British Journal of Pharmacology (1998) 125, 193–201
http://www.stockton-press.co.uk/bjp

Cytokine induction of NO synthase II in human DLD-1 cells: roles of the JAK-STAT, AP-1 and NF-κB-signaling pathways

[1,4]Hartmut Kleinert, [1]Thomas Wallerath, [2]Gerhard Fritz, [1]Irmgard Ihrig-Biedert, [1]Fernando Rodriguez-Pascual, [3]David A. Geller & [1]Ulrich Förstermann

Departments of [1]Pharmacology and [2]Applied Toxicology, Johannes Gutenberg University, Obere Zahlbacher Strasse 67, D-55101 Mainz, Germany and [3]Department of Surgery, University of Pittsburgh, 497 Scaife Hall, Pittsburgh, Pennsylvania 15261, USA

1 In human epithelial-like DLD-1 cells, nitric oxide synthase (NOS) II expression was induced by interferon-γ (100 u ml^{-1}) alone and, to a larger extent, by a cytokine mixture (CM) consisting of interferon-γ, interleukin-1β (50 u ml^{-1}) and tumor necrosis factor-α (10 ng ml^{-1}).

2 CM-induced NOS II expression was inhibited by tyrphostin B42 (mRNA down to 1%; nitrite production down to 0.5% at 300 μM) and tyrphostin A25 (mRNA down to 24%, nitrite production down to 1% at 200 μM), suggesting the involvement of janus kinase 2 (JAK-2). Tyrphostin B42 also blocked the CM-induced JAK-2 phosphorylation (kinase assay) and reduced the CM-stimulated STAT1α binding activity (gel shift analysis).

3 CM reduced the nuclear binding activity of transcription factor AP-1. A heterogenous group of compounds, that stimulated the expression of c-fos/c-jun, enhanced the nuclear binding activity of AP-1. This group includes the protein phosphatase inhibitors calyculin A, okadaic acid, and phenylarsine oxide, as well as the inhibitor of translation anisomycin. All of these compounds reduced CM-induced NOS II mRNA expression (to 9% at 50 nM calyculin A; to 28% at 500 nM okadaic acid; to 18% at 10 μM phenylarsine oxide; and to 19% at 100 ng ml^{-1} anisomycin) without changing NOS II mRNA stability. In cotransfection experiments, overexpression of c-Jun and c-Fos reduced promoter activity of a 7 kb DNA fragment of the 5′-flanking sequence of the human NOS II gene to 63%.

4 Nuclear extracts from resting DLD-1 cells showed significant binding activity for transcription factor NF-κB, which was only slightly enhanced by CM. The NF-κB inhibitors dexamethasone (1 μM), 3,4-dichloroisocoumarin (50 μM), panepoxydone (5 μg ml^{-1}) and pyrrolidine dithiocarbamate (100 μM) produced no inhibition of CM-induced NOS II induction.

5 We conclude that in human DLD-1 cells, the interferon-γ–JAK-2-STAT1α pathway is important for NOS II induction. AP-1 (that is downregulated by CM) seems to be a negative regulator of NOS II expression. NF-κB, which is probably important for basal activity of the human NOS II promoter, is unlikely to function as a major effector of CM in DLD-1 cells.

Keywords: nitric oxide synthase II; janus kinase 2; STAT1α; AP-1; NF-κB

Introduction

High output NO production by the inducible isoform of nitric oxide synthase (NOS II) in macrophages and many other cell types appears to control the replication of intracellular bacteria, parasites and tumor cells. However, NOS II-derived NO can also convey inflammatory damage to host cells as has been documented for various inflammatory conditions (Kröncke *et al*., 1995), including inflammatory bowel disease (Singer *et al*., 1996). Therefore, we studied the mechanisms controling the expression of NOS II in the colon epithelial-derived cell line DLD-1.

Most human cells require a mixture of cytokines (CM) usually composed of interferon-γ (INF-γ) tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) for NOS II induction. Exposure of cells to INF-γ can result in the activation of protein tyrosine kinases such as the janus kinases JAK-1 and JAK-2 which phosphorylate and activate the signal transducer and activator of transcription 1α (STAT1α). The activated transcription factor STAT1α translocates into the nucleus and induces transcription of INF-γ regulated genes (Schindler & Darnell, 1995). Mutation of the INF-γ-activated site (GAS) in the *murine* NOS II promoter reduced INF-γ-induced promoter activity (Gao *et al*., 1997) and INF-γ-induced NOS II expression is paralleled by STAT1α activation in adult *rat* ventricular myocytes, cardiac microvascular endothelial cells and *rat* C6 glioma cells (Nishiya *et al*., 1997; Singh *et al*., 1996). JAK-2 is also likely to be involved in INF-γ-induced NOS II expression in *rat* glial cells (Kitamura *et al*., 1996) and herbimycin A has been shown to inhibit JAK-2 tyrosine phosphorylation and the subsequent NOS II expression in *rat* C6 glioma cells (Nishiya *et al*., 1995).

Signal transduction pathways activated by TNF-α and IL-1β are still somewhat controversial (Thanos & Maniatis, 1995). However, in many cell systems, transcription factor NF-κB is activated by these cytokines, and the known promoter sequence of the *human* NOS II gene contains several consensus sequences for the binding of NF-κB (Linn *et al*., 1997; Spitsin *et al*., 1996). Both cytokines have also been shown to stimulate activator protein 1 (AP-1) (Hattori *et al*., 1993; Jaattela *et al*., 1996). However, data generated with the *murine* NOS II promoter have indicated that AP-1 may be an inhibitory transcription factor for this gene (Lowenstein *et al*., 1993).

Therefore, the current study was designed to analyse the molecular mechanism involved in NOS II mRNA induction in *human* DLD-1 cells.

[4] Author for correspondence.

Methods

Reagents

Human INF-γ, IL1-β and TNF-α were purchased from PAN Systems. Actinomycin D, dexamethasone, 3,4-dichloroisocoumarin (DCI), phenylarsine oxide (PAO), protein A-Sepharose and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma, Deisenhofen, Germany. Tyrphostin A25 (AG82, α-cyano-[3,4,5-trihydroxy]cinnamonitrile), and tyrphostin B42 (AG490, α-cyano-[3,4-dihydroxy]-N-benzylcinnamide), okadaic acid (OA), and calyculin A were purchased from Calbiochem, Bad Soden, Germany. Isotopes were obtained from NEN/Dupont, Köln, Germany. Restriction enzymes, polynucleotide kinase, Taq polymerase, T3 and T7 RNA polymerase, dNTPs and oligo-dT primer were purchased from Pharmacia, Freiburg, Germany. RNase A, RNase T1 and DNase I were obtained from Boehringer Mannheim, Mannheim, Germany. Superscript reverse transcriptase was purchased from Gibco/BRL, Eggenstein, Germany. A rabbit polyclonal anti-JAK-2 antibody and a murine monoclonal anti-tyrosine phosphate antibody were purchased from Santa Cruz, Heidelberg, Germany. Human interferon-γ, tumor necrosis factor-α and interleukin-1β were purchased from PAN-Systems, Nürnberg, Germany. Panepoxydone (Erkel *et al*., 1996) was a generous gift of Dr T Anke, University of Kaiserslautern, Germany. The plasmids pCMV-jun and pCMV-fos were a gift from Dr T Curran, Dept. of Molecular Oncology, Nutely, NJ, U.S.A.

Cell culture, cytokine and inhibitor treatment, RNA isolation and measurement of NO production

Human DLD-1 cells (ATCC, #CCL-221; stable colon adenocarcinoma cell line) were grown in RPMI (Gibco/BRL) with 10% fetal bovine serum, 2 mM L-glutamine, penicillin and streptomycin. Eighteen hours before induction, the cells were washed and incubated with DMEM (PAN Systems) without phenol red containing 2 mM L-glutamine. For NOS II mRNA and NO production analyses, DLD-1 cells were induced for 8 h with the cytokines INF-γ, (100 u ml^{-1}), IL-1β (50 u ml^{-1}) and TNF-α (10 ng ml^{-1}) alone or in combination. In some experiments, anisomycin (1–100 ng ml^{-1}), calyculin A (1–50 nM), dexamethasone (0.01–1 μM), DCI (50 μM), OA (1–500 nM), PAO (0.1–25 μM), panepoxydone (0.05–5 μg ml^{-1}), PDTC (100 μM), tyrphostin A25 (2–200 μM) or tyrphostin B42 (2–350 μM) were present during the induction. For determination of the stability of the NOS II mRNA in the presence of OA or PAO, CM-induced cells were incubated with 10 μg ml^{-1} actinomycin D and PAO (10 μM) or OA (500 nM) for different periods of time. Total RNA was isolated from DLD-1 cells by guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski & Sacchi, 1987). Briefly, cells were lysed by adding 600 μl GIT-buffer (4 M guinidiniumiosthiocyanate, 25 mM sodium citrate pH 7.0, 0.5% laurylsarcosine, 0.1 M β-mercaptoethanol) per 30 mm tissue culture plate. The cells were scraped off with a rubber policeman, the lysates were transferred into Eppendorf tubes and 60 μl of sodium acetate (pH 4.0) was added. Then 600 μl of water-saturated phenol and 120 μl of chloroform was added. The solution was mixed and incubated for 10 min at 4°C. After a centrifugation at 13,000 × *g* for 10 min at 4°C, the upper aqueous phase was transfered to a new Eppendorf tube and the RNA was precipitated by adding 1 ml of 2-propanol. After an incubation at −20°C for 1 h , the RNA was collected by centrifuagtion at 13,000 × *g* for 20 min at 4°C. The pellet was washed twice with 70% ethanol, dried and dissolved in DEPC-treated water. NO_2^- concentrations in cell supernatants were determined by the Griess reaction (Green *et al*., 1982).

*Cloning of human c-*jun *and c-*fos *cDNA fragments*

C-*jun* and c-*fos* cDNA fragments were obtained by RT–PCR using the following oligonucleotide primers: ATGACTG-CAAAGATGGAAACGACC (sense) and TGCCCGTTG-CTGGACTGG (antisense) for c-*jun* (corresponding to positions 1261–1284 and 1503–1520 of the human c-*jun* gene (Hattori *et al*., 1988)), and GAGTCTGAGGAGGCCTT-CACCC (sense) and TCACAGGGCCAGCAGCGTG (antisense) for c-*fos* (representing positions 2886–2907 and 3311–3329 of the human c-*fos* gene (van Straaten *et al*., 1983)). The amplified cDNA fragments (c-*jun* 260 bp, c-*fos* 438 bp) were cloned into the *Eco*RV site of pCR-Script (Stratagene, Heidelberg, Germany) generating the cDNA-clones pCR_c-*jun*_human and pCR_c-*fos*_human. DNA sequences of the cloned PCR products were determined from plasmid templates using the dideoxy chain termination method with the ^{T7}Sequencing Kit (Pharmacia).

Preparation of antisense RNA probes

To generate radiolabeled human NOS II, β-actin, c-*jun*, c-*fos* or hCAT-1 antisense RNA probes for RNase protection assays, the plasmids pCR_NOS_II_human, pCR_β-actin_human, (Kleinert *et al*., 1996a), pCR_c-*jun*_human, pCR_c-*fos*_human or phCAT-1 (Albritton *et al*., 1992) were linearized with *Sma*I, *Bst*EII, *Nco*I, *Hind*III and *Nco*I, respectively, extracted with phenol/chloroform and concentrated by ethanol precipitation. One half of a microgram of this DNA was *in vitro* transcribed using T7 or T3 RNA polymerase (Pharmacia) and α-^{32}P-UTP. After a 1 h incubation, the template DNA was degraded with DNase I for 45 min. The radiolabeled RNA was purified using NucTrap probe purification columns (Stratagene).

RNase protection analyses

RNase protection assays were performed with a mixture of RNase A and RNase T1 as previously described (Kleinert *et al*., 1998; Kleinert *et al*., 1996b). Briefly, following denaturation, 20 μg of total RNA (prepared as described above) were hybridized with 200,000 c.p.m. labeled NOS II-, c-*jun*-, c-*fos*- or hCAT-1 antisense RNA probe and 20,000 c.p.m. labeled β-actin antisense RNA probe overnight, digested with RNase A and T1, and the products analysed by electrophoresis on denaturing urea-polyacrylamide gels (8 M urea, 6% PAGE). Densitometric analyses were performed using a PhosphoImager (BioRad, München, Germany). The protected DNA fragments of NOS II, β-actin, c-*jun*, c-*fos* and hCAT-1 were 386 nt, 108 nt, 260 nt, 199 nt and 205 nt, respectively.

Immunoprecipitation using an antibody against JAK-2

DLD-1 cells were incubated with CM or CM with tyrphostin B42 for 5–30 min at 37°C. Cells were scraped and lysed in 1 ml RIPA-buffer (10 mM Tris-HCl, pH 8; 140 mM NaCl; 1% Triton; 1% desoxycholate; 0.1% SDS) containing 10 μg ml^{-1} pepstatin, 10 μg ml$^-$ leupeptin, 20 u ml^{-1} aprotinin, 0.2 mM phenylmethylsulfonyl fluoride and 1 mM Na_3VO_4. Three milligrams of protein A-Sepharose (preincubated with BSA/low fat milk powder) and 2 μg antibody were added and the

lysates were incubated at 4°C overnight. The lysates were centrifuged (14,000 r.p.m., 4°C) and the pellets were washed with RIPA buffer containing 1 mM Na_3VO_4. After a second centrifugation the pellets were washed with TEM (10 mM Tris-HCl, pH 8; 0.1 mM EDTA, 1 mM $MgCl_2$) containing 1 mM Na_3VO_4. After a third centrifugation, the immunoprecipitated proteins were dissolved in Laemmli sample buffer.

Immunoblot assay with a monoclonal anti-phosphotyrosine antibody

Western blotting was performed as described (Kleinert *et al*., 1996a). Briefly, protein samples were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE, 7.5%). The proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by electroblotting (BioRad). Blots were blocked for 30 min with 3%, w/v, low fat milk powder, 0.05%, v/v, Tween 20 and 10% goat serum in TBS (10 mM Tris-HCl pH 7.4, 150 mM NaCl, w/v) at room temperature and then incubated for phosphotyrosine detection with a monoclonal mouse anti-phosphotyrosine antibody (Santa Cruz, dilution 1 : 200) in TBS containing 1%, w/v, BSA and 0.1%, w/v, Tween 20 overnight at 4°C. After washing in TBS/low fat milk powder/Tween, the blots were incubated with horseradish peroxidase-conjugated second antibodies diluted 1 : 1500 in TBS/low fat milk powder/Tween for 30 min. The blots were washed stepwise with TBS/low fat milk powder/Tween, TBS/Tween, and TBS alone. Immunocomplexes were developed using an enhanced horseradish peroxidase/luminol chemiluminescence reagent (NEN/Dupont) according to the manufacturer's instruction.

Electrophoretic mobility shift assays (gelshift assays)

Binding activities of AP-1 and STAT1α in the nuclei of DLD-1 cells was determined by electrophoretic mobility shift assays using the Promega (Promega, Mannheim, Germany) gel shift assay system. Nuclear proteins were extracted from DLD-1 cells by detergent lysis (Osborn *et al*., 1989). Ten μg of nuclear protein were incubated with 17.5 fmol ^{32}P-labeled double stranded oligonucleotide containing a consensus STAT1α binding motif (5′-CATGTTATGCAT**ATTCCTGTAAG**TTG-3′; Santa Cruz) or an AP-1 binding motif (5′-CGCTTGAT**GACTCA**GCCGGAA-3′; Santa Cruz). Specificity of binding was determined by adding excess (1.75 pmol) unlabeled oligonucleotide. DNA-protein complexes were analysed on 5% polyacrylamide gels (buffer 6.7 mM Tris/HCl, pH 7.5; 3.3 mM Na acetate; 1 mM EDTA). The gels were dried and autoradiographed on X-ray film or a Phospho-Imager screen (BioRad).

Cotransfections and reportergene assay

DLD-1 cells were plated in (1.5×10^5 cells per 30 mm cell culture dish) 24 h before transfection. The cells were transfected by lipofection with DOTAP according to the manufacturer's recommendations (Boehringer Mannheim). The following plasmids were cotransfected: (i) 2.5 μg of plasmid pXP2 (Nordeen, 1988), containing a promoterless luciferase reportergene, (ii) plasmid pNOS2(7.0)Luc (de Vera *et al*., 1996), containing a 7 kb DNA fragment of the 5′-flanking sequence of the human NOS II gene cloned into pXP2, and 2.5 μg pCMV-*jun* and 2.5 μg pCMV-*fos*, containing the full length *rat* c-*jun* or c-*fos* cDNAs under the control of a cytomegalovirus (CMV) enhancer/promoter, (iii) plasmid pNOS2(7.0)Luc and 5 μg pRc-CMV, containing the CMV enhancer/promoter alone (In-vitrogen, NV Leek, The Netherlands). Two point five micrograms of pCH110 (Pharmacia, containing the β-galactosidase gene driven by an SV40 promoter) were cotransfected in all cases for normalization. The cells were washed with culture medium 18 h after transfection and incubated in medium for an additional 24 h. Then extracts (200 μl) were prepared using the reporter lysis buffer (Promega). The luciferase and β-galactosidase activities of the extracts were determined using the Luciferase Assay System (Promega) and the Galacto-Light System (Tropix/PE Applied Biosystems, Weiterstadt, Germany) as described (Kleinert *et al*., 1996a). The light units (LU) of the luciferase assay were normalized by the LU of the β-galactosidase assay after subtraction of extract background.

Statistical analyses

For statistical analyses of data, ANOVA followed by Fisher's PLSD test was used.

Results

INF-γ alone induced submaximal NOS II mRNA expression in DLD-1 cells which was enhanced by TNF-α and IL-1β

Quantitative RNase protection analysis detected maximal expression of NOS II mRNA in DLD-1 cells incubated with a cytokine mixture (CM, 100%, $n=12$) consisting of 100 μ ml^{-1} INF-γ, 50 u ml^{-1} IL-1β and 10 ng ml^{-1} TNF-α. Virtually no NOS II mRNA was found in non-induced DLD-1 cells ($4.5 \pm 1.4\%$, $n=12$), or DLD-1 cells incubated with 10 ng ml^{-1} TNF-α ($0.1 \pm 0.1\%$, $n=12$) or 50 u ml^{-1} IL-1β ($2.5 \pm 1.3\%$, $n=12$; Figure 1) alone or in combination ($5.3 \pm 2.7\%$; $n=12$). Incubation with 100 u ml^{-1} INF-γ, however, resulted in a significant NOS II mRNA expression ($33.7 \pm 5.7\%$, $n=12$). Induced NOS activity mirrored the NOS II mRNA levels. INF-γ alone stimulated $26.7 \pm 7.9\%$ of the maximal NO_2^- production induced by CM ($100\% = 4.0 \pm 0.2$ μM nitrite, $n=12$, non-induced control cells 0.22 ± 0.05 μM nitrite, $n=12$).

The specific JAK-2 protein tyrosine kinase inhibitor tyrphostin B42 inhibited NOS II mRNA expression, JAK-2 phosphorylation and CM-induced enhancement of STAT1α binding activity in nuclear extracts of DLD-1 cells

Incubation of CM-induced DLD-1 cells with tyrphostin B42 (synonymous with compound AG490), a specific inhibitor of the INF-γ-activated JAK-2 protein tyrosine kinase (Meydan *et al*., 1996), and the protein tyrosine kinase inhibitor tyrphostin A25 inhibited NOS II mRNA expression in a concentration-dependent manner (Figure 2). Both inhibitors alone did not induce any NOS II mRNA ($n=3$, data not shown). Similarly, nitrite production of CM-induced DLD-1 cells was inhibited to $0.5 \pm 0.4\%$ ($n=7$) by tyrphostin B42 (300 μM) and to $1.4 \pm 1.2\%$ by tyrphostin A25 (200 μM) ($n=7$).

CM-incubation of DLD-1 cells resulted in tyrosine-phosphorylation of JAK-2, which was inhibited by tyrphostin B42 as shown by immunoprecipitation/Western-blot analyses (Figure 3a). CM-incubation also resulted in an enhancement of STAT1α binding activity in nuclear extracts of DLD-1 cells (Figure 3b). This binding activity was also prevented by tyrphostin B42 (Figure 3b).

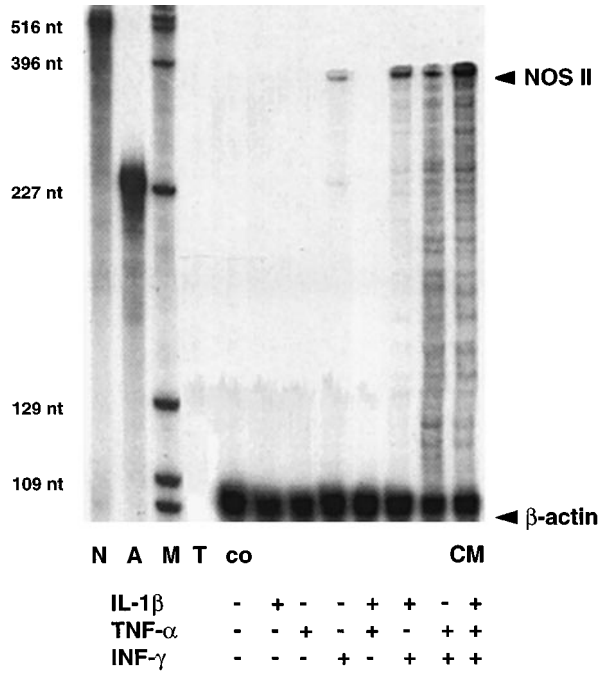

Figure 1 Cytokine-induced expression of NOS II mRNA in DLD-1 cells. RNase protection analyses were performed using antisense RNA probes for human NOS II and β-actin (for standardization). RNAs were prepared from untreated DLD-1 cells (co) or cells treated with 50 u ml^{-1} interleukin-1β (IL-1β), 10 ng ml^{-1} tumor necrosis factor-α (TNF-α), and 100 u ml^{-1} interferon-γ (INF-γ), alone or in combination (CM = mixture of these three cytokines). (N = NOS II antisense probe; A = β-actin antisense probe; M = molecular weight standard, pGl_2-Basic restricted with *Hinf*I; T = tRNA). The gel is representative of twelve similar gels. Results of densitometric analyses of these twelve gels (means ± s.e.mean) are given in the text.

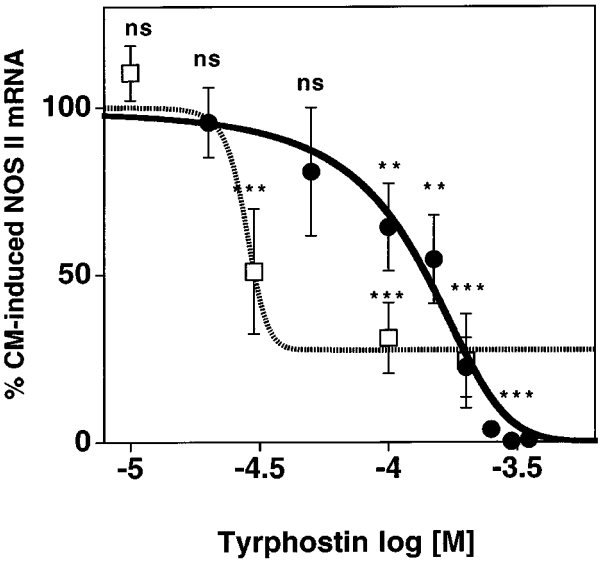

Figure 2 Inhibition of CM-induced NOS II mRNA expression in DLD-1 cells by the protein tyrosine kinase (PTK) inhibitors tyrphostin A25 and B42. RNase protection analyses using antisense RNA probes for human NOS II and β-actin (for standardization). Each data point represent the results of densitometric analyses of seven different gels (means ± s.e.mean). RNAs were prepared from DLD-1 cells stimulated with a cytokine mixture (CM, see Figure 1) in the presence of the indicated concentrations of tyrphostin B42 (—●—) or tyrphostin A25 (–□–); **, $P < 0.01$ vs CM; ***, $P < 0.001$ vs CM; ns: not significantly different from CM.

*Compounds that induced the expression c-*fos *and c-*jun*, and/or enhanced the DNA binding of transcription factor AP-1 inhibited CM-induced NOS II induction*

Incubation of DLD-1 cells with the serine/threonine phosphatase inhibitors calyculin A or okadaic acid (OA), inhibited the CM-induced expression of NOS II mRNA (Figure 4a). Similarly, nitrite production of CM-induced DLD-1 cells (3.8 ± 0.4 μM nitrite, $n = 6$) was inhibited to $9.5 \pm 2.3\%$ ($n = 6$) by calyculin A (50 nM) and to $17.5 \pm 4.1\%$ ($n = 6$) by okadaic acid (500 nM). Both compounds markedly enhanced c-*jun* and c-*fos* mRNA expression in CM-induced DLD-1 cells (Figure 4b) as described for other cell models (Haby *et al*., 1994; Park *et al*., 1992). In actinomycin D experiments (see Materials and methods), the half-life of NOS II mRNA ranged from 7.8–9.9 h ($n = 3$) under control conditions and from 8.3–12.8 h ($n = 3$) in the presence of 500 nM OA.

Also the inhibitor of tyrosine phosphatases, PAO (Garcia *et al*., 1990), reduced the CM-induced NOS II mRNA induction

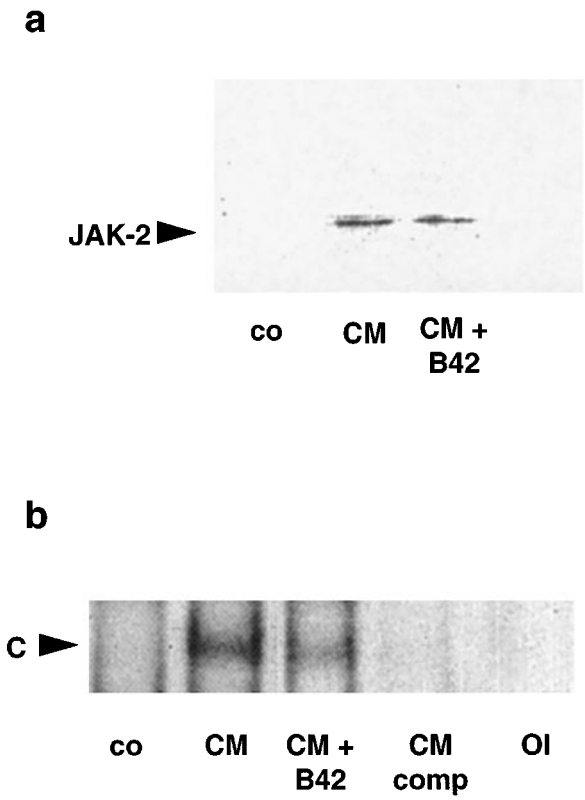

Figure 3 Induction of janus kinase 2 (JAK-2) phosphorylation and enhanced STAT1α binding activity in nuclear extracts of DLD-1 cells stimulated with CM. Panel a shows immunoprecipitation/Western blot analyses using a rabbit polyclonal anti-JAK-2 antibody for immunoprecipitation and a murine monoclonal anti-tyrosine phosphate antibody for immunoblotting. Extracts for immunoprecipitation were prepared from untreated DLD-1 cells (co) or cells incubated for 5 min with a cytokine mixture (CM) in the absence or presence of 200 μM tyrphostin B42 (CM + B42). The blot shown is representative of three blots yielding similar results. Panel b shows gelshift experiments using a radiolabeled oligonucleotide containing a consensus STAT1α binding site and nuclear extracts from untreated DLD-1 cells (co) or nuclear extracts from CM-treated cells incubated for 2 h in the absence (CM) or presence of 200 μM tyrphostin B42 (CM + B42). (CM comp: competition of the CM-induced signal with 50-fold excess of unlabeled oligonucleotide; OI: radiolabeled oligonucleotide alone; C: DNA-protein complex). The gel is representative of three gels showing similar results.

in a concentration-dependent fashion (Figure 4a). The nitrite production of CM-induced cells was reduced to 1.5% ± 1.5% ($n=6$) by PAO (10 μM). PAO also enhanced c-*fos* and c-*jun* mRNA-expression (Figure 4b). In actinomycin D experiments, 10 μM PAO even increased the half-life of NOS II mRNA from 7.8 – 9.9 h under control conditions to 17.1 – 19.4 h after PAO ($n=3$).

Anisomycin reduced NOS II induction in a concentration-dependent manner (Figure 4a). Also, nitrite production of CM-induced DLD-1 cells was inhibited to 3.5 ± 1.3% ($n=6$) by anisomycin (100 ng ml^{-1}). Anisomycin enhanced c-*fos* and c-*jun* mRNA expression (Figure 4b) as in other cell models (Messina, 1990). NOS II induction was inhibited about 80% with 100 ng ml^{-1} anisomycin (Figure 4a), a concentration reported not to inhibit protein synthesis (Oguchi *et al*., 1994). Conversely, cycloheximide, another inhibitor of translation, did not prevent NOS II mRNA induction in concentrations up to 10 μg ml^{-1}, at lower concentrations (1 μg ml^{-1}) even enhanced NOS II mRNA expression ($n=6$, not shown). Cycloheximide did not induce c-*fos* or c-*jun* mRNA expression ($n=6$, data not shown).

Calyculin A, OA, PAO and anisomycin did not change the mRNA expression of an unrelated gene, the human L-arginine transporter hCAT-1 ($n=3$, data not shown).

Gelshift experiments demonstrated decreased AP-1 binding activity of nuclear extracts of DLD-1 cells after CM-incubation (Figure 5). The phosphatase inhibitors PAO (10 μM) or calyculin A (50 nM) increased AP-1 binding activity (Figure 5).

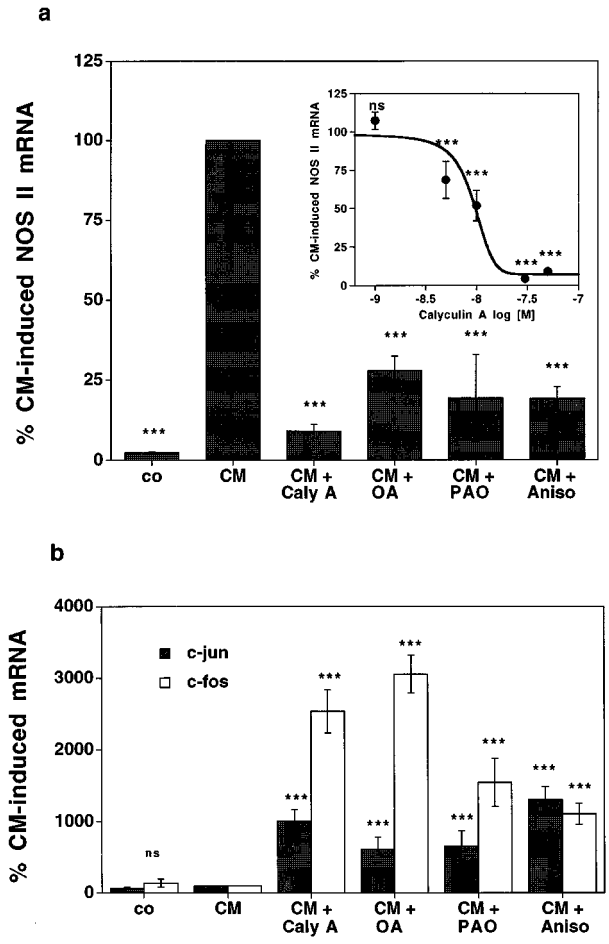

Figure 4 Inhibition of CM-induced NOS II mRNA expression in DLD-1 cells by the c-*fos*/c-*jun*-inducing compounds calyculin A, okadaic acid, phenylarsine oxide and anisomycin. Panel a shows RNase protection analyses preformed with antisense RNA probes for human NOS II and β-actin (for standardization). The figure shows densitometric analyses of six different gels (means ± s.e.mean). RNAs were prepared from untreated DLD-1 cells (co) or cells stimulated with a cytokine mixture (CM, see Figure 1). Concentration-inhibition curves were generated with 1 – 50 nM calyculin A (Caly A, —●—, inset), 1 – 500 nM okadaic acid (OA), 0.1 – 10 μM phenylarsine oxide (PAO), or 1 – 100 ng ml^{-1} anisomycin (Aniso). Columns represent the relative NOS II mRNA levels at the highest concentration of the respective inhibitor. Panel b depicts the induction of c-*fos* and c-*jun* mRNAs by the same maximum concentrations of the inhibitors; ***, $P<0.001$ vs CM; ns: not significantly different from CM.

*Cotransfection of c-*jun *and c-*fos *expression plasmids reduced the promoter activity of a 7 kb promoter fragment of the human NOS II gene*

Transient transfection of DLD-1 cells with pNOS2(7.0)Luc (de Vera *et al*., 1996) resulted in a significant luciferase activity of the extracts of transfected cells. This activity was not significantly enhanced by CM-induction of the transfected cells ($n=6$, data not shown).

Cotransfection of expression plasmids containing the full length c-*jun* (pCMV-*jun*) and c-*fos* (pCMV-*fos*) cDNAs under the control of the CMV enhancer/promoter significantly reduced the luciferase activity in pNOS2(7.0)Luc transfected cells (Figure 6).

Lack of involvement of NF-κ*B in cytokine induction of NOS II in DLD-1 cells*

Co-incubation of CM-treated DLD-1 cells with four chemically distinct inhibitors of NF-κB activation, namely PDTC (up to 100 μM), DCI (up to 50 μM), dexamethasone (up to 1 μM) (Kleinert *et al*., 1996a) and panepoxydone (up to

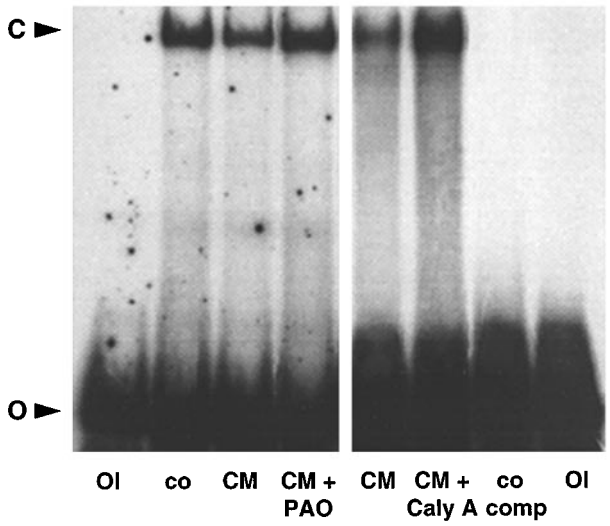

Figure 5 Stimulation of AP-1 binding activity in nuclear extracts of DLD-1 cells by the c-*fos*/c-*jun*-inducing agents phenylarsine oxide (PAO) and calyculin A (Caly A). Gelshift experiments using a radiolabeled oligonucleotide containing a consensus AP-1 binding site and nuclear extracts from untreated DLD-1 cells (co) or cells stimulated with a cytokine mixture (CM, see Figure 1) with or without 10 μM PAO, or 50 nM calyculin A. To determine the specificity of the oligonucleotide-AP-1-protein complex, nuclear extracts of untreated cells were incubated with the radiolabeled oligonucleotide and a 50-fold excess of unlabeled oligonucleotide (co comp). (Ol: radiolabeled oligonucleotide alone; C: DNA-protein complex; O: free oligonucleotide). The gels shown are representative for three gels showing similar results.

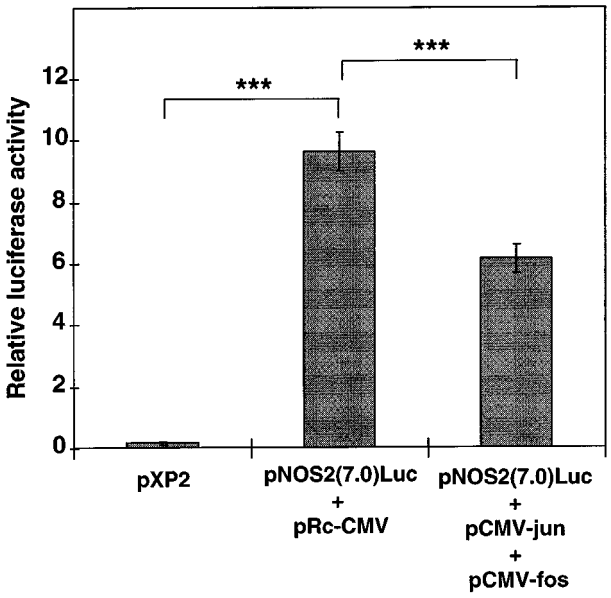

Figure 6 Inhibition of the promoter activity of a 7 kb DNA-fragment of the 5′-flanking sequence of the human NOS II gene by cotransfection of c-*jun*/c-*fos*. Human DLD-1 cells were transfected with pXP2 (containing a promoterless luciferase gene) or *pNOS2(7.0)Luc* (containing a 7 kb NOS II promoter fragment cloned before the luciferase gene). pCMV-jun and pCMV-fos (containing full length cDNA fragments of *rat* c-*jun* and c-*fos* under the control of the CMV enhancer/promoter, respectively) or pRc-CMV (containing only the CMV enhancer/promoter) and pCH110 (containing the β-galactosidase gene) were cotransfected. After overnight incubation with the DNA/DOTAP mixture, the cells were further incubated for 24 h. Then, the cells were lysed and light units determined. The relative luciferase activity (corrected for β-galactosidase activity) was taken as a measure of NOS II promoter activity. Bars represent means $\pm$ s.e.mean of six independent experiments (***, $P<0.001$).

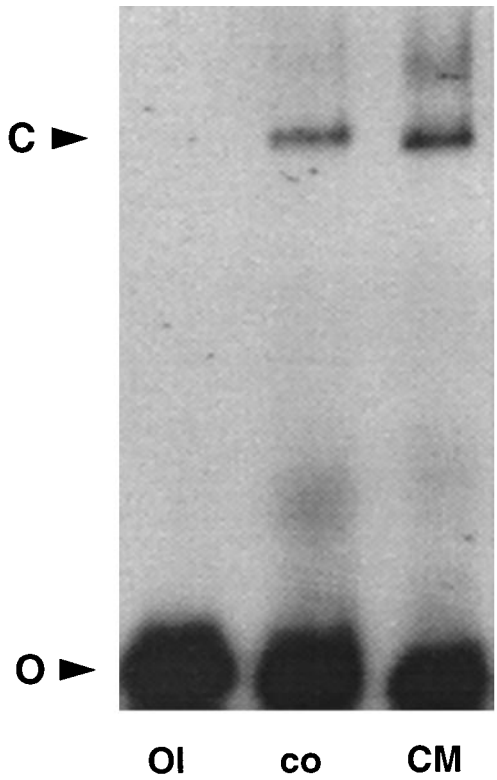

Figure 7 NF-κB-binding activity of nuclear extracts of untreated and CM-stimulated DLD-1 cells. Gelshift experiments using a radiolabeled oligonucleotide containing a consensus NF-κB binding site and nuclear extracts from untreated DLD-1 cells (co), or DLD-1 cells stimulated with a cytokine mixture (CM, see Figure 1) (Ol: radiolabeled oligonucleotide alone; C: DNA-protein complex; O: free oligonucleotide). The gel is representative of four gels showing similar results.

5 μg ml^{-1}) (Erkel *et al*., 1996) did not block CM-induced NOS II mRNA expression (Table 1).

Gelshift experiments demonstrated that DLD-1 cells contained high basal levels of activated NF-κB which were only slightly enhanced by CM (Figure 7).

Discussion

Human cells generally require a cytokine mixture typically consisting of INF-γ, IL-1β, and TNF-α for NOS II induction (Kleinert *et al*., 1996a; Sherman *et al*., 1993). However, human chondrocytes (Charles *et al*., 1993) and human primary hepatocytes (Geller *et al*., 1995) can be stimulated with IL-1β alone to express significant levels of NOS II. In human DLD-1 cells (Jin *et al*., 1996; Kleinert *et al*., 1998) (Figure 1), INF-γ alone can produce about 30% (both in terms of NOS II mRNA and NOS activity) of the maximal rate induced by CM (TNF-α and IL-1β alone or in combination were ineffective). However, in all cases, expression is further enhanced by the other cytokines mentioned. Therefore, transcription factors induced by INF-γ alone are responsible for at least 30% of the transcription of the human NOS II gene in DLD-1 cells. For maximal transcription, additional transcription factors regulated by TNF-α and IL-1β seem to be required. The human NOS II promoter contains consensus sequences for the binding of transcription factors, including INF-γ regulatory factor-1 (IRF-1), STAT binding to GAS, AP-1, NF-κB, and others (Linn *et al*., 1997; Spitsin *et al*., 1996).

Table 1 Effect of inhibitors of NF-κB-activation on NOS II mRNA expression induced by a cytokine mixture (CM) consisting of 50 u ml^{-1} interleukin-1β, 10 ng ml^{-1} tumor necrosis factor-α, and 100 u ml^{-1} interferon-γ

	NOS II mRNA expression (% of CM)	
Treatment of cells	*Mean*	*s.e.m.*
Untreated	0.43	0.31
CM (INF-γ, TNF-α, IL 1β	100.00	–
CM + 100 μM pyrrolidine dithiocarbamate	106.12	20.71
CM + 50 μM 3,4-dichloroisocoumarin	118.24	17.81
CM + 1 μM dexamethasone	95.11	9.20
CM + 5 μg ml^{-1} panepoxydone	108.21	18.89

Values represent the results of densitometric analyses of eight different RNase protection assays. NOS II mRNA expression is shown relative to CM-induced NOS II mRNA expression. RNAs were prepared from untreated DLD-1 cells and cells stimulated with CM in the absence and presence of the NF-κB inhibitors indicated. None of the inhibitors produced any significant change in NOS II mRNA levels.

Incubation of cells with cytokines or growth factors usually results in tyrosine-phosphorylation of different proteins including the cytokine- or growth factor receptors themselves. Protein tyrosine kinase activity may be intrinsic to activated receptors (for example the PDGF- or EGF receptors). Other

receptors, such as cytokine receptors, are phosphorylated by non-receptor protein tyrosine kinases (Heldin, 1995). In both kinds of receptors, tyrosine phosphorylation is essential for dimerization and the subsequent signal transduction. In the present study, incubation of CM-induced DLD-1 cells with the protein tyrosine kinase inhibitors tyrphostin B42 and A25 inhibited NOS II mRNA expression in a concentration-dependent manner (Figure 2). Tyrphostin B42 has been described as a specific JAK-2 inhibitor (Meydan *et al*., 1996). JAK-2 is a member of the protein tyrosine kinase family activated by INF-γ, the essential cytokine for NOS II induction in DLD-1 cells. After INF-γ incubation, JAK-2 is tyrosine-phosphorylated and tyrosine-phosphorylates and activates STAT1α. Activated STAT1α then translocates to the nucleus and binds to GAS elements in INF-γ regulated genes. As shown in Figure 3a, CM-incubation induced tyrosine-phosphorylation of JAK-2 which was inhibited by tyrphostin B42. Figure 3b demonstrates that CM-incubation went along with an enhancement of STAT1α binding activity in nuclear extracts of DLD-1 cells. This binding activity was also prevented by tyrphostin B42. Therefore, as described for rodent cells (Gao *et al*., 1997; Kitamura *et al*., 1996; Nishiya *et al*., 1997; Singh *et al*., 1996) the INF-γ-JAK-2-STAT1α-pathway seems to be an important pathway for NOS II induction in human DLD-1 cells.

Deletion of the AP-1 binding site at position -1062 of the *murine* NOS II promoter leads to increased promoter activity in *murine* macrophages (Lowenstein *et al*., 1993). This result indicates a negative role of the transcription factor AP-1 in the regulation of the NOS II transcription. Serine/threonine phosphatase inhibitors like calyculin A and OA have been shown to enhance the expression of c-*fos* and c-*jun* mRNA (coding for the two components of transcription factor AP-1) (Haby *et al*., 1994; Park *et al*., 1992). In addition, OA has been found to increase binding activity of this transcription factor (Haby *et al*., 1994; Park *et al*., 1992). OA has previously been shown to prevent NOS II induction in *murine* macrophages (Dong *et al*., 1995; Singh *et al*., 1996). In our DLD-1 cell model, calyculin A and OA inhibited the CM-induced expression of NOS II mRNA (Figure 4a) and enhanced c-*jun* and c-*fos* mRNA expression (Figure 4b).

Also the inhibitor of tyrosine phosphatases, PAO (Garcia *et al*., 1990), reduced the CM-induced NOS II mRNA induction in a concentration-dependent fashion (Figure 4a) and also increased c-*jun* and c-*fos* mRNA (Figure 4b). OA and PAO did not reduce the stability of the NOS II mRNA in DLD-1 cells. Therefore, reduced transcription is the most likely cause of the inhibitory effects of the phosphatase inhibitors (shown in Figure 4).

Anisomycin, an inhibitor of translation, has also been described to enhance c-*jun* and c-*fos* expression and/or AP-1 binding activity (Messina, 1990). This compound reduced NOS II induction in a concentration-dependent manner (Figure 4a) and enhanced (as expected) c-*jun* and c-*fos* mRNA expression (Figure 4b).

Subsequent gelshift experiments demonstrated that the phosphatase inhibitors also increased AP-1 binding activity of nuclear extracts of DLD-1 cells (Figure 5). Interestingly, the gelshift experiments also showed decreased AP-1 binding activity after CM (Figure 5) which is consistent with an inhibitory role of this transcription factor.

The promoter activity of a 7 kb human NOS II promoter fragment transfected into DLD-1 cells was not significantly enhanced by CM-induction. This is in accordance with data by Linn *et al*. (1997), who also found no CM-induction of a similar 7 kb human NOS II promoter fragment transfected into DLD-1 cells. However, AKN cells transfected with the same NOS II promoter luciferase construct and incubated with CM displayed a fivefold induction of luciferase activity (de Vera *et al*., 1996). Therefore, the human NOS II promoter seems to be regulated differently in different cells.

Cotransfection of expression plasmids containing the full length c-*jun* (pCMV-*jun*) and c-*fos* (pCMV-*fos*) cDNAs under the control of the CMV enhancer/promoter reduced the luciferase activity in pNOS2(7.0)Luc transfected cells (Figure 6). Thus, overexpression of c-Jun and c-Fos (AP-1) in DLD-1 cells resulted in a reduction of promoter-activity of the cotransfected 7 kb NOS II promoter. Therefore, the transfected human NOS II promoter fragment was negatively regulated by AP-1, as was the endogenous NOS II mRNA (Figure 4).

NF-κB has been shown to be functionally important for the activity of the NOS II promoter in *murine* (Xie *et al*., 1994) and human cells (Kleinert *et al*., 1996a; Nunokawa *et al*., 1996). However, different inhibitors of NF-κB activation (PDTC, DCI, dexamethasone and panepoxydone) did not block CM-induced NOS II mRNA expression in DLD-1 cells (Table 1). The same inhibitors have previously been found to block NOS II induction in A549/8 cells (Kleinert *et al*., 1996a), thereby demonstrating their effectiveness. Gelshift experiments then showed that DLD-1 cells contained high basal levels of activated NF-κB which were only slightly enhanced by CM (Figure 7). Since NOS II was not pre-induced in our DLD-1 cells (*c.f*. Figure 1) and cytokines did not enhance the levels of NF-κB in any significant way, NF-κB is unlikely to act as an important effector of CM-stimulated NOS II induction in these cells. This is at variance with other human cells, such as A549/8 (Kleinert *et al*., 1996a) or AKN-1 cells (Geller *et al*., 1997), in which cytokine induction of NF-κB resulted in enhanced NOS II expression. Interestingly, OA can activate NF-κB (Menon *et al*., 1995; Natarajan *et al*., 1996), but the compound still inhibited NOS II expression in DLD-1 cells, obviously due to other mechanisms. Nevertheless, NF-κB is not irrelevant for the expression of human NOS II because a 3-bp mutation of the NF-κB binding site of the human NOS II promoter (positions -115 to -106) markedly reduced promoter activity (Nunokawa *et al*., 1996). Therefore, in DLD-1 cells, the role of NF-κB may be that of a 'basal' transcription factor of the NOS II promoter (similar to the 'TATA box'). Apparently, the levels of active NF-κB remaining in the presence of PDTC and other inhibitors (which do not block completely) may still be sufficient to drive 'basal' promoter activity.

In conclusion, we have demonstrated that CM-induction of DLD-1 cells activated JAK-2 kinase and STAT1α DNA binding. Both these effects, and the induction of NOS II, was inhibited by the JAK inhibitor tyrphostin B42. Thus, the INF-γ-JAK-2-STAT1α pathway seems to be an important activator of NOS II transcription in DLD-1 cells. Cytokines downregulated AP-1 thereby producing disinhibition of NOS II transcription. Compounds enhancing AP-1 expression/activity inhibited NOS II expression. Therefore, transcription factor AP-1 seems to be a negative regulator of NOS II expression. DLD-1 cells contained high basal levels of activated NF-κB (not significantly enhanced by cytokine incubation) and different inhibitors of NF-κB activation did not block CM-induced NOS II mRNA expression. Therefore, NF-κB is unlikely to act as an important effector of CM-stimulated NOS II induction in these cells.

This work was supported by Grant 8312-38 62 61 from the Innovation Foundation of the State of Rhineland-Palatinate (to HK and UF) and by SFB 553, Project A1 (to UF) from the Deutsche Forschungsgemeinschaft, Bonn, Germany. Fernando Rodriguez-Pascual is the recipient of a FEBS Long-Term Fellowship. The expert technical assistance of Bärbel Hering and Kerstin Masch is gratefully acknowledged. The plasmids pCMV-*jun* and pCMV-*fos* were generous gifts of Dr T. Curran, Dept. of Molecular Oncology, Nutely, NJ, U.S.A.

References

ALBRITTON, L.M., BOWCOCK, A.M., EDDY, R.L., MORTON, C.C., TSENG, L., FARRER, L.A., CAVALLI SFORZA, L.L., SHOWS, T.B. & CUNNINGHAM, J.M. (1992). The human cationic amino acid transporter (ATRC1): physical and genetic mapping to 13q13-q14. *Genomics,* **12,** 430–434.

CHARLES, I.G., PALMER, R.M.J., HICKERY, M.S., BAYLISS, M.T., CHUBB, A.P., HALL, V.S., MOSS, D.W. & MONCADA, S. (1993). Cloning, characterization, and expression of a cDNA encoding an inducible nitric oxide synthase from the human chondrocyte. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 11419–11423.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* **162,** 156–159.

DE VERA, M.E., SHAPIRO, R.A., NÜSSLER, A.K., MUDGETT, J.S., SIMMONS, R.L., MORRIS, JR. S.M., BILLIAR, T.R. & GELLER, D.A. (1996). Transcriptional regulation of human inducible nitric oxide synthase (NOS2) gene by cytokines: initial analysis of the human NOS2 promoter. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 1054–1059.

DONG, Z.Y., YANG, X.L., XIE, K.P., JUANG, S.H., LLANSA, N. & FIDLER, I.J. (1995). Activation of inducible nitric oxide synthase gene in murine macrophages requires protein phosphatases 1 and 2A activities. *J. Leukocyte Biol.,* **58,** 725–732.

ERKLE, G., ANKE, T. & STERNER, O. (1996). Inhibition of NF-κB activation by panepoxydone. *Biochem. Biophys. Res. Commun.,* **226,** 214–221.

GAO, J., MORRISON, D.C., PARMELY, T.J., RUSSELL, S.W. & MURPHY, W.J. (1997). An interferon-gamma-activated site (GAS) is necessary for full expression of the mouse iNOS gene in response to interferon-gamma and lipopolysaccharide. *J. Biol. Chem.,* **272,** 1226–1230.

GARCIA, M.P., MINAMI, Y., LUONG, E., KLAUSNER, R.D. & SAMELSON, L.E. (1990). Tyrosine phosphorylation in T cells is regulated by phosphatase activity: studies with phenylarsine oxide. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 9255–9259.

GELLER, D.A., DE VERA, M.E., RUSSELL, D.A., SHAPIRO, R.A., NUSSLER, A.K., SIMMONS, R.L. & BILLIAR, T.R. (1995). A central role for IL-1 beta in the in vitro and in vivo regulation of hepatic inducible nitric oxide synthase. IL-1 beta induces hepatic nitric oxide synthesis. *J. Immunol.,* **155,** 4890–4898.

GELLER, D.A., MELLOTT, J., TAYLOR, B.S., NICK, H.S., BILLIAR, T.R. & CHESROW, S.E. (1997). Transcriptional regulation of the human inducible nitric oxide synthase (iNOS) gene. *Jap. J. Pharmacol.,* **75** (suppl 1), 6P.

GREEN, L.C., WAGNER, D.A., GLOGOWSKI, J., SKIPPER, P.L., WISHNOK, J.S. & TANNENBAUM, S.R. (1992). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal. Biochem.,* **126,** 131–138.

HABY, C., AUNIS, D. & ZWILLER, J. (1994). Okadaic acid induces activator protein 1 activity and immediate early gene transcription in rat pheochromocytoma cells. Mechanism of action. *Biochem. Pharmacol.,* **418,** 819–825.

HATTORI, K., ANGEL, P., LE BEAU, M.M. & KARIN, M. (1988). Structure and chromosomal localizatioan of the functional intronless human JUN protooncogene. *Proc. Natl. Acad. Sci. U.S.A.,* **85,** 9148–9152.

HATTORI, M., TUGORES, A., WESTWICK, J.K., VELOZ, L., LEFFERT, H.L., KARIN, M. & BRENNER, D.A. (1993). Activation of activating protein 1 during hepatic acute phase response. *Am. J. Physiol.,* **264,** G95–G103.

HELDIN, C.H.. (1995). Dimerizatiaon of cell surface receptors in signal transduction. *Cell,* **80,** 213–223.

JAATTELA, M., MOURITZEN, H., ELLING, F. & BASTHOLM, L. (1996). A20 zinc finger protein inhibits TNF and IL-1 signaling. *J. Immunol.,* **156,** 1166–1173.

JIN, Y., HECK, D.E., DEGEORGE, G., TIAN, Y. & LASKIN, J.D. (1996). 5-fluorouracil suppresses nitric oxide biosynthesis in colon carcinoma cells. *Cancer Res.,* **56,** 1978–1982.

KITAMURA, Y., TAKAHASHI, H., NOMURA, Y. & TANIGUCHI, T. (1996). Possible involvement of Janus kinase Jak2 in interferon-gamma induction of nitric oxide synthasein rat glial cells. *Eur. J. Pharmacol.,* **306,** 297–306.

KLEINERT, H., EUCHENHOFER, C., FRITZ, G., IHRIG-BIEDERT, I. & FÖRSTERMANN, U. (1989). Involvement of protein kinases in the induction of NO synthase II in human DLD-1 cells. *Brit. J. Pharmacol.,* **123,** 1716–1722.

KLEINERT, H., EUCHENHOFER, C., IHRIG-BEIDERT, I. & FÖRSTERMANN, U. (1996a). Glucocorticoids inhibit the induction of nitric oxide synthase II by down-regulating cytokine-induced activity of transcription factor nuclear factor-κB. *Mol. Pharmacol.,* **49,** 15–21.

KLEINERT, H., EUCHENHOFER, C., IHRIG-BIEDERT, I. & FÖRSTERMANN, U. (1996b). In murine 3T3 fibroblasts, different second messenger pathways resulting in the induction of NO synthase II (iNOS) converge in the activation of transcription factor NF-κB. *J. Biol. Chem.,* **271,** 6039–6045.

KRÖNCKE, K.D., FEHSEL, K. & BOLB-BACHOFEN, V. (1995). Inducible nitric oxide synthase and its product nitric oxide, a small molecule with complex biological activities. *Biol. Chem. Hoppe Seyler,* **376,** 327–343.

LINN, S.C., MORELLI, P.J., EDRY, I., COTTONGIM, S.E., SZABO, C. & SALZMAN, A.L. (1997). Transcriptional regulation of human inducible nitric oxide synthase gene in an intestinal epithelial cell line. *Am. J. Physiol.,* **272,** G1499–G1508.

LOWENSTEIN, C.J., ALLEY, E.W., RAVAL, P., SNOWMAN, A.M., SNYDER, S.H., RUSSEL, S.W. & MURPHY, W.J. (1993). Macrophage nitric oxide synthase gene – two upstream regions mediate induction by interferon-gamma and lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 9730–9734.

MENON, S.D., GUY, G.R. & TAN, Y.H. (1995). Involvement of a putative protein-tyrosine phosphatase and I kappa B-alpha serine phosphorylation in nuclear factor kappa B activation by tumor necrosis factor. *J. Biol. Chem.,* **270,** 18881–18887.

MESSINA, J.L. (1990). Insulin's regulation of c-*fos* gene transcription in hepatoma cells. *J. Biol. Chem.,* **265,** 11700–11705.

MEYDAN, N., GRUNBERGER, T., DADI, H., SHAHAR, M., ARPAIA, E., LAPIDOT, Z., LEEDER, J.S., FREEDMAN, M., COHEN, A., GAZIT, A., LEVITZKI, A. & ROIFMAN, C.M. (1996). Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor. *Nature,* **379,** 645–648.

NATARAJAN, K., SINGH, S., BURKE, JR. T.R., GRUNBERGER, D. & AGGARWAL, B.B. (1996). Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 9090–9095.

NISHIYA, T., UEHARA, T., EDAMATSU, H., KAZIRO, Y., ITOH, H. & NOMURA, Y. (1997). Activation of Stat1 and subsequent transcription of inducible nitric oxide synthase gene in C6 glioma cells is independent of interferon-gamma-induced MAPK activation that is mediated by p21ras. *FEBS Lett.,* **408,** 33–38.

NISHIYA, T., UEHARA, T. & NOMURA, Y. (1995). Herbimycin a suppresses NF-κB activation and tyrosine phosphorylation of JAK2 and the subsequent induction of nitric oxide synthase in c6 glioma cells. *FEBS Lett.,* **371,** 333–336.

NORDEEN, S.K. (1988). Luciferase reporter gene vectors for analysis of promoters and enhancers. *Biotechniques,* **6,** 454–458.

NUNOKAWA, Y., OIKAWA, S. & TANAKA, S. (1996). Human inducible nitric oxide synthase gene is transcriptionally regulated by nuclear factor-κB dependent mechanism. *Biochem. Biophys. Res. Commun.,* **223,** 347–352.

OGUCHI, S., WEISZ, A. & ESUMI, H. (1994). Enhancement of Inducible-type NO synthase gene transcription by protein synthesis inhibitors – activation of an intracellular signal transduction pathway by low concentrations of cycloheximide. *FEBS Lett.,* **338,** 326–330.

OSBORN, L., KUNKEL, S. & NABEL, G.J. (1989). Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc. Natl. Acad. Sci. U.S.A.,* **86,** 2336–2340.

PARK, K., CHUNG, M. & KIM, S.J. (1992). Inhibition of myogenesis by okadaic acid, an inhibitor of protein phosphatases, 1 and 2A, correlates with the induction of AP1. *J. Biol. Chem.,* **267,** 10810–10815.

SCHINDLER, C. & DARNELL, J.E. (1995). Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu. Rev. Biochem.,* **64,** 621–651.

SHERMAN, P.A., LAUBACH, V.E., REEP, B.R. & WOOD, E.R. (1993). Purification and cDNA sequence of an inducible nitric oxide synthase from a human tumor cell line. *Biochemistry,* **32,** 11600–11605.

SINGER, I.I., KAWKA, D.W., SCOTT, S., WEIDNER, J.R., MUMFORD, R.A., RIEHL, T.E. & STENSON, W.F. (1996). Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. *Gastroenterology,* **111,** 871–885.

SINGH, K., BALLINGAND, J.L., FISCHER, T.A., SMITH, T.W. & KELLY, R.A. (1996). Regulation of cytokine-inducible nitric oxide synthase in cardiac myocytes and microvascular endothelial cells – role of extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2) and STAT1 alpha. *J. Biol. Chem.,* **271,** 1111–1117.

SPITSIN, S.V., KOPROWSKI, H. & MICHAELS, F.H. (1996). Characterization and functional analysis of the human inducible nitric oxide synthase gene promoter. *Mol. Medicine,* **2,** 226–235.

THANOS, D. & MANIATIS, T. (1995). NF-kappa B: a lesson in family values. *Cell,* **80,** 529–532.

VAN STRAATEN, F., MULLER, R., CURRAN, T., VAN BEVEREN, C. & VERMA, I.M. (1983). Complete nucleotide sequence of a human c-onc gene: deduced amino acid sequence of the human c-fos protein. *Proc. Natl. Acad. Sci. U.S.A.,* **80,** 3183–3187.

XIE, Q.W., KASHIWABARA, Y. & NATHAN, C. (1994). Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J. Biol. Chem.,* **269,** 4705–4708.

(Received April 15, 1998
Revised June 9, 1998
Accepted June 19, 1998)