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Suppression of nitric oxide formation by tyrosine kinase inhibitors in murine N9 microglia

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1 Microglial cells represent the first line of defence in the brain against infection and damage. However, under conditions of chronic inflammation and neurodegeneration, excessive activation of microglia can contribute to the neurodegenerative process by releasing a cornucopia of potentially cytotoxic substances including the cytotoxic free radical nitric oxide (NO). Although the cell signalling events implicated in NO formation in peripheral macrophages are well defined, events occurring in the phenotypically homologous cerebral microglial cell are not yet fully characterized.

2 In the present study, a cloned murine microglial cell line (N9), stimulated with combined lipopolysaccharide/interferon-γ (LPS/IFN) incubation, was shown to produce a significant increase in NO formation, as measured by medium nitrite levels, during 8–72 h exposure.

3 LPS/IFN-stimulated NO production was partially inhibited with the nitric oxide synthase (NOS) competitive antagonists; N^{ω}-nitro-L-arginine methyl ester and N^{ω}-nitro-L-arginine. The ability of the selective inducible (iNOS) inhibitor, aminoguanidine, but not the selective 'neuronal-type' constitutive (cNOS) inhibitor 7-nitroindazole, to inhibit NO production suggested a primary role of iNOS in this response and was confirmed by immunolabelling of activated cells with a specific iNOS antibody.

4 A series of tyrosine kinase inhibitors, herbimycin A, genestein, tyrphostins, AG-126, AG-556 and the tyrosine phosphatase inhibitors, sodium orthovanadate and phenylarsine oxide, significantly attenuated LPS/IFN-mediated NO production. The serine/threonine kinase inhibitors, staursporine (protein kinase C), H-9 (cyclic GMP/cyclic AMP-dependent kinase) or serine/threonine phosphatase inhibitors, cyclosporin A (phosphatase 2B) and okadaic acid (phosphatase 1/2A), reduced NO formation by an apparent cytostatic mechanism, as determined by cellular reduction of 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyl-tetrazolium bromide (MTT).

5 The present results suggest that the co-ordinated activation of protein tyrosine kinases/phosphatases, and proximal signalling events implicating the interplay between serine-threonine kinases/phosphatases, is intricately linked with inflammatory mediated mechanisms of iNOS activation in microglial cells by regulating the activation of the transcription factor NFκB.

Keywords: Microglia; nitric oxide; tyrosine kinase; cytokine; nitric oxide synthase; neurodegeneration; inflammation

Introduction

The CNS inflammatory response is an essential damage-limiting process, restoring homeostatic conditions to specific brain regions following host infection and trauma. Characteristics of the CNS inflammatory response involve microglial cell activation, astrogliosis and recruitment of blood-borne monocytes from the periphery via disruption of the blood brain barrier (BBB). Astrocytes, when stimulated, produce various neurotrophic factors such as neuronal growth factor (NGF) (Gadient *et al*., 1990), thereby supporting neuronal growth and survival. The principal role of microglia includes the removal of cellular debris and micro-organisms by phagocytosis, lipid recycling and the secretion of a wide spectrum of cytokines possessing trophic, mitogenic, chemotactic and cytotoxic properties (Giulian, 1987; Gehrmann *et al*., 1995; Kreutzberg, 1996).

Hyperactivation of microglia and astrocytes can occur resulting in excessive release of potentially cytotoxic factors (Théry *et al*., 1991; Piani *et al*., 1991; 1992; Banati *et al*., 1993; Dickson *et al*., 1993). However, in the CNS, post-mitotic neurones once destroyed cannot be replaced, consequently, inflammation in the CNS carries a substantial pathogenic risk leading to potential neurodegenerative mechanisms. Dickson *et al*. (1993), detected activated microglia invading areas of cortical neuronal loss in Alzheimer's diseased (AD) brains and clustered microglia have been observed to infiltrate and surround senile plaques in brain tissue from AD patients (Haga *et al*., 1989). As activated microglia have been observed in numerous other neurodegenerative conditions, including the AIDS dementia complex, amyotrophic lateral sclerosis, Down's syndrome, Parkinson's disease and Huntington's chorea (Dickson *et al*., 1993; Lees, 1993), it appears that stimulated microglia while attempting to minimize cell damage actually contribute to the neurodegenerative process. Indeed activation of resident microglia is modified via a number of mediators ranging from 6-hydroxydopamine chemical lesions, contamination with HIV-1, tumour growth, amyloid proteins and ischaemia (reviewed by Wood, 1994). Microglia infiltrate rapidly into the traumatized region and along with astrocytes create a glial cell barrier aimed at destroying micro-organisms, removing harmful debris around the lesion site, initiating tissue repair and subsequently re-establishing cellular homeostasis (Perry *et al*., 1993). Microglial and astrocyte activaiton generally occur in concerted action during CNS injury with a spatially and temporally distinct pattern, which seems to be under the control of mainly T-cell and microglial derived cytokines. Indeed, interleukin-1 (IL-1) released from microglia can activate astrocytes, thereby inducing growth factor production and subsequent cellular repair, and leads to the

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release of protein mitogens (Giulian *et al*., 1993), which in turn stimulate microglial activity.

The debate concerning the origins of microglia is still somewhat controversial; microglia may derive from either the monocyte lineage or from neuroepithelial stem cells (Jordan & Thomas, 1988). Nevertheless, microglia are phenotypically more closely related to peripheral macrophages than to any other cells of the CNS. Indeed, microglia have been referred to as the 'ontogenic and functional equivalent of mononuclear phagocytes in somatic tissues' (Giulian *et al*., 1988), and share similar characteristics with peripheral macrophages, including cytokine production and respiratory burst activity. The activation of peripheral macrophages or monocytes, and the subsequent induction of new protein synthesis, correlates with the activation of certain transcription factors, notably nuclear factor-κB (NF-κB) and NF-AT. When activated these transcription factors translocate from the cytosol into the nucleus and bind to DNA in upstream regulatory transcriptional consensus sequences of numerous stress response genes, including IL-1, tumour necrosis factor-α (TNF-α), IL-6, ICAMs and nitric oxide synthase (EC 1.14.13.39) (Lenardo & Baltimore, 1989; Baeuerle & Henkel, 1994; Trenckner *et al*., 1994). Inducible NOS (iNOS), as well as the constitutive 'neuronal' and 'endothelial' NOS (cNOS) isoforms, catalyse the biochemical conversion of L-arginine to L-citrulline, releasing NO as a by-product. However, NO production induced by iNOS is both continuous and highly amplified (nmol NO), compared to cNOS catalysed release of NO (pmol). Activation of iNOS is intrinsic to activated immune cells and therefore essential to their respiratory-burst and phagocytotic action (Anggard, 1994). Nitric oxide (NO) along with the other identified products released from microglia, such as the superoxide radical (O_2^-) (leading to subsequent radical reactions e.g. formation of hydroxyl (OH) and the peroxynitrite ($ONOO^-$) radicals), are neurotoxic when secreted in sufficient levels in primary neuronal/microglial co-cultures (Théry *et al*., 1991; Piani *et al*., 1991; 1992; Chao *et al*., 1992; Banati *et al*., 1993; Dickson *et al*., 1993).

In an attempt to study such a process we investigated the release of cytokine-mediated NO release from a murine N9 microglia cloned cell line received from P. Castagnoli (Milan, Italy) and obtained by immortalization of E13 mouse embryonic cultures with the 3RV retrovirus carrying an activated *v-myc* oncogene (Righi *et al*., 1989). This cell line has previously been demonstrated to produce substantial amounts of NO and various cytokines after stimulation (Righi *et al*., 1989; Corradin *et al*., 1993; Meda *et al*., 1995). Based on the similarities between peripheral monocytes/macrophages and microglial functions, a parallel scheme of NF-κB-based induction of iNOS in peripheral immune cells can be envisaged for microglia. Indeed inducible NF-κB has been detected in microglial cells (Kaltschmidt *et al*., 1993). Consequently, we investigated the regulation of cell signalling events induced by various protein kinases/phosphatases, by using selective inhibitors, that may be implicated in inflammatory-mediated activation of iNOS in microglial cultures.

Methods

N9-microglial cell cultures

N9 murine microglia (obtained from P. Castagnoli, Milan, Italy) were immortalized from isolated microglia derived from day-13 embryonic rat brain cultures by transfection with 3RV retrovirus carrying an activated *v-myc* oncogene, as previously described by Righi *et al*. (1989). Cells were grown to confluence in DMEM/F-12 medium supplemented with 10% FCS, in 75 cm^2 culture flasks (Costar), at 37°C/5%CO_2 humidified atmosphere. Confluent microglial cells were treated with trypsin briefly (2–5 min), and centrifuged at 800 r.p.m. 15 min^{-1}. Cultures were used for a maximum of twenty five passages.

Detached cells were plated at 5×10^4 cells/well in 100 μl of DMEM/F-12/well in the central 60 wells of 96-well poly-D-lysine-coated plates (TPP), and maintained in identical atmospheric conditions for 24 h before each experiment. Resulting cultures exhibited in excess of 99% staining when labelled with fluorescein isithiocyanate (FITC)-labelled *Bandeiraea simplicifolia BS-II* lectin, as previously described (Streit, 1990).

Immunofluorescence labelling

N9 microglia were plated onto poly-D-lysine-coated cover-slips at approximately 1×10^5 cells/well in 24-well plates (Costar), and grown to confluence for 7–10 days. Culture medium was then removed and cells rinsed several times with phosphate buffered saline (PBS), pH 7.4. Cells were fixed with freshly hydrolyzed paraformaldehyde 3.7% (w/v) (Sigma), (depolymerized by heating to 60°C in the presence of concentrated NaOH and buffered with ×10 PBS, pH 7.4) for 30 min at room temperature. Following incubation, fixed cells were rinsed several times with PBS and stored at −20°C.

Fixed microglial cultures were rinsed with PBS (3 × 5 min) and then made permeable by incubation for 30 min with 3% (v/v) non-specific-goat-serum (NSGS, Sigma), 0.4% (v/v) Triton X-100, in PBS. Cells were then rinsed several times with PBS and incubated with a rabbit polyclonal murine anti iNOS (0.5 μg ml^{-1}) (Tebu, France), for 18 h at +4°C. Cells were then rinsed (3 × 10 min) in PBS and incubated for a further 1.5 h with FITC-labelled anti-rabbit IgG (whole molecule) F(ab′)$_2$ fragment (1 : 100; Sigma). Labelled cells were washed briefly (3 × 10 min), air dried and mounted on slides with a drop of mounting medium (Vectashield, Vector Lab). Coverslips were sealed with varnish and then viewed with u.v. optics to analyse qualitatively the extent of fluorescent labelling. Control background labelling was assessed by the omission of primary anti-iNOS antibody.

Product/exposure protocols

In order to determine optimum inflammatory priming conditions of confluent cells (5×10^4/well), culture medium was removed and replaced with 200 μl of fresh DMEM/F-12, containing designated dosed product concentrations of lipopolysaccharide (LPS) (0.01–100 μg ml^{-1}) and/or murine recombinant interferon-γ (1–500 u ml^{-1}) and/or murine recombinant interleukin-1β (0.5–100 ng ml^{-1}). Treated plates were then incubated for 24 h at 37°C/5%CO_2.

Protocols based on co-administration of inhibitors involved removal of culture medium and replacement with 200 μl DMEM/F-12 containing dosed product, followed by a 15 min incubation period. Cells were then stimulated with LPS (0.5 μg ml^{-1})/IFN-γ (100 u ml^{-1}) for a 24 h incubation period. Fresh product samples were used for all protocols.

Medium nitrite determination

Quantitative analysis for the level of NO release from cultured microglia and primary glial cultures was determined with the Greiss reagent by estimation of medium nitrite levels as

previously described (Kong *et al*., 1996). Unstable NO is readily oxidized to the stable end-products nitrites and nitrates and the aforementioned test accurately and rapidly assays these adducts. An equal volume of Greiss reagent (0.1% (w/v) N-(I-naphthyl)-ethylenediamine (Sigma), plus 1% (w/v) sulphanilamide (Sigma) prepared in 5% H_3PO_4 (v/v)) is added to individual culture supernatant samples and read within 1 h at 540 nm with an automatic plate reader (SLC 340ATTC, Labinstruments) against a standard curve of sodium nitrite (1–100 μM).

Cell viability assay

Cytotoxicity of resident microglial cells was assessed with the 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyl-tetrazolium bromide (MTT)-reduction assay (Mosmann, 1983). Cultures (50 μl), were treated with MTT (4 mg ml^{-1}) 5 μl/well and incubated at 37°C/5%CO_2 for 3–4 h. The resultant formazan crystals were solubilised by the addition of 1 M HCl (Prolabo)/4%(v/v) isopropyl alcohol (50 μl/well) and briefly agitated to dissolve remaining formazan crystals. Absorption readings were taken at 540 nm with an automatic plate reader (SLC 340 ATTC, Labinstruments).

Materials

Lipopolysaccharide (LPS) (*E. coli*, L-8274), murine recombinant interferon-gamma (IFN-γ), murine recombinant interleukin 1-β (IL-1β), genestein, herbimycin A, staurosporine, okadaic acid, N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{ω}-nitro-L-arginine (L-NOARG) and aminoguanidine were all supplied by Sigma/Aldrich Chemical Co. (L'Isle D'Abeau Chesnes, France). Cyclosporin A, 7-nitroindazole (7-NI), H-9 dihydrochloride (N-(2-aminoethyl)-5-isoquinolinesulphonamide), were obtained from RBI Biochemicals Int. (Bioblock Scientific, Illkirch, France). Tyrophostin AG-126 (2-(3-hydroxy-r-nitro-benzylidene)-malonitrile) was obtained from Calbiochem Co. (La Jolla, CA, U.S.A). Tyrophostins AG-556 (2-cyano-3-(3,4-dihydroxy-phenyl)-N-(4-phenyl-butyl)-acylamide) and AG-1290 (2-cyano-3-(3,4-dihydroxy-5-nitro-phenyl) acylic acid) were obtained from Dr A. Gaviz (Hebrew University, Jerusalem, Israel).

Results

N9 microglia stimulation

Qualitative analysis of cultured N9 microglia indicated that $\geqslant$98% of N9 microglia cultures were labelled with the selective microglia marker *BS-II* lectin (FITC-labelled). Co-incubation of N9 cells with a fixed LPS concentration of 50 μg ml^{-1} and administered IFN-γ (1–500 u ml^{-1}) demonstrated a dose-dependent increase in NO formation at concentrations of IFN-γ ranging from 1 u ml^{-1} up to 100 u ml^{-1} (Figure 1a). Maximum stimulation (approx. 48 μM nitrite) was observed between 100–500 u ml^{-1} of IFN-γ. In a reciprocal experiment, N9 cells were stimulated with a fixed concentration of IFN-γ (100 u ml^{-1}) and increasing doses of LPS (0.01–50 μg ml^{-1}). In this case, LPS induced a dose-dependent increase in IFN-γ-stimulated NO release with maximum stimulation (40 μM) observed between 0.5–50 μg ml^{-1} of LPS (Figure 1b). On the other hand, IL-1β (0.25–100 ng ml^{-1}) failed to stimulate medium nitrite levels above control values, and similarly, IL-1β (2.5–100 ng ml^{-1}) did not produce a statistically significant increase in NO synthesis when co-incubated with either IFN-γ (100 u ml^{-1}) alone or in combination with IFN-γ (100 u ml^{-1}) and LPS (0.5 μg ml^{-1}) (data not shown).

In order to study the kinetics of NO synthesis, microglia cultures were stimulated with IFN/LPS and cultured supernatant was then sampled at hourly periods for up to 8 h, then at subsequent 24 h, 48 h and 72 h stages. An 8 h lag-period was apparent before the appearance of any detectable levels of nitrite (6$\pm$1 μM; n=5) in the culture medium. Thereafter, significant levels of nitrite were detected at 24 h (40$\pm$4 μM) with saturation levels detected between 48 h (64$\pm$10 μM) and 72 h (71$\pm$11 μM). Under similar experimental conditions, we failed to observe any detectable increase in medium nitrite levels in stimulated primary astrocyte cultures of the rat (data not shown).

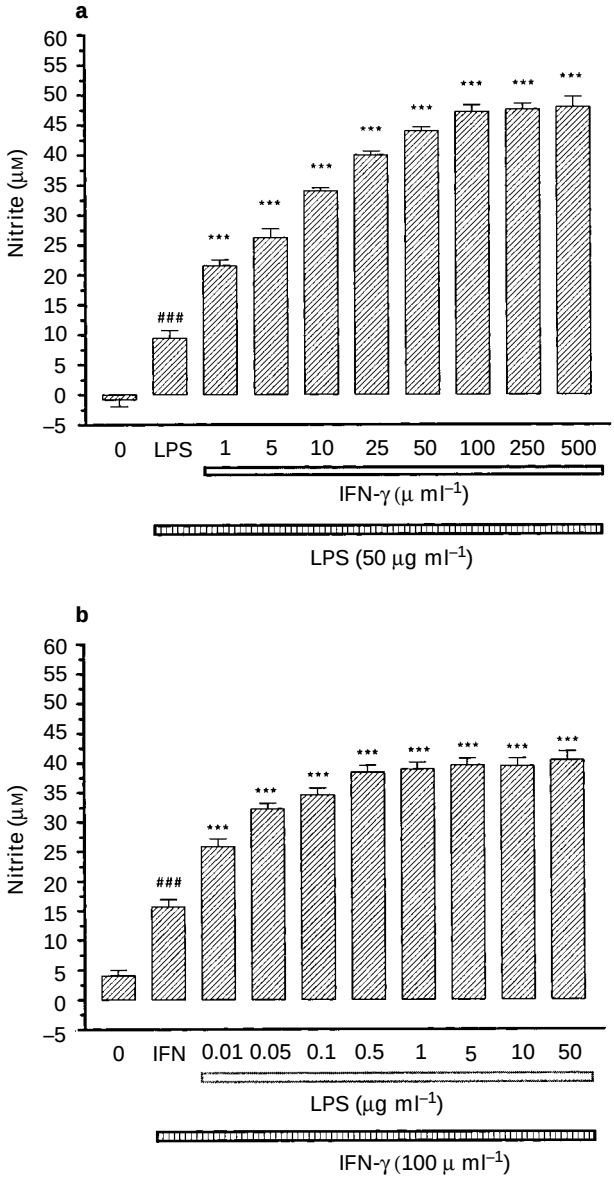

Figure 1 Synergistic effect of LPS and IFN-γ on stimulated NO production. Murine N9 microglia (3–4 d.i.v.) were stimulated with (a) LPS (50 μg ml^{-1}) plus IFN-γ (1–500 u ml^{-1}) (n=4). (b) IFN-γ (100 u ml^{-1}) plus LPS (0.01–100 μg ml^{-1}) (n=6). Culture medium (100 μl) was then assayed for nitrite/nitrate levels with the Greiss reagent after 24 h exposure. Data are mean$\pm$s.e.mean of 6 samples per condition; n=number of experiments. Statistical significance vs (#) untreated controls or vs (*) LPS or IFN alone. *P<0.05; **P<0.01; ***P<0.001; ###P<0.001, (Student's t test).

NOS inhibitors

As previously stated, NO production is a by-product of nitric oxide synthase (NOS) activity in the conversion of L-arginine to L-citrulline. Consequently, we analysed the inhibitory affect of two L-arginine analogues (L-NAME, L-NOARG), on LPS/IFN-stimulated NO production in N9 microglia. Cells were pre-incubated for 15 min with the different antagonists, and for 24 h in the presence of LPS/IFN. L-NAME and L-NOARG produced significant inhibition of NO synthesis between 250–1000 μM with maximal inhibition of 39% at 1000 μM L-NAME and 49% inhibition at 1000 μM L-NOARG (Figure 2a and b). For both L-arginine analogues no significant decrease in the redox state of the cells was observed, as evidenced by the lack of a significant decrease in MTT reduction relative to LPS/IFN controls (Figure 2a and b). However, it is important to note that in the majority of our experiments with LPS/IFN co-stimulation conditions, an approximate 10–20% decrease in MTT reduction was often observed between LPS/IFN activated cells, compared to unstimulated control cells. Microscopic analysis of cultures under phase-contrast optics indicated no significant alterations in cellular morphology following LPS/IFN exposure, albeit the presence of more flattened and convoluted cells relative to controls (data not shown).

Having established that IFN/LPS-stimulated microglial NO production was indeed related to NOS activity, we then proceeded to determine which NOS isoform was implicated in the reaction. Aminoguanidine (5–1000 μM), a selective inhibitor of iNOS, demonstrated a potent inhibitory effect on nitrite production (85% decrease), with an IC_{50} of 275 μM (Figure 3a). Assay of cellular MTT reduction indicated the lack of any significant drug-mediated cytostatic/toxic effect (Figure 3a). Interestingly, increasing doses of aminoguanidine did induce a moderate but non-significant enhancement of MTT reduction compared to LPS/IFN controls, and re-established cellular MTT reduction levels to untreated control values (Figure 3a). The selective neuronal cNOS inhibitor, 7-nitroindazole (7-NI) did not inhibit LPS/IFN-induced NO synthesis in N9 microglia at concentrations ranging from 0.5–200 μM (Figure 4b). Moreover, a marked up-regulation (+13%–+48%) in NO production with increasing 7-NI concentrations (0.3–100 μM) was observed. However, between 100–200 μM of 7-NI, the increase in medium nitrite levels was reduced to +24% of LPS/IFN control values. In general, MTT values were relatively stable across the entire 7-NI dose range with negligible ($\geqslant$5%) fluctuation, indicating no drug-induced decrease in cellular redox activity (Figure 3b). Immunolabelling of N9 microglia with a specific iNOS antibody indicated a qualitative increase in the number of labelled cells in LPS/IFN-treated cells compared to control cultures (compare (a) and (b) in Figure 4).

Protein kinase inhibitors

In an attempt to dissect part of the potential intracellular signalling mechanism(s) that may be implicated in the activation of iNOS, the inhibitory action of selective classes of protein kinase inhibitors on LPS/IFN-induced NO release was investigated. Genestein, a selective receptor-mediated tyrosine kinase inhibitor, produced a marked decrease in nitrite production (IC_{50}=37 μM), culminating in an 85% reduction in medium nitrite levels at drug levels of 100 μM (Figure 5a). The genestein-mediated inhibition of NO synthesis was contiguous with decreased cellular MTT reduction. However, only at 100 μM genestein was NO inhibition (85%) related to a significant inhibition of MTT reduction (57%). Interestingly, at 25 μM and 50 μM genestein a significant inhibition of NO production was observed (32%, 62%, respectively) whereas MTT reduction (+14%, 14%, respectively) was not significantly altered from controls (Figure 5a). Phase-contrast microscopic observation of genestein-treated cells indicated no evident cytotoxic action of the drug, suggesting that genestein-mediated inhibition of MTT cellular reduction may relate to a cytostatic rather than a cytotoxic effect (data not shown). The fungal antibiotic herbimycin A, a broad-range receptor and non-receptor linked protein tyrosine

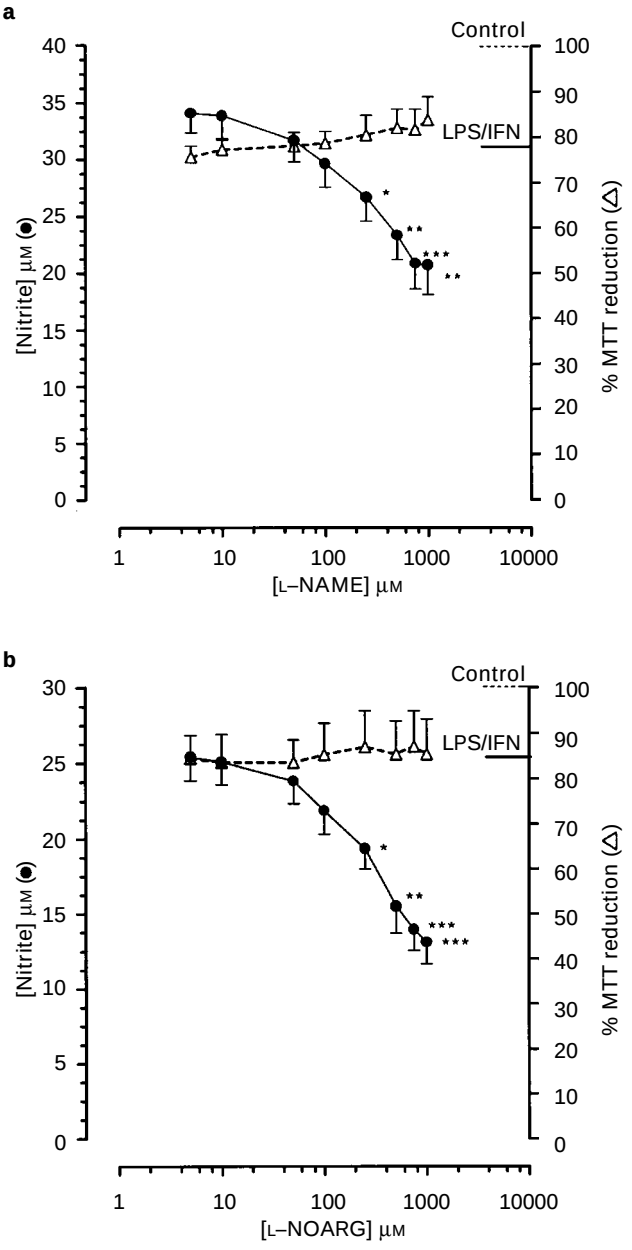

Figure 2 Effect of L-NAME and L-NOARG on LPS/IFN-stimulated NO release from microglia. Murine N9 microglia (3–4 d.i.v.) were incubated with IFN-γ (100 u ml^{-1}) plus LPS (0.5 μg ml^{-1}) in the presence of increasing concentrations of (a) L-NAME (10–1000 μM) or (b) L-NOARG (10–1000 μM). Culture medium (100 μl) was then sampled after 24 h exposure and analysed for nitrite/nitrate levels with the Greiss reagent. Cultures were then assessed for MTT reductive capacity. Data are mean of 6 samples per condition (number of experiments=6) and vertical lines show s.e.mean. Statistical significance vs LPS/IFN controls; *$P<0.05$; **$P<0.01$; ***$P<0.001$ (Student's t test).

kinase inhibitor, demonstrated a highly potent attenuation of LPS/IFN-stimulated NO synthesis ($IC_{50}=4.3$ μM) with maximum inhibition (70%) at 10 μM (Figure 5b). Furthermore, herbimycin A, over a 200 fold concentration range, did not significantly modify cellular MTT redox activity (Figure 5b).

The efficacy of two novel non-receptor linked tyrosine kinase inhibitors; the tyrphostins AG-126 and AG-556 was also examined. AG-126 had a potent inhibitory effect upon nitrite production ($IC_{50}=60$ μM), with significant inhibition observed between 10–25 μM, increasing to approximately 95% inhibition at 100 μM (Figure 5c). Tyrphostin AG-556 had a slightly less potent effect ($IC_{50}=70$ μM) with 72% nitrite inhibition at 100 μM (Figure 5d). In all three cases, cellular MTT reduction remained virtually unchanged, compared to LPS/IFN controls, between 0.5–100 μM. Interestingly, a moderate but reproducible increase in MTT reduction (9–15%) was observed between 25–50 μM of AG-126 and AG-556 (Figure 5c–d).

Having established that tyrosine kinases were likely to be involved in the transduction process implicated in LPS/IFN-mediated induction of iNOS, we analysed the roles of a second major class of intracellular kinases, notably the serine/threonine kinases. Staurosporine, a selective inhibitor of protein kinase C (PKC) produced a potent dose-related attentuation ($IC_{50}=0.2$ μM) of NO production with maximal (92%) inhibition detected at 1 μM (Figure 6a). This effect was coupled with a significant dose-dependent loss of cellular MTT reduction culminating in a 35% decrease in cellular redox activity at 1 μM drug levels (Figure 6a). Microscopic analysis of drug-treated cells indicated significant cellular retraction but no gross morphological damage.

H-9 preferentially inhibits cyclic GMP/AMP-dependent (cyclic nucleotide, PKA) protein kinases but also possesses moderate inhibitory activity for PKC. At high doses, between 100–200 μM, a sharp decrease in medium nitrite levels (60% inhibition at 200 μM) was detected (Figure 6b) and correlated with a 22% decrease in MTT reduction at similar drug concentrations. Microscopic observation revealed no significant levels of cellular damage. Based on these observations it is

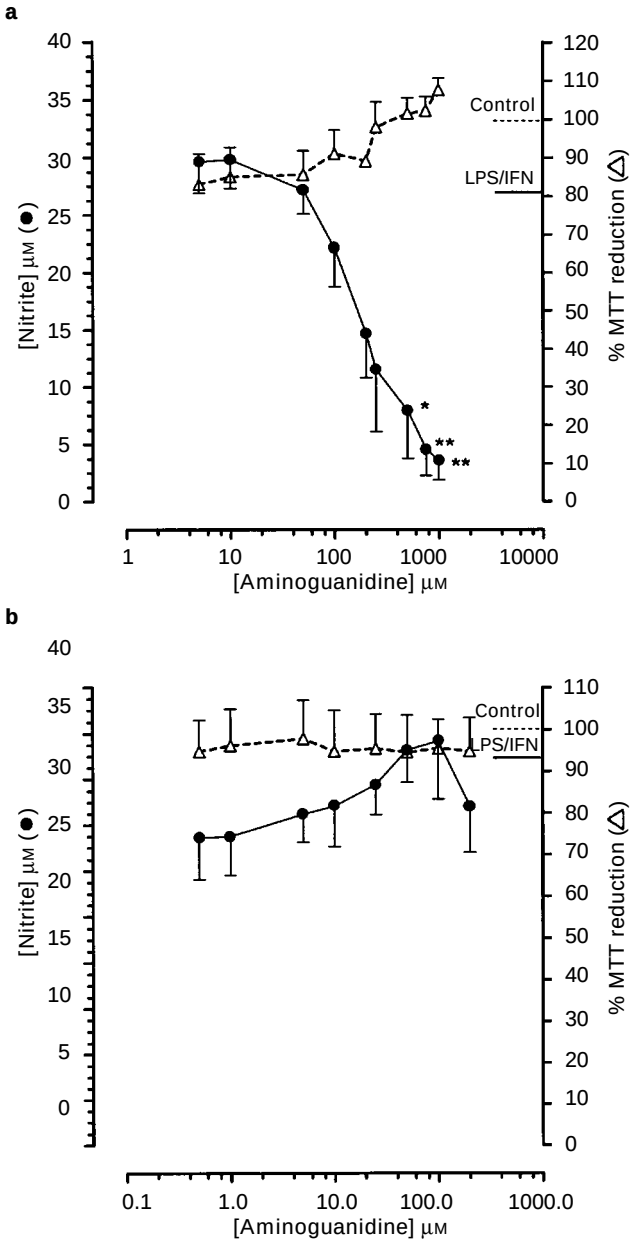

Figure 3 Effect of NOS inhibitors aminoguanidine and 7-nitroindazole on LPS/IFN-stimulated NO release from N9 microglia. Cultures (3–4 d.i.v.) were incubated with IFN-γ (100 u ml^{-1}) plus LPS (0.5 μg ml^{-1}) in the presence of increasing concentrations of (a) aminoguanidine (10–1000 μM) ($n=4$–6) or (b) 7-nitroindazole (10–200 μM) ($n=5$–7). Culture medium (100 μl) was then sampled at 24 h exposure and analysed for nitrite/nitrate levels with Greiss reagent. Cultures were then assessed for cellular MTT reductive capacity. Data are mean of 6 samples per condition (n=number of experiments) and vertical lines show s.e.mean. Statistical significance vs LPS/IFN controls; *$P<0.05$; **$P<0.01$; ***$P<0.001$ (Student's t test).

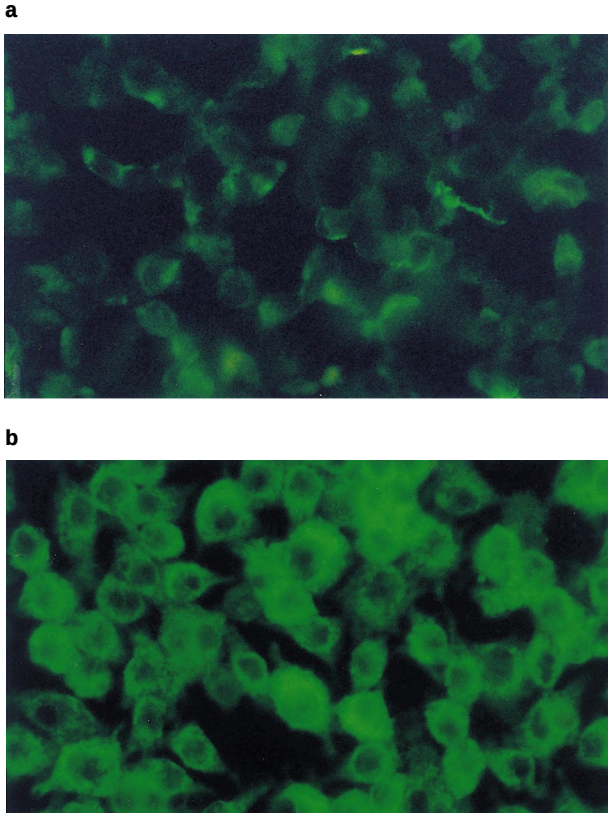

Figure 4 Immunofluoresence labelling of N9-stimulated cells with iNOS antibody. N-9 microglial cells were incubated in the (a) absence or (b) presence of LPS/IFN for 24 h, and then processed for immunofluorescence microscopy as described in Methods and incubated with an iNOS specific antibody. Cells were photographed under u.v.-optics and photographed with Kodak-GOLD ASA-400 film. Magnification $\times 380$.

likely that the ability of H9 to inhibit NO production was, once again, probably a result of a drug-induced cytostatic effect.

Protein phosphatase inhibitors

With regard to the intricate coordinated action of protein tyrosine kinases and phosphatases in cell signalling regulatory mechanisms, we examined the effect of tyrosine phosphatase inhibitors on LPS/IFN-stimulated NO synthesis in N9 microglia. Two relatively broad-range protein tyrosine phosphatase inhibitors, sodium orthovanadate and phenylarsine oxide, produced two very different inhibitory profiles. Sodium orthovanadate produced a dose-dependent inhibition of NO release ($IC_{50}=150$ μM) with no significant cytostatic or cytotoxic action (Figure 7a). A potent inhibitory effect of phenylarsine oxide ($IC_{50}=8$ μM) without significant cytostatic or cytotoxic effect was observed up to 10 μM (Figure 7b). However, between 10–200 μM the inhibitory action of phenylarsine oxide on NO formation (97% inhibition at 50 μM) correlated with a significant cytotoxic action (IC_{50} MTT = 26 μM: MTT 86% inhibition at 50 μM) (Figure 7b).

Okadaic acid, a selective protein phosphatase inhibitor, specific for phosphatase 1/2A isoforms produced a significant decrease ($IC_{50}=0.09$ μM) in nitrite production at drug concentrations ranging from 0.1–0.2 μM (Figure 7c). However, within the same concentration range, a significant decline in redox activity was detected (27%), suggesting that okadaic acid reduces NO production via a moderate but significant cytostatic effect on cultured microglia. Figure 7d describes the effect of a selective protein phosphatase 2B(calcineurin) inhibitor, cyclosporin A. Significant inhibition of nitrite production ($IC_{50}=8$ μM) was observed at drug levels between 1–2.5 μM. This inhibition was dose-dependent reaching a maximal 57% NO inhibition at 10 μM of cyclosporin A. A close relationship between nitrite inhibition and the decline of MTT, with a corresponding 46% MTT inhibition at 10 μM

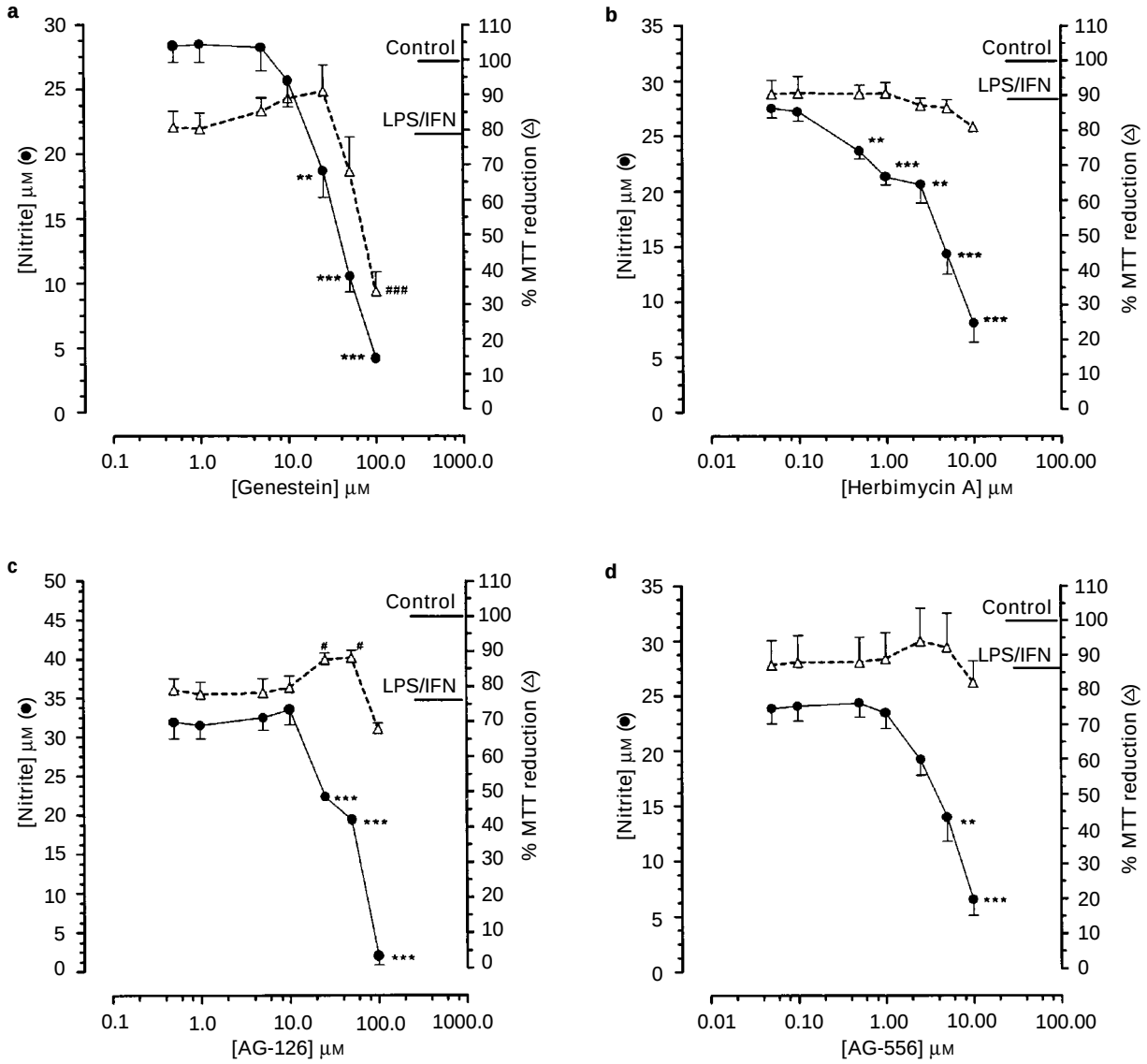

Figure 5 Effect of receptor-linked and receptor/non-receptor-linked tyrosine kinase inhibitors on LPS/IFN-stimulated NO release from N9 microglia. Cultures (3–4 d.i.v.) were incubated with IFN-γ (100 u ml^{-1}) plus LPS (0.5 μg ml^{-1}) in the presence of increasing concentrations of (a) genestein (1–100 μM) ($n=5$), (b) herbimycin-A (0.05–10 μM) ($n=3$–6), (c) AG-126 (0.5–100 μM) ($n=6$) and (d) AG-556 (0.5–100 μM) ($n=8$). Culture medium (100 μl) was then sampled after 24 h exposure and analysed for nitrite/nitrate levels with Greiss reagent. Cultures were then assessed for cellular MTT reductive capacity. Data are mean of 6 samples per condition (n = number of experiments) and vertical lines show s.e.mean. Statistical significance vs LPS/IFN controls, (*) nitrite; (#) MTT; *$P<0.05$; **$P<0.01$; ***$P<0.001$ (Student's t test).

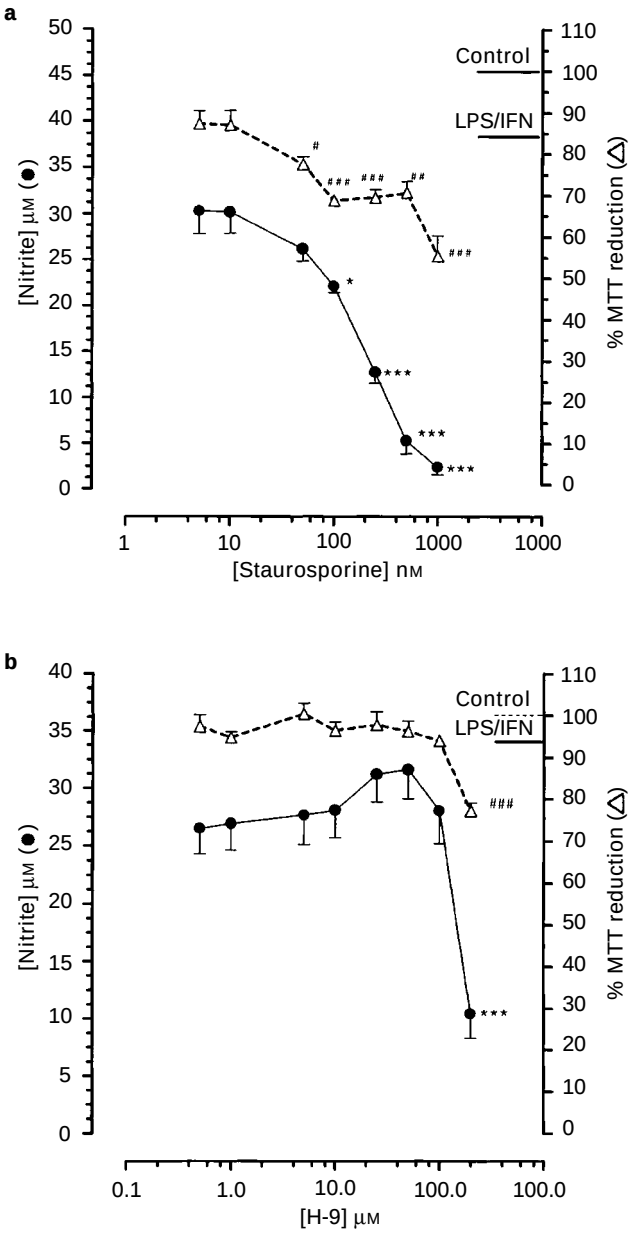

Figure 6 Effect of serine/threonine kinase inhibitors on LPS/IFN-stimulated NO release. Cultures (3–4 d.i.v.) were incubated with IFN-γ (100 u ml^{-1}) plus LPS (0.5 μg ml^{-1}) in the presence of increasing concentrations of (a) staurosporine (PKC) (5–1000 nM; $n=6$) or (b) H-9 (cyclic AMP/cyclic GMP-dependent kinase) (0.5–100 μM; $n=5$). Culture medium (100 μl) was sampled after 24 h exposure and analysed for nitrite/nitrate levels with Greiss reagent and cultures were assessed for cellular MTT reductive capacity. Data are mean of 6 samples per condition (n = number of experiments) and vertical lines show s.e.mean. Statistical significance vs LPS/IFN controls (*) nitrite; (#) MTT; *$P<0.05$; **$P<0.01$; ***$P<0.001$ (Student's t test).

was observed (Figure 7d). It is therefore, likely that cyclosporin A-mediated inhibition of LPS/IFN-stimulated NO synthesis is related to drug-induced growth inhibitory effects.

Cells incubated with inhibitors alone (genestein (25 μM); herbimycin (5 μM); orthovanadate (100 μM); AG-556 (50 μM); H-9 (50 μM); cyclosporin A (5 μM); okadaic acid (0.1 μM) and staurosporine (0.1 μM), failed to demonstrate any detectable increase in nitrite levels in the medium compared to untreated controls (data not shown).

Discussion

In the present study, LPS-induced NOS activity in N9 microglia, as expressed by assay of medium nitrite levels, was potentiated by IFN-γ in a concentration-dependent manner and demonstrated a $\geqslant$8 h lag-phase before the appearance of detectable levels of NO_2^- in the culture medium. The L-arginine analogues L-NAME and L-NOARG, competitive inhibitors of NOS, only produced a partial (approx. 50%) inhibition of LPS/IFN-stimulated microglia NOS activity at maximum concentrations of 1 mM, which may result from the levels of L-arginine (0.7 mM) present in the cell culture medium. On the other hand, inhibition of LPS/IFN induced NO production was obtained with the selective iNOS inhibitor aminoguanidine, whereas, the 'neuronal-like' cNOS inhibitor, 7-nitroindazole was inactive. This implicates iNOS as the major NOS isoform involved in NO synthesis in microglia. The qualitative increase in immunofluorescence labelling of LPS/IFN-stimulated microglia, compared to controls, with a selective iNOS antibody further supports these observations. Previous studies have also demonstrated that NO production in transformed or primary microglial cultures implicates primarily the iNOS isoform (Corradin *et al*., 1993) and is a result of an increased rate of NOS transcription.

In the present study the ATP analogue, genestein, a non-selective inhibitor of receptor-linked protein tyrosine kinases, including the EGF, HER-2/neu/c-erb-2 and PDGF receptors (reviewed by Levitzki & Gazit, 1995), inhibited LPS/IFN-stimulated NO synthesis. At high doses genestein appeared to reduce iNOS activation as a result of cellular growth arrest, based on its ability to produce a contiguous decrease in cellular MTT activity. Genestein, by acting as an ATP analogue, is not necessarily specific for protein tyrosine kinases but may also inhibit other kinases (Akiyama *et al*., 1987), and is consequently relatively toxic to cells. It is evident that at lower doses, genestein inhibited LPS/IFN-stimulated NO release without any statistically significant inhibition of MTT reduction, suggesting that at least in this dose range the effect is not via a cytotoxic/static action. An ATP antagonist is likely to be highly toxic to cells because it would interfere with a myriad of intracellular metabolic and signalling processes. On the other hand, herbimycin A, an ATP analogue and broad spectrum tyrosine kinase inhibitor is recognised as a general inhibitor of receptor (EGF, HER-2/neu/c-erb-2 and PDGF) and non-receptor-linked (src, BCR-ABL, JAK) tyrosine kinases (Levitzki & Gazit, 1995). In the present study, herbimycin A produced a potent inhibition of LPS/IFN-stimulated NO production without any significant effect on cellular MTT reductive capacity.

These results clearly suggest that both receptor-linked and intracellular tyrosine kinases are likely to be involved in IFN/LPS-stimulated NO production. This result is further supported by evidence that two selective intracellular tyrosine kinase inhibitors, the tyrphostins AG-126 and AG-556, were potent inhibitors of NO production without inducing any significant cytostatic effect. Tyrphostins, act at the substrate sub-site of tyrosine kinases, and are thus potentially less toxic and more selective for protein tyrosine kinases (Levitzki, 1992; Levitzki & Gazit, 1995). Tyrphostins AG-126 and AG-556 were previously shown to reduce LPS lethality in mice and attenuate NO and TNF-α production by inhibiting the phosphorylation of a p42 MAP kinase (Novogrodsky *et al*., 1994). These tyrphostins were shown to have no effect on EGF, Her-2/neu or the PDGFβ receptors at concentrations as high as 100 μM, and are more likely to interact with the *src* family of intracellular tyrosine kinases (Novogrodsky *et al*., 1994).

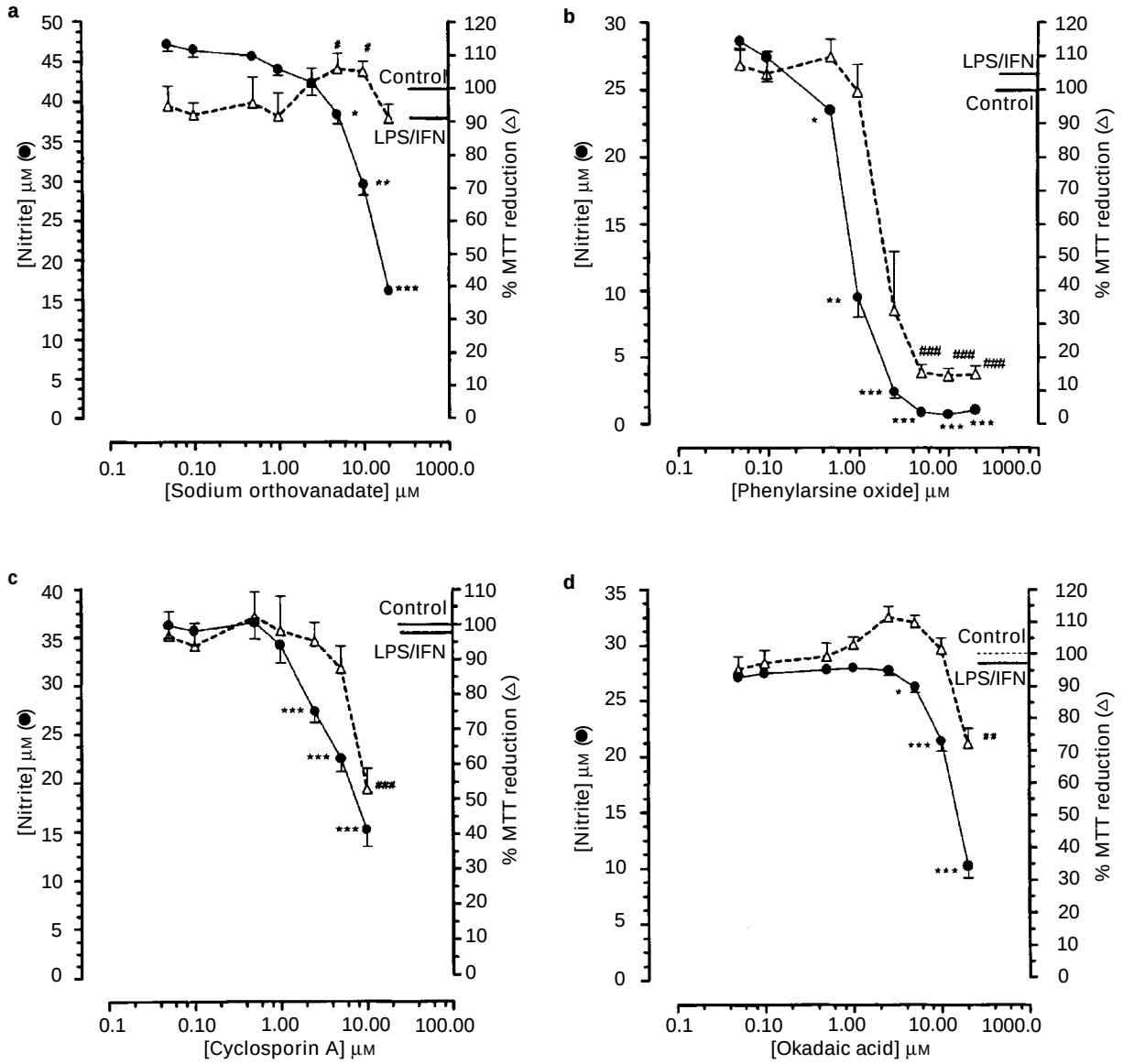

Figure 7 Effect of protein tyrosine phosphatase and serine/threonine phosphatase inhibitors on LPS/IFN-stimulated NO release from N9 microglia. Cultures (3–4 d.i.v.) were incubated with IFN-γ (100 u ml^{-1}) plus LPS (0.5 μg ml^{-1}) in the presence of increasing concentrations of (a) sodium orthovanadate (0.5–200 μM) ($n=5$), (b) phenylarsine oxide (0.5–200 μM) ($n=2$), (c) cyclosporin-A (0.05–10 μM) ($n=6$) or (d) okadaic acid (0.5–200 nM) ($n=4-9$). Culture medium (100 μl) was then sampled after 24 h exposure and analysed for nitrite/nitrate levels with Greiss reagent. Cultures were then assessed for cellular MTT reductive capacity. Data are mean of 6 samples per condition ($n=$number of experiments) and vertical lines show s.e.mean. Statistical significance vs LPS/IFN controls (*) nitrite; (#) MTT; *$P<0.05$; **$P<0.01$; ***$P<0.001$ (Student's t test).

Results from previous studies have demonstrated that similar tyrosine kinase cell signalling mechanisms regulating iNOS activity exist in peripheral macrophages. The tyrosine kinase inhibitor genestein was previously shown to inhibit LPS or cytokine-induced nitrite accumulation in J774.2 macrophages (Akarasereenont *et al*., 1994). Indeed previous studies have demonstrated that genestein, herbimycin A and tyrphostin, at similar doses to those used in the present study, reduced LPS/IFN-stimulated NO release from murine macrophages (Dong *et al*., 1993a), and that genestein suppresses the expression of iNOS activity (Paul *et al*., 1995). Furthermore, herbimycin A and genestein inhibit LPS-induced TNF-α and IL-6 protein and mRNA production in human monocytes (Shapira *et al*., 1994; Beaty *et al*., 1994). Similarly, LPS has been shown to induce the tyrosine phosphorylation of a group of 39–45 kDa proteins and activation of MAP kinases in murine macrophages, and both effects were blocked by tyrosine kinase inhibitors (Weinstein *et al*., 1991; Dong *et al*., 1993b). It is therefore likely, based on the phenotypic homologies between peripheral macrophages and microglia, that protein tyrosine phosphorylation is an integral component of the signal transduction pathways involved in the regulation of microglial activation. Indeed, a recent study, demonstrating that protein tyrosine kinase inhibitors, genestein and tyrphostin A-25 inhibit LPS/γIFN-induced NO production in mixed glia cultures (Kong *et al*., 1996), supports these conclusions.

Protein tyrosine kinases do not exert their effects in isolation but rather co-ordinate their effects with protein tyrosine phosphatases (Sun & Tonks, 1994). Indeed tyrosine phosphatases may inactivate tyrosine phosphorylated protein or activate constitutively phosphorylated protein. The inhibitory action of two broad-range tyrosine phosphatase inhibitors, Na_3VO_4 and phenylarsine oxide, in the present study, suggests a co-ordinated role of tyrosine phosphatases in the signalling pathway implicated in IFN/LPS-stimulated NO formation.

Tyrosine kinases can mediate intracellular signalling cascades by phosphorylating tyrosine residues on substrate proteins, such as intracellular protein kinases including Raf1 kinase, mitogen activated protein (MAP) kinase and protein kinase C (Weinstein *et al*., 1991; Pazin & Williams, 1992; Pelech & Sanghera, 1992; Dong *et al*., 1993b). The data obtained indicated that inhibition of PKC with staurosporine induced significant inhibition of NO formation, with a moderate but significant decline in cellular MTT reduction. Indeed, LPS has been demonstrated to activate simultaneously a Ca^{2+}-independent PKC and p42 and p44 MAP kinases in mononuclear phagocytes via signalling through the CD-14 receptor (Liu *et al*., 1994). Although the results obtained suggest a PKC-mediated step in the activation of iNOS by IFN/LPS, non-specific inhibition of unrelated PKC-mediated cellular metabolic mechanisms cannot be ignored. H-9 which inhibits cyclic AMP and cyclic GMP-dependent kinases, as well as PKC, also inhibited NO production, and caused a similar decrease in MTT activity as staurosporine.

Treatment of LPS/IFN activated N9 microglia with the phosphatase 1/2A phosphatase inhibitor, okadaic acid produced a potent reduction of NO production, contiguous with a significant decrease in cellular MTT reductive capacity. Although okadaic acid was shown to induce NF-κB activation (Thévinin *et al*., 1991), our findings indicate that pertubation of phosphatase 1/2A systems during LPS/IFN cellular activation tends to inhibit cell signalling events implicated in iNOS induction. In addition, cyclosporin A appeared to inhibit NO formation by an apparent cytostatic mechanism. The immunosuppressive action of cyclosporin A appears to reside in the ability of the cyclophilin-cyclosporin A complex to bind to a calcium/calmodulin-dependent serine/threonine phosphatase (type 2B) termed calcineurin (Maggon, 1994). Inhibition of calcineurin prevents dephosphorylation and nuclear translocation, and thus activation of the transcription factor NF-AT which induces transcription of a number of cytokine genes (Rao, 1994). Furthermore, NF-AT can bind to certain NF-κB sites indicating a dual-regulatory role for these transcription factors in mediating stress/inflammatory responses. Previously described growth inhibitory effects of cyclosporin A occur in the micromolar range but may not be exclusively related to inhibition of calcineurin (Richter *et al*., 1995).

In toto, these results argue in favour of a PKC or related kinase-mediated step in the activation of iNOS. Indeed, this conclusion is supported by previous findings demonstrating that H-7 blocked NF-κB activation (Tong-Starken *et al*., 1989). In addition, Shapira *et al*. (1994) have shown that H-7 and staurosporine inhibit LPS induced TNF-α and IL-1β production in human monocytes, and suggested that two proximal intracellular pathways involving PTKs and PKC are involved in LPS-mediated cell signalling mechanisms. Although specific MAP kinases inhibitors are not yet available, a recent study describes a specific inhibition of human monocyte cytokine production with a series of pyridinyl-imidazole compounds which appear to target a pair of serine/threonine kinases homologous to MAP kinases (Lee *et al*., 1994).

The regulation of NOS activity by direct phosphorylation is not entirely clear. To our knowledge no results appear in the literature demonstrating a direct tyrosine phosphorylation of NOS enzyme, although putative phosphorylation sites for tyrosine kinases, and different classes of protein kinases; PKC, PKA (cyclic GMP-dependent kinase), CamKII exist on NOS (Bredt *et al*., 1992). As iNOS most likely represents the major source of NO synthesis in stimulated N9 microglia, our results suggest that LPS/IFN activation of iNOS may occur via a cell signalling event involving tyrosine and serine/threonine kinase/phosphatase activity. Upstream coding sequences for the iNOS gene indicate the presence of a consensus sequence for the NF-κB transcription factor (Baeuerle & Henkel, 1994). The activation of the pleiotropic intracellular transcription factor NF-κb, an essential mediator of the induction of iNOS, and its translocation into the nucleus is regulated via the coordinated activation of an as yet unidentified protein kinases/phosphatases. Moreover, PTKs appear to be implicated in the upstream activation of NF-κB, as herbimycin A was shown to inhibit IL-1-mediated activation of NF-κB (Bomsztyk *et al*., 1991). Many kinases have been shown to activate NF-κB-1κB complexes, including tyrosine kinases of the *src* family, PKC, cyclic AMP-dependent kinase, haeme-regulated kinase and raf-1 kinase (Baeuerle & Henkel, 1994). However, it has not yet been established if these mechanisms are involved in the direct phosphorylation of the I-κB complex or via upstream signalling mechanisms. Consensus data suggest that I-κB is phosphorylated on a serine residue (32, 36) by an unidentified serine/threonine kinase, or may be constitutively phosphorylated, and is thus activated by a highly specific phosphatase. In either case it is apparent that upstream tyrosine kinases/phosphatases and serine/threonine kinase/phosphatases are involved in this activation process.

NF-κB and NF-AT play a pivotal role in inflammatory-mediated-responses in peripheral macrophages (Baeuerle & Henkel, 1994; Rao, 1994). From the phenotypic homology that exists between peripheral macrophages and microglia (Jordan & Thomas, 1988) it is evident that inhibition of microglial inflammatory mechanisms could represent an important therapeutic potential in the treatment of neurodegenerative disorders. Ultimately, as human macrophages appear to lack a high-output iNOS system (Peterson *et al*., 1994), a comparative study with cloned human microglial cells must be performed in order to delineate a potential role of NO released from microglia in human neurodegenerative disorders.

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