# Role of L-citrulline transport in nitric oxide synthesis in rat aortic smooth muscle cells activated with LPS and interferon-γ

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1

# Abstract

1. L-citrulline, a co-product of nitric oxide synthase (NOS)-catalysed metabolism of L-arginine to nitric oxide (NO), is an important intermediate of the urea cycle and a precursor for L-arginine biosynthesis in vascular cells.

2. In the present study, we have examined the characteristics of L-citrulline transport, regulation by lipopolysaccharide (LPS) and interferon-γ (IFN-γ) and the ability of L-citrulline to sustain NO synthesis in rat cultured aortic smooth muscle cells.

3. L-citrulline transport was saturable with an apparent $K_m = 1.6 \pm 0.2$ mM and $V_{max} = 5.9 \pm 0.6$ pmol $\mu g^{-1}$ protein $min^{-1}$. Transport was pH-insensitive, partially $Na^+$-dependent and markedly inhibited by substrates selective for amino acid transport systems L and N but not by L-arginine or substrates for systems A, ASC, $x_c^-$ or $X_{AG}$. Moreover, transport was not altered in cells treated with LPS (100 $\mu g$ $ml^{-1}$) and IFN-γ (50 U $ml^{-1}$) for 0-24 h.

4. Unlike L-arginine, L-citrulline could not sustain maximal NO production in cells expressing iNOS.

5. Our findings provide the first evidence in vascular smooth muscle cells that L-citrulline transport is mediated via a low affinity carrier with characteristics resembling systems L and N. Moreover, in L-arginine deprived rat aortic smooth muscle cells, L-citrulline cannot sustain maximal NO release via iNOS.

**Key words**: L-citrulline, Transport, Nitric oxide synthesis, Inducible nitric oxide synthase, Rat aortic smooth muscle cells

**Abbreviations used**: AL, argininosuccinate lyase; AS, argininosuccinate synthase; BCH, 2-aminobicycloheptane-2-carboxylic acid; CATs, cationic amino acid transporters; DMEM, Dulbecco's Modified Eagle's Medium; DON, 6-diazo-5-oxo-L-norleucine; IFN-$\gamma$, interferon-$\gamma$; iNOS, inducible nitric oxide synthase; LPS, bacterial lipopolysaccharide; MeAIB, 2-methylaminoisobutyric acid; RASMC, rat cultured aortic smooth muscle cells; SMC, smooth muscle cells

# Introduction

Nitric oxide is synthesised from the cationic amino acid L-arginine via a series of oxidation reactions which produce L-citrulline as a co-product. In rat cultured aortic smooth muscle cells (RASMC), the metabolism of L-arginine to NO and L-citrulline is mediated by $Ca^{2+}$/calmodulin-insensitive NO synthase (iNOS) (Knowles & Moncada, 1994; Alderton *et al.*, 2001), which is induced following exposure of these cells to pro-inflammatory mediators (Wileman *et al.*, 1995). Once induced, sustained synthesis of NO by iNOS is critically dependent on the availability of extracellular L-arginine (Escobales *et al.*, 2000; Schott *et al.*, 1993) and, as a consequence, supply of L-arginine may become limiting for NO production.

Under physiological conditions most plasma L-arginine is derived from metabolism of L-citrulline by the kidneys (Windmueller & Spaeth, 1981; Dhanakoti *et al.*, 1990), and is transported into cells via selective cationic amino acid transporters (Devés & Boyd, 1998; Palacin *et al.*, 1998; Closs & Mann, 1999; Mann *et al.*, 2003). In vascular smooth muscle, L-arginine can also be generated endogenously from L-citrulline. These cells, although lacking a complete urea cycle, express argininosuccinate synthase (AS) and argininosuccinate lyase (AL) (Hattori *et al.*, 1994), which respectively catalyse the synthesis of argininosuccinate from L-citrulline and the cleavage of argininosuccinate to L-arginine and fumarate (Meijer *et al.*, 1990). The capacity of these enzymes to regenerate L-arginine from L-citrulline may represent a rate-limiting mechanism in smooth muscle cells for maintaining substrate supply during sustained NO synthesis. Of direct relevance to this is the fact that AS, the rate limiting enzyme in the pathway, is markedly enhanced in smooth muscle cells expressing iNOS (Hattori *et al.*, 1994). Moreover, the induction of AS parallels that of iNOS indicating coordinated regulation of both proteins with perhaps a close functional relationship where enhanced AS expression drives the metabolism of L-citrulline to L-arginine, sustaining substrate supply

for iNOS. In agreement with this hypothesis, L-citrulline has been reported to induce NO-dependent vasorelaxation in denuded aortic rings isolated from endotoxin treated rats (Raghavan & Dikshit, 2001) and to sustain maximal rates of NO production in cultured aortic smooth muscle cells activated with bacterial lipopolysaccharide (LPS) and interferon-γ (IFN-γ) (Hattori *et al.*, 1994). In the latter study, it is worth noting that concentrations of L-citrulline required ($EC_{50}$ = 600 μM) for optimal iNOS activity were however 20-fold higher than the predicted $K_m$ for purified AS (Meijer *et al.*, 1990). This finding, together with the fact that vascular responses to L-citrulline developed slowly, requiring 45 min to reach a maximum (Raghavan & Dikshit, 2001), potentially highlights a limitation in cellular uptake of L-citrulline.

Currently, transport of L-citrulline into vascular cells is poorly understood, with only two reports describing distinct pathways for L-citrulline transport in macrophages (Baydoun *et al.*, 1994) and neural cell cultures (Schmidlin *et al.*, 2000). To our knowledge the mechanisms mediating L-citrulline transport in vascular smooth muscle cells have not been investigated. In view of the potential role of L-citrulline in regulating NO synthesis and vascular tone under physiological and pathophysiological conditions, we have characterized the system(s) mediating L-citrulline transport into smooth muscle cells cultured from rat aorta and investigated the dependency of iNOS on extracellular L-arginine and L-citrulline. Furthermore, we have examined whether L-citrulline transport is regulated by pro-inflammatory mediators by examining the effects of LPS in combination with IFN-γ.

# Materials and Methods

*Materials*

Tissue culture reagents were purchased from Gibco (Paisley, UK). Recombinant murine IFN-γ was from Genzyme (Cambridge, UK). Monoclonal anti-α smooth muscle actin, L-citrulline, and LPS from *Escherichia coli* (serotype 0111:B4) were obtained from Sigma (Poole, UK). Other chemicals were from Sigma or BDH and of the highest analytical grade obtainable. Radioactive tracers, L-[*ureido*-$^{14}$C]citrulline (57.8 mCi mmol$^{-1}$) and D-[$^{3}$H]mannitol (49.3 mCi mmol$^{-1}$) were obtained from New England Nuclear, Dreieich, Germany.

*Cell culture*

Smooth muscle cells (SMC) were isolated from rat aortic explants as described previously (see Wileman *et al.*, 1995). Briefly, male Sprague-Dawley rats (250-300 g) were stunned and exsanguinated and the thoracic aorta dissected in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4.4% NaHCO$_3$, penicillin (100 units ml$^{-1}$) and streptomycin (100 mg ml$^{-1}$). Following removal of the adventitia and endothelium, each aorta was cut into 2 mm$^2$ segments and placed in a T-25 tissue culture flask containing DMEM, supplemented with 2 mM glutamine and 10 % foetal calf serum. Explants were left in culture for 14 days, after which migrating and rapidly dividing cells were harvested with trypsin/EDTA (0.01/0.02%) and cultured to confluence in a T-75 flask. Cell were identified and characterized as SMC by immunostaining of smooth muscle α-actin, using mouse anti-α-actin antibody and anti-mouse IgG FITC conjugate (Sigma, Poole, Dorset, UK), as described by Skalli et al. (1986). All experiments were performed using cells between passages 4 and 7.

*Activation of cells with lipopolysacchride and interferon-γ*

Rat aortic SMC were plated into 96 well plates at a seeding density of $8\times10^3$ cells per well and allowed to grow to confluence. The culture medium was then replaced either with fresh medium or with medium containing LPS (100 μg ml$^{-1}$) plus IFN-γ (50 U ml$^{-1}$). Cells were incubated for a further 24 h, after which the supernatant was removed from each well and analysed for nitrite, and transport of L-[$^{14}$C]citrulline (1 μCi ml$^{-1}$, 37°C, 0-10 min) determined in cell monolayers incubated with Krebs solution (Wileman *et al.*, 1995).

To investigate the dependency of NO synthesis on extracellular L-arginine or L-citrulline, confluent SMC monolayers in 96 well plates were activated for 24 h with LPS and IFN-γ in the presence of increasing concentrations of either L-arginine (0-1 mM) or in L-arginine-free medium supplemented with increasing concentrations of L-citrulline (0-1 mM). Culture medium was removed after the incubation period and analysed for nitrite. Total protein content of each cell monolayer was determined using Brilliant Blue G reagent (Bradford, 1976).

*L-citrulline transport assay*

Cell monolayers were rinsed twice with a Hepes-buffered Krebs solution (mM: NaCl: 131; KCl: 5.5; MgCl$_2$: 1; CaCl$_2$: 2.5; NaHCO$_3$: 25; NaH$_2$PO$_4$: 1; D-glucose: 5.5; Hepes: 20; pH 7.4) maintained at 37$^o$C. As described previously (Wileman *et al.*, 1995), transport of L-[$^{14}$C]citrulline (1 μCi ml$^{-1}$, 0.1 mM) was measured in confluent cell monolayers in the absence or presence of a 10-fold excess of a given inhibitor (1 mM). Kinetics of L-citrulline transport were assessed over a wide range of substrate concentrations (0.05 – 5 mM). The effects of pH on L-citrulline transport (0.1 mM) were examined in Krebs solution titrated with 0.1N HCl or 5N NaOH to achieve pH values ranging between pH 5 - 8. In sodium-free experiments, Krebs buffer was modified by replacing NaCl, NaHCO$_3$ and NaH$_2$PO$_4$ with choline chloride, choline bicarbonate and KH$_2$PO$_4$, respectively. In some experiments an extracellular reference tracer,

D-[³H]mannitol, was included in the incubation medium and in these studies < 0.01% of D-mannitol applied was recovered in cell lysates.

Tracer uptake was terminated by placing the 96 well plates on ice and rinsing the cell monolayers three times with 200 μl ice-cold Krebs containing 10 mM unlabelled L-citrulline. Cell protein was determined using Brilliant Blue G and radioactivity (dpm) in formic acid digests of the cells was determined by liquid scintillation counting. Transport was then calculated and expressed as pmol μg protein$^{-1}$ min$^{-1}$.

*Nitrite analysis*

Nitrite accumulation in the culture medium was determined colorimetrically by a diazotization reaction using the standard Griess reagent (Green *et al.*, 1982; Wileman *et al.*, 1995).

*Analysis of intracellular amino acid concentrations*

L-arginine and L-citrulline concentrations in rat cultured aortic smooth muscle cells were determined by reversed-phase hplc. Confluent monolayers from unstimulated or LPS (100 mg ml$^{-1}$) plus IFN-γ (50 U ml$^{-1}$) treated cells were incubated in either L-arginine-containing or L-arginine-deprived medium.  Following a 24 h incubation period, the culture medium was removed, and the cell monolayer washed twice with ice-cold PBS, lysed with 0.5 ml ice-cold methanol and stored at -20°C for analysis, as described previously (Baydoun *et al.*, 1990).

*Statistics*

Data are expressed as means ± S.E.M. of measurements in at least three different cell cultures with 5-6 replicates per experiment. Statistical analyses were performed using either an unpaired Student's *t*-test or by analysis of variance when necessary with the overall confidence levels set at 95% (0.05).

## Results

*Time-course and kinetics of L-citrulline transport*

Transport of L-citrulline (0.1 mM) into rat aortic smooth muscle cells (RASMC) was time-dependent and linear for up to 30 min (see Fig. 4B in Wileman *et al.*, 1995). Cells accumulated <0.5 pmol L-citrulline per µg protein in 1 min. In all subsequent experiments, transport was measured over a 10 min incubation period to maximize tracer activity in smooth muscle cell digests. In unstimulated smooth muscle cells, transport of L-citrulline (0.05 − 5 mM) was fitted best by a single Michaelis-Menten equation, with an apparent $K_m$ of $1.6 \pm 0.2$ mM and $V_{max}$ of $5.9 \pm 0.6$ pmol µg$^{-1}$ protein min$^{-1}$ (Fig. 1). Pretreatment of smooth muscle cells with LPS (100 mg ml$^{-1}$) plus IFN-γ (50 U ml$^{-1}$) for 24 h had no significant effect on either $V_{max}$ ($6.9 \pm 0.9$ pmol µg$^{-1}$ protein min$^{-1}$) or $K_m$ ($2.4 \pm 0.4$ mM).

*Characteristics of L-citrulline transport*

In both unstimulated and LPS/IFN-γ activated cells, transport of L-citrulline was partially dependent on extracellular Na$^+$ but unaffected by changes in extracellular pH ranging from 5-8 (Table 1). Transport was significantly inhibited by a 10-fold excess of unlabelled L-citrulline ($K_i = 0.4$ mM), 2-aminobicycloheptane-2-carboxylic acid (BCH; model substrate for system L; $K_i = 0.2$ mM) or 6-diazo-5-oxo-L-norleucine (DON; model substrate for system N; $K_i = 0.8$ mM). In contrast, 2-methylamino-isobutyric acid (MeAIB) and L-glutamate (L-Glu), substrates for systems A and $x_c^-/X_{AG}$ respectively, were ineffective inhibitors, whilst L-arginine, a substrate for cationic amino acid transporters (CATs), caused only marginal (18 %) inhibition (Table 2).

Detailed analysis of the inhibition of L-citrulline transport by L-citrulline, BCH or DON revealed that each compound inhibited transport in a concentration-dependent manner, reducing L-citrulline uptake by $67 \pm 4\%$, $76 \pm 2\%$ and $80 \pm 0.4\%$ at 5 mM (Fig 2). In contrast, the

9

marginal inhibition caused by 1 mM L-arginine was not further enhanced by increasing extracellular L-arginine to 5 mM (Fig 2).

*Dependency of nitrite production on extracellular L-citrulline*

To investigate whether extracellular L-citrulline could sustain NO production by iNOS, RASMCs were activated with LPS (100 μg ml$^{-1}$) and IFN-γ (50 U ml$^{-1}$) for 24 h in an L-arginine-free medium containing increasing concentrations of L-citrulline (0 -1000 μM). In parallel experiments, the effects of extracellular L-arginine were examined over a similar concentration range. There was no detectable nitrite in the culture medium obtained from LPS/IFN-γ activated smooth muscle cells in the absence of extracellular L-arginine or L-citrulline.

Activation of cells in the presence of exogenous L-arginine caused a concentration-dependent increase in accumulated nitrite levels. This response was near maximal at plasma L-arginine concentrations (~100 μM, see Baydoun *et al.*, 1990; Hallemeesch *et al.*, 2002; Mann *et al.*, 2003), leveling off at 300 μM nitrite (Fig 3). In contrast to L-arginine, increasing concentrations of L-citrulline failed to sustain maximal NO production. More importantly, there was no detectable nitrite produced at plasma concentrations of L-citrulline (~30 μM, see Castillo *et al.*, 1993) and at 1 mM, the maximum levels of nitrite detected in the culture medium were approximately one third those produced in the presence of the same concentration of L-arginine (Fig 3).

Chromatographic analyses of cell lysates obtained from smooth muscle cells deprived of L-arginine for 24h revealed a decrease in intracellular L-arginine levels from 1.43 mM to 260 μM (Table 3). In cells activated with LPS and IFN-γ for 24h, intracellular L-arginine levels decreased to 760 μM whilst intracellular L-citrulline levels increased to 3.19 mM, consistent

with an enhanced production of NO via iNOS. In the absence of extracellular L-arginine, intracellular L-arginine and L-citrulline levels decreased and were maintained at 300 μM and 40 μM, respectively following activation (Table 3).

## Discussion

L-citrulline has emerged as an important amino acid both as a product of the urea cycle and as a precursor for L-arginine biosynthesis within the vasculature. *In vivo* citrulline is synthesized and released from the intestine as an end product of L-glutamine nitrogen metabolism (Windmueller & Spaeth, 1981). Once in the circulation L-citrulline is rapidly cleared by the kidney and subsequently metabolised and released as L-arginine (Windmueller & Spaeth, 1981; Dhanakoti *et al.*, 1990). In addition to this pathway, various cells within the vasculature possess a truncated urea cycle, expressing both argininosuccinate synthase and argininosuccinate lyase, which respectively catalyze the metabolism of citrulline to argininosuccinate and then L-arginine. The presence of these enzymes has led to the suggestion that cells may be able to recycle L-citrulline, thereby maintaining cellular concentrations of L-arginine for sustained NO synthesis (Hattori *et al.*, 1994; Raghavan & Dikshit, 2001). Consistent with this hypothesis, conversion of L-citrulline to L-arginine is enhanced in endothelial cells stimulated to release NO (Hecker *et al.*, 1990a; Hecker *et al.*, 1990b), and in macrophages (Baydoun *et al.*, 1994; Wu & Brosnan, 1992) and smooth muscle cells (Hattori *et al.*, 1994) generating NO following activation with pro-inflammatory mediators.

It would appear however that the effectiveness of L-citrulline to maintain adequate substrate supply for NOS may be limited by its ability to enter cells. Transport of L-citrulline into rat aortic smooth muscle cells (RASMC) is slow, remaining linear over a period of up to 30 min. At physiological plasma concentrations, initial rates of L-citrulline transport are 3-fold lower than rates measured previously for L-arginine (Wileman *et al.*, 1995). Unlike L-arginine, kinetic analysis revealed that transport is mediated via a low affinity carrier ($K_m$ ~1.6 mM), which may limit uptake from plasma in the presence of other circulating competitor amino acids. The lower rate of transport together with the relatively low affinity of

the transporter could explain the slow onset of action of L-citrulline in inducing relaxation of preconstricted aortic rings (Raghavan & Dikshit, 2001) and could also account for the significantly high concentrations of exogenous L-citrulline required to sustain maximal iNOS activity in cultured smooth muscle cells (Hattori *et al.*, 1994).

Kinetic cross-inhibition experiments suggest that the transporter for L-citrulline in RASMC is distinct from the cationic amino acid transporters mediating L-arginine entry in this cell type. First, transport of L-citrulline was not significantly altered by a 10-50 fold excess of L-arginine. Second, transport was also unaffected by LPS and IFN-γ, which are both known to increase L-arginine transport in various cell types including rat aortic smooth muscle cells (Wileman *et al.*, 1995; Durante *et al.*, 1995). The insensitivity of L-citrulline transport to pro-inflammatory mediators is consistent with our previous findings in J774 macrophages (Baydoun *et al.*, 1994) and with reports in neuronal cell cultures (Schmidlin *et al.*, 2000).

The fact that L-arginine failed to cause a significant modulation of L-citrulline transport in RASMC also rules out a role for the broad-scope amino acid transporters such as the $Na^+$-dependent system $B^{o,+}$ or the $Na^+$-independent system $b^{o,+}$ capable of transporting both neutral and basic amino acids (Van Winkle *et al.*, 1988; Van Winkle *et al.*, 1985). Moreover, the low affinity for L-citrulline transport excludes a significant contribution of the high affinity system $y^+L$ identified in several different cell types (reviewed by Devés & Boyd, 1998; Devés & Boyd, 2000; Wagner *et al.*, 2001) including a recent study in human endothelial cells (Sala *et al.*, 2002). Instead, our findings suggest that transport of L-citrulline in RASMC may be mediated predominantly via a carrier with characteristics similar to system L.

The classical $Na^+$-independent transport system L is most reactive with branched chain and aromatic neutral amino acids and is often characterised using the selective

nonmetabolisable analog BCH (Christensen *et al.*, 1969; Shotwell *et al.*, 1983). Transport via system L is *trans*-stimulated by intracellular substrates of this carrier and in some cases may be increased by lowered extracellular pH. Studies in rat glioma cells, primary astroglial cells and lymphocytes suggested that 4F2hc serves as a necessary component for expression of system L-like transport activity (Bröer *et al.*, 1995). Co-expression of 4F2hc and LAT-1 or LAT-2 (system L transporters) in oocytes induces $Na^+$-independent transport with a broad specificity for small and large zwitterionic amino acids and sensitivity to *trans*-stimulation (Kanai *et al.*, 1998; Pineda *et al.*, 1999). The marked inhibition of L-citrulline transport by BCH and the trend for increased transport at lowered pH implicates system L as the major transporter L-citrulline uptake in RASMC. This may not however be the only system that is involved. Depletion of $Na^+$ from the transport buffer resulted in a reduction (33%) in L-citrulline influx indicating a partially $Na^+$-dependent mechanism of entry. This was further investigated by examining the effects of substrates specific for known $Na^+$-dependent transporters including DON (system N, see Goldstein, 1975), MeAIB (system A; Gazzola *et al.*, 1980) and L-glutamate (systems $x_c^-$/ $X_{AG}$, see Dall'Asta *et al.*, 1983). L-citrulline transport was virtually unaffected by a 10-fold excess of either MeAIB or L-glutamate, markedly inhibited by DON and not decreased at lowered extracellular pH. It is therefore likely that the $Na^+$-sensitive component of L-citrulline transport was mediated via system N. Inhibition of L-citrulline transport by L-glutamine and inhibitory actions of L-glutamine (and its metabolite glucosamine) on the pentose cycle (Wu *et al.*, 2001) may well account for the reduction of NO synthesis reported in both intact cell systems (Hecker *et al.*, 1990a; Wu & Meininger, 1993) and blood vessels (Swierkosz *et al.*, 1990).

Entry via systems L and N or, an as yet unidentified, carrier with properties similar to system L and/or N, could explain why, in the absence of extracellular L-arginine, L-citrulline was not able to maintain maximal rates of NO production in our studies. Several other amino

acids utilise systems L and N for entry into cells. Some of these amino acids, including L-phenylalanine and L-glutamine are present in high concentrations in various culture media, including Dulbecco's modified Eagle's medium. Thus under standard experimental conditions, uptake of L-citrulline will be impaired by competing amino acids, with their combined effect causing marked inhibition of L-citrulline transport. Under these conditions, the capacity of cells to recycle L-arginine from L-citrulline would also be limited because other neutral amino acids will inhibit uptake of L-citrulline by competing for the same transporter(s) that facilitate entry of citrulline into cells. As a result, regeneration of L-arginine would not be efficiently maintained to match the consumption by NOS, even if the capacity of argininosuccinate synthase and argininosuccinate lysase to generate L-arginine from L-citrulline may be sufficient for maintaining maximal rates of NO synthesis (Hattori *et al.*, 1994). The finding that in LPS and IFN-γ activated cells with depleted intracellular L-citrulline and L-arginine levels (Table 3), only limited nitrite is released in the presence of 1 mM extracellular L-citrulline (Fig. 3), indicates that L-citrulline transport cannot maintain maximal NO synthesis.  It is also possible that L-citrulline available to arginosuccinate synthase and arginosuccinate lyase is compartmentalized and not readily exchangable with transported L-citrulline.

Activation of L-arginine transport in vascular smooth muscle cells by pro-inflammatory cytokines (Wileman *et al.*, 1995; Durante *et al.*, 1995) provides a mechanism for sustaining L-arginine supply during enhanced utilisation by iNOS.  Even though low intracellular L-arginine levels (300 μM) in L-arginine deprived cells would be expected to sustain NO synthesis, it is possible that as in macrophages (Closs *et al.*, 2000) a non-freely exchangeable L-arginine pool is not accessible to iNOS. Under these conditions, and as demonstrated in the present study, transport of L-arginine becomes rate-limiting for NO production. This hypothesis is further supported by the finding that iNOS mediated NO

production is reduced significantly in macrophages from CAT2⁻ᐟ⁻ mice (Nicholson *et al.*, 2001). This report and our present findings strongly suggest a functional association between CAT2 and iNOS, with a critical dependency of NO production on L-arginine delivery.

The inability of cytokines to stimulate L-citrulline transport in RASMC suggests that delivery of L-citrulline will not occur at a sufficiently high rate to maintain adequate levels of L-arginine for optimal iNOS activity. L-citrulline would therefore be far less effective than L-arginine in supporting NO synthesis, maintaining, at best, a fraction of the maximal rate of NO synthesis induced by L-arginine, as demonstrated in this and other studies in macrophages (Baydoun *et al.*, 1994; Wu & Brosnan, 1992). The lack of potentiation of L-citrulline transport by pro-inflammatory cytokines is another limiting factor worth taking into account when evaluating the potential of L-citrulline in maintaining the generation of NO via iNOS.

In summary, our findings establish that uptake of L-citrulline by RASMC is mediated via amino acid transport systems with properties characteristic of systems L and N. Inhibition by circulating amino acids will restrict the entry of L-citrulline into vascular cells and thereby limit the amount of intracellular L-arginine generated from this source. As a result, L-citrulline has only a limited ability to sustain maximal rates of NO synthesis both *in vitro* and *in vivo*.

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**Table 1.  The effects of extracellular sodium and external pH on L-citrulline transport.**

| Conditions | L-citrulline transport (% Control) |
|---|---|
| Na$^+$-free | $67 \pm 5$* |
| pH 5 | $112 \pm 6$ |
| pH 6 | $106 \pm 5$ |
| pH 8 | $89 \pm 1$ |

Dependency of L-citrulline transport on extracellular sodium or varying pH was examined using either nominally Na$^+$-free Krebs or buffers with pH values ranging from 5-8. Transport of L-citrulline (0.1 mM) was measured in unstimulated cells over a 10 min period. Data are expressed as a % of the influx rate obtained in normal Na$^+$-containing Krebs maintained at pH 7.4 (100% = 0.31 $\pm$ 0.04 pmol $\mu$g protein$^{-1}$ min$^{-1}$). Values denote the means $\pm$ S.E.M. of experiments in 3 different cell cultures with 5 replicates in each experiment. *P<0.05 compared to control.

**Table 2. Selectivity of L-citrulline transport in rat aortic smooth muscle cells.**

| Inhibitor (1 mM) | System Selectivity | L-citrulline transport (% Control) |
|---|---|---|
| L-arginine | $y^+$ | $82 \pm 4^*$ |
| L-citrulline | ? | $62 \pm 4^{**}$ |
| BCH | L | $32 \pm 2^{**}$ |
| MeAIB | A | $92 \pm 2$ |
| DON | N | $46 \pm 2^{**}$ |
| L-glutamate | $x_c^-$ or $X_{AG}$ | $99 \pm 3$ |

Transport of L-citrulline (0.1 mM) was measured over 10 min in the absence or presence of a 10 fold excess (1 mM) of various system selective amino acid substrates. Data are expressed as a % of the influx rate in control cells (100 % = $0.44 \pm 0.05$ pmol µg protein$^{-1}$ min$^{-1}$). Values denote the means $\pm$ S.E.M. of experiments in 3 different cell cultures with 5 replicates in each experiment. $^*$P<0.05 and $^{**}$P<0.01 compared to control.
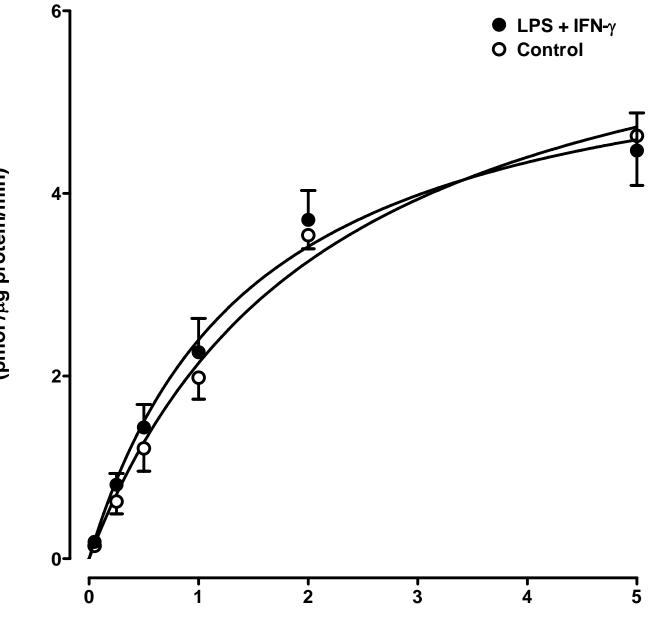
**Table 3.  Intracellular concentrations of L-citrulline and L-arginine in unstimulated and LPS/interferon-γ stimulated aortic smooth muscle cells.**

| Amino Acid | Control | Control (- L-arginine) | LPS + IFN-γ | LPS + IFN-γ (- L-arginine) |
|---|---|---|---|---|
| L-arginine (mM) | $1.43 \pm 0.36$ | $0.26 \pm 0.01$* | $0.76 \pm 0.10$ | $0.30 \pm 0.03$◆ |
| L-citrulline (mM) | $0.16 \pm 0.03$ | $0.49 \pm 0.29$ | $3.19 \pm 0.11$* | $0.04 \pm 0.04$◆ |

Unstimulated and activated aortic smooth muscle cells (100 μg ml$^{-1}$ LPS + 50 U ml$^{-1}$ IFN-γ, 24 h) were extracted and intracellular L-arginine and L-citrulline concentrations determined by reverse phase hplc. Results are expressed as intracellular amino acid concentration (mM) assuming an intracellular water space of 1 pl ml$^{-1}$. Data denote the means ± S.E.M. of determinations in 3 different cell cultures. *$P < 0.05$ versus control, ◆$P <0.05$ versus LPS + IFN-γ.

**Figure legends**

**Figure 1.   Kinetics of L-citrulline transport in rat aortic smooth muscle cells.**

Kinetics of L-citrulline transport (0.05 - 5 mM) were examined over a 10 min incubation period in unstimulated aortic smooth muscle cells and following activation of cells with LPS(100 μg ml$^{-1}$) plus IFN-γ (50 U ml$^{-1}$) for 24 h.   Values denote the means ± S.E.M. of experiments in 4 different cell cultures with 5 replicate measurements in each experiment.

**Figure 2.   Specificity of L-citrulline transport in rat aortic smooth muscle cells.**

Inhibition of L-citrulline transport was measured in unstimulated cells incubated with Na$^{+}$ containing Krebs buffer with increasing concentrations (0.25 - 5 mM) of either L-arginine, L-citrulline, DON or BCH. Transport of L-citrulline (0.1 mM) was measured over 10 min, and expressed as a % of the transport rate determined in the absence of an inhibitor amino acid. Values denote the means ± S.E.M. of experiments in 3 different cell cultures with 5 replicate measurements in each experiment.

**Figure 3.  Dependency of LPS plus interferon-γ stimulated nitrite release from rat aortic smooth muscle cells on extracellular L-arginine or L-citrulline.**
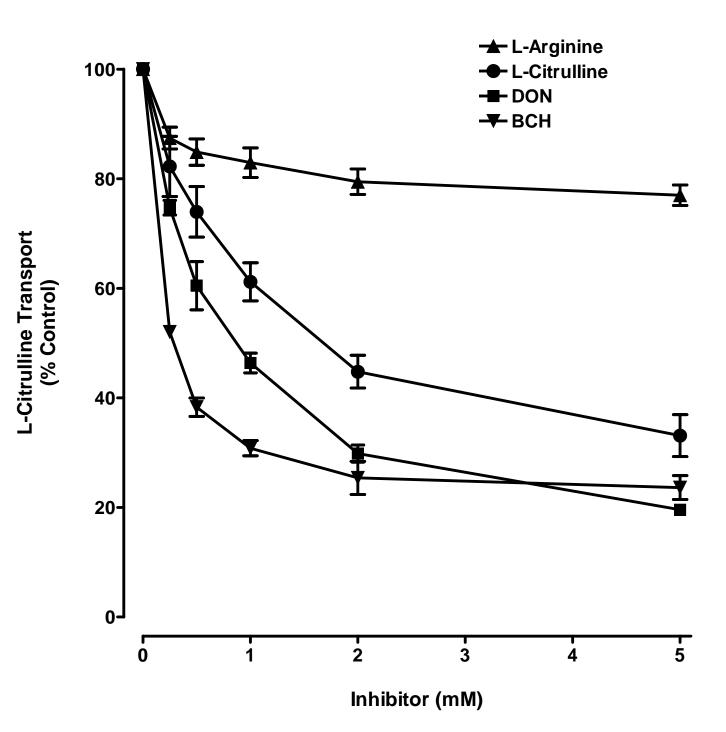
Confluent smooth muscle cells were activated with LPS (100 μg ml$^{-1}$) and IFN-γ (50 U ml$^{-1}$) for 24 h in L-arginine-depleted DMEM supplemented with increasing concentrations of either L-arginine or L-citrulline.  Nitrite accumulation in the culture medium was assayed using the Griess reaction.  Values denote the means ± S.E.M. of experiments in 3 different cell cultures with 5 replicate measurements in each experiment.
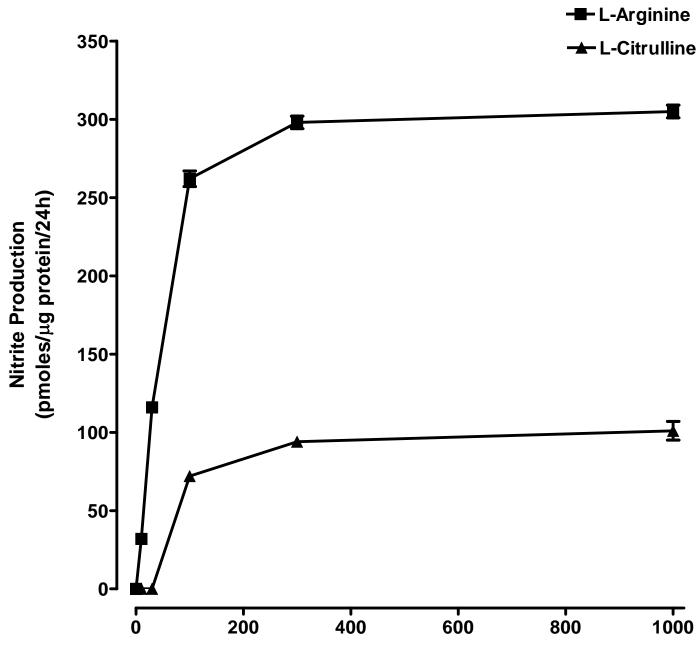
Fig 1

Fig 2

Fig 3