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Induction of L-arginine transport and nitric oxide synthase in vascular smooth muscle cells: synergistic actions of pro-inflammatory cytokines and bacterial lipopolysaccharide

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1 The interactions between pro-inflammatory cytokines and bacterial lipopolysaccharide (LPS) on L-arginine transporter and inducible nitric oxide synthase (iNOS) activities were examined in rat cultured aortic smooth muscle cells.

2 LPS induced a concentration (0.01-100 $\mu g\ ml^{-1}$) and time (8-24 h)-dependent stimulation of nitrite production which was accompanied by a parallel increase in L-arginine transport.

3 Unlike LPS, activation of smooth muscle cells with either interferon-γ (IFN-γ, 100 u ml^{-1}), tumour necrosis factor-α (TNF-α, 300 u ml^{-1}) or interleukin-1α (IL-1α, 100 u ml^{-1}) failed to stimulate L-arginine transport or increase nitrite accumulation.

4 When applied in combination with LPS (100 $\mu g\ ml^{-1}$) both IFN-γ and TNF-α, but not IL-1α, enhanced the effects observed with LPS alone. Furthermore, activation of cells with LPS and IFN-γ had no effect on uptake of the neutral amino acid L-citrulline but selectively increased the V_{max} for L-arginine transport 2.8 fold and nitrite levels from 24 ± 7 to 188 ± 14 pmol μg^{-1} protein 24 h^{-1}.

5 The substrate specificity, Na^+ and pH-independence of saturable L-arginine transport in both unactivated ($K_m = 44\ \mu M$, $V_{max} = 3$ pmol μg^{-1} protein min^{-1}) and activated ($K_m = 75\ \mu M$, $V_{max} = 8.3$ pmol μg^{-1} protein min^{-1}) smooth muscle cells were characteristic of the cationic amino acid transport system y^+.

6 Cycloheximide (1 μM) abolished induction of L-arginine transport and nitrite accumulation in response to LPS and IFN-γ. In contrast, the glucocorticoid dexamethasone (10 μM, 24 h) selectively inhibited nitrite production.

7 Our results demonstrate that pro-inflammatory mediators selectively enhance transport of L-arginine under conditions of sustained NO synthesis by vascular smooth muscle cells. In addition, the differential inhibition of iNOS and L-arginine transporter activity by dexamethasone suggests that distinct signalling pathways mediate induction of the cationic transport protein and iNOS. The close coupling between substrate supply and NO production may have important implications in the pathogenesis of several disease states including endotoxin shock.

Keywords: Aorta; smooth muscle cells; nitric oxide; L-arginine transport; bacterial lipopolysaccharide; endotoxin shock; interferon-γ; tumour necrosis factor-α; interleukin-1α; dexamethasone; cycloheximide

Introduction

Over production of nitric oxide (NO) by vascular smooth muscle cells (Knowles *et al.*, 1990; Rees *et al.*, 1990; Fleming *et al.*, 1990; 1991) has been implicated as the major cause of the sustained hypotension (Kilbourn *et al.*, 1990; Thiemermann & Vane, 1990; Wright *et al.*, 1992) and hyporeactivity to various vasoconstrictor agents (Julou-Schaeffer *et al.*, 1990; Fleming *et al.*, 1990; Gray *et al.*, 1991) in endotoxaemia. Under these conditions, synthesis of NO from its precursor L-arginine is catalysed by the calcium/calmodulin-insensitive nitric oxide synthase (iNOS), which, unlike the constitutive endothelial calcium/calmodulin-dependent isoform (eNOS), is induced by pro-inflammatory cytokines and bacterial lipopolysaccharide (LPS; see Moncada *et al.*, 1991). Expression of iNOS is time-dependent and susceptible to inhibition by cycloheximide, indicating a requirement for *de novo* protein synthesis (Knowles *et al.*, 1990; Rees *et al.*, 1990; see Moncada *et al.*, 1991).

Once induced, production of NO by this enzyme is blocked by inhibitors of NO synthase, including N^G-monomethyl-L-arginine (L-NMMA) (Kilbourn *et al.*, 1990; Thiemermann & Vane, 1990; Wright *et al.*, 1992), and its activity appears to be critically dependent on the availability of extracellular arginine (Beasley *et al.*, 1991; Schott *et al.*, 1993). Thus, the L-arginine transport system(s) may be a key target for regulating NO synthesis via the inducible L-arginine-nitric oxide pathway. In this regard we have shown previously that LPS induces a time- and dose-dependent stimulation of iNOS and L-arginine transporter activity in cultured J774 macrophages thereby providing a possible mechanism for sustaining substrate supply during periods of enhanced NO synthesis (Bogle *et al.*, 1992a; Baydoun *et al.*, 1993a).

In this study we have further investigated this phenomenon and examined the effects of LPS in combination with other pro-inflammatory mediators, including the cytokines interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) on L-arginine transport in correlation with the expression of iNOS in rat aortic smooth muscle cells in culture. Alterations in the kinetics and specificity of L-arginine transport were also examined in unactivated and LPS and/or cytokine activated cells. A preliminary account of this work has been presented in abstract form (Baydoun *et al.*, 1993b).

Methods

Cell culture

Vascular smooth muscle cells were cultured from rat aortic explants as described by Campbell & Campbell (1993). Male Sprague-Dawley rats (250-300 g) were stunned and ex-

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sanguinated 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 $\mu g\ ml^{-1}$). 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. Cells were passaged weekly and used between passages 4 and 14. All isolates were identified as smooth muscle cells by phase contrast microscopy and immunostaining of smooth muscle α-actin, with mouse anti-α-actin antibody and anti-mouse IgG FITC conjugate (Sigma Chemical Co.; Skalli *et al.*, 1986).

Incubation of cells with LPS and/or cytokines

Cells were plated at a density of 8×10^3 cells per well into 96 well plates for assays of nitrite and L-arginine transport. Confluent monolayers were activated either with a fixed concentration of LPS (100 $\mu g\ ml^{-1}$) for various time points or with varying concentrations of LPS (0.01 – 100 $\mu g\ ml^{-1}$) for 24 h. In another series of experiments cells were exposed to IFN-γ (50 and 100 u ml^{-1}), TNF-α (100 and 300 u ml^{-1}) or IL-1α (50 and 100 u ml^{-1}) alone and in combination with LPS (100 $\mu g\ ml^{-1}$). The effects of dexamethasone (10 μM) and the protein synthesis inhibitor cycloheximide (1 μM) on transporter activity and expression of iNOS were examined over a 24 h period in cells activated with LPS (100 $\mu g\ ml^{-1}$) and IFN-γ (50 u ml^{-1}). The supernatant was removed from each well at the end of the incubation period, assayed for nitrite accumulation and L-arginine transport was then measured in the cell monolayer incubated with Krebs solution.

Nitrite accumulation

Nitrite accumulation in the culture medium was determined colorimetrically by a diazotization reaction using the standard Griess reagent (Green *et al.*, 1982), as described previously (Bogle *et al.*, 1992a; Baydoun *et al.*, 1993a).

Measurement of L-arginine transport

Unidirectional transport of L-arginine was measured in confluent smooth muscle cell monolayers. Cells 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_2PO_4 1, D-glucose 5.5, HEPES 20, pH 7.4) maintained at 37°C. In some experiments the Krebs solution was titrated with 0.1 N HCl or 5 N NaOH to achieve pH values ranging between pH 5–8. In sodium-free experiments, the Krebs was modified by replacing NaCl, $NaHCO_3$ and NaH_2PO_4 with choline chloride, choline bicarbonate and KH_2PO_4, respectively.

Uptake was initiated by addding 50 μl of Krebs (37°C) containing 100 μM L-[^{3}H]-arginine (2 $\mu Ci\ ml^{-1}$) to each well. Transport was linear for up to 3 min (see Figure 4), and influx was measured over 30 s. Plates were then placed on ice and cells rinsed three times with 200 μl ice-cold Krebs containing 10 mM unlabelled L-arginine. When the extracellular tracer D-[^{14}C]-mannitol was included in the incubation medium, recovery in cell lysates was $<0.01\%$. Cell protein was determined with Brilliant Blue G (Bradford, 1976), and radioactivity (d.p.m.) in cell digests was measured by liquid scintillation counting. Transport was expressed in pmol μg^{-1} protein min^{-1}.

Selectivity and kinetics of L-arginine transport

Inhibition of L-arginine transport by competitor amino acids and L-arginine analogues was investigated by incubating cells with Krebs solution containing 100 μM L-[^{3}H]-arginine in the absence or presence of a 10 fold excess of a given inhibitor (1 mM). In other experiments we examined the concentration-dependent (0.1 – 5 mM) inhibition of L-arginine (100 μM) transport by L-citrulline (a coproduct of NO synthesis).

In kinetic experiments cells were incubated for 30 s with Krebs containing Na^+ and increasing concentrations of L-arginine (0.005 – 1 mM). Data were analyzed with the computer programmes Enzfitter and Ultra Fit (Elsevier, Biosoft) and fitted best by a Michaelis-Menten equation plus a non-saturable linear component.

Materials

Tissue culture reagents were from Gibco (Paisley, U.K.). Recombinant murine IFN-γ was from Genzyme (Cambridge, U.K.). Human recombinant IL-1α and TNF-α were from British Bio-technology (Abingdon, U.K.). Monoclonal anti-α smooth muscle actin, dexamethasone, cycloheximide, L-arginine, N^G-nitro-L-arginine, N^G-nitro-L-arginine methylester and LPS from *Escherichia coli* (serotype 0111 : B4) were obtained from Sigma (Poole, U.K.). Other chemicals were from Sigma or BDH and of the highest analytical grade obtainable. Radioactive tracers, L-[2,3-^{3}H]-arginine (36.1 Ci $mmol^{-1}$) and D-[^{14}C]-mannitol (49.3 mCi $mmol^{-1}$) were obtained from New England Nuclear, Dreieich, Germany. N^G-monomethyl-L-arginine and N^G-iminoethyl-L-ornithine were gifts from Wellcome Research Laboratories, Beckenham, U.K.

Statistics

All values are means ± s.e. of measurements in at least three different cell cultures with 5–6 replicates per experiment. Statistical analyses were performed with either an unpaired Student's *t* test or a multiple means comparison test (Harper, 1984) validated by comparison with the Newman-Keuls multiple range test in the statistical package SPP (Royston, 1984) with the overall confidence levels set at 95% (0.05).

Results

Induction of iNOS and L-arginine transport by LPS

Incubation of smooth muscle cells with increasing concentrations of LPS (0.01 – 100 $\mu g\ ml^{-1}$) for 24 h resulted in a concentration-dependent accumulation of nitrite in the culture medium. The minimum concentration of LPS required to elicit this response was 10 $\mu g\ ml^{-1}$ with 100 $\mu g\ ml^{-1}$ increasing nitrite levels from a basal value of 7.4 ± 1.7 to 318 ± 26 pmol μg^{-1} protein 24 h^{-1} (Figure 1a). In parallel experiments, nitrite accumulation was detectable after 8 h (14 ± 5 pmol μg^{-1} protein) and increased to 221 ± 22 pmol μg^{-1} protein after 24 h exposure to LPS (Figure 2a).

In the same cell cultures, L-arginine transport was mediated against a 14 fold concentration gradient (data not shown) and enhanced in a concentration- and time-dependent manner by LPS (0.1 – 100 $\mu g\ ml^{-1}$). At 100 $\mu g\ ml^{-1}$, LPS increased transport from 6.8 ± 0.5 to 9.5 ± 0.4 pmol μg^{-1} protein min^{-1} (Figure 1b). Stimulation of transport was evident after a lag phase of 8 h, reaching a maximum at 12 h and sustained over a 24 h incubation period (Figure 2b). It is worth noting that, although the above effects were reproducible, particular batches of the same serotype (0111 : B4) of LPS consistently failed to induce nitrite accumulation or L-arginine transport, presumably due to a variation in the content of endotoxin in different batches of LPS. This could account for the differences in sensitivity to LPS observed in our study and indeed explain the discrepancy in the literature regarding the ability of LPS alone to induce expression of iNOS in various cell types.

Effects of cytokines and LPS on iNOS and L-arginine transport activity

Unlike LPS, incubation of smooth muscle cells with maximal concentrations of either IFN-γ (100 u ml^{-1}), TNF-α, (300 u ml^{-1}) or IL-1α (100 u ml^{-1}) failed to cause any significant changes in nitrite levels or L-arginine transport (Table 1). In contrast, both accumulation of nitrite (310% increase above basal) and L-arginine transport (63% increase above basal) were markedly enhanced by IFN-γ (100 u ml^{-1}) in combination with TNF-α (100 u ml^{-1}), to a lesser extent by IFN-γ (100 u ml^{-1}) and IL-1α (100 u ml^{-1}), but not by TNF-α (100 u ml^{-1}) and IL-1α (100 u ml^{-1}) (Table 1). All three cytokines in combination increased nitrite production and L-arginine transport to a similar extent as that caused by IFN-γ and TNF-α.

When applied together with LPS (100 μg ml^{-1}), both IFN-γ (100 u ml^{-1}) and TNF-α (300 u ml^{-1}), but not IL-1α (100 u ml^{-1}), enhanced the effects observed with LPS alone. Under these conditions, elevations in nitrite accumulation (Figure 3a) and L-arginine transport (Figure 3b) induced by LPS and IFN-γ were significantly higher than rates induced by either LPS and TNF-α (Figure 3a and 3b) or TNF-α in combination with IFN-γ (Table 1).

In order to establish whether LPS and IFN-γ selectively increased L-arginine transporter activity in vascular smooth muscle cells, we compared the time courses of L-arginine uptake and that of the neutral amino acid L-citrulline, in unstimulated and LPS/IFN-γ activated cells. Unlike L-arginine, uptake of L-citrulline was unaffected following 24 h treatment with LPS (100 μg ml^{-1}) and IFN-γ (50 u ml^{-1}) (Figure 4). Furthermore, in contrast to our findings in J774 macrophages

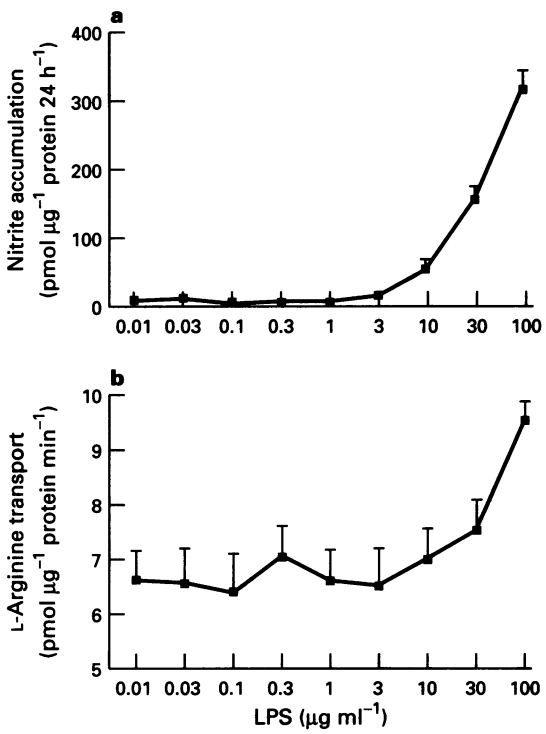

Figure 1 Concentration-dependent effects of LPS on nitrite accumulation (a) and L-arginine transport (b). Cells were exposed to LPS (0.01–100 μg ml^{-1}) and the accumulation of nitrite in the culture medium assayed 24 h later. Transport of 100 μM L-arginine was then measured over 30 s in the cell monolayer incubated with Krebs solution. Values are the mean ± s.e. of 3 different cell cultures.

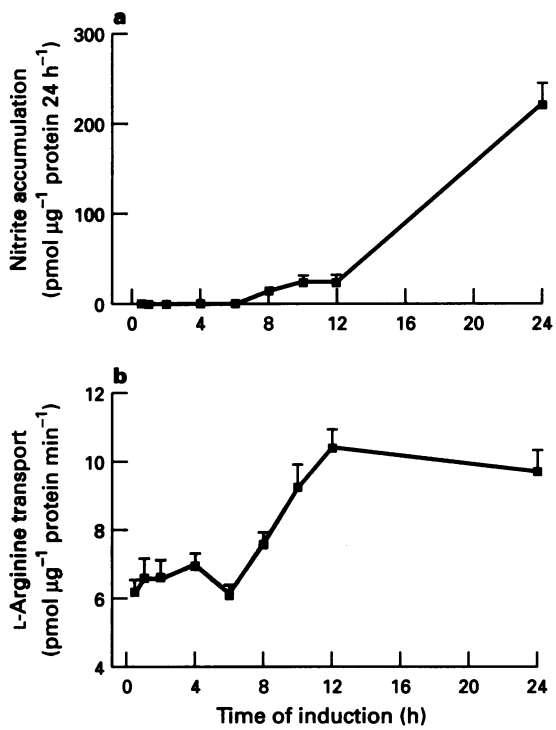

Figure 2 Time course of induction of nitrite accumulation (a) and L-arginine transport (b) in cells treated with LPS (100 μg ml^{-1}). Nitrite accumulation was measured at each point and transport of 100 μM L-arginine was then measured over 30 s in the cell monolayer incubated with Krebs. Values are means ± s.e. of 3 different cell cultures.

Table 1 Cytokine-mediated induction of nitrite accumulation and L-arginine transport

	Nitrite accumulation (pmol μg^{-1} protein 24 h^{-1})	*L-Arginine transport* (pmol μg^{-1} protein min^{-1})
Control	22 ± 2.7	4.2 ± 0.6
LPS	44 ± 3.3*	4.8 ± 0.5
TNF-α	9 ± 5.4	4.3 ± 0.3
Il-1α	15 ± 6.5	4.5 ± 0.3
IFN-γ	12 ± 5.9	3.8 ± 0.4
TNF-α + IFN-γ	91 ± 24.3*	7.0 ± 0.8
TNF-α + IL-1α	7 ± 43	4.8 ± 0.7
IFN + IL-1α	25 ± 12.3	5.8 ± 0.8
TNF-α + IFN-γ + IL-1α	106 ± 24.2*	7.1 ± 1.1

Cells were exposed to LPS (100 μg ml^{-1}), TNF-α (300 u ml^{-1}), IL-1α (100 u ml^{-1}) and IFN-γ (100 u ml^{-1}) either alone or in combination. Accumulation of nitrite in the culture medium was measured after 24 h, and transport of L-arginine was then determined over 30 s in the cell monolayers. Values are the means ± s.e. of 3 different cell cultures, *$P < 0.05$ compared to control.

(Baydoun *et al.*, 1994b), rates of L-citrulline transport were significantly lower than those of L-arginine in smooth muscle cells.

Dependency of iNOS and L-arginine transport induction on de novo *protein synthesis*

In order to determine whether enhanced nitrite accumulation and L-arginine transport were dependent on *de novo* protein synthesis, cells were incubated for 24 h with LPS (100 μg ml^{-1}) and IFN-γ (50 u ml^{-1}) in the absence or presence of cycloheximide (1 μM, 24 h). The effects of the glucocorticoid, dexamethasone (10 μM, 24 h), were also examined, since in the murine macrophage cell line J774 induction of iNOS and L-arginine transport were regulated differentially (Baydoun *et al.*, 1993a). The stimulatory effects of LPS and IFN-γ on nitrite accumulation and L-arginine transport were abolished by cycloheximide, whereas dexamethasone only inhibited nitrite accumulation without significantly altering enhanced rates of L-arginine transport (Table 2).

Effects of LPS and IFN-γ on kinetics of L-arginine transport

Kinetic analysis of L-arginine transport was carried out to determine whether the enhanced uptake was due to a change in the velocity of transport (V_{max}) or apparent affinity (K_m) of the transporter for L-arginine. In these experiments the overall rates of L-arginine transport in control and LPS/IFNγ stimulated cells were fitted best by a Michaelis-Menten equation plus a linear non-saturable component (Figure 5). Activation of cells increased the V_{max} for L-arginine transport 2.8 fold,

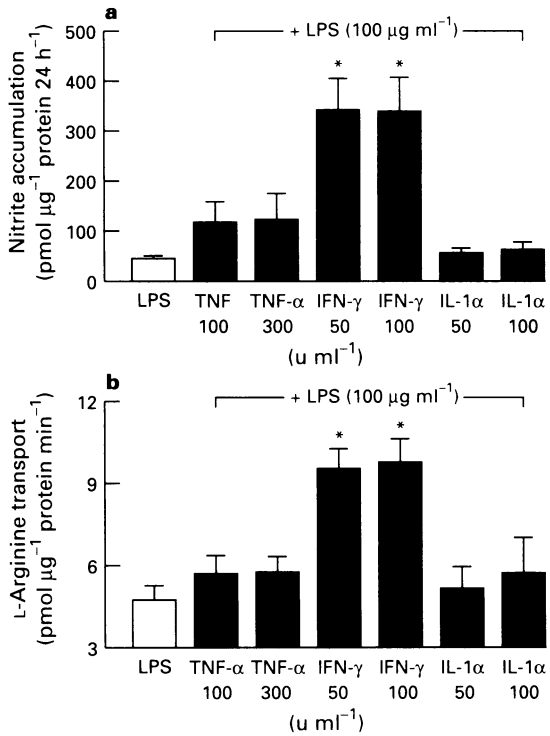

Figure 3 Effects of LPS on cytokine-stimulated changes in nitrite accumulation (a) and L-arginine transport (b). Cells were stimulated for 24 h with LPS (100 μg ml^{-1}) alone or in combination with TNF-α (100 and 300 u ml^{-1}), IL-1α (50 and 100 u ml^{-1}) and/or IFN-γ (50 and 100 u ml^{-1}). Nitrite accumulation in the culture medium was determined over 24 h and transport of L-arginine was measured in the cell monolayers. Values are mean ± s.e. of 3 different cell cultures, $*P<0.05$ compared to LPS alone.

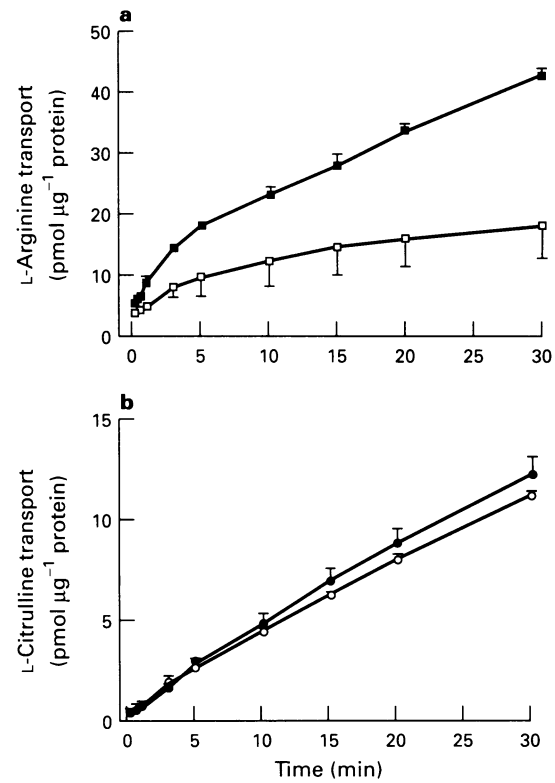

Figure 4 Differential regulation of L-arginine and L-citrulline uptake by LPS and IFN-γ. The time course of 100 μM L-arginine (a) and L-citrulline (b) uptake was compared in unstimulated cells (□, ○) and cells activated (■, ●) with LPS (100 μg ml^{-1}) and IFN-γ (50 u ml^{-1}) for 24 h. Values are the means ± s.e. of 3 different cell cultures.

Table 2 Effects of cycloheximide or dexamethasone on nitrite accumulation and L-arginine transport in smooth muscle cells activated with LPS and IFN-γ

	Nitrite accumulation (pmol μg^{-1} protein 24 h^{-1})	*L-Arginine transport* (pmol μg^{-1} protein min^{-1})
Control	24.2 ± 7.5	3.9 ± 0.3
LPS + IFN-γ	187.9 ± 13.8*†	7.8 ± 0.6*,NS
LPS + IFN-γ + cycloheximide	22.8 ± 4.1	4.6 ± 0.7
LPS + IFN-γ + dexamethasone	53.1 ± 6.2*	6.3 ± 0.4*

Cells were incubated with LPS (100 μg ml^{-1}) and IFN-γ (50 u ml^{-1}) in the absence or presence of cycloheximide (1 μM) or dexamethasone (10 μM). Accumulation of nitrite in the culture medium was determined after 24 h and transport of L-arginine was measured over 30 s in the cell monolayers. Values are the means ± s.e. of 4 different cell cultures. $*P<0.05$ compared to control values. ^{NS}not significantly different, and $\dagger P<0.05$ compared to LPS + IFN-γ + dexamethasone-treated cells.

with no significant ($P>0.05$) change in K_m (Table 3). The value of K_D (non-saturable component) was also not increased significantly in activated cells (Table 3).

Although entry of L-arginine was also mediated by a non-saturable component at higher substrate concentrations, the saturable component of transport accounted for ~75% of the total influx in unstimulated and LPS/IFN-γ stimulated cells at physiological L-arginine concentrations (100 μM).

Characteristics of L-arginine transport in unstimulated and LPS/IFN-γ stimulated cells

In both cell types, L-arginine transport was temperature-dependent and unaffected by changes in extracellular Na^+ or pH (data not shown). As shown in Table 4, the specificity of L-arginine transport was indistinguishable in unstimulated and LPS/IFN-γ activated cells. L-Lysine, L-ornithine and L-arginine analogues, including the cationic NO synthase inhibitors L-NMMA and L-NIO, were the most effective inhibitors. In contrast, L-citrulline (Figure 6) and other neutral amino acid analogues, selective for transport systems A (2-methylaminoisobutyric acid), L (phenylalanine, leucine), N (6-diazo-5-oxo-L-norleucine, glutamine) or ASC (cysteine), were poor inhibitors. As in endothelial cells (Bogle *et al.*, 1992b) and J774 macrophages (Baydoun & Mann, 1994), the neutral NO synthase inhibitors L-NNA and its methyl ester, L-NAME, were less effective inhibitors of L-arginine transport in both control and activated smooth muscle cells (Table 4).

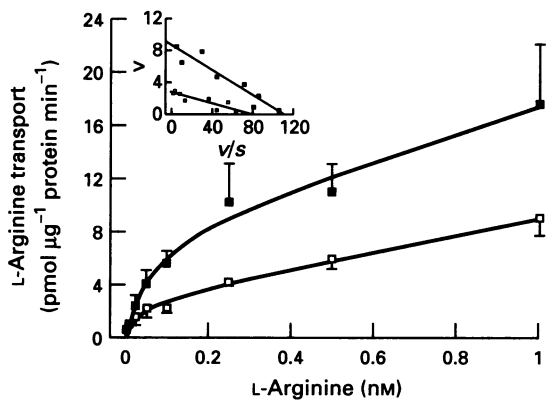

Figure 5 Effects of LPS and IFN-γ on the kinetics of L-arginine transport. Rates of total L-arginine transport (0.005–1 mM) were measured in unstimulated cells (□) and cells activated (■) with LPS (100 μg ml^{-1}) and IFN-γ (50 u ml^{-1}) for 24 h. The inset shows Eadie-Hofstee plots of saturable transport in unstimulated and activated cells, where V is the initial velocity (pmol μg^{-1} protein min^{-1}) and S is the substrate concentration (mM). Rectangular hyperbolae were fitted to the mean influx values weighted for the reciprocal standard error. Values are the means ± s.e. of 4 different cell cultures.

Discussion

This study has examined the effects of pro-inflammatory mediators, including LPS and the cytokines IFN-γ, TNF-α and IL-1α on the inducible L-arginine nitric oxide pathway in cultured rat aortic smooth muscle cells and has correlated changes in L-arginine transporter activity with expression of iNOS. The results obtained suggest that L-arginine transport and iNOS are coinduced in cells activated with LPS alone or in combination with pro-inflammatory cytokines. These observations extend a recent report showing that cytokines stimulate uptake of L-arginine and nitrite production in rat aortic smooth muscle cells (Durante *et al.*, 1995) and are consistent with findings that iNOS is induced in these cells either by LPS alone (Marczin *et al.*, 1993a,b) or in combination with cytokines (Nakayama *et al.*, 1992; 1994; Sirsjö *et al.*, 1994). More importantly, our results demonstrate that induction of NOS in

Table 3 Kinetic parameters for L-arginine transport in unstimulated and LPS/IFN-γ activated smooth muscle cells

	Unstimulated	*LPS/IFN-γ*
K_m (μM)	44.0 ± 6.0-	75.0 ± 9.0
V_{max} (pmol μg^{-1} protein min^{-1})	3.0 ± 0.2	8.3 ± 0.7*
K_D (pmol μg^{-1} protein min^{-1} mM^{-1})	6.3 ± 0.6	9.3 ± 2.1

Cells were exposed to LPS (100 μg ml^{-1}) and IFN-γ (50 u ml^{-1}) for 24 h. Transport of L-arginine was then measured over 30 s in both unstimulated and activated cells. Values are the means ± s.e. of 4 different cell cultures, *$P<0.001$.

Table 4 Selectivity of L-arginine transport in unstimulated and LPS/IFN-γ activated smooth muscle cells

	Initial rate of L-arginine transport (% control)	
	Unactivated	*LPS + IFN-γ*
L-Arginine	40 ± 2*	39 ± 3*
D-Arginine	87 ± 3	94 ± 4
L-Lysine	49 ± 4*	45 ± 1*
L-Ornithine	43 ± 3*	48 ± 5*
L-Citrulline	95 ± 3	99 ± 6
L-Glutamine	106 ± 20	108 ± 14
6-Diazo-5-oxo-L-norleucine	94 ± 13	105 ± 9
2-Methylaminoisobutyric acid	104 ± 19	111 ± 8
L-Cysteine	117 ± 17	114 ± 11
L-Phenylalanine	86 ± 3*	93 ± 5
L-Leucine	73 ± 5*	87 ± 3
N^G nitro-L-arginine methyl ester	97 ± 5	97 ± 2
N^G nitro-L-arginine	84 ± 2*	89 ± 4
N^G iminoethyl-L-ornithine	47 ± 7*	40 ± 5*
N^G monomethyl-L-arginine	45 ± 10*	43 ± 7*

Transport of 100 μM L-arginine was measured over 30 s in the absence or presence of a 10 fold excess (1 mM) of a given inhibitor in both unactivated cells and cells activated with LPS (100 μg ml^{-1}) and IFN-γ (50 u ml^{-1}) for 24 h. Data are expressed as a % of the respective control influx in unactivated (3.1 ± 0.2 pmol μg^{-1} protein min^{-1}) and LPS/IFN-γ activated (7.7 ± 0.5 pmol μg protein min^{-1}) cells. Values are the means ± s.e. of 3 different cell cultures, *$P<0.05$.

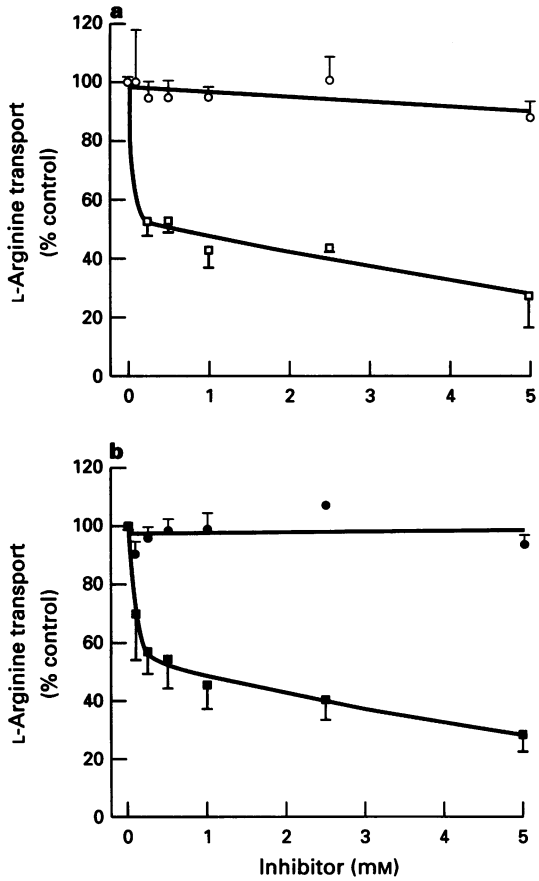

Figure 6 Self- and cross-inhibition of L-arginine transport in (a) unstimulated and (b) LPS ($100\,\mu g\,ml^{-1}$) and IFN-γ ($50\,u\,ml^{-1}$) activated smooth muscle cells. Transport of $100\,\mu M$ L-arginine (30 s) was measured in the absence or presence of increasing concentrations (0.1–5 mM) of either L-citrulline (○, ●) or L-arginine (□, ■). Values are the means ± s.e. of 3 different cell cultures.

rat aortic smooth muscle cells is accompanied by a parallel increase in transport and thus supply of L-arginine into activated cells generating NO.

We highlighted this phenomenon in a previous study in which we demonstrated that LPS-stimulated macrophages showed an enhanced uptake of L-arginine (Bogle *et al.*, 1992a; Baydoun *et al.*, 1993a). We have now identified significant differences in the response of J774 macrophages and rat aortic smooth muscle cells to LPS and cytokines. Firstly, unlike J774 cells, in which activation of L-arginine transport was detectable at doses of LPS that did not stimulate nitrite release, the increase in L-arginine transport in smooth muscle cels was always paralleled by an increase in nitrite accumulation, suggesting a closer coupling between induction of the transporter and iNOS in these cells. Secondly, incubation of smooth muscle cells with LPS and either IFN-γ or TNF-α synergistically enhanced the actions of LPS, with IFN-γ and LPS being the most potent. In addition, L-arginine transport in these cells was also stimulated (63–136%) by cytokines but only when applied in combination. These findings are in contrast to those of Durante *et al.* (1995), who reported that IL-1β and TNF-α independently enhance (10–30%) uptake of L-arginine in rat aortic smooth muscle cells with no apparent synergism between cytokines.

Indeed, although several reports have shown that expression of iNOS in vascular smooth muscle cells can also be induced by individual cytokines such as TNF-α (Busse & Mülsch., 1990; Koide *et al.*, 1993; 1994; Nakayama *et al.*, 1992), IL-1β (Beasley *et al.*, 1991; Kanno *et al.*, 1993; Marumo *et al.*, 1993), IL-α (Pomerantz *et al.*, 1993; Marczin *et al.*, 1993b) or IFN-γ (Koide *et al.*, 1993; 1994), we did not detect measurable nitrite release from smooth muscle cells incubated with either TNF-α, IL-1α or IFN-γ alone. Although the reasons for these differences are unclear, it is worth noting that the presence of contaminating traces of endotoxin in normal culture medium may synergise with added cytokines, giving rise to measurable nitrite levels.

Consistent with our findings, other studies with rat aortic smooth muscle cells have reported that combinations of cytokines and/or LPS are essential for production of NO (Gross & Levi, 1992; Geng *et al.*, 1992; Marumo *et al.*, 1993; Hattori *et al.*, 1994; Sirsjö *et al.*, 1994), and in human hepatocytes a combination of IFN-γ, TNF-α and IL-1β together with LPS appears to be mandatory for activation of iNOS (Nussler *et al.*, 1993). Furthermore, when applied alone neither TNF-α (Marumo *et al.*, 1993), IL-1α (Marumo *et al.*, 1993), IL-1β (Nakayama *et al.*, 1992) nor IFN-γ (Sirsjö *et al.*, 1994) were able to induce iNOS mRNA in rat aortic or pulmonary artery smooth muscle cells. Taken together these findings strongly suggest that induction of iNOS and L-arginine transporter activity are critically dependent on the stimulus used, with an adequate combination of cytokines and/or LPS being essential for full activation of one or both pathways. These observations reflect possible differences in the molecular mechanism(s) mediating the actions of the various pro-inflammatory mediators, with their synergistic actions indicating that distinct signal transduction pathways may be used by each agent to induce iNOS and/or stimulate L-arginine transporter activity.

Whilst the signalling events mediating activation of L-arginine transport await further investigation, considerable progress has been made in elucidating those associated with expression of iNOS. The gene for iNOS has been cloned in several different cell types (see Knowles & Moncada, 1994; Sessa, 1994), including rat aortic smooth muscle cells (Nunokawa *et al.*, 1993; Geng *et al.*, 1994), and contains response elements for certain pro-inflammatory mediators such as IFN-γ (Xie *et al.*, 1993; Lowenstien *et al.*, 1993; Martin *et al.*, 1994). Cytokines and LPS regulate expression of iNOS largely at the transcriptional level (Xie *et al.*, 1992; Lorsbach *et al.*, 1993; Martin *et al.*, 1994) and do so either via the induction of an intermediary protein (Koide *et al.*, 1994) or by activating nuclear regulatory factors such as the interferon regulatory factor-1 (Martin *et al.*, 1994) which enhance iNOS mRNA expression (Koide *et al.*, 1993; 1994; Martin *et al.*, 1994). This hypothesis, substantiated by the fact that cycloheximide selectively inhibits TNF-α but not IFN-γ induced iNOS mRNA (Koide *et al.*, 1994), could explain the marked synergism observed in our study between TNF-α, LPS and IFN-γ.

It is evident from our present results and our previous study in macrophages (Baydoun *et al.*, 1993a) that the pathways responsible for the enhanced transporter activity are in part distinct from those leading to expression of iNOS. Dexamethasone selectively inhibits production of nitrite, whilst having no significant effect on transport, indicating that the gene for the L-arginine transporter is not sensitive to regulation by glucocorticoids. Similar to the enhanced production of NO, activation of L-arginine transport is sensitive to cycloheximide, demonstrating that *de novo* protein synthesis is essential for enhanced transporter activity. As in J774 cells (Bogle *et al.*, 1992a), the increased V_{max} of L-arginine transport in activated rat aortic smooth muscle cells is consistent with an increased expression of transporter proteins. The fact that transport of L-citrulline is unaltered by LPS and IFN-γ indicates that the increased expression of carriers for L-arginine is selective for cationic amino acid transporter proteins.

L-Arginine entry in both unstimulated and LPS/IFN-γ activated smooth muscle cells was inhibited selectively by other cationic amino acids and by the NOS inhibitors, L-NMMA and L-NIO. Neutral L-arginine analogues, L-NNA and L-NAME were less effective inhibitors of L-arginine influx, confirming our previous study in J744 macrophages (Baydoun & Mann, 1994) and similar findings in RAW 264.7 macrophages (Schmidt *et al.*, 1994). The transport selectivity in our study, which also employed transport system specific non-

metabolized amino acid analogues, confirms recent reports for L-lysine and L-arginine in rat cultured aortic smooth muscle cells (Low *et al.*, 1993; Durante *et al.*, 1995). The characteristics, kinetic properties and pH- and Na^+-independence of L-arginine transport in unstimulated and activated smooth muscle cells in our study suggest that transport is mediated by the classical cationic amino acid transport system y^+ (see review by White, 1985), recently cloned from murine fibroblasts (MCAT-1, $K_m = 0.14-0.25$ mM) and expressed in *Xenopus* oocytes (Kim *et al.*, 1991; Wang *et al.*, 1991). It is worth noting, however, that two further highly homologous cationic amino acid transporters (MCAT-2A and MCAT-2B), with high selectivity but different affinity for L-arginine, have been identified in other cell types (Closs *et al.*, 1993a,b). Functionally, these proteins are distinguishable only by their affinity for L-arginine with MCAT-2B ($K_m = 0.25-0.38$ mM) exhibiting a 10 fold higher affinity for L-arginine than MCAT-2A ($K_m > 4$ mM). Thus the kinetic properties of MCAT-2B are difficult to discriminate from system y^+ by simple kinetic analysis (Closs *et al.*, 1993b).

Although our data are consistent with L-arginine transport being mediated by system y^+ in both unstimulated and activated smooth muscle cells, we cannot exclude the involvement of MCAT-2B nor can we account for total transport (saturable and linear) via system y^+ alone. The apparently non-saturable component of transport noted in our study may occur via a low affinity cationic transport system, such as MCAT-2A. Although another study has identified two saturable components for L-arginine transport in unstimulated rat aortic smooth muscle cells (Durante *et al.*, 1995), we were unable to confirm the presence of two saturable pathways for L-arginine entry in either unstimulated or activated smooth muscle cells. Moreover, it is worth noting that Low *et al.* (1993) could identify only one saturable transport system for another cationic substrate, L-lysine.

In conclusion, the ability of LPS and cytokines to enhance transport of L-arginine under conditions of increased NO production provides a unique mechanism for sustaining NO synthesis and may have important implications in the pathogenesis of endotoxin shock. Furthermore, the observation that dexamethasone selectively inhibits iNOS expression but not L-arginine transport has led to the realisation that the signalling pathways leading to the production of NO and enhanced transporter activity are distinguishable. Further studies of the signalling pathways mediating activation of L-arginine transport may permit the targeting of specific inhibitors to the cationic amino acid transporter(s), thereby providing a novel therapeutic approach for the management or prevention of the hypotension associated with an overproduction of NO generated following activation of iNOS.

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References

BAYDOUN, A.R., BOGLE, R.G., PEARSON, J.D. & MANN, G.E. (1993a). Selective inhibition by dexamethasone of induction of NO synthase, but not of induction of L-arginine transport, in murine macrophage J774 cells. *Br. J. Pharmacol.*, **110**, 1401–1406.

BAYDOUN, A.R., BOGLE, R.G., PEARSON, J.D. & MANN, G.E. (1994). Discrimination between L-citrulline and L-arginine transport in activated murine macrophages: inefficient synthesis of NO from recycling of citrulline to arginine. *Br. J. Pharmacol.*, **112**, 487–492.

BAYDOUN, A.R. & MANN, G.E. (1994). Selective targeting of nitric oxide synthase inhibitors to system y^+ in activated macrophages. *Biochem. Biophys. Res. Commun.*, **200**, 726–731.

BAYDOUN, A.R., WILEMAN, S.M., JAY, M.T. & MANN, G.E. (1993b). Induction of L-arginine transport and nitric oxide synthase in cultured rat aortic smooth muscle cells activated with bacterial endotoxin. *J. Physiol.*, **475**, 46P.

BEASLEY, D., SCHWARTZ, J.H. & BRENNER, B.M. (1991). Interleukin 1 induces prolonged L-arginine-dependent cyclic guanosine monophosphate and nitrite production in rat vascular smooth muscle cells. *J. Clin. Invest.*, **87**, 602–608.

BOGLE, R.G., BAYDOUN, A.R., PEARSON, J.D., MONCADA, S. & MANN, G.E. (1992a). L-Arginine transport is increased in macrophages generating nitric oxide. *Biochem. J.*, **284**, 15–18.

BOGLE, R.G., MONCADA, S., PEARSON, J.D. & MANN, G.E. (1992b). Identification of inhibitors of nitric oxide synthase that do not interact with the endothelial cell L-arginine transporter. *Br. J. Pharmacol.*, **105**, 768–770.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

BUSSE, R. & MÜLSCH, A. (1990). Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett.*, **275**, 87–90.

CAMPBELL, J.H. & CAMPBELL, R.G. (1993). Culture techniques and their applications to studies of vascular smooth muscle. *Clin. Sci.*, **85**, 501–513.

CLOSS, E.I., ALBRITTON, L.M., KIM, J.W. & CUNNINGHAM, J.M. (1993a). Identification of a low affinity, high capacity transporter of cationic amino acids in mouse liver. *J. Biol. Chem.*, **268**, 7538–7544.

CLOSS, E.I., LYONS, C.R., KELLY, C. & CUNNINGHAM, J.M. (1993b). Characterization of the third member of the MCAT family of cationic amino acid transporters. *J. Biol. Chem.*, **268**, 20796–20800.

DURANTE, W., LIAO, L. & SCHAFER, A.I. (1995). Differential regulation of L-arginine transport and inducible NOS in cultured vascular smooth muscle cells. *Am. J. Physiol.*, **268**, H1158–H1164.

FLEMING, I., GRAY, G.A., JULOU-SCHAEFFER, G., PARRATT, J.R. & STOCLET, J-C. (1990). Incubation with endotoxin activates the L-arginine pathways in vascular tissue. *Biochem. Biophys. Res. Commun.*, **171**, 562–568.

FLEMING, I., GRAY, G.A., SCHOTT, C.A. & STOCLET, J-C. (1991). Inducible but not constitutive production of nitric oxide by vascular smooth muscle cells. *Eur. J. Pharmacol.*, **200**, 375–376.

GENG, Y-J., HANSSON, G.K. & HOLME, E. (1992). Interferon-γ and tumour necrosis factor synergize to induce nitric oxide production and inhibit mitochondrial respiration in vascular smooth muscle cells. *Circ. Res.*, **71**, 1268–1276.

GENG, Y-J., ALMQVIST, M. & HANSSON, G. (1994). cDNA cloning and expression of inducible nitric oxide synthase from rat vascular smooth muscle cells. *Biochim. Biophys. Acta*, **1218**, 421–424.

GRAY, G.A., SCHOTT, C., JULOU-SCHAEFFER, G., FLEMING, I., PARRATT, J.R. & STOCLET, J-C. (1991). The effects of inhibitors of the L-arginine/nitric oxide pathway on endotoxin-induced loss of vascular responsiveness in anaesthetized rats. *Br. J. Pharmacol.*, **103**, 1218–1224.

GREEN, L.C., WAGNER, D.A., GLOGOWSKI, J., SKIPPER, P.L., WISHNOK, J.S. & TANNENBAUM, S.R. (1982). Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal. Biochem.*, **126**, 131–138.

GROSS, S.S. & LEVI, R. (1992). Tetrahydrobiopterin synthesis: An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. *J. Biol. Chem.*, **267**, 25722–25729.

HARPER, J.F. (1984). BASIC programme of a robust multiple comparison test for statistical analysis of all differences among group means. *Comput. Biol. Med.*, **14**, 437–444.

HATTORI, Y., CAMPBELL, E.B. & GROSS, S.S. (1994). Arginosuccinate synthetase mRNA and activity are induced by immunostimulants in vascular smooth muscle. *J. Biol. Chem.*, **269**, 9405–9408.

JULOU-SCHAEFFER, G., GRAY, G.A., FLEMING, I., PARRATT, J.R. & STOCLET, J-C. (1990). Loss of vascular responsiveness induced by endotoxin involves the L-arginine pathway. *Am. J. Physiol.*, **259**, H1038–H1043.

KANNO, K., HIRATA, Y., IMAI, T. & MARUMO, F. (1993). Induction of nitric oxide synthase gene by interleukin in vascular smooth muscle cells. *Hypertension*, **22**, 34–39.

KILBOURN, R.G., JUBRAN, A., GROSS, S.S., GRIFFITH, O.W., LEVI, R., ADAMS, J. & LODATO, R.F. (1990). Reversal of endotoxin-mediated shock by N^G-methyl-L-arginine, an inhibitor of nitric oxide synthesis. *Biochem. Biophys. Res. Commun.*, **172**, 1132–1138.

KIM, J.W., CLOSS, E.I., ALBRITTON, L.M. & CUNNINGHAM, J.M. (1991). Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. *Nature*, **352**, 725–728.

KNOWLES, R.G. & MONCADA, S. (1994). Nitric oxide synthases in mammals. *Biochem. J.*, **298**, 249–258.

KNOWLES, R.G., SALTER, M., BROOKS, S.L. & MONCADA, S. (1990). Anti-inflammatory glucocorticoids inhibit the induction by endotoxin of nitric oxide synthase in the lung, liver and aorta of the rat. *Biochem. Biophys. Res. Commun.*, **172**, 1042–1048.

KOIDE, M., KAWAHARA, Y., TSUDA, T., NAKAYAMA, I. & YOKOYAMA, M. (1994). Expression of nitric oxide synthase by cytokines in vascular smooth muscle cells. *Hypertension*, **23**, 145–148.

KOIDE, M., KAWAHARA, Y., TSUDA, T. & YOKOYAMA, M. (1993). Cytokine-induced expression of an inducible type of nitric oxide synthase gene in cultured vascular smooth muscle cells. *FEBS Lett.*, **318**, 213–217.

LORSBACH, R.B., MURPHY, W.J., LOWENSTEIN, C.J., SNYDER, S.H. & RUSSELL, S.W. (1993). Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. *J. Biol. Chem.*, **268**, 1908–1913.

LOW, B.C., ROSS, I.K. & GRIGOR, M.R. (1993). Characterization of system L and system y^+ amino acid transport activity in cultured vascular smooth muscle cells. *J. Cell. Physiol.*, **156**, 626–634.

LOWENSTEIN, C.J., ALLEY, E.W., RAVAL, P., SNOWMAN, A.M., SNYDER, S.H., RUSSELL, S.W. & MURPHY, W.J. (1993). Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 9730–9734.

MARCZIN, N., PAPAPETROPOULOS, A. & CATRAVAS, J.D. (1993a). Tyrosine kinase inhibitors suppress endotoxin- and IL-1β-induced NO synthesis in aortic smooth muscle cells. *Am. J. Physiol.*, **265**, H1014–H1018.

MARCZIN, N., PAPAPETROPOULOS, A., JILLING, T. & CATRAVAS, J.D. (1993b). Prevention of nitric oxide synthase induction in vascular smooth muscle cells by microtubule depolymerizing agents. *Br. J. Pharmacol.*, **109**, 603–605.

MARTIN, E., NATHAN, C. & XIE, Q-W. (1994). Role of interferon regulatory factor 1 in induction of nitric oxide synthase. *J. Exp. Med.*, **180**, 977–984.

MARUMO, T., NAKAKI, T., NAGATA, K., MIYATA, M., ADACHI, H., ESUMI, H., SUZUKI, H., SARUTA, T. & KATO, R. (1993). Dexamethasone inhibits nitric oxide synthase mRNA induction by interleukin-1α and tumor necrosis factor-α in vascular smooth muscle cells. *Jpn. J. Pharmacol.*, **63**, 361–367.

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

NAKAYAMA, D.K., GELLER, D.A., DI SILVIO, M., BLOOMGARDEN, G., DAVIES, P., PITT, B.R., HATAKEYAMA, K., KAGAMIYAMA, H., SIMMONS, R.L. & BILLIAR, T.R. (1994). Tetrahydrobiopterin synthesis and inducible nitric oxide production in pulmonary artery smooth muscle. *Am. J. Physiol.*, **266**, L455–L460.

NAKAYAMA, D.K., GELLER, D.A., LOWNSTEIN, C.J., DAVIES, P., PITT, B.R., SIMMONS, R.L. & BILLIAR, T.R. (1992). Cytokines and lipopolysaccharide induce nitric oxide synthase in cultured rat pulmonary artery smooth muscle. *Am. J. Respir. Cell Mol. Biol.*, **7**, 471–476.

NUNOKAWA, Y., ISHIDA, N. & TANAKA, S. (1993). Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **191**, 89–94.

NÜSSLER, A.K., GELLER, D.A., SWEETLAND, M.A., DI SILVIO, M., BILLIAR, T.R., MADARIAGA, J.B., SIMMONS, R.L. & LANCASTER, J.R. (1993). Induction of nitric oxide synthesis and its reactions in cultured human and rat hepatocytes stimulated with cytokines plus LPS. *Biochem. Biophys. Res. Commun.*, **194**, 826–835.

POMERANTZ, K.B., HAJJAR, D.P., LEVI, R. & GROSS, S.S. (1993). Cholesterol enrichment of arterial smooth muscle cells upregulates cytokine-induced nitric oxide synthesis. *Biochem. Biophys. Res. Commun.*, **191**, 103–109.

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 endotoxin shock. *Biochem. Biophys. Res. Commun.*, **173**, 541–547.

ROYSTON, J.P. (1984). *SPP – Statistical Package for Personal Computers.* TCL., 40B Royal Hill, London, SE10 8RT. UK.

SCHOTT, C.A., GRAY, G.A. & STOCLET, J-C. (1993a). Dependence of endotoxin-induced vascular hyporeactivity on extracellular L-arginine. *Br. J. Pharmacol.*, **108**, 38–43.

SCHMIDT, K., KLATT, P. & MAYER, B. (1994). Uptake of nitric oxide synthase inhibitors by macrophage RAW 264.7 cells. *Biochem. J.*, **301**, 313–316.

SESSA, W.C. (1994). The nitric oxide synthase family of proteins. *J. Vasc. Res.*, **31**, 131–143.

SIRSJO, A., SODERKVIST, P., SUNDQVIST, T., CARLSSON, M., OST, M. & GIDLOF, A. (1994). Different induction mechanisms of mRNA for inducible nitric oxide synthase in rat smooth muscle cells in culture and in aortic strips. *FEBS Lett.*, **338**, 191–196.

SKALLI, O., ROPRAZ, P., TRZECIAK, A., BENZONANA, G., GILLESSEN, D. & GABBIANI, G. (1986). A monoclonal antibody against α-smooth muscle actin: A new probe for smooth muscle differentiation. *J. Cell Biol.*, **103**, 2787–2796.

THIEMERMANN, C. & VANE, J. (1990). Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat in vivo. *Eur. J. Pharmacol.*, **182**, 591–595.

WANG, H., KAVANAUGH, M.P., NORTH, R.A. & KABAT, D. (1991). Cell-surface receptor for ecotropic murine retroviruses is a basic amino acid transporter. *Nature*, **352**, 729–731.

WHITE, M.F. (1985). The transport of cationic amino acids across the plasma membrane of mammalian cells. *Biochim. Biophys. Acta*, **822**, 355–374.

WRIGHT, C.E., REES, D.D. & MONCADA, S. (1992). Protective and pathological roles of nitric oxide in endotoxin shock. *Circ. Res.*, **26**, 48–57.

XIE, Q-W., CHO, H.J., CALAYCAY, J., MUMFORD, R.A., SWIDEREK, K.M., LEE, T.D., DING, A., TROSCO, T. & NATHAN, C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science*, **256**, 225–228.

XIE, Q-W., WHISNANT, R. & NATHAN, C. (1993). Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and lipopolysaccharide. *J. Exp. Med.*, **177**, 1779–1784.

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