries were not affected by chronic L-NAME treatment (Fig. 2A, B). On the other hand, neurogenic contractions in the pulmonary artery were enhanced at higher frequencies of stimulation. The increased contractile responses observed in the pulmonary artery of L-NAME-treated animals did not reflect a nonspecific effect of smooth muscles since the contractile response to KCl, a vasoconstrictor agent acting through a non-receptor mechanism, was similar in the control and L-NAME treated puppies (Fig. 3). MacLean *et al.* (1993) observed that removal of the endothelium did not enhance the neurogenic contractions in pulmonary artery, i.e. after L-NAME treatment, an extraendothelial site for NO synthesis in this artery could be supposed. Since NO synthase was proven in nerve fibres innervating coronary and pulmonary vessels (Klimaschewski *et al.* 1992), one cannot exclude that chronic NO synthase inhibition could compromise the "nitroxidergic nerves" (Toda and Okamura 1992) and enhance neurogenic contractions.

Small alterations in vascular responses after six-week treatment of newborn dogs with L-NAME probably reflects the preservation of high levels of nitric oxide synthase activity in the arterial wall. Furthermore, the contribution of EDHF to the marked residual acetylcholine-induced relaxation cannot be excluded.

Acknowledgments

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