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Citrulline does not relax isolated rat and rabbit vessels

[1]Stephen Marx, *,[1]Yuri Vedernikov, [1]George R. Saade & [1]Robert E. Garfield

[1]Department of Obstetrics & Gynecology, Divisions of Reproductive Sciences and Maternal-Fetal Medicine, The University of Texas Medical Branch, 301 University Boulevard, Galveston, Texas, TX 77555-1062, U.S.A.

1 The study was prompted by the report of Ruiz E. & Tejerina T., 1998 describing endothelium-independent relaxation by L-citrulline *via* activation of particulate guanylate cyclase.

2 We compared the effects of L-citrulline and L-arginine in isolated aortic rings of rats and in isolated aortic, carotid and femoral artery rings of rabbits.

3 No significant relaxation to either L-citrulline or L-arginine was found in the concentration range of 10^{-12} to 10^{-3} M, while 3-morpholinosydnonimine hydrochloride (SIN-1, 10^{-6} M) relaxed vascular tissues.

4 This study does not support the conclusion that L-citrulline has direct vasorelaxing action on vascular smooth muscle.

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Abbreviations: ACh, acetylcholine; IND, indomethacin; KHS, Krebs-Henseleit solution; NA, noradrenaline; NLA, N^{ω}-nitro-L-arginine; NO-nitric oxide; SIN-1, 3-morpholinosydnonimine hydrochloride

Introduction

L-arginine is a precursor for nitrate/nitrite synthesis (Hibbs *et al*., 1987; Palmer *et al*., 1988) and the endothelium-derived relaxing factor/nitric oxide (Furchgott & Zawadski, 1980). Data on the acute relaxing effect of L-arginine are variable from weak and mild to very pronounced (Alonso *et al*., 1998; Briones *et al*., 1999) in cerebral vessels, especially in hypertensive rats. L-arginine relaxes aortic rings from hypertensive, but not from normotensive rats (Puci *et al*., 1995; Wu *et al*., 1996). The abnormal state of iNOS was suggested as a substrate for L-arginine effects (Wu *et al*., 1996).

L-arginine relaxes isolated mammalian vessels after prolonged, but not short-term incubation in physiological buffers (Schini *et al*., 1990; Wood *et al*., 1990). This discrepancy was initially attributed to depletion of L-arginine, but now is explained by induction of iNOS in the vascular smooth muscle by endotoxin present in the incubation medium (Rees *et al*., 1990; Moncada *et al*., 1991). Infusion of L-arginine, however, decreases arterial blood pressure in healthy (Bode-Boger *et al*., 1998, 1999) and hypertensive humans (Hagashi *et al*., 1999; Marin & Rodrigues-Martinez, 1997).

L-citrulline is a by-product of the formation of NO from L-arginine (Moncada *et al*., 1991). L-citrulline may be recycled to L-arginine, thereby maintaining adequate L-arginine levels required for the production of NO (Hecker *et al*., 1990; Sessa *et al*., 1990; Nussler *et al*., 1994; Norris *et al*., 1995; Wu & Morris, 1998).

Recently inhibition of noradrenaline-induced contraction in endothelium denuded rabbit aortic rings by L-citrulline *via* activation of particulate guanylate cyclase was reported (Ruiz & Tejerina, 1998). This study may have important implications as L-citrulline could represent an important vasoregulatory mechanism.

Prompted by these results, we aimed to confirm the Ruiz & Tejerina study and to determine if a similar mechanism of citrulline-induced relaxation exists in isolated vessels from other species. The experiments were designed to study the potential mechanisms of L-citrulline-induced relaxation in isolated rat thoracic aorta as well as rabbit aortas, carotid and femoral arteries.

*Author for correspondence.

Methods

Five nonpregnant female Sprague-Dawley rats (9-weeks-old, 250–300 g weight) were euthanized by CO_2 inhalation. Four white male New Zealand rabbits (2.5–3.5 kg) were euthanized by translocation of the cervical spine followed by exsanguination from the common carotid artery. All procedures were approved by the Animal Care and Use Committee of the University of Texas Medical Branch at Galveston.

Rat thoracic aorta was excised, placed in cold Krebs-Henseleit solution (KHS), cleaned of surrounding tissue and cut into eight rings ($\sim$ 4 mm in width). Rings of rabbit aorta, carotid and femoral arteries were similarly prepared. Vascular rings were positioned in 10 ml organ chambers between two tungsten wire (250 μm diameter) stirrups. One stirrup was fixed to the bottom of the chamber, and the other was connected to the isometric force transducer (Harvard Apparatus, South Natick, MA, U.S.A.). Rings were equilibrated at 2 g passive tension for 60 min in KHS. The organ chambers were thermostatically maintained at 37°C and continuously bubbled with 5% carbon dioxide in air (pH $\sim$ 7.4). The bathing solution was changed every 15 min during the equilibration period.

Changes in isometric tension were recorded on-line and stored in a desktop computer using DI-220 data acquisition system and Windaq/200 software (Data Q Instruments Inc., Akron, OH, U.S.A.).

Experimental design

Following a protocol that was similar to that of Ruiz & Tejerina (1998), rings of rat aorta were contracted with noradrenaline (NA, 10^{-7} M) and the integrity of the endothelium was checked by the ability of acetylcholine (ACh,

10^{-6} M) to induce relaxation. The rings were then washed, given 60 min rest, incubated for an additional 45 min with inhibitors of cyclo-oxygenase (indomethacin; IND, 10^{-5} M) and/or nitric oxide synthase (N^{ω}-nitro-L-arginine; NLA; 10^{-4} M). The rings were then contracted with noradrenaline (10^{-6} M) and the relaxation induced by cumulative concentrations of L-citrulline was determined and compared with those of L-arginine and D-arginine. Agents were added to the organ chamber in the volume of 10 μL, except for the final concentration which was 90 μL. After completion of the concentration response experiments, 3-morpholinosydnonimine hydrochloride (SIN-1, 10^{-6} M final concentration in the organ chamber) was added.

The experimental protocol employed in the study of rabbit vascular rings was similar except the rings were not preincubated with the cyclo-oxygenase or nitric oxide synthase inhibitors and only the relaxant effects of L-citrulline and L-arginine in noradrenaline (10^{-6} M) contracted tissues were studied.

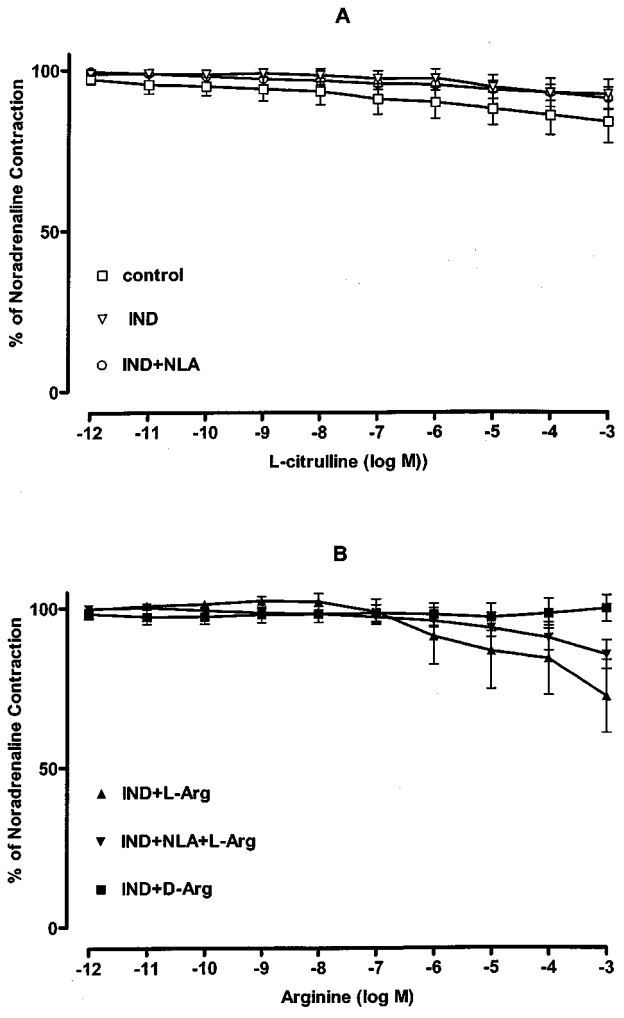

Figure 1 Efects of L-citrulline (A) and L-arginine and D-arginine (B) in rat aortic rings contracted with noradrenaline (NA, 10^{-6} M). (A) Concentration-response curves (CRC) to L-citrulline in the absence or presence of indomethacin (IND, 10^{-5} M) or indomethacin + N^{ω}-nitro-L-arginin (10^{-4} M, IND + NLA) Relaxation induced by SIN-1 (10^{-6} M) after completion of CRC was 82.8 ± 5.7% of NA tension (pooled data). (B) CRC to L-arginine in the presence of IND or IND + NLA, and to D-arginine in the presence of IND, in rat aortic rings precontracted with NA (10^{-6} M). Relaxation induced by SIN-1 (10^{-6} M) in after completion of CRC was 82.8 ± 5.7% of NA tension (pooled data).

Data analysis and statistics

Relaxation is expressed as per cent decrease in tension induced by noradrenaline. Results are presented as means ± s.e.mean. The statistical comparisons of the means were made by unpaired Student's *t*-test (GraphPad Prizm 2, San Diego, CA, U.S.A.). A $P<0.05$ was used to indicate a significant effect of the agent.

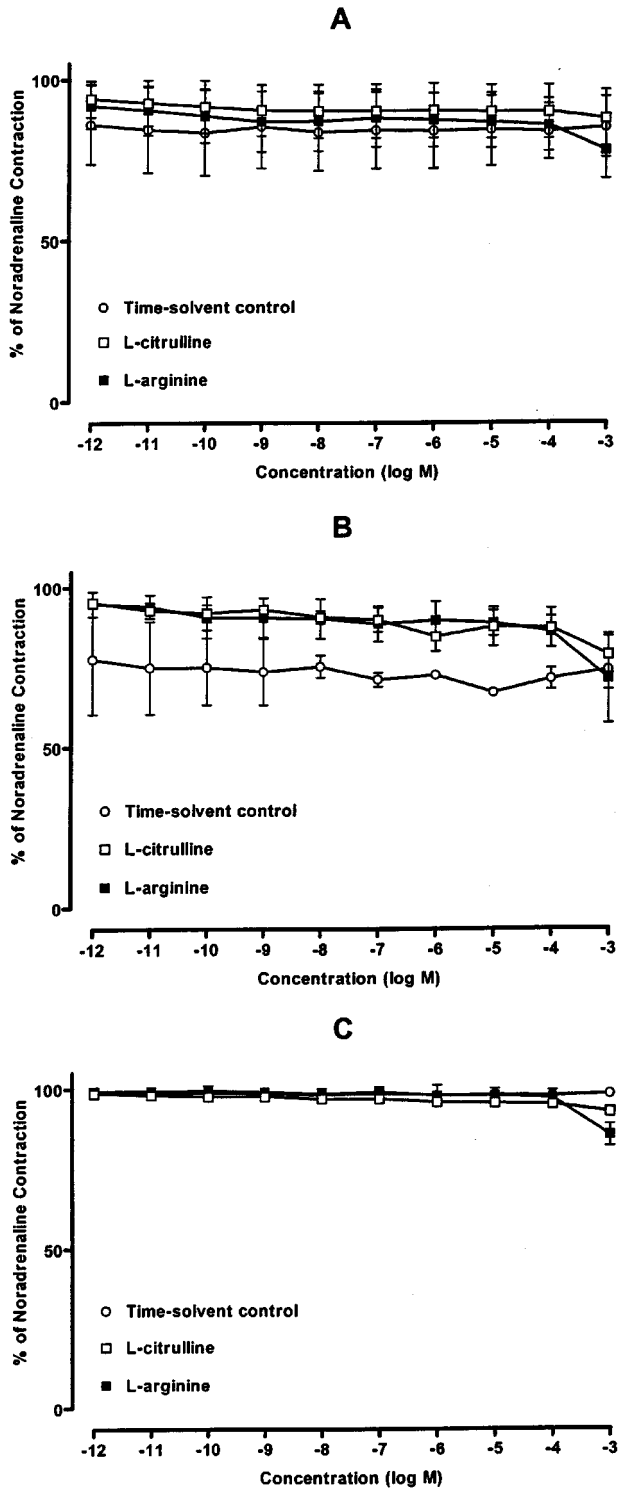

Figure 2 Concentration-response curves (CRC) to L-citrulline, L-arginine in noradrenaline (NA, 10^{-6} M)-contracted rabbit aortic (A), carotid (B) and femoral (C) artery rings. Relaxation induced by SIN-1 (10^{-6} M) after completion of CRC was 80.4 + 12.3% of NA tension (pooled data).

Drugs and solutions

KHS contained (in mM): sodium chloride 119.0, potassium chloride 4.7, potassium phosphate 1.2, sodium bicarbonate 25, magnesium sulphate 1.2, calcium chloride 2.5, sodium ethylenediamine tetra acetic acid 0.026, glucose 11.5. Noradrenaline,[(−)-arterenol bitartrate], L-arginine, D-arginine, L-citrulline, indomethacin, N^{ω}-nitro-L-arginine and all the ingredients of Krebs buffer were purchased from Sigma Chemical Co. (St. Louis, MO). 3-morpholinosydnonimine hydrochloride (SIN-1) was purchased from RBI (Natick, MA, U.S.A.). Stock solution of noradrenaline (10^{-2} M) was made with 0.1% ascorbic acid and the needed dilutions were made using Krebs buffer containing 0.01% ascorbic acid. All solutions were freshly prepared before the experiment.

Results

L-citrulline in the concentration range 10^{-12} to 10^{-3} M had no significant relaxing effect in rat aortic rings contracted with noradrenaline (Figure 1A). Similar results were seen with L-arginine and D-arginine (Figure 1B). The nitric oxide donor SIN-1, however, relaxed rat aortic rings by ~89% of noradrenaline-induced tension. In addition, neither L-citrulline nor L-arginine had any relaxing effects on rabbit aortic, femoral or carotid artery rings, while SIN-1 relaxed them by ~80% (Figure 2).

Discussion

The results of the present study indicate that L-citrulline lacks a vascular relaxant effect in rat and rabbit vessels.

The absence of relaxation of vascular rings by L-arginine and its inactive stereoisomere D-arginine was expected given the previous reports in fresh isolated vascular smooth muscle (Moncada *et al*., 1991). The data also demonstrate that it is unlikely that L-citrulline is a direct vascular relaxant.

In other experiments using isolated perfused rat uterine vascular beds we also were unable to detect an inhibitory effects of either L-citrulline or L-arginine (10^{-3} M) on intraluminal pressure increased by phenylephrine, or L-NAME. However, both L-citrulline and L-arginine decreased systolic blood pressure measured by tail cuff method in spontaneously hypertensive and L-NAME treated rats (unpublished data).

In contrast, Ruiz & Tejerina reported a maximal inhibitory response to L-citrulline of about 70% at a concentration of 10^{-8} M (Ruiz & Tejerina, 1998), a concentration of L-citrulline even lower than its plasma level in adult rats (6.2×10^{-6} M; Wu & Morris, 1998).

The discrepancy between our results and the data presented by Ruiz & Tejerina may be due to some differences in the methodology. The following are some of the points which may account for the differences between the two studies: (1) The difference in the time of equilibration period, 60 min in our experiments and 90–120 min in Ruiz & Tejerina's study, may not result in differences in the responsiveness. However, the bathing solution was changed every 15 min during the equilibration period in our experiments. Changing the bathing solution during the equilibration period is an essential requirement in isolated vessel studies in order to remove metabolites formed by the tissue during the adaptation to a new environment. (2) The difference in the composition of the solution used for incubation of the tissues may have a very significantly impact on the responses. The solution Ruiz & Tejerina used does not contain phosphates, an important pH buffer. The authors did not specify what was their solution's pH. In a solution with low buffering capacity pH becomes unstable. (3) The Ruiz & Tejerina's organ chamber solution was aerated with the gas mixture of 95% O_2-5% CO_2. This would have resulted in a partial oxygen pressure about 650 mm Hg. In our solution, aerated with 5% CO_2 in air, partial oxygen pressure would have been about 150 mmHg. (4) The content of $CaCl_2$ in Ruiz & Tejerina's solution is half that in modified Krebs-Henseleit solution used in our experiments. Ruiz & Tejerina used Godfraind's solution. This solution is not often used in physiological-pharmacological experiments, but has been used particularly in studies of calcium channel blockers. (5) In our experiments indomethacin was added to the solution in order to block cyclooxygenase activity, a routine approach when investigating the nitric oxide-cyclic GMP pathway involvement. Dexamethasone was used by Ruiz & Tejerina to prevent possible induction of iNOS. (6) Ruiz & Tejerina write 'The aorta rings were mounted under 2 g tension. Each preparation was allowed to equilibrate for 90–120 min.' It is not clear whether or not passive tension applied was readjusted to 2 g during the equilibration period, or the rings were placed at 2 g tension and then allowed to equilibrate. In our experiments, the tension was gradually adjusted during the equilibration period in order to reach a final passive tension of 2 g. (7) Our experiments were carried out in endothelium-preserved rings. Removal of the endothelium shifts the concentration-relaxation relationships to nitric oxide to the left (Shirasaki & Su, 1985; Shirasaki *et al*., 1986), most likely secondary to removal of inhibitory effect of basaly released nitric oxide. Thus the lack of a direct relaxing effect by L-arginine or L-citrulline could not have been due to inhibition by the presence of endothelium. Moreover, chemical inactivation of endothelial NOS did not influence the responses to either L-citrulline or L-arginine. (8) Instability of the noradrenaline due to oxidation in hyperoxygenated solution and changes in pH after addition of L-citrulline may have caused a decaying contraction. Although Ruiz & Tejerina noted that '10^{-4} M of ascorbic acid was added to each daily prepared solution of noradrenaline'. In our experiments, stock solutions of noradrenaline were prepared in 0.1% solution of ascorbic acid in double distilled water and the subsequent dilutions were made using 0.01% ascorbic acid in Krebs buffer containing 0.026 mM of sodium ethylenediamine tetra acetic acid (also an antioxidant).

The possible reason for decay in contraction may also be dilution of noradrenaline after addition of L-citrulline solution (the volume of the solution added was not specified in the paper).

Thus considering the aforementioned criticisms, we therefore conclude that L-citrulline does not have a direct relaxing effect in rat and rabbit aortic rings and rabbit femoral and carotid artery rings contracted by noradrenaline and bathed in KHS.

References

ALONSO, M.J., RODRIGUEZ-MARTINEZ, A., MARTINEZ ORGADO, J., MARIN J. & SALAICES M. (1998). The L-arginine inhibition of rat middle cerebral artery contractile response is mediated by inducible nitric oxide synthase. *J. Auton. Pharmacol.,* **18,** 105–113.

BODE-BOGER, S., BOGER, R.H., GALLAND, A., TSIKAS, D. & FROLICH, J.C. (1998). L-arginine-induced vasodilation in healthy humans: Pharmacokinetic-pharmacodynamic relationship. *Br. J. Clin. Pharmacol.,* **46,** 489–497.

BODE-BOGER, S., BOGER, R.H., LOFFLER, M., TSIKAS, D., BRABAND, G. & FROLICH, J.C. (1999). L-arginine stimulates NO-dependent vasodilation in healthy humans-Effect of somatostatin pretreatment. *J. Investig. Med.,* **47,** 43–50.

BRIONES, A.M., ALONSO, M., MARIN, J. & SALAICES, M. (1999). Role of iNOS in the vasodilator responses induced by L-arginine in the middle cerebral artery from normotensive and hypertensive rats. *Br. J. Pharmacol.* **126,** 111–120.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature,* **288,** 373–376.

HAGASHI, Y., OSHIMA, T., OZONO, R., MATSUURA, H., KAMBE, M. & KAJIYAMA, G. (1999). Effect of l-arginine Hypertens. **12,** 8–15.

HECKER, M., SESSA, W.C., HARRIS, H.J., ANGGARD, E.E. & VANE, J.R. (1990). The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 8612–8616.

HIBBS, J.B.N., TAINTOR, R.R. & VARVIN, Z. (1987). Macrophage cytotoxicity: role for L-arginine deaminase activity and imino nitrogen oxidation to nitrite. *Science,* **235,** 473–476.

MARIN, J. & RODRIGUEZ-MARTINEZ, M.A. (1997). Role of nitric oxide in physiological and pathological conditions. *Pharmacol. Ther.,* **75,** 111–134.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: Physiology, Pathophysiology, and Pharmacology. *Pharmacol Rev.,* **43,** 109–142.

NORRIS, K.A., SHRIMPF, J.E., FLYNN, J.L. & MORRIS, JR S.M.. (1995). Enhancement of macrophages microbicidal activity: Supplemental arginine and citrulline augment nitric oxide production in murine peritoneal macrophages and promote intracellular killing of Trypanosoma cruzi. *Infect. Immunol.* **63,** 2793–2796.

NUSSLER, A.K., BILLAR, T.R., LIU, Z.Z. & MORRIS, JR S.M.. (1994). Coinduction of nitric oxide synthase and argininosuccinate synthase in murine macrophage cell line. *J. Biol. Chem.,* **269,** 1257–1261.

PALMER, R.M.J., REES, D.D. & MONCADA, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature (London),* **333,** 664–666.

PUCI, M.L., DICK, L.B., MILLER, K.B., SMITH, C.J. & NASJLETTI, A. (1995). Enhanced responses to L-arginine in aortic rings from rats with angiotensin-dependent hypertension. *J. Pharmacol. Exp. Ther.,* **274,** 1–7.

REES, D.D, CELLEK, S., PALMER, R.M.J. & MONCADA, S. (1990). Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxic shock. *Biochem. Biophys. Res. Commun.,* **173,** 541–547.

RUIZ, E. & TEJERINA, T. (1998). Relaxant effects of L-citrulline in rabbit vascular smooth muscle. *Br. J. Pharmacol.,* **125,** 186–192.

SESSA, W.C., HECKER, M., MITCHELL, J.A. & VANE, J.R. (1990). The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: L-glutamine inhibits the generation of L-arginine by cultured endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 8607–8611.

SCHINI, V.B., BOND, R.A. & VANHOUTTE, P.M. (1990). l-arginine induces endothelium-dependent and independent relaxations of tolerant rat aortas. *Arch. Int. Pharmacol. Ther,* **305,** 227.

SHIRASAKI, Y. & SU, C. (1985). Endothelium removal augments vasodilation by sodium nitroprusside and sodium nitrate. *Eur. J. Pharmacol.,* **114,** 93–96.

SHIRASAKI, Y., S.U, C., LEE, T.J.-F., KOLM, P., CLINE, JR. W.H. & NICKOLS, G.A. (1986). Endothelial modulation of vascular relaxation to nitrovasodilators in aging and hypertension. *J. Pharmacol. Exp. Ther.,* **239,** 861–866.

WOOD, K.S., BUGA, C.M., BYRNS, R.E. & IGNARRO, L.J. (1990). Vascular smooth muscle-derived relaxing factor (mdrf) and its close similarity to nitric oxide. *Biochem. Biophys. Res. Commun.,* **170,** 80–87.

WU, C.-C. HONG, H.-J., CHOU, T.C., DING, Y.-A. & YEN, M.-H. (1996). Evidence for inducible nitric oxide synthase in spontaneously hypertensive rats. *Biochem. Biophys. Res.Commun.,* **228,** 459–466.

WU, G. & MORRIS, S.M. (1998). Arginine metabolism: nitric oxide and beyond. *Biochem. J.,* **336,** 1–17.

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