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Involvement of protein kinases in the induction of NO synthase II in human DLD-1 cells

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1 Protein phosphorylation is involved in the induction of nitric oxide synthase II (NOS II, iNOS) in several types of animal cells. Here we have investigated the possible involvement of major protein kinases in the induction of NOS II expression in human DLD-1 cells.

2 In DLD-1 cells, interferon-γ alone induced a submaximal NOS II expression; a cytokine mixture consisting of interferon-γ, tumour necrosis factor-α and interleukin-1β produced maximal NOS II induction.

3 Activators of protein kinase A (forskolin, 8-dibutyryl-cyclic AMP), of protein kinase C (tetradecanoylphorbol-13-acetate), and of protein kinase G (8-bromo cyclic GMP) did not induce NOS II mRNA by themselves, nor did they alter NOS II mRNA induction in response to cytokines.

4 Inhibitors of protein kinase A (compound H89), of protein kinase C (bisindolylmaleimide, chelerythrine or staurosporine), of phosphatidylinositol 3-kinase (wortmannin), of p38 mitogen-activated protein kinase (compound SB 203580) and of extracellular signal-regulated kinase (compound PD 98059) also had no influence on basal or cytokine-induced NOS II mRNA expression.

5 Immunoprecipitation kinase assays showed no activation of extracellular signal-regulated kinase or p38 mitogen-activated protein kinase in cytokine-incubated DLD-1 cells. The c-Jun NH_2-terminal kinase was activated by cytokines, but the most efficacious cytokine was tumour necrosis factor-α which did not induce NOS II by itself.

6 In contrast, the protein tyrosine kinase inhibitor tyrphostin B42 (a specific inhibitor of interferon-γ-activated janus kinase 2) and the protein tyrosine kinase inhibitor tyrphostin A25 both reduced CM-induced NOS II mRNA expression in a concentration-dependent manner.

7 These results suggest that activation of NOS II expression in DLD-1 cells is independent of the activities of protein kinases A, C and G, phosphatidylinositol 3-kinase, extracellular signal regulated kinase and p38 mitogen-activated protein kinase, but seems to require protein tyrosine kinase activity, especially the interferon-γ-activated janus kinase 2.

Keywords: Nitric oxide synthase II; protein kinases; tyrphostins; janus kinase 2

Introduction

The expression of the inducible isoform of nitric oxide synthase (NOS II, iNOS) is regulated mainly at the level of transcription (Förstermann & Kleinert, 1995). Most human cells require a complex mixture of cytokines, usually composed of interferon-γ (INF-γ), tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), for NOS II induction. However, in human chondrocytes (Charles *et al*., 1993) and human DLD-1 epithelial-like cells (Jin *et al*., 1996) NOS II expression can be stimulated with only IL-1β or INF-γ, respectively. In both cell types, NOS II expression is further enhanced by the other cytokines mentioned.

Protein phosphorylation plays a cardinal role in regulating signal transduction pathways in eucaryotes. These processes are reversibly controlled by protein kinases and protein phosphatases (Hunter, 1995). The involvement of protein kinases in the signal transduction pathways leading to NOS II expression seems to differ markedly between cell types and species.

Activators of protein kinases A, C and G (PKA, PKC and PKG) have been shown to induce or enhance NOS II expression in various cell systems (Eberhardt *et al*., 1994; Geng *et al*., 1994; Jun *et al*., 1994; Marumo *et al*., 1995; Kleinert *et al*., 1996b). However, in rat RINm5F insulinoma cells, lipophilic adenosine 3′:5′-cyclic monosphosphate (cyclic AMP) analogues inhibited NOS II expression (Messmer & Brüne, 1994).

Wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase (PI3-kinase), inhibited NOS II induction in chick isolated macrophages (Yang *et al*., 1996) and in rat mesangial cells (Donaldson *et al*., 1996). In contrast, in human colonic HT29 cells, the inhibition of NOS II induction by IL-13 has been attributed to an enhanced PI3-kinase activity (Wright *et al*., 1997).

Cytokines can stimulate signal cascades involving the activation of mitogen activated protein kinases (MAP kinases, MAPKs). MAPKs are a family of protein kinases divided into different subgroups. In mammals, the extracellular signal-regulated kinases (ERKs) are probably the best-studied subgroup (Davis, 1994). ERKs are activated by dual specificity MAPK-kinases in response to diverse stimuli. These MAPK-kinases are specifically inhibited by compound PD 98059 (Alessi *et al*., 1995). This compound has recently been shown to block IL-1β-induced NOS II expression in rat cardiac microvascular endothelial cells (Singh *et al*., 1996). Other members of the MAP kinase family (p38 MAP kinase and c-Jun NH_2-terminal kinase; JNK) are also activated by cytokines and may be involved in NOS II induction (Davis, 1994).

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In many cell types analysed, protein tyrosine kinases are involved in NOS II induction and induction is inhibited by protein tyrosine kinase inhibitors such as genistein, herbimycin A or tyrphostins (Feinstein *et al*., 1994; Geng *et al*., 1995; Kong *et al*., 1996).

The large majority of the studies mentioned above has been performed in animal cells. Therefore, the current study was designed to investigate, in human DLD-1 epithelial-like cells, the effect of activators and inhibitors of all major protein kinases on the induction of NOS II mRNA. In cases where specific kinase inhibitors were not available, we tested whether the respective kinases were activated by the cytokines stimulating NOS II expression. The experiments showed that PKA, PKC, PKG, MAPK and PI3-kinase are unlikely to play a role for the signal transduction leading to NOS II mRNA expression in DLD-1 cells. On the other hand, protein tyrosine phosphorylation (most probably by janus kinase 2, JAK-2) is likely to be involved in cytokine induction of NOS II in DLD-1 cells.

Methods

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

Human epithelial-like colon carcinoma DLD-1 cells (ATCC) were grown in RPMI (Gibco) with 10% foetal bovine serum, 2 mM L-glutamine, penicillin and streptomycin (Kleinert *et al*., 1996a). Eighteen hours before cytokine induction, the cells were washed and the medium was changed to DMEM containing 2 mM L-glutamine, but no phenol red (phenol red can interfere withe Griess assay, see below). For NOS II mRNA and NO_2^- 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 (Kleinert *et al*., 1996a). In the different series of experiments, 8-bromo-cyclic GMP (1 mM), bisindolylmaleimide I (1 μM), chelerythrine (2 μM), chrysin (2.5 to 50 μM), dibutyryl-cyclic AMP (100 μM), forskolin (100 μM), compound H89 (10 μM), compound PD 98059 (0.5 to 50 μM), compound SB 203580 (5 μM), staurosporine (10 to 100 nM), TPA (50 ng ml^{-1}), tyrphostins A25 and B42 (10 to 200 μM) or wortmannin (0.5 to 500 nM) were present during the 8 h induction period. In other experiments, some of these agents were added to the cells in the absence of cytokines. Total RNA was isolated from DLD-1 cells by guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski & Sacchi, 1987). NO synthase activity was determined by measuring NO_2^- concentrations in the supernatant of the cells by the Griess reaction (Green *et al*., 1982; Kleinert *et al*., 1996b).

Preparation of DNA and antisense RNA probes

To generate DNA probes for S1-nuclease protection analyses, the cDNA-clones pCR-NOS II-Hu and pCR-β-actin-Hu (Kleinert 1996a), containing human NOS II- and β-actin-cDNA fragments, respectively, were restricted with *Sma*I and *Sty* I, respectively. They were dephosphorylated (calf intestinal alkaline phosphatase, CIAP, Boehringer-Mannheim), extracted with phenol/chloroform and concentrated by ethanol precipitation. Fifty nanograms of this DNA was labelled with γ-[^{32}P]-ATP by use of polynucleotide kinase (PNK, Pharmacia). The radiolabelled DNA was separated from unincorporated radioactivity by use of NucTrap probe purification columns (Stratagene).

To generate radiolabelled antisense RNA probes for RNase protection assays, pCR-NOS II-Hu and pCR-β-actin-Hu were linearized with *Sma* I or *Bst* II, extracted with phenol/chloroform and concentrated by ethanol precipitation. One half of a microgram of this DNA was *in vitro* transcribed by use of 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 radiolabelled RNA was purified with NucTrap probe purification columns (Stratagene).

S1-nuclease protection analyses and RNase protection analyses

S1-nuclease protection analyses were performed as previously described (Kleinert & Benecke, 1988; Kleinert *et al*., 1996a). Briefly, after denaturation at 85°C for 30 min, 20 μg of total RNA (prepared as described above) were hybridized at 52°C for 16 h with 150,000 c.p.m. labelled NOS II DNA probe and 30,000 c.p.m. labelled β-actin DNA probe in hybridization buffer (40 mM PIPES pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% formamide) in a volume of 30 μl. The S1-nuclease digestion was started by adding 310 μl of digesting buffer (280 mM NaCl, 4.5 mM $Zn(CH_3COO^-)_2$, pH 4.5, 30 mg ml^{-1} denatured salmon sperm DNA, and 300 u ml^{-1} S1-nuclease). After 20 min at 37°C, the reaction was stopped by adding 65 μl stop-buffer (2.5 M NH_4-acetate, 50 mM EDTA), followed by a phenol/chloroform extraction. The reaction products were concentrated by ethanol precipitation and analysed by electrophoresis in denaturing urea-polyacrylamide gels (8 M urea, 6% PAGE). The electrophoresis buffer was 1 × TBE (1.08% Tris, pH 8.3, 0.55% boric acid, and 20 mM EDTA). The gels were electrophoresed for 2–3 h, dried and exposed to X-ray films. Densitometric analyses were performed with a Phospho-Imager (BioRad). The protected DNA fragments of NOS II and β-actin were 380 nt and 110 nt, respectively.

RNase protection assays were performed with a mixture of RNase A and RNase T1 according to the manufacturer's instructions (Boehringer Mannheim). Briefly, following denaturation, 20 μg of total RNA (prepared as described above) were hybridized with 200,000 c.p.m. labelled NOS II antisense RNA probe and 20,000 c.p.m. labelled β-actin antisense RNA probe at 51°C for 16 h in a volume of 40 μl hybridization buffer (40 mM PIPES, pH 6.7, 1 mM EDTA, 400 mM NaCl, 50% formamide). Then the mixture was digested by adding 300 μl digestion buffer (10 mM Tris/HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA) containing 3.5 mg RNase A and 37.5 u RNase T1 for 30 min at 30°C. The reaction was stopped by proteinase K digestion (70 mg/sample in 70 μl 7.15 mM Tris/HCl, pH 7.4, 7.15 mM EDTA, 2.85% SDS; 15 min at 37°C) and phenol extraction. The reaction products were concentrated by ethanol precipitation and analysed by electrophoresis on denaturing urea-polyacrylamide gels (8 M urea, 6% PAGE). The electrophoresis buffer was 1 × TBE (1.08% Tris, pH 8.3, 0.55% boric acid, and 20 mM EDTA). The gels were electrophoresed for 1–2 h, dried and exposed to X-ray films. Densitometric analyses were performed with a Phospho-Imager (BioRad). The protected DNA fragments of NOS II, and β-actin were 386 nt and 108 nt, respectively.

Immune-complex kinase assay for extracellular signal-regulated kinases 2 (ERK2), p38 mitogen activated protein kinase (MAPK) and c-Jun NH_2-terminal kinase (JNK)

ERK2-, p38 MAP kinase- and JNK activities were determined by immune complex kinase assays by use of kinase-specific

rabbit polyclonal antibodies (Santa Cruz) for immunoprecipitation. Briefly, phosphorylation reactions were performed in 40 μl kinase buffer for 30 min at 30°C in the presence of specific substrates (see below). For the ERK2 kinase assay, the buffer consisted of 20 mM HEPES, pH 7.1; 10 mM $MgCl_2$; 1 mM Na_3VO_4; 25 μM ATP; 1 μCi of γ-[^{32}P]-ATP. For the p38 MAPK and JNK assays, the buffer consisted of 25 mM HEPES, pH 7.6; 20 mM $MgCl_2$; 20 mM β-glycerolphosphate; 0.1 mM Na_3VO_4; 2 mM DTT; 25 μM ATP and 1 μCi of γ-[^{32}P]-ATP. In the ERK2 assay, 1 μg of myelin basic protein (Sigma) was used as the substrate. For the JNK assay, 1 μg of GST-Jun (1/166) was used as the substrate. The p38 MAPK assay was performed with 1 μg of GST-ATF-2 as the substrate. The kinase reactions were terminated by addition of Laemmli sample buffer (Laemmli, 1970) and heating (5 min, 95°C). After separation by SDS-PAGE (10%), the gels were dried and subjected to autoradiography.

Reagents

Human INF-γ, IL-1β, and TNF-α were purchased from PAN-Systems. 8-Bromo-cyclic GMP, dibutyryl-cyclic AMP, chrysin, forskolin, tetradecanoylphorbol-13-acetate (TPA), as well as protein A-Sepharose were purchased from Sigma. Bisindolylmaleimide I, chelerythrine, staurosporine, 2′-amino-3′-methoxyflavone (compound PD 98059), 4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)1H-imidazole (compound SB 203580), tyrphostins A25 and B42, N-(2-[{*p*-bromocinnamyl}amino]ethyl)-5-isoquinolinesulphonamide (compound H89) and wortmannin were purchased from Calbiochem. Isotopes were obtained from NEN/Dupont. Restriction enzymes, polynucleotide kinase, Taq polymerase, T3 and T7 RNA polymerase, dNTPs and oligo-dT primer were purchased from Pharmacia. RNase A, RNase T1 and DNase I were obtained from Boehringer Mannheim. Superscript reverse transcriptase was purchased from Gibco/BRL. Rabbit polyclonal anti-JNK-, anti-p38 MAP kinase-, and anti-ERK2 antibodies was purchased from Santa Cruz.

Statistical analyses

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

Results

Cytokines inducing NOS II in DLD-1 cells

Quantitative S1- or RNase protection analyses detected no NOS II mRNA in untreated DLD-1 cells or in DLD-1 cells incubated for 8 h with 10 ng ml^{-1} TNF-α or 50 u ml^{-1} IL-1β alone or in combination ($n \geqslant 10$, data not shown). However, incubation with 100 u ml^{-1} INF-γ resulted in a significant NOS II mRNA expression (Figure 1). Incubation of DLD-1 cells with a triple cytokine mixture (CM) consisting of IL-1β, TNF-α TNF-α and INF-γ (at the concentrations mentioned above) resulted in maximal NOS II expression (Figure 1). INF-γ alone produced about 30% of this maximal NOS II mRNA induction.

No NO_2^- production was measurable in the supernatant of non-induced DLD-1 cells (detection limit 100 nM NO_2^-). DLD-1 cells treated for 8 h with CM generated an NO_2^- concentration of 3.7$\pm$0.2 μM (mean$\pm$s.e.mean, n=10) in the supernatant at the end of this period of time. INF-γ alone also stimulated about 30% (1.1$\pm$0.1 μM NO_2^-) of the NO_2^- production induced by CM, whereas IL-1β and TNF-α alone had no measurable effect.

PKA, PKC, PKG, ERK2, p38 MAP kinase, JNK, and PI3-kinase are unlikely to be involved in the signal transduction pathways resulting in NOS II mRNA induction

Activation of PKA with forskolin (100 μM) or dibutyryl-cyclic AMP (100 μM) did not result in NOS II mRNA expression in

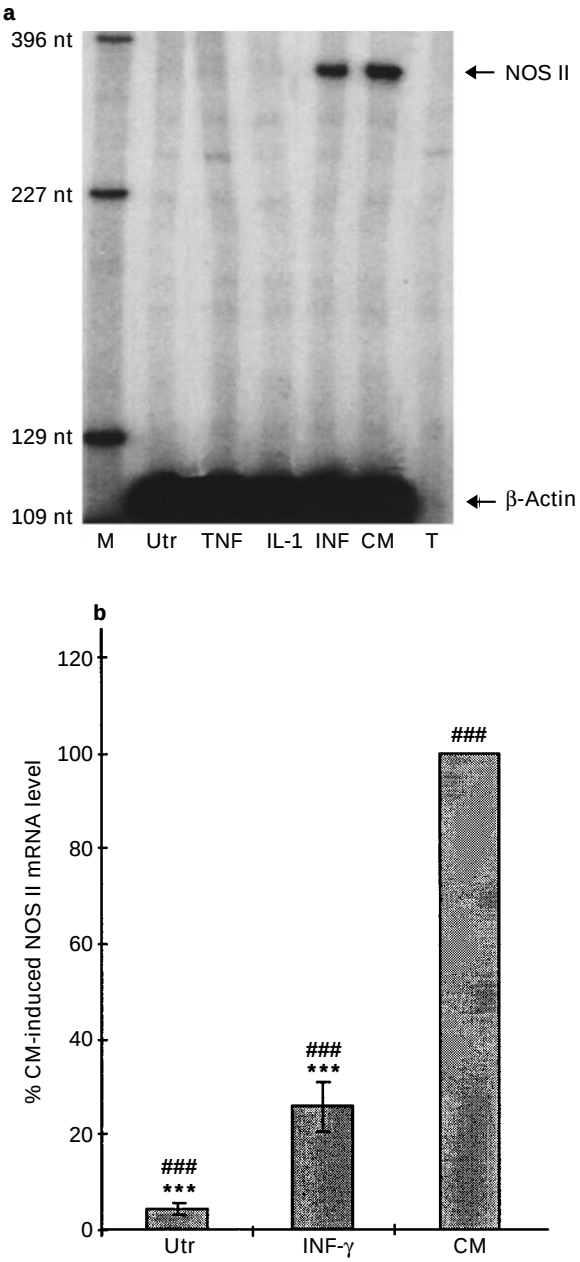

Figure 1 S1 nuclease protection analyses using antisense probes to human NOS II and β-actin (for standardization). RNAs were prepared from untreated DLD-1 cells (Utr), cells treated with 10 ng ml^{-1} tumour necrosis factor-α (TNF), 50 u ml^{-1} interleukin-1β (IL-1), 100 u ml^{-1} interferon-γ (INF), or a cytokine mixture (CM) consisting of 100 u ml^{-1} interferon-γ, 50 u ml^{-1} interleukin-1β and 10 ng ml^{-1} tumour necrosis factor-α. (a) An autoradiograph of a representative gel (T=tRNA; M=molecular weight standard; pG1$_2$-Basic restricted with *Hinf* I; N=NOS II antisense probe; A=β-actin antisense probe). (b) Densitometric analyses (means$\pm$s.e.mean) of 6 different gels. **P<0.01 vs CM; ***P<0.001 vs CM; ###P<0.001 vs Utr).

DLD-1 cells. Furthermore, neither the PKA activators forskolin and dibutyryl-cyclic AMP nor the PKA inhibitor compound H89 (10 μM) modified the CM-induced NOS II mRNA expression (Figure 2). The same concentrations of forskolin and cyclic AMP analogues have previously been shown to induce NOS II mRNA expression in murine 3T3 fibroblasts (Kleinert *et al*., 1996b).

Activation of PKC with TPA (50 ng ml^{-1}) or inhibition of the kinase with bisindolylmaleimide I (1 μM), chelerythrine (2 μM), or staurosporine (100 nM) did not modify CM-induced NOS II mRNA expression in DLD-1 cells (Figure 3). However, in previous experiments, the same concentration of TPA produced a marked NOS II mRNA induction in murine 3T3 fibroblasts (Kleinert *et al*., 1996b).

Incubation of DLD-1 cells with 1 mM 8-bromo-cyclic GMP did not induce NOS II mRNA expression by itself and also did not modify CM-induced NOS II mRNA expression (Figure 4).

CM incubation of DLD-1 cells did not activate ERK as measured by a ERK2 immune-complex kinase assay with a specific anti-ERK2 antibody ($n=3$, data not shown). Also compound PD 98059, a specific inhibitor of MAPK-kinase (which activates ERK) did not induce NOS II mRNA in DLD-1 cells and also had no effect on CM-induced NOS II mRNA expression (Figure 5). In p38 MAP immune-complex kinase assays with a specific anti-p38 MAP kinase antibody, no difference in the p38 MAP kinase activity could be detected between untreated and CM-induced DLD-1 cells ($n=3$, data not shown). Also incubation of CM-induced cells with 5 μM of the p38 MAP kinase inhibitor compound SB 203580 did not modify NOS II mRNA induction (Figure 5). Incubation of DLD-1 cells with wortmannin, a specific PI3-kinase inhibitor, also had no effect on basal or CM-induced NOS II expression (Figure 5).

JNK immune-complex kinase assays with a specific anti-JNK antibody demonstrated an activation of JNK activity in extracts from CM incubated DLD-1 cells (Figure 6a). When the single cytokine components of CM were tested for their JNK-activating effect, TNF-α proved to be the most efficacious cytokine (Figure 6b). IL-1β and INF-γ gave much smaller signals (Figure 6b) ($n=3$).

Inhibition of cytokine-induced NOS II mRNA expression by the protein tyrosine kinase inhibitors tyrphostins A25 and B42

Incubation of DLD-1 cells with tyrphostin B42, a specific inhibitor of INF-γ-activated JAK2, and the protein tyrosine kinase inhibitor tyrphostin A25 did not induce any NOS II

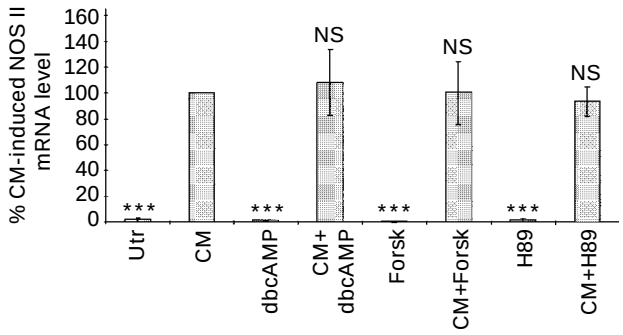

Figure 2 RNase protection analyses with antisense RNA probes to human NOS II and β-actin (for standardization). Columns represent the results of densitometric analyses of four different gels (means $\pm$s.e.mean). RNAs were prepared from untreated DLD-1 cells (Utr) or cells stimulated with a cytokine mixture (CM, see Figure 1). Non-induced cells and CM-induced cells were treated with 100 μM dibutyryl-cyclic AMP (dbcAMP), 100 μM forskolin (Forsk) or 10 μM compound H89. ***$P<0.001$ vs CM; NS: not significantly different from CM.

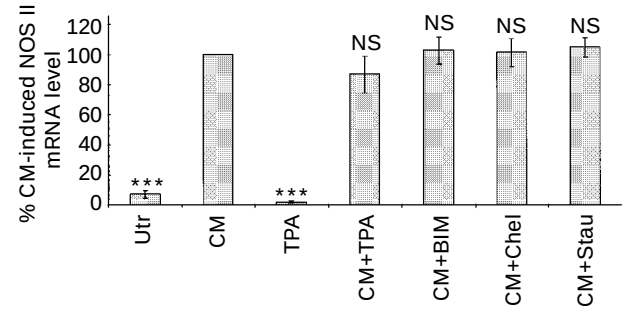

Figure 3 RNase protection analyses with antisense RNA probes to human NOS II and β-actin (for standardization). Columns represent the results of densitometric analyses of six different gels (means $\pm$s.e.mean). RNAs were prepared from untreated DLD-1 cells (Utr), cells treated with 50 ng ml^{-1} TPA (TPA) and cells stimulated with a cytokine mixture (CM, see Figure 1) alone or in the presence of 50 ng ml^{-1} TPA (CM + TPA), 1 μM bisindolylmaleimide I (CM + BIM), 2 μM chelerythrine (CM + Chel) or 100 nM staurosporine (CM + Stau). ***$P<0.001$ vs CM; NS: not significantly different from CM.

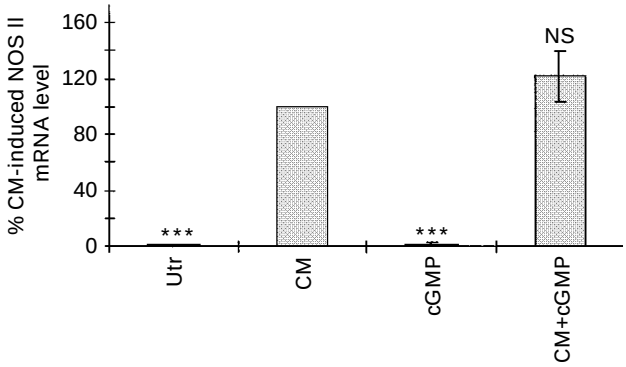

Figure 4 RNase protection analyses with antisense RNA probes for human NOS II and β-actin (for standardization). Columns represent the results of densitometric analyses of three different gels (means $\pm$s.e.mean). RNAs were prepared from untreated DLD-1 cells (Utr), cells treated with 1 mM 8-bromo-cyclic GMP (cGMP) and cells stimulated with a cytokine mixture (CM, see Figure 1) with or without 1 mM 8-bromo-cyclic GMP (CM + cGMP). ***$P<0.001$ vs CM; **$P<0.01$ vs CM; NS: not significantly different from CM.

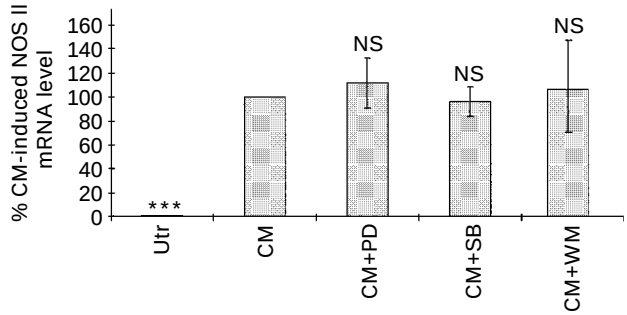

Figure 5 RNase protection analyses with antisense RNA probes for human NOS II and β-actin (for standardization). Columns represent the results of densitometric analyses of seven different gels (means $\pm$s.e.mean). RNAs were prepared from untreated DLD-1 cells (Utr) and cells stimulated with a cytokine mixture (CM, see Figure 1) in the absence or presence of 50 μM compound PD 98059 (CM + PD), 5 μM compound SB 203580 (CM + SB) or 500 nM wortmannin (CM + WM). ***$P<0.001$ vs CM; NS: not significantly different from CM.

mRNA ($n=3$, not shown). However, both inhibitors reduced the CM-induced NOS II mRNA expression in a concentration-dependent manner (Figure 7). Tyrphostins B42 and A25 (200 μM, each) also reduced the NOS II induction of INF-γ alone (see Figure 1) by $67\pm6\%$ (mean $\pm$ s.e.mean, $n=3$, data not shown). Similarly, chrysin, a compound described to inhibit INF-γ-induced gene transcription (Hecker *et al*., 1996), inhibited NOS II mRNA expression in DLD-1 cells in a concentration-dependent manner ($n=3$, data not shown).

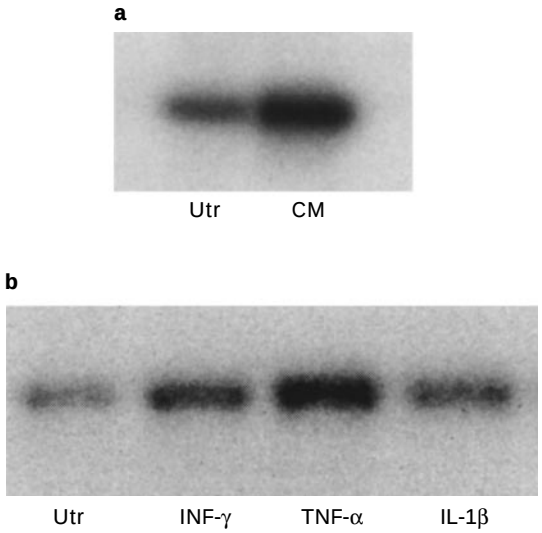

Figure 6 c-Jun NH2-terminal kinase (JNK) immune complex kinase assay with extracts from untreated DLD-1 cells (Utr) or cells incubated for 30 min with a cytokine mixture (CM, see Figure 1) (a), or the single components of CM, 100 u ml^{-1} INF-γ (INF-γ), 10 ng ml^{-1} TNF-α (TNF-α) or 50 u ml^{-1} IL-1β (IL-1β) (b). Extracts were prepared and JNK activity determined as described in Methods. The gels shown are representative of three assays each yielding similar results.

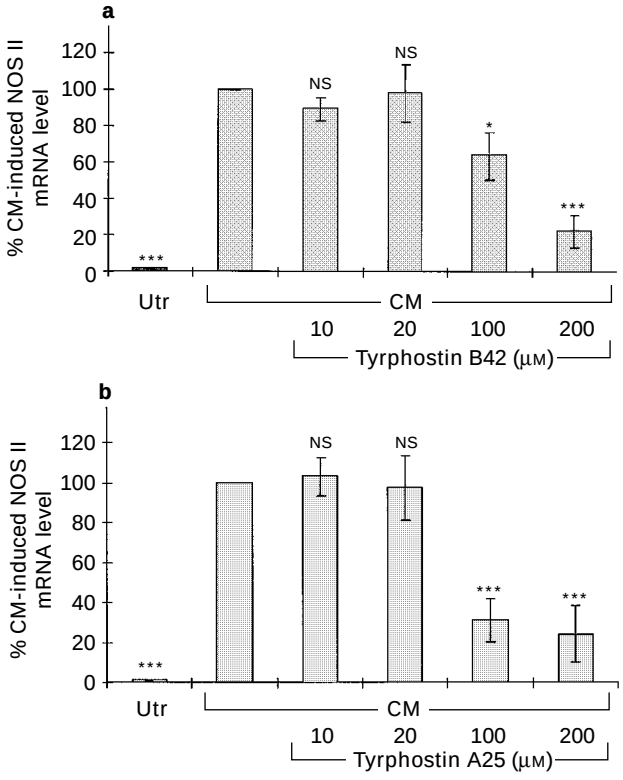

Figure 7 RNase protection analyses with antisense RNA probes for human NOS II and β-actin (for standardization). Columns represent the results of densitometric analyses of five different gels (means $\pm$ s.e.mean). RNAs were prepared from untreated DLD-1 cells (Utr) or cells stimulated with a cytokine mixture (CM, see Figure 1). The experiments were performed in the absence or presence of 10 to 200 μM tyrphostin B42 (a) or tyrphostin A25 (b). *$P<0.05$ vs CM; ***$P<0.001$ vs CM; NS: not significantly different from CM.

Discussion

Protein phosphorylation plays a crucial role in signal transduction pathways leading to the induction of different genes. The phosphorylation of the signal transduction components involved is reversibly controlled by protein kinases and protein phosphatases (Hunter, 1995). Analyses of the signalling pathways leading to NOS II induction have shown major differences between cell types and species (cf Introduction). The present study investigated major protein kinase pathways for their involvement in the induction of NOS II in human DLD-1 cells.

Similar to other human cells (Sherman *et al*., 1993; Kleinert *et al*., 1996a), the DLD-1 cells used here required a mixture of cytokines for maximal NOS II induction. Of the cytokines involved (INF-γ, TNF-α and IL-1β) only INF-γ was able to induce the DLD-1 cells by itself. Neither TNF-α nor IL-1β alone or in combination induced NOS II expression. However, they both enhanced the INF-γ response (Figure 1). Both in terms of NOS II mRNA and NOS activity, INF-γ alone produced about 30% of the maximal rate induced by CM. For maximal transcription, additional signalling pathways and transcription factors (activated by TNF-α and IL-1β) seem to be required.

Despite the multifactorial nature of NOS II induction in human DLD-1 cells, most of the kinase pathways tested turned out not to be involved in this process. Activation or inhibition of PKA did not induce NOS II mRNA expression and did not modify CM-induced NOS II mRNA expression in DLD-1 cells (Figure 2). This is at variance with the induction of NOS II by forskolin and lipophilic cyclic AMP analogues in murine 3T3 fibroblasts (Kleinert *et al*., 1996b) and in rat mesangial cells (Eberhardt *et al*., 1994).

Activators of PKC have been shown previously to enhanced NOS II in murine peritoneal macrophages (Jun *et al*., 1994), and PKC inhibitors blocked NOS II expression in RINm5F insulinoma cells (Messmer & Brüne, 1994). Conversely, PKC stimulators inhibited cytokine-induced NOS II mRNA expression in rat vascular smooth muscle cells (Geng *et al*., 1994). In the present study, activation of PKC with TPA or inhibition with specific PKC inhibitors did not modify CM-induced NOS II mRNA expression in DLD-1 cells (Figure 3). Therefore, the involvement of PKC in NOS II induction is unlikely in these cells.

In rat vascular smooth muscle cells, the IL-1β/TNF-α-induced NOS II expression is enhanced by activation of PKG (Marumo *et al*., 1995). On the other hand, in IL-1β-induced rat cardiac myocytes, NOS II expression was not influenced by dibutyryl-cyclic GMP (LaPointe & Sitkins, 1996). In our DLD-1 cell model, 1 mM 8-bromo-cyclic GMP did not induce NOS II mRNA expression by itself and also did not modify CM-induced NOS II mRNA expression (Figure 4). Therefore, PKG seems to have no role in the signal transduction pathways leading to NOS II induction.

MAP kinase cascades are involved in the expressional regulation of many genes induced by cytokines. ERK1 and ERK2 are members of this kinase family. They are phosphorylated and activated by MAPK kinases (Davis,

1994). The activity of these MAPK kinases can be inhibited by compound PD 98059 (Alessi *et al*., 1995). In rat cardiac microvascular endothelial cells, this inhibitor prevents the IL-1β-induced NOS II expression (Singh *et al*., 1996). In our study, CM incubation did not activated ERK as measured by an ERK2 immune complex kinase assay. Accordingly, the MAPK kinase inhibitor compound PD 98059 did not induce NOS II mRNA in DLD-1 cells and also had no effect on CM-induced NOS II mRNA expression. (Figure 5). Thus, there is no evidence for the involvement of this MAPK family in NOS II induction in DLD-1 cells.

Another member of the MAP kinase family is the p38 MAP kinase (Davis, 1994). In rat glomerular mesangial cells, the p38 MAP kinase seems to regulate negatively NOS II induction (Guan *et al*., 1997). This MAP kinase can be activated by IL-1β and inhibited by compound SB 203580 (Badger *et al*., 1996). However, in DLD-1 cells, IL-1β alone was not able to induce NOS II mRNA expression in DLD-1 cells. In a p38 MAP immune-complex kinase assays, no difference in p38 MAPK activity could be detected between untreated and CM-induced DLD-1 cells. Also incubation of CM-induced cells with the p38 MAP kinase inhibitor compound SB 203580 did not modify the NOS II mRNA induction (Figure 5). Therefore, in contrast to rat glomerular mesangial cells (Guan *et al*., 1997), p38 MAPK is unlikely to be involved in the induction of NOS II in DLD-1 cells.

The JNK is a member of the third subgroup of the MAP kinases also named stress activated protein kinases (SAPK). Apart from u.v.-light, JNKs are activated by pro-inflammatory cytokines such as TNF-α and IL-1β (Davis, 1994). JNK immune-complex kinase assays demonstrated an enhancement of JNK activity in response to CM. TNF-α was the cytokine mainly responsible for this effect (Figure 6). However, as mentioned above, TNF-α alone was not able to induce NOS II mRNA expression in DLD-1 cells. Recent evidence suggests that PI3-kinase activation mediates JNK/SAPK activation in response to various stimuli (Fritz & Kaina, 1997; Logan *et al*., 1997). Wortmannin indirectly inhibited JNK/SAPK activity in these experiments. Therefore our negative results with wortmannin (Figure 5) also argue against an involvement of JNK/SAPK in NOS II induction. Interestingly, the phosphatase inhibitor okadaic acid has also been shown to activate JNK/SAPK in mesangial cells (Guan *et al*., 1996). However, okadaic acid is an inhibitor of CM-induced NOS II expression in murine macrophages (Dong *et al*., 1995; Singh *et al*., 1996) and in DLD-1 cells (Kleinert *et al*., 1996c). Based on these data it seems unlikely that JNK/SAPK activation is involved in NOS II induction in DLD-1 cells.

Wortmannin, a specific PI3-kinase inhibitor, had no effect on the CM-induced NOS II expression in DLD-1 cells (Figure 5). This is in contrast to rat mesangial cells (Donaldson *et al*., 1996), chicken macrophages (Yang *et al*., 1996) and human colonic HT29 cells (Wright *et al*., 1997) where PI3K seems to be involved in NOS II induction.

Incubation of cells with cytokines or growth factors often 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 (for example cytokine receptors) are phosphorylated by non-receptor protein tyrosine kinases (Heldin, 1995). In both types of receptor, tyrosine phosphorylation is essential for dimerization and the subsequent signal transduction. NOS II induction is inhibited by protein tyrosine kinase inhibitors such as genistein, herbimycin A or tyrphostins in many animal cells (Geng *et al*., 1995; Nishiya *et al*., 1995; Kong *et al*., 1996). In the present study, incubation of CM-induced DLD-1 cells with the protein tyrosine kinase inhibitors tyrphostin A25 and B42 inhibited NOS II mRNA expression in a concentration-dependent manner (Figure 7). 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, which is the essential cytokine for NOS II induction in DLD-1 cells (see Figure 1). Following INF-γ incubation, JAK-2 is tyrosine-phosphorylated and tyrosine phosphorylates and activates the transcription factor STAT1α. Binding of STAT1α to the NOS II promoter has been suggested to be required for optimal induction of the NOS II gene in murine RAW 264.7 macrophages (Gao *et al*., 1997). On the other hand, Hecker *et al*. (1996) have shown in RAW 264.7 macrophages that chrysin inhibits NOS II induction and attenuates the DNA binding of interferon-regulatory factor 1 (IRF-1) which has also been implicated in NOS II transcription (Martin *et al*., 1994). Further analyses are required to elucidate which of these transcription factors is important for NOS II transcription in human DLD-1 cells.

In conclusion, we have demonstrated that activators and/or inhibitors of many established protein kinase pathways are without effect on NOS II expression in DLD-1 cells and do not modulate the inducing effect of cytokines. However, a protein tyrosine kinase pathway, most probably phosphorylation by JAK-2, seems to be involved in cytokine-activated NOS II induction.

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