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L-Citrulline mediated relaxation in the control and lipopolysaccharide-treated rat aortic rings

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Abstract

The present study was undertaken to investigate relaxant effect of L-citrulline in phenylephrine precontracted endothelium intact thoracic aortic rings obtained from control or lipopolysaccharide (1 mg/kg)-treated rats. L-Citrulline produced $40 \pm 3\%$ ($n = 36$) and $60 \pm 5\%$ ($n = 24$) relaxations in control and lipopolysaccharide-treated rings, respectively. Nitric oxide (NO) release and cyclic guanosine-3′,5′-monophosphate levels from the rings were also increased following treatment with L-citrulline. Inhibition of guanylate cyclase, L-citrulline recycling to L-arginine or denudation of the endothelium, significantly reduced L-citrulline-induced relaxations both in control and lipopolysaccharide-treated rings. Treatment of rings with protein synthesis inhibitors prevented relaxations to L-citrulline. Inhibitor of Ca^{2+}-activated K^+ channels, tetrabutylammonium or precontraction of the rings with KCl (80 mM), significantly attenuated L-citrulline mediated relaxations in control and lipopolysaccharide-treated rings. Thus, L-citrulline seems to exert significant relaxation by supplementing the release of NO due to its recycling to L-arginine, which gets further augmented after lipopolysaccharide treatment.
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Keywords: L-Citrulline; L-Arginine; Nitric Oxide (NO); Vascular relaxation; Lipopolysaccharide; Aortic ring, rat

1. Introduction

Role of nitric oxide (NO) formed from semi-essential amino acid L-arginine on conversion to L-citrulline in the presence of enzyme, nitric oxide synthase (NOS) is well established in various physiological systems (Moncada et al., 1991; Anggard, 1994). Endogenous NO subserves important mechanisms of circulatory control by maintaining blood pressure within the physiological range. Though involvement of endogenous NO in regulating the vascular tone is well defined (Moncada et al., 1991), the role of L-citrulline, the co-product of NO synthesis, however remains controversial (Schini and Vanhoutte, 1991; Ruiz and Tejerina, 1998; Marx et al., 2000).

Relaxations to L-citrulline were observed in the rat aorta (Ruiz and Tejerina, 1998), while others failed to observe such relaxations in the isolated rat or rabbit arteries (Schini and Vanhoutte, 1991; Marx et al., 2000). Moreover, there are no reports describing the physiological role of L-citrulline in endotoxic shock, a condition that leads to substantial NO production and concomitantly, equimolar amounts of L-citrulline.

Hence, the present study was undertaken to investigate the effect of L-citrulline in control and lipopolysaccharide-treated rat aortic rings. The results will help to clarify the controversy underlying the relaxation potential of L-citrulline in isolated rat thoracic aortic rings and might also help in understanding the role of L-citrulline in endotoxaemia.

2. Methods

2.1. Experimental protocol

Male Sprague–Dawley rats (National Laboratory Animal Centre of Central Drug Research Institute) weighing 200–225 were given by intraperitoneal injection, lipopolysaccharide in a dose of 1 mg/kg, 4 h prior to in vitro vascular reactivity. Control animals received equivalent volumes of vehicle or 0.9% normal saline. Protocols used in the present study were in accordance with European Community guidelines for the use of experimental animals and were approved by the Institutional Ethics Committee.

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2.2. In vitro vascular reactivity

Rats were anaesthetized by inhalation of ether. After opening the chest thoracic aorta was excised and immediately placed in ice-cold Krebs bicarbonate medium of the following composition (mM): NaCl 118; KCl 5; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25; Glucose 11; disodium EDTA 0.030; pH 7.4. It was dissected free of connective tissue and fat and was cut into rings (5 mm in length). Rings of the same animal were divided into two groups and in one group the endothelium was denuded by rubbing the intimal surface of the aorta with a fine forceps. The rings were mounted vertically between two stirrups in organ chambers filled with 10 ml Krebs solution maintained at 37 ± 0.5 °C, constantly bubbled with 95%O_2–5%CO_2. One stirrup was connected to an anchor and the other to a force transducer for recording of isometric tension (FT 030, Grass Instruments, USA). The rings were equilibrated for 90 min, during which the bathing fluid was changed every 15 min and the tissue was kept under a constant tension of 2 g through out the experiment (Hegde et al., 1998; Srivastava et al., 1998; Imaoka et al., 1999).

After equilibration, the rings were evaluated for the presence of a functional endothelium. The aortic rings were contracted with submaximal concentration of phenylephrine [10^{-7} M, approximate EC_{80} evaluated from concentration response curve to phenylephrine (10^{-9} to 10^{-5} M)] and relaxation responses to acetylcholine (10^{-6} M) were obtained. Rings with functional endothelium exhibited significant relaxation whereas endothelium denuded rings lacked any relaxation to acetylcholine.

The aortic rings were then contracted with phenylephrine (10^{-7} M), when the plateau was achieved; L-citrulline (10^{-13} M to 10^{-5} M) was added in a cumulative manner. The plateau responses obtained with phenylephrine remained stable over at least 60 min and additions of equal volumes of vehicle did not produce significant relaxations. The same protocol was adopted for the endothelium denuded rings and in the rings from lipopolysaccharide-treated animals. Relaxation responses were also evoked for L-arginine according to the same protocol. In one group of experiments, the rings were incubated with 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (1 μM, a soluble guanylyl cyclase inhibitor) for 30 min prior to the contraction by phenylephrine and relaxation responses were obtained. In one group of experiments, the aortic rings were incubated in Krebs solution containing the cyclooxygenase inhibitor, indomethacin (10^{-5} M), and L-citrulline-induced relaxations were carried out in these rings.

Aortic rings from control and lipopolysaccharide-treated rats were incubated with L-glutamine (10 mM), an inhibitor of argininosuccinate synthetase (Sessa et al., 1990), for 30 min prior to the relaxation by L-citrulline 10^{-13} to 10^{-5} M. Similarly, the aortic rings were incubated with inhibitors of protein synthesis, corticosterone (1 μM) and cycloheximide (100 μM) (Eckly-Michel et al., 1999), and inhibitor of iNOS, aminoguanidine (300 μM) (Das et al., 1999), and relaxations to L-citrulline were evoked.

In another group of experiments, to check the participation of K^+ channels in the mechanism of relaxation of these agents, the aortic rings from control rats were contracted with KCl (80 mM) solution (obtained by equimolar replacement of NaCl with KCl in the Krebs bicarbonate solution) and cumulative relaxation responses were obtained by adding aliquots of L-citrulline/L-arginine from 10^{-13} to 10^{-5} M. Aortic rings were also incubated with a blocker of Ca^{2+}-activated K^+ channels, tetra butyl ammonium (1 mM), for 30 min prior to the phenylephrine precontraction.

2.3. Measurement of NO released from rat aortic rings

Nitrite, the metabolite of NO, was measured according to the method of Castillo et al. (1999). Aortic rings were washed in Krebs solution and divided into different experimental groups, which were treated with L-citrulline (100 μM) or L-arginine (100 μM). Superoxide dismutase (100 U/ml) and catalase (100 U/ml) were added to the rings and incubated at 37 °C in a shaker for 30 min. After reduction of NO_3^- to NO_2^- using nitrate reductase (0.1 U) over 30 min, the ring supernatants were passed through a 0.45-μm Whatman membrane filters. Equal volumes of Griess reagent (1% sulfanilamide and 0.1% naphthylethylene diamine in 5% phosphoric acid) were added and the mixture was incubated at 37 °C for 30 min; absorbance was read at 548 nm on a spectrophotometer to obtain the total nitrite content. Data are expressed as nM/mg dry weight of tissue over 30 min. Nitrite contents were also measured in some rings incubated with L-glutamine (10 mM) alone or both L-glutamine (10 mM) and L-citrulline (100 μM).

2.4. Measurement of cGMP levels

After obtaining the maximal relaxations to L-citrulline/L-arginine, the aortic rings were removed, frozen in liquid nitrogen, weighed and stored at −70 °C until homogenized in 0.5 ml of 10% trichloroacetic acid. The homogenate was centrifuged at $10{,}000 \times g$ 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 enzyme immunoassay kit of Cayman Chemical (USA). Data were expressed as fM cGMP/mg of tissue.

2.5. Chemicals

Corticosterone HBC complex was obtained from RBI. All other reagents used in this study were obtained from Sigma (St. Louis, MO, USA). Phenylephrine, L-citrulline, L-arginine were prepared as 10-mM stock solutions and

necessary dilutions were carried out at the time of the study. Corticosterone HBC complex equivalent to corticosterone, cycloheximide, indomethacin were weighed and diluted to the required concentration in the Krebs solution.

2.6. Statistical analysis

The contraction obtained to 10^{-7} M phenylephrine was considered as 100%. Subsequent relaxations observed on cumulative additions of L-citrulline were expressed as % of this phenylephrine precontraction. All values used in the analyses represent mean ± S.E.M. of at least eight rats in each group. Comparison between relaxations at individual concentrations for different groups under study were performed by Student's '*t*'-test and one-way ANOVA followed by Bonferroni multiple comparison test and differences were considered significant when $P < 0.05$.

3. Results

3.1. L-Citrulline-induced relaxations in control and lipopolysaccharide-treated aortic rings

Addition of L-citrulline (10^{-13} to 10^{-5} M) to the phenylephrine precontracted rings produced relaxation in a concentration-dependent manner (Fig. 1A), the maximum relaxation being 40 ± 3% ($n = 36$) in endothelium intact rat thoracic aortic rings observed approximately over a duration of 45 min. Pre-treatment of the aortic rings with a NO-sensitive guanylyl cyclase inhibitor, ODQ (1 μM), significantly attenuated the sensitivity and maximal relaxations to L-citrulline, the maximal relaxations being 15 ± 2% (Fig. 1A), indicating the involvement of a NO-sensitive guanylyl cyclase in these relaxations (Table 1). However, indomethacin pre-treatment did not alter the relaxations to L-citrulline (data not shown). Isolated aorta from the lipopolysaccharide-treated rats showed augmented relaxations to L-citrulline, the maximal relaxations being 60 ± 5% (Fig. 1A). Denudation of the endothelium only partially lowered the relaxations in control but significantly attenuated the maximal relaxations to L-citrulline in lipopolysaccharide-treated rings and the sensitivity of the rings to L-citrulline was also significantly reduced in the denuded rings from lipopolysaccharide-treated rats (Table 1 and Fig. 1B). Inhibition of NO-sensitive guanylyl cyclase in the lipopolysaccharide-treated rings significantly inhibited the maximal relaxations to L-citrulline and the relaxation profiles were similar to those from control rats.

3.2. Release of NO from control and lipopolysaccharide-treated rat aortic rings

Nitrite, an indicator of NO production, was significantly increased in L-citrulline (100 μM)-treated rings in comparison to the control rings. Levels of nitrite on L-citrulline treatment were comparable to the L-arginine (100 μM)-treated rings (Fig. 2). Nitrite content was increased further in the lipopolysaccharide-treated rat aortic rings following L-citrulline or L-arginine treatment in comparison to their respective controls (Fig. 2).

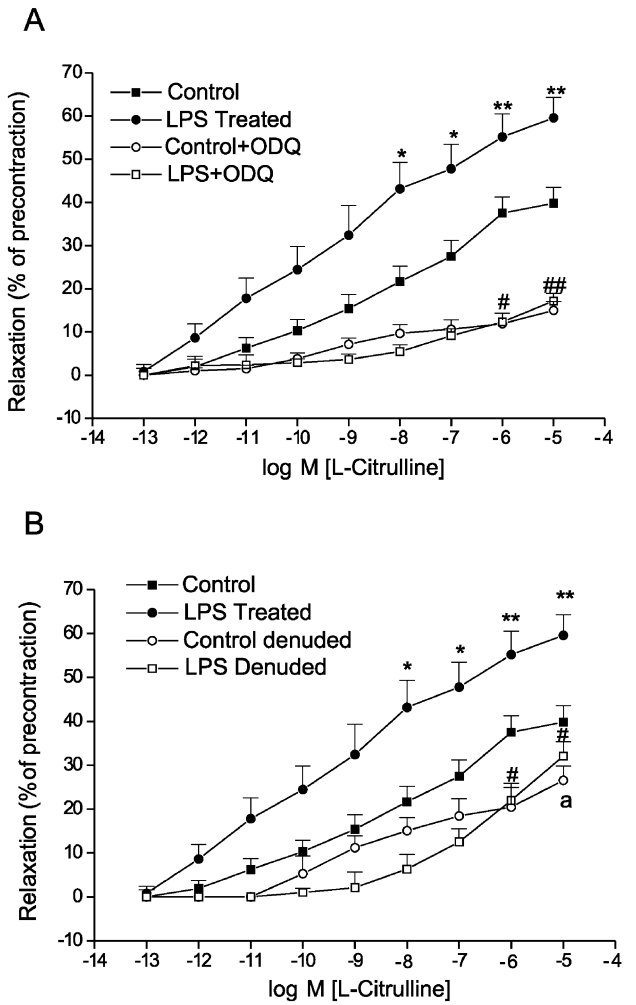

Fig. 1. (A) Concentration–relaxation response curves to L-citrulline (10^{-13} to 10^{-5} M) in phenylephrine (10^{-7} M) precontracted endothelium intact control rings, rings from lipopolysaccharide (LPS)-treated rats and in rings pretreated with ODQ from both control and lipopolysaccharide-treated rats. Each point represents the mean ± S.E.M of at least 10 experiments from different rats. $^{*}P < 0.05$, $^{**}P < 0.01$ in lipopolysaccharide-treated rings compared to normal rats. $^{\#,\#\#}P < 0.05$, $P < 0.01$ respectively on ODQ treatment compared to the respective controls. (B) Concentration–relaxation curves to L-citrulline (10^{-13} to 10^{-5} M) in endothelium intact and denuded rings from control and lipopolysaccharide-treated rat aortic rings precontracted with phenylephrine (10^{-7} M). Each point represents the mean ± S.E.M of at least eight experiments from different rats. $^{*}P < 0.05$, $^{**}P < 0.01$ in endothelium intact lipopolysaccharide treated rat aortic rings compared to intact control rat aortic rings. $^{\#}P < 0.05$ in endothelium denuded rat aortic rings in comparison to endothelium intact rings from lipopolysaccharide-treated rats. $^{a}P < 0.05$ in endothelium denuded aortic rings in comparison to endothelium intact rings from control rats.

Table 1
EC_{50} comparisons of the relaxation profiles of L-Citrulline and L-Arginine (10^{-13} M to 10^{-5} M) in (1×10^{-7} M) phenylephrine precontracted rings

Interventions (number of experiments)	EC_{50} (nM) (mean ± S.E.M.)
L-Citrulline (36)	13.29 ± 1.1
Lipopolysaccharide treatment + L-citrulline (18)	11.5 ± 3.1
Endothelial denudation + L-citrulline (12)	13.37 ± 5.6
Endothelial denudation on lipopolysaccharide treatment + L-citrulline (8)	102.50 ± 48[a]
L-Glutamine pretreatment + L-citrulline (8)	317.361 ± 28.1[b]
Lipopolysaccharide-treated rats + L-glutamine pre-treatment + L-citrulline (8)	44.89 ± 20.4[a]
L-arginine (24)	22.44 ± 5.75
Lipopolysaccharide treatment + L-arginine (12)	8.49 ± 3.3[c]
Endothelial denudation + L-arginine (8)	80.47 ± 29.8[d]
Denudation from lipopolysaccharide pre-treated rats + L-arginine (8)	93.55 ± 43.07[d]

[a] $P < 0.01$, 0.001 increase in EC_{50} compared to control L-citrulline values, respectively.
[b] $P < 0.01$, 0.001 increase in EC_{50} compared to control L-citrulline values, respectively.
[c] $P < 0.05$ decrease in EC_{50} compared to control L-arginine values.
[d] $P < 0.05$ increase in EC_{50} compared to control L-arginine values.

3.3. L-Arginine-induced relaxations

Cumulative concentration-dependent relaxation responses to L-arginine (10^{-13} to 10^{-5} M) were also generated in normal and lipopolysaccharide-treated rat thoracic aortic rings (Fig. 3). Surprisingly, the relaxation curves to L-arginine (10^{-13} to 10^{-5} M) were almost similar to that of L-citrulline. Relaxations to L-arginine (10^{-13} to 10^{-5} M) were significantly inhibited on ODQ (1 μM) treatment and attenuated on endothelial denudation (Table 1 and Fig. 3). Indomethacin did not significantly alter the relaxations to L-arginine as well. However, induction of NOS after lipopolysaccharide treatment significantly augmented L-arginine-induced relaxations, the maximal relaxation being 71 ± 8% in endothelium intact aortic rings. Denudation of

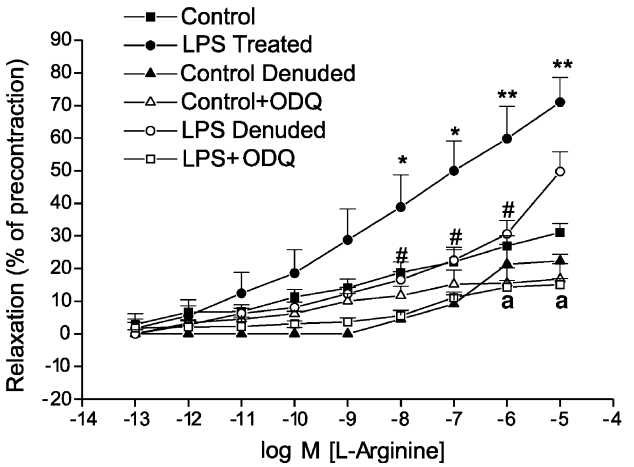

Fig. 3. Concentration–response curves to L-arginine (10^{-13} to 10^{-5} M) in phenylephrine (10^{-7} M) precontracted endothelium intact, endothelium denuded rings from control and lipopolysaccharide-treated rats and in control rings pre-treated with ODQ (1 μM) from both control and lipopolysaccharide (LPS)-treated rats. Each point represents the mean ± S.E.M. of at least eight experiments from different rats. $^{*}P < 0.05$, $^{**}P < 0.01$ in lipopolysaccharide-treated rings compared to normal rats. $^{a}P < 0.05$ in ODQ-treated rings from lipopolysaccharide-treated rats in comparison to their controls. $^{\#}P < 0.05$ in denuded rings compared to endothelium intact rings from LPS-treated rats.

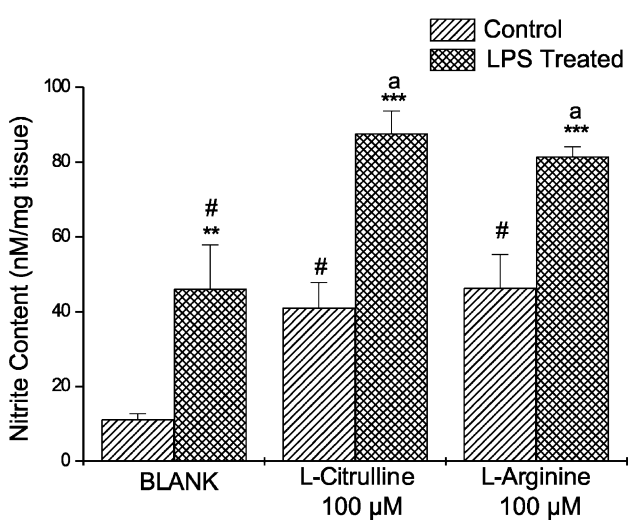

Fig. 2. Bar diagram representing the amount of nitrite released from endothelium intact rat aortic rings in blank (Basal), L-citrulline (L-Cit, 100 μM) and L-arginine (L-Arg, 100 μM) in normal rats and in lipopolysaccharide-treated rat aortic rings. Each bar represents the mean ± S.E.M. of at least eight experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ in lipopolysaccharide (LPS)-treated rings compared to the respective treatment in normal rats. $^{\#}P < 0.01$, $^{a}P < 0.01$ in the treated rings compared to basal nitrite levels in control and lipopolysaccharide treated rings, respectively.

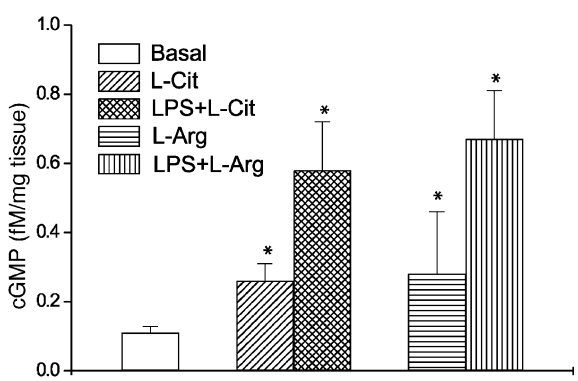

Fig. 4. Bar diagram representing levels of cGMP after the addition of blank, L-citrulline (L-Cit, 10^{-13} to 10^{-5} M) or L-arginine (L-Arg, 10^{-13} to 10^{-5} M) on phenylephrine (1×10^{-7} M) precontracted endothelium intact control or lipopolysaccharide (LPS)-treated rat aortic rings. Bars show the mean ± S.E.M. of three experiments. $^{*}P < 0.05$ in comparison to basal levels.

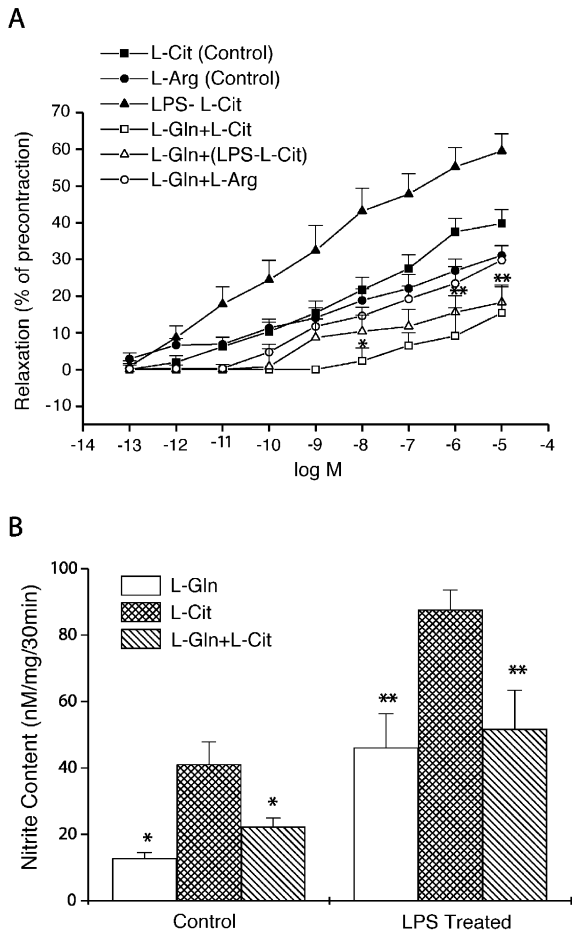

Fig. 5. (A) Concentration–response curves to L-citrulline (L-Cit) in endothelium intact rat aortic rings in control and after L-glutamine (L-Gln, 10 mM), lipopolysaccharide (LPS) treatment and lipopolysaccharide + L-Glutamine treatment. Each point represents the mean ± S.E.M. of at least eight experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ in glutamine-treated rings compared to normal in both control and lipopolysaccharide-treated rats. (B) Bar diagram representing the amount of nitrite released in aortic rings from control and lipopolysaccharide-treated rats from blank, after L-citrulline (L-Cit, 100 μM) and L-glutamine (L-Gln, 10 mM) + L-citrulline treatment. Each point represents the mean ± S.E.M. of at least four experiments. $^{*}P < 0.05$, $^{**}P < 0.001$ in basal and L-Gln + L-Cit-treated rings in comparison to L-citrulline-treated rings in both control and lipopolysaccharide-treated rats.

the endothelium in lipopolysaccharide-treated rat aortic rings significantly attenuated the L-arginine-induced relaxations, though the maximal relaxations still remained higher than those from control rings (Table 1 and Fig. 3).

3.4. Effects of L-citrulline and L-arginine on cGMP levels

L-Citrulline (10^{-13} to 10^{-5} M) and L-arginine (10^{-13} to 10^{-5} M) induced significant increases in cGMP levels in normal rats in comparison to the basal values. Levels of cGMP were further augmented in lipopolysaccharide-treated rings in comparison to control and from rings of normal rats treated with L-citrulline (Fig. 4).

3.5. Effect of L-glutamine on L-citrulline-induced relaxations and nitrite content

Relaxations to L-citrulline were significantly inhibited with increasing concentrations of L-glutamine and maximal inhibition was observed at a concentration of 10 mM (Fig. 5A). L-Glutamine (10 mM) also significantly inhibited the relaxations to L-citrulline in lipopolysaccharide-treated rat aortic rings. However, L-glutamine treatment did not affect the L-arginine-induced relaxations and the maximal relaxations and sensitivity to L-arginine were not significantly different from both normal and lipopolysaccharide-treated rats (Fig. 5A).

L-Glutamine (10 mM) alone did not produce any change in the nitrite content. Addition of L-citrulline (100 μM) to the rings increased nitrite content as observed earlier; however, addition of L-citrulline (100 μM) in presence of L-glutamine (10 mM) did not cause rise in nitrite levels (Fig. 5B). Similar pattern was also observed with lipopolysaccharide-treated rat aortic rings in the presence of L-glutamine (Fig. 5B).

3.6. Effect of corticosterone, aminoguanidine and cycloheximide on L-citrulline-induced relaxations

Rat aortic rings were pre-treated with inhibitors, corticosterone or cycloheximide and also with aminoguanidine. The maximal relaxations to L-citrulline was 28 ± 2%, 17 ± 4% and 13 ± 3%, respectively, in the presence of cycloheximide (100 μM), corticosterone (1 μM) or aminoguanidine (300 μM), though the sensitivity to L-citrulline was not significantly altered with any of these inhibitors (Fig. 6).

3.7. Involvement of K^+ channels in L-citrulline-induced relaxations

Relaxations produced by L-citrulline or L-arginine was significantly inhibited (Fig. 7) in the KCl (80 mM Krebs

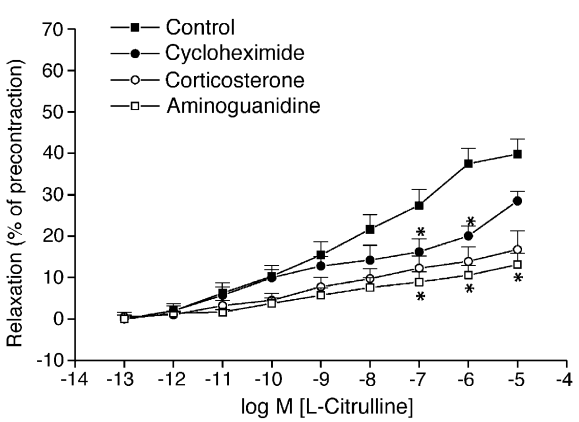

Fig. 6. Cumulative–concentration responses to L-citrulline (10^{-13} to 10^{-5} M) in control, corticosterone (1 μM), aminoguanidine (300 μM) and cycloheximide (100 μM) pre-treated aortic rings precontracted with phenylephrine (10^{-7} M). Each point represents the mean ± S.E.M. of eight experiments. $^{*}P < 0.05$ in the presence of the inhibitors compared to control.

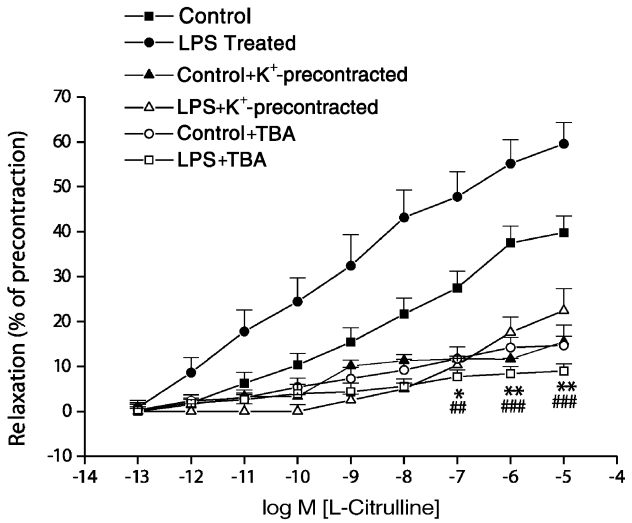

Fig. 7. Concentration–response curves to L-citrulline (10^{-13} to 10^{-5} M) in endothelium intact rat aortic rings contracted with KCl (80 mM) PSS or in phenylephrine precontracted rings in the presence of tetrabutylammonium (TBA, 1 mM) in both control and lipopolysaccharide-treated rats. Each point represents the mean ± S.E.M. of at least eight experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{\#\#}P < 0.01$, $^{\#\#}P < 0.001$ in tetrabutylammonium-treated, KCl precontracted rings as compared to control rings from normal and lipopolysaccharide-treated rats contracted with phenylephrine.

solution) precontracted endothelium intact or denuded rings of normal or lipopolysaccharide-treated rats. Maximal relaxations to L-citrulline or L-arginine were $16 \pm 4\%$ and $12 \pm 5\%$, respectively (Fig. 7). In addition, contraction with KCl (80 mM) also significantly attenuated the L-citrulline-induced relaxations in lipopolysaccharide-treated rat aortic rings as well, the maximal relaxations being $21 \pm 5\%$. Tetrabutylammonium, an inhibitor of K^+ channels (Ca^{2+} activated), significantly inhibited the maximal relaxations to L-citrulline in both control and lipopolysaccharide-treated rat aortic rings, the maximal relaxations being $14 \pm 2\%$ and $10 \pm 2\%$, respectively (Fig. 7).

4. Discussion

Results of the present study demonstrate significant vasorelaxant effect of L-citrulline in the isolated rat thoracic aortic rings, which was further potentiated after endotoxin treatment.

Ruiz and Tejerina (1998) observed relaxations to L-citrulline in isolated rabbit aortic rings, which were dependent on particulate guanylyl cyclase and Ca^{2+}-activated K^+ channels. Marx et al. (2000) recently reported that L-citrulline did not exert direct vasorelaxing action on the vascular smooth muscle. However, either of these reports did not investigate the possibility of induction of NO in the vascular smooth muscle.

L-Citrulline-induced relaxations in endothelium intact rat aortic rings were significantly reduced following guanylyl cyclase inhibition with ODQ. Moreover, removal of endothelium also attenuated the relaxations to L-citrulline, suggesting involvement of NO. Relaxations to L-citrulline were significantly increased in lipopolysaccharide-treated endothelium intact aortic rings. Administration of lipopolysaccharide to rats in addition to the induction of NOS in smooth muscle (Gardiner et al., 1995, 1996), has also been found to increase the synthesis of endothelium-derived NO by the activation of endothelial NOS through protein tyrosine kinase (Rees et al., 1990; Salvemini et al., 1990; Dudek et al., 1992; Huang et al., 1998). Denudation of endothelium in the lipopolysaccharide-treated rings attenuated the maximal relaxations to L-citrulline compared to the intact rings, indicating partial dependence of L-citrulline on the endothelial-derived factors. However, relaxations to L-citrulline were significant even on denudation, suggesting involvement of other factors, which might be NOS induction in the smooth muscle. Hence, a relaxation to L-citrulline seems to be mediated by both endothelial-dependent and -independent factors.

Involvement of NO in the L-citrulline-induced relaxations was further confirmed by an increase in nitrite content. Induction of NOS on lipopolysaccharide treatment significantly elevated the basal nitrite content, which was further increased after L-citrulline addition. Increase in nitrite content after L-citrulline treatment was comparable to the increase observed with L-arginine. Similarly cGMP content was also augmented after treatment with L-citrulline. cGMP levels were increased further in the lipopolysaccharide-treated rings, indicating the involvement of NO-mediated activation of guanylate cyclase in the L-citrulline-induced relaxations.

Studies on the endotoxin-mediated induction of NOS were mostly conducted at high doses of lipopolysaccharide (5–20 mg/kg) and duration of treatment was mostly more than 6 h (Gardiner et al., 1995; Erol and Kosay, 2000; Mitchell et al., 2000). Under these conditions, responses to contractile agents were significantly attenuated possibly due to the opposing vasodilatory action of NO and its effect on the contractile machinery. While significant increase in nitrite content after lipopolysaccharide treatment in the present study suggests that 1 mg/kg dose of lipopolysaccharide was sufficient to induce NOS, however, contractile response to phenylephrine remains unaffected. Augmentation of relaxations to L-citrulline in lipopolysaccharide-treated rats seems to be partially dependent on the endothelium, suggesting that NOS induced in the vascular smooth muscle might also account for the observed relaxations.

Endothelial cells of the vasculature recycle L-citrulline to L-arginine during the biosynthesis of endothelium-derived relaxing factor and regulate the excess nitrogen (Hecker et al., 1990; Sessa et al., 1990; Wu and Meininger, 1993). Involvement of NO in the L-citrulline-mediated relaxations thus suggested involvement of "citrulline-NO cycle" (Nussler et al., 1994), implicating formation and

participation of L-arginine as an intermediate. Hence, responses to L-arginine were evoked under similar conditions of study and were compared with L-citrulline.

Relaxation curves to L-arginine exhibited marked similarity to that of L-citrulline, with the relaxations being potentiated on lipopolysaccharide treatment and significantly inhibited on guanylyl cyclase inhibition. Relaxations to L-arginine in both endothelium intact and denuded rings of rat aorta, rat cerebral vessels and also in humans have been reported (Schini and Vanhoutte, 1991; Alonso et al., 1998; Bode-Boger et al., 1998; Briones et al., 1999). Moritoki et al. (1991, 1992) observed detectable relaxations to L-arginine after 2-h incubation with L-arginine and exhibited involvement of iNOS in these relaxations (Moritoki et al., 1992). In agreement to these reports, in the present study L-arginine-induced relaxations was potentiated on lipopolysaccharide treatment, indicating that iNOS seems to be responsible for the relaxations.

Furchgott (1996) has reported that rat aortic rings with or without endothelium are more prone to induction of NOS in the smooth muscle cells over the course of long experiments (6–10 h). Also, Eckly-Michel et al. (1999) observed that pre-treatment of rat aorta with vasoconstrictors could induce vascular NOS, which results in an increased relaxation to the relaxing agents. In the present study, after equilibration of the rings, relaxation to L-citrulline or L-arginine was taken only after confirming the presence of a functional endothelium. Rings also had a brief exposure to the contracting stimulus prior to relaxations by the agents used. Hence, exposure of the rings to this contractile stimulus might have induced NOS in the vasculature (Eckly-Michel et al., 1999). Moreover, total duration of the present experiment was around 6–8 h, thus suggesting the involvement of iNOS in our experiments.

Lipopolysaccharide treatment also induces enzymes argininosuccinate synthetase and argininosuccinate lyase, which recycle L-citrulline to L-arginine (Hattori et al., 1995; Nagasaki et al., 1996). Nussler et al. (1994) reported that endogenous arginine synthesis is coupled to NO synthesis and hence induction of NOS in our experiments might have resulted in the activation of argininosuccinate synthetase and argininosuccinate lyase resulting in augmented relaxations to L-citrulline.

L-Glutamine hampers recycling of L-citrulline to L-arginine by inhibiting uptake of argininosuccinate and also inhibits the activity of argininosuccinate synthetase (Sessa et al., 1990; Hecker et al., 1990; Wu and Meininger, 1993; Wu and Morris, 1998). L-Glutamine did not affect significantly the pre-contractions to phenylephrine or the relaxations to NO and L-arginine. However, L-citrulline response in control rat aortic rings was significantly shifted to the right and maximal relaxation was also inhibited, thus confirming the involvement of arginine synthesis in L-citrulline induced relaxation. Pre-treatment of lipopolysaccharide-treated rings with L-glutamine also shifted relaxation curves to the right in a similar manner as observed in control rings. Reduction in nitrite content in the L-glutamine pre-treated rings further confirmed the inhibition of L-arginine synthesis and subsequent inhibition of NO formation.

Subsequently, to confirm our hypothesis that prolonged incubations with contractile agent could have caused induction of iNOS and enzymes involved in the recycling of L-citrulline to L-arginine, we used inhibitors of protein synthesis as well as iNOS and estimated the L-citrulline-induced relaxations. Corticosterone suppresses both induction of iNOS and argininosuccinate synthetase (Moritoki et al., 1992; Hattori et al., 1994), whereas cycloheximide is found to induce iNOS (Hattori et al., 1994; Morris and Billiar, 1994) and modestly suppress argininosuccinate synthetase (Meijer et al., 1990). In agreement to the above-mentioned reports, corticosterone significantly inhibited relaxations to L-citrulline, whereas cycloheximide caused a moderate inhibition only. Aminoguanidine also significantly inhibited relaxations to L-citrulline, suggesting that iNOS seems to be involved in L-citrulline-induced relaxations.

L-Citrulline-induced release of NO activates guanylyl cyclase and leads to the elevation of cGMP levels, which could act through various mechanism to produce relaxations, the most predominant being activating cGMP kinases and hyperpolarization through K^+ channels (Lincoln and Cornwell, 1993; Ruiz and Tejerina, 1998). To investigate the possibility of hyperpolarization in L-citrulline relaxations, we used a high K^+-containing Krebs solution. 80 mM K^+ containing Krebs solution significantly inhibited the relaxations to L-citrulline and L-arginine. Amplitude of the endothelium-dependent hyperpolarization of smooth muscle is related nonlinearly to extracellular K^+ ion concentration (Chen and Suzuki, 1989) and K^+ overload has been reported to abolish the endothelium-dependent hyperpolarization (Ruiz and Tejerina, 1998). Hence, L-citrulline-induced relaxation could partly be elicited by hyperpolarization of the smooth muscle. To characterize the K^+ channel, we used a Ca^{2+}-activated K^+ channel blocker, tetrabutylammonium (Muller et al., 1998; Misurski et al., 2000). Inhibition of relaxations to L-citrulline by tetrabutylammonium in both control and lipopolysaccharide-treated aortic rings suggests the involvement of Ca^{2+} activated K^+ channels.

Thus, the results of the present study clearly indicate that L-citrulline possesses a vasorelaxing effect and to our knowledge is the first report implicating importance of L-citrulline in endotoxaemia. During endotoxaemia or sepsis, iNOS is induced in the smooth muscle cells of the vasculature, concomitant induction of argininosuccinate synthetase leads to recycling of L-citrulline to L-arginine and subsequently increased formation of NO as also suggested by our study. Relaxation to L-citrulline are slow developing and depends on the induction of NOS in smooth muscle, which is evident from the augmented relaxations in lipopolysaccharide-treated rat aorta. NO

formed following treatment with L-citrulline acts, at least in part, by opening Ca^{2+}-activated K^+ channels; however, steps mediating activation need to be further explored.

Infusion of L-arginine decreases arterial blood pressure (Bode-Boger et al., 1998; Hagashi et al., 1999; Mitchell et al., 2000) in animals and healthy humans. However, in pathological conditions such as endotoxaemia, significant decrease in blood pressure is observed due to the induction of iNOS, which leads to the increased formation of L-citrulline along with NO. L-Citrulline under these conditions might get recycled to L-arginine owing to the induction of argininosuccinate synthetase and thus increased L-arginine and NO formation. Regulating the levels of L-citrulline in addition to scavenging or inhibiting the formation of NO therefore seems to be an important target in endotoxaemia.

We conclude that L-citrulline might play an important role in the regulation of vascular tone by relaxing the vascular smooth muscle. In endotoxaemia, the vasorelaxing action of L-citrulline attains prominence by the increased recycling of L-citrulline to L-arginine and subsequent release of NO as well as hyperpolarization of the smooth muscle. The present study is yet another evidence of the complementary action of L-citrulline and the endothelium-derived relaxing factor, NO.

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