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L-Arginine does not improve endothelium-dependent relaxation in *in vitro* Watanabe rabbit thoracic aorta

L. Caparrotta[1], L. Pandolfo[1], A. Chinellato[1], E. Ragazzi[1], G. Froldi[1], G. Aliev[2], and G. Fassina[1]

[1] Department of Pharmacology, University of Padova, Padova, Italy
[2] Department of Electron Microscopy, Ivanovo Medical Institute, Ivanovo, Russia

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Summary. The findings that even a single *in vitro* addition of L-arginine is able to normalize endothelium function in cerebral vessel from diet-induced hypercholesterolemic rabbits prompted us to investigate if similar results could be obtained on Watanabe rabbits thoracic aorta, in which we previously demonstrated low content of the amino acid.

L-Arginine (1 mM) preincubated for 45 minutes before the addition of drugs for studing endothelium-dependent vascular relaxation, did not modify the effect of acetylcholine on aortic isolated preparations. The lack of any effect by L-arginine indicates that the amino acid deficiency is not main cause of the impairment of endothelium function. The muscarinic receptor functionality affected by atherosclerotic process and/or the increased synthesis of EDCFs could account for the reduced endothelium-dependent relaxation.

Keywords: Amino acids – Atherosclerosis – Endothelium – Watanabe heritable hyperlipidemic rabbit (WHHL) – L-Arginine

Introduction

Nitric oxide (NO) is synthesized by the vascular endothelium from the amino acid L-arginine and accounts for the biological activities of endothelium-derived relaxing factor (EDRF) (Palmer et al., 1988; Schmidt et al., 1988). NO causes vascular smooth muscle relaxation interacting with soluble guanylate cyclase that leads to increased cGMP levels (Arnold et al., 1977; Waldman and Murad, 1987). In cholesterol-fed rabbits, atherosclerosis is associated with reduced endothelium-dependent vascular relaxation (Jayakody et al., 1985; Verbeuren et al., 1986; Ragazzi et al., 1989a). It has been demonstrated that also in aortas from Watanabe heritable hyperlipidemic (WHHL) rabbit

endothelium-dependent relaxation is impaired in comparison to normocholesterolemic New Zealand rabbit (Ragazzi et al., 1989b; Kolodgie et al., 1990; Chinellato et al., 1991). WHHL rabbit presents a genetic lack of low-density lipoprotein receptors, similarly to familial hyperlipidemia in humans (Watanabe, 1980). A reduced synthesis and/or inactivation of EDRF have been suggested to be involved in the impaired endothelium-dependent relaxation in WHHL rabbit aorta (Tagawa et al., 1991). We recently demonstrated that the vascular content of L-arginine is significantly lower in WHHL rabbit, if compared to New Zealand rabbits (Chinellato et al., 1992).

It has been recently reported that *in vitro* and *in vivo* L-arginine is able to normalize endothelial function in vessels from diet-induced hypercholesterolemic rabbits (Rossitch et al., 1991; Girerd et al., 1990). Conversely, other authors did not find restoration of endothelial functionality in cholesterol-fed rabbit aorta after *in vitro* L-arginine addition (Mügge and Harrison, 1991). The purpose of the present study was to examine the possibility that in vitro exposure of WHHL rabbit aorta to the amino acid L-arginine might affect endothelium-dependent relaxation in an animal model of severe atherosclerosis. The specificity of arginine was also investigated by using arginine D-stereoisomer.

Material and methods

An inbred colony of WHHL rabbits (breeding stock obtained from Professor Y. Watanabe, Kobe University, Japan), bred at the Fidia Research Laboratories (Abano Terme, Italy) was used. Experiments were carried out on WHHL rabbits of both sexes (12 months of age, 2.5–3 kg body weight). Age-matched New Zealand rabbits were used as controls. The rabbits were fed a standard cholesterol-free 2RB15-GLP diet (Mucedola, Milan, Italy) and water *ad libitum*.

After deep anaesthesia with pentobarbital (25 mg/kg i.v.), the thoracic aorta was removed and cleaned of adjacent tissue. Special care was taken to avoid contact with the luminal surface in order to preserve the endothelium. The descending thoracic aorta was cut into transverse rings approximately 3 mm thick. Two consecutive rings were tied together with silk thread, thus creating a 2-ring chain, in order to increase the mechanical response and the signal/noise ratio. The preparation was suspended in a 30-ml tissue bath containing modified Krebs-bicarbonate solution of the following composition (mmol/l): NaCl 116.0; KCl 5.4; $CaCl_2$ 1.2; $MgCl_2$ 1.2; NaH_2PO_4 1.2; $NaHCO_3$ 22.0; glucose 10.1; ascorbic acid 1.1, and equilibrated with a 95% O_2–5% CO_2 gas mixture, pH 7.4, at 37°C.

Isometric tension was recorded by means of force transducers (Type DYO Basile, Comerio, Italy) connected to a chart recorder (Unirecord Basile, Comerio, Italy). The aortic chains were held at a resting tension of 35 mN and allowed to equilibrate at optimal length for 60–90 min before experiments were started, the buffer being changed every 15 min.

Experimental protocol

All aorta preparations were tested with the approximate EC_{50} of noradrenaline (0.3 μM), washed and equilibrated for at least 60 min before experiments were started. This procedure was found to increase and stabilize any subsequent contractile response to noradrenaline.

One aorta preparation was used to determine the cumulative concentration-response curve of noradrenaline in order to calculate the EC_{50} of the contractile agonist for each rabbit. For relaxation studies, acetylcholine or L-arginine were cumulatively added to aorta rings precontracted with EC_{50} noradrenaline to steady-state tension. In other aorta preparations, L-arginine or D-arginine were preincubated for 45 minutes before EC_{50} noradrenaline

addition and subsequent cumulative acetylcholine response curve. This incubation time is considered sufficient for extracellular L-arginine penetration into the endothelial cells and use as substrate for the nitric oxide synthetase (Mügge and Harrison, 1991).

At the end of the experiments, preparations were washed, reequilibrated for 1 hour and tested with EC_{50} noradrenaline to verify contraction stability. The spontaneous relaxation following noradrenaline contraction was subtracted from the relaxation caused by the vasodilator agonists, to calculate the net effect of the agonists.

Electron microscopy examination

WHHL and New Zealand rabbits were anaesthetized with pentobarbital (30 mg/Kg i.v.) and the abdominal aorta was cannulated retrogradely with a cannula of which the exit diameter was no less than two-thirds of the arterial diameter. The jugular vein was opened and the vascular system was perfused with medium 199, containing 1/10 parts of polyglycin and heparin (10 IU/ml) under a pressure of 100–110 mmHg for 60 s. Fixation was then maintained with Karnowsky's solution. The proximal and terminal tracts of thoracic aorta were cut out and fixed in the same fixator for 2 hours.

Post-fixation for scanning electron microscopy (SEM) lasted 24 hours. After washing with medium 199, samples were post-fixed with a 1% water solution of OsO_4, briefly washed again and put into a 1% water solution of tannic acid for 30 minutes. After further washing, they were post-fixed with OsO_4 again for 30 minutes, dehydrated in graded concentrations of ethanol and absolute acetone, and dried to the critical point. Samples were then dissected under stereomicroscopy with a razor blade along the longitudinal axis of the aorta, coated with gold in an EICO-III sputtering device, and examined under Hitachi S-507 or Philips SEM 505 scanning electron microscopes operating at 20 and 30 KV, respectively.

Drugs and reagents

Noradrenaline bitartrate (NA), acetylcholine bromide (ACh), L-arginine and D-arginine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

All drugs were dissolved daily in saline solution. Aliquots were added to the tissue bath fluid to obtain the final desired concentration.

Statistical analysis

The percentage of relaxation by each drug was calculated taking as 100%, the EC_{50} noradrenaline-induced maximal contraction. Data were expressed as means $\pm$ SE. Statistical significance was assessed by using Student's test for unpaired data.

Results

Scanning and electron microscopy

In order to verify the presence and/or characterize the status of vascular endothelium, scanning electron microscopy (SEM) examination of WHHL rabbit aorta was performed.

The luminal surface of thoracic aorta from WHHL rabbits showed the presence of characteristic atherosclerotic lesions, particularly plaques close to the entry of intercostal arteries (Fig. 1 A). In the plaque areas, the endothelial monolayer was present but in disorganized form (Fig. 1 B); endothelial cells were well defined but their long axes were sometimes not parallel with the axis of the artery. On the endothelial layer surface, besides adhering leukocytes, microthrombi were also observed (Fig. 1 A, B).

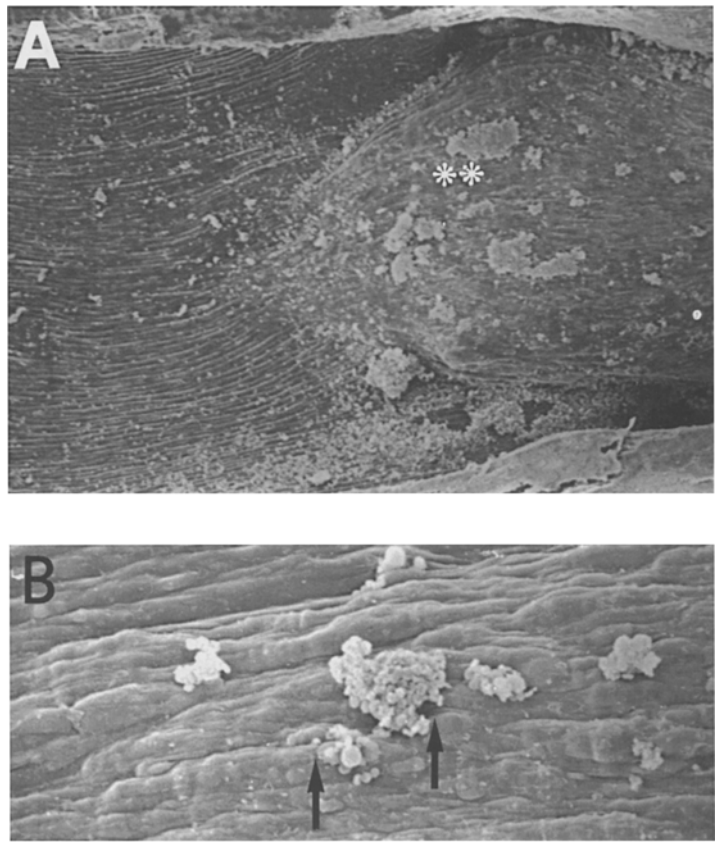

Fig. 1. Scanning electron microscopy from thoracic aorta of WHHL rabbit. A) Characteristic atherosclerotic plaque (**), particularly close to entry of intercostal artery orifice (SEM × 35). B) Blood cell adhesion (↑) (mainly monocytes) in atheroclerotic plaque zone (SEM × 220)

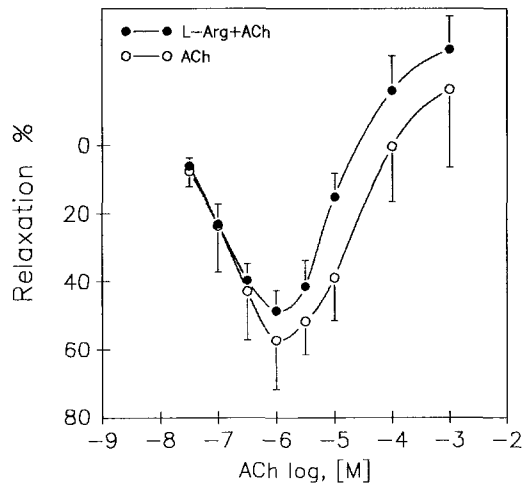

Fig. 2. Response to ACh in thoracic aorta rings from New Zealand rabbit, in presence or absence of 1 mM L-arginine. Each point is mean value ± SE from 5 rabbits

Functional aortic response

L-Arginine and D-Arginine (10 nM–1 mM) did not induce any significant functional effect on noradrenaline-precontracted aortic preparations from WHHL and New Zealand rabbits (data not shown). L- and D-arginine 1 mM were therefore pre-incubated with aorta rings 45 minutes before ACh-induced relaxation.

Figure 2 shows that the relaxation induced by ACh (30 nM–1 mM) in aorta from New Zealand rabbit was not significantly modified by 1 mM L-arginine addition.

In WHHL rabbit aorta, the ACh-induced relaxation was severely impaired (Fig. 3) and the addition of 1 mM L-arginine did not restore or modify the endothelium-dependent response to ACh (Fig. 3).

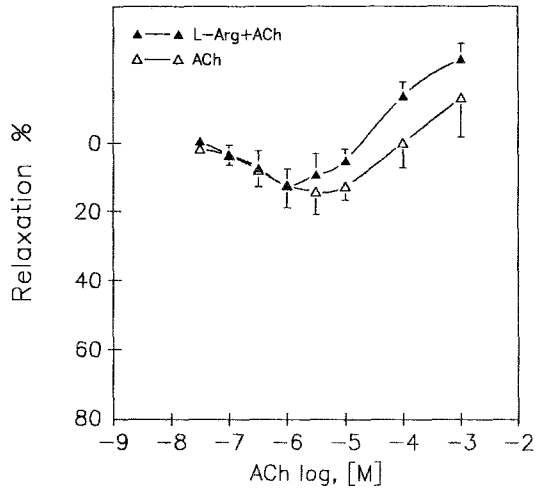

Fig. 3. Response to ACh in thoracic aorta rings from WHHL rabbit, in presence or absence of 1 mM L-arginine. Each point is mean value ± SE from 5 rabbits

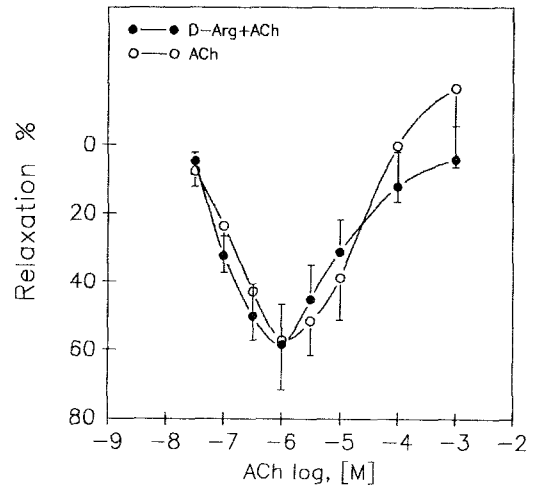

Fig. 4. Response to ACh in thoracic aorta rings from New Zealand rabbit, in presence or absence of 1 mM D-arginine. Each point is mean value ± SE from 5 rabbits

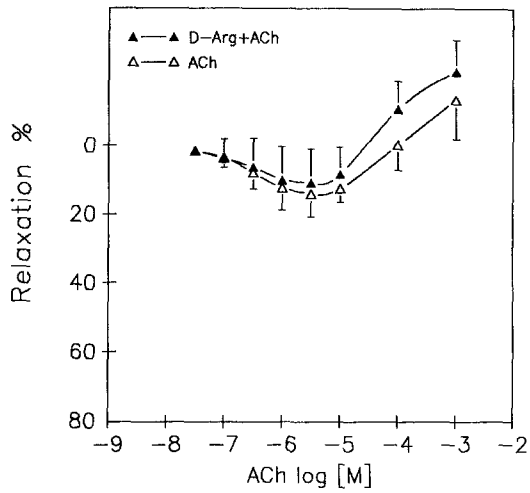

Fig. 5. Response to ACh in thoracic aorta rings from WHHL rabbit, in presence or absence of 1 mM D-arginine. Each point is mean value ± SE from 5 rabbits

The stereospecificity of arginine was also investigated by using the D-arginine stereoisomer. Both in New Zealand and WHHL rabbit aorta, 1 mM D-arginine did not modify the vasodilation response to ACh at all the considered concentrations (Figs. 4 and 5).

Discussion

The present study indicates that the impaired endothelium-dependent relaxation to acetylcholine of WHHL rabbit aorta is not affected by single *in vitro* exposure to L-arginine. D-Arginine stereoisomer of L-arginine was also uneffective on vascular response to acetylcholine both in New Zealand and WHHL rabbit aorta.

Electron microscopy morphology of thoracic aorta from WHHL rabbit indicated that, despite numerous atheromatous plaques, endothelium layer was present and covering aortic lesions. Disorganization and dystrophic changes of the endothelial cells were also observed. We therefore investigated the *in vitro* reactivity of endothelium with respect of the action of L-arginine a well known precursor in the NO-synthesis pathway (Palmer et al., 1988).

According to previous results (Ragazzi et al., 1989b; Chinellato et al., 1991), aorta from WHHL rabbit showed severe impairment of endothelium-dependent relaxation to acetylcholine, when compared to normocholesterolemic New Zealand rabbit aorta.

Moncada et al. (1989) demonstrated that nitric oxide (NO), which accounts for the biological activity of EDRF, is synthesized by the vascular endothelium from the amino acid L-arginine. It has been shown that in normolipemic New Zealand rabbits, L-arginine does not affect vascular endothelial reactivity to acetylcholine (Thomas et al., 1989; Rossitch et al., 1991). Also in our experiments L-arginine did not alter aortic endothelial response suggesting that under physi-

ological condition endothelial stores of L-arginine are sufficient for the nitric-oxide synthesis.

We have recently demonstrated that the vascular content of L-arginine is significantly lower in WHHL rabbit thoracic aorta, if compared to New Zealand normocholesterolemic rabbit (Chinellato et al., 1992). The findings that even a single *in vitro* addition of L-arginine was able to normalize endothelial function in cerebral vessels from diet-induced hypercholesterolemic rabbits (Rossitch et al., 1991) suggested the possibility that also in Watanabe rabbit aorta a similar effect could be obtained. By contrast we did not find any improvement of acetylcholine-mediated vascular relaxation after 45-minute incubation with L-arginine. This result indicates that the amino acid deficiency cannot be considered the main cause of the impairment in muscarinic relaxation. Several mechanisms were hypothesized (Rubanyi, 1991) to explain the impairment of endothelium-dependent relaxation in hypercholesterolemia-induced vascular damage: reduced synthesis of EDRF(s), impaired diffusion or increased inactivation of EDRF(s) in the subendothelial layer, alterations of receptors modulating EDRF(s) synthesis, increased synthesis and release of endothelium-derived contracting factors (EDCFs). The present experiments seem to exclude a role for reduced availability of EDRF, since the addition of the direct precursor of NO, L-arginine, does not improve the aortic endothelium-dependent relaxation. There are also data excluding an impaired diffusion or increased inactivation of EDRF since the activation of NO pathway by calcimycin (non-receptor-mediated endothelium-dependent vasodilating agent) is not affected by experimental hypercholesterolemia (Bossaller et al., 1987). Considering these data and the present findings we agree with the hypothesis that vascular muscarinic receptor function could be affected by atherosclerotic process (Fig. 6) (Bossaller et al., 1987; De Meyer et al., 1991). Alternatively and/or concomitantly, increased synthesis of EDCFs could account for the reduced endothelium-dependent vasodilation (Fig. 6).

In conclusion, *in vitro* addition of L-arginine does not modify the reduced aortic endothelium-dependent relaxation in heritable hyperlipidemic Watanabe rabbit. The deficiency of L-arginine therefore cannot be considered as the main cause of aortic endothelial dysfunction at least in Watanabe rabbit. Further investigation is required in order to verify the hypothesis of a muscarinic receptor alteration linked to the evolution of atherosclerotic process.

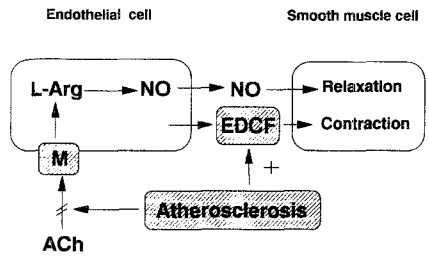

Fig. 6. Schematic diagram of the sites affected by atherosclerosis in WHHL rabbit aorta. *ACh* Acetylcholine, *L-Arg* L-Arginine, *NO* Nitric Oxide, *EDCF* Endothelium Derived Contracting Factor

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Authors' address: Prof. L. Caparrotta, Department of Pharmacology, University of Padova, Largo E. Meneghetti 2, I-35131 Padova, Italy.

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