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Role of Transcriptional Activation of IκBα in Mediation of Immunosuppression by Glucocorticoids

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Glucocorticoids are potent immunosuppressive drugs, but their mechanism is poorly understood. Nuclear factor kappa B (NF-κB), a regulator of immune system and inflammation genes, may be a target for glucocorticoid-mediated immunosuppression. The activation of NF-κB involves the targeted degradation of its cytoplasmic inhibitor, IκBα, and the translocation of NF-κB to the nucleus. Here it is shown that the synthetic glucocorticoid dexamethasone induces the transcription of the IκBα gene, which results in an increased rate of IκBα protein synthesis. Stimulation by tumor necrosis factor causes the release of NF-κB from IκBα. However, in the presence of dexamethasone this newly released NF-κB quickly reassociates with newly synthesized IκBα, thus markedly reducing the amount of NF-κB that translocates to the nucleus. This decrease in nuclear NF-κB is predicted to markedly decrease cytokine secretion and thus effectively block the activation of the immune system.

Glucocorticoids (GCs) have been used for decades as clinical tools to suppress both the immune response and the processes of inflammation (*1*), yet the immunosuppressive mechanism by which these drugs act is poorly understood. GCs bind to a cytoplasmic glucocorticoid receptor (GR), a member of the steroid hormone receptor superfamily, which then translocates to the nucleus as a transcription factor (*2*). Transcriptional activation of cytokine and cell adhesion genes is critical in the activation of the immune and inflammation systems and is repressed by GCs (*3*). GC repressive elements, however, have not been found in cytokine promoters. In addition, the GR is able to repress the transcription factor AP-1 through a cross-coupling mechanism (*4*), yet AP-1 regulates only a small number of GC-sensitive cytokine promoters. GCs can also repress members of the NF-κB–Rel transcription factor family (*5*, *6*). NF-κB–responsive elements are required for the function of many cytokine promoters (*7*), and NF-κB–responsive elements in the interleukin-6 (IL-6) and IL-8 promoters have been implicated in GC-mediated suppression (*5*). A major form of NF-κB is composed of a dimer of p50 and p65 (RelA) subunits, and this complex is retained in the cytoplasm by repressor molecules that contain ankyrin repeat motifs (*7*). These inhibitory molecules include the IκB family: IκBα, β, and γ, as well as the NF-κB precursor molecules p105 (NF-κB1) and p100 (NF-κB2) (*7*–*9*). Although a cross-coupling mechanism of inhibition exists between NF-κB and the GR (*5*, *6*), it cannot fully explain the ability of GCs to inhibit NF-κB. Here it is shown that GCs induce the transcription of the gene encoding IκBα. The increase in IκBα mRNA results in increased IκBα protein synthesis, which effectively inhibits NF-κB activation.

We initiated this study by extending our analysis of the ability of the synthetic GC dexamethasone (DEX) to block the induction of NF-κB–like DNA binding activities in several cell types and by several different inducers. As shown previously (*6*), DEX blocks the induction of NF-κB by tumor necrosis factor α (TNF-α) in HeLa cells (Fig. 1A, left panel) as well as induction by IL-1 (*10*). The identity of this complex as NF-κB was determined by supershift experiments (*10*). DEX inhibited the induction of NF-κB in the monocytic cell line THP-1, mediated by TNF-α (Fig. 1A, middle panel) and by lipopolysaccharide (LPS) (*10*). DEX also inhibited the TNF-α–mediated induction of NF-κB in the murine T cell hybridoma 2B4 (Fig. 1A, right panel). In addition, thymocytes and lymph nodes derived from mice treated with monoclonal antibody (mAb) to CD3 in combination with DEX no longer express NF-κB activity as compared with mice treated with CD3 mAb alone (*11*). This effect of DEX requires protein synthesis, as it is blocked by cycloheximide (CHX) (Fig. 1A, right panel). DEX treatment results in a reduction in NF-κB–mediated gene expression as measured by transfected reporter constructs (*5*, *6*, *11*). Previous work indicated that the inhibition of NF-κB was the result in part of a decrease in nuclear translocation after stimulation with TNF-α.

We wanted to determine if the DEX-mediated reduction of nuclear p65 translocation, after TNF-α stimulation, correlated with an increase in IκBα. IκBα is rapidly degraded after TNF-α addition (*7*, *12*) (Fig. 1B, top left panel) and this loss correlates with the appearance of nuclear p65 (Fig. 1B, bottom left panel). After 1 hour in the presence of TNF-α, IκBα protein begins to reappear as a result of the induction of gene transcription by NF-κB (Fig. 1B, top panel, lane 5). After pretreating HeLa cultures with DEX, we observed a small but measurable increase in IκBα protein (Fig. 1B, top panels; compare lanes 1 and 6). The average increase in IκBα protein was measured as 1.5-fold ($n = 10$). DEX pretreatment had no effect on cytoplasmic p65 amounts (*10*). In addition, DEX pretreatment slowed the disappearance of IκBα mediated by TNF-α. After 1 hour of TNF-α treatment, the amount of translocated nuclear p65 was reduced approximately 50% in DEX-treated cultures as compared with untreated cultures (Fig. 1B, bottom panels; compare lanes 5 and 10). THP-1 cultures induced with TNF or LPS gave similar results (*10*). We then considered whether DEX might induce other NF-κB–sequestering molecules such as the recently cloned IκBβ (*13*). HeLa cells cultured with DEX for 5 hours were compared with untreated HeLa cultures, and no differences were found in amounts of IκBβ (*10*). Whereas IκBβ is insensitive to TNF treatment, LPS induction for 2 hours is sufficient to induce release of NF-κB and IκBβ degradation (*13*). Pretreatment with DEX had no effect on either IκBβ amounts or LPS-induced IκBβ

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loss (*10*). Thus, DEX selectively increases the abundance and overall stability of the IκBα population.

In order to determine if the GC-mediated increase in IκBα protein was preceded by an increase in IκBα mRNA, Northern (RNA) blot analysis was done on mRNA derived from HeLa cultures treated with DEX for increasing periods of time. DEX induced a marked increase in IκBα mRNA abundance, which peaked by 1 to 2 hours (Fig. 2A) and in some cases remained elevated for over 20 hours (*10*). A similar pattern was observed with THP-1 cultures (*10*) and is consistent with other studies (*11*). We tested whether protein synthesis was necessary for this induction of the IκBα message. HeLa cultures were treated with DEX in the absence or presence of CHX, and IκBα mRNA amounts were compared by Northern blot analysis. CHX alone induced IκBα mRNA, as has been observed for other messages (Fig. 2B, lane 2). CHX plus DEX superinduced IκBα mRNA (Fig. 2B, lane 4), indicating that DEX does not function by inducing the synthesis of an intermediary factor which must then induce the IκBα message. These data suggest that GCs directly activate IκBα gene transcription. We tested this hypothesis by performing run-on transcription assays on HeLa and THP-1 cultures after DEX treatment for various periods of time (Fig. 2C). DEX clearly increased RNA polymerase occupancy on the IκBα gene within 15 min and for at least 2 hours. In order to quantitate the effect, we normalized the IκBα signal to an actin control, thereby measuring the increase in transcription as almost 10-fold (Table 1). We cannot discount the possibility that actin transcription may be slightly inhibited by DEX treatment, thus decreasing the amount of the induction, but the interpretation of the data remains unaffected. In addition, DEX had no effect on transcription of genes encoding c-Myc or TNF-α (*10*). TNF-α treatment, in comparison, induces both IκBα and IL-8 transcription (Fig. 2C and Table 1). No change in IκBα mRNA stability was detected as measured after actinomycin D treatment in the absence or presence of DEX (*10*). Preliminary data indicated that a IκBα promoter construct extending to position −600 was not activated by DEX (*10*), which suggests that the putative GC-responsive element is located further upstream or within the gene. Taken together, these data suggest that DEX increases IκBα protein abundance through an increase in gene transcription.

The increase in IκBα gene transcription induced by DEX was much greater than the average increase in IκBα protein abundance as measured by protein immunoblot. We thus wanted to determine if the rate of IκBα protein synthesis increased in a manner similar to that of the mRNA population. We metabolically labeled HeLa cells in the absence or presence of DEX, prepared whole-cell extracts, and immunoprecipitated with an antibody specific for either IκBα or p65. DEX induced a three- to fivefold increase in the rate of incorporation of ^{35}S-labeled methionine into the IκBα pool (Fig. 3A, compare lanes 1 and 3). The p65 antiserum immunoprecipitated almost as much labeled IκBα as did the IκBα antiserum, suggesting that the majority of newly synthesized IκBα was associated with p65 (Fig. 3A, compare lanes 3 and 4). Detection of coimmunoprecipitated p65 by the IκBα antiserum was obscured by a comigrating nonspecific band (Fig. 3A, lanes 1 to 3). In addition, the degree of incorporation of label into the p65 pool was

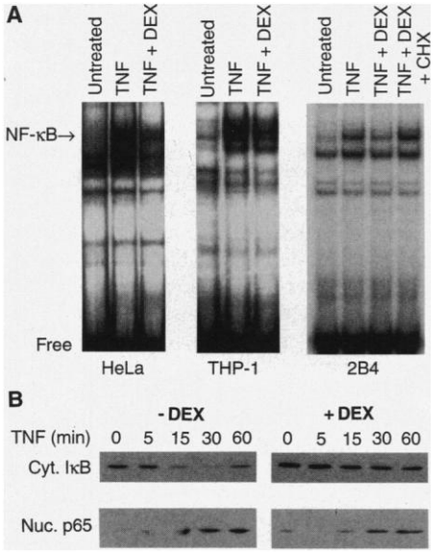

Fig. 1. DEX-mediated repression of NF-κB. (**A**) HeLa cultures were pretreated for 12 hours with 10^{-7} M DEX and stimulated for 1 hour with TNF-α (1 ng/ml). THP-1 cells and 2B4 murine T cell hybridomas were pretreated for 5 hours with 10^{-7} M DEX and stimulated for 1 hour with TNF-α (1 ng/ml). Nuclear extracts were analyzed by electrophoretic mobility-shift assay with the murine major histocompatibility complex class I NF-κB DNA binding probe UV21 as described (*6*). Data are representative of six independent experiments. (**B**) HeLa cultures were pretreated with 10^{-7} M DEX for 2 hours and stimulated with TNF-α (1 ng/ml) for periods of time shown at the top of the figure. Cytoplasmic (Cyt.) and nuclear (Nuc.) extracts were normalized for protein amounts and analyzed by immunoblotting with IκBα and p65 antibodies to peptide (obtained from Rockland, Boyertown, Pennsylvania).

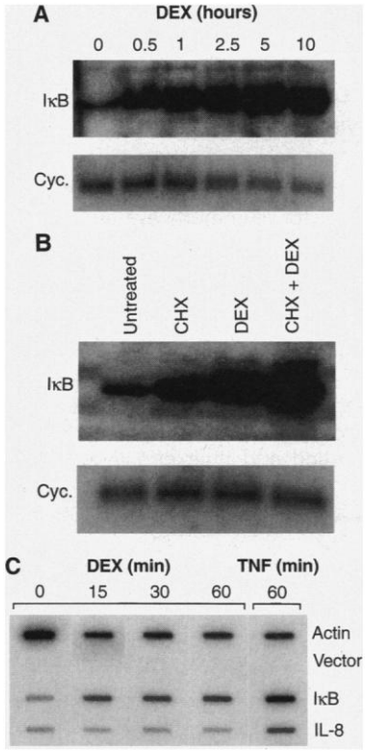

Fig. 2. DEX induces IκBα gene transcription. (**A**) Northern blot analysis of HeLa total RNA. HeLa cultures were treated for increasing periods of time with 10^{-7} M DEX and harvested for RNA. RNA was prepared as described (*17*). Equal amounts of RNA (10 μg) as determined by ethidium-stained 28*S* RNA were size separated by formaldehyde acrylamide gel electrophoresis, blotted to Zetaprobe (Bio-Rad), and processed according to the manufacturer's instructions. The blot was probed first with a *MAD-3*–IκBα complementary DNA (cDNA) (*8*) labeled by random priming. Subsequently, the blot was reprobed with a cyclophilin (Cyc.) cDNA (labeled as above) as a control RNA. Prehybridization and washing were done according to Bio-Rad's recommendations. (**B**) Cultures were treated with CHX (10 ng/ml) for 1 hour; 10^{-7} M DEX was then added to appropriate cultures for an additional 2.5 hours, and RNA was harvested and analyzed by Northern blot as described above. (**C**) Run-on transcription assays were done on cultures treated with either 10^{-7} M DEX or TNF-α (1 ng/ml) for varying periods of time as shown. Nuclei were isolated, and run-on transcriptions were done as described (*18*). The filters were exposed to a PhosphoImager (Molecular Dynamics) screen, and the bands were quantitated. IκBα and IL-8 transcription were normalized to actin transcription. The data are representative of four independent experiments.

Table 1. Fold activation of transcription normalized to actin. Number of experiments, *n*.

Condition	Average* (±SD) IκBα	Experiments (*n*)
Control	1.00 (0.07)	3
DEX (15 min)	9.88 (1.73)	2
DEX (30 min)	10.06 (3.74)	4
DEX (1 hour)	11.08 (4.66)	4
DEX (2 hours)	7.54 (0.59)	2
TNF-α (1 hour)	23.5 (17.77)	2

*Experiments such as those shown in Fig. 2C were quantitated with a PhosphoImager. Radioactivity was normalized to actin and expressed as fold increase over control.

much reduced as compared with IκBα, even though the two proteins contained similar numbers of methionines, indicating that the rate of p65 synthesis was low. These data indicate that newly synthesized IκBα is incorporated into preexisting NF-κB complexes, which is consistent with previous results (*14*). We tested this hypothesis further by labeling HeLa cultures for short periods of time and determining the efficiency of immunoprecipitation of IκB by the two antisera. After a 15-min pulse, extract was prepared and divided into identical aliquots, one of which was incubated with the IκBα antisera and the other with the p65 antisera. Once again, the IκBα signal derived from the p65 immunoprecipitation was similar to that derived from the IκBα immunoprecipitation (Fig. 3B, lanes 1 and 2). Analysis of the supernatants indicated that the antibodies accounted for all of the signal (Fig. 3B, Sup. 1 and Sup. 2). Thus, all of the surviving newly synthesized IκBα protein was rapidly associated with NF-κB. This is consistent with the observation that free IκBα is intrinsically unstable and rapidly degraded (*14*).

In the absence of DEX, TNF-α treatment results in the transient loss of IκBα, which allows NF-κB to translocate to the nucleus (*12*). We wanted to observe the effect of TNF-α treatment on the DEX-induced pool of newly synthesized IκBα associated with NF-κB. To this end, HeLa cultures were metabolically labeled in the absence or presence of DEX. TNF-α was then added and extracts were prepared at various times. The extracts were immunoprecipitated with the p65-specific antibody, and the resultant material was analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). As expected, TNF-α treatment in the absence of DEX resulted in the rapid loss of p65-associated IκBα (Fig. 4A). In the presence of DEX, the amount of newly synthesized IκBα associated with p65 was greatly increased (Fig. 4A; compare lanes 2 and 6). When treated with TNF-α and DEX, the amount of newly synthesized IκBα associated with p65 remained elevated (Fig. 4A). Thus, DEX treatment stabilizes the overall association of p65 with the pool of newly synthesized IκBα even in the presence of an inducer like TNF-α.

This result could be explained either by the inhibition by DEX of the TNF-α–mediated release of NF-κB from IκBα (for example, by affecting IκBα phosphorylation) or by the induction of the reassociation of newly synthesized IκBα with newly released NF-κB. These possibilities can be distinguished by performing a pulse-chase experiment and treating HeLa cultures with TNF-α during the chase. Under chase conditions, newly synthesized IκBα protein has a much lower specific activity. Thus, if DEX blocks the TNF-α–mediated release of NF-κB, the IκBα signal should remain relatively unchanged. If, in the presence of TNF-α, newly synthesized IκBα (low specific activity) replaces preexisting IκBα (high specific activity), then the IκBα signal should sub-

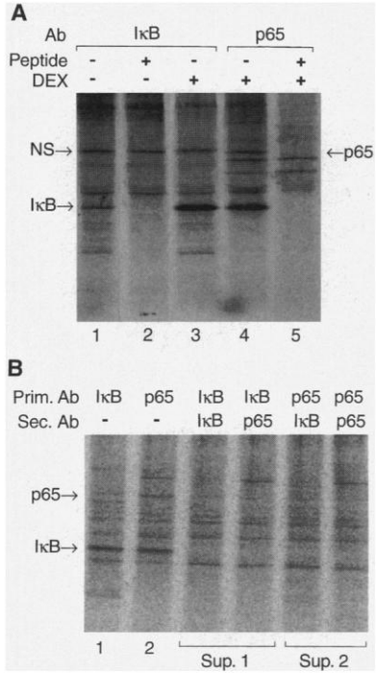

Fig. 3. DEX induces new IκB protein synthesis and association with preexisting p65. (**A**) HeLa cells were metabolically labeled for 2 hours in the absence or presence of 10^{-7} M DEX, and extracts were prepared as described (*19*). Immunoprecipitations were done overnight with 1 μl of IκBα or p65 antiserum (Ab) to peptide plus 20 μl of protein A–Sepharose (PAS) either in the absence or presence of 1 μl of competing peptide (1 mg/ml) as indicated. NS, nonspecific. Data are representative of seven independent experiments. (**B**) Six HeLa cultures were metabolically labeled for 15 min, and extracts were pooled and divided into two equal portions. Each was immunoprecipitated overnight with antiserum to peptide as above. PAS was pelleted and supernatants were collected, and each was divided into two equal portions. Supernatants derived from the IκBα immunoprecipitation (Sup. 1) and the p65 immunoprecipitation (Sup. 2) were reimmunoprecipitated as above [second antibody (Sec. Ab)]. Immunoprecipitations were analyzed as in (A).

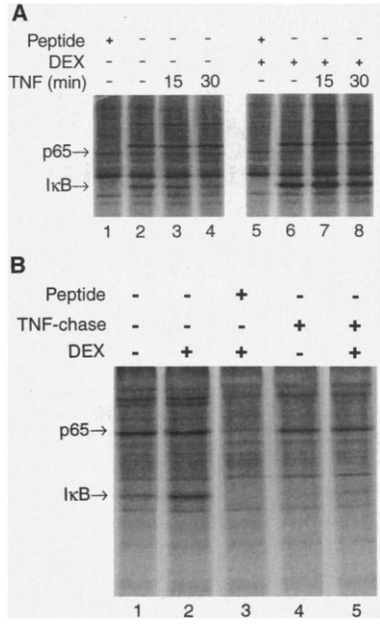

Fig. 4. Newly synthesized IκBα reassociates with preexisting NF-κB after TNF-α stimulation. (**A**) HeLa cultures were metabolically labeled in the presence or absence of 10^{-7} M DEX for 2 hours. TNF-α (1 ng/ml) was then added in the presence of carrier bovine serum albumin. Extracts were prepared by lysis in RIP buffer as described (*19*) and incubated with 1 μl of antiserum to p65 in the absence or presence of 1 μl of competing peptide as shown at the top of the panel. PAS pellets were extensively washed, and immunoprecipitated proteins were analyzed by SDS-PAGE. (**B**) HeLa cultures were metabolically labeled in the presence or absence of 10^{-7} M DEX as described above. Plates were washed with PBS and either harvested or treated with chase medium containing TNF-α (1 ng/ml). After 30 min, plates were harvested and extracts were incubated with antiserum to p65 and processed as described above.

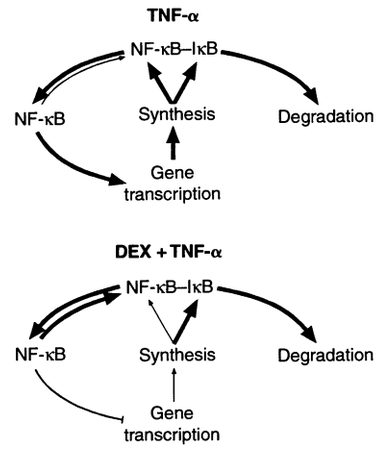

Fig. 5. A model illustrating how DEX treatment affects the NF-κB system as a whole. TNF-α induction results in the rapid degradation of IκBα and the nuclear translocation of NF-κB (upper diagram). NF-κB either reassociates with a newly synthesized IκBα molecule (thin arrow) or translocates to the nucleus. Nuclear NF-κB induces transcription of both p50 and IκBα genes, as well as effector molecules regulating the biological response. In the presence of DEX (lower diagram), IκBα synthesis is increased as a result of increased gene transcription. TNF-α–mediated nuclear translocation of NF-κB is inhibited by the reassociation of NF-κB with newly synthesized IκBα. In addition, DEX renders the remaining NF-κB that translocates to the nucleus unable to bind DNA (*5, 6*). Thick arrows represent induced pathways; thin blocked line represents an inhibited pathway.

stantially decrease. HeLa cultures were metabolically labeled in the absence or presence of DEX for several hours. The labeling medium was then replaced with chase medium containing DEX and TNF-α. Cultures were harvested and extracts were immunoprecipitated with the p65-specific antibody. Under these conditions, the IκBα signal rapidly disappeared (Fig. 4B). Thus, DEX does not appear to alter the signal transduction pathway leading to the induced dissociation of NF-κB and IκBα.

Together, these data indicate that DEX treatment induces the transcription of the IκBα gene. This induction results in the increased synthesis of IκBα protein. This increase in protein synthesis leads to the rapid turnover of IκBα protein associated with preexisting NF-κB complexes. In the presence of an activator such as TNF-α, newly released NF-κB reassociates with the DEX-induced IκBα and thus reduces the amount of NF-κB translocating to the nucleus. Additionally, newly synthesized IκBα may enter the nucleus and inhibit NF-κB DNA binding (*15*). A model of this process is shown in Fig. 5. Consistent with this model, we show that the GC-mediated inhibition of NF-κB induction by means of TNF-α is blocked by CHX (Fig. 1). Previously we and others showed that activated GR could physically associate with NF-κB subunits and that DEX represses the DNA binding activity of nuclear NF-κB (*5*, *6*). Here we demonstrate a second independent mechanism through which the NF-κB and GC signal transduction systems interact. As NF-κB is a critical regulator of cytokine genes, the inhibition of the activity of this transcription factor would effectively block cytokine secretion, thus explaining an immunosuppressive function of GCs. It has also been reported recently that salicylates, at concentrations corresponding to doses prescribed for arthritis patients, also block NF-κB activity (*16*). Thus, NF-κB activation serves as a target for two distinct immunosuppressive therapies. The presence of multiple levels of interaction between the NF-κB and GC systems suggests that these interactions may have evolved to serve a physiological role in the development of the immune system and in modulation of the immune response.

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Immunosuppression by Glucocorticoids: Inhibition of NF-κB Activity Through Induction of IκB Synthesis

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Glucocorticoids are among the most potent anti-inflammatory and immunosuppressive agents. They inhibit synthesis of almost all known cytokines and of several cell surface molecules required for immune function, but the mechanism underlying this activity has been unclear. Here it is shown that glucocorticoids are potent inhibitors of nuclear factor kappa B (NF-κB) activation in mice and cultured cells. This inhibition is mediated by induction of the IκBα inhibitory protein, which traps activated NF-κB in inactive cytoplasmic complexes. Because NF-κB activates many immunoregulatory genes in response to pro-inflammatory stimuli, the inhibition of its activity can be a major component of the anti-inflammatory activity of glucocorticoids.

Glucocorticoids (GCs) are physiological inhibitors of inflammatory responses and are widely used as immunosuppressive and anti-inflammatory agents (*1*). Interference with GC action or synthesis increases animal mortality after challenge with bacterial superantigens (*2*). GCs induce lymphocyte apoptosis (*1*, *3*) and inhibit synthesis of lymphokines (*4*) and cell surface molecules required for immune functions (*5*). In spite of the widespread use of GCs, the molecular mechanisms that underlie their therapeutic effects are poorly understood (*1*). GCs induce target genes through the glucocorticoid receptor (GR), a ligand-activated transcription factor (*6*). GCs repress gene expression through transcriptional interfer-

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