

Involvement of p38 mitogen-activated protein kinase in lipopolysaccharide-induced iNOS and COX-2 expression in J774 macrophages

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SUMMARY

Both the nitrite and prostaglandin E₂ (PGE₂) release caused by lipopolysaccharide (LPS) in J774 macrophages are inhibited by SB 203580, a specific p38 mitogen-activated protein kinase (MAPK) inhibitor, in a concentration-dependent manner. The 50% inhibitory concentration (IC₅₀) for nitrite and PGE₂ responses was 1 μM and 0.5 μM, respectively. Inhibition was marked following simultaneous treatment with SB 203580 and LPS, and was much reduced when SB 203580 was added 6 hr after LPS treatment. In parallel, LPS induction of inducible NO synthase (iNOS) and cyclo-oxygenase-2 (COX-2) proteins and their steady-state levels of mRNA were reduced by SB 203580. LPS activation of nuclear factor-kappa B (NF-κB), activator protein-1 (AP-1) and p38 MAPK was also inhibited by SB 203580. These results suggest a crucial role of p38 MAPK in regulation of the transcriptional level of endotoxin LPS-induced iNOS and COX-2 protein expression.

INTRODUCTION

p38 mitogen-activated protein kinase (MAPK) is a member of a family of enzymes activated by the dual phosphorylation of threonyl and tyrosyl residues separated by a single amino acid.^{1–3} The gene for p38 MAPK was originally cloned from *Saccharomyces cerevisiae* (HOG1)⁴ but homologues were subsequently found in *Xenopus*,⁵ murine (p38)¹ and human (CSBP1 and CSBP2) tissues.² In view of its conservation and wide distribution, it would appear that p38 serves an important function in cellular responses. This kinase is activated by stressful stimuli (e.g. high osmolarity, H₂O₂, ultraviolet light and shock),⁴ endotoxin,^{6,7} inflammatory cytokines, interleukin-1 (IL-1),^{8,9} tumour necrosis factor-α (TNF-α)^{3,10} and thrombin.¹¹ p38 MAPK may help cells resist thermal stress¹² and may be involved in the synthesis of IL-1, TNF-α,^{2,13} interleukin-8 (IL-8),^{14,15} interleukin-6 (IL-6),^{9,16} cyclo-oxygenase-2 (COX-2),^{8,9} collagenase-1⁹ and stromelysin-1⁹ in different cell types. Moreover, it provides a crucial signal for platelet aggregation,¹⁷ neutrophil chemo-

taxis,^{7,18} cytosolic phospholipase A₂ (cPLA₂) activation,¹⁹ E-selectin expression,¹⁰ cytosolic alkalization²⁰ and cell apoptosis.^{21,22} More recently, this kinase has been identified in macrophages,² but its regulation and physiological role in these cells have not been clearly explored.

Different regulatory actions of p38 MAPK on protein synthesis, both transcription and translation, have been proposed. For example, in human fibroblasts and umbilical vein endothelial cells stimulated by IL-1, p38 MAPK plays an important role in the regulation of the transcription of COX-2, collagenase-1 and stromelysin-1 genes, while it regulates IL-6 production at the translational level.⁹ In contrast, in L929 cells, it modulates TNF-induced IL-6 synthesis at the transcriptional level.¹⁶ In monocytes, p38 MAPK is also required for the lipopolysaccharide (LPS)-induced synthesis of IL-1 and TNF-α and acts at the translational level.^{1,2,23} In contrast to its general up-regulatory effect for several proteins in a variety of cells, as mentioned above, p38 MAPK has also been suggested to down-regulate IL-1-induced inducible NO synthase (iNOS) mRNA expression and nitric oxide (NO) biosynthesis in glomerular mesangial cells.⁸

To date, the roles of p38 MAPK in endotoxin-elicited iNOS and COX-2 induction have not been clearly identified, particularly in macrophages, the major source of NO and eicosanoids during inflammation. Although LPS can stimulate p38 MAPK and induce iNOS and COX-2 expression in a variety of cell types, including macrophages,^{1,6} the causal relationship between these two events is still unclear. Thus, the aim of this study was to determine the role of p38 MAPK activation, either at the transcriptional or translational level, in LPS-induced iNOS and COX-2 expression and NO and prostaglandin E₂ (PGE₂) production.

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Abbreviations: AP-1, activator protein-1; COX-2, cyclo-oxygenase-2; cPLA₂, cytosolic phospholipase A₂; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; IL-1, interleukin-1; IL-6, interleukin-6; IL-8, interleukin-8; iNOS, inducible NO synthase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; NF-κB, nuclear factor-kappa B; NO, nitric oxide; PGE₂, prostaglandin E₂; TNF-α, tumour necrosis factor-α.

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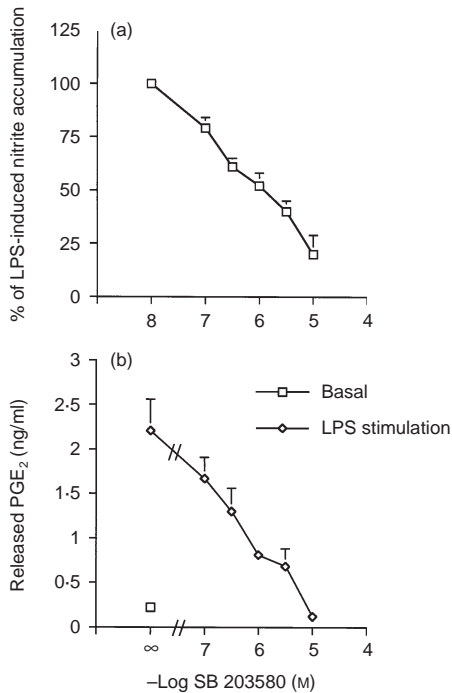


Figure 1. Concentration-dependent inhibition of LPS-induced nitrite and PGE₂ formation by SB 203580. Cells were incubated with 1 µg/ml LPS in the presence or absence of SB 203580, at the concentrations indicated, for 24 hr before assaying for nitrite (a) or PGE₂ (b). The data represents the mean ± SEM of three experiments performed in duplicate.

MATERIALS AND METHODS

Reagents

Rabbit and mouse antibodies specific for iNOS and COX-2 were purchased from Transduction Laboratories (Lexington, KY). Rabbit antibody specific for p38 MAPK was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). iNOS and COX-2 cDNA and the enzyme immunoassay (EIA) kit for PGE₂ assay were obtained from Cayman Chemical (Ann Arbor, MI). The sequences of the double-stranded oligonucleotides (the binding sites are underlined) used to detect the DNA-binding activities of nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) were, respectively, 5'GAT-CAG-TTG-AGG-GGA-CTT-TCC-CAG-GC3' and 5'GAT-CCG-CTT-GAT-GAC-TCA-GCC-GGA-A3'. RNAzol was obtained from Tel-Test Inc. (Friendswood, TX). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin and streptomycin were obtained from Gibco BRL (Grand Island, NY). [α -³²P]ATP (3000 Ci/mmol), [γ -³²P]ATP (5000 Ci/mmol), horseradish peroxidase-coupled second antibody, and the enhanced chemiluminescence (ECL) detection agent were purchased from Amersham International (Arlington Heights, IL). SB 203580 was obtained from Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma (St Louis, MO). Protein A-Sepharose beads were purchased from Transduction Laboratories (Lexington, KY).

Activation of J774 cells

The mouse macrophage cell line, J774, obtained from ATCC (Rockville, MD), was cultured, as described previously,²⁴ in

DMEM containing 10% fetal bovine serum and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin). Cells in 0.5 ml of DMEM were seeded into 24-well plates and, at confluence, 0.5 ml of fresh culture medium, with or without drugs, was added to each well to activate the cells. After 24 hr (unless otherwise indicated) the medium was collected for nitrite and PGE₂ assays. As described below, for the electrophoretic mobility shift assay (EMSA), immunoblot and RNA-blot analysis, cells were grown in 10-cm Petri dishes.

Nitrite production and PGE₂ assay

Nitrite was measured by adding 100 µl of Griess reagent (1% sulphanylamine and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100-µl samples of culture medium. The optical density at 550 nm (OD₅₅₀) was measured using a microplate reader, and the nitrite concentration calculated by comparison with the OD₅₅₀ produced using standard solutions of sodium nitrite in culture medium. PGE₂ was measured using an EIA kit from Cayman, following the manufacturer's instructions.

Immunoblot analysis of iNOS and COX-2

After drug treatment, cells were washed twice in ice-cold phosphate-buffered saline (PBS), then solubilized in 20 mM Tris-HCl, 0.5 mM EGTA, 2 mM EDTA, 2 mM DDT, 0.5 mM p-methylsulphonyl fluoride and 10 µg/ml of leupeptin, pH 7.5. Samples of equal amounts of protein (50–100 µg) were applied to 7.5% (for iNOS) and 9% (for COX-2) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and electrophoresed under reducing conditions. The separated proteins were transferred onto a nitrocellulose membrane, incubated in TBST buffer (150 mM NaCl, 20 mM Tris, 0.02% Tween-20, pH 7.4) containing 5% milk, and then probed with antibodies specific for iNOS or COX-2, followed by a second antibody. Immunoreactivity was detected by ECL, following the manufacturer's instructions.

RNA blotting

Confluent cells, grown in 10-cm Petri dishes, were treated with pharmacological agents for different periods and harvested. Equal amounts (about 50 µg) of total RNA, purified using RNAzol reagent, were applied to each lane of 1.2% (w/v) formaldehyde-agarose gels, electrophoresed, and transferred to Immobilon-N membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). After ultraviolet cross-linking and pre-hybridization for 2 hr at 42°, the membranes were probed for 16–24 hr with iNOS or COX-2 cDNA probes labelled with [α -³²P]dATP by random primer (*c.* 2 × 10⁸ c.p.m./µg). Hybridization reactions were performed in 50% formamide, 5 × SSPE, 10 × Denhardt's solution, 0.5% SDS and 0.1 mg/ml of salmon sperm DNA. The membranes were washed twice in 2 × SSC, 0.1% SDS at room temperature for 20 min, then twice in 0.1 × SSC, 0.1% SDS at 65° for 30 min before exposure to film, using intensifying screens. Densitometric analyses were performed on a Molecular Dynamics (Sunnyvale, CA) densitometer.

Preparation of nuclear extracts and EMSA

Nuclear extracts from stimulated or unstimulated cells were prepared by lysing the cells in 100 µl of 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF,

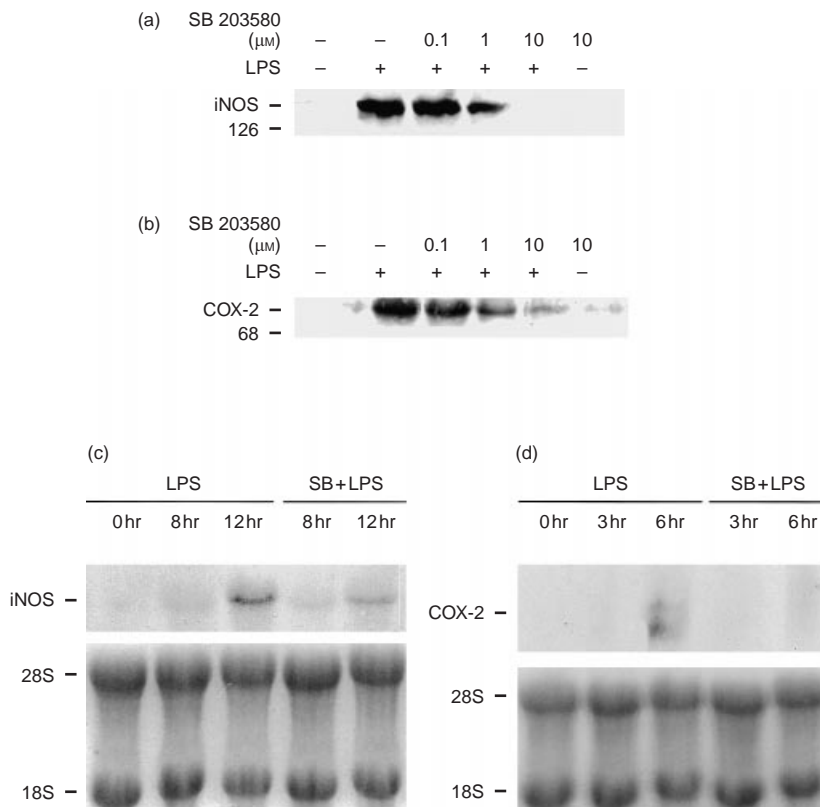


Figure 2. Concentration-dependent inhibition of LPS-induced iNOS and COX-2 expression by SB 203580. iNOS (a) and COX-2 (b) proteins were detected on Western blots by ECL after treatment of the cells with LPS (1 µg/ml) and/or SB 203580 (0.1–10 µM) for 24 hr. Northern blot analysis for iNOS (c) and COX-2 (d) mRNA expression after LPS (1 µg/ml) and SB 203580 (3 µM) co-stimulation is indicated. The example is representative of three independent experiments.

followed by vigorous vortexing for 15 seconds, standing at 4° for 10 min, and centrifugation at 2700 *g* for 2 min. The pelleted nuclei were resuspended in 30 µl of 20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF for 20 min on ice, then the lysates were centrifuged at 20 000 *g* for 3 min. For the EMSA, reaction mixtures (15 µl) contained 0.25 µg of poly(dI-dC) (Amersham Pharmacia Biotech, Uppsala, Sweden) and 20 000 d.p.m. of ³²P-labelled DNA probe in binding buffer consisting of 10 mM Tris, pH 7.5, 1 mM EDTA, 4% Ficoll, 1 mM DTT and 75 mM KCl; the binding reaction was started by the addition of cell extracts and continued for 30 min. Samples were analysed on native 5% polyacrylamide gels.

p38 MAPK assay

Equal amounts of protein (about 1500 µg) from cytosolic lysates prepared in immunoprecipitation buffer (20 mM Tris, pH 7.5, 1 mM MgCl₂, 125 mM NaCl, 1% Triton-X-100, 1 mM PMSF, 10 µg/ml of leupeptin, 10 µg/ml of aprotinin, 50 mM NaF, 25 mM β-glycerophosphate, 100 mM Na₃VO₄) were incubated with anti-p38 MAPK antibody and protein A-Sepharose beads overnight at 4°, then the beads were washed three times with 1 ml of ice-cold immunoprecipitation buffer and the bound immune complex assayed for p38 kinase activity at 30° for 30 min in 20 µl of kinase reaction buffer [25 mM HEPES, pH 7.4, 20 mM MgCl₂, 0.1 mM Na₃VO₄, 2 mM DTT, 50 µg/ml of myelin basic protein (MBP), 100 µM ATP and 10 µCi

[γ-³²P]ATP]. The reaction was terminated with 5 µl 5 × Laemmli sample buffer, the products resolved by 15% SDS-PAGE gel electrophoresis, and the phosphorylated MBP visualized by autoradiography. A PhosphorImager (Molecular Dynamics) was used to quantify band intensity.

Statistical evaluation

Values are expressed as the mean ± standard error of the mean (SEM) of at least three experiments. The Student's *t*-test was used to assess the statistical significance of the differences; *P*-values less than 0.05 were considered statistically significant.

RESULTS

Inhibition of LPS-induced NO and PGE₂ formation by SB 203580

Incubation of cultured J774 macrophages with LPS (1 µg/ml) for 24 hr caused increases in nitrite and PGE₂ accumulation in the cell culture medium from 8 ± 2 µM to 40 ± 5 µM (*n* = 5) and from 250 ± 27 pg/ml to 2250 ± 370 pg/ml (*n* = 3), respectively. On simultaneous co-incubation with SB 203580, both responses were inhibited by SB 203580 in a concentration-dependent manner over the range of 0.1–10 µM, the 50% inhibitory concentration (IC₅₀) for the nitrite and PGE₂ responses being 1.0 ± 0.2 µM and 0.5 ± 0.2 µM, respectively (Fig. 1).

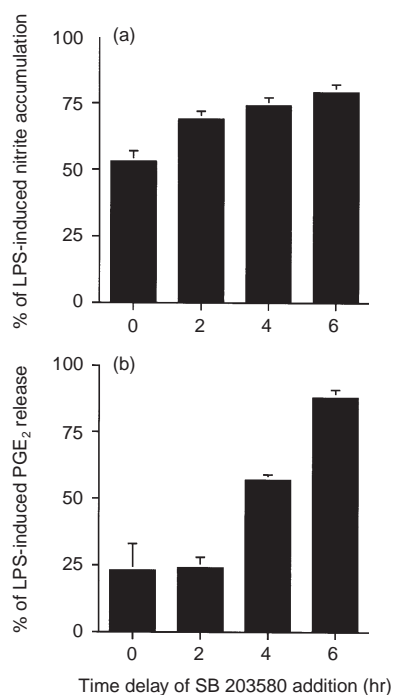


Figure 3. Time-dependent effects of SB 203580 on LPS-induced nitrite and PGE₂ production. SB 203580 (3 μ M) was added to the cell cultures at the same time as, or 2, 4 or 6 hr after, LPS (1 μ g/ml). Twenty-four hours after LPS addition, nitrite (a) and PGE₂ (b) production in the medium was determined. The data represents the mean \pm SEM of three experiments.

Inhibition of LPS-induced iNOS and COX-2 expression by SB 203580

As shown in Fig. 2a,b, co-incubation of SB 203580 (0.1–10 μ M) with 1 μ g/ml of LPS inhibited the LPS-mediated induction of iNOS and COX-2 proteins in a concentration-dependent manner. Northern blot analysis of the steady-state levels of iNOS and COX-2 mRNA showed that the induction of iNOS and COX-2 mRNA after LPS treatment for, respectively, 12 and 6 hr was clearly inhibited by the simultaneous addition of SB 203580 (3 μ M) (Fig. 2c,d). These results suggest an essential role for p38 MAPK at the transcriptional level of iNOS and COX-2 induction.

Transcriptional inhibition by SB 203580

To confirm the transcriptional role of p38 MAPK further, we added SB 203580 (3 μ M) at different intervals following treatment with LPS. As shown in Fig. 3, the inhibitory effects of SB 203580 on nitrite (Fig. 3a) and PGE₂ (Fig. 3b) formation decreased with time after delayed addition of SB 203580.

Because activation of transcription factors NF- κ B and AP-1 has been shown previously to be indispensable for induction of iNOS and COX-2 gene activation by various stimuli,^{25,26} we explored the possible role of p38 MAPK in the activation of NF- κ B and AP-1. Direct EMSA measurement of the activation of transcription factors revealed that the activation of NF- κ B and AP-1 by LPS (1 μ g/ml) was abolished by co-addition of SB 203580 (10 μ M) (Fig. 4a,b).

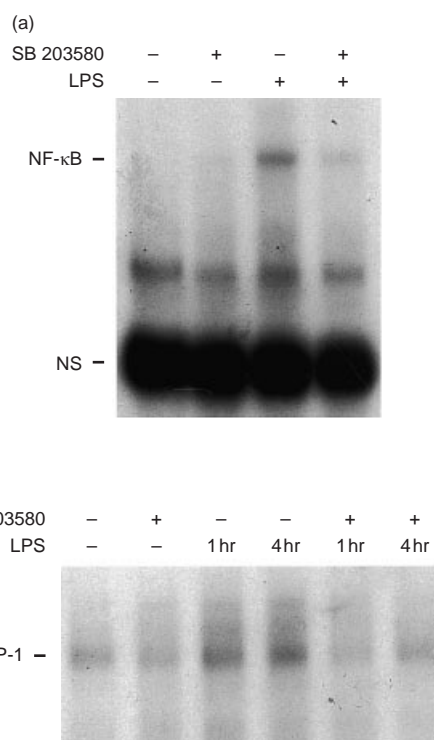


Figure 4. Effects of SB 203580 on LPS-induced transactivation of NF- κ B and AP-1. Cells were either untreated or preincubated with SB 203580 (10 μ M) for 20 min, then incubated with or without, LPS (1 μ g/ml) for 1 or 4 hr. NF- κ B activation at 1 hr (a) and AP-1 activation at either 1 or 4 hr (b) were determined by EMSA. Typical traces, representative of three experiments, are shown.

p38 MAPK activation by LPS

To determine whether p38 MAPK was directly responsive to LPS, J774 cells were treated with LPS (1 μ g/ml) for 5 min in the presence or absence of SB 203580 and the cytosolic lysates analysed by the immune complex kinase assay. As shown in Fig. 5, LPS was able to increase p38 MAPK activity by $61 \pm 7\%$ ($n=3$) at 5 min, and this effect was inhibited in a concentration-dependent manner by SB 203580.

DISCUSSION

Using a class of pyridinyl imidazoles, including SB 203580,¹³ that are highly specific p38 MAPK inhibitors, evidence has been accumulated that suggests p38 MAPK activation may be essential for some cellular responses associated with acute or chronic inflammation. In the present study, the role of p38 MAPK in LPS-elicited iNOS and COX-2 induction, leading to the generation of NO and PGE₂, was investigated in murine J774 macrophages.

From the results, several conclusions can be drawn regarding the involvement of p38 MAPK in the transcriptional regulation of macrophage iNOS and COX-2 induction by LPS. Firstly, SB 203580 over the range of 0.1–10 μ M potently attenuated the induction of the iNOS and COX-2 proteins, and this was related to its ability to inhibit p38 MAPK activity. Secondly, SB 203580 almost completely inhibited nitrite and PGE₂ formation when given together with LPS, but the inhibition diminished when the inhibitor was added 2–4 hr

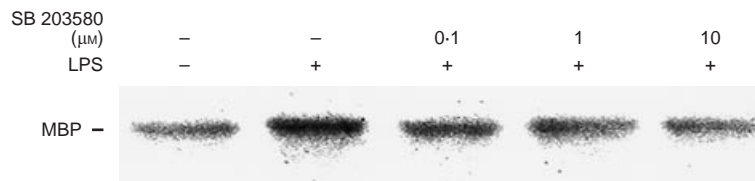


Figure 5. Inhibition of LPS-induced p38 MAPK activation by SB 203580. J774 cells were pretreated with vehicle or SB 203580 at the concentrations indicated; they were then treated with vehicle or 1 $\mu\text{g}/\text{ml}$ LPS for 5 min, extracted and subjected to the immune complex kinase assay using anti-p38 antibody. p38 activity was measured by phosphorylation of MBP as described in the Materials and Methods.

after stimulus induction, suggesting a role for p38 MAPK in the transcriptional processes of iNOS and COX-2 induction. To assess this possibility further, the effects of SB 203580 on the activation of the transcription factors NF- κ B and AP-1, which act, respectively, on the two key promoter regions of iNOS and COX-2 and are responsible for initiation of iNOS and COX-2 mRNA transcription in response to LPS,²⁵⁻²⁸ were studied; the fact that the LPS-induced DNA binding of both NF- κ B and AP-1 was attenuated by SB 203580 indicates a transcriptional role of p38 MAPK in iNOS and COX-2 induction. Thirdly, consistent with the ability of LPS to activate p38 MAPK in other cells, such as human neutrophils,⁷ B cells,⁶ murine RAW 264.7 macrophages,⁶ HeLa, Chinese hamster ovary and transfected COS-1 cell lines,³ LPS increased SB 203580-susceptible p38 MAPK activity in J774 macrophages.

In this study, we show, for the first time, that SB 203580 can inhibit LPS-mediated NF- κ B and AP-1 activation, suggesting a crucial role for a p38-dependent signalling mechanism upstream of the activation of both transcription factors. These findings are in contrast to those seen for TNF- α , the action of which, on NF- κ B activation in L929 cells, was not modulated by 10 μM SB 203580.⁶ Thus, it appears that multiple signalling cascades converge to elicit NF- κ B activation, depending on the stimuli and/or cell systems studied, and further studies are needed to link the action of p38 MAPK to NF- κ B and AP-1 activation.

Taken together, our data suggest that the p38 MAPK-NF- κ B/AP-1-iNOS/COX-2 cascade is an important signalling pathway involved in the regulation of nitric oxide and prostaglandin biosynthesis by bacterial endotoxin.

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