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Analysis of the signal transduction in the induction of nitric oxide synthase by lipoteichoic acid in macrophages

Muralitharan Kengatharan, Sjef J. De Kimpe & [1]Christoph Thiemermann

The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London, EC1M 6BQ

1 This study investigates the signal transduction mechanisms leading to the enhanced formation of nitric oxide (NO) due to the induction of NO synthase (iNOS) in murine J774.2 macrophages in culture activated with lipoteichoic acid (LTA), a cell wall component of the gram-positive bacterium *Staphylococcus aureus*.

2 LTA (10 $\mu g\ ml^{-1}$) caused within 24 h an enhanced accumulation of nitrite (an indicator of NO biosynthesis) in the supernatant of J774.2 macrophages which was prevented by the non-selective NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA; IC_{50}: 35μM) or by the iNOS-selective NOS inhibitor, aminoethyl-isothiourea (AE-ITU; IC_{50}: 6 μM). The inhibition of nitrite formation afforded by these agents was prevented by excess L-arginine (3–30 mM), but not by D-arginine (3–30 mM). Furthermore, the degree of iNOS inhibition was similar when these NOS inhibitors were added to the macrophages 10 h after LTA.

3 Pretreatment of J774.2 macrophages with cyclohexamide or dexamethasone prevented the enhanced formation of nitrite caused by LTA. This inhibition did not occur when dexamethasone or cyclohexamide were added to the cells 10 h after LTA. The increase in nitrite formation stimulated by LTA (10 $\mu g\ ml^{-1}$) was not affected by polymyxin B (0.05–0.5 $\mu g\ ml^{-1}$), an agent which binds and inactivates endotoxin.

4 A specific inhibitor of phosphatidylcholine-phospholipase C (PC-PLC), D609, prevented the increase in nitrite formation (IC_{50} = 20 $\mu g\ ml^{-1}$) caused by LTA. The inhibition afforded by D609 was significantly smaller when this agent was added to the cells 10 h after LTA.

5 The structurally distinct tyrosine kinase inhibitors, erbstatin, genistein, and tyrphostin AG126 prevented the formation of nitrite caused by LTA. The inhibition afforded by these compounds was significantly attenuated when they were added to the cells 10 h after LTA. In contrast, daidzein or tyrphostin A-1, which are inactive analogues of genistein and tyrphostin (up to a concentration of 10 μM) did not affect the nitrite formation caused by LTA.

6 Inhibitors of the activation of the nuclear transcription factor NF-κB such as pyrrolidine dithiocarbamate (PDTC; an antioxidant and a metal chelator), butylated hydroxyanisole (BHA; an antioxidant), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), calpain inhibitor I (both IκB-protease inhibitors), or rotenone (an antioxidant which inhibits electron transport) prevented the nitrite formation stimulated by LTA. The inhibition afforded by these agents was significantly smaller when they were added to the macrophages 10 h after LTA.

7 Incubation of J774.2 cells with LTA over 24 h resulted in the expression of iNOS protein (130 kDa) as identified by Western blot analysis. The expression of iNOS protein by LTA was significantly attenuated by cyclohexamide, D609, tyrphostin AG126, PDTC or by TPCK.

8 Thus, the signal transduction leading to the expression of iNOS protein and activity caused by LTA in murine J774.2 macrophages involves (i) the activation of PC-PLC, (ii) phosphorylation of tyrosine kinase, and (iii) the activation of the transcription factor NF-κB.

Keywords: Nitric oxide synthase; lipoteichoic acid; macrophages; PC-PLC; tyrosine kinase; NF-κB

Introduction

Nitric oxide (NO) is a gaseous autacoid with potent vasodilator and cytotoxic effects (for reviews see, Moncada *et al.*, 1991; Moncada & Higgs, 1993). NO is formed from one of the terminal guanidino nitrogen atoms of L-arginine by nitric oxide synthase (NOS). NOS exists as three distinct isoforms, namely (i) endothelial NOS (eNOS), (ii) brain NOS (bNOS) and (iii) cytokine inducible NOS (iNOS) (Dinerman *et al.*, 1993; Moncada & Higgs, 1993). Activation of eNOS and bNOS requires an increase in the intracellular concentration of calcium, whereas iNOS does not (Cho *et al.*, 1992). Exposure of cells in culture to lipopolysaccharide (LPS or endotoxin, derived from the cell wall of gram-negative bacteria), alone or together with pro-inflammatory cytokines such as interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) or interferon-γ (IFN-γ) causes the expression of iNOS. Over-production of NO following induction of iNOS by LPS or cytokines contributes importantly to the bacteriocidal and tumouricidal effects of macrophages (Drapier *et al.*, 1988). Furthermore, an enhanced formation of NO contributes importantly to the delayed circulatory failure (excessive vasodilatation and vascular hyporeactivity to vasoconstrictor agents) associated with gram-negative sepsis and haemorrhagic shock (for review, see Thiemermann, 1994).

The iNOS gene from murine macrophages has been cloned and characterized (Lowenstein *et al.*, 1992; Lyons *et al.*, 1992), but little is known about the intracellular signal transduction mechanisms involved in the expression of this enzyme. Glucocorticoids inhibit the expression of iNOS in endothelial cells stimulated with LPS (Radomski *et al.*, 1990), and this effect is mediated by lipocortin-1 (Wu *et al.*, 1995). Activation of tyrosine kinases by cytokines has been suggested as a key event in the signal transduction pathway that mediates induction of

[1]Author for correspondence.

iNOS by LPS in murine macrophage cells (Akarasereenont *et al.*, 1994). Furthermore, transcription of a variety of cellular genes such as the gene responsible for the expression of iNOS is regulated by NF-κB/Rel (NF-κB) proteins (Baeuerle & Henkel, 1994; Xie *et al.*, 1994). The promoter of the murine gene encoding iNOS contains a sequence for a NF-κB binding site (Xie *et al.*, 1994) and inhibitors of the activation of NF-κB suppress the induction of iNOS in LPS and IFN-γ stimulated macrophages (Sherman *et al.*, 1993) and in IL-1β stimulated rat mesangial cells (Eberhardt *et al.*, 1994). However, the functional role of NF-κB in the induction of iNOS by LPS or cytokines is based mainly on the use of single agents the specificity of which is often not certain. Phosphatidylcholine-specific phospholipase C (PC-PLC) controls the activation of NF-κB in response to the binding of TNF-α to its surface receptor (Schütze *et al.*, 1994) and, on the other hand, the inhibition of tyrosine kinase with genistein inhibits the activation of NF-κB (Read *et al.*, 1992).

Although the incidence of gram-positive sepsis has risen considerably over the last decade (Bone, 1994), our knowledge regarding the mechanisms underlying the circulatory failure in gram-positive sepsis and shock is still very limited. Lipoteichoic acid (LTA), a cell wall component of the gram-positive organisms such as *Staphylococcus aureus* (a bacterium without endotoxin) enhances the production of NO through expression of iNOS in murine macrophages (Cunha *et al.*, 1993) and in vascular smooth muscle cells in culture (Auguet *et al.*, 1992; Lonchampt *et al.*, 1992). In addition, an enhanced formation of NO due to induction of iNOS is responsible for the delayed circulatory failure caused by LTA in a rodent model of septic shock (De Kimpe *et al.*, 1995). However, the signal transduction events leading to the expression of iNOS by LTA are unclear.

Here, we investigate the role of (i) PC-PLC, (ii) tyrosine kinase and (iii) the transcription factor NF-κB in the expression of iNOS induced by LTA in J774.2 macrophages. Part of this work has been communicated to the British Pharmacological Society (Kengatharan *et al.*, 1995).

Methods

Cell culture

Murine macrophages (J774.2; The European Collection of Animal Cell Culture; Salisbury, UK) were cultured in 96-well plates with Dulbecco's Modified Eagle's Medium (DMEM; 200 μl/well) containing 10% foetal calf serum (Gibco) and 4 mM L-glutamine. Cells were incubated at 37°C in a humidified incubator.

Measurement of nitrite

NOS activity was assessed by measuring the accumulation of nitrite in the supernatant of J774.2 macrophages by the Griess reaction adapted for a 96-well plate reader (Gross *et al.*, 1990). Briefly, after cells reached confluence (approximately 60,000 cells per well), iNOS activity was induced by adding fresh culture medium to the cells containing LTA (0.01–30 μg ml^{-1}). As a concentration of 10 μg ml^{-1} of LTA caused (i) a submaximal increase in nitrite in the supernatant of activated macrophages, (ii) an increase in the nitrite levels which was similar to the increase afforded by a 10 fold lower concentration of endotoxin (Szabo *et al.*, 1993) and, (iii) a small but significant reduction (≈25%) in cell viability which enabled us to study the potential contribution of NO from iNOS to cell death; this concentration of LTA was used in all subsequent studies. Nitrite accumulation in the medium was measured at 24 h after the addition of LTA. Nitrite levels were measured by adding 100 μl Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 μl of sample culture medium. The optical density at 550 nm–650 nm (OD_{550}-OD_{650}) was measured with a Molecular Devices microplate reader (Richmond, CA, U.S.A.). Nitrite concentrations were calculated by comparison with OD_{550}-OD_{650} of standard solutions of sodium nitrite prepared in culture medium. The detection limit for nitrite measurement in culture medium by the Griess method was 1 μM.

In order to elucidate whether the inhibition of nitrite formation by various agents in J774.2 macrophages activated with LTA is due to inhibition of the expression of iNOS, or of its activity, separate experiments were performed in which agents were given either 15 min before LTA or at 2, 6 or 10 h after LTA. Agents which inhibit the expression of iNOS lose over time their ability to inhibit the increase in nitrite formation afforded by LTA, because the expression of iNOS in these cells is maximal after 6 to 10 h (Szabo *et al.*, 1993). Agents which inhibit the activity of iNOS work after early or late addition.

Experimental protocol

In order to confirm that the rise in nitrite afforded by LTA is due to NO from iNOS, cells were treated with LTA in the absence (control) or presence of the NOS inhibitors, N^G-monomethyl-L-arginine (L-NMMA, 1–1000 μM) or aminoethyl-isothiourea (AE-ITU, 1–1000 μM; Garvey *et al.*, 1994; Southan *et al.*, 1995), dexamethasone (0.01–10 μM) or cyclohexamide (0.01–3 μg ml^{-1}), the latter two of which prevent the expression of iNOS caused by LPS (Radomski *et al.*, 1990; Rees *et al.*, 1990).

Three distinct tyrosine kinase inhibitors, genistein, erbstatin (Imoto *et al.*, 1987; Marczin *et al.*, 1993) or tyrphostin AG126 (Novogrodsky *et al.*, 1994) or the inactive analogues daidzein or tyrphostin A-1, were used to elucidate whether any increase in nitrite by LTA involves the activation of tyrosine kinases, while tricyclodecan-9-yl-xanthogenate (D609), a relatively specific inhibitor of PC-PLC (Schütze *et al.*, 1992), was used to evaluate the role of PC-PLC in the increase in nitrite caused by LTA.

Activation of NF-κB by LPS can be inhibited by PDTC (Sherman *et al.*, 1993), and involves the formation of reactive oxygen intermediates (Baeuerle & Henkel, 1994). To elucidate the potential role of NF-κB in the increase in nitrite caused by LTA, cells were activated with LTA in the absence (control) or presence of PDTC or the antioxidants rotenone or butylated hydroxyanisole (BHA); both of which prevent activation of NF-κB (Baeuerle & Henkel, 1994). To confirm whether IκB-protease, an enzyme necessary for the activation of NF-κB (Miyamoto *et al.*, 1994; Lin *et al.*, 1995) is involved in the increase in nitrite caused by LTA we have used calpain inhibitor I (Miyamoto *et al.*, 1994; Lin *et al.*, 1995) and a cysteine and serine protease inhibitor, L-1-tosylamido-2-phenylethyl chloroemethyl ketone (TPCK; Henkel *et al.*, 1993). Chymostatin, a cysteine and serine protease inhibitor that is structurally similar to calpain inhibitor I and which does not inhibit activation of NF-κB (Lin *et al.*, 1995) was used as a negative control.

Measurement of cell viability

Cell respiration, an indicator of cell viability, was assessed by the mitochondrial-dependant reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Mossman, 1983). At the end of each experiment, cells in 96-well plates were incubated (37°C) with MTT (0.4 mg ml^{-1}, dissolved in culture medium) for 30 min after which time, the medium was removed by aspiration and cells were solubilized in dimethylsulphoxide (DMSO, 200 μl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of optical density at 550 nm–650 nm (OD_{550}-OD_{650}) using a Molecular Devices microplate reader (Richmond, CA, U.S.A.).

Immunoblot (Western blot) analysis

J774.2 macrophages were cultured in 6-well culture plates. After reaching confluence, the wells were treated with vehicle

(control), with LTA alone (10 μg ml^{-1}) or with LTA in the presence of cyclohexamide (0.3 μg ml^{-1}), D609, an inhibitor of PC-PLC (30 μg ml^{-1}; Schütze *et al.*, 1992), the tyrosine kinase inhibitor tyrphostin AG126 (10 μM; Novogrodsky *et al.*, 1994), an inhibitor of NF-κB activation, PDTC (25 μM; Schreck *et al.*, 1992) or an inhibitor of IκB-protease, TPCK (100 μM; Baeuerle & Henkel, 1994) and incubated in a humidified incubator at 37°C. After 24 h, the cells were washed with phosphate buffered saline (PBS; pH 7.4) and incubated at room temperature (2 min) with 250 μl of extraction buffer (Tris, 50 mM; EDTA, 10 mM; Triton X-100, 1% v/v; phenylmethylsulphonyl fluoride; PMSF, 1 mM; pepstatin A, 0.05 mM and leupeptin, 0.2 mM) with gentle shaking. The cell extract was snap frozen in liquid nitrogen and stored at −80°C. On a subsequent day the cell extract was thawed before being boiled (10 min) in a ratio of 1:1 with gel loading buffer (Tris 50 mM, SDS 10% w/v, glycerol 10% v/v, 2-mercaptoethanol 10% v/v and bromophenol blue 2 mg ml^{-1}, pH 6.8). Samples were then centrifuged at 10,000 *g* for 2 min prior to resolving by one dimensional gel electrophoresis (7.5% SDS gel) with molecular weight markers (SDS-7B; Sigma). After transfer to nitrocellulose by electrophoresis, the membranes were primed overnight at 4°C with a polyclonal antibody raised to macrophage iNOS developed in rabbits (a generous gift from Dr Claire Bryant, William Harvey Research Institute, UK) (Bryant *et al.*, 1995). The blots were then incubated as appropriate with anti-rabbit IgG linked to horseradish peroxidase. All antibodies were used at a 1:1000 dilution. Subsequently, the Western blots were developed with diaminobenzamine used as a substrate.

Materials

Lipoteichoic acid (from *Staphyloccocus aureus*), polymyxin B, DMSO, phosphate buffered saline (PBS; pH 7.4), Trizma base, EDTA, triton X-100, phenylmethylsulphonyl fluoride (PMSF), pepstatin A, leupeptin, glycerol, 2-mercaptoethanol, bromophenol blue, sulphanilamide, naphthlethylenediamide, phosphoric acid, sodium nitrite, sodium dodecyl sulphate (SDS), anti-rabbit IgG antibody, D609, PDTC, TPCK, genistein, BHA, chymostatin and MTT were supplied by Sigma Chemical Company (Poole, UK). Erbstatin, tyrphostin AG126, daidzein, tyrphostin A-1 and calpain inhibitor I were purchased from Calbiochem Novabiochem (Nottingham, UK). Dulbecco's modified Eagle's medium (DMEM) was obtained from Flow Laboratories. L-Glutamine was obtained from B.D.H (Dagenham, U.K.) and foetal calf serum was obtained from Gibco BRL (U.K.). Pure nitrocellulose membrane (0.45 μm) and filter paper were purchased from BIO-RAD (Hertfordshire, U.K.).

Unless otherwise stated, stock solutions of agents which were dissolved in saline were sterilised by filtration through a filter (pore size: 0.22 micron) after which serial dilutions were made in culture medium before added to the cells under sterile conditions.

Statistical analysis

Results shown are mean ± s.e.mean from triplicate determinations (wells) from 3–5 separate experiments. One way or two way analysis of variance (ANOVA) followed by, when appropriate, Bonferroni multiple range test were used to determine the statistical significance in the difference between means. A *P*-value of less than 0.05 was taken as statistically significant.

Results

The effect of polymyxin B on the formation of nitrite induced by LTA and LPS in J774.2 macrophages

LTA (0.01–30 μg ml^{-1}) induced within 24 h a concentration-dependent increase in the concentration of nitrite in the culture medium of J774.2 macrophages from 2 ± 1 (baseline) to 39 ± 3 μM (n = 12; Figure 1a). The increase in NO accumulation was maximal at 24 h (Figure 1b). Activation of J774.2 macrophages with LPS (1 μg ml^{-1}) similarly increased the accumulation of nitrite by more than 20 fold to 40 ± 3 μM (n = 12). Significant loss of cell viability in the treated compared to untreated cells was observed at the highest concentration (that used) of LTA (10 μg ml^{-1}; 75 ± 3 % of control) and LPS (1 μg ml^{-1}; 68 ± 4% of control). Addition of polymyxin B (0.05–0.5 μg ml^{-1}), an agent which binds and inactivates endotoxin, together with LTA or LPS significantly prevented the nitrite formation induced by LPS without affecting the rise in nitrite caused by LTA (Figure 1c).

The effect of NOS inhibitors and protein synthesis inhibitors on the nitrite formation induced by LTA in J774-2 macrophages

Addition to the cells of the NOS inhibitors, N^G-monomethyl-L-arginine (L-NMMA; IC_{50}; 35 μM) or aminoethyl-isothiourea (AE-ITU; IC_{50}: 6 μM) concentration-dependently prevented the nitrite accumulation induced by LTA (10 μg ml^{-1}) (Figure 2a). When these inhibitors were added to the cells 10 h after the addition of LTA, the degree of inhibition was similar to that observed when these inhibitors were given to the cells together with LTA (Figure 2a). Neither AE-ITU nor L-

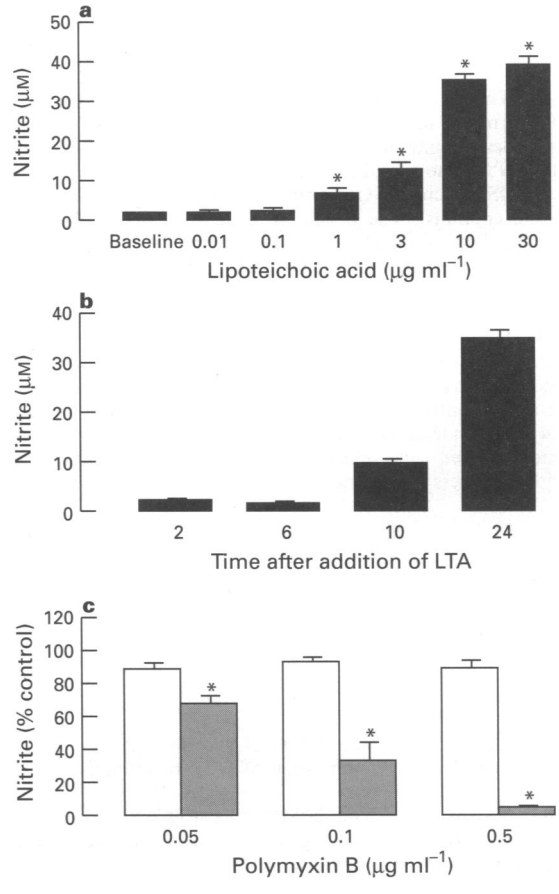

Figure 1 (a) Lipoteichoic acid (from *Staphylococcus aureus*) causes within 24 h a concentration-dependent accumulation of nitrite in the supernatant of J774.2 macrophages. (b) The increased nitrite accumulation by LTA (10 μg ml^{-1}) is maximal at 24 h. (c) The nitrite formation induced by LPS (1 μg ml^{-1}; stippled columns), but not LTA (10 μg ml^{-1}; open columns) is concentration-dependently inhibited by polymyxin B, an agent which binds and inactivates LPS. Data are expressed as mean ± s.e.mean from triplicate determinations (wells) from 4–5 separate experimental days (n = 12–15). *P < 0.05 represents (a) significant difference compared to the untreated cells or (c) a significant difference in the nitrite production by LPS between cells treated with and without polymyxin B.

NMMA affected the conversion of NO to nitrite, for these agents did not attenuate the formation of nitrite by the NO donor SIN-1 (0.1–1 mM) in the culture medium (results not shown). Furthermore, the inhibition of nitrite formation afforded by AE-ITU (10 μM) or L-NMMA (100 μM) was concentration-dependently reversed by addition of L-arginine (3–30 mM) but not D-arginine (3–30 mM) (Figure 2b). Neither of the NOS inhibitors used affected the cell viability.

Treatment of cells with cyclohexamide or dexamethasone concentration-dependently prevented the increase in nitrite elicited by LTA (IC_{50}s: 0.1 μg ml^{-1} for cyclohexamide, and 0.05 μM for dexamethasone). However, when these agents were given to the cells 6 or 10 h after LTA, the inhibition or nitrite formation afforded by cyclohexamide (0.3 μg ml^{-1}) or dexamethasone (1 μM) was significantly less (Table 1). Pretreatment of cells with dexamethasone, but not with cyclohexamide, prevented the reduction in cell viability caused by LTA (results not shown). In contrast, the loss of cell respiration caused by LTA was not affected when dexamethasone was given to the cells 10 h after LTA.

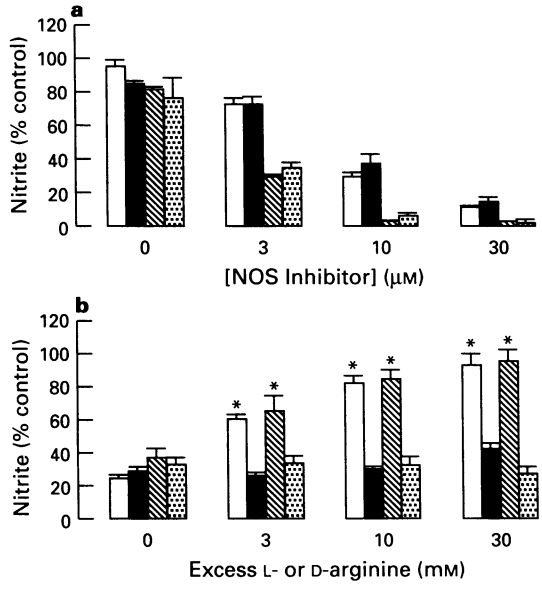

Figure 2 The increase in nitrite formation caused within 24 h by LTA (10 μg ml^{-1}) in cultured J774.2 macrophages is (a) concentration-dependently inhibited by the non-isoenzyme selective NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA; open columns) or by the iNOS selective NOS inhibitor, aminoethyl-isothiourea (AE-ITU; solid columns). The profile of inhibition was similar when L-NMMA hatched columns) or AE-ITU (stippled columns) was added to the macrophages 10 h after LTA. (b) The inhibition by L-NMMA (100 μM) of the nitrite formation afforded by LTA is reversed by excess L-arginine (hatched columns), but not by D-arginine (solid columns). Similarly, the inhibition afforded by AE-ITU (10 μM) of the increase in nitrite caused by LTA is reversed by excess L-arginine (hatched columns), but not by D-arginine (stippled columns). Data (nitrite as % control) are expressed as mean ± s.e.mean of 12 wells from 4 independent experiments. *$P<0.05$ represents significant difference between cells subjected to LTA plus NOS inhibitor with and without excess L-arginine.

The effect of the inhibitors of PC-PLC or tyrosine kinase on the increase in nitrite formation caused by LTA in J774.2 macrophages

D609 concentration-dependently prevented the nitrite accumulation induced by LTA in J774.2 macrophages (IC_{50}; 20 μg ml^{-1}; Figure 3). The inhibition afforded by D609 (30 μg ml^{-1}) was significantly less when D609 was added to the cells at 6 or 10 h after LTA (Table 1). The tyrosine kinase inhibitors, erbstatin, genistein or tyrphostin AG126 also pre-

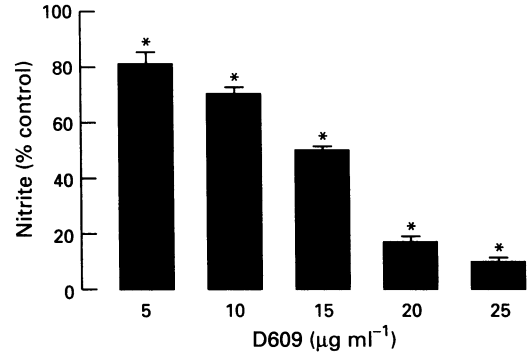

Figure 3 Effect of increasing concentrations of the PC-PLC specific inhibitor, D609, on the nitrite formation induced by LTA (10 μg ml^{-1}) in J774.2 cells. Data (nitrite as % control) are expressed as means ± s.e.mean of 9 wells from 3 independent experiments. *$P<0.05$ represents significant difference between cells subjected to LTA with and without (control) D609.

Table I Inhibitors of iNOS induction lose over time their ability to inhibit the increase in nitrite formation stimulated by LTA (10 μg ml^{-1}, at time 0) in J774.2 macrophages

Inhibitor	*Mode of inhibition*	*Concentration*	*Nitrite (% control) at 24 h for agents given at various time points after the addition of LTA*			
			−15 min	*2 h*	*6 h*	*10 h*
Dexamethasone	Inhibition of protein synthesis	1 μM	33.5 ± 3.5	54.2 ± 3.3*	76.2 ± 2.6*	93.8 ± 1.9*
Cyclohexamide	Inhibition of protein synthesis	0.3 μg ml^{-1}	16.2 ± 1.9	12.6 ± 1.8	43.0 ± 4.0*	90.8 ± 2.1*
D609	Inhibition of PC-PLC	30 μg ml^{-1}	16.3 ± 2.3	10.6 ± 2.2	20.2 ± 3.0*	64.5 ± 2.5*
Genistein	Inhibition of tyrosine kinase	100 μM	12.4 ± 0.7	25.4 ± 2.4	42.7 ± 5.4*	86.3 ± 4.3*
Erbstatin	Inhibition of tyrosine kinase	10 μM	19.3 ± 2.1	40.6 ± 0.4*	74.3 ± 1.5*	98.3 ± 5.2*
Tyrphostin AG126	Inhibition of tyrosine kinase	10 μM	24.1 ± 2.3	57.3 ± 1.1*	91.3 ± 2.7*	96.2 ± 3.0*
PDTC	Inhibition of NF-κB activation	25 μM	10.4 ± 1	26.8 ± 5.3*	46.4 ± 3.8*	85.9 ± 1.5*
Rotenone	Inhibition of NF-κB activation	30 μM	13.7 ± 1.5	16.1 ± 1.1	56.3 ± 3.1*	91.9 ± 5.0*
BHA	Inhibition of NF-κB activation	30 μM	37.4 ± 4.0	80.8 ± 3.8*	88.9 ± 2.9*	95.3 ± 5.4*
TPCK	Inhibition of IκB-protease	30 μM	8.9 ± 1.4	41.3 ± 7.6*	71.5 ± 3.5*	92.6 ± 4.2*
Calpain inhibitor I	Inhibition of IκB-protease	30 μM	13.5 ± 1.4	52.3 ± 2.6*	81.4 ± 4.5*	95.5 ± 4.1*

Data (nitrite as % control) are expressed as mean ± s.e.mean from triplicate determinations (wells) from 3 separate experimental days ($n=9$). Baseline nitrite values (% of 24 h control) were 5.5 ± 0.4 at time 0, 7.0 ± 0.6 at time 2 h, 5.7 ± 0.4 at time 6 h and 28.3 ± 0.5 at time 10 h after the addition of LTA. *$P<0.05$ represents significant difference between nitrite formation in cells pretreated with the inhibitors and nitrite formation in cells treated with the inhibitors at various time points after the addition of LTA.

vented nitrite formation induced by LTA in J774.2 cells in a concentration-dependent manner, the maximal inhibition afforded by erbstatin and tyrphostin AG126 being at 10 μM (Figure 4), while the maximal inhibition of nitrite formation afforded by genistein was at 100 μM (Table 1). Tyrphostin A-1, an inactive analogue of tyrphostin AG126 or daidzein, an inactive analogue of genistein (both used up to a concentration of 10 μM) did not significantly alter the formation of nitrite (Figure 4). The comparable maximal inhibition caused by the tyrosine kinase inhibitors was significantly less when they were given 10 h after addition of LTA to the cells (Table 1). The vehicle used to dissolve the tyrosine kinase inhibitors, DMSO, at a maximum concentration of 0.05% used in the wells, did not affect the nitrite formation induced by LTA. At the highest concentration used in this study, tyrosine kinase inhibitors but not D609 prevented the reduction in cell viability induced by LTA.

The effect of inhibitors of NF-κB activation on the increase in nitrite formation caused by LTA in J774.2 macrophages

The increase in nitrite formation caused by LTA was concentration-dependently prevented by pyrrolidine dithiocarbamate (PDTC; IC_{50}: 15 μM, Figure 5a). Furthermore, rotenone, BHA, calpain inhibitor I or TPCK also concentration-dependently prevented the increased nitrite formation induced by LTA. In contrast, however, chymostatin (up to a concentration of 30 μM) did not affect the nitrite formation induced by LTA (Figure 5b). The maximum amount of DMSO (0.17%) used to dissolve these compounds also did not affect the nitrite accumulation induced by LTA. The inhibition afforded by all these compounds was significantly less when they were given to the cells 10 h after LTA (Table 1). None of these inhibitors of NF-κB activation, at the concentrations used, prevented the loss in cell viability induced by LTA.

The effect of the inhibitors of protein synthesis, PC-PLC, tyrosine kinase and NF-κB on the expression of iNOS protein in LTA-treated J774.2 macrophages

J774.2 cells activated with LTA (10 μg ml^{-1}) contained a protein of approximately 130 kDa (identified from molecular weight standards; lane a) which was recognised by a specific antibody to macrophage iNOS (Figure 6; lanes c and g). In contrast, untreated J774.2 macrophages contained no detectable iNOS protein (lane b). The expression of iNOS protein by LTA was reduced by cyclohexamide (0.3 μg ml^{-1}; Figure 6; lane d), D609 (30 μg ml^{-1}; Figure 6; lane e), tyrphostin AG126 (10 μM; Figure 6; lane f), TPCK (100 μM; Figure 6; lane h) or PDTC (25 μM; Figure 6; lane i).

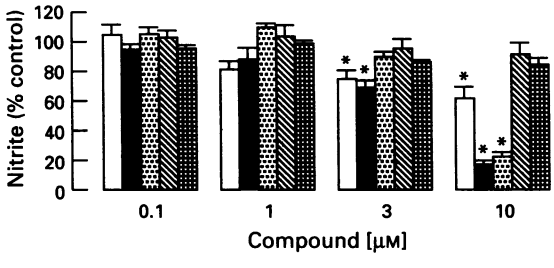

Figure 4 Increasing concentrations of the tyrosine kinase inhibitors genistein (open columns), erbstatin (solid columns), tyrphostin AG126 (stippled columns) but not daidzein (hatched column; an inactive analogue of genistein or tyrphostin A-1 (squared columns; an inactive analogue of tyrphostin) inhibited the nitrite formation induced by LTA (10 μg ml^{-1}) in J774.2 macrophages. Data (nitrite as % control) are expressed as means ± s.e.mean of 9–15 wells from 3–5 independent experiments. *$P<0.05$ represents significant difference between cells stimulated with LTA in the presence or absence (control) of the respective tyrosine kinase inhibitors or the inactive analogues.

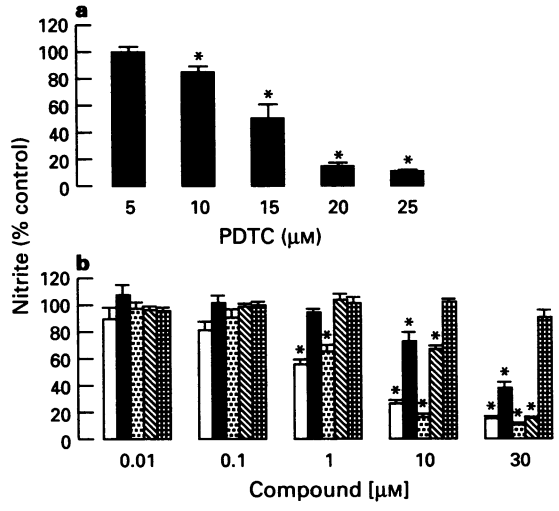

Figure 5 The nitrite formation in the supernatant of J774.2 macrophages activated with LTA (10 μg ml^{-1}) for 24 h is (a) concentration-dependently inhibited by PDTC, a relatively specific inhibitor of the transcription factor NF-κB. (b) The nitrite formation induced by LTA was also concentration-dependently inhibited by rotenone, an antioxidant which inhibits electron transport (open columns), the antioxidant butylated hydroxyanisole (BHA) (solid columns) or by the IκB-protease inhibitors, L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) (stippled columns) or calpain inhibitor I (hatched columns), but not by chymostatin (squared columns). Data (nitrite as % control) are expressed as means ± s.e.mean of 9–12 wells from 3–4 independent experiments *$P<0.05$ represents significant difference between cells stimulated with LTA in the presence or absence of (control) the respective inhibitors of NF-κB activation.

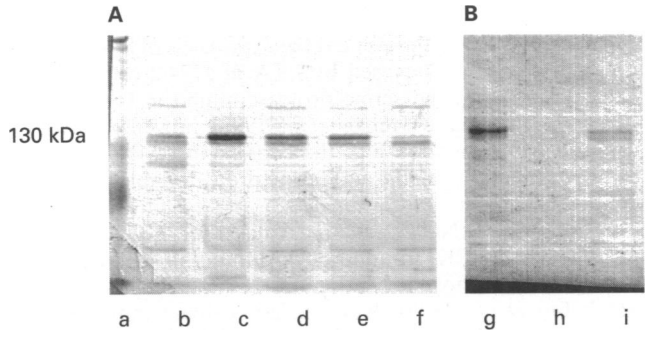

Figure 6 Depicted are Western blots using polyclonal antibodies to inducible nitric oxide synthase (iNOS) in cell extracts from J774.2 macrophages treated with LTA (10 μg ml^{-1}) in the presence or absence of various agents. In all lanes equal amounts of protein from J774.2 macrophages were loaded. Extracts prepared from macrophages activated with LTA (10 μg ml^{-1} for 24 h) contained iNOS protein (lane c and g) compared to extracts prepared from untreated macrophages (lane b). The induction of iNOS by LTA was reduced by cyclohexamide (0.3 μg ml^{-1}; lane d), tricyclodecan-9-yl-xanthogenate (D609, 30 μg ml^{-1}; lane e), tyrphostin AG126 (10 μM; lane f), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK, 100 μM; lane h) or by pyrrolidine dithiocarbamate (PDTC, 25 μM; lane i). Standard molecular weight markers are shown in lane (a). Similar results were obtained with cell extracts from 3 separate batches of cells.

Discussion

This study demonstrates that the increase in the NO formation caused by LTA in J774.2 macrophages is a consequence of the induction of iNOS and indicates that PC-PLC, tyrosine kinase and the transcription factor NF-κB are involved in the signal transduction leading to the expression of iNOS caused by LTA in these cells.

The increase in nitrite elicited by LTA was due to an enhanced formation of NO, for (i) it was abolished by two different NOS inhibitors, L-NMMA and AE-ITU, and (ii) the inhibition afforded by these NOS inhibitors was restored by L-arginine but not by D-arginine. Furthermore, the enhanced formation of NO was due to induction of iNOS, for (i) LTA caused the expression of iNOS protein in J774.2 macrophages, and (ii) both the increase in nitrite as well as the expression of iNOS protein were attenuated by dexamethasone or cyclohexamide. Thus, LTA increases NO formation by causing the expression of iNOS in J774.2 macrophages (this study, Auguet *et al.*, 1992; Lonchampt *et al.*, 1992) and in vascular smooth muscle cells (Cunha *et al.*, 1993). The loss in cell viability caused by LTA was not due to an enhanced formation of NO, for it was not attenuated by the NOS inhibitors.

What then, is the mechanism by which LTA causes the expression of iNOS protein? Clearly, the induction of iNOS caused by LTA is not due to contamination with LPS, for polymyxin B, an agent which binds and inactivates LPS, abolished the increase in nitrite caused by LPS, but not by LTA. Expression of proteins including iNOS by stimulation with pro-inflammatory cytokine agents, is mediated by transcription factors such as NF-κB (Xie *et al.*, 1994). NF-κB is constitutively present in cells as a heterodimer, consisting of a p50 DNA-binding subunit and a p65 trans-activating subunit. NF-κB is normally held in the cytoplasm in an inactivated state by the inhibitor protein IκB-α (Grimm & Baeuerle, 1993; Baeuerle & Henkel, 1994). Pro-inflammatory cytokines activate cell surface receptors leading to (i) dissociation of IκB-α from the NF-κB complex following phosphorylation of IκBα (ii) proteolytic degradation of the IκB-α by IκB-α protease, (iii) translocation of the activated NF-κB into the nucleus and attachment to relevant DNA binding sites on the promoter region of genes, and (iv) initiation of transcription. For activation of NF-κB, degradation of IκB-α by the enzyme IκB-α protease is essential, for agents that inhibit this enzyme without affecting the phosphorylation of IκB-α, prevent stimulation of NF-κB (Miyamoto *et al.*, 1994; Lin *et al.*, 1995).

We show that PDTC which inhibits NF-κB activation (Schreck *et al.*, 1992) prevented the expression of iNOS protein and activity (nitrite) caused by LTA in J774.2 cells. In addition, the cysteine and serine protease inhibitor TPCK, which inhibits IκB protease (Henkel *et al.*, 1993), prevented the LTA-induced NO formation and the expression of iNOS protein. However, not all cysteine and serine protease inhibitors inhibit NF-κB activation (Lin *et al.*, 1995). Indeed, chymostatin did not inhibit the iNOS induction caused by LTA. It is now evident that TPCK, in addition to inhibiting IκB-α protease, also prevents phosphorylation of IκB-α (Miyamoto *et al.*, 1994; Lin *et al.*, 1995). Nevertheless, calpain inhibitor I (an agent structurally similar to chymostatin), which inhibits IκB-α protease but not the signal induced phosphorylation of IκB-α (Lin *et al.*, 1995), effectively prevented iNOS induction stimulated by LTA. This implies that activation of IκB-α protease is important in the induction of iNOS caused by LTA. The inhibition by PDTC, TPCK or calpain inhibitor I, of the increase in nitrite stimulated by LTA was significantly less when they were added to the macrophages 10 h after LTA: thus, PDTC, TPCK or calpain inhibitor I inhibit the induction, but not the activity, of iNOS.

A number of events precede the activation of NF-κB after stimulation of cells by LPS or cytokines. For instance, PC-PLC controls the activation of NF-κB in response to the binding of TNF-α to the TNF-α receptor (Schütze *et al.*, 1992). D609 which inhibits PC-PLC without affecting phosphatidylinositol-specific PLC, phospholipase A_2 or phospholipase D, suppresses the TNF-α induced release of diacylglycerol (DAG), a potent activator of protein kinase C (PKC) and acid-sphingomyelinase (Schütze *et al.*, 1992). We show here that induction of iNOS protein caused by LTA was prevented by D609 indicating that PC-PLC plays a role in the signal transduction leading to the expression of iNOS. This is consistent with the finding that the induction of iNOS afforded by LPS together with IFN-γ in macrophages also depends on the activation of PC-PLC (Tschaikowsky *et al.*, 1994).

One of the determinants of NF-κB activation is the redox status of the cell (Schieven *et al.*, 1993; Baeuerle & Henkel, 1994). This status is determined by the concentration of reactive oxygen species such as superoxide, hydrogen peroxide, hydroxy radicals and singlet oxygen which are frequently produced as intermediates in the reduction of dioxygen to water during mitochondrial respiration (Schreck & Baeuerle, 1991). There is ample evidence to suggest that cytokines increase the formation of cellular reactive oxygen intermediates by causing an alteration of electron flow in the mitochondria (Baeuerle & Henkel, 1994). The antioxidants rotenone (Schulze-Osthoff *et al.*, 1992) or BHA, prevented the iNOS induction by LTA (this study) suggesting that reactive oxygen species are generated by activation of macrophages by LTA. Thus, reactive oxygen intermediates, presumably by their ability to activate NF-κB, play an important role in the series of events leading to the expression of iNOS induced by LTA.

Inhibition of tyrosine kinase with genistein inhibits the activation of NF-κB implying that tyrosine kinase activation precedes NF-κB activation (Read *et al.*, 1993). Indeed, the tyrosine kinase inhibitor, herbmycin A, interferes with the activation of NF-κB by IL-1 (Iwasata *et al.*, 1992). We demonstrate here that three structurally distinct tyrosine kinase inhibitors, genistein (competitive inhibitor at the ATP-binding site), erbstatin or tyrphostin AG126 (both competitive inhibitors at the substrate binding site) (Casnellie, 1991; Levitzki & Gilon, 1991; Novogrodsky *et al.*, 1994) inhibit the expression of iNOS caused by LTA clearly showing that tyrosine phosphorylation plays an important part in the signal transduction leading to the expression of iNOS by LTA. Similarly, the induction of iNOS activity caused by IL-1β (Marczin *et al.*, 1993) or by LPS (Akarasereenont *et al.*, 1995) is prevented by inhibitors of tyrosine kinase.

There is substantial evidence to suggest that LPS uses a receptor protein (CD14) to initiate the induction of iNOS (Wright *et al.*, 1990). It is not clear whether LTA uses a similar CD14-like protein for the induction of iNOS. Nevertheless, LTA does bind to a 70 kDa protein on macrophages (Dziarski *et al.*, 1994). Interestingly, the expression of the adhesion molecule VCAM-1 induced by LTA, which is dependent on activation of NF-κB (Baeuerle & Henkel, 1994), is inhibited by monoclonal antibodies to CD14 (Renzi & Lee, 1995).

In conclusion, our results imply that the signal transduction events leading to the expression of iNOS by LTA involve (i) the activation of PC-PLC, (ii) the phosphorylation of tyrosine kinase, and (iii) the activation of the transcription factor NF-κB. Further studies are necessary to elucidate which protein acts as a 'receptor' for LTA on the surface of macrophages, and whether cytokines such as TNF-α, which is released by macrophages following stimulation by LTA (Takada *et al.*, 1995), contributes to the induction of iNOS afforded by LTA.

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