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Impaired response to interferon-γ in activated macrophages due to tyrosine nitration of STAT1 by endogenous nitric oxide

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1 Inducible NO synthase (iNOS) expression and activity were measured in the mouse macrophage cell line J774 after exposure to bacterial lipopolysaccharide (LPS) with or without interferon-γ (IFN-γ).

2 Inhibition of NOS activity by concomitant N^G-monomethyl-L-arginine (L-NMMA) treatment further increased iNOS protein levels, with a substantial increase in iNOS half-life.

3 Western blotting and ELISA demonstrated that several cell proteins were tyrosine-nitrated when iNOS activity was high.

4 Rapid IFN-γ-induced phosphorylation of STAT1 was reduced by about 40% when cells were pretreated to induce iNOS, unless L-NMMA was present during the pretreatment period. 2D gel electrophoresis demonstrated the presence of nitrotyrosine in STAT1 after iNOS induction, and confirmed the reduction in phospho-STAT1 on subsequent stimulation with IFN-γ for 15 min and its partial restoration when L-NMMA was present during the pretreatment period.

5 We did not detect tyrosine nitration of the upstream kinase JAK2 in LPS + IFN-γ pretreated cells, but JAK2 activity was also impaired, and was partially restored by concomitant L-NMMA pretreatment.

6 We conclude that endogenous production of NO induces feedback inhibition of signalling pathways activated by IFN-γ, at least in part by nitrating tyrosine residues in STAT1 which prevents phosphorylation.

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Introduction

Oxidative stress has been implicated in many disease processes including inflammation, reperfusion, atherosclerosis, ageing and cancer (Sies, 1991). One of the important agents that mediate damage induced by oxidative stress is peroxynitrite, a potent oxidation product of the reaction between superoxide and NO (Beckman *et al*., 1990). Peroxynitrite can react irreversibly with tyrosine residues of proteins to form 3-nitrotyrosine (Ischiropoulos *et al*., 1992). This 'marker' molecule has been detected at inflammatory sites, as well as in body fluids (e.g. Kaur & Halliwell, 1994; Haddad *et al*., 1994) leading to the suggestion that peroxynitrite contributes to tissue damage.

In murine macrophages, proinflammatory cytokines such as gamma-interferon (IFN-γ), and also lipopolysaccharide (LPS) induce the expression of nitric oxide synthase (iNOS) (Xie *et al*., 1992; Lorsbach *et al*., 1993) which is able to produce relatively large quantities of NO for long periods of time (hours). iNOS can generate both superoxide and NO in L-arginine depleted macrophages, with the subsequent formation of peroxynitrite (Xia & Zweier, 1997). This may contribute to one of the important macrophage functions: killing infectious agents (Nathan & Hibbs, 1991), as well as providing antitumour cell cytotoxicity (Hibbs *et al*., 1987). It has also been shown that endogenous NO production in activated macrophages and in endothelial cells directly regulates mitochondrial respiration (reviewed by Brown, 2000).

Tyrosine phosphorylation is an important regulator of cellular signal transduction, and therefore nitrotyrosine formation may interfere with normal activation pathways. Chemically produced tyrosine nitration has been shown to inactivate nearly 140 mammalian proteins whose activity is dependent on tyrosine residues (Nielsen, 1995). Kong *et al*. (1996) demonstrated that the nitration of a single tyrosine residue in the cell cycle kinase cdc2 prevents tyrosine phosphorylation. Takakura *et al*. (1999) showed that several protein tyrosine phosphatases are inactivated by exogenous peroxynitrite, though this is due to nitrosylation of essential free thiols, rather than tyrosine nitration. Gow *et al*. (1996) described that the exposure of endothelial cells to peroxynitrite produced from SIN-1, an NO donor, resulted in protein tyrosine nitration. This interfered with phosphorylation and also targeted proteins for degradation.

The functional roles of physiological tyrosine nitration due to endogenous peroxynitrite generation have, however, as yet hardly been studied. A function has been implied from the

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finding that the major tyrosine-nitrated protein in human pancreatic adenocarcinoma *in vivo* is the tyrosine kinase c-Src (MacMillan-Crow *et al*., 2000). Zou *et al*. (1998) showed that endogenous peroxynitrite formation in mesangial cells interfered with prostacyclin synthesis, apparently by nitrating prostacyclin synthase, and it has been suggested that peroxynitrite is an endogenous activator of c-Jun kinase in response to shear stress acting on endothelial cells (Go *et al*., 1999).

Intracellular signalling in response to IFN-γ involves two cytoplasmic tyrosine kinases that are members of the Janus (JAK) family of non-receptor protein tyrosine kinases (Ihle *et al*., 1995; Taniguchi, 1995). After tyrosine phosphorylation JAK1 and JAK2 are activated, leading to the tyrosine phosphorylation of the 91,000 Mr DNA-binding protein STAT1. Phosphorylated STAT1 is dimerized and translocated to the nucleus where it modulates gene expression (Darnell *et al*., 1994). We therefore induced, in murine macrophages, a high level of endogenous NO production and then determined the effect this had on the JAK-STAT signalling pathway by analysing STAT1 phosphorylation/nitration and JAK2 activity.

Methods

Cell culture

The mouse macrophage cell line J774 was routinely cultured in spinner flasks with Dulbecco's medium (DMEM; Sigma) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (Sigma), 100 μg ml^{-1} streptomycin and 100 units ml^{-1} penicillin at 37°C in 5% CO_2/95% humidified air. All experiments were performed with cells seeded from the stock in 6-well-plates (3×10^6 cells per well) or 60-cm tissue culture dishes (6×10^6 cells per dish) and cultured overnight. Non-adherent cells were removed and pre-warmed medium was added containing the different stimulants to be tested: 50 U ml^{-1} mouse IFN-γ (Genzyme), 5 μg ml^{-1} lipopolysaccharide (LPS from *E.coli* 026:B6, Difco Laboratories), or their combinations. In some experiments 1 mM N^G-monomethyl-L-arginine monoacetate (L-NMMA, BioMol) was added in combination with the stimulants mentioned above. Cells were incubated for different time periods, and in some experiments the cells were re-stimulated with IFN-γ (100 U ml^{-1}) for 15 min before harvesting. 3-Morpholinosydnonimine (SIN-1, BioMol) at 0.1 mM was used for 4 h and subsequently the cells were stimulated with 100 U ml^{-1} IFN-γ for 15 min, before harvesting.

Determination of nitrite accumulation

Nitrite (NO_2^-) accumulation in the supernatant of cultured cells was used as an indication of NO production and was determined by a modification of the Griess reaction (Kolb *et al*., 1994) using sodium nitrite as standard.

Antibodies

Polyclonal rabbit antiserum against iNOS and immunopurified rabbit anti-nitrotyrosine Ig were obtained as described by Hamid *et al*. (1993) and Beckman *et al*. (1994a) respectively. Monoclonal antibodies against nitrotyrosine and polyclonal rabbit antiserum against JAK2 were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). A monoclonal anti-phosphotyrosine antibody was purchased from Santa Cruz Biotechnology. Rabbit polyclonal antiserum against phospho-STAT1 was from New England Biolabs Ltd. Monoclonal antibody against STAT1 was purchased from Transduction Laboratories.

Immunoprecipitation and Western blot analysis

After treatment with stimulants, cells were solubilized in lysis buffer (1% Triton X-100, 0.15 M NaCl, 50 mM Tris pH 8.0, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM orthovanadate) at 4°C and centrifuged for 10 min at $14{,}000 \times g$. The lysate supernatant was then pre-cleared with 1 μl of normal mouse serum or normal rabbit serum and 10 μl of Protein A/G Plus-agarose beads (Santa Cruz Biotechnology) mixed for 1 h at 4°C. After separation of the agarose beads by centrifugation, the lysate was incubated with either rabbit anti-JAK2, mouse anti-STAT1 or rabbit anti-iNOS, all used at about 1 μg ml^{-1} for 2–4 h at 4°C. Protein A/G Plus-agarose was added and incubated overnight at 4°C to isolate the immune complexes. The bound immunocomplexes were washed three times in lysis buffer and boiled in SDS sample buffer.

Samples were analysed by 7.5 or 10% SDS–PAGE, followed by electrophoretic transfer to nitrocellulose membranes (Sigma). The membranes were blocked with 5% skimmed milk in Tris-buffer saline-Tween 20 (TBST) (20 mM Tris pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 12–16 h at 4°C. The membranes were then washed in TBST and incubated for 2 h at room temperature with 3% bovine serum albumin in TBST containing the primary antibody normally diluted 1 : 1000 or 1 : 500. The membranes were again washed in TBST and incubated for 1 h with horseradish peroxidase conjugate of a suitable secondary antibody (anti-rabbit or anti-mouse, Pierce) diluted as recommended by the supplier. After washing as above the immunoblots were developed with enhanced chemiluminescence (ECL) detection reagents, using Hyperfilm™ ECL (Amersham Pharmacia Biotech). To perform other immunodetections on the same membrane, the bound antibodies were stripped by incubating the membranes in 2% SDS, 100 mM β-mercaptoethanol, and 62.5 mM Tris-HCl (pH 6.8) for 30 min at 50°C. After extensive washing and reblocking, the membranes were reprobed with another antibody. When anti-phosphotyrosine antibody was used skimmed milk was replaced by bovine serum albumin (ICN). Anti-nitrotyrosine immunodetection was performed following the procedure recommended by the supplier.

2-D gel electrophoresis

Immunoprecipitated samples of STAT1 were analysed by two-dimensional polyacrylamide gel electrophoresis as described by Görg *et al*. (1995) using Immobiline DryStrip™ pH range 3–10 and ExcelGel SDS 8–18% (Pharmacia Biotech). After running the second dimension, gels were transferred to nitrocellulose membranes following Pharmacia's protocol and analysed as described above for Western blotting.

JAK2 in vitro *kinase assay*

JAK2 was immunoprecipitated from 1 mg of total protein as described above. The immunoprecipitates were washed in kinase assay buffer (50 mM HEPES pH 7.3, 100 mM NaCl, 0.1% Triton X-100, 6.25 mM $MnCl_2$, 0.5 mM DTT, 0.1 mM sodium orthovanadate) and resuspended in the same buffer containing 25 μM ATP and 5 μCi of [γ-^{32}P]-ATP. The reaction mixture was incubated for 30 min at 30°C and the immunoprecipitate washed several times with lysis buffer prior to the addition of SDS sample buffer for further analysis by SDS–PAGE analysis and autoradiography (Kodak Biomax MR-1 films).

[^{35}S]-Methionine pulse-chase incorporation

Cells were stimulated as described earlier and incubated for 30 min in methionine-free DMEM, containing 10% dialysed foetal bovine serum, followed by the addition of 100 μCi ml^{-1} of L-[^{35}S]-methionine (>1000 mCi mmol^{-1}; Amersham) and incubated for 30 min at 37°C. Labelled cells were washed and incubated in label-free medium containing 0.2 mM methionine for various lengths of time, after which the cells were lysed. An equal amount of protein for each sample was used for immunoprecipitation, followed by SDS–PAGE, and autoradiography (Kodak Biomax MR-1 films).

Densitometric analysis

The films were analysed by a CCD video camera system GDS-7500 from UVP (Cambridge, U.K.), using the image analysis software supplied by the makers. The data are expressed as per cent of the relative absorbance value, specifying in each case which condition was taken as 100%.

Quantification of nitrotyrosine formation

A direct binding ELISA was developed. Adherent cells cultured in 96-well plates had the culture medium removed, washed with PBS and stored at −20°C until use. The cells were disrupted by addition of 50 μl 1 M KSCN, followed by three cycles of freezing and thawing on dry ice. Soft U-shaped Dynatech microtitre plates were coated overnight at 4°C with either: (1) standard nitrotyrosine-albumin (1–125 ng protein per well), starting in the first well with a 1 : 1 mixture of 1 M KSCN and 0.1 M Na bicarbonate pH 9.6, and subsequent doubling dilutions were made in bicarbonate buffer; or (2) 50 μl of the sample to be tested, lysed as above, added to 50 μl bicarbonate buffer and doubling dilutions performed as above. Next day the wells were washed five times with 0.05% Tween 20/PBS. Fifty μl of affinity purified anti-nitrotyrosine antibody (Venkataprasad *et al*., 1999) diluted 1 : 20,000 in 3% skimmed milk/0.05%Tween/PBS were added to each well and incubated for 1 h at 37°C. After washing, commercial goat anti-rabbit antibody conjugated to alkaline phosphatase (Sigma, diluted according to the manufacturer's instructions) in 3% milk/Tween/PBS was added to each well. The plates were incubated for 1 h at 37°C and washed as before. The colour reaction was developed by adding 100 μl/well of 1 mg ml^{-1} *p*-nitrophenyl phosphate in 0.1 M ethanolamine pH 9.6, incubated for 1 h at 37°C and read immediately in a Titertek Multiskan Plus MK II instrument, using a 405 nm filter. The value obtained was used to calculate the amount of nitrotyrosine, after correcting for the dilution of the sample, using as standard nitrotyrosine-albumin. The standard had a ratio of 10 nitrotyrosine molecules per molecule of albumin (i.e. 3.42 ng nitrotyrosine per 100 ng albumin). It was kindly supplied by Dr V. Darley-Usmar.

Results

iNOS expression and NO production in stimulated J774 cells

In initial experiments we determined the time course of iNOS expression and activity in J774 cells stimulated with either LPS (5 μg ml^{-1}), IFN-γ (50 U ml^{-1}), or their combinations. In agreement with previously published data (Bogle *et al*., 1992; Lorsbach *et al*., 1993; Jesch *et al*., 1997), LPS or IFN-γ alone induced a similar increase in iNOS expression and activity, which was augmented more than 4 fold when both were added, yielding nitrite levels of 135±6 (mean±s.e.mean; $n=3$) nmol per 10^6 cells after 24 h. The iNOS inhibitor L-NMMA substantially enhanced the effect of LPS plus IFN-γ on iNOS expression. For example, after 24 h, a further 3 fold increase was measured, while NO production was inhibited by >80%, in agreement with the results of Weisz *et al*. (1996).

To assess whether the observed effects were due to changes in either *de novo* synthesis or stability of iNOS, we performed pulse-chase experiments using [^{35}S]-methionine. Cells were stimulated with IFN-γ alone or with LPS for 32 h, labelled for 30 min, and radioactivity in immunoprecipitated iNOS determined by autoradiography after SDS–PAGE, 0, 6, 12 and 24 h later. Densitometric estimation of iNOS half life ($t_{1/2}$) indicated that when induced by IFN-γ alone $t_{1/2}$ was ~8 h, while induction by IFN + LPS increased $t_{1/2}$ to ~30 h. In the presence of L-NMMA there was a further increase in both iNOS synthesis and stability, consistent with the accumulation of iNOS protein described above.

Protein tyrosine nitration

J774 cell extracts were analysed after incubation for 32 h with different stimulants. Densitometric analysis of Western blots reacted with anti-nitrotyrosine antibody showed that IFN-γ or LPS treatment induced a measurable increase in the nitration of several proteins. The combined treatment of LPS plus IFN-γ produced a synergistic increase in protein nitration, in parallel with the NO production. Both increases were inhibited by L-NMMA (Figure 1).

Quantification of nitrotyrosine by direct ELISA corroborated the results observed by Western analysis (Table 1). Cells stimulated with LPS plus IFN-γ showed a large increase in tyrosine nitration. This was inhibited by 50% after addition of L-NMMA.

Effect of LPS and IFN-γ on STAT1 expression

By Western blotting, we detected an increase in STAT1 expression after 4 h of stimulation with IFN-γ, reaching a maximum after 32 h (Figure 2). LPS treatment of J774 cells also stimulated STAT1 expression, but to a much lower level

Table 1 3-Nitrotyrosine accumulation in J774 cells

48 h treatment	*Nitrotyrosine* (ng/10^6 cells)
None	0.07
LPS	3.60
LPS + IFN	8.60
LPS + IFN + L-NMMA	4.40

Representative results from one of at least six independent determinations, with equivalent results. Values cannot be pooled and analysed together because different degrees of cell death were observed.

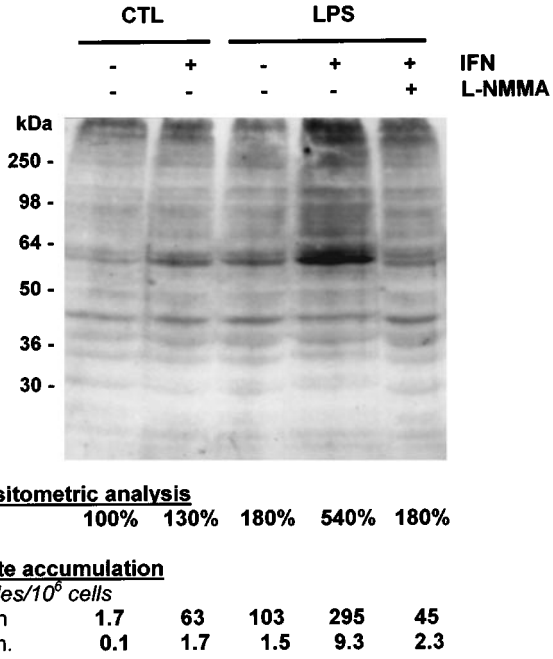


Figure 1 Nitrotyrosine immunoreactivity in stimulated J774 cells. Cells were incubated for 32 h with IFN-γ (50 U ml^{-1}), LPS (5 μg ml^{-1}) or LPS + IFN-γ in the presence or absence of L-NMMA (1 mM) and lysed. CTL = control cells. Equal amounts of protein for each sample (80 μg) were resolved by SDS–PAGE, Western blotting and nitrotyrosine immunodetection (see Methods). Densitometric analysis of the blot is shown at the bottom of the Figure, expressed as per cent of control lane. The nitrite accumulated in the culture medium after incubation was measured in triplicate determinations ($n = 3$). The blot shown is representative of three independent experiments.

and a plateau was reached after 24 h of incubation. Upon addition of both stimulants, STAT1 expression was intermediate between that produced by IFN-γ and LPS individually. Unlike its effect on iNOS, L-NMMA had no detectable effect on STAT1 expression. STAT1 *de novo* synthesis, analysed by stimulating J774 cells with LPS and IFN-γ for different lengths of time followed by a pulse of [^{35}S]-methionine, peaked within 6 h and declined thereafter, while chase experiments indicated that STAT1 was stable for at least 24 h under all the conditions tested (data not shown).

STAT1 activation: phosphorylation

Phospho-STAT1 was detectable within 5 min of IFN-γ stimulation. The maximum phosphorylation was achieved

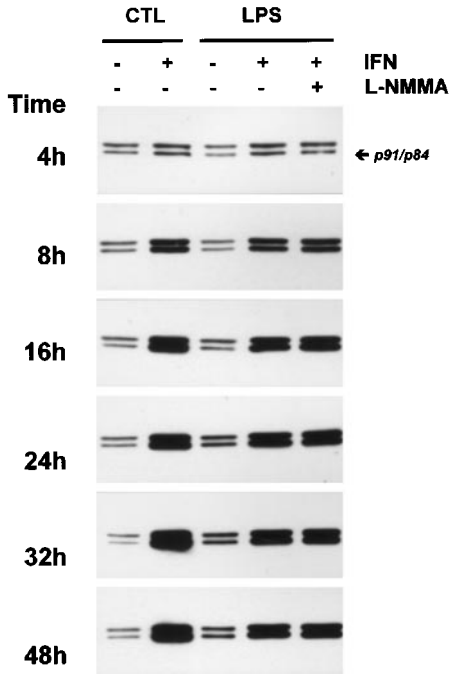


Figure 2 Kinetics of STAT1 accumulation in stimulated J774 cells. Immunoblot analysis of STAT1 after treatment of J774 cells for various time periods with either IFN-γ, LPS or LPS + IFN-γ in the presence or absence of L-NMMA (see Methods; CTL = control cells). Representative blots are shown from three independent experiments.

after 15 min (data not shown). Figure 3 shows that pre-treatment of J774 cells with LPS for either 16 or 32 h produced a time-dependent impairment in the phosphorylation induced by 15 min stimulation with IFN-γ (Figure 3, panel a, lanes 2, 4, 7). Pre-treatment with LPS plus IFN-γ for 32 h further impaired IFN-stimulated STAT1 phosphorylation (Figure 3, panel a, lanes 9, 10 and 2). However, in cells pre-treated with LPS or with LPS plus IFN-γ in the presence of L-NMMA, the impairment of STAT1 phosphorylation was substantially reversed. The blot shown in Figure 3 panel a was stripped and re-probed for STAT1. Panel b confirms that (as in Figure 2) pre-treatment with LPS and IFN-γ for 32 h increased STAT1 expression to a similar extent in the presence or absence of L-NMMA. These results strongly suggest that NO production directly or indirectly interferes with the ability of STAT1 to be phosphorylated.

Nitration of STAT1

To analyse further whether the reduction in phosphorylation of STAT1 observed after LPS addition was directly NO-mediated, immunoprecipitation of STAT1 from cell homogenates was carried out. The samples were analysed by 2-D gel electrophoresis and Western blotting. Figure 4 shows that in control cells STAT1 was highly phosphorylated after 15 min treatment with IFN-γ, with barely detectable levels of nitrotyrosine (panels d and a – the densitometric ratio was 15.2). The reverse was true in cells pretreated for 32 h with LPS + IFN-γ (panels e and b – densitometric ratio 0.6). Pretreatment in the presence of L-NMMA partially reversed the effect of LPS + IFN-γ (panels

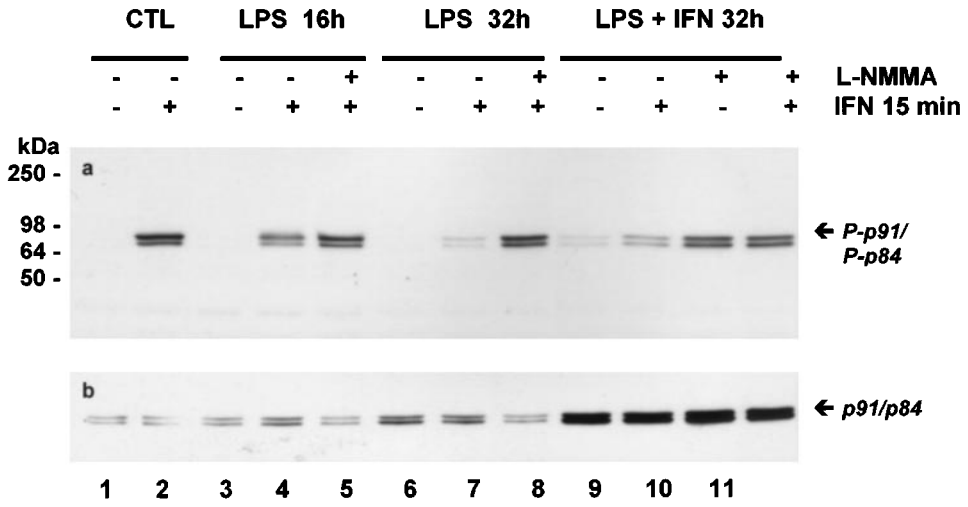
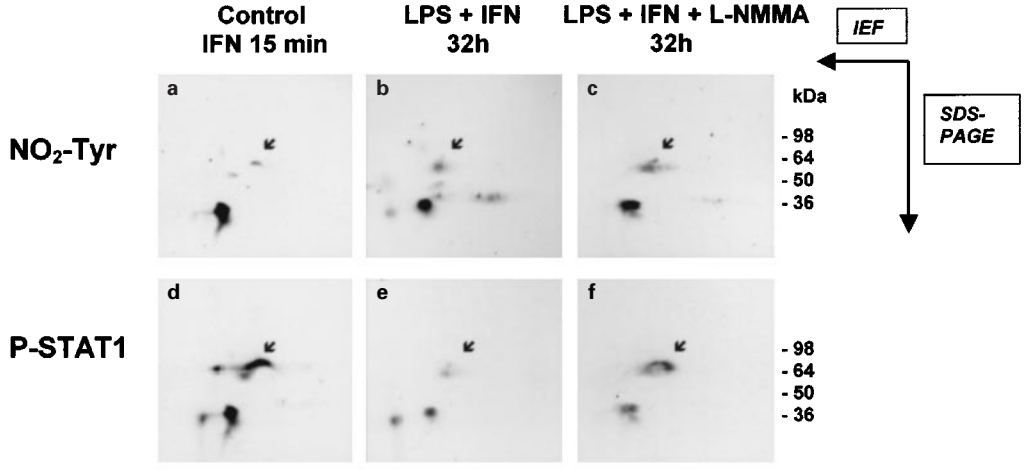


Figure 3 STAT1 phosphorylation after activation of J774 cells with IFN-γ. Cells either untreated or pretreated with LPS (16 h and 32 h) or LPS + IFN-γ (32 h) in the presence or absence of L-NMMA, were stimulated for 15 min with 100 U ml^{-1} of IFN-γ and lysed. CTL = control cells. Equal amounts of protein (50 μg) were analysed by Western blotting and probed with anti-phospho-STAT1 specific antibody (see Methods) (panel a). The blot was stripped and re-probed with anti-STAT1 antibody (panel b) to check STAT1 protein expression. The blots shown are representative of four independent experiments.



Densitometry
(arbitrary units)

	Control	LPS + IFN	LPS + IFN + L-NMMA
NO_2-Tyr	3816	10395	8243
P-STAT1	57396	6219	31631
Ratio phosphorylation / nitration			
	15.2	0.6	3.8

Figure 4 STAT1 nitration and phosphorylation analysed by 2-D PAGE. J774 cells pretreated for 32 h with LPS plus IFN-γ in the presence or absence of L-NMMA, as well as control cells, were stimulated for 15 min with IFN-γ (100 U ml^{-1}) and lysed. STAT1 was immunoprecipitated and analysed by 2-D PAGE, transferred to nitrocellulose membranes and immunodetected for nitrotyrosine (panels a, b, c). The same membranes were stripped and reprobed with phospho-STAT1 specific antibody (panels d, e, f). Arrows indicate the position of STAT1 in the 2-D gels. Low molecular weight dots are the result of the secondary antibody reacting with IgGs used for immunoprecipitation.

f and c – densitometric ratio 3.8). This experiment was repeated three times and the calculated ratios in each case were of a similar magnitude.

To investigate if the addition to J774 cells of an external source of NO and superoxide had comparable effects on STAT1 phosphorylation, we performed experiments using the

NO-donor SIN-1. A 35% decrease in STAT1 phosphorylation was observed when cells were pre-incubated for 4 h with 0.1 mM SIN-1 prior to 15 min activation by IFN-γ and analysed by Western blotting and densitometry (data not shown).

JAK2 activity

Untreated cells had a detectable basal JAK2 kinase activity, which was activated after 15 min in the presence of IFN-γ (Figure 5, panel a, lanes 1 and 2). Incubation with LPS alone for 32 h decreased the basal activity, but IFN-γ treatment still induced activation (Figure 5, panel a, lanes 3 and 4). When LPS was added in combination with IFN-γ a greater reduction of JAK2 activity was observed (30% of the control, lane 6), which was partially reversed by the presence of L-NMMA (lane 7). JAK2 expression remained relatively constant after all treatments (Figure 5, panel b).

Discussion

Several *in vitro* studies, using NO donors or peroxynitrite, have demonstrated that NO can inhibit enzymes and cell-signalling proteins, due either to S-nitrosylation or to tyrosine nitration (e.g. Frears *et al*., 1996; Trotti *et al*., 1996; Estrada *et al*., 1997; Li *et al*., 1998), which has led to the hypothesis that endogenous production of NO modulates cellular signalling pathways by these reactions. However, there have been few previous direct tests of this hypothesis. By immunohistochemistry, tyrosine nitration has been detected in human atherosclerotic lesions (Beckman *et al*., 1994b; Buttery *et al*., 1996), inflammatory processes (Bachmaier *et al*., 1997), neurodegenerative disorders (Bagasra *et al*, 1995; Smith *et al*., 1997) and in skeletal muscle due to ageing (Viner *et al*., 1996), but not in normal tissues. These results give strong indirect support to the notion that local production of NO may induce cellular damage. In the present study, we have demonstrated that a high production of NO in the macrophage cell line J774 results in increased protein tyrosine nitration and in the impairment of the IFN-γ signalling cascade: the JAK-STAT pathway.

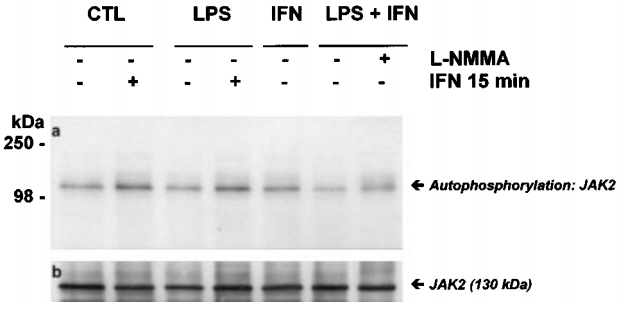


Figure 5 JAK2 kinase activity. Untreated J774 cells or pretreated for 24 h with LPS, IFN-γ or LPS plus IFN-γ, in the presence or absence of L-NMMA and some, as indicated, stimulated for 15 min with IFN-γ were lysed as described. CTL = control cells. JAK2 was immunoprecipitated from equal amounts of total protein. *In vitro* kinase assay was performed using γ-[^{32}P]-ATP (see Methods). The samples were analysed by electrophoresis, transferred to nitrocellulose membrane and autoradiography (panel a). Subsequently, the membrane was probed with anti-JAK2 antibody to assess expression and quantity of protein immunoprecipitated (panel b). Three independent experiments were performed with similar results.

It is well established that iNOS activity in macrophages is induced by IFN-γ and LPS individually, and that in combination they cause a synergistic stimulation of iNOS induction and enzyme activity (Bogle *et al*., 1992; Lorsbach *et al*., 1993; Jesch *et al*., 1997). We confirmed this, and in addition observed that inhibition of NO synthase activity by concomitantly adding L-NMMA significantly increased iNOS expression, reaching a maximum of 10 fold after 48 h. A similar observation has been reported by Luss *et al*. (1994), who observed a dramatic increase in iNOS mRNA and protein after long term NOS inhibition in a model of chronic liver inflammation. Weisz *et al*. (1996) have also reported that L-NMMA enhances induction of the iNOS gene promoter by IFN-γ and LPS. Reinheckel *et al*. (1998) showed that NO inhibits proteasome 26S, which is involved in the activation of NFκB through the degradation of its inhibitor IκB, and Sekkaï *et al*. (1998) reported that NO produced by iNOS inhibits NFκB activation. Our results are in agreement with this, though the pulse-chase analysis indicates that L-NMMA, by inhibiting NO production, additionally stabilises iNOS protein, by mechanisms yet to be investigated. In contrast to these results, Mühl & Pfeilschifter (1995) found that NO enhanced iNOS expression in IL-1 stimulated rat mesangial cells. The reason for this discrepancy is unclear, though it may relate to the relative balance of NO and superoxide (and hence also the level of peroxynitrite) produced under different experimental conditions, since the same group subsequently showed that superoxide also enhanced iNOS expression in IL-1 stimulated mesangial cells (Beck *et al*., 1998).

Protein tyrosine nitration is the result of the generation of reactive nitrogen species, peroxynitrite and nitryl (nitronium) chloride (Halliwell, 1997). Direct addition of peroxynitrite to cultured cells produces a rapid increase in protein tyrosine nitration (Li *et al*., 1998). Our results show that endogenous generation of NO from iNOS in J774 cells causes increased nitration of tyrosine in several cellular proteins, detected by Western blot and quantified by ELISA. The amount of nitration correlated with the NO produced, and inhibition of iNOS activity by L-NMMA reduced nitrotyrosine and nitrite levels.

STAT1 has been reported to be a primary response gene to cytokines in macrophages; specifically IFN-γ up-regulates STAT1 expression (Lehtonen *et al*., 1997). Here we confirm these results and show that LPS also increases the accumulation of STAT1, but to a lower degree than IFN-γ (Figures 2 and 3b). Our results also suggest that LPS reduces IFN-γ-induced STAT1 protein expression. Therefore, LPS may interfere with the IFN-γ signalling involved in the expression and *de novo* synthesis of STAT1. L-NMMA did not alter STAT1 expression, suggesting that it is not affected by NO.

Prior activation of iNOS by LPS treatment of J774 cells caused a reduction of the ability of IFN-γ to phosphorylate STAT1, an effect that is apparently NO-mediated since if L-NMMA was also present no decrease was evident. This suggests that LPS, by stimulating NO production, interferes with STAT1 phosphorylation. Using 2-D gel electrophoresis and Western blotting we were able to demonstrate directly that LPS plus IFN-γ treatment led to detectable STAT1 tyrosine nitration and reduced STAT1 phosphorylation,

partially reversed by L-NMMA, strongly suggesting that a direct chemical modification of tyrosine residues in STAT1 by NO impairs its phosphorylation. Other reactive nitrogen species such as nitryl chloride may also be formed in oxidative conditions (Stamler & Singel, 1992), and Eiserich *et al*. (1998) have shown that *in vivo* this leads to chlorination of tyrosine residues, but this possibility cannot easily be explored at present due to the lack of commercially available anti-chloro-tyrosine antibody.

The decrease in STAT1 phosphorylation might additionally be due to inactivation of JAK2, the protein tyrosine kinase responsible for STAT1 phosphorylation. We analysed JAK2 activity in cells with high NO production, and found a substantial decrease in JAK2 autophosphorylation. However, we did not detect nitrotyrosine in immunoprecipitated JAK2. It is widely accepted that proteins with catalytic activity can be inhibited by nitration in residues other than tyrosine, such as sulphydryl groups (Stamler & Singel, 1992). It is also possible that other modifications such as peroxidation and/or chlorination might be involved in JAK2 inactivation. An alternative possibility is that a fast onset of degradation might occur after JAK2 nitration, as it has been reported to occur in other systems (Gow *et al*., 1996). This seems to be unlikely since a fairly constant amount of JAK2 was visible after all treatments.

In summary, we have demonstrated that induction of iNOS activity in J774 cells results in nitration of cellular proteins and in the impairment of the JAK-STAT pathway. This is due, at least in part, to direct nitration of STAT1 which impairs its ability to be activated by phosphorylation. We also noted that LPS increases STAT1 expression, suggesting a further mechanism by which this bacterial component exerts its modulatory role on the macrophage response to cytokines.

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