British Journal of Pharmacology (1997) 120, 1439–1444

Dissociation of lipopolysaccharide-mediated induction of nitric oxide synthase and inhibition of DNA synthesis in RAW 264.7 macrophages and rat aortic smooth muscle cells

[1]Andrew Paul, *Clare Bryant, †Mark F. Lawson, †Edwin R. Chilvers & Robin Plevin

Department of Physiology & Pharmacology, University of Strathclyde, Royal College, 204 George Street, Glasgow G1 1XW, *Department of Biochemical Pharmacology, The Medical College, St. Bartholomews Hospital, Charterhouse Square, London EC1M 6BQ and †Respiratory Medicine Unit, Department of Medicine (RIE), Rayne Laboratory, University of Edinburgh, Royal Infirmary, Lauriston Place, Edinburgh EH3 9YW

1 The active component of endotoxin, lipopolysaccharide (LPS), inhibited basal DNA synthesis in both RAW 264.7 macrophages (IC_{50} 0.05 ± 0.03 $\mu g\ ml^{-1}$) and rat aortic smooth muscle cells (RASMC) (IC_{50} 9.7 ± 0.4 $\mu g\ ml^{-1}$).

2 In both cell types, serum differentially affected LPS-stimulated inhibition of DNA synthesis. In RAW 264.7 macrophages the presence of serum reduced the IC_{50} for LPS-stimulated inhibition of DNA synthesis (1.4 ± 0.85 ng ml^{-1}). However, in RASMC serum stimulated DNA synthesis and further increased the IC_{50} value for LPS-stimulated inhibition of thymidine incorporation (57.3 ± 7.8 $\mu g\ ml^{-1}$).

3 LPS also stimulated the induction of nitric oxide synthase (NOS) in RAW 264.7 macrophages with maximal expression at concentrations of $1-3$ $\mu g\ ml^{-1}$. This was wholly dependent upon the presence of serum. In RASMC LPS alone, up to concentrations of 100 $\mu g\ ml^{-1}$, did not induce nitric oxide synthase and required co-incubation with the direct activator of adenylyl cyclase, forskolin. Under these conditions stimulated expression of NOS was inhibited by the presence of serum.

4 Incubation with the nitric oxide synthase inhibitors N^{ω}-nitro-L-arginine methyl ester (L-NAME) and L-canavanine did not reverse the inhibition of [^{3}H]-thymidine incorporation in response to LPS but prevented the formation of nitrite in both cell types.

5 These results indicate that the effects of LPS upon cell growth are independent of the induction of the 130 kDa isoform of nitric oxide synthase and nitric oxide formation in both RAW 264.7 macrophages and RASMC.

Keywords: DNA synthesis; lipopolysaccharide

Introduction

The active component of endotoxin, lipopolysaccharide (LPS), has major effects upon cells of the vasculature causing systemic hypotension through initiation of smooth muscle relaxation and impairment of endothelial cell function (see Moncada *et al*., 1991). LPS also profoundly affects immune cell function and illicits a non-specific response, particularly in macrophages which represent one of the main cellular targets for LPS. The effects of LPS are achieved primarily through the induction of a number of new genes particularly a number of cytokines and chemokines (Sunderkotter *et al*., 1994). This also includes the stimulated induction of the 130 kDa isoform of nitric oxide synthase (iNOS), leading to the subsequent generation of nitric oxide (NO) (Schmidt & Walter, 1994). Generation of NO by macrophages contributes to their cytotoxic activity towards bacteria whilst in smooth muscle NO initiates smooth muscle relaxation mediated by guanylyl cyclase activation and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) formation (Schmidt & Walter, 1994).

An additional component of LPS action may involve the regulation of cell growth and division. Cytokines such as interferon (IFN)γ have previously been shown to inhibit DNA synthesis and cell growth and division by a NO-dependent mechanism (Garg & Hassid, 1989; Nunokawa & Tanaka, 1992). This is presumed to be due to a cyclic GMP/protein kinase G mediated inhibition of DNA synthesis (Nunokawa & Tanaka, 1992). In addition, it has recently been shown that in murine peritoneal macrophages and RAW 264.7 macrophages, NO itself is the cellular mediator of apoptosis and may be reflected in the restriction of cellular proliferation (Albina *et al*., 1993; Mebmer *et al*., 1994). However, in rat peritoneal macrophages both bacterial derived lipopeptides stimulate cellular apoptosis/cell death in a manner independent of induced NO production (Terenzi *et al*., 1995). Thus, the role of NOS induction and NO formation and its relationship to inhibition of cell growth and/or apoptosis remains controversial.

In this study we show that LPS inhibits both basal and serum-stimulated DNA synthesis in cultured RAW 264.7 macrophages and rat aortic smooth muscle cells. These effects can be dissociated from the induction of iNOS by a number of different approaches and are distinct from induced cellular apoptosis. This may indicate the involvement of different or divergent signalling pathways in each functional response upon stimulation with LPS.

Methods

Culture of RAW 264.7 macrophages and rat aortic smooth muscle cells

RAW 264.7 murine macrophages were obtained from the European Cell Culture Collection. Primary cultures of vascular smooth muscle cells were established from thoracic aortae excised from 180–240 g Wistar rats and enzymically dispersed by collagenase (1 mg ml^{-1}) and elastase (0.1 mg ml^{-1}) in the presence of soya bean trypsin inhibitor (1 mg ml^{-1}). Both RAW 264.7 macrophages and RASMC were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) foetal calf serum (FCS), 2 mM glutamine, 250 iu ml^{-1} penicillin and 250 $\mu g\ ml^{-1}$ streptomycin at 37°C in a humidified atmosphere of air/CO_2 (19:1).

[1] Author for correspondence.

[^{3}H]-thymidine incorporation

Smooth muscle cells were seeded onto 24 well culture dishes and when near confluent were rendered quiescent by serum deprivation for 48 h. Agonists were subsequently added and the cells incubated for a further 24 h. Macrophages were grown to near confluence and maintained in serum free media for 12 h before being stimulated with LPS in the presence or absence of 2% (v/v) FCS for 24 h. In each cell type [^{3}H]-thymidine (0.1 μCi ml^{-1}) was added for the final 14 h of the incubation. Cells were washed twice in phosphate buffered saline (PBS), three times in 5% (w/v) trichloroacetic acid (TCA), twice with ethanol and then solubilized in 0.3 M NaOH. [^{3}H]-thymidine incorporation in the recovered solubilized extracts was assessed by liquid scintillation counting.

Immunological detection of iNOS

Following stimulation cells were washed twice with ice-cold 20 mM HEPES buffer, pH 7.4 containing 150 mM NaCl, 50 mM NaF, 10 mM $Na_4P_2O_7$, 4 mM EDTA, 2 mM EGTA and Na_3VO_4 (HPFEV) then solubilized in hot (70°C) SDS–PAGE (sodium dodecyl sulphate) sample buffer. The samples were dispersed by repeated passage through a 21G needle and then transferred to Eppendorf tubes. Subsequent to boiling, the samples were stored at −20°C until analysis. Aliquots (15–100 μg protein) were subjected to SDS–PAGE on 7.5% polyacrylamide slab gels and then blotted onto nitrocellulose. The nitrocellulose membranes were incubated for 3 h in 150 mM NaCl, 20 mM Tris, 0.02% (v/v) Tween-20 pH 7.4 (NaTT), containing 3% (w/v) BSA then incubated overnight in NaTT containing 0.2% (w.v) BSA and 1 μg ml^{-1} of anti-NOS antibody. Following six washes in NaTT, the membranes were incubated with anti-mouse Ig-HRP (horseradish peroxidase) for 90 min and then washed a further 6 times in NaTT. The immunoblots were developed with the enhanced chemiluminence (ECL) detection system (Amersham).

Assessment of NOS activity

NOS activity was estimated by a modification of the method of Mitchell *et al.* (1992) as previously described (Paul *et al.*, 1995). Cells were harvested from six well plates and centrifuged for 2 min at 1000 g. The supernatant was removed and the pellet resuspended in 25 mM HEPES buffer pH 7.5, containing 25 mM β-mercaptoethanol, 10 μM pepstatin, 10 μM leupeptin, 1% (v/v) Triton X-100 and incubated on ice for 30 min. The cell extracts were incubated in 10 mM HEPES buffer pH 7.4 containing 1 mM NADPH, 10 μM biopterin, 10 μM FAD (flavin adenine dinucleotide), 10 μM FMN (flavin mononucleotide), and 10 μM L-arginine/[^{3}H]-L-arginine (specific activity = 37 MBq ml^{-1}, 0.1 μCi per tube) for 15 min at 37°C. [^{3}H]-L-citrulline was separated by Dowex cation-exchange chromatography and measured by liquid scintillation counting.

Assessment of NO production: detection of cellular nitrite release

NO production was measured indirectly by assessing the accumulation of nitrite. The supernatants of cultured macrophages and RASMC were removed and assayed for nitrite accumulation by the Greiss reaction as an indication of iNOS activity (Gross *et al.*, 1991). Briefly, an equal volume of Greiss Reagent (4% (w/v) sulphanilamide and 0.2% (w/v) napthylenediamide in 10% (v/v) phosphoric acid) was added to an equal volume of sample and colorimetric difference in the optical density at 540 and 620 nm recorded immediately. The values obtained were compared to standards of sodium nitrite dissolved in DMEM and calculated as μM concentrations of nitrite release.

Statistics

Statistical analysis of the data was performed by an unpaired *t* test. Analysis of the concentration-response curves was performed by an iterative curve fitting procedure (Delean *et al.*, 1980).

Materials

Mouse monoclonal antibodies to the 130 kDa inducible macrophage-type isoform of NOS were purchased from Affiniti Research Products (Exeter, U.K.). [^{3}H]-thymidine (specific activity 1.4 TMBq mmol^{-1}) and [^{3}H]-L-arginine (specific activity 1.3 TMBq mmol^{-1}) were purchased from N.E.N. (Stevenage, U.K.). ECL detection reagents were purchased from Amersham International (Bucks., U.K.) and all cell culture reagents were supplied by Gibco (Paisley, Scotland). All other chemicals were of the highest commercial grade available.

Results

The effect of LPS upon [^{3}H]-thymidine incorporation in RAW 264.7 macrophages and rat aortic smooth muscle cells (RASMC) is shown in Figure 1. In macrophages, LPS alone reduced basal [^{3}H]-thymidine incorporation by approximately 80–85% and corresponded to an IC_{50} value of 0.05 ± 0.03 μg ml^{-1} ($n = 3$). This effect was enhanced in the presence of serum and generated an IC_{50} value of 1.4 ± 0.85 ng ml^{-1}. It should be noted that in some experiments serum also inhibited [^{3}H]-thymidine incorporation. Similarly in RASMC, LPS reduced both basal and serum-stimulated [^{3}H]-thymidine incorporation (Figure 1b). However, in the presence of serum the inhibitory effect of LPS upon thymidine incorporation was reduced (Figure 1b). These represented IC_{50} values of 9.7 ± 0.4 μg ml^{-1} and 57.3 ± 7.8 μg ml^{-1} in the presence or absence of serum, respectively. Although LPS maximally stimulated inhibition of basal thymidine incorporation, low concentrations of LPS were also observed to potentiate serum stimulated thymidine incorporation.

In RAW 264.7 macrophages, LPS stimulated the induction of immunologically detectable iNOS over a 24 h period in a concentration-dependent manner (Figure 2a). This coincided with a marked increase in recoverable NOS activity as observed previously (Paul *et al.*, 1995; $EC_{50} = 68.7 \pm 23$ ng ml^{-1}). In RASMC, LPS alone, up to concentrations of 100 μg ml^{-1}, did not stimulate an increase in the expression of the 130 kDa isoform of NOS. However, upon co-incubation with 10 μM forskolin, a direct activator of adenylyl cyclase, a marked increase in NOS induction was observed (Figure 2b), maximal induction apparent in the presence of 100 μg ml^{-1} LPS. Forskolin alone did not induce NOS protein expression or NOS activity (results not shown).

LPS-stimulated expression of iNOS in myeloid cells is well recognised to require the integral involvement of a serum-derived LPS binding protein (LBP) that mediates binding of LPS to and interaction with CD14 (Schumann *et al.*, 1990; Martin *et al.*, 1992; Schumann, 1992), the receptor for bacterial LPS (Wright *et al.*, 1990). Figure 3 shows the effect of serum upon LPS-induced expression of iNOS in RAW 264.7 macrophages and RASMC. In macrophages, serum was an absolute requirement for the stimulated expression of NOS in response to LPS (Figure 3a). However, in RASMC co-incubation with 2% FCS reduced stimulated expression of NOS by LPS and forskolin in combination (Figure 3b).

In order to examine further the potential role of NO formation in the inhibition of cell growth, the effect of two inhibitors of NOS activity, N^G-nitro-L-arginine-methyl ester (L-NAME, 100 μM) and L-canavanine (300 μM), upon LPS-induced inhibition of [^{3}H]-thymidine incorporation were examined. Both compounds failed to reverse the inhibitory effect of LPS on [^{3}H]-thymidine in RAW 264.7 macrophages and

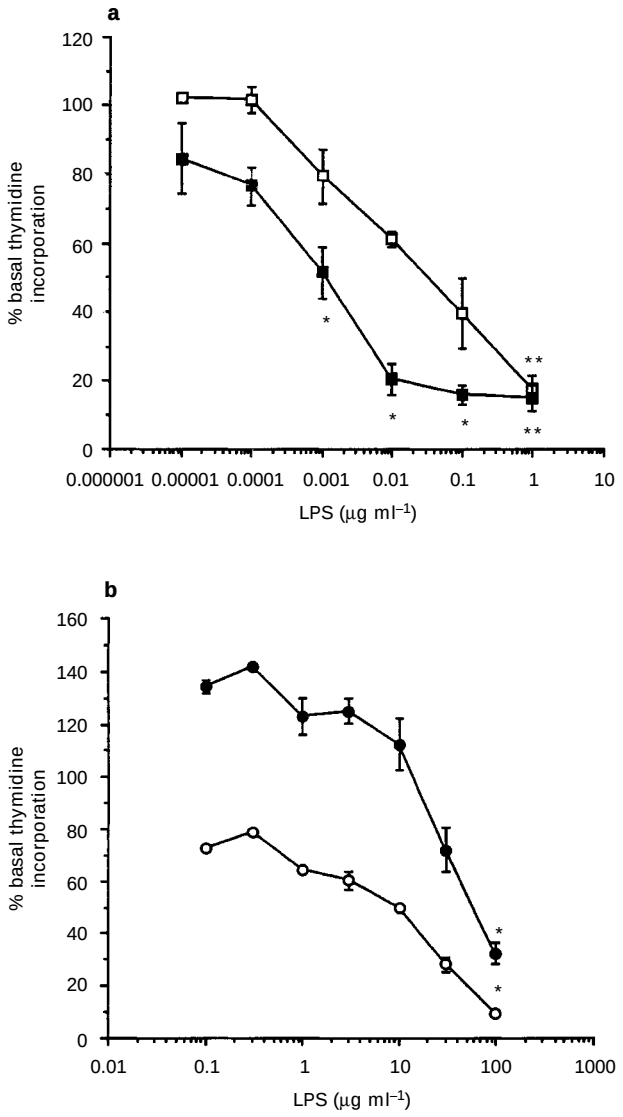


Figure 1 The effect of LPS upon [^{3}H]-thymidine incorporation in RAW 264.7 macrophages and RASMC. Cells were cultured as outlined in the Methods section. In (a) RAW 264.7 macrophages were incubated with LPS in the absence (□) or presence (■) of 2% serum for 24 h. In (b) RASMC were treated with LPS in the absence (○) or presence (●) of 2% serum for 24 h. During the final 14 h 0.1 μCi ml^{-1} of [^{3}H]-thymidine was added and incorporation measured at the end of the 24 h period as outlined in the Methods section. Each value is the mean of duplicate determinations from at least 3 independent experiments; vertical lines show s.e.mean. In (a) *significantly different from corresponding LPS concentration in the absence of FCS, $P<0.05$; **significantly different from controls in the absence or presence of FCS, $P<0.05$. In (b) *significantly different from controls in the absence or presence of FCS, $P<0.05$. Representative control values (100%, d.p.m. $\pm$ s.e.mean) for basal thymidine incorporation in RAW 264.7 macrophages and RASMC were 87699 $\pm$ 1720 and 12531 $\pm$ 141, respectively.

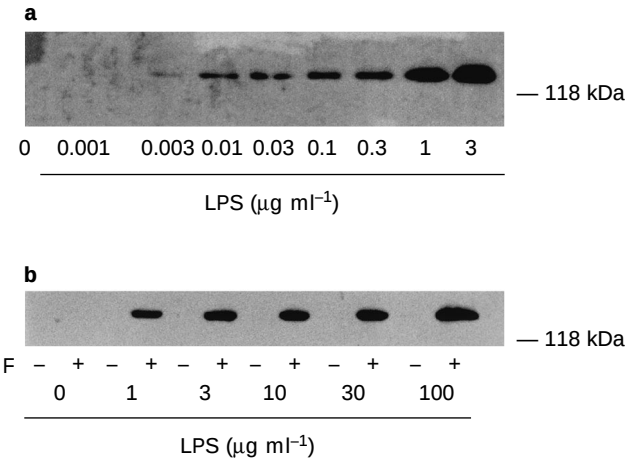


Figure 2 LPS-stimulated induction of NOS in RAW 264.7 macrophages and RASMC. In (a) RAW 264.7 macrophages were incubated with increasing concentration of LPS for 24 h. In (b) RASMC were incubated with increasing concentrations of LPS in the absence (−) or presence (+) of 10 μM forskolin (F) for 24 h. Upon termination of reactions, expression of iNOS was determained by Western blotting as outlined in the Methods section. Each blot is representative of at least three others.

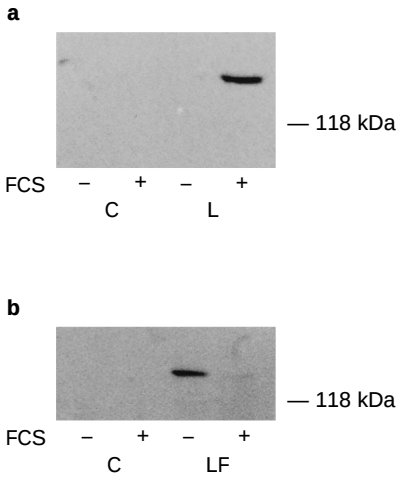


Figure 3 The effect of serum on LPS-stimulated induction of NOS in RAW 264.7 macrophages and RASMC. In (a) RAW 264.7 macrophages were incubated with LPS alone (L) in the absence (−) or presence (+) of 2% serum for 24 h. In (b) RASMC were treated additionally with 10 μM forskolin (F). Upon termination of reactions, expression of iNOS was determined by Western blotting as outlined in the Methods section. Each blot is representative of at least four others.

RASMC (Figure 4a–d). However, the LPS-induced NOS activity was abolished, as assessed by an *in vitro* assay (data not shown). These compounds did not prevent the induction of NOS in either cell type (Figure 5a, b). However, they inhibited the formation of nitrite in RAW 264.7 macrophages (Figure 5c; IC_{50} values of 46 $\pm$ 15 μM and 90 $\pm$ 45 μM, respectively) and RASMC (data not shown). In addition, virtual abolition of LPS-stimulated nitrite production was only apparent if both L-NAME and L-canavanine were added before the period of LPS stimulation (Table 1).

Nitric oxide formation itself has previously been shown to possess antitumour properties and also mediate growth arrest, cell necrosis and apoptosis (Nathan, 1992). Inhibition of cell growth in these studies was not a manifestation of stimulated cellular apoptosis. Little or no chromatin condensation and DNA fragmentation, characteristic of cells undergoing apoptosis, were observed under the conditions of LPS stimulation described here (data not shown), only supramaximal stimulation (10 μg ml^{-1} LPS + 100 u ml^{-1} IFNγ) of RAW 264.7 macrophages did result in cellular apoptosis (data not shown). No apparent DNA fragmentation and apoptosis was observed in RASMC under any of the conditions of cell stimulation (data not shown).

Discussion

In this study we showed that LPS stimulates the inhibition of DNA synthesis in both macrophages and in vascular smooth muscle cells via a mechanism which is dissociated from the

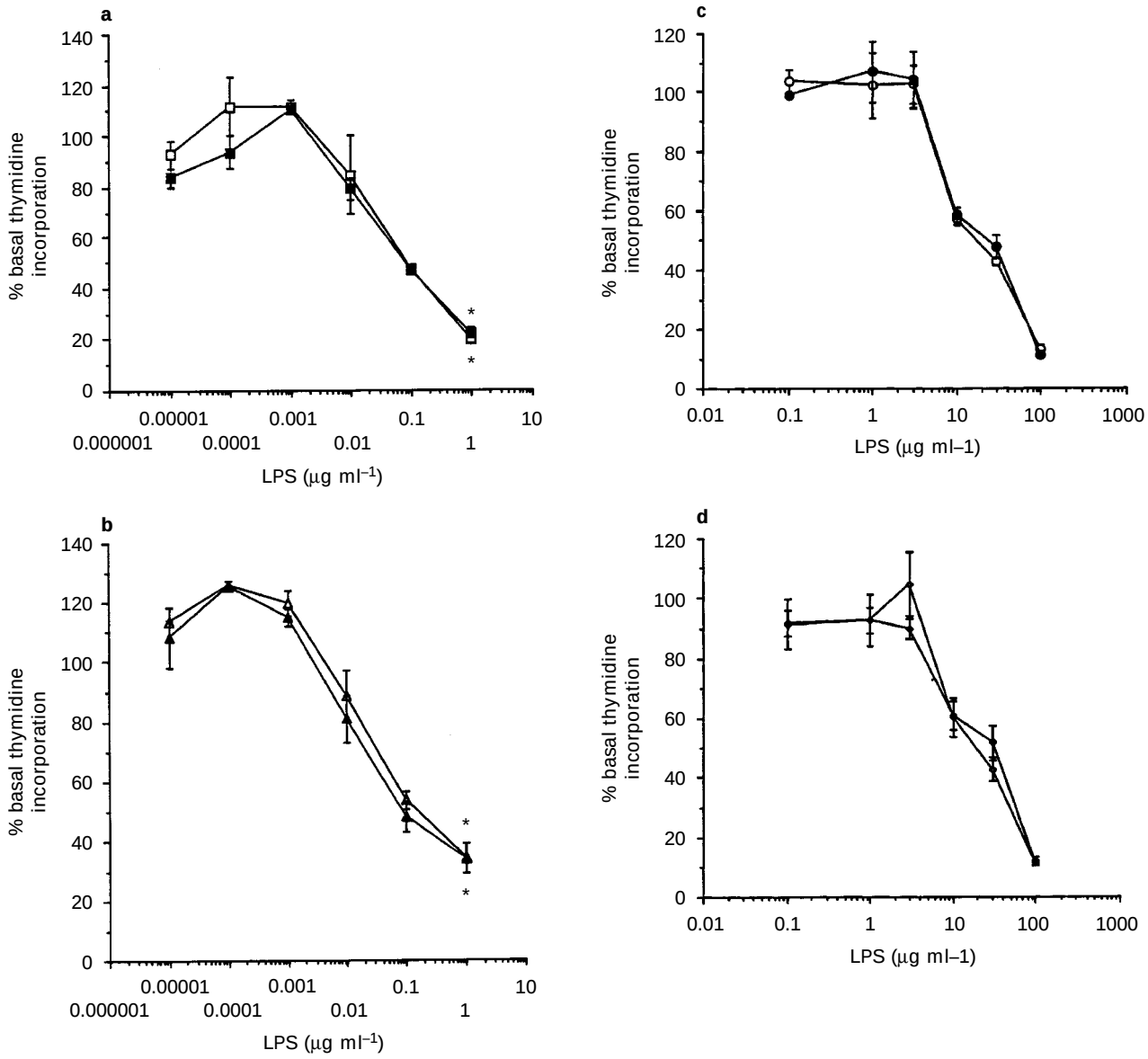
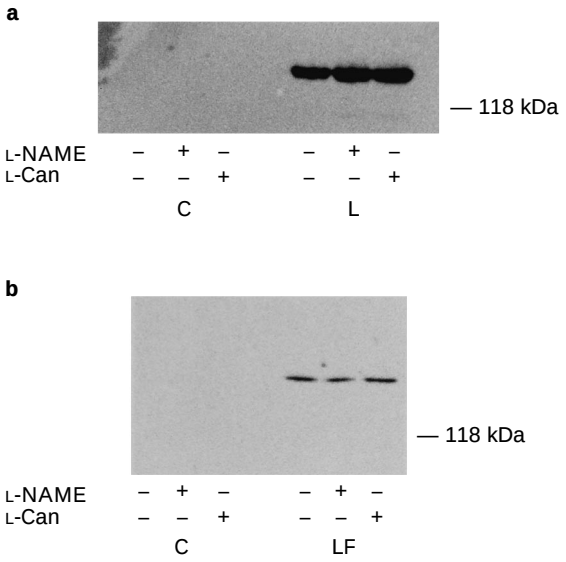


Figure 4 The effect of L-NAME and L-canavanine on LPS-mediated inhibition of [^{3}H]-thymidine incorporation in RAW 264.7 macrophages and RASMC. In (a) RAW 264.7 macrophages were preincubated for 30 min in the absence (□) or presence (■) of 100 μM L-NAME before stimulation with increasing concentrations of LPS for 24 h. In (b) RAW 264.7 macrophages were preincubated for 30 min in the absence (△) or presence (▲) of 300 μM L-canavanine before stimulation with increasing concentrations of LPS for 24 h. In (c) RASMC were preincubated for 30 min in the absence (○) or presence (●) of 100 μM L-NAME before stimulation with increasing concentrations of LPS for 24 h. In (d) RASMC were preincubated for 30 min in the absence (◇) or presence (◆) of 300 μM L-canavanine before stimulation with increasing concentrations of LPS for 24 h. In each case, cells were assayed for [^{3}H]-thymidine incorporation as described in the Methods section. Each value is the mean of duplicate determinations from at least three independent experiments and vertical lines show s.e.mean. In (a–d): *significantly different from controls in the absence or presence of L-NAME/L-canavanine, $P<0.05$. Comparison of individual LPS concentrations either in the presence and absence of inhibitor compounds showed no significant difference, $P<0.05$ for all points in either cell type.

induction of nitric oxide synthase. The potential for the formation of NO to have an effect upon smooth muscle and macrophage cell division has not been extensively documented. However, it has been assumed that NO will decrease cell growth through effects upon cellular levels of cyclic GMP which has been previously shown to be associated with an inhibition of cell growth (Garg & Hassid, 1989; Nunokawa & Tanaka, 1992). It is also possible that NO may play a role in the regulation of cellular apoptosis (Albina *et al*., 1994; Mebmer *et al*., 1994). However, evidence in favour of this hypothesis is not overwhelming.

Although LPS stimulated the expression of the 130 kDa isoform of NOS in both RAW 264.7 macrophages and RASMC, several observations presented in this study do not support the involvement of NOS in the actions of LPS upon cell growth. Both parameters could, for example, be distinguished according to serum conditions. In RAW 264.7 macrophages we found that LPS reduced basal DNA synthesis independently of the presence of serum while serum alone was also able to inhibit basal DNA synthesis. The effects of serum alone on cell growth may represent the inability of the cells to re-enter the cell cycle after a 24 h period of 'growth-factor' depletion or potentially due to an unknown concentration of endotoxin in the serum. However, irrespective of this, we and others have previously shown that the presence of serum is required for the induction of NOS activity in this cell line (Paul *et al*., 1995), consistent with the participation of a serum-derived LPS-binding protein in the initiation of LPS binding to its putative receptor CD14 (Martin *et al*., 1992). Here we show that the effect of serum upon LPS-induced NOS activity is mediated at the level of protein expression, thus confirming



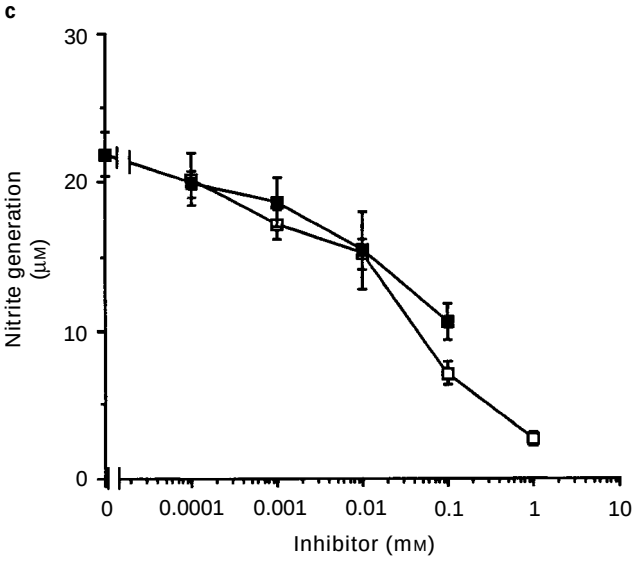


Figure 5 The effect of L-NAME and L-canavanine on LPS-stimulated expression of NOS in RAW 264.7 macrophages and RASMC and LPS-mediated nitrite formation in RAW 264.7 macrophages. RAW 264.7 macrophages (a) and RASMC (b) were preincubated in the absence (−) or presence (+) of vehicle, 100 μM L-NAME or 300 μM L-canavanine as indicated for 30 min before stimulation with LPS or LPS in combination with 10 μM forskolin (F) for 24 h. Upon termination of reactions, expression of NOS was determined by Western blotting as outlined in the Methods section. Each blot is representative of at least three others. In (c) RAW 264.7 macrophages were preincubated with vehicle or increasing concentrations of L-NAME (□) or L-canavanine (■) for 30 min before stimulation with LPS (1 μg ml^{-1}). After 24 h generation of nitrite was assessed in recovered culture supernatants as described in the Methods section. Each value is the mean of triplicate determinations from a single experiment representative of three; vertical lines show s.e.mean.

Table 1 The effect of L-NAME or L-canavanine addition pre- or post-incubation with vehicle or LPS

Addition	*Nitrite production (μM) at 24 h* *LPS + vehicle*	*LPS + inhibitor*
Vehicle or L-NAME before stimulation period	20 ± 1.6	2.95 ± 0.3
Vehicle or L-canavanine before stimulation period	22 + 1.5	13.1 ± 0.6
Vehicle or L-NAME 22 h after stimulation period	20 + 1.7	2.7 ± 2.0
Vehicle or L-canavanine 22 h after stimulation period	22 ± 1.0	10.6 ± 1.2

RAW 264.7 macrophages were pretreated with vehicle, L-NAME (100 μM) or L-canavanine (300 μM) before stimulation with LPS (1 μg ml^{-1}) for 24 h or with vehicle, L-NAME (100 μM) or L-canavanine (300 μM) for 2 h following stimulation with LPS (1 μg ml^{-1}) or LPS + inhibitor for 22 h. In both cases nitrite formation as assessed after 24 h as described in the Methods section. Values represent mean ± s.e.mean from three independent experiments performed in duplicate.

that the effects of LPS on NOS induction can be dissociated from the effects on DNA synthesis by this intervention. These findings suggest that LPS is mediating its effects on cellular growth and DNA synthesis via an NO-independent signalling pathway.

LPS displayed inhibitory affects upon basal DNA synthesis in vascular smooth muscle cells, which was also apparent in serum-stimulated cells, with only a small decrease in the apparent IC_{50} values (approximately 5 fold) recorded for the effect of LPS in either condition. However, in this cell system LPS alone, at concentrations which inhibited DNA synthesis, did not stimulate the expression of either NOS or NOS activity, but required the presence of forskolin. The lack of effect of LPS alone on the induction of NOS in cultured smooth muscle remains unclear and the molecular basis for this phenomenon is at present unknown. However, since protein kinase C (PKC) isoforms, particularly PKCε, may also play some tonic inhibitory role in the regulation of interleukin-1β (IL-1β)-mediated NOS induction in rat renal mesangial cells (Muhl & Pfielschiffter, 1994) and LPS-stimulated NOS induction in RASMC (Paul & Plevin, 1995; Paul *et al*., unpublished observations), the finding that PKCε is highly expressed in cultured RASMC may represent a possible explanation.

It is also apparent that the inhibition of cell growth was not as a result of stimulated apoptosis. Indeed, results described here were contrary to previous studies conducted in RAW 264.7 macrophages whereby stimulated NO-dependent apoptosis was observed (Mebmer *et al*., 1994). Stimulated apoptosis only occurred in response to LPS and IFNγ added in combination. However, these represent concentrations (10 μg ml^{-1} LPS + 100 u ml^{-1} IFNγ) far in excess of those observed to be maximal (1 μg ml^{-1} LPS) for stimulated expression of the 130 kDa isoform of NOS in these murine-derived macrophages (Paul *et al*., 1995). This may further indicate the dissociation of NO production from the inhibition of cell growth. Moreover, this may support the proposal that LPS can differentially regulate cell cycle events including cycle arrest and different types of cell death; either necrosis or apoptosis. Therefore, LPS may stimulate varying cell cycle responses in either an NO-dependent or independent manner and these responses may be selectively dependent upon the degree of LPS/cytokine exposure and NO-generation thereafter.

A previous study has implicated a role for NOS and NO generation in IFNγ-stimulated inhibition of DNA synthesis in RASMC as co-incubation with IFNγ dose-dependently inhibited basal thymidine incorporation (Nunokawa & Tanaka, 1992). This effect of IFNγ upon cellular proliferation was inhibited by prior incubation of these cells with N^G-nitro-L-arginine, an iNOS inhibitor compound (Nunokawa & Tanaka, 1992). We therefore examined the effect of similar NOS inhibitors, L-NAME and L-canavanine, upon the ability of LPS to inhibit DNA synthesis.

Pre-incubation with these NOS inhibitor compounds could not reverse the effects of LPS (Figure 4). Further, this was also true of LPS stimulation of both cell types in the presence of serum (data not shown). This was not due to the inability of these compounds to cross the plasma membrane since these compounds prevented the formation of nitrite in both cell types (Figure 5c; data not shown). It is possible that prolonged

incubation with these compounds may have potential non-specific effects, however, we found that both agents did not prevent the induction of NOS over the 24 h time period (Figure 5a, b). Taken together these results strongly argue against a role for NO in the inhibitory effect of LPS upon cellular DNA synthesis.

While these results indicate that the inhibition of DNA synthesis and induction of NOS are distinct cellular events activated in response to LPS, it is at present unclear which cellular signalling pathways may be involved. In addition this study further exemplifies and is consistent with the recent finding that genetic mice lacking the iNOS gene exhibit both iNOS-dependent and iNOS-independent routes leading to LPS-stimulated hypotension and death (MacMicking *et al*., 1995). Therefore, in RAW 264.7 macrophages and RASMC, both NO-dependent and independent pathways are relevant to the regulation of cell cycle responses induced by LPS. In particular, LPS-stimulated inhibition of cell growth is mediated in an NO-independent manner.

This work was funded by a Wellcome Trust Grant to R.P.. E.R.C. is a Wellcome Senior Clinical Research Fellow.

References

ALBINA, J.E., CUI, S., MATEO, R.B. & REICHNER, J.S. (1994). Nitric oxide mediated apoptosis in murine peritoneal macrophages. *J. Immunol*., **150,** 5080 – 5085.

DELEAN, A., MUNSON, P.J. & RODBARD, D. (1980). Simultaneous analysis of a family of sigmoidal curves: application to bioassay and physiological dose response curves. *Am. J. Physiol*., **235,** E99 – E102.

GARG, U.T. & HASSID, A. (1989). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest*., **83,** 1744 – 1777.

GROSS, S.S., JAFFE, E.A., LEVI, R. & KILBOURN, R.G. (1991). Cytokine-activated endothelial cells express an isotype of nitric oxide synthase which is tetrahydrobiopterin-dependent, calmodulin-dependent and inhibited by arginine analogs with a rank-order potency characteristic of activated macrophages. *Biochem. Biophys. Res. Commun*., **178,** 823 – 829.

MACMICKING, J.D., NATHAN, C., HOM, G., CHARTRAIN, N., FLETCHER, D.S., TRUMBAUER, M., STEVENS, K., XIE, Q.-W., SOKOL, K., HUTCHISON, N., CHEN, H. & MUDGETT, J.S. (1995). Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell*, **81,** 641 – 650.

MARTIN, T.R., MATHISON, J.C., TOBIAS, P.S., LETURCQ, D.J., MORIARTY, A.M., MAUNDER, R.J. & ULEVITCH, R.J. (1992). Lipopolysaccharide binding protein enhances the responsiveness of alveolar macrophages to bacterial lipopolysaccharide. *J. Clin. Invest*., **90,** 2209 – 2219.

MEBMER, U.K., ANKARCRONA, M., NICOTERA, P. & BRUNE, B. (1994). p53 expression in nitric oxide-induced apoptosis. *FEBS Lett*., **355,** 23 – 26.

MITCHELL, J.A., KOLHAAS, K.L., MATSUMOTO, T., POLLOCK, J.S., FORSTERMANN, U., WARNER, T.D., SCHMIDT, H.H.W. & MURAD, F. (1992). Induction of NADPH-dependent diaphorase and nitric oxide synthase activity in aortic smooth muscle and cultured macrophages. *Mol. Pharmacol*., **41,** 1163 – 1168.

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

MUHL, H. & PFEILSCHIFTER, J. (1994). Possible role of protein kinase C-ε isoenzyme in inhibition of interleukin 1β induction of nitric oxide synthase in rat renal mesangial cells. *Biochem. J*., **303,** 607 – 612.

NATHAN, C. (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB J*., **6,** 3051 – 3059.

NUNOKAWA, Y. & TANAKA, S. (1992). Interferon-γ inhibits proliferation of rat vascular smooth muscle cells by nitric oxide generation. *Biochem. Biophys. Res. Commun*., **188,** 409 – 415.

PAUL, A., PENDREIGH, R.H. & PLEVIN, R. (1995). Protein kinase C and tyrosine kinase pathways regulate lipopolysaccharide-induced nitric oxide synthase activity in RAW 264.7 macrophages. *Br. J. Pharmacol*., **114,** 482 – 488.

PAUL, A. & PLEVIN, R. (1995). Cell signalling events involved in mediating the induction of nitric oxide synthase in macrophages and vascular smooth muscle cells. *Biochem. Soc. Trans*., **23,** 1029 – 1033.

PAUL, A., DOHERTY, K. & PLEVIN, R. (1997). Differential regulation by Protein Kinase C isoforms of nitric oxide synthase induction in RAW 264.7 macrophages and rat aortic smooth muscle cells. *Br. J. Pharmacol*., (in press).

SCHMIDT, H.H.H.W. & WALTER, U. (1994). NO at work. *Cell*, **78,** 919 – 925.

SCHUMANN, R.R. (1992). Function of lipolysaccharide (LPS)-binding protein (LBP) and CD14, the receptor for LPS/LBP complexes: a short review. *Res. Immunol*., **143,** 11 – 15.

SCHUMANN, R.R., LEONG, S.R., FLAGGS, G.W., GRAY, P.W., WRIGHT, S.D., MATHISON, J.C., TOBIAS, P.S. & ULEVITCH, R.J. (1990). Structure and function of lipopolysaccharide binding protein. *Science*, **249,** 1429 – 1431.

SUNDERKOTTER, C., STEINBRINK, K., GOEBELER, M., BHARDWAJ, R. & SORG, C. (1994). Macrophages and angiogenesis. *J. Leukoc. Biol*., **55,** 410 – 422.

TERENZI, F., DIAZ-GUERRA, M.J.M., CASADO, M., HORTELANO, S., LEONI, S. & BOSCA, L. (1995). Bacterial lipopeptides induce nitric oxide synthase and promote apoptosis through nitric oxide-independent pathways in rat macrophages. *J. Biol. Chem*., **270,** 6017 – 6021.

WRIGHT, S.D., RAMOS, R.A., TOBIAS, P.S., ULEVITCH, R.J. & MATHISON, J.C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science*, **249,** 1431 – 1433.

(Received October 15, 1996
Accepted January 14, 1997)