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Relaxant effects of L-citrulline in rabbit vascular smooth muscle

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1 Vascular endothelium plays a pivotal role in the control of vascular tone through the release of vasoactive factors such as EDRF (NO).

2 The aim of this study was to investigate whether the addition of exogenous L-citrulline, the byproduct of the NO-synthesis, could relax vascular smooth muscle.

3 L-citrulline relaxed both endothelium-denuded and endothelium-intact rabbit aortic rings precontracted with noradrenaline 10^{-6} M (maximum relaxations induced by L-citrulline 10^{-8} M were $74.1 \pm 5.2\%$ vs $51.3 \pm 2.8\%$ in endothelium-denuded and endothelium-intact arteries, respectively).

4 This relaxant effect was enhanced by zaprinast (a phosphodiesterase type 5 inhibitor) and inhibited by HS-142-1 (a particulate guanylate cyclase inhibitor) and by apamin (a K_{Ca}-channel blocker).

5 L-citrulline (10^{-13}–10^{-8} M) increased cGMP levels in aortic rings (maximum value with L-citrulline 10^{-8} M was 0.165 ± 0.010 pmol cGMP mg^{-1} of tissue vs 0.038 ± 0.009 pmol mg^{-1} of tissue in basal).

6 L-citrulline as well as NO were released from endothelial cells in culture stimulated with ACh. The values were 6.50 ± 0.50 μM vs 2.30 ± 0.20 μM (stimulated with ACh and basal respectively) for L-citrulline and 4.22 ± 0.10 μM vs 0.87 ± 0.26 μM (stimulated with ACh and basal respectively) for NO.

7 These results suggest that L-citrulline could be released together with NO from endothelium and may have actions complementary to those of NO in the control of vascular smooth muscle relaxation.

Keywords: L-citrulline; nitric oxide; vascular smooth muscle; rabbit aorta; vascular relaxation

Introduction

Vascular endothelial cells modulate arterial and venous tone through the release of several vasoactive factors, including the Endothelium-derived Relaxing Factor (EDRF) (Furchgott & Zawadzki, 1980). To date, at least one EDRF, namely nitric oxide (NO) (Ignarro *et al*., 1987; Palmer *et al*., 1987), has been recognized. The Endothelium-dependent Hyperpolarizing Factor (EDHF) has not yet been identified. It seems possible that the EDHF could be a cytochrome P_{450} derived arachidonic acid metabolite which hyperpolarizes the underlying smooth muscle cell layer by opening K^+_{Ca} channels (Hecker *et al*., 1994), thus causing relaxation.

NO biosynthesis is catalyzed by NO synthase (NOs). Constitutive and inducible isotypes of NOs have been differentiated on the basis, of calcium-calmodulin dependence (Gross *et al*., 1990). The initial step in the reaction is the hydroxylation of the nitrogen in the guanidino group of L-arginine (Palmer *et al*., 1988). The process incorporates molecular oxygen into NO and citrulline. The reaction, which is a five-electron oxidation, requires reduced pyridoxine nucleotides, reduced biopteridines and calmodulin. It is well known that in cultured endothelial cells from bovine aorta, the byproduct of the synthesis, L-citrulline, is recycled back to L-arginine by the incorporation of a nitrogen (Hecker *et al*., 1990).

Several findings demonstrate that the NO-generating enzyme system in the bovine aortic endothelium is membrane-bound and may be associated with the plasma membrane of the endothelial cells (Boje & Fung, 1990). In addition, Fostermann *et al*. (1991) have reported that in endothelial cells, a particulate Ca/calmodulin regulated enzyme accounts for more than 95% of the total NOs activity. Moreover, Pollock *et al*. (1991) have purified this constitutive particulate NOs and characterized the enzyme as NADPH- and (6R)-5,6,7,8-tetrahydrobiopterin-dependent. On the other hand, Busconi & Mitchel (1993) have reported that N-terminal myristoylation of the endothelial NOs may provide a potential point of regulation in the biological function of EDRF *in situ*.

The purpose of this study was to investigate the possibility that L-citrulline might not merely be a byproduct of the NO-synthesis but might also play a role in the modulation of vascular tone by endothelial cells. We have studied the possible relaxant properties of L-citrulline on isolated rabbit aorta and have also attempted to develop our understanding of the mechanism of action of that response. In addition, we tested whether L-citrulline together with NO were released from endothelial cell cultures.

Methods

General procedure

Male New Zealand White rabbits weighing 2.5–3.0 kg were obtained from Biocentre SA (Barcelona, Spain). The animals were anesthetized with ethyl ether and killed by exsanguination from the common carotid.

Isolated aortic preparations

The thoracic aorta was rapidly removed and placed in Godfraind solution of the following composition (mM): NaCl 121, KCl 5.8, $NaHCO_3$ 14.9, $MgCl_2$ 1.22, glucose 11 and $CaCl_2$ 1.25. Adherent fat and surrounding tissue were cleaned off and the arteries were cut into rings approximately 2–3 mm wide. The rings were then suspended between two stainless steel hooks in organ baths containing 10 ml of Godfraind solution. The solution was kept at 36 ± 0.5°C and gassed continuously with a 95% O_2-5% CO_2 gas mixture. The aorta rings were mounted under 2 g tension. Each preparation was allowed to equilibrate for 90–120 min. Contractile

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responses were measured isometrically by means of force-displacement transducers (Grass FT 03) and were recorded on a Grass polygraph as previously described (Tejerina *et al*., 1988). The isometric force was also digitalized by a MacLab A/D converter (Chart v3.2, A.D Instruments Pty. Ltd., Castle Hill, Australia) and stored and displayed on a Mackintosh computer.

The arteries were divided into two groups and the endothelium was removed in one of these two groups (−E arteries). The endothelium-denuded arteries were prepared mechanically by inserting a stainless-steel rod into the rings and rubbing the rings gently with the fingers, following the procedure described by Furchgott & Zawadzki (1980), while in the other group the endothelium was kept intact (+E arteries). Endothelium functionality was tested in both groups of arteries by precontracting with noradrenaline (NA) (10^{-6} M) and when the plateau was reached, acetylcholine (ACh) (10^{-6} M) was added to the bath. We considered +E arteries, arteries those in which ACh caused more than 50% relaxation and −E arteries those in which ACh caused between 5–10% relaxation. After equilibration the following experiments were carried out.

Experimental protocol

In both groups of arteries (+E or −E), contractions induced by noradrenaline 10^{-6} M were carried out, when the plateau was reached, L-citrulline from 10^{-13}–10^{-8} M was added in a cumulatively manner.

In order to rule out any effect of L-citrulline on NOs expressed in vascular smooth muscle cells, the chambers were sterilized and the arteries (−E) were incubated during the experiment (from the isolation of the arteries) in the presence of dexamethasone 10^{-6} M and the previous procedure repeated. Moreover, in the other group of experiments, aortic rings were incubated with N^{ω}-nitro-L-arginine methyl ester (L-NAME) (10^{-4} M) (a NOs inhibitor) for 45 min and the relaxation procedure induced by L-citrulline was repeated.

In another group of experiments we tested whether cGMP formation was implicated in the relaxant effect of L-citrulline. For this, we repeated the relaxant procedure with L-citrulline mentioned above, after this, the arteries were washed out and then were incubated in the presence of zaprinast 10^{-5} M (a phosphodiesterase type 5 inhibitor) for 30 min. We also studied the possibility that L-citrulline might act on the soluble and particulate guanylate cyclase enzymes, which synthesize cGMP from GTP. The −E arteries were contracted with noradrenaline 10^{-6} M and when the plateau was reached, cumulative concentrations of L-citrulline from 10^{-13}–10^{-8} M were added, then the rings were washed out and incubated in the presence of either ODQ 10^{-6} M (a selective inhibitor of soluble guanylate cyclase) or HS-142-1 10^{-5} M (an inhibitor of particulate guanylate cyclase) (Matsuda & Morishita, 1993) for 45 min and the relaxant procedure induced by L-citrulline was repeated as mentioned above.

In another group of experiments, −E arteries were precontracted with KCl 80 mM and a cumulative relaxation curve made by adding aliquots of L-citrulline from 10^{-13}–10^{-8} M. The aim of this experiment was to check if a K^+ channel was implicated in the mechanism of the relaxant effect of L-citrulline. We studied this subject in more detail by precontracting the −E arteries with NA 10^{-6} M and then inducing a relaxation by L-citrulline (10^{-13}–10^{-8} M). The arteries were washed out and then incubated in the presence of either glibenclamide 10^{-5} M (a K_{ATP}-channel blocker), charybdotoxin 10^{-8} M (a large-conductance K^+_{Ca}-channel blocker) or apamin 10^{-6} M (a small-conductance K^+_{Ca}-channel blocker) for 45 min and the previous procedure repeated.

Measurements of cGMP levels

In the light of these results, we studied whether L-citrulline was able to increase cGMP levels in aortic rings (−E arteries). After an equilibration period of 90 min, a contraction with noradrenaline 10^{-6} M was induced and the relaxing protocol with cumulative concentrations of L-citrulline (10^{-13}–10^{-8} M) described above was carried out removing the arteries rapidly after each concentration of L-citrulline. These were dried, weighed and frozen in liquid nitrogen and stored at −70°C until homogenized in 0.5 ml of 10% trichloroacetic acid (TCA) using a Potter glass homogenizer. The homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant was removed and extracted three times with four volumes of diethyl ether. The cyclic GMP content was then assayed using the [$^{-3}$H]cGMP radioimmunoassay kit of Amersham Int. as described in the manufacturer's instructions. Data were expressed as pmol cGMP/mg of tissue.

Cell culture

We also tested whether L-citrulline in cell culture was released from endothelial cells together with NO. For this, we used bovine endothelial aortic cell (BEA) culture.

BEA cells were isolated from bovine thoracic aorta by enzymatic dissociation. Cells were maintained in Iscove-F12 (1 : 1) medium (Gibco) supplemented with New born calf serum (Gibco). The purity of BEA cells was confirmed immunochemically using anti-VIII Factor antibody and characterized by cobblestone morphology.

Measurements of NO and L-citrulline released from endothelial cells

L-citrulline and nitrite production, as indicators of NO synthesis, were measured in the supernatant of (bovine aortic endothelial) BEA cells. Briefly, BEA cells were cultured in 24-well plates (obtained from passage 2) with 1 ml of culture medium until cells reached confluence. L-citrulline and nitrite accumulation in the cell culture was measured after 2 min stimulation with acetylcholine 10^{-6} M.

The supernatant was removed for quantification of L-citrulline by HPLC analysis. These extracts were filtered using 0.2 μM nylon syringe filters (Scientific Resources INC.). A 40 μl aliquot of filtered sample was derivatized (prior to column injection) by mixing with 20 μl o-phtaldialdehyde (OPA) solution. The reagent had the following composition: 5 mg OPA (Sigma), 15 μl mercaptoethanol, 1785 μl 0.1 M borate buffer (pH = 11); 200 μl methanol. The derived sample (50 μl) was injected into the HPLC system *via* a Hamilton syringe and amino acids were detected using a Perkin Elmer LC240 fluorescence detector (excitation wavelength 340 nm, emission wavelength 455 nm). Elution of L-citrulline from a Nova-Pak C_{18} column (3.9 × 15 mm. Waters) was achieved using a stepped gradient mobile phase; ranging from 20% (v/v) methanol, 0.05 M sodium acetate buffer (pH = 6.4) plus 1% THF (solution A) to 80% methanol (solution B) and a flow rate of 1 ml min^{-1} was maintained throughout. Peak areas were quantified with a chromatography computing integrator working with an external standard curve.

Nitrites were measured by adding 100 μl of Griess reagent (Green *et al*., 1982) (1% sulfanilamide and 0.1% naphtyethylene diamine in 5% phosphoric acid) to 100 μl samples of

phosphate buffer saline (PBS). Optical density at 550 nm (OD_{550}) was measured with a microplater reader. Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrate prepared in PBS.

Drugs

The following drugs were used: L-citrulline (Sigma), L-noradrenaline bitartrate (Sigma), potassium chloride (Merck), acetylcholine chloride (Sigma), N-nitro-L-arginine methyl ester (Sigma), dexamethasone (Sigma), glibenclamide (Sigma), apamin (Sigma), charybdotoxin (Sigma), zaprinast (Sigma). HS-142-1 was supplied by Kyowa Hakko Kogyo Co., Ltd. and 1H-(1,2,4,) oxadiazolo (4,3-a) quinoxaline-1-one (ODQ) (Tocris Cookson (Bristol, U.K.)). Stock (0.1 M) solutions of ODQ and glibenclamide were dissolved in DMSO 50% and ethanol respectively. The rest of the compounds were prepared by dissolution in distilled water. Working solutions were made in Godfraind solution; since control experiments had demonstrated that the highest ethanol and DMSO levels used at 10^{-6} and 10^{-8} M respectively had no effect on vascular smooth muscle contraction. The concentrations for each chemical or drug were expressed as final concentration in the chamber in terms of the salt. Ascorbic acid (10^{-4} M) was added to each daily prepared solution of noradrenaline.

Statistical analyses

All values used in the analyses represent means ± s.e.mean of 6–8 rabbits in each group. Concentration-response curves were used to determine the concentration of L-citrulline producing 50% inhibition of the maximal contractile response (IC_{50}), using linear regression analysis over the response range of 20–80% of the maximal inhibition. Comparisons between the different groups were performed by two way ANOVA tests for paired data and by Student's *t*-test for unpaired data. Differences were considered significant when $P < 0.05$.

All protocols concerning animals were approved by the Complutense University of Madrid (EEC official registration 28079-15ABC).

Results

Relaxation induced by L-citrulline

When −E arteries aortic rings were exposed to noradrenaline 10^{-6} M a contraction of 5.6 ± 0.7 g was evoked. In −E arteries as well as in +E arteries (Figure 1), L-citrulline (10^{-13}–10^{-8} M) caused a relaxation in a concentration-dependent manner, the maximum relaxations being 74.1 ± 5.2% (−E arteries, $n = 15$), and 51.3 ± 2.8% (+E arteries, $n = 15$).

Effect of dexamethasone and L-NAME on the relaxation induced by L-citrulline in −E arteries

When the arteries were incubated with dexamethasone or L-NAME, noradrenaline 10^{-6} M induced a contraction of 7.1 ± 1.2 g or 6.0 ± 1.3 g respectively (Figure 2). We did not find any differences in the relaxant response of L-citrulline either in the presence of dexamethasone or L-NAME, the maximum relaxant effect induced by L-citrulline 10^{-8} M being 74.0 ± 4.2% and 74.1 ± 10.0% in the presence of dexamethasone and L-NAME respectively vs 74.1 ± 5.2% in control as shown in Figure 2.

Effect of zaprinast on the relaxation induced by L-citrulline

Figure 3 shows the effect of zaprinast 10^{-5} M (a phosphodiesterase type 5 inhibitor) on the relaxation induced by L-citrulline. When the arteries (−E) were incubated in the presence of zaprinast, the relaxant response of L-citrulline was enhanced (the EC_{50} were $1.2 \times 10^{-9} \pm 0.14$ M in control and $1.9 \times 10^{-10} \pm 0.18$ M in zaprinast-treated arteries, $P < 0.001$, $n = 7$)

Effect of L-citrulline on cGMP levels in aortic rings

We also studied cGMP levels directly in aortic rings stimulated with L-citrulline 10^{-13}–10^{-8} M for 15 min. As shown in Figure 4, L-citrulline induced an increase in cGMP levels (with respect to basal levels) reaching statistical significance at the concentration of L-citrulline of 10^{-13} M ($P < 0.01$, $n = 7$).

Effect of ODQ and HS-142-1 on the relaxation induced by L-citrulline

Taking into account the last two results, it seems clear that during the relaxation induced by L-citrulline in aortic rings,

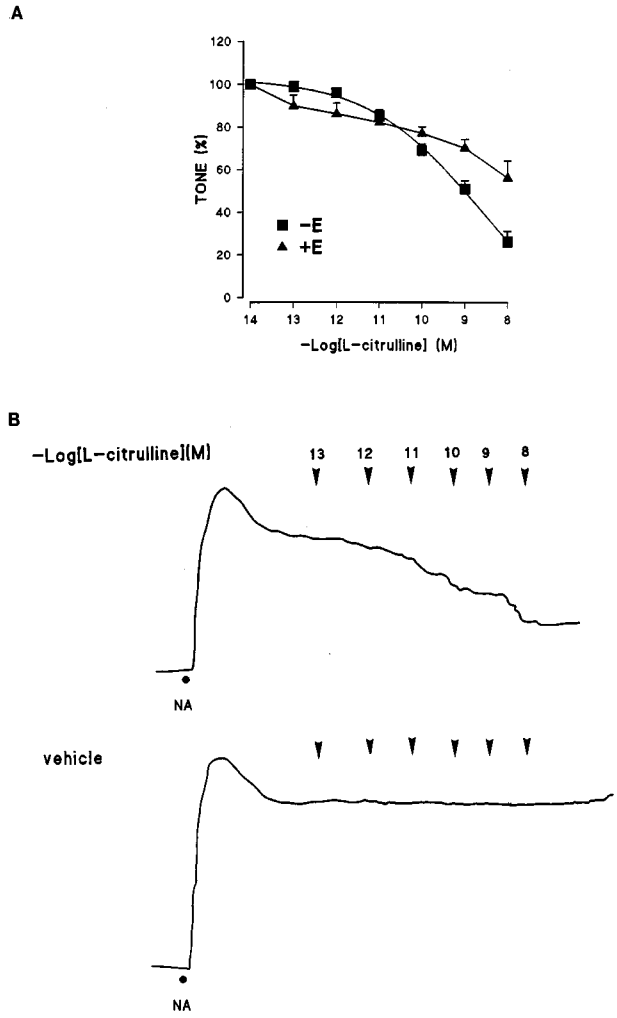

Figure 1 (A) Concentration-response curve to L-citrulline (10^{-13}–10^{-8} M) in rabbit isolated aorta artery with and without endothelium precontracted with noradrenaline 10^{-6} M. (B) Representative traces showing the relaxation induced by L-citrulline (upper panel) and vehicle (lower panel) in denuded rabbit isolated aorta precontracted with NA 10^{-6} M. Each point represents the mean ± s.e. mean of 15 experiments.

there was an increase in cGMP levels. Consequently, we tried to investigate whether this increase was through the stimulation of either the soluble or the particulate isoenzyme of guanylate cyclase by L-citrulline. Figure 5 shows the effect of ODQ 10^{-6} M (a soluble guanylate cyclase inhibitor) and HS-142-1 10^{-5} M (a particulate guanylate cyclase inhibitor) on the relaxant response induced by L-citrulline. We did not find any change in this effect when the −E arteries were incubated with ODQ whereas HS-142-inhibited the relaxation induced by L-citrulline. The maximum relaxation decreased from $74.1 \pm 5.2\%$ in control to $25.2 \pm 5.2\%$ ($P < 0.001$, $n = 6$) in HS-142-1-treated arteries.

Measurements of L-citrulline and NO levels

L-citrulline and NO levels in BEA cells were measured after 2 min of stimulation with ACh 10^{-6} M. As shown in Figure 6, L-citrulline increased from (μM) 2.30 ± 0.20 (basal level) to 6.50 ± 0.50 ($P < 0.001$, $n = 3$ duplicated) after 2 min of stimulation. Moreover, NO levels also increased from 0.87 ± 0.03 (basal level) to 4.22 ± 0.10 ($P < 0.001$, $n = 3$ duplicated) after 2 min of stimulation.

Implication of a K^+ channel

When the arteries (−E) were contracted with KCl 80 mM, L-citrulline ($10^{-13} - 10^{-8}$ M) was not able to cause a relaxation (as shown in Figure 7) which indicates that a K^+-channel is implicated in the mechanism of action of L-citrulline. We investigated the effect of several K^+-channel blockers on the relaxant responses to L-citrulline. Figure 8 shows the effects of glibenclamide 10^{-6} M, charybdotoxin 10^{-8} M and apamin 10^{-6} M on the relaxant response induced by L-citrulline. When the −E arteries were incubated with glibenclamide or charybdotoxin, L-citrulline ($10^{-13} - 10^{-8}$ M) caused the same relaxation as in control. The maximum effect induced by L-citrulline 10^{-8} M was of $70.1 \pm 4.7\%$ and $63.4 \pm 6.2\%$ in glibenclamide and charybdotoxin-incubated arteries respectively vs $74.1 \pm 5.2\%$ in control. Apamin 10^{-6} M significantly decreased the relaxation induced by L-citrulline ($10^{-13} - 10^{-8}$ M). Thus, the maximum relaxation was $74.1 \pm 5.2\%$ in control and $23.1 \pm 2.3\%$ ($P < 0.001$, $n = 7$) in apamin-incubated arteries.

Discussion

The major findings in this study can be summarized as follows: (1) the addition of exogenous L-citrulline directly causes

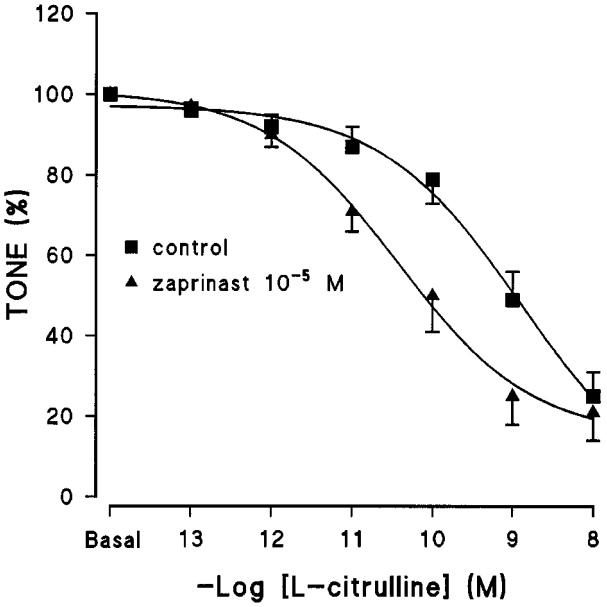

Figure 3 Effect of zaprinast 10^{-5} M on relaxation induced by L-citrulline in endothelium-denuded aorta arteries precontracted with noradrenaline 10^{-6} M. The contractions induced by noradrenaline 10^{-6} M in control and after incubation with zaprinast were 5.6 ± 0.7 g and 7.0 ± 1.3 g respectively. Each point represents the mean ± s.e.mean of seven experiments.

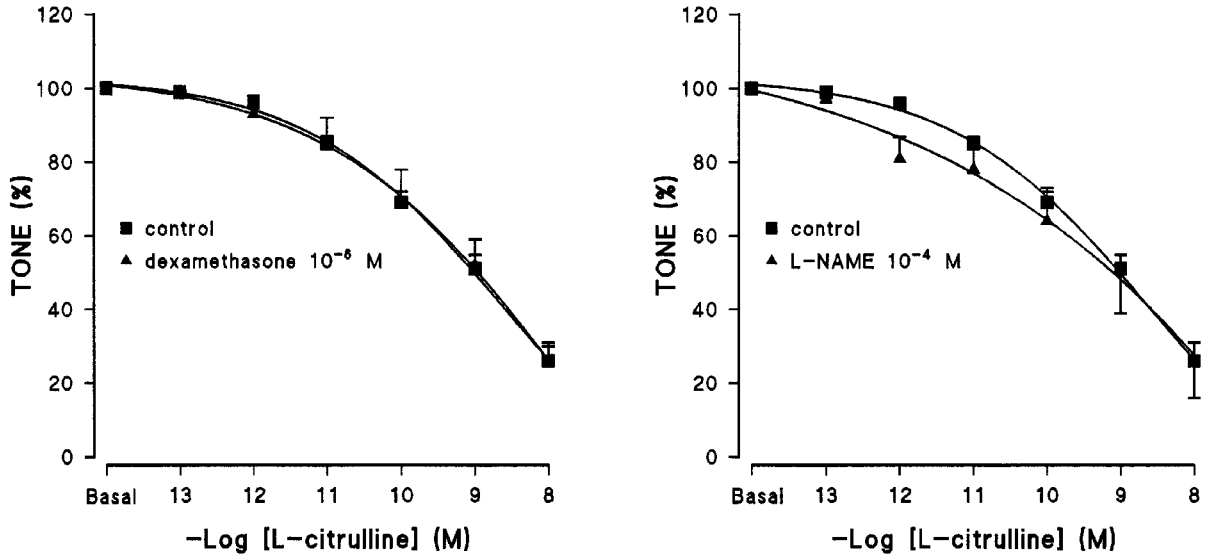

Figure 2 Effect of dexamethasone 10^{-6} M (left panel) and L-NAME 10^{-4} M (right panel) on relaxation induced by L-citrulline in endothelium-denuded aorta arteries precontracted with noradrenaline 10^{-6} M. The contractions induced by noradrenaline 10^{-6} M in control and after incubation with dexamethasone or L-NAME were 5.6 ± 0.7 g; 7.1 ± 1.2 g and 6.0 ± 1.3 g respectively. Each point represents the mean ± s.e.mean of seven experiments respectively.

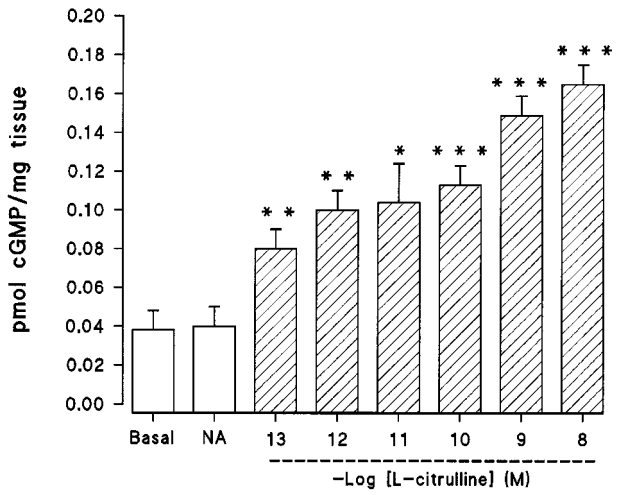

Figure 4 Levels of cGMP in aortic rings after the addition of L-citrulline ($10^{-13}-10^{-8}$ M) on a contraction induced by noradrenaline 10^{-6} M. Bars show the mean±s.e.mean of six duplicated experiments. $^{*}P<0.05$; $^{**}P<0.01$, $^{***}P<0.001$ with respect to basal situation.

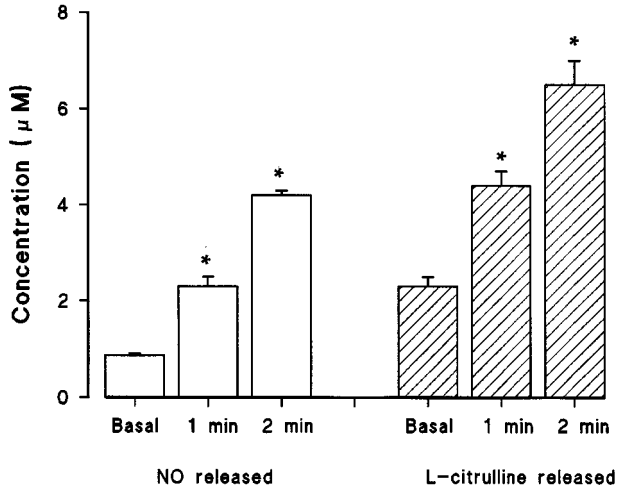

Figure 6 Concentration of L-citrulline and NO released from endothelium after 2 min stimulation with ACh 10^{-6} M. Each data represent the mean±s.e.mean of three experiments performed in duplicate. $^{*}P<0.001$.

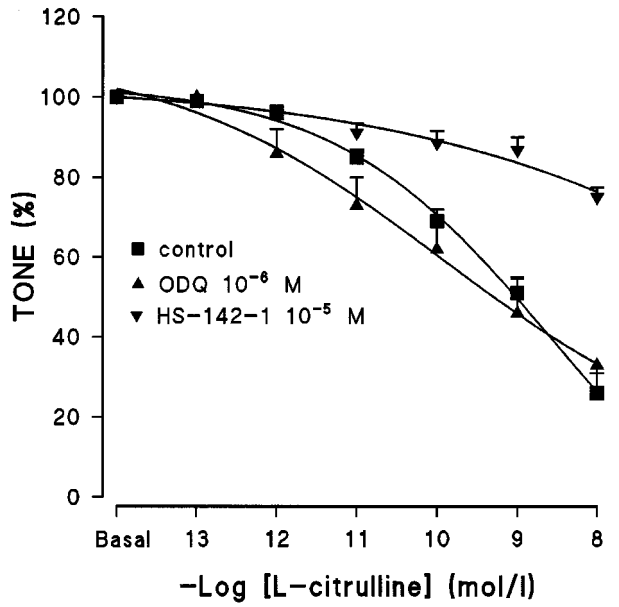

Figure 5 Effect of ODQ 10^{-6} M or HS-142-1 10^{-5} M on relaxation induced by L-citrulline in endothelium-denuded aorta arteries precontracted with noradrenaline 10^{-6} M. The contractions induced by noradrenaline 10^{-6} M in control and after incubation with ODQ or HS-142-1 were 5.6±0.7 g, 5.3±1.2 g and 6.05±1.2 g respectively. Each point represents the mean±s.e.mean of seven experiments.

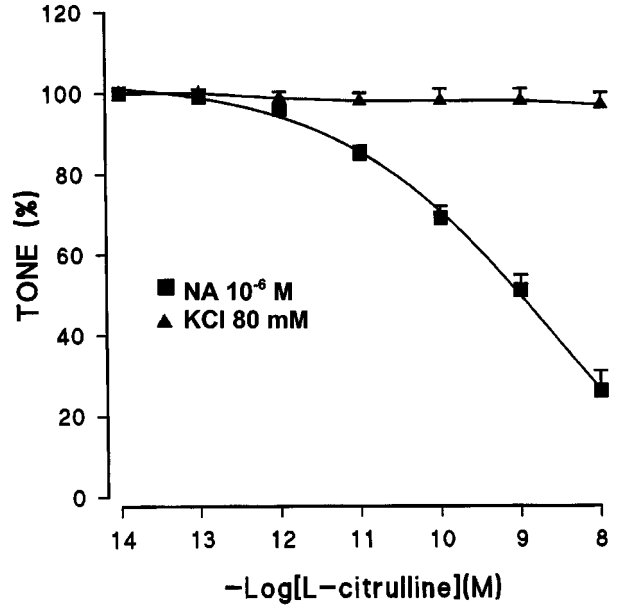

Figure 7 Relaxation induced by L-citrulline ($10^{-13}-10^{-8}$ M) in −E arteries precontracted either noradrenaline 10^{-6} M or KCl 80 mM. Each point represents the mean±s.e.mean of seven experiments.

relaxation in rabbit aorta arteries since neither dexamethasone nor L-NAME were able to block this effect; (2) that relaxation is mediated by the generation of cGMP in vascular smooth muscle and this cGMP is generated by particulate but not soluble guanylate cyclase and; (3) the cGMP formed, probably acts at least in part, by opening K_{Ca} channels leading to hyperpolarization of vascular smooth muscle cells and reduction of $[Ca^{2+}]_i$.

To our knowledge, this is the first report on the role of L-citrulline in the relaxation of vascular smooth muscle cells. We found that L-citrulline is released from endothelium in the same proportion as NO (Figure 6) as was previously described by Moncada & Palmer (1990), and the addition of exogenous L-citrulline causes relaxation in isolated rabbit aorta with or without endothelium. This fact implies that L-citrulline acts directly on vascular smooth muscle cells. It could be explained if we take into account that a particulate NO-synthase has been characterized (Pollock *et al.*, 1991). Thus, NO together with L-citrulline might be released toward the extracellular side of endothelial cells, close to vascular smooth muscle cells. This mechanism would be an easy way for endothelial cells to ensure that NO, a highly reactive compound, could exert its action on vascular smooth muscle cells modulating the vascular tone.

We tried to study in more detail the mechanism of action of L-citrulline on vascular smooth muscle and we found a cGMP-dependent mechanism. Moreover, the cGMP generated by the action of L-citrulline is not due to soluble guanylate cyclase. As

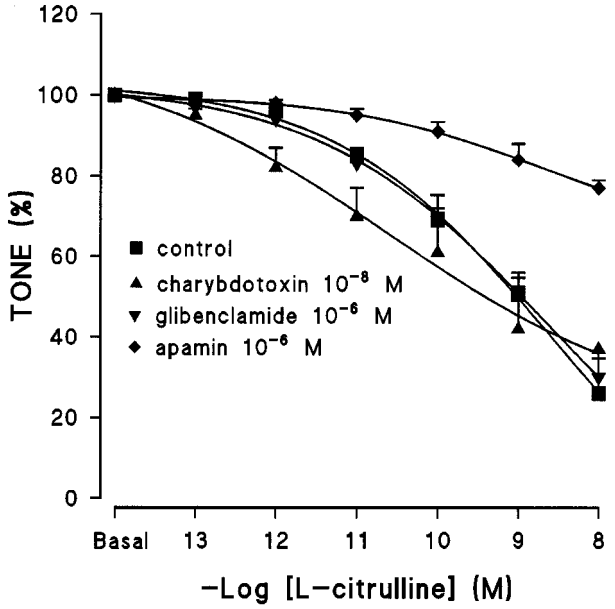

Figure 8 Effect of glibenclamide 10^{-6} M, charybdotoxin 10^{-8} M and apamin 10^{-6} M on the relaxation induced by L-citrulline in endothelium-denuded aorta arteries precontracted with noradrenaline 10^{-6} M. The contraction induced by noradrenaline 10^{-6} M in control and after incubation with glibenclamide, charybdotoxin and apamin were 5.6 ± 0.7 g, 6.7 ± 1.1 g, 8.2 ± 0.8 g and 7.2 ± 2.2 g respectively. Each point represents the mean$\pm$s.e.mean of seven experiments.

shown in Figure 5, ODQ, a specific inhibitor of that enzyme, did not affect the relaxation induced by L-citrulline. However, zaprinast (a phosphodiesterase type 5 inhibitor) enhanced this effect. Moreover, we found an increase in cGMP levels in aortic rings when they were exposed to L-citrulline (Figure 4). Although the increase in cGMP levels was statistically significant from the first concentration of L-citrulline used (10^{-13} M), onwards at this concentration we did not find a relaxant effect of L-citrulline. The lack of correlation between relaxation and cGMP-levels could be due to the existence of a threshold in the cGMP-levels. The last facts indicate that L-citrulline produces an increase in the cGMP account of vascular smooth muscle and that this increase is not due to soluble guanylate cyclase.

In most cell types, guanylate cyclase is present in two locations. The guanylate cyclase isoenzyme associated with the membrane fraction is known as the particulate isoenzyme, whereas the soluble isoenzyme is present in the cytosol (Waldman & Murad, 1988). The increase in cGMP in vascular smooth muscle cells caused by L-citrulline seems to be mediated by the action of the particulate guanylate cyclase since HS-142-1 (a specific inhibitor of the particulate guanylate cyclase) decreased the relaxant effect of L-citrulline. Particulate guanylate cyclase is the target of natriuretic peptides. These compounds comprise a family of distinct peptides: atrial (ANP), brain (BNP), myocardial (Needleman *et al.*, 1989; Ogawa *et al.*, 1991) and C-type (CNP) natriuretic peptides of endothelial cells (Heublein *et al.*, 1992). Specifically, ANP and BNP are ligands for the natriuretic peptide-A receptor while CNP is the ligand for the natriuretic peptide-B receptor. Thus, these peptides may function in parallel with the L-arginine-NO pathway (Supaporn *et al.*, 1996).

Molecular targets for cGMP include protein kinases (cGMP kinases) and ion channels (Lincoln & Cornwell, 1993). It appears that no single mechanism explains all of the effects of cGMP on relaxation in the variety of systems examined. One of these mechanisms could be stimulation of K^+-channels leading to hyperpolarization of the smooth muscle cell membrane and reduction of $[Ca^{2+}]_i$ (Chen & Rembold, 1992).

Endothelium-dependent hyperpolarization of smooth muscle is produced by an increase in K^+ conductance of the cell membrane (Bolton *et al.*, 1984; Chen & Suzuki, 1989) and the amplitude of the hyperpolarization is related, although nonlinearly, to extracellular $[K^+]$. K^+-channel activity is the main determinant of membrane potential, and K^+ efflux, resulting from K-channel opening, inhibits voltage-gated Ca^{2+}-channels and promotes relaxation. In this paper we have shown that the relaxation induced by L-citrulline is abolished when there is an extracellular potassium overload. As shown in Figure 7, when the aortic rings were precontracted with KCl 80 mM, L-citrulline was not able to produce relaxation. Several experiments suggest that hyperpolarization of vascular smooth muscle is a mechanism shared by endothelium-dependent relaxing factors. NO itself hyperpolarizes vascular smooth muscle in many (Tare *et al.*, 1990; Krippeit-Drews *et al.*, 1992) but not all (Komori *et al.*, 1988; Brayden, 1990) studies. In this paper we investigated which type of K^+-channel was implicated in the relaxant effect of L-citrulline on vascular smooth muscle and we found that K_{Ca}-channels seem to be implicated in this effect since apamin, a specific small-conductance K_{Ca}-channel blocker, significantly decreased the relaxation induced by L-citrulline, whereas glibenclamide, an ATP-dependent K^+ channel blocker, and charybdotoxin, a large-conductance K_{Ca}-channel blocker, did not affect the relaxant effect of L-citrulline.

Finally, L-citrulline, the byproduct in the synthesis of NO, may play a role in the control of vascular tone since its causes relaxation of vascular smooth muscle. These facts may indicate that L-citrulline acts by itself or strengthens the action of other compounds, such as relaxant factors which activate particulate guanylate cyclase. We conclude that L-citrulline causes relaxation directly in rabbit aorta arteries since neither dexamethasone nor L-NAME were able to block this effect; this relaxation is mediated by the generation of cGMP in rabbit aortic rings and this cGMP is generated by particulate guanylate cyclase. Moreover, the cGMP formed acts, at least in part, by opening K_{Ca} channels leading to hyperpolarization of vascular smooth muscle cells and reduction of $[Ca^{2+}]_i$. The above mentioned fact is further evidence that both compounds (NO and L-citrulline) might have complementary actions.

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