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Glucocorticoid-mediated Repression of NFκB Activity in Endothelial Cells Does Not Involve Induction of IκBα Synthesis*

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Christine Brostjan‡, Josef Anrather, Vilmos Csizmadia, Deborah Stroka, Miguel Soares, Fritz H. Bach, and Hans Winkler

From the Sandoz Center for Immunobiology, Deaconess Hospital, Harvard Medical School, Boston, Massachusetts 02215

Repression of NFκB-dependent gene expression is one of the major elements of immunosuppression by glucocorticoids. Protein-protein interactions between the glucocorticoid receptor and NFκB have been characterized and shown to be a possible mechanism of mutual inhibition of transactivation properties. More recently, glucocorticoid-mediated induction of IκBα, an inhibitor of NFκB, has been described in monocytes and lymphocytes; an increase in IκBα mRNA and protein resulted in inactivation and cytosolic retention of NFκB. Thus, rather than the physical interaction between the glucocorticoid receptor and NFκB, the up-regulation of IκBα was presented as the key element in immunosuppression by glucocorticoids. In contrast, we show that the IκBα pathway is not involved in glucocorticoid-mediated inhibition of NFκB activity in endothelial cells. Although transcriptional activation by NFκB was significantly reduced in the presence of glucocorticoids, we did not detect induction of IκBα protein that could prevent nuclear translocation of NFκB upon stimulation with lipopolysaccharide or tumor necrosis factor α. Furthermore, treatment with glucocorticoids did not seem to affect the transcription rate or mRNA stability of IκBα. We therefore conclude that, although induction of IκBα expression by glucocorticoids seems to be of importance in monocytes and lymphocytes, it cannot explain inhibition of NFκB-dependent gene expression in endothelial cells. Our results emphasize the relevance of physical interaction between the glucocorticoid receptor and NFκB in endothelial cells and thus in suppression of inflammation by glucocorticoids.

Glucocorticoids are potent immunosuppressive agents (1) with the potential to inhibit expression of several cytokines and adhesion molecules involved in inflammatory immune responses (2–8). Their effect is mediated by the cytosolic glucocorticoid receptor (GR),[1] which, upon binding of ligand, translocates into the nucleus and engages in regulation of gene expression (9, 10). The GR has been described as carrying out a variety of functions by several different mechanisms, some of which are posttranscriptional (11, 12) but most of which are at the level of transcriptional control. Activation of gene expression by glucocorticoids generally requires binding of a receptor dimer to a specific site on the DNA (13, 14). However, transcriptional repression by the GR may be based on protein-protein interactions between the GR and other transcription factors, without the requirement for DNA binding by the GR (15). The latter seems to be of importance in the glucocorticoid-mediated modification of the immune response. Induction of cytokine gene expression is mostly dependent on the activation of the transcription factors AP1 and/or NFκB, both of which have been shown to interact physically with the GR, resulting in mutual inhibition of DNA binding and transactivation properties (16–22).

NFκB was originally identified as a heterodimer consisting of NFκB1 (p50) and RelA (p65) (23, 24). NFκB is constitutively present in the cytosol but is kept inactive by association with inhibitors of the IκB family (25). Upon exposure to phorbol esters or to inflammatory stimuli such as lipopolysaccharide (LPS) or tumor necrosis factor α (TNFα), IκBα is rapidly degraded, allowing NFκB to translocate into the nucleus and induce transcription by binding to defined promoter elements.

Recently published results on the repression of NFκB activity by dexamethasone (DEX), a synthetic glucocorticoid and anti-inflammatory drug, focused on the effects of glucocorticoids on IκBα (26, 27). DEX was shown to induce the synthesis of IκBα mRNA and protein in monocytic cell lines as well as in T lymphocytes *in vitro* and *in vivo*, thus leading to increased levels of inhibitor in the cytosol preventing the release and therefore activation of NFκB upon signaling with TNFα or phorbol esters. The data presented led to the conclusion that rather than physical interaction between NFκB and the GR, the up-regulation of IκBα by DEX and consequently the cytosolic retention of NFκB represent the main mechanism underlying immunosuppression by glucocorticoids.

To study the effect of glucocorticoids on endothelial cells, we analyzed DEX-dependent regulation of IκBα expression and NFκB inhibition in these cells. A small increase in IκBα mRNA and protein levels was noted upon treatment with DEX. However, this increase was insufficient to compensate for degradation of IκBα or to prevent nuclear translocation of NFκB upon stimulation of endothelial cells with either LPS or TNFα. At the same time, NFκB activity was greatly reduced: we observed DEX-mediated repression of NFκB-dependent gene expression. We conclude that although induction of IκBα synthesis by DEX may play a crucial role in preventing NFκB activation in monocytes and T lymphocytes, it cannot account for glucocorticoid-mediated repression of NFκB-dependent gene expression in endothelial cells, which emphasizes the importance of protein-protein interactions between NFκB and the GR in immunosuppression.

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‡ To whom correspondence should be addressed: Sandoz Center for Immunobiology, Deaconess Hospital, Harvard Medical School, 99 Brookline Ave., Boston, MA 02215. Tel.: 617-632-0876; Fax: 617-632-0880.

[1] The abbreviations used are: GR, glucocorticoid receptor; LPS, lipopolysaccharide; TNFα, tumor necrosis factor α; DEX, dexamethasone; PAEC, porcine aortic endothelial cell; BAEC, bovine aortic endothelial cell; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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MATERIALS AND METHODS

Cell Culture and Transient Transfections—Porcine or bovine aortic endothelial cells (PAECs and BAECs, respectively) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin G (50 units/ml), and streptomycin (50 μg/ml). Experiments were carried out on day 1–3 postconfluence. HeLa cells were grown under conditions identical to those applied for primary endothelial cells. THP1 cells were cultured in RPMI 1640 containing 10% fetal calf serum, penicillin G (50 units/ml), and streptomycin (50 μg/ml). For transfection experiments, BAECs were grown without antibiotics and seeded at 3×10^5 cells/30-mm well. 24 h later, the cells were exposed to 1.5 μg of DNA (0.7 μg of reporter plasmid, 0.5 μg of GR expression plasmid, and 0.3 μg of β-galactosidase control plasmid) and 4 μl of LipofectAMINE (Life Technologies, Inc.) in Dulbecco's modified Eagle's medium without fetal calf serum for 5 h. After addition of fetal calf serum to a final concentration of 10%, the cells were allowed to recover for 2 days. DEX was added at 0.1 μM 1 h before incubation with human recombinant TNFα (Sandoz Pharma, Basel, Switzerland) at 25 ng/ml or LPS (Sigma) at 1 μg/ml for 7 h. Cell extracts were prepared by repeated freeze-thaw lysis in phosphate buffer and assayed for luciferase (28) and β-galactosidase activity in an EG&G Berthold MicroLumat LB96P luminometer. The respective substrates were obtained from Sigma and Tropix, Inc. (Bedford, MA). Each experiment was done in triplicate, and several experiments have been performed with plasmids from different DNA preparations to assess the influence of DNA quality.

Reporter Construct and Expression Plasmids—The NFκB-dependent reporter construct (3 × NFκB/TK/Luc) was based on plasmid 34, kindly provided by A. Palmetshofer. The vector is a pBS SK^+ derivative comprising the cDNA sequence of the firefly luciferase under the control of the herpes simplex virus thymidine kinase minimal promoter starting at position −82 with respect to the transcription initiation site. The plasmid was cut with *Xho*I and *Nhe*I within the polylinker region preceding the thymidine kinase minimal promoter and ligated blunt ended with a double-stranded oligonucleotide (Midland Certified Reagent Co., Midland, TX) containing three binding motifs for NFκB (top strand: 5′-CT **GGGAATTCCT** CTGCT **GGGAAACTTT** CTGCT **GGGAATTCCT** CTGT-3′). The binding sites for NFκB were derived from the positive regulatory domains κB-2 and κB-3 of the porcine E-selectin promoter (GenBank™ U58943 (29)). The expression plasmid for the human GR was a generous gift from A. C. Cato, Karlsruhe, Germany (15). A vector expressing β-galactosidase under the control of the Rous sarcoma virus promoter was used as an internal control in transient transfection assays.

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)—PAECs or BAECs were either left untreated or incubated with DEX (0.1 μM) for 1 h and then stimulated with TNFα (25 ng/ml) or LPS (1 μg/ml) for 1 h. Nuclear proteins were extracted as described (30), and protease inhibitors were included in all buffers (see "Western Blot Analysis"). The double-stranded oligonucleotide NFκB (top strand: 5′-CTGCT **GGGAATTCCT** CTGTA-3′) contains a NFκB binding site (κB-3) from the porcine E-selectin promoter (GenBank™ U58943 (29)) and was end labeled using T4 polynucleotide kinase and γ-[^{32}P]ATP. 5-μg protein equivalents of nuclear extract were incubated with 100,000 cpm of radiolabeled NFκB oligonucleotide at room temperature for 30 min, and the resulting complexes were separated on a 5% polyacrylamide gel in Tris/glycine/EDTA buffer at pH 8.5. A 500-fold molar excess of unlabeled oligonucleotide was included in competition experiments. The unspecific competitor oligonucleotide comprises the NF-ELAM1 sequence (top strand: 5′-GATTC **TGACATCA** CCGTA-3′) from the porcine E-selectin promoter (GenBank™ U58943 (29)).

Western Blot Analysis—PAECs were pretreated with and without 0.1 μM DEX for 1 h before stimulation with LPS (1 μg/ml) for 0, 10, 20, 30, and 60 min. Cytosolic extracts were prepared as described (30) with the addition of further protease inhibitors: 0.1 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone, 0.1 mM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 1 μg/ml aprotinin, 1 μg/ml leupeptin. 15 μg of protein extract were separated by SDS-polyacrylamide gel electrophoresis (10%), transferred to an Immobilon P polyvinylidene difluoride membrane by electroblotting (Trans-Blot S.D. Semi-Dry Transfer Cell, Bio-Rad) and probed with the polyclonal antibody sc371 directed against a C-terminal epitope of human IκBα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Bands were visualized using horseradish peroxidase conjugated donkey anti-rabbit IgG (Pierce) and the Enhanced Chemiluminescence assay (Amersham Life Science, Inc.) according to the manufacturer's instructions.

Northern Blot Analysis—PAECs or BAECs were pretreated without and with DEX at a final concentration of 0.01, 0.1, or 1 μM for 1 h before addition of TNFα (25 ng/ml) or LPS (1 μg/ml). 2 h later total RNA was extracted using TRIzol (Life Technologies, Inc.) according to the manufacturer's instructions. 20 μg of RNA were separated on an agarose gel containing formaldehyde, transferred to a Hybond-N nylon membrane (Amersham Life Science, Inc.) and analyzed by hybridization to radiolabeled cDNA probes of porcine IκBα (gift from R. de Martin (31)) and porcine E-selectin (29). Quantitation of bands was routinely performed with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). All membranes were probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA to correct for unequal loading (32), and all quantitated IκBα and E-selectin transcript levels were adjusted accordingly.

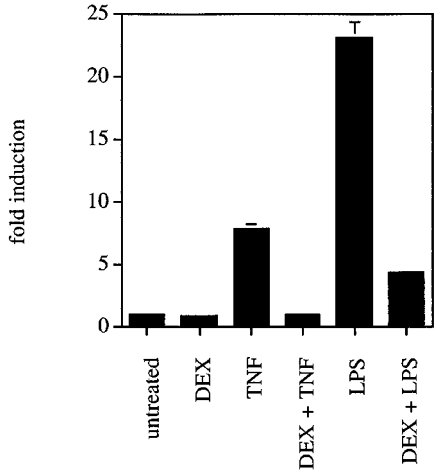

FIG. 1. **Effect of DEX on NFκB-dependent gene expression as demonstrated by reporter assay.** BAECs were transiently transfected with 0.7 μg of reporter plasmid (3 × NFκB/TK/Luc), 0.5 μg of GR expression vector, and 0.3 μg of β-galactosidase control plasmid using LipofectAMINE (Life Technologies, Inc.). 2 days later, cells were incubated with and without DEX (0.1 μM) for 1 h before addition of TNFα (25 ng/ml) or LPS (1 μg/ml) for 7 h. Cell extracts were prepared and assayed for luciferase and β-galactosidase activity. Luciferase relative light units were corrected according to β-galactosidase measurements, and values are given in -fold induction as compared to unstimulated cells. The results shown are the mean of three samples ± S.D. and are representative for several identical experiments performed.

RESULTS AND DISCUSSION

When exposed to inflammatory stimuli, endothelial cells rapidly activate NFκB leading to induced expression of cytokines and adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and E-selectin (33). While it has been shown for other cell types that glucocorticoids interfere with NFκB-mediated transcriptional activation (19–21), we investigated the effect of DEX on NFκB-dependent gene expression in primary endothelial cells.

BAECs were transiently transfected with the reporter construct 3xNFκB/TK/Luc (Fig. 1). The plasmid contains three binding sites for NFκB that were placed in front of an otherwise inactive minimal core promoter, resulting in strictly NFκB-dependent expression of the reporter gene, firefly luciferase. When cells were treated with TNFα or LPS, measured luciferase activity was highly increased (8- and 23-fold, respectively, in one representative experiment). Addition of DEX (at 0.1 μM) inhibited induction by 85–100%. The data demonstrate that NFκB-dependent gene expression is significantly repressed in endothelial cells treated with glucocorticoids.

To determine whether the loss of function was based on reduced binding of NFκB to DNA, we exposed BAECs, as well as PAECs, to LPS or TNFα, with and without prior treatment with DEX. Nuclear proteins were isolated and tested for NFκB binding activity by EMSA (Fig. 2*A*). Binding of NFκB to the radiolabeled DNA probe was observed with nuclear extracts from cells incubated with LPS or TNFα. Pretreatment with DEX did not result in a reduction of bound protein. The binding

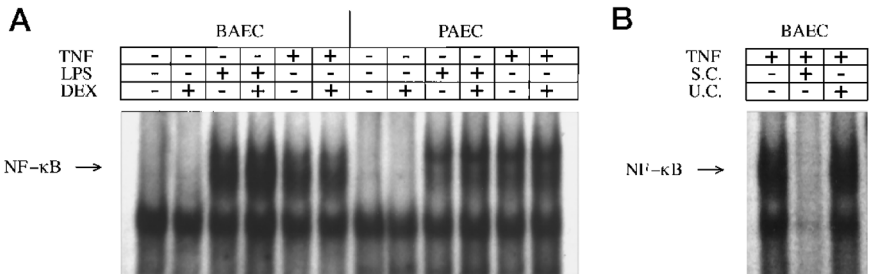

FIG. 2. **Effect of DEX on DNA binding activity of NFκB in nuclear extracts of stimulated endothelial cells.** *A*, PAECs or BAECs were either left untreated or incubated with DEX (0.1 μM) for 1 h and then stimulated with TNFα (25 ng/ml) or LPS (1 μg/ml) for 1 h. Nuclear proteins were extracted and incubated with radiolabeled oligonucleotides containing a binding site for NFκB. Resulting complexes were analyzed by EMSA. *B*, specificity of binding was demonstrated by competition with unlabeled oligonucleotides of identical (*S.C.*, specific competitor) or unrelated (*U.C.*, unspecific competitor) sequence.

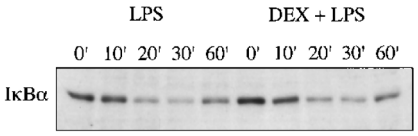

FIG. 3. **Degradation of IκBα protein in the absence or presence of DEX.** PAECs were pretreated with and without 0.1 μM DEX for 1 h before stimulation with LPS (1 μg/ml) for 0, 10, 20, 30, and 60 min. Cytosolic extracts were prepared and subjected to Western blot analysis with anti-IκBα antibody.

activity was specific in that it was inhibited by a 500-fold molar excess of unlabeled NFκB probe, but not by an oligonucleotide of unrelated DNA sequence (Fig. 2*B*). All experiments performed with nuclear extracts isolated at 1, 2, 3, 6, and 24 h after addition of stimulus gave identical results (data not shown).

Since the EMSA revealed comparable amounts of NFκB present in the nucleus of activated endothelial cells with or without treatment with glucocorticoids, we concluded that DEX did not prevent nuclear translocation of NFκB or binding of NFκB to DNA. Similar results have been described by van de Stolpe *et al.* (34, 35) regarding the effect of glucocorticoids on the expression of ICAM-1 in U937 cells. The ICAM-1 promoter element mediating inhibition by DEX was identified as the binding site for NFκB. Also, in agreement with our data discussed above, these authors could not detect any difference in NFκB binding activity of nuclear extracts from cells treated with TNFα or TNFα plus DEX. They proposed a mechanism of protein-protein interactions between NFκB and the GR that did not alter NFκB binding assayed by EMSA but resulted in repression of the transactivation property of NFκB.

In contrast, Scheinman *et al.* (26) and Auphan *et al.* (27) have recently proposed that DEX acts by inducing IκBα, resulting in cytosolic retention of NFκB as demonstrated by EMSA as well as by immunofluorescence staining. These investigators noted DEX-induced synthesis of IκBα mRNA and protein; the rapid degradation of IκBα protein seen upon stimulation of monocytic (THP1) and lymphocytic (Jurkat GR^+) cell lines with TNFα or phorbol esters could be prevented by treatment with DEX. Basal levels of cytosolic IκBα were clearly increased, and although some loss of protein occurred upon incubation with the stimulus, the amount of IκBα never fell below the level seen in untreated cells.

To investigate any effect glucocorticoids might have on the expression of IκBα in endothelial cells, we treated PAECs with and without 0.1 μM DEX for 1 h and then challenged the cells with 1 μg/ml LPS for different periods of time. Cytosolic extracts were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-IκBα antibody. As shown in Fig. 3, DEX pretreatment slightly increased the basal amount

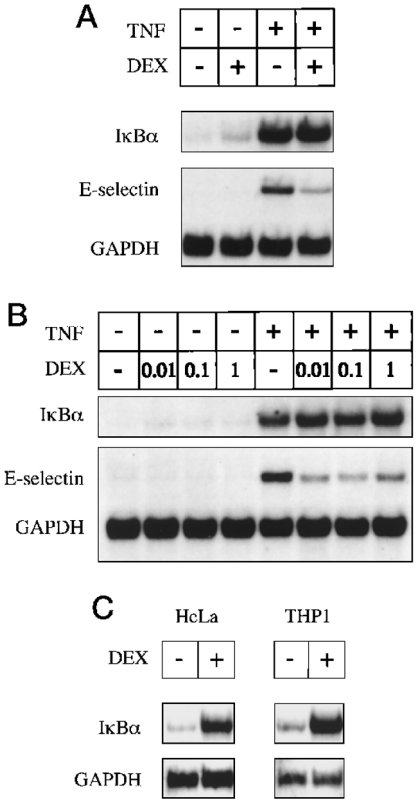

FIG. 4. **Effect of DEX on IκBα steady state mRNA level.** *A*, PAECs were either left untreated or incubated with DEX (0.1 μM) for 3 h. Where indicated, TNFα (25 ng/ml) was given 1 h after addition of DEX. *B*, BAECs were treated without and with DEX at final concentrations of 0.01, 0.1, and 1 μM. 1 h later, TNFα (25 ng/ml) was added as indicated and the incubation was continued for 2 h. *C*, HeLa and THP1 cells were cultured in the absence or presence of DEX (0.1 μM) for 1.5 h. Total RNA was extracted, Northern blotting was performed, and membranes were probed for mRNA of IκBα, E-selectin, and GADPH.

of IκBα present in the cytoplasm but did not prevent the significant loss of IκBα protein seen at 20 and 30 min of incubation with LPS. After 60 min, IκBα levels started to increase again, due to the usually observed autoregulatory induction of IκBα synthesis by activated NFκB (36).

Since the increase in IκBα protein observed in HeLa, THP1, and Jurkat GR^+ cell lines treated with DEX was mainly based on enhanced transcription of IκBα (26, 27), we examined IκBα mRNA levels in primary endothelial cells (Fig. 4). When incubated with DEX for 3 h, cells showed little or no increase in IκBα mRNA. Stimulation with TNFα resulted in a 12–16-fold induction of IκBα transcript. DEX given 1 h before addition of TNFα for 2 h did not modify the accumulation of IκBα mRNA

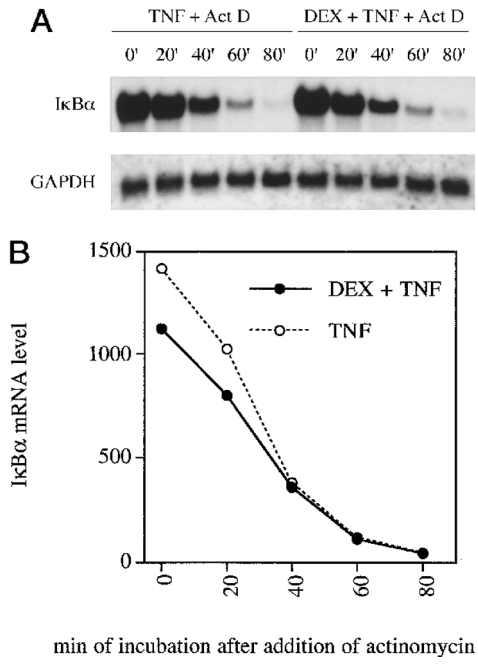

FIG. 5. **Effect of DEX on IκBα mRNA stability.** PAECs were incubated with and without 0.1 μM DEX for 1 h, then stimulated with TNFα (25 ng/ml) for 2 h before addition of actinomycin D (*Act D*, 10 μg/ml) for 0–80 min. Isolated total RNA was analyzed by Northern blotting and probed for mRNA of IκBα as well as GAPDH. IκBα bands shown in the autoradiogram (*A*) were quantitated with a PhosphorImager (Molecular Dynamics) and adjusted to variations in GAPDH levels. The graphic representation of IκBα mRNA decay is given in *B*.

seen with TNFα alone. Experiments performed with LPS yielded similar results (data not shown).

The observation that IκBα transcript levels in endothelial cells are unaltered by treatment with DEX was noted in both porcine (Fig. 4*A*) and bovine (Fig. 4*B*) aortic endothelial cells. Furthermore, varying the concentration of DEX from 10 nM to 1 μM (Fig. 4*B*) and the time of incubation from 1 to 2, 3, 6, or 24 h (data not shown) did not lead to any changes in the effects described.

To demonstrate that endothelial cells were responsive to DEX under the tested conditions, the same Northern blot membranes were reprobed for mRNA of E-selectin, an adhesion molecule the expression of which is reportedly inhibited by glucocorticoids (8). TNFα-induced transcript levels were suppressed by pretreatment with DEX by 62–78%. Furthermore, we confirmed that under the culture conditions applied, HeLa, as well as THP1, cells (Fig. 4*C*) responded readily to DEX treatment: mRNA for IκBα was induced by 9–10-fold as previously reported (26).

Thus, we show that primary endothelial cells do not up-regulate IκBα protein or mRNA upon incubation with glucocorticoids, as has been described for monocytic (26) and T-cell lines (27). Scheinman *et al.* (26) demonstrated a parallel increase of IκBα mRNA steady state levels and transcription rate in HeLa and THP1 cells, thereby reducing the possibility of regulation by changes in mRNA stability. We wondered whether the lack of an effect of DEX on IκBα transcripts that we observed could be explained by an increase in IκBα transcription rate, but simultaneous decrease in mRNA stability that would result in an apparently unchanged level of IκBα transcripts in endothelial cells. Therefore, we performed mRNA stability tests with RNA isolated from PAECs that were induced with TNFα (Fig. 5) or LPS (data not shown) for 2 h and subsequently incubated with actinomycin D (10 μg/ml) for 0–80 min. We observed no difference in the rate of degradation of IκBα mRNA in the presence or absence of DEX (0.1 μM), which led us to conclude that DEX has no effect on the stability of IκBα mRNA. To test separately for any influence DEX might have on the transcription rate of IκBα, transient transfection assays of BAECs were carried out with a reporter construct expressing the firefly luciferase under the control of the porcine IκBα promoter region −600 to +20 (31). Expression of luciferase was inducible by LPS or TNFα but not by DEX (data not shown).

Taken together, these experiments confirm that in endothelial cells, DEX has no influence on the transcription rate or on mRNA stability of IκBα transcripts, resulting in a comparatively unchanged level of IκBα protein present in the cytoplasm. Furthermore, in accordance with the noted lack of IκBα up-regulation, NFκB is translocated to the nucleus at normal rates in the presence of DEX. Thus, direct interaction between the glucocorticoid receptor and NFκB, as characterized for the transcriptional repression of IL-6 (20), is more likely to be the mechanism underlying inhibition of NFκB activity in endothelial cells. While data on monocytic cell lines and T lymphocytes clearly demonstrate the up-regulation of IκBα and subsequent cytoplasmic retention of NFκB by treatment with DEX, we show that this mechanism is not involved in inhibition of NFκB-mediated gene expression in endothelial cells and is thus not a universal mechanism that can explain NFκB repression relevant to immunosuppression by glucocorticoids.

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