

LPS Induction of I κ B- α Degradation and iNOS Expression in a Skin Dendritic Cell Line Is Prevented by the Janus Kinase 2 Inhibitor, Tyrphostin B42

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Received June 16, 2000, and in revised form October 11, 2000; published online January 25, 2001

The Janus kinase (JAK) family of protein tyrosine kinases are activated in response to a wide variety of external stimuli. Here we have investigated whether the janus kinase 2 (JAK2) is involved in the induction of nitric oxide synthase type II (iNOS) expression in a mouse fetal skin dendritic cell line (FSDC). In FSDC the expression of iNOS protein and nitric oxide production, in response to the lipopolysaccharide (LPS) stimulus (5 μ g/ml), is inhibited by the specific inhibitor of the JAK2, tyrphostin B42 with an half maximal inhibitory concentration (IC₅₀) of 9.65 μ M. The antioxidant compound pyrrolidinedithiocarbamate (PDTC) inhibits both the nitrite production with an IC₅₀ of 16.6 μ M and the iNOS protein expression in FSDC. In addition, LPS induces the activation of NF- κ B, and tyrphostin B42 prevents the degradation of the cytosolic factor I κ B- α and blocks the translocation of the NF- κ B p65 protein subunit into the nucleus. These results indicate that the JAK family of protein kinases and the transcription factor NF- κ B are involved in the induction of iNOS protein expression in FSDC stimulated with LPS. © 2001 Academic Press

Key Words: nitric oxide; nitric oxide synthase; dendritic cells; JAK2; NF- κ B; signal transduction.

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Nitric oxide (NO)² can be synthesized by several cell types in mammalian tissues, and the mechanism of synthesis and functions of NO have been the focus of wide investigation (1). NO plays a role as an autocrine and paracrine mediator in diverse physiological and pathological conditions, namely in wound healing (2, 3) and skin inflammatory processes (4–7). The expression of the inducible isoform of NO synthase (iNOS), or NOS type II, has been demonstrated in keratinocytes, dendritic cells (DC), and Langerhans cells (2, 8, 9), but the mechanisms by which the bacterial lipopolysaccharide (LPS) is involved in the iNOS expression are not completely understood.

LPS is a common initiator of inflammation, and one of the earliest signalling events following cellular contact with this endotoxin seems to be tyrosine phosphorylation and activation of mitogen-activated protein kinases (MAPKs) (10). Activated MAPKs phosphorylate and regulate a variety of transcrip-

² Abbreviations used: DC, dendritic cell; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FSDC, fetal skin dendritic cell line; IC₅₀, half maximal inhibitory concentration; IFN- γ , interferon γ ; IKK, I κ B kinase; iNOS, inducible nitric oxide synthase; JAK, Janus kinase; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; MEKK-1, mitogen activated protein kinase kinase 1; NF- κ B, transcription nuclear factor kappa B; NO, nitric oxide; NO₂⁻, nitrite; PDTC, pyrrolidinedithiocarbamate; STAT, signal transducer and activator of transcription.

tion factors, which lead to inflammatory gene expression (11).

In several cell types, LPS also activates the transcription nuclear factor kappa B (NF- κ B) and regulates the iNOS gene expression (12). In resting cells, NF- κ B is localized in the cytoplasm as an heterodimer, composed by two polypeptides of 50 kDa (p50) and 65 kDa (p65), which are associated with an inhibitory protein, generically called I κ B (13). Treatment of cells with various inducers, including LPS, results in the degradation of the I κ B protein, thus releasing the bound p50/p65 complex, which translocates to the nucleus and upregulates gene expression (14, 15).

In response to a variety of inflammatory inducers, the Janus kinase (JAK) family of protein tyrosine kinases is activated, leading to the subsequent activation of the signal transducer and activator of transcription (STATs). Activated STATs form dimers that translocate to the nucleus and bind to response elements to induce transcription (16). However, the interactions between STATs and other transcription factors have not yet been described (17).

In our previous work, we observed that a fetal skin dendritic cell line (FSDC) produces NO in response to LPS and to cytokines by a mechanism involving the activation of JAK2 and NF- κ B (18). Therefore the aim of this study was to assess whether the JAK2 specific inhibitor, known as AG-490 or tyrphostin B42 (19), and the antioxidant pyrrolidinedithiocarbamate (PDTC) (14, 20), inhibits the iNOS expression and NO production induced by LPS in FSDC. The results demonstrated that inhibition of the JAK2 prevents the degradation of the cytosolic factor I κ B- α , and the subsequent translocation of the NF- κ B p65 protein subunit into the nucleus, thus inhibiting the NF- κ B binding to DNA.

MATERIALS AND METHODS

Materials

The rabbit anti-mouse iNOS polyclonal antibody was purchased from Transduction Laboratories (Lexington, KY), the rabbit anti-human NF- κ B p65 was from Serotec (Oxford, England), and the rabbit anti I κ B- α was from New England BioLabs Inc. (Beverly, MA). Mouse monoclonal antibody against

actin and the protease inhibitor cocktail were purchased from Boehringer-Mannheim (Carnaxide, Portugal). The NF- κ B consensus oligonucleotide and the rabbit anti-human NF- κ B p50 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 32 P-Labeled γ -ATP was obtained from Amersham Life Sciences (Buckinghamshire, England), and the T4 polynucleotide kinase and poly(dI-dC) were from Pharmacia Biotech (Carnaxide, Portugal). The horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin was from DAKO (Copenhagen, Denmark). LPS from *Escherichia coli* (serotype 026:B6) was obtained from Sigma Chemical Co. (St. Louis, MO). Tyrphostin B42 was obtained from RBI (Natick, MA), fetal calf serum was from Biochrom KG (Berlin, Germany), and trypsin from Gibco (Paisley, UK). The ECL Western blotting analysis system and the X-ray films were from Amersham Life Sciences (Buckinghamshire, England). All other reagents were from Sigma Chemical Co.

Cell Culture

The fetal mouse skin dendritic cell line FSDC was kindly supplied by Dr. G. Girolomoni (21). The cells were cultured in Iscove's medium supplemented with 10% fetal calf serum, 1% glutamine, 100 μ g/ml streptomycin, and 100 U/ml penicillin.

Nitrite Measurement

The production of NO was assessed as the accumulation of nitrite (NO $_2^-$) in the culture supernatants, using a colorimetric reaction with the Griess reagent (22). Briefly, after stimulation for 48 h, the culture supernatants were collected and diluted with equal volumes of the Griess reagent (0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride, 1% sulfanilamide, and 5% H $_3$ PO $_4$), during 10 min. The absorbance at 550 nm was measured after 10 min incubation in an automated plate reader (SLT, Austria). The nitrite concentration was determined from a sodium nitrite standard curve.

Western Blot Analysis

For immunodetection of iNOS, FSDC were plated at 2×10^6 cells/well in six-well culture dishes for

24 h prior to treatment. The cells were pretreated for 2 h with culture medium, in the presence or absence (control) of 30 μ M PDTC or 50 μ M tyrphostin B42. Next, the cells were treated with culture medium (control), or with LPS (5 μ g/ml), in the presence or in the absence of the inhibitors, for 24 h. After treatment, the cells were washed twice with phosphate-buffered saline and lysed with 200 μ l of lysis buffer (phosphate-buffered saline containing 10 mM EDTA, 1% Triton X-100, and the protease inhibitor cocktail).

For immunodetection of I κ B- α , FSDC were plated at 2×10^6 cells/well in six-well culture dishes for 24 h before treatment. Next, the cells were treated with culture medium (control) or with LPS (5 μ g/ml) for 5 min, 15 min, 30 min, 1 h, and 2 h. The cells were also pretreated for 2 h with 50 μ M tyrphostin B42 and subsequently stimulated with 5 μ g/ml LPS for 30 min. After treatment, the cells were washed twice with phosphate-buffered saline and lysed with 200 μ l of the lysis buffer described above.

For immunodetection of p65, FSDC were plated at 2×10^6 cells/well in six-well culture dishes for 24 h before treatment. Cytosolic fraction proteins were obtained after cells harvest in 10 mM Tris-HCl containing 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40, 1 mM dithiothreitol (DTT), and the protease inhibitor cocktail. The lysates were incubated on ice for 15 min and centrifuged at 2300g for 10 min. The supernatant contained the cytosolic proteins. In brief, protein samples (3.6 μ g protein for iNOS detection and 25 μ g protein for I κ B- α and p65 detection) were separated on a 10% (for iNOS detection) or 15% (for I κ B- α and p65 detection) sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The membrane was blocked with 5% dry milk in Tris-buffered saline with 0.1% Tween 20 for 1 h. The levels of iNOS protein, p65 protein, and I κ B- α protein were detected using 1:2000 dilution of a rabbit polyclonal anti-mouse iNOS antibody, 1:1000 of a rabbit polyclonal anti-human p65 antibody and 1:1000 of a rabbit polyclonal anti-I κ B- α antibody, respectively, for 1 h, followed by incubation with a 1:1000 dilution of the horseradish peroxidase-conjugated swine anti-rabbit antibody for 1 h. The immunocomplexes were visualized by the ECL chemiluminescence method. To demonstrate equiv-

alent protein loading the membrane was stripped and reprobed with anti-actin antibody (1:10,000).

Electrophoretic Mobility Shift Assay (EMSA)

FSDC cells were plated at 2×10^6 cells/well in six-well culture dishes for 24 h before treatment. Then the cells were pretreated for 2 h with or without 50 μ M tyrphostin B42. After this period the cells were treated for 30 min with LPS (5 μ g/ml) in the presence of the JAK2 inhibitor. Cells were washed and lysed in 10 mM Tris-HCl (pH 7.5) containing 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40, 1 mM DTT, and the protease inhibitor cocktail. The lysates were incubated on ice for 15 min and centrifuged at 2,300g for 10 min. The pellet obtained was resuspended in 20 mM Hepes buffer (pH 7.5) containing 300 mM NaCl, 3 mM MgCl₂, 20% glycerol, 1 mM DTT, 0.2 mM EDTA and the protease inhibitor cocktail, incubated on ice for 1 h, and centrifuged at 12,000g for 20 min. The supernatant containing the nuclear proteins was collected, and protein concentration was determined. The EMSA method used was that described previously (14), with slight modifications. The probe consisted of a double-stranded oligonucleotide containing the consensus binding sequence of NF- κ B (5'-AGT TGA GGG GAC TTT CCC AGG C-3') end-labeled with [γ -³²P]ATP using T4 polynucleotide kinase. Typical binding reactions consisted of 12 μ g of nuclear extract, \sim 200,000 cpm of ³²P-labeled oligonucleotide, 100 μ g/ml poly(dI-dC) in a buffer containing 20 mM Hepes (pH 7.9), 1 mM MgCl₂, 4% Ficoll 400, 0.5 mM DTT, 50 mM KCl, and 1 mg/ml BSA and were incubated at room temperature for 30 min. Binding reactions were separated on 10% nondenaturing polyacrylamide gels in a buffer system containing 0.044 M Tris-Base (pH 8.0), 4.45 mM boric acid, 1 mM EDTA, at a constant voltage of 150 V, for 2 h, at room temperature. The gels were transferred to Whatman paper, dried, and subjected to autoradiography. In competition experiments, unlabeled oligonucleotide was added to the nuclear extracts for 10 min prior to addition of radiolabeled probe.

Data Analysis

Results are presented as mean \pm SEM of the indicated number of experiments. Mean values were

compared using one-way ANOVA and the Bonferoni's multiple comparison test. The significance level was 0.05.

RESULTS

Tyrphostin B42 and PDTC Inhibit NO Production and iNOS Protein Expression in FSDC Stimulated with LPS

Several concentrations of LPS (from 0 to 10 $\mu\text{g/ml}$) were tested for the measurement of NO production by FSDC. As indicated in Fig. 1a, LPS (10 $\mu\text{g/ml}$) caused a dose-dependent increase in nitrite concentration, from 1.0 ± 0.2 to 9.3 ± 2.2 μM , reflecting an increase in NO production.

To investigate whether protein tyrosine kinase JAK2 is involved in LPS-mediated NO production, we examined the effect of tyrphostin B42, a specific inhibitor of the JAK2 (19), on the nitrite production upon stimulation of the cells with LPS (Fig. 1b). Tyrphostin B42 abolished the LPS-induced nitrite production in a dose-dependent manner, with an half maximal inhibitory concentration (IC_{50}) value of 9.65 μM and a maximal inhibitory effect at 50 μM of tyrphostin B42 (Fig. 1b). The contribution of NF- κB in LPS-induced NO production, was examined by measuring the effect of PDTC, an antioxidant inhibitor of the transcription factor NF- κB (14, 20), on the nitrite production upon stimulation of the cells with LPS (Fig. 1c). The results indicate that PDTC elicited a concentration-dependent inhibition of LPS-induced nitrite formation in FSDC cells, with an IC_{50} value of 16.6 μM , as calculated by the Hill plots (Fig. 1c). The assay of cellular MTT reduction indicated the lack of any significant toxic effect induced by tyrphostin B42 and PDTC for the concentrations used in the experiments above (data not shown).

Western blot was used to characterize iNOS protein levels in control cells and in cells treated with LPS (5 $\mu\text{g/ml}$), in the presence or absence of tyrphostin B42 (50 μM) or PDTC (30 μM) (Fig. 2). Nonactivated cells did not express iNOS protein (Fig. 2, lane 1). The specific protein iNOS (130 kDa) was detected in cells stimulated with 5 $\mu\text{g/ml}$ LPS (Fig. 2, lane 2) for 24 h. Both 50 μM tyrphostin B42 and 30 μM PDTC inhibited the expression of iNOS in cells stimulated with LPS (Fig. 2, lanes 3 and 4, respectively).

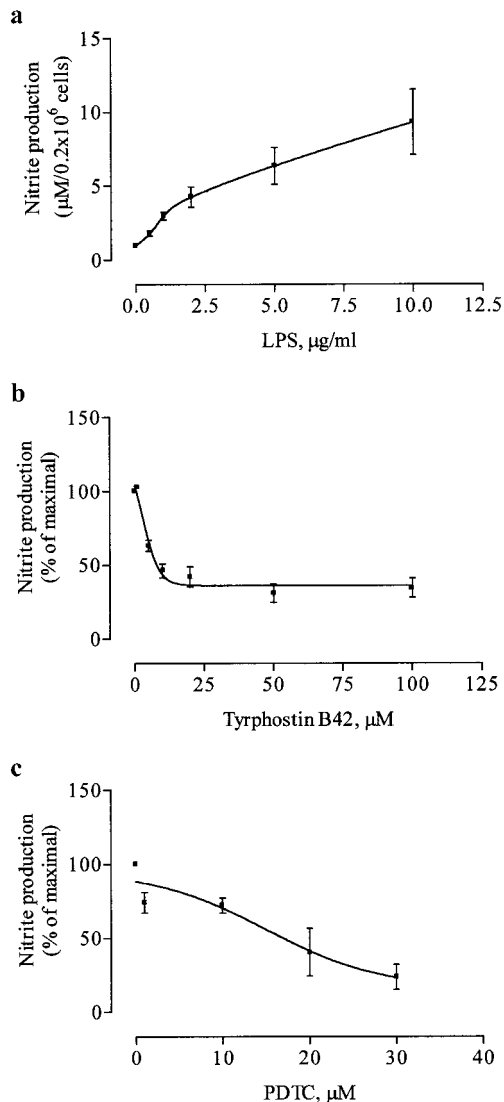


FIG. 1. Dose-dependent effect of tyrphostin B42 and PDTC on LPS-induced nitrite production in FSDC. (a) FSDC were incubated with different concentrations of LPS, for 48 h. (b) FSDC cells were incubated with LPS (5 $\mu\text{g/ml}$), in the presence or in the absence of the indicated concentrations of tyrphostin B42, for 48 h. Results are expressed as percentage of maximal nitrite accumulation on the supernatants of the cells maintained in culture medium in the presence of LPS and in the absence of inhibitor. (c) FSDC cells were incubated with LPS (5 $\mu\text{g/ml}$) and different concentrations of PDTC, for 48 h. Results are expressed as percentage of maximal nitrite production by cells maintained in culture medium in the presence of LPS and in the absence of PDTC. Nitrite levels in the culture supernatants were detected by the Griess reaction as described under Materials and Methods. Each value represents the mean \pm SEM from six experiments, performed in duplicate.

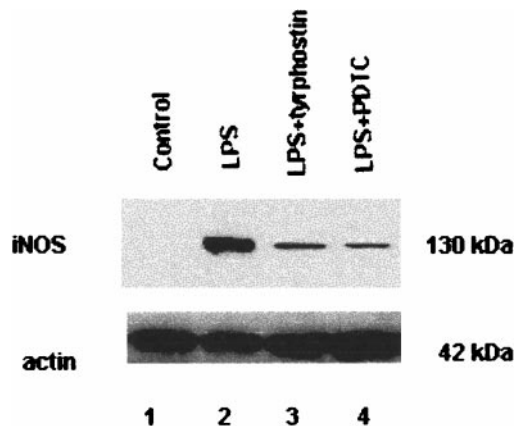


FIG. 2. Effect of tyrphostin B42 and PDTC on LPS-induced iNOS protein expression in FSDC. FSDC cells (2×10^6 cells) were incubated in culture medium alone (control, lane 1), or in the presence of 5 μ g/ml LPS for 24 h (lanes 2–4). Stimulation with LPS was carried out in the absence (lane 2) or in the presence of 50 μ M tyrphostin B42 (lane 3) for 24 h, or 30 μ M PDTC (lane 4). When the effect of tyrphostin B42 or of PDTC was tested, the cells were preincubated with antagonists for 2 h before stimulation with LPS. Total cell extracts were electrophoresed through SDS-PAGE, transferred to PVDF membranes, and subjected to Western blot analysis using anti-iNOS as described under Materials and Methods. The membrane was stripped and reprobed with anti-actin antibody to confirm equal protein loading. The blot shown is representative of three blots yielding similar results.

This decrease in protein expression caused by tyrphostin B42 and PDTC correlated well with the maximal inhibitory effect on NO production, as shown above (Figs. 1b and 1c).

Tyrphostin B42 Blocks the p65 Translocation into the Nucleus and Blocks the Activation of NF- κ B in FSDC Stimulated with LPS

The effect of tyrphostin B42 (50 μ M) on the p65 protein present in the cytosolic extracts was examined by Western blot analysis. As shown in Fig. 3a (lane 2), treatment of FSDC with LPS (5 μ g/ml) significantly reduced the level of p65 in the cytosol. Pretreatment of cells with tyrphostin B42 inhibited the disappearance of p65 induced by LPS (Fig. 3a, lane 3).

To determine whether tyrphostin B42 prevented the NF- κ B binding to DNA in cells stimulated with LPS, FSDC were preincubated or not for 2 h with 50 μ M tyrphostin B42, treated with LPS (5 μ g/ml) for 30 min, and then cell nuclear extracts were exam-

ined by EMSA for NF- κ B binding activity. The results in Fig. 3b indicate that tyrphostin B42 abolished the LPS-dependent activation of NF- κ B. As control for the gel shift assays, unlabeled oligonucleotide (100-fold in excess) inhibited the formation of the NF- κ B probe complex (lane 4).

Tyrphostin B42 Inhibited the Cytosolic I κ B- α Degradation in FSDC Stimulated with LPS

Phosphorylation and subsequent proteolytic degradation of I κ B- α is a critical step for the translocation of NF- κ B subunits to the nucleus (13). To determine whether the suppressive effect of tyrphostin B42 on LPS-induced p65 nuclear translocation was due to modulation of I κ B- α degradation, the levels of I κ B- α on the cell extracts were examined by Western blot analysis. As shown in Fig. 4, treatment of cells with 5 μ g/ml LPS caused the degradation of I κ B- α at 15 and 30 min. The results obtained at 1 h after the initial stimulus indicate that newly synthesized I κ B- α accumulates in the cytoplasm. However, when the cells were pretreated with tyrphostin B42 and then exposed to LPS for 30 min, no proteolytic degradation of I κ B- α was found (Fig. 4, lane 5).

DISCUSSION

In the present report, we investigated whether JAK2 is involved in LPS-induced NF- κ B activation and iNOS expression in a fetal skin-derived dendritic cell line (FSDC). Our results indicated that the specific JAK2 inhibitor, tyrphostin B42, and the antioxidant inhibitor of NF- κ B, PDTC, inhibited in a dose-dependent fashion the iNOS expression and the nitrite production induced by LPS in FSDC (Figs. 1b and 1c). Moreover, tyrphostin B42 blocked the cytoplasmic degradation of I κ B- α (Fig. 4), and inhibited the NF- κ B (p65) translocation to the nucleus (Fig. 3a), thus preventing the NF- κ B binding to DNA (Fig. 3b). These data suggest that the tyrphostin B42 inhibited iNOS gene expression by transcriptional mechanisms involving the NF- κ B/I κ B pathway.

The intracellular signalling events involved in iNOS expression are not well understood, and the knowledge of the mechanisms involved in the control of NO synthesis by different cell types is a sub-

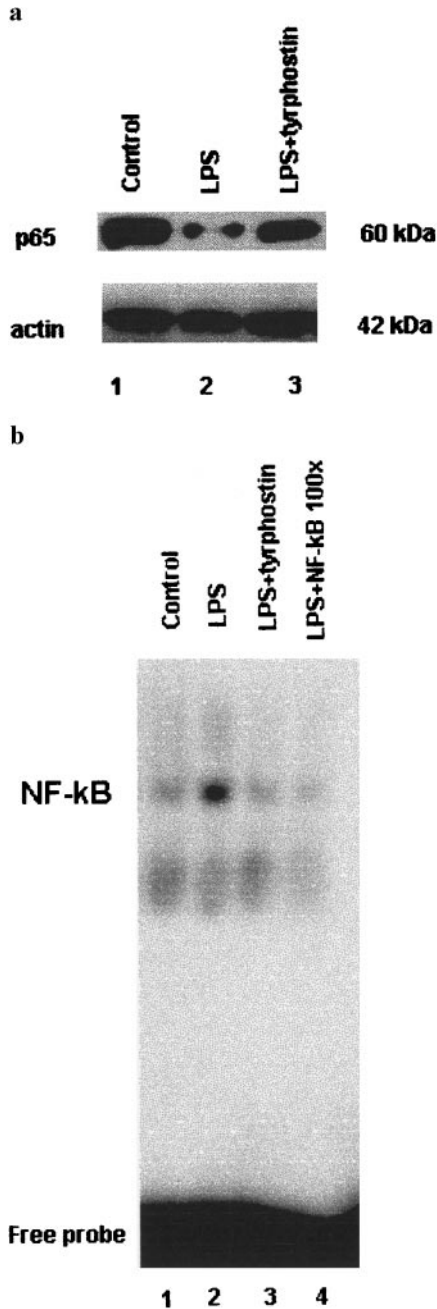


FIG. 3. Effect of tyrphostin B42 on the NF- κ B-p65 protein subunit translocation into the nucleus and on the NF- κ B binding to DNA in LPS-stimulated FSDC. FSDC cells (2×10^6 cells) were incubated in culture medium alone (control, lane 1) or in the presence of $5 \mu\text{g/ml}$ LPS for 30 min (lanes 2–3). Where indicated (lane 3), the cells were stimulated with LPS in the presence of $50 \mu\text{M}$ tyrphostin B42, after 2 h preincubation with the inhibitor. (a) Cytosolic extracts were electrophoresed through SDS-PAGE, transferred to PVDF membranes, and subjected to Western blot analysis using anti-p65 as described under Materials and Methods. The membrane was stripped and reprobred with anti-actin

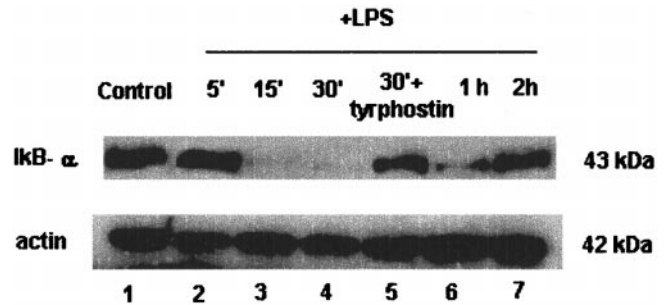


FIG. 4. Effect of tyrphostin B42 on I κ B- α degradation in cytosol of FSDC-stimulated with LPS. FSDC cells (2×10^6 cells) were incubated with culture medium alone (control, lane 1) or with LPS ($5 \mu\text{g/ml}$) for the time periods indicated in the figure. Lane 5 corresponds to cells preincubated with $50 \mu\text{M}$ tyrphostin B42 for 2 h and then stimulated with $5 \mu\text{g/ml}$ LPS for 30 min. Total cell extracts were electrophoresed through SDS-PAGE transferred to PVDF membranes and subjected to Western blot analysis using anti-I κ B- α as described under Materials and Methods. The membrane was stripped and reprobred with anti-actin antibody to confirm equal protein loading. The blot shown is representative of three blots yielding similar results.

ject of current interest. Although the expression of iNOS appears to be regulated in a cell-specific manner, it has been observed that cyclic adenosine monophosphate, protein tyrosine kinases, and protein kinase C are important regulators of iNOS gene expression in a variety of cell types (23–25).

Here, we observed that LPS increases the expression of iNOS in FSDC by a mechanism involving the activation of JAK2, thereby leading to the production of NO. Accordingly, in both glial cells and epithelial-like DLD-1 cells, the expression of iNOS stimulated by interferon γ (IFN- γ) and by LPS has been reported to require tyrosine kinase activity, specifically JAK2 (26, 27). In human intestinal epithelial cells, JAK2 was also involved in iNOS expression induced by cytokines (28) and an IFN- γ activated site (GAS) was necessary for full expression of the mouse *iNOS* gene in response to

antibody to confirm equal protein loading. The blot shown is representative of three blots yielding similar results. (b) Nuclear extracts were subjected to EMSA analysis as described under Materials and Methods. To demonstrate specificity of induced bands, binding was carried out in the presence of molar excess (100 \times) of nonradioactive NF- κ B consensus containing oligonucleotide (lane 4). The gel shown is representative of three gels yielding similar results.

IFN- γ and LPS (29). In contrast, the JAK/STAT pathway suppresses, rather than activates, IFN- γ and LPS-stimulated iNOS induction in vascular smooth muscle cells (30).

NF- κ B regulates the expression of a variety of genes essential for cellular immune response, inflammation, growth, and development (13). Transcriptionally active NF- κ B dimers are formed through the combinatorial assembly of the five monomeric polypeptides, p50, p65, p52, c-Rel, and RelB (13). Dendritic cells have been found to express high levels of all these NF- κ B/Rel proteins, with nuclear activity residing primarily within RelB, p50, and p65 (31). Of particular interest was our finding demonstrating that the inhibitor of JAK2, tyrphostin B42, inhibited the I κ B- α degradation induced by LPS (Fig. 4), which shows that the JAK2 activity is required for phosphorylation and subsequent degradation of I κ B- α . Phosphorylation at serine-32 and serine-36, that precedes degradation of I κ B- α , is known to cause a decrease in the mobility of I κ B- α on SDS-PAGE. No decrease in gel mobility was found on the band of I κ B- α seen on electrophoresis (Fig. 4), suggesting that phosphorylation at tyrosine residues on I κ B- α did not occur, and that tyrphostin B42 probably acts at a step before serine phosphorylation. In fact, it has been described that extracellular stimuli trigger various signal transduction cascades which converge at the phosphorylation of I κ B at specific amino-terminal serine residues (Ser-32 and Ser-36 for I κ B α , Ser-19, and Ser-23 for I κ B β) in complex with a dimer of NF- κ B (13).

The transcription factor NF- κ B has been shown to be required for iNOS induction by LPS in different cell types (12, 14, 32, 33). Our results show that PDTC prevented LPS-induced iNOS expression (Fig. 2) and nitrite production by FSDC (Fig. 1c), indicating that NF- κ B participates in LPS-induced iNOS expression. These results strongly suggest that the production of large amounts of NO in FSDC, induced by LPS, involves the activation of JAK2. The signal is then probably passed on to an I κ B kinase (IKK), which would phosphorylate I κ B and lead to its degradation. IKK is composed of three subunits—IKK α and IKK β , which are highly similar protein kinases, and IKK γ , a regulatory sub-

unit (34). TNF- α and IL-1 β initiate a signalling cascade leading to activation IKK α and IKK β , which phosphorylate I κ B at specific amino-terminal serine residues (35). Direct activators of IKK complex include mitogen activated protein kinase kinase 1 (MEKK-1) and NF- κ B-inducing kinase (NIK), although the precise molecular mechanisms underlying this regulation are unknown (34).

It has been demonstrated that JAK activates the STAT transcription factors as well as several other signalling cascades, including the phosphatidylinositol 3-kinase (PI-3K) and the Ras pathway (17, 36). More recently a direct link from PI-3K/Akt to NF- κ B activation via IKK α -IKK β was also demonstrated (37). These findings suggesting that JAK2 may activate kinases, which in turn activate the IKK complex, are correlated with our results, demonstrating that the JAK family of protein kinases and the NF- κ B are involved in the activation of NO/iNOS pathway induced by LPS in FSDC.

ACKNOWLEDGMENTS

We thank to Dr. G. Girolomoni (Laboratory of Immunology, Istituto Dermopatico dell'Immacolata, IRCCS, Rome, Italy) for the kind gift of the fetal skin derived dendritic cell line (FSDC). This work was supported by Praxis/P/SAU/126/96.

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