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Glucocorticoids Inhibit the Induction of Nitric Oxide Synthase II by Down-regulating Cytokine-Induced Activity of Transcription Factor Nuclear Factor-κB

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SUMMARY

Incubation of human A549/8 cells with human interleukin-1β (50 units/ml), interferon-γ (100 units/ml), and tumor necrosis factor-α (10 ng/ml) (cytomix) resulted in a marked expression of the mRNA of the inducible nitric oxide synthase (NOS II). This induction was prevented by cycloheximide. Dexamethasone markedly reduced cytokine-induced NOS II mRNA concentrations; this reduction was prevented by RU 38486 (mifepristone). Pyrrolidine dithiocarbamate, an inhibitor of nuclear factor-κB (NF-κB) activation, also significantly decreased cytomix-induced NOS II mRNA levels. When A549/8 cells were transfected with a construct containing 1570-bp 5′-flanking sequence of the murine NOS II gene cloned before a reporter gene, the murine NOS II promoter was induced up to 20-fold with cytomix but not with bacterial lipopolysaccharide. Dexamethasone as well as pyrrolidine dithiocarbamate inhibited this induction. In electrophoretic mobility shift assays, nuclear protein extracts from cytomix-induced, but not from unstimulated cells, significantly slowed the migration of an oligonucleotide containing the NF-κB-binding site. This band shift was markedly reduced by dexamethasone. On the other hand, cytomix-induced nuclear protein content of NF-κB p65 and NF-κB p50 was not reduced by dexamethasone (as analyzed by Western blot). Dexamethasone also did not reduce cytomix-induced expression of NF-κB p65 mRNA or enhance the expression of NF-κB inhibitor mRNA. The human and murine NOS II promoters also contain consensus sequences for activating protein-1 (AP-1) binding. However, AP-1 binding activity of nuclear extracts of A549/8 cells was not enhanced by cytomix or inhibited by dexamethasone. These data suggest that the activated glucocorticoid receptor prevents (by a protein/protein interaction) the binding of transcription factor NF-κB, but not AP-1, to the NOS II promoter, thereby inhibiting the induction of NOS II transcription.

NOS (L-arginine, NADPH:oxygen oxidoreductase, nitric oxide forming; EC 1.14.13.39) catalyzes the production of NO, a short-lived radical gas with physiological or pathophysiological functions in nearly every organ system. NOS II is a high-output isoform compared with the two constitutive NOS isoforms (1). In uninduced cells, expression of NOS II is usually very low or absent. NOS II induction by LPS and/or cytokines was first demonstrated in macrophages, but other cell types can also be induced to express this isoform (1). NO produced by NOS II is likely to mediate much of the antimicrobial activity of macrophages against certain fungal, helminthic, protozoal, and bacterial pathogens, but it is also considered to be a mediator of autoimmune and inflammatory responses and septic shock (1–3).

Glucocorticoids are potent inhibitors of immune responses, inflammation, and endotoxic shock. This occurs in part through an inhibition of the synthesis of proinflammatory enzymes and cytokines. One of the target enzymes of glucocorticoid inhibition is NOS II (4–10). Despite numerous reports on the inhibition of NOS II induction by glucocorticoids, the molecular mechanism of this effect has not been elucidated.

A portion of the 5′-flanking region of the murine NOS II gene has been cloned (11–13).[1] The promoter of the murine gene contains a TATA box and consensus sequences for the binding of transcription factors associated with stimuli that induce NOS II expression (12). Functional analysis of the murine NOS promoter revealed two important regions. The first region (positions −48 to −209) was responsive to LPS

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[1] H. Kleinert, C. Euchenhofer, I. Ihrig-Biedert, and U. Förstermann, unpublished observations.

ABBREVIATIONS: AP-1, activating protein-1; GRE, glucocorticoid responsive element; I-κB, inhibitor of nuclear factor-κB; IL, interleukin; INF-γ, interferon-γ; LPS, bacterial lipopolysaccharide; NOS, nitric oxide synthase; NF, nuclear factor; PDTC, pyrrolidine dithiocarbamate; TNF-α, tumor necrosis factor-α; NO, nitric oxide; NOS II, inducible NOS; IRF-E, interferon regulatory factor binding site; RT, reverse transcriptase; PCR, polymerase chain reaction; bp, basepair(s); nt, nucleotide(s); LU, light units; TBS, Tris-buffered saline.

and contains putative binding sites for NF/IL-6 and NF-κB (13). Oligonucleotide probes containing the NF-κB site bound proteins that appeared in the nuclei of LPS-induced murine macrophages (14). The NF-κB inhibitor PDTC blocked the expression of NOS II mRNA and the activation of proteins binding to the NF-κB binding site, indicating that NF-κB activation is important for transcription of the NOS II gene in murine macrophages (14). The second region (positions −913 to −1029) mediated the IFN-γ potentiation of the LPS induction (13). This region displays an IRF-E (15). Site-specific mutagenesis of two nucleotides within the IRF-E abolished the enhancement of transcription by IFN-γ. Gel shift analyses demonstrated IRF-E-binding protein(s) in the nuclei of IFN-γ-treated macrophages, one of which was immunochemically identified as IRF-1 (15). GREs have not been found in the known sequence of the murine NOS II promoter.

Fragments of the 5′-flanking region of the human NOS II gene have also been cloned (16, 17).[2] They show significant structural similarity with the murine NOS II promoter sequence, including a TATA box and several consensus sequences for the binding of transcription factors involved in the cytokine-mediated induction of other genes (IFN-γ-RE, NF/IL-6- and NF-κB-binding motifs). Also, the known human NOS II promoter sequence is devoid of a GRE motif.

Glucocorticoids modulate biological activities in cells by activating their cognate glucocorticoid receptor. In most cases, the occupied glucocorticoid receptor mediates transcriptional effects. It induces the expression of certain genes and the repression of other genes (18, 19). Gene repression by activated glucocorticoid receptor may be caused by binding to negative GREs (20) or by interference with other stimulating transcription factors. This interference can occur through overlapping response elements (21), by which the activated glucocorticoid receptor inhibits the binding of another transcription factor. Recently, inhibitory protein/protein interactions have been described between the activated glucocorticoid receptor and other transcription factors, e.g., AP-1 (18) or NF-κB (22, 23)

In the current study, we analyzed the mechanism of repression of NOS II mRNA induction and the regulation of the NOS II promoter by dexamethasone in human alveolar epithelium-like A549/8 cells. We observed that dexamethasone decreased the activity of the NOS II promoter and reduced the formation of cytokine-induced NF-κB complexes that bind to the NF-κB site in the human NOS II promoter.

Materials and Methods

Reagents. Human INF-γ, human IL-1β, and human TNF-α were purchased from Genzyme. LPS (*Escherichia coli* 026:B6), PDTC, dexamethasone, and cycloheximide were purchased from Sigma Chemical Co. Isotopes were obtained from Amersham Corp. Restriction enzymes, polynucleotide kinase, *Taq* polymerase, S1-nuclease, dNTPs, and oligo-dT primer were obtained from Pharmacia. Superscript RT was obtained from GIBCO-BRL. The luciferase assay system and galacto-light β-galactosidase reporter assay were obtained from Serva/Promega. The NF-κB p50 and p65 antibodies were purchased from Santa Cruz Biotechnology. The enhanced horseradish peroxidase/luminol chemiluminescence reaction kit was purchased from DuPont-NEN. The glucocorticoid receptor antagonist RU 38486 (mifepristone) was obtained from Roussel-Uclaf.

Cell Culture. The human alveolar epithelium-like A549/8 cells (24), human hepatocellular carcinoma cells Hep G2, and murine fibroblasts NIH/3T3 (all from American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (GIBCO) with 10% fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin. For induction, confluent A549/8 cells were incubated for 3–14 hr with the cytokines INF-γ, (100 units/ml), IL-1β (50 units/ml), and TNF-α (10 ng/ml) alone or in combination, and/or LPS (1 μg/ml). In some experiments, dexamethasone (0.1–10 μM) or PDTC (100 μM) was present during the induction.

Cloning of a human NOS II and a human β-actin cDNA fragment. Total RNA was isolated from uninduced Hep G2 cells by guanidinium thiocyanate/phenol/chloroform extraction (25). Then, 2 μg of this RNA was annealed with 0.5 μg of an oligo-dT primer (Pharmacia) and reverse-transcribed with Superscript RT (GIBCO-BRL) following the manufacturer's instructions. RT-generated cDNAs encoding for human NOS II and human β-actin were amplified using PCR. Oligonucleotide primers for NOS II were CCATCCTTGCATCCTCATCG (sense) and TCCAGGGTGCTACTTGTTAGGAG (antisense) corresponding to positions 3128–3148 and 3828–3851 of the human hepatocyte NOS II cDNA (26). Oligonucleotide primers for β-actin were ACCAACTGGGACGACATGGAG (sense) and CGTGAGGATCTTCATGAGGTAGTC (antisense) representing positions 270–291 and 599–623 of the human β-actin cDNA (27). PCR was performed in a 100-μl volume containing 1× *Taq* polymerase buffer (Pharmacia), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2 units *Taq* polymerase, 50 pmol oligonucleotide primers, and RT products (1/10 of the RT reaction). After an initial denaturation step at 95° for 5 min, 30 cycles were performed (1 min at 95°, 1 min at 60°, and 1 min at 72°) followed by a final 10-min extension step at 72°. The PCR products (30 μl) were analyzed on a 1.5% agarose gel containing 0.1 μg/ml ethidium bromide. The amplified cDNA fragments (NOS II, 726 bp; β-actin, 354 bp) were cloned into the *Eco* RV site of pCR-Script (Stratagene) using the Sure Clone Ligation Kit (Pharmacia), generating the cDNA clones pCR-NOS, II-Hu, and pCR-βactin Hu. DNA sequences of the cloned PCR products were determined from plasmid templates using the dideoxy chain termination method with the sequencing kit (Pharmacia).

Cloning of the 5′-flanking region from the human and murine NOS II genes. Chromosomal DNA was isolated from human Hep G2 or murine NIH/3T3 cells by RNase/proteinase digestion and phenol/chloroform extraction as described (28). This DNA was used for amplification of the 5′-flanking DNA of the human or murine NOS II gene. The PCR reaction was performed as described above using the following oligonucleotides as primers: CATATGTATGGGAATACTGTATTTCAGGC (5′) and CTTGAGAACTTCGGGACTGTCTAG (3′) for the human NOS II promoter and ATCCATAAGCTGTGTGTGTGTGCAAG (5′) and TGAACAAGACCCAAGCGTGAG (3′) for the murine NOS II promoter. The sequences were based on published 5′-flanking sequences of the human (16) or murine (12, 13) NOS II genes. The amplified DNA fragments (human: 1221 bp, positions −1090 to +131; murine: 1711 bp, positions −1570 to +141) were cloned into the *Sma*I site of pUC 18, generating pUC-MNOS II-5′ and pUC-Hu NOS II-5′. The DNA sequence of the cloned PCR products were determined using the sequencing kit (Pharmacia). The human and murine NOS II 5′-flanking sequences were then inserted into the luciferase gene containing plasmid pGl2-Basic (Promega), generating pGL-Hu NOS II-5′-Luc and pGL-MNOS II-5′-Luc.

Preparation of probes. To generate DNA probes for S1-nuclease protection analyses, plasmids containing the NOS II/, NF-κB p65/, I-κB/ or β-actin/cDNA fragments were restricted with *Sma*I, *Bst*EII, *Asp*I, or *Sty*I, respectively. They were dephosphorylated (calf intestinal alkaline phosphatase; Boehringer-Mannheim), extracted with phenol/chloroform, and concentrated by ethanol precipitation. Fifty nanograms of this DNA was labeled with γ^{32}P-ATP using polynucleotide kinase (Pharmacia). The radiolabeled DNA was separated from

[2] H. Kleinert, C. Euchenhofer, I. Ihrig-Biedert, and U. Förstermann, unpublished observations.

unincorporated radioactivity by using NucTrap probe purification columns (Stratagene).

S1-nuclease protection analyses. S1-nuclease protection analyses were performed as described (29). Briefly, 20 μg total RNA isolated by the guanidinium thiocyanate/phenol/chloroform extraction method (25) was hybridized at 52° for 16 hr in parallel with 75,000 cpm labeled NOS II, NF-κB p65, or I-κB probe and 30,000 cpm labeled β-actin probe in hybridization buffer (40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% formamide) after a denaturation step at 85° for 30 min in a volume of 30 μl. The S1-nuclease digestion was started by the addition of 310 μl of digesting buffer (280 mM NaCl, 4.5 mM Zn-acetate, pH 4.5, 30 μg/ml denatured salmon sperm DNA, 300 units/ml S1-nuclease). After 20 min at 37°, the reaction was stopped by the addition of 65 μl stop-buffer (2.5 M NH_4-acetate, 50 mM EDTA) followed by phenol/chloroform extraction. The reaction products were concentrated by ethanol precipitation and analyzed by electrophoresis in denaturing urea polyacrylamide gels (8 M urea, 6% polyacrylamide). The electrophoresis buffer was 1× TBE (1.08% Tris, pH 8.3, 0.55% boric acid, and 20 mM EDTA), and the gels were electrophoresed for 2 hr, dried, and exposed to X-ray film or a Phospho-Imager screen (Bio-Rad) for quantification. The protected DNA fragment of the NOS II probe was 338 nt; the protected fragment of the NF-κB p65 probe was 307 nt; the protected fragment of the I-κB probe was 561 nt; and the protected fragment of the β-actin probe was 113 nt.

Transient transfection of A549/8 cells and luciferase/β-galactosidase assays. A549/8 cells were plated in 100-mm cell culture dishes at least 24 hr before transfection. The cells (~80% confluent) were transfected by a modification of the Ca^{2+} phosphate coprecipitation method (28) with 10 μg of pGL-Hu NOS II-5′-Luc, pGL-MNOS II-5′-Luc, or pGL2 Basic and 10 μg of pCH110 (Pharmacia) for normalization of the transfection efficiency. The cells were washed with culture medium at 16 hr after transfection and induced with cytokines and/or LPS at 24 hr after transfection. Extracts (300 μl) were prepared 24 hr later using the reporter lysis buffer (Promega). The luciferase and β-galactosidase activities of the extracts were determined using the Luciferase Assay System and the Galacto-Light System (Promega). Forty microliters of extract were assayed for luciferase activity in a luminometer (Berthold). The reaction was started by the injection of 100 μl luciferase assay buffer (Promega). The same extract was used for determination of the β-galactosidase activity. After incubation at 50° for 1 hr (for reduction of the endogenous β-galactosidase activity), 20 μl extract was incubated with 70 μl Galacto-Light reaction buffer (Promega) at room temperature for 1 hr. Then, the reaction was started by the injection of 100 μl accelerator (Promega), and β-galactosidase activity was assayed in a luminometer. The LU of the luciferase assay were normalized by the LU of the β-galactosidase assay after subtraction of extract background: $(LU_{Luc} - \text{background})/(LU_{\beta\text{-Gal}} - \text{background}) \times 100$.

Electrophoretic mobility shift assay. NF-κB and AP-1 binding activity in nuclei of uninduced and induced cells was determined by electrophoretic mobility shift assays using the Promega gel shift assay system. Nuclear proteins were extracted from A549/8 cells by detergent lysis (30). Ten micrograms of nuclear protein were incubated with 17.5 fmol ^{32}P-labeled double-stranded oligonucleotide containing the NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) or AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) binding motifs. The specificity of binding was determined by the addition of excess (1.75 pmol) of the same unlabeled oligonucleotide. Competition assays were performed with an unlabeled oligonucleotide containing the NF-κB binding motif in the human NOS II promoter (5′-CAAGCTGGGGACACTCCCTTTG-3′). The DNA/protein complexes were analyzed on 5% polyacrylamide gels (buffer: 6.7 mM Tris·HCl, pH 7.5, 3.3 mM sodium acetate, 1 mM EDTA). The gels were dried and autoradiographed on X-ray film or a Phospho-Imager screen (Bio-Rad).

Western blots. Nuclear proteins isolated from untreated cells, cytomix-induced cells, and cells treated with cytomix in the presence of dexamethasone were separated by polyacrylamide (10%) gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell) by electroblotting (Bio-Rad). The blots were blocked at 4° in Blotto (10 mM Tris·HCl, pH 8.0, 150 mM NaCl, 5% nonfat dried milk, 0.05% Tween-20) for 1 hr. All subsequent steps were performed at room temperature. The blots were incubated with 2 μg/ml of anti-NF-κB p65 or anti-NF-κB p50 antibody (Santa Cruz Biotechnology) in Blotto for 45 min and then washed twice (7 min each) in TBS (150 mM NaCl buffered with 10 mM Tris·HCl, pH 8.0) containing 0.05% Tween-20. Subsequently, the blots were incubated for 30 min with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:2000 in Blotto. The blots were washed three times (5 min each) in TBS containing 0.05% Tween-20, followed by two washes (5 min each) in TBS alone. The immunocomplexes were developed using an enhanced horseradish peroxidase/luminol chemiluminescence reaction (DuPont-NEN) according to the manufacturer's instructions.

Results

NOS II mRNA expression in A549/8 cells. In contrast to most animal cells, LPS (10 ng/ml to 1 μg/ml) did not induce NOS II mRNA expression in human A549/8 cells as measured by S1-nuclease protection analysis. Also, there was no detectable NOS II mRNA expression in A549/8 cells incubated for 14 hr with human IFN-γ (100 units/ml) or human TNF-α (10 ng/ml) alone. Human IL-1β (50 units/ml) sometimes produced a faint signal. Combinations of IL-1β (50 units/ml) with INF-γ (100 units/ml) and IL-1β (500 units/ml) with TNF-α (10 ng/ml) resulted in a measurable NOS II mRNA expression (34 ± 9% and 8 ± 4% of the maximum response, respectively). Maximum expression was seen with a mixture of IL-1β (50 units/ml), INF-γ (100 units/ml), and TNF-α (10 ng/ml) (cytomix) (Fig. 1).

Inhibitors of NOS II mRNA expression. Dexamethasone significantly reduced cytomix-induced NOS II mRNA levels. The maximum inhibitory effect (inhibition to 29 ± 1% of the NOS II mRNA levels of cytomix-induced cells) was seen at 1 μM (Fig. 2). This inhibition was reversed by the glucocorticoid receptor antagonist RU 38486 (mifepristone, 30 μM) (Fig. 3). The protein synthesis inhibitor cycloheximide (10 μg/ml) reduced NOS II mRNA expression to 14 ± 4% of the level of cytomix-induced cells. Also, PDTC (100 μM), an inhibitor of the activation of NF-κB, inhibited NOS II mRNA expression to 66 ± 14% of the level of cytomix-induced cells. Dexamethasone or PDTC alone did not induce NOS II mRNA (Fig. 2).

Activities of the murine and human NOS II promoters in human A549/8 cells. The activity of the human and murine NOS II promoters was analyzed in A549/8 cells transiently transfected with the plasmid pGL-Hu NOS II-5′-Luc or pGL-MNOS II-5′-Luc (containing the 5′-flanking sequence of the human or murine NOS II promoters inserted before the luciferase reporter gene). Similar to the endogenous human NOS II promoter, the murine promoter was not inducible by LPS in A549/8 cells. On the other hand, 3T3 fibroblasts from which the murine NOS II promoter was derived express significant levels of NOS II mRNA in response to LPS alone (not shown). A ≤20-fold induction of the promoter activity of the murine 5′-flanking sequence was obtained in A549/8 cells incubated with cytomix (Fig. 3). The activity of the human promoter fragment was induced ~2-fold by this cytokine mixture; this small induction was also totally prevented by

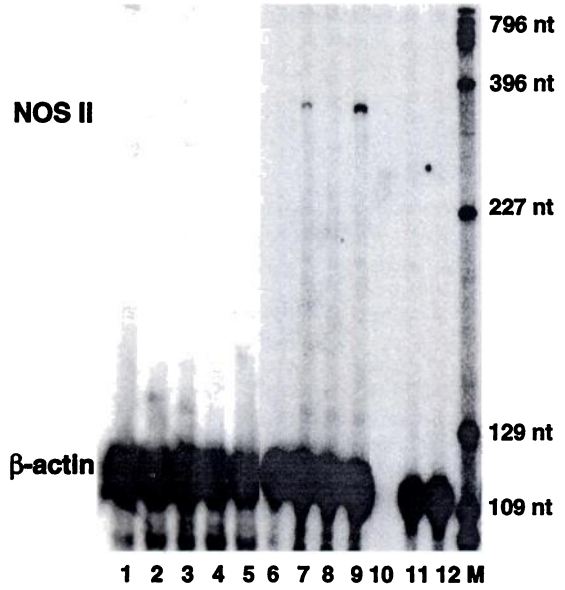

Fig. 1. S1-nuclease protection analysis using a human NOS II cDNA probe and RNAs obtained from untreated A549/8 cells (controls: *lanes 1, 11, and 12*) and A549/8 cells induced with various combinations of LPS and cytokines. *Lane 2*, LPS (1 μg/ml). *Lane 3*, LPS (1 μg/ml) and IFN-γ (100 units/ml). *Lane 4*, LPS (1 μg/ml) and IL-1β (500 units/ml). *Lane 5*, IL-1β (500 units/ml). *Lane 6*, IL-1β (50 units/ml). *Lane 7*, IL-1β (50 units/ml) and INF-γ (100 units/ml). *Lane 8*, IL-1β (50 units/ml) and TNF-α (10 ng/ml). *Lane 9*, IL-1β (50 units/ml), INF-γ (100 units/ml), and TNF-α (10 ng/ml) (cytomix). *Lane 10*, tRNA control. *M*, molecular weight markers (pGl2-Basic restricted with *Hinf*I).

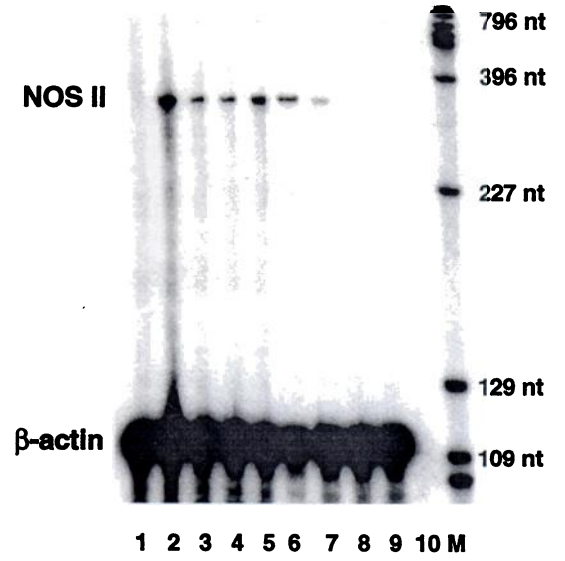

Fig. 2. S1-nuclease protection analysis of RNAs from A549/8 cells using a human NOS II cDNA probe. Cells received the following treatments before RNA was prepared. *Lane 1*, untreated control cells. *Lane 2*, cells induced with IL-1β (50 units/ml), INF-γ (100 units/ml), and TNF-α (10 ng/ml) (cytomix). *Lanes 3–5*, cells incubated with cytomix in the presence of dexamethasone (0.1, 1.0, and 10 μM, respectively). *Lane 6*, cells incubated with cytomix in the presence of PDTC (100 μM). *Lane 7*, cells incubated with cytomix in the presence of cycloheximide (10 μg/ml). *Lane 8*, cells incubated with dexamethasone (10 μg/ml) alone. *Lane 9*, cells incubated with PDTC (100 μM) alone. *Lane 10*, tRNA control. *M*, molecular weight markers (pGl2-Basic restricted with *Hinf*I).

dexamethasone (5 μM; three experiments. Data not shown). Consequently, the murine promoter/reporter gene construct was used in subsequent experiments. The induction of the

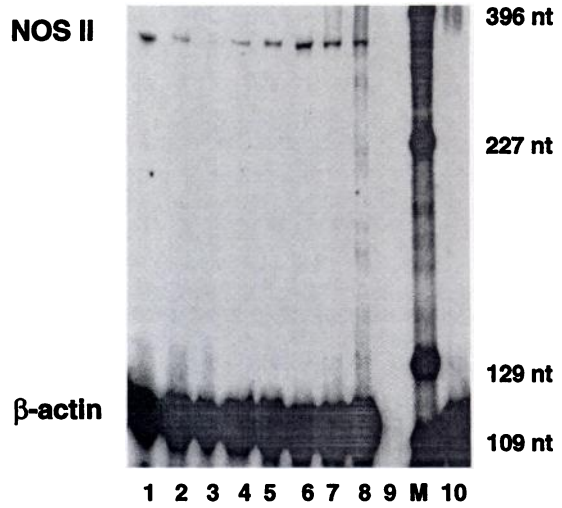

Fig. 3. S1-nuclease protection analysis of RNAs from A549/8 cells using a human NOS II cDNA probe. Cells received the following treatments before RNA was prepared. *Lane 1*, cells induced with IL-1β (50 units/ml), INF-γ (100 units/ml), and TNF-α (10 ng/ml) (cytomix). *Lanes 2–4*, cells incubated with cytomix in the presence of dexamethasone (0.1, 1.0, and 10 μM, respectively). *Lane 5*, cells incubated with the glucocorticoid receptor antagonist RU 38486 (mifepristone, 30 μM). *Lanes 6–8*, cells incubated with cytomix in the presence of dexamethasone (0.1, 1.0, and 10 μM, respectively) and RU 38486 (30 μM). *Lane 9*, tRNA control. *Lane 10*, untreated control cells. *M*, molecular weight markers (pGl2-Basic restricted with *Hinf*I).

murine NOS II promoter in A549/8 cells was inhibited in a concentration-dependent manner by dexamethasone and by PDTC (Fig. 4).

Cytokine-induced A549/8 cells expressed nuclear proteins with NF-κB binding activity, inhibition by dexamethasone. Electrophoretic mobility shift assays using a consensus oligonucleotide for NF-κB binding showed a low level of NF-κB binding activity in untreated A549/8 cells. Incubation of A549/8 cells with cytomix for 3 hr markedly increased this activity (Fig. 5). The addition of excess unlabeled consensus oligonucleotide completely prevented the band shifts, demonstrating the specificity of the protein/DNA

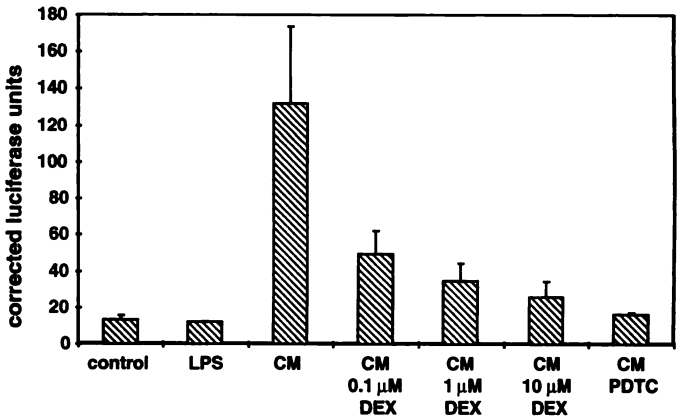

Fig. 4. Luciferase assay of transfected A549/8 cells. *Ordinate*, corrected luciferase activity of A549/8 cells transfected with both pGL-MNOS II-5′-Luc and, for normalization, pCH110. Transfected cells were incubated with the following agents: culture medium alone (*control*); LPS (1 μg/ml); IL-1β (50 units/ml), INF-γ (100 units/ml), and TNF-α (10 ng/ml) (cytomix [*CM*]); cytomix in the presence of dexamethasone ([*DEX*] 0.1, 1.0, and 10 μM, respectively); and cytomix in the presence of PDTC (100 μM).

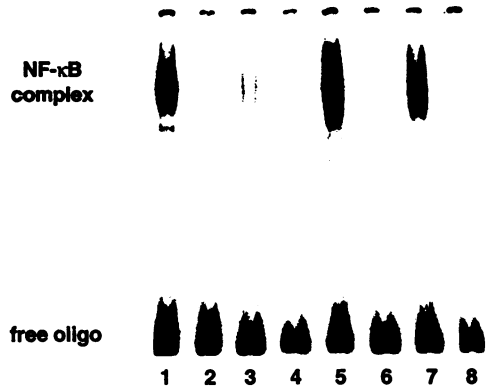

Fig. 5. Electromobility shift assay using a 5′-end-labeled consensus oligonucleotide for NF-κB binding and nuclear extracts from HeLa cells (which are known to express significant NF-κB-binding activity; *lanes 1 and 2*) and A549/8 cells (*lanes 3–8*). The specificity of complex formation was verified in each case by displacement with a 100-fold excess of the same unlabeled oligonucleotide (*lanes 2, 4, 6, and 8*). *Lanes 3 and 4*, untreated A549/8 cells were used. *Lanes 5 and 6*, A549/8 cells induced for 3 hr with IL-1β (50 units/ml), INF-γ (100 units/ml), and TNF-α (10 ng/ml) (cytomix) were used. *Lanes 7 and 8*, A549/8 cells treated for 3 hr with cytomix in the presence of dexamethasone (5 μM) were used.

interaction (Fig. 5). The band shift was also prevented by a double-stranded oligonucleotide containing the putative NF-κB binding motif of the human NOS II promoter (Fig. 6). The stimulation of specific NF-κB binding by cytokines was markedly inhibited by coincubation with dexamethasone (1–5 μM) (Figs. 5 and 6).

Cytokines and/or dexamethasone did not change the expression by A549/8 cells of nuclear proteins with AP-1 binding activity. Incubation of A549/8 cells with cytomix and coincubation with dexamethasone (5 μM) did not change the low level AP-1-binding activity expressed in the nuclei of these cells (Fig. 7).

Dexamethasone did not change the nuclear content of NF-κB p65 or NF-κB p50 immunoreactivities in cytokine-induced A549/8 cells. Incubation of A549/8 cells with cytomix for 4 hr increased the nuclear content of NF-κB p65 and p50 immunoreactivities as determined by Western blot analysis using polyclonal anti-NF-κB p65 and p50 antibodies (Fig. 8).

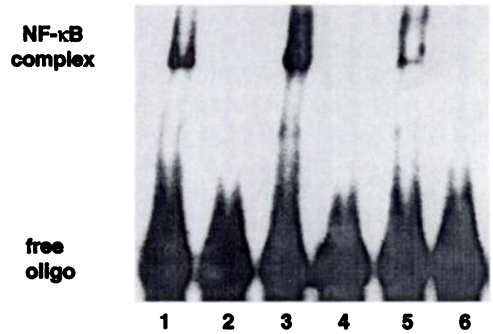

Fig. 6. Electromobility shift assay using a 5′-end-labeled consensus oligonucleotide for NF-κB binding and nuclear extracts from A549/8 cells. *Lanes 1 and 2*, untreated A549/8 cells were used. *Lanes 3 and 4*, A549/8 cells induced for 3 hr with IL-1β (50 units/ml), INF-γ (100 units/ml), and TNF-α (10 ng/ml) (cytomix) were used. *Lanes 5 and 6*, A549/8 cells treated for 3 hr with cytomix in the presence of dexamethasone (5 μM) were used. In all three cases, complex formation was completely prevented with an unlabeled oligonucleotide containing the putative NF-κB binding site of the human NOS II promoter (100-fold excess; *lanes 2, 4, and 6*).

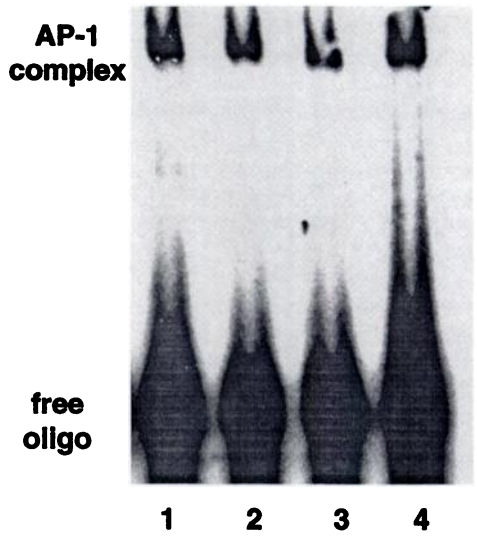

Fig. 7. Electromobility shift assay using a 5′-end-labeled consensus oligonucleotide for AP-1 binding and nuclear extracts from A549/8 cells. *Lane 1*, untreated A549/8 cells were used. *Lane 2*, A549/8 cells induced for 3 hr with IL-1β (50 units/ml), INF-γ (100 units/ml), and TNF-α (10 ng/ml) (cytomix) were used. *Lane 3*, A549/8 cells treated for 3 hr with cytomix in the presence of dexamethasone (5 mM) were used. *Lane 4*, nuclear extracts from HeLa cells were used as controls (HeLa cells express significant levels of AP-1-binding activity).

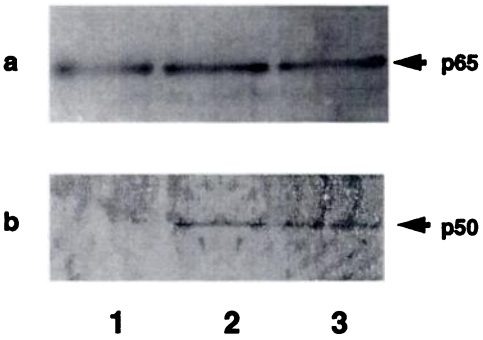

Fig. 8. Western blots of nuclear extracts of A549/8 cell using a polyclonal anti-NF-κB p65 antibody (a) or a polyclonal anti-NF-κB p50 antibody (b). *Lane 1*, untreated A549/8 cells were used. *Lane 2*, A549/8 cells induced for 3 hr with IL-1β (50 units/ml), INF-γ (100 units/ml), and TNF-α (10 ng/ml) (cytomix) were used. *Lane 3*, A549/8 cells treated for 3 hr with cytomix in the presence of dexamethasone (1 μM)were used.

Dexamethasone (1 μM) treatment of the cells had no effect on the amount of either NF-κB monomer (Fig. 8).

Dexamethasone incubation did not change the cytokine-induced expression of NF-κB p65 mRNA or I-κB mRNA. Incubation of A549/8 cells with cytomix for 14 hr increased the expression of NF-κB p65 mRNA and I-κB mRNA as measured by S1-nuclease protection analyses. The addition of dexamethasone (0.1, 1.0, or 10 μM) to cytomix-treated A549/8 cells did not change the level of expression of either mRNA (Fig. 9).

Discussion

In murine and rat macrophages, NOS II expression can be induced with LPS alone or in combination with cytokines (31, 32). Many human cells (e.g., hepatocytes (26) and DLD-1 adenocarcinoma cells (33)) do not respond to LPS and require a cytokine mixture typically consisting of IL-1β, IFN-γ, and TNF-α for induction. Similarly, the human A549/8 cells used

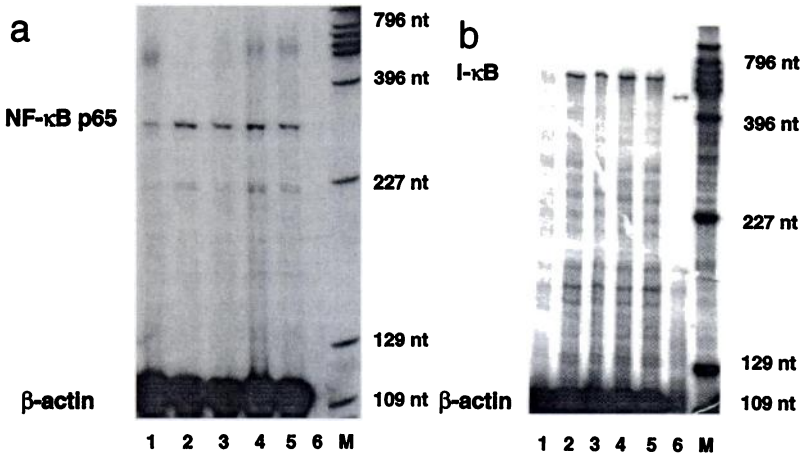

Fig. 9. S1-nuclease protection analyses of RNAs from A549/8 cells using a human NF-κB p65 cDNA probe (A) or a human I-κB cDNA probe (B). Cells received the following treatments before RNA was prepared. *Lane 1*, untreated control cells. *Lane 2*, cells induced with IL-1β (50 units/ml), INF-γ (100 units/ml), and TNF-α (10 ng/ml) (cytomix). *Lanes 3–5*, cells incubated with cytomix in the presence of dexamethasone (0.1, 1.0, and 10 μM, respectively). *Lane 6*, tRNA control. *M*, molecular weight markers (pGl2-Basic restricted with *Hinf*I).

in the current study required human IL-1β (50 units/ml), INF-γ (100 units/ml), and TNF-α (10 ng/ml) (cytomix) for maximum induction (see Ref. 34). LPS was without effect. Of these cytokines, IL-1β appeared to be the most important because low levels of NOS II mRNA expression were detected in A549/8 cells incubated with IL-1β (50 units/ml) alone. INF-γ or TNF-α alone did not produce such a signal. The induction of NOS II mRNA expression in response to cytomix was sensitive to cycloheximide, indicating that *de novo* protein synthesis (presumably of transcription factors) is required for the induction of the NOS II gene in these cells. This is in contrast to certain fibroblasts (3T3 cells) for which NOS II has been described as a primary response gene (8).

Functional analysis of the murine NOS II promoter (position −1570 to +141) in human A549/8 cells by transfection and luciferase assay of the pGL-MNOS II-5′-Luc promoter-reporter gene construct demonstrated that the compounds required for induction of the promoter were dependent on the human host cell, and not the murine cell from which the promoter had been cloned. LPS was not able to induce the murine NOS II promoter in the human A549/8 cell, although in many murine cells the murine promoter sequence allows NOS II induction by LPS. Apparently, the transcriptional machinery of human cells does not allow LPS activation of the murine promoter. The low level of induction by cytomix of the transfected human promoter fragment in A549/8 cells indicates that the cloned human 5′-flanking sequence is not sufficient to convey significant reporter gene activity.

Similar to many other cell types (4–10), dexamethasone inhibited NOS II mRNA induction in A549/8 cells. The cloned murine and human 5′-flanking sequences of the NOS II gene both contain consensus sequences for transcription factors involved in gene induction (e.g., AP-1, NF-κB, IRF-E), but there is no good homology to the consensus sequence of the GRE in either promoter. Despite the lack of a GRE, the activation of the introduced murine promoter (and also the endogenous human promoter) was prevented by dexamethasone in A549/8 cells.

The repression by dexamethasone of the induction of the transfected murine NOS II promoter is unlikely to result merely from an inhibition of protein synthesis because expression of the cotransfected β-galactosidase gene was not changed by dexamethasone.

Mechanisms other than binding of the occupied glucocorticoid receptor to the GRE of a target gene can mediate glucocorticoid effects. The inhibition of expression of the collagen gene by dexamethasone is brought about by a direct interaction of the activated glucocorticoid receptor with the AP-1 components c-*jun* and c-*fos* (18). Touray *et al.* demonstrated that activated glucocorticoid receptors can form stable complexes with c-*jun* or c-*fos* in intact cells (35). A similar mechanism is operative in the repression by glucocorticoids of the IL-1α-induced IL-8 expression in human T98G glioblastoma cells (22). In this case, the activated glucocorticoid receptor interferes with the binding of the essential transcription factor NF-κB to its cognate *cis* element, thereby suppressing transcription of the IL-8 gene (22). Electrophoretic mobility shift assays (22) revealed decreased binding of NF-κB complexes to the NF-κB-binding site of the IL-8 promoter in cells treated with dexamethasone. Interestingly, the promoter of the IL-8 gene does contain a consensus GRE that is unlikely to be involved in this inhibition (22). In HeLa cells, it has been shown that the p65 subunit of NF-κB is physically associated with, and can be cross-linked to, the activated glucocorticoid receptor. Overexpression of the glucocorticoid receptor inhibited p65-induced reporter gene expression. Conversely, overexpression of the p65 protein prevented the expression of a reporter gene induced by the activated glucocorticoid receptor (23).

Functional analysis of the murine NOS II promoter in RAW 264.7 cells demonstrated that a deletion fragment without the AP-1 site at position −1126 to −1120 displayed slightly more promoter activity than the wild-type promoter (13). Electrophoretic mobility shift assays performed in the current study showed no effect of cytomix on the low level AP-1 binding activity in nuclei of A549/8 cells. Also, dexamethasone had no significant effect on nuclear AP-1 binding activity of these cells. Thus, in human A549/8 cells, AP-1 is unlikely to represent an essential transcription factor for the NOS II gene.

On the other hand, there was a marked induction of NF-κB binding activity in the nuclei of cytomix-induced A549/8 cells. This increase in NF-κB binding activity was prevented by dexamethasone. Dexamethasone did not reduce the nuclear content of NF-κB p65 or NF-κb p50 proteins. Furthermore, there was no inhibition by dexamethasone of the expression of NF-κB p65 mRNA. I-κB is a cytosolic inhibitor of NF-κB and prevents the translocation of the transcription factor into the nucleus. Dexamethasone also did not increase the expression of I-κB mRNA. Thus, NF-κB expression and nuclear

translocation is likely to remain unchanged after dexamethasone treatment of A549/8 cells. Thus, in A549/8 cells, the dexamethasone-activated glucocorticoid receptor is likely to interact with the cytokine-activated NF-κB complex, thereby repressing the binding of this complex to the NF-κB-RE in the 5′-flanking sequence of the NOS II gene. Similar to the inhibition by dexamethasone of the IL-1α-induced expression of the IL-8 gene (22), the reduction in NF-κB binding activity is likely to decrease the transcriptional activity of the NOS II promoter.

We conclude that dexamethasone (presumably via the occupied glucocorticoid receptor) prevents the binding of the essential transcription factor NF-κB to the NOS II promoter, which in turn leads to decreased transcription of the NOS II gene.

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