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Effects of L-Arginine Analogues in Isolated Cat Cerebral Arteries

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The possible contribution of nitric oxide (NO) to the endothelium-dependent relaxation of isolated cat cerebral arteries was studied by examining the effects of the L-arginine (L-ARG) analogues L-canavanine and N^G-nitro-L-arginine (L-NOARG) on acetylcholine-induced relaxation. L-canavanine (100 μM) as well as L-NOARG (10 μM) decreased significantly the relaxant response of acetylcholine, their effect being significantly reversed by L-arginine (10 μM) but not by D-arginine (10 μM). In resting conditions, L-NOARG (10 μM) elicited a contraction of 314 ± 42 mg in arteries endowed with endothelium that was significantly diminished by endothelium removal to 88 ± 35 mg, and by (10 μM) L-arginine to 135 ± 54 mg. L-canavanine (100 μM) induced contractions in arteries with and without endothelial cells which were not statistically different. The results suggest that the endothelium of cat cerebral arteries has the ability to synthesize nitric oxide from L-arginine and to release it, even in the absence of a stimulus such as that of acetylcholine. L-NOARG seems to be a specific and powerful inhibitor of nitric oxide synthesis.

Key words: L-canavanine, N^G-nitro-L-arginine, Nitric oxide, Cerebral arteries.

Endothelium mediates vasoactive responses of a variety of humoral agents by releasing one or more labile substances called endothelium-derived relaxing factors (4-6). Several in vitro studies support that NO* or a nitrogen oxide-related compound is one of the EDRFs or at least

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* *Abbreviations:* ACh, acetylcholine; L-ARG, L-arginine; L-CAN, L-canavanine; EDRF, endothelium-derived relaxing factor; NO, nitric oxide; L-NOARG, N^G-nitro-L-arginine; $PGF_{2\alpha}$, Prostaglandin $F_{2\alpha}$.

the major factor accounting for the biological activity of EDRF (15, 20, 25). Since L-ARG is the physiological precursor of NO formation in endothelial cells, chemical analogues of L-ARG, such as N^G-monomethyl-L-arginine, L-NOARG and L-CAN, are potentially specific inhibitors of EDRF-mediated effects on vascular tone. L-CAN inhibits the endothelium-dependent relaxation elicited by ATP and ACh in rat thoracic aorta rings (24, 25). On the other hand, L-NOARG reduces NO synthesis in the cytosol of freshly harvested porcine aortic endothelial cells and inhibits the endothelium-dependent dilations to ACh of rabbit femoral arteries, all these effects being attenuated by L-ARG (17).

Studies carried out in brain vessels have shown that ACh evokes endothelium-dependent relaxation in rabbit (18), mouse (27), cat (2), and man (9) but not in dog (8). It has been suggested that the functional mechanism by which ACh produces relaxation of both rabbit (21) and cat cerebral arteries (13) is by releasing NO from the endothelium.

Previous reports from our laboratory indicated that the endothelium of isolated human and cat cerebral arteries can modulate the vasodilation elicited by ACh but not the responses to other vasoactive agents and platelets (3, 11). The effect to ACh is probably mediated by endothelium-derived NO since it was enhanced by L-ARG whereas the aminoacid did not modify a response independent of endothelium such as that of serotonin. L-ARG, however, could not by itself stimulate the production of EDRF (3).

The present study was, therefore, undertaken to elucidate the contribution of NO to the ACh-induced relaxation in isolated cat cerebral arteries. To achieve this, we investigated whether L-CAN and L-NOARG were able to inhibit the endothelium-dependent relaxation induced by ACh and whether their effects were reverted by L-ARG.

Materials and Methods

Mongrel cats of either sex, weighing 2.0-2.5 kg, were used in the present study. The animals were housed in the proper facilities complying with the European Community directive 86/609/CEE, and Spanish legislation (R.D. 223/1988) regarding the care of the animals used in experimentation and other scientific purposes. The experiments reported here were aproved by the Biosafety and Animal Care Unit Committee of the Faculty of Medicine of Autónoma University of Madrid. The animals were anesthetized with sodium pentobarbitone (35 mg/kg i.p.) and exsanguinated. The brain was removed, placed in a dissection dish containing cold oxygenated Krebs-Henseleit solution (for composition, see below), and both middle cerebral arteries (0.4-0.5 mm in outer diameter) were dissected. Experiments were performed either on fresh arteries or on arteries stored in Krebs-Henseleit solution at 4 °C for no longer than 24 h. Since no major difference was observed in the behaviour of fresh and stored arteries the results were pooled.

Isometric tension recording. — Vascular cylindrical segments, 2 mm in length, were cut and set up in organ baths for isometric recording of tension according to the technique adapted (3) from that described by NIELSEN and OWMAN (19). The recording system included a force displacement transducer (FT 03 Grass, Quincy, USA) and a polygraph (Grass Model 7-E). The organ bath contained 6 ml of Krebs-Henseleit solution maintained at 37 °C, by means of a water jacket, and bubbled continuously with a 95 % O_2-5 % CO_2 gas mixture to keep the pH at 7.4. The vessels were allowed a 60-90 min equilibration period with repeated renewal of the bath medium and adjustment of the resting tension to 300 mg.

Endothelial cells. — Endothelium re-

moval was performed by gentle rubbing of the intimal surface with a stainless steel rod of appropriate diameter inserted through the lumen. The presence or absence of endothelial cells was checked in each preparation by analyzing the relaxing effect of ACh (0.1 μM-10 μM) after inducing a contraction with $PGF_{2\alpha}$ (10 μM). Those arteries showing a relaxation to ACh greater than 60 % of this tone were considered as endowed with endothelium whereas those showing less than 10 % as endothelium deprived (3).

Dose-response curves. — Successive dose-response curves to ACh were performed after bringing about a contraction with $PGF_{2\alpha}$. The first curve was used as control (see above). L-CAN (100 μM) or L-NOARG (10 μM, 100 μM) was added, 2 h or 10 min, respectively, before performing the second dose-response curve to ACh. To control any change in sensitivity of the vascular segments to ACh, the same procedure was followed simultaneously in another group of arterial preparations but omitting L-CAN or L-NOARG. After renewing several times the bath medium during a 60 min period, a third curve to ACh was obtained to demonstrate the reversibility of any of the effects observed.

In some segments, L-ARG (10 μM) or D-ARG (10 μM) was added to the bath solution 10 min before undertaking the second dose-response curve to ACh in the presence of the potential inhibitors of NO biosynthesis.

Statistical analysis. — Data are expressed as means ± S.E.M. and the relaxant responses given as percentage of the contracting tone obtained with $PGF_{2\alpha}$. Statistical analysis of differences between groups was determined by Student's *t* test for paired and unpaired observations. A probability value less than 0.05 was considered enough to reject the null hypothesis.

Chemicals and solutions. — The composition of the Krebs-Henseleit solution was (in mM): NaCl, 115; KCl, 4.6; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; and glucose, 11.1. Acetylcholine chloride, prostaglandin $F_{2\alpha}$, L-arginine hydrochloride, D-arginine hydrochloride, and L-canavanine sulphate were purchased from Sigma, and N^G-nitro-L-arginine from Aldrich. All drugs were dissolved in isotonic saline solution (0.9 % w/v NaCl), except $PGF_{2\alpha}$ stock solution that was dissolved in absolute ethanol. N^G-nitro-L-arginine was dissolved in saline solution following sonication. Stock solution of ACh (8 mM) was distributed into aliquots and kept at −15 °C until its use. Drug solutions were made fresh daily, maintained on ice throughout the experiment and discarded at the end of the day.

Concentrations of drugs are expressed as their final molar concentration in the bath medium.

Results

Effect of L-canavanine in cat cerebral arteries. — In resting conditions, L-CAN (100 μM) induced contractions in arteries endowed with endothelial cells (208 ± 78 mg, n = 35) which appeared unmodified in arteries without endothelium (151 ± 29 mg, n = 27; $p > 0.05$).

In precontracted unrubbed cerebral arteries, the maximal relaxing effect induced by ACh was 73 ± 7 % (n = 12) over a tone of 751 ± 71 mg. This dilator response to ACh was markedly reduced in the presence of L-CAN (100 μM) at the first two doses used (fig. 1, upper plot), the maximal relaxation obtained being 42 ± 8 % ($p < 0.001$ vs control). The inhibition of the endothelium-dependent relaxation induced by ACh was also found at all doses used ($p < 0.001$) when the third dose-response curve to ACh was performed after changing several times the bath medium

during 60 min. When unrubbed vascular segments were challenged a second time to ACh their sensitivity to this agent remained unaffected (results not shown). In the endothelium-denuded preparations, the addition of L-CAN (100 μM) to the organ bath did not change the slight relaxation evoked by ACh (fig. 1, lower plot).

The inhibition of the ACh-induced relaxations by L-CAN (100 μM) was reversed in the presence of L-ARG (10 μM) (fig. 2, upper plot). No difference was found in the relaxation elicited by ACh in the presence of both L-CAN and L-ARG when compared to the control curve. In contrast (fig. 2, lower plot), D-ARG (10 μM) did not revert the inhibitory effect of L-CAN (100 μM), i.e., the dilator effect of ACh was markedly reduced ($p < 0.05$; $n = 15$) in the presence of both L–CAN and D-ARG when compared to the control dose-response curve to ACh.

Effect of N^G-nitro-L-arginine in cat cerebral arteries. — L-NOARG (10 μM) brought about a contraction of 314 ± 42 mg, $n = 29$, in unrubbed cat arteries submitted only to resting tension. This contractile response appeared significantly reduced when L-NOARG (10 μM) was added to segments without endothelium (88 ± 35 mg, $n = 13$, $p < 0.002$).

The contraction induced by L-NOARG in unrubbed segments under

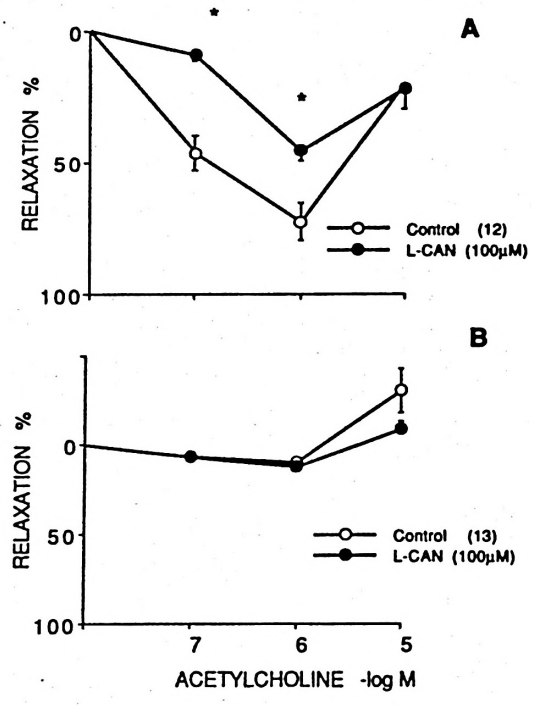

Fig. 1. *Effect of L-CAN on the relaxation induced by acetylcholine in cat cerebral arteries with (A) and without (B) endothelium.*
Data are expressed as percentages of the contraction induced by 10 μM $PGF_{2\alpha}$. Relaxation to acetylcholine was tested in the absence (control, -o-) and in the presence of L-CAN (-•-). Values are means ± S.E.M. Number of experiments are given in parentheses. *$p < 0.05$ (paired t-test) vs. control.

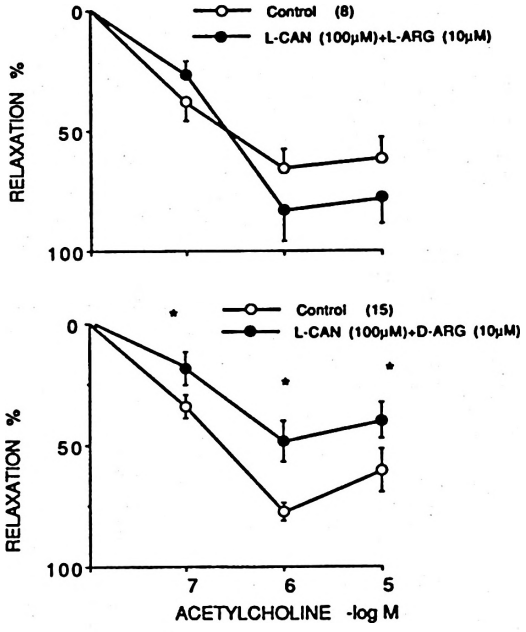

Fig. 2. *Effect of L-arginine and D-arginine on the inhibitory effect of L-CAN on acetylcholine-induced endothelium-dependent relaxation.*
Legend as in figure 1.

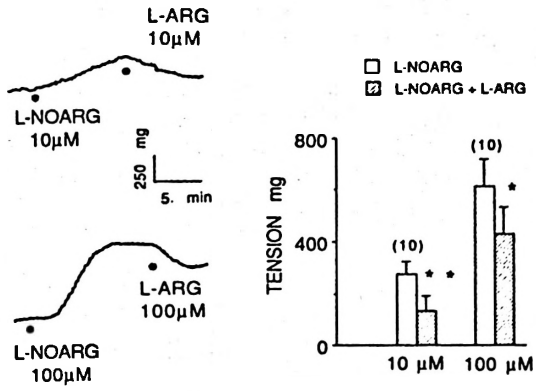

Fig. 3. *Effect by L-ARG on the contractile response induced by L-NOARG on resting cat cerebral arteries with endothelium.*
Left panel: Representative cases from two isolated cerebral segments. *Right panel:* Summary of the experiments exposed in the left panel. Values are means ± S.E.M. Numbers of experiments are given in parentheses. ** $p < 0.001$, * $p < 0.02$ (paired *t*-test) vs. control.

resting conditions seems to be concentration-related and significantly decreased by L-ARG. Figure 3 (left panel) shows a typical recording obtained from arterial cylinders where the addition of L-ARG

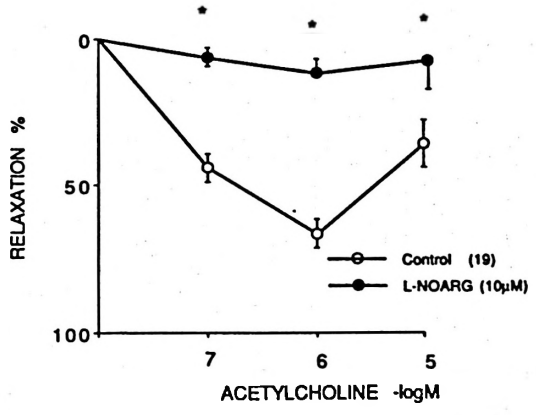

Fig. 4. *Effect of L-NOARG on the relaxation induced by acetylcholine in cat cerebral arteries with endothelium.*
Legend as in figure 1.

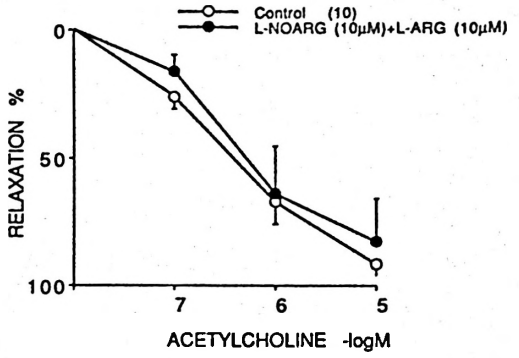

Fig. 5. *Reversal by L-ARG of the inhibitory effect of L-NOARG on acetylcholine-induced endothelium-dependent relaxation.*
Legend as in figure 1.

counteracted the contractile responses evoked by L-NOARG at two different concentrations. The mean contractile effect induced by 10 μM L-NOARG (275 ± 59 mg; n = 10) was significantly decreased by 10 μM L-ARG to 135 ± 54 mg ($p < 0{,}001$) (fig. 3, right panel). Similarly, 100 μM L-ARG reduced significantly (n = 10, $p < 0.02$) the contraction elicited by 100 μM L-NOARG from 612 ± 100 mg to 430 ± 100 mg.

In precontracted unrubbed cerebral arteries, the vasodilation induced by ACh was significantly reduced in the presence of 10 μM L-NOARG at all doses used and the maximal relaxing response obtained under those circumstances was 12 ± 4 % instead of 67 ± 5 %, over a tone of 620 ± 62 mg ($p < 0.001$). This inhibitory effect of L-NOARG persisted in spite of repeated washouts for up to 60 min. On the other hand, the addition of L-NOARG (10 μM) to the organ bath did not modify the slight relaxation induced by ACh in rubbed arteries (fig. 4).

The inhibitory effect of L-NOARG (10 μM) on the relaxation induced by ACh was overcome by the addition of L-ARG (10 μM) to the organ bath (fig. 5). No difference was found in the relaxation elicited by ACh in the presence of both L-

NOARG and L-ARG when compared to the control curve ($p > 0.05$; $n = 10$).

Discussion

The results reported here confirm that the endothelium-dependent relaxation elicited by ACh in isolated cat cerebral arteries is mediated by the L-ARG-NO pathway. The NO synthase inhibitors L-CAN and L-NOARG (16, 25) decreased it significantly, and their action was counteracted by L-ARG, the biological precursor of NO, but not by D-ARG. The ability of ACh to release NO from the vascular endothelium has been already described in the cerebral circulation (1, 21) as well as in other vascular beds (7, 12, 20, 22, 23, 26).

In the present experiments, L-NOARG appears to be a better inhibitor of endothelial NO synthase than L-CAN. This is suggested by the fact that L-NOARG (10 μM) reduced the maximal ACh-induced vasodilation by an 80 % whereas L-CAN (100 μM) did it only by a 45 %. Experiments performed in non-cerebral arteries, such as perfused rabbit femoral arteries (17), isolated rabbit aorta (16), and bovine aortic endothelial cells (7) also support that L-NOARG is a rather potent and specific inhibitor of NO biosynthesis.

On the other hand, L-NOARG brought about a contraction of isolated cat cerebral arteries under resting conditions, which was diminished by endothelium removal and partially decreased by L-ARG. These results might indicate the existence of a tonic NO release from the endothelial cells that would be suppressed by L-NOARG. They seem to disagree with previous results showing that L-ARG was unable to modify either the resting tension or the contraction induced by $PGF_{2\alpha}$ in cat cerebral vessels (3). The explanation might be that the levels of L-ARG in these endothelial cells are already so high that NO synthase is in saturating conditions and, therefore, an increase in the substrate concentration would not affect NO production. Such a mechanism was suggested by MITCHELL *et al.* (14) in bovine aortic endothelial cells in culture. An interference with the basal release of NO from endothelial cells by L-NOARG has also been reported in isolated rabbit aorta (16) and in intact rabbit coronary vascular bed (10). L-CAN (100 μM), however, induced a contraction of the isolated cat cerebral arteries, which was unaffected by endothelium removal, indicating a direct action on vascular smooth muscle. This inability of L-CAN in modifying the basal release of NO is probably due to its poor inhibitory effect on NO synthesis.

Our results show, then, that the endothelial cells lining the cat cerebral arteries have the ability to synthesize NO from L-ARG and to release it as any other vascular endothelial layer. Although NO appears to be liberated tonically from the endothelium, these cells do not seem to play a role in modulating the mechanical responses of vasoactive agents with the exception of ACh.

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Resumen

Se estudia la posible contribución del óxido nítrico (NO) en la relajación dependiente de endotelio de arterias cerebrales de gato, analizando los efectos de los análogos de L-arginina, L-canavanina y N^G-nitro–L-arginina (L-NOARG) sobre la relajación inducida por acetilcolina. L-canavanina (100 μM) y L-NOARG (10 μM) disminuyen significativamente la respuesta relajante de la acetilcolina, siendo estos efectos significativamente revertidos por la L-arginina (10 μM), pero no por la D-arginina (10 μM). En condiciones de tensión basal, L-NOARG (10 μM) produce una contracción de

314 ± 42 mg en arterias con células endoteliales que es significativamente menor tanto en arterias desprovistas de endotelio (88 ± 35 mg) como por el efecto de L-arginina (10 μM; 135 ± 54 mg). L-canavanina (100 μM) induce contracción independiente de endotelio. Estos resultados sugieren que el endotelio de arterias cerebrales de gato puede sintetizar y liberar óxido nítrico a partir de L-arginina, incluso en ausencia de acetilcolina. La síntesis de óxido nítrico en este lecho vascular parece inhibirse de una forma más específica por L-NOARG que por L-canavanina.

Palabras clave: L-canavanina, N^G-nitro-L-arginina, Oxido nítrico, Arterias cerebrales.

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