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IκBα-independent downregulation of NF-κB activity by glucocorticoid receptor

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IκBα is an inhibitor protein that prevents nuclear transport and activation of the transcription factor NF-κB. In acute inflammation, NF-κB is activated and increases the expression of several pro-inflammatory cytokine and chemokine genes. Glucocorticoids counteract this process. It has been proposed that the glucocorticoid-dependent inhibition of NF-κB activity is mediated by increased synthesis of IκBα which should then sequester NF-κB in an inactive cytoplasmic form. Here, we show by the use of a mutant glucocorticoid receptor and steroidal ligands that hormone-induced IκBα synthesis and inhibition of NF-κB activity are separable biochemical processes. A dimerization-defective glucocorticoid receptor mutant that does not enhance the IκBα level is still able to repress NF-κB activity. Conversely, glucocorticoid analogues competent in enhancing IκBα synthesis do not repress NF-κB activity. These results demonstrate that increased synthesis of IκBα is neither required nor sufficient for the hormone-mediated downmodulation of NF-κB activity.

Keywords: androgen receptor/anti-inflammation/ dissociating glucocorticoids/glucocorticoid receptor dimerization/oestrogen receptor

Introduction

NF-κB/Rel is a family of transcription factors that participate in the activation of immune-regulatory genes including cytokine, cell surface receptor and acute-phase genes (for reviews, see Grimm and Baeuerle, 1993; Verma *et al.*, 1995). Each member of the family has an ~300 amino acid Rel homology region with which it binds to DNA, dimerizes with other members or with itself, and interacts with inhibitory IκB proteins.

The IκB proteins share characteristic ankyrin repeat motifs which interact with the Rel proteins to mask their nuclear translocation signal and to keep them in an inactive cytoplasmic form (Beg *et al.*, 1992; Beg and Baldwin, 1993). The IκB proteins also constitute a family with their individual members differing in the spectrum of Rel dimers they bind to and in their response to various inducing signals (for reviews, see Thanos and Maniatis, 1995; Verma *et al.*, 1995; Baeuerle and Baltimore, 1996). With respect to NF-κB function, the most extensively studied protein in this family is IκBα. It interacts with all NF-κB/Rel heterodimers, as well as RelA and c-Rel homodimers (Urban and Baeuerle, 1990; Zabel and Baeuerle, 1990; Davis *et al.*, 1991; Kerr *et al.*, 1991; Liou *et al.*, 1992; Wulczyn *et al.*, 1992) and responds to all known NF-κB inducing signals (reviewed by Thanos and Maniatis, 1995). IκBα plays the major role in the cytoplasmic retention of NF-κB as haematopoietic tissues from mice in which the IκBα gene has been disrupted by homologous recombination show elevated levels of nuclear NF-κB and spontaneous transcription of several genes regulated by NF-κB (Beg *et al.*, 1995).

NF-κB inducing agents, e.g. double-stranded RNA, phorbol esters, tumour necrosis factor-α (TNFα), interleukin-1 (IL-1) and lipopolysaccharide (LPS), accelerate the degradation of cytosolic IκBα, thereby promoting the nuclear translocation of NF-κB/Rel dimers (Beg and Baldwin, 1993; Beg *et al.*, 1993; Grimm and Baeuerle, 1993; Liou and Baltimore, 1993). Following such an inducer-mediated stimulation, IκBα becomes hyperphosphorylated. This does not impair its ability to associate with NF-κB, but represents a signal for subsequent ubiquitination and degradation by the proteasome pathway (Finco *et al.*, 1994; Traenckner *et al.*, 1994; Alkalay *et al.*, 1995; Lin *et al.*, 1995). Degradation of IκBα and release of NF-κB/Rel dimers lead to transactivation of genes including MAD3, the IκBα gene itself. This therefore establishes an autoregulatory loop in which newly synthesized IκBα is thought to restore the cytoplasmic pool of latent NF-κB (Brown *et al.*, 1993; Grimm and Baeuerle, 1993; Le Bail *et al.*, 1993; Sun *et al.*, 1993).

Among the genes that are transcriptionally activated by NF-κB/Rel dimers are those encoding several inflammatory cytokines and chemokines such as IL-2, IL-6, IL-8, RANTES, and enzymes such as the inducible form of nitric oxide synthase, all of which feature prominently in inflammation. NF-κB is therefore a transcription factor central to many inflammatory processes (for a review, see Barnes and Adcock, 1993; Cato and Wade, 1996). Downmodulation of the activity of this transcription factor should ideally be useful in the treatment of many inflammatory processes (Neurath *et al.*, 1996).

Glucocorticoid hormones exert their potent anti-inflammatory action through inhibition of cytokine gene expression and inhibition of the synthesis of relevant adhesion molecules resulting in, for example, redistribution of lymphocyte traffic. Their anti-inflammatory potential correlates with their ability to interfere with the activity of transcription factors such as AP-1 and NF-κB (reviewed by Cato and Wade, 1996). For example, they inhibit the NF-κB-regulated expression of the gene that encodes intercellular adhesion molecule 1, a key protein in many inflammatory processes (Caldenhoven *et al.*, 1995). Elevated levels of NF-κB/Rel A also inhibit the activity of

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glucocorticoid receptor (GR) regulated genes (Ray and Prefontaine, 1994). The mutual nature of this cross-talk between the GR and NF-κB, as well as *in vitro* co-precipitation experiments, have suggested that these processes occur through GR–NF-κB interactions (Ray and Prefontaine, 1994). Interestingly, glucocorticoids also increase the synthesis of IκBα (Auphan *et al.*, 1995; Scheinman *et al.*, 1995a) which would possibly sequester NF-κB in an inactive cytoplasmic form.

To distinguish which of these two putative mechanisms is responsible for the glucocorticoid-dependent repression of NF-κB activity, we examined whether we can dissociate NF-κB repression from hormone-induced IκBα synthesis. We show that a mutant GR that does not enhance the synthesis of IκBα is still able to repress an NF-κB regulated reporter gene. Other steroids like oestradiol and androgen, that do not enhance IκBα synthesis, repress NF-κB regulated genes. Conversely, IκBα synthesis is enhanced by glucocorticoid analogues that cannot repress NF-κB activity. Together, these results demonstrate that hormone-mediated enhanced expression of IκBα and hormone-induced repression of NF-κB activity are two events that are neither related nor dependent on each other.

Results

Mutant glucocorticoid receptors

To investigate whether GR-induced synthesis of IκBα can contribute to repression of NF-κB activity, we analysed the transrepression function of two receptor mutants that are defective in transactivation: LS-7 which contains the double mutation P493R, A494S in the rat GR (Schena *et al.*, 1989; Helmberg *et al.*, 1995), and GR(D4X) which contains the exchanges N454D, A458T, R460D and D462C in the dimerization region of the human GR (Heck *et al.*, 1994).

These mutant receptors or their wild-type counterparts were stably transfected into ΩE fibroblasts that lack endogenous GR, and several clones were isolated and examined for their ability to repress NF-κB activity. The transrepression experiments were performed by transient transfection of the ΩE clones with an indicator construct containing multimerized NF-κB binding sites derived from the HIV 1-LTR linked to a minimal promoter driving a luciferase gene. NF-κB activity was induced by TNFα in the presence or absence of the activated receptor. The GR was activated by the synthetic glucocorticoid dexamethasone. Figure 1A shows the results of these experiments where the ratio of activity of the indicator plasmid in the presence of dexamethasone over its activity in the absence of hormone has been plotted. Both mutant receptors repressed NF-κB activity to almost the same extent as their wild-type counterparts.

If repression of NF-κB were caused by GR-mediated synthesis of IκBα, the mutant receptors that repress NF-κB activity should both induce IκBα synthesis. Immunoblots were performed as described by Auphan *et al.* (1995) after the cells had been treated with dexamethasone for 2.5 h and with TNFα for 0.5 h where indicated (Figure 1B). The treatment with TNFα amplifies the effect of the hormone as it transiently reduces the basal as well as the induced IκBα levels. In the immunoblot assays, we found the following: first, in cells transfected with the wild-type human and rat GRs, dexamethasone enhanced IκBα synthesis by a factor of 2–3 (Figure 1B, compare lanes 5 and 6 and lanes 13 and 14). This increase was evident even in the presence of TNFα (Figure 1B, compare lane 7 with 8 and lane 15 with 16). Second, cells containing the LS-7 mutant showed a reduced glucocorticoid-mediated activation of IκBα synthesis. This weak activation was not observed in the presence of TNFα treatment (Figure 1B, lanes 9–12). Third, cells containing the mutant GR(D4X) and the empty expression vector did not show any increase in IκBα synthesis in the presence of dexamethasone irrespective of treatment with TNFα (Figure 1B, lanes 1–4 and 17–20).

Thus, repression appears to occur in the absence of IκBα induction. As the data of Figure 1A show transcriptional activity over 30 h, while the immunoblot assays provide information on IκBα levels only at fixed time points, we performed kinetic experiments to examine the level of synthesis of IκBα over the whole period of 30 h incubation of the transrepression assay. Representative examples of these results are shown in Figure 1C and D. These results demonstrate that the glucocorticoid-mediated increase in IκBα synthesis is long-lived. In clones containing the wild-type GR, dexamethasone induced IκBα synthesis over 15 h (Figure 1C, lanes 1–9). This effect tapers off gradually, but an increase is still noticeable at 30 h of treatment (Figure 1C, compare lane 10 with lane 11). Similar kinetic results were obtained from clones containing the LS-7 mutant, although the level of dexamethasone-induced synthesis of IκBα was reduced (results not shown). In contrast, clones containing the GR(D4X) mutant receptor or the empty receptor expression vector showed no hormone-mediated increase in IκBα synthesis over the same period of time (Figure 1C, lanes 1–11). As the ΩE cells were treated simultaneously with dexamethasone and TNFα in the transrepression assay, we examined the effect these conditions would have on IκBα synthesis. TNFα first causes a rapid decrease of the IκBα level (0.5 h), followed by an increase (1.5–4 h). There is a second phase of IκBα reduction (6–15 h) before steady-state levels are reached. We have no explanantion for the second downregulation (Figure 1D, lanes 1–11). Simultaneous treatment of the clones transfected with the wild-type GR with dexamethasone and TNFα did not drastically alter the overall IκBα protein level (Figure 1D, lanes 1–11). (The reduced IκBα level in lane 11 of the experiment in Figure 1D is due to a protein blotting failure.) The results of these kinetic studies show that IκBα levels stay at almost the same level all through the 30 h incubation period in clones containing the wild-type (Figure 1D) and the LS-7 mutant receptors (results not shown). In clones containing the mutant GR(D4X), the kinetics of IκBα synthesis after simultaneous treatment with dexamethasone and TNFα did not differ from the results with TNFα alone (Figure 1D, lanes 1–11).

The inability of the two GR mutants to elevate IκBα levels, as shown in the immunoblot experiments, prompted us to examine their ability to enhance the activity of a glucocorticoid-responsive mouse mammary tumour virus (MMTV) reporter construct transiently transfected into the ΩE clones. In these experiments, the LS-7 transfected cells showed a severely reduced yet measureable activity in enhancing expression at the MMTV promoter (Figure

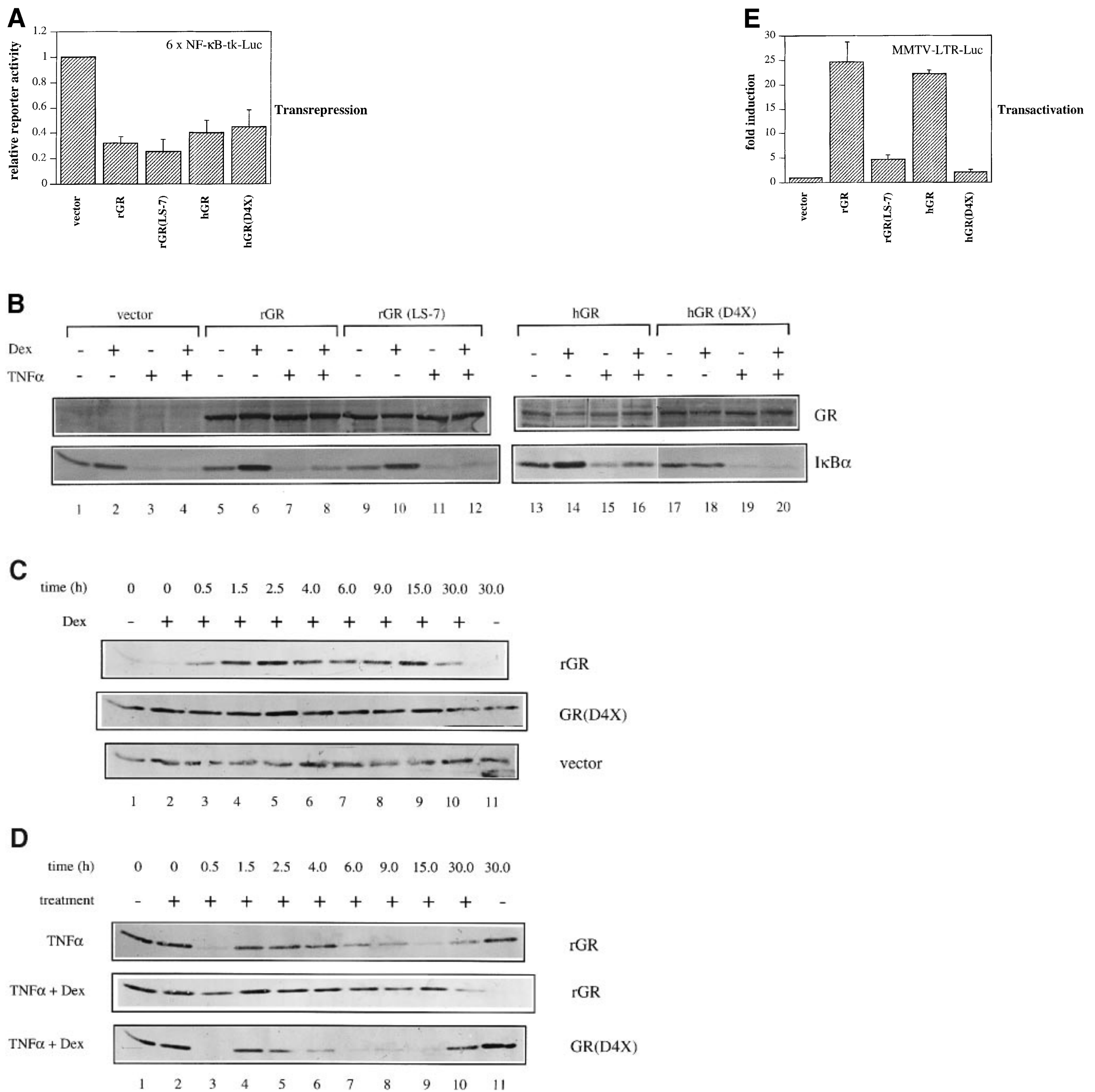


Fig. 1. Dexamethasone-induced synthesis of IκBα is not necessary for repression of NF-κB activity. ΩE cells were stably transfected with expression vectors which were either empty or contained cDNAs encoding the wild-type rat GR, mutant rat GR (LS-7), wild-type human GR or mutant human GR (D4X) sequences. Single clones were isolated and analysed by immunoblotting or transient transfection assay. (**A**) Transrepressing activities of the wild-type and mutant GR expressing clones. Transrepression experiments were performed by transfecting 7×10^5 ΩE cells/9 cm culture dish with 5 μg of the construct 6xNF-κB-tk-Luciferase. The transfected cells were treated with TNFα (5 ng/ml) in the presence or absence of 10^{-7} M dexamethasone. The cells were harvested 30 h after treatment and luciferase assays were performed with equal amounts of protein. The results are derived from the ratio of the level of luciferase activity induced by TNFα in the presence of dexamethasone over the value in the absence of this hormone. This ratio was expressed relative to unity which was the value assigned to the results obtained in cells containing the empty expression vector. The bar charts are the average values and standard deviations of the relative reporter activity measured in at least three independent experiments. (**B**) Immunoblot analysis of protein extracts from the indicated stably transfected ΩE cells. The cells were either treated for 2.5 h with 10^{-7} M dexamethasone or pre-treated for 2 h with dexamethasone and then TNFα (5 ng/ml) was added for 0.5 h before the end of the hormone treatment. The cells were harvested and disrupted, and protein extracts were analysed for the presence of IκBα and GR by the immunoblotting technique. The IκBα antibody was used at a dilution of 1:1000 and the GR antibody at 1:100. (**C**) and (**D**) Immunoblot analysis of protein extracts from the indicated stably transfected ΩE cells with an antibody against IκBα (1:1000). The cells were treated without or with 10^{-7} M dexamethasone and/or TNFα (5 ng/ml) for the indicated time. Thereafter, they were harvested and disrupted. Extracts with equal amounts of protein were analysed for the presence of IκBα by the immunoblotting technique. (**E**) Transactivation activities of the wild-type and mutant GR-expressing clones. 7×10^5 ΩE cells/9 cm culture dish were transiently transfected with 9 μg of the MMTV-LTR-Luc reporter construct pHC_{wt}-Luc and treated without or with 10^{-7} M dexamethasone. Thirty hours after treatment, the cells were harvested and disrupted, and cellular extracts with equal amounts of protein were used for luciferase assay. The results are presented as the level of induced expression of the reporter construct relative to unity which was the value obtained in cells containing the empty expression vector. The bar chart represents the mean and standard deviation of at least three independent experiments.

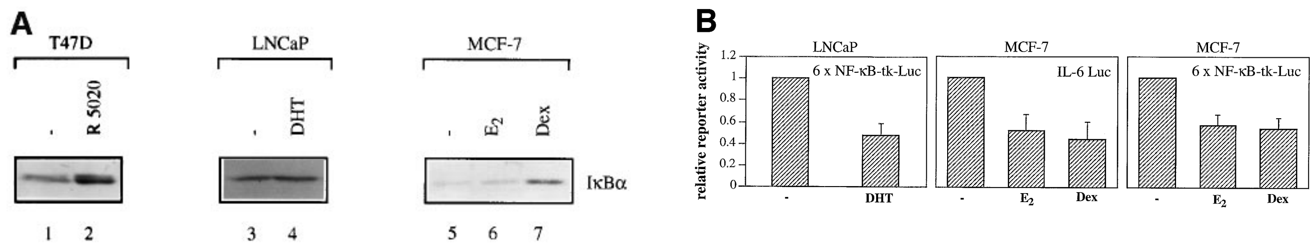


Fig. 2. Effect of progestin, androgen and oestradiol on synthesis of IκBα and repression of NF-κB regulated genes. (**A**) Immunoblot analysis with protein extracts from T47D, LNCaP and MCF-7 cells. T47D cells were treated with 10^{-7} M of the progestin R5020, LNCaP cells with 10^{-7} M dihydrotestosterone (DHT) and MCF-7 cells with 10^{-8} M 17β-oestradiol (E_2) or 10^{-7} M dexamethasone (Dex) for 2.5 h. The cells were harvested and disrupted, and the protein extracts were analysed for the level of IκBα with an anti-IκBα antibody (1:1000). (**B**) Transrepression of NF-κB regulated genes by DHT and E_2. 7×10^5 LNCaP cells in 9 cm culture dishes were transiently transfected with 5 μg of the 6xNF-κB-tk-Luciferase reporter plasmid. The transfected cells were treated with TNFα (5 ng/ml) in the presence or absence of 10^{-7} M DHT. Thirty hours after treatment, the cells were harvested, cellular extracts were prepared and equal amounts of protein were used for luciferase activity measurements. In the case of MCF-7 cells, the same numbers of cells were transiently transfected with 10 μg of the IL-6-Luciferase construct –158/+11 IL-6-Luc or with 5 μg of the 6xNF-κB-tk Luciferase reporter plasmid. Twenty-four hours after transfection, the cells were pre-treated for 30 min with or without E_2 (10^{-8} M) or dexamethasone (10^{-7} M). Thereafter, TNFα was added to a final concentration of 5 ng/ml. Eight hours later, the cells were harvested and cellular extracts with equal amounts of protein were employed for assay of luciferase activities. The results are calculated from the ratio of the level of luciferase activity induced by TNFα in the presence of hormone over the value in the absence of hormone. This ratio was presented relative to unity, which was the value assigned to the luciferase activity measured in cells treated with TNFα in the absence of hormone. The bar charts represent the averages and standard deviations of results from at least three independent experiments. Cells that received no hormone treatment were exposed to the same amount of vehicle (80% ethanol solution) as that used in the hormone treatment.

1E). The LS-7 mutant is, therefore, not completely defective in transactivation. Cells transfected with the dimerization-defective GR(D4X) mutant, on the other hand, exhibited an almost insignificant effect on MMTV promoter activity compared with cells containing the empty expression vector (Figure 1E). This agrees with the inability of the GR(D4X) mutant to enhance the synthesis of IκBα. As this mutant is nevertheless fully competent in repressing the activity of a NF-κB regulated gene (Figure 1A), we conclude that an increased level of IκBα is not necessary for the repression of NF-κB activity.

Repression by 17β-oestradiol and dihydrotestosterone

Nuclear receptors share the ability to repress genes that are regulated by AP-1 and NF-κB (Jonat *et al.*, 1990; Schüle *et al.*, 1990; Yang-Yen *et al.*, 1990; Zhang *et al.*, 1991; Caldenhoven *et al.*, 1995; Stein and Yang, 1995; Kalkhoven *et al.*, 1996; Keller *et al.*, 1996). Thus, the GR-mediated repression of NF-κB and increased synthesis of IκBα are expected to be mimicked by other nuclear receptors if IκBα synthesis were required for repression. In immunoblot assays, the progestin R5020 indeed enhanced IκBα synthesis in the progesterone receptor-positive human mammary cell line T47D (Figure 2A, compare lanes 1 and 2) which correlates with its negative effect on NF-κB activity (Kalkhoven *et al.*, 1996). In contrast, the androgen dihydrotestosterone (DHT) did not enhance IκBα synthesis in human prostatic carcinoma LNCaP cells, nor did 17β-oestradiol enhance the synthesis of IκBα in human mammary tumour MCF-7 cells, although both cell lines contain functional androgen and oestrogen receptors (Figure 2A, compare lane 3 with 4 and lane 5 with 6). As a control, the MCF-7 cells were treated with dexamethasone as these cells contain a functional GR in addition to oestrogen receptor and this treatment enhanced the level of IκBα (Figure 2A, compare lanes 5 and 7).

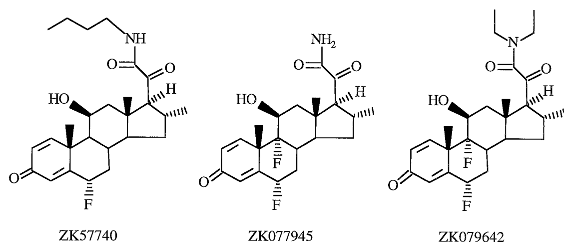
To demonstrate that DHT and oestradiol repress NF-κB activity despite the fact that they do not induce synthesis of IκBα, we investigated the ability of these two hormones to repress the activity of NF-κB regulated indicator genes. To this end, we transiently transfected a tk-luciferase gene construct driven by a multimerized NF-κB site or a NF-κB regulated IL-6 luciferase construct into the LNCaP and MCF-7 cells. Although DHT and oestradiol do not induce IκBα synthesis, they both repressed NF-κB activity (Figure 2B). In the case of oestradiol, the level of repression was almost the same as that achieved by dexamethasone. These results indicate that the repression of NF-κB activity by androgen and oestradiol does not involve a steroid hormone-mediated increase in IκBα synthesis.

Dissociating glucocorticoids

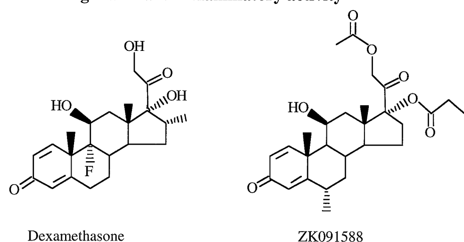
A third approach to demonstrate that hormone-induced IκBα synthesis and repression of NF-κB are unrelated events also proved successful. We were able to establish the reverse situation where hormone-induced synthesis of IκBα occurs in the absence of downregulation of NF-κB regulated genes. We achieved this by the use of steroids that transactivate but do not transrepress.

Three glucocorticoid analogues (ZK57740, ZK077945 and ZK079642) (Figure 3) that had been selected on the basis of their lacking anti-inflammatory activity in an animal model (H.Zentel, unpublished) were examined for their ability to transactivate and to transrepress. This was achieved in Northern blot analyses carried out with different cell lines. For transactivation, glucocorticoid-induced transcription of metallothionein IIa (MTIIa) (Karin *et al.*, 1984) in HeLa cells was analysed. For transrepression, downregulation of both TPA-induced collagenase I RNA in Hela cells (Jonat *et al.*, 1990) and LPS-induced transcription of IL-1β in THP-1 human monocytic cells (Lee *et al.*, 1988) was measured. The collagenase I gene requires the transcription factor AP-1 for its function. The IL-1β gene is regulated by the transcription factors C/EBPβ and CREB. Both genes are negatively regulated by the GR (Angel *et al.*, 1987; Lee *et al.*, 1988; Jonat *et al.*, 1990; Shirakawa *et al.*, 1993; Tsukada *et al.*, 1994). In the transactivation studies, ZK57740 and ZK077945 significantly enhanced the expression of MTIIa albeit with a reduced efficiency

Glucocorticoid analogs: no anti-inflammatory activity



Glucocorticoid analogs: with anti-inflammatory activity



Glucocorticoid antagonists: no anti-inflammatory activity

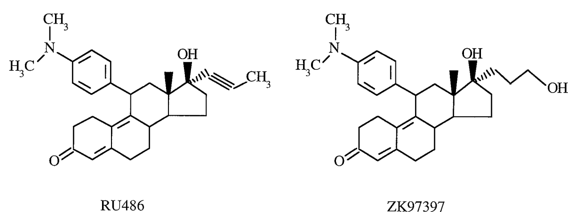


Fig. 3. Structure of various steroid analogues. The structures are presented of various steroid analogues classified into three groups based on their anti-inflammatory activities as determined by the croton oil-induced oedema test of the ear of rats (Schering AG, unpublished results). In the case of ZK091588 (methylprednisolone aceponate), its anti-inflammatory activity has previously been published (Zentel and Töpert, 1994). Information on RU486 and ZK97397 as GR antagonists with relatively high affinity for the receptor can be obtained from Wehle *et al.* (1995).

compared with dexamethasone (Figure 4A). ZK079642 did not alter the transactivation potential of the GR (Figure 4A). In the repression studies, ZK57740 and ZK077945 were found to be severely defective in their ability to downregulate collagenase I and IL-1β expression, whereas ZK079642 did not repress at all (Figure 4B and C). These transrepression experiments, together with the transactivation results, indicate that ZK57740 and ZK077945 partially dissociate transactivating and transrepressing properties of the endogenous GR.

ZK57740, ZK077945 and other glucocorticoid analogues from different classification groups (Figure 3) were analysed for their abilities to enhance the synthesis of IκBα protein and the promoter activity of MMTV, as well as to repress NF-κB activity in the same cell line. In an immunoblot assay with protein extracts from ΩE cells stably transfected with the wild-type rat GR, the two dissociating ligands (ZK57740 and ZK077945), as well as ZK091588 and dexamethasone, enhanced the synthesis of IκBα after 2.5 h. [In kinetic experiments in the presence of TNFα, no overall increased synthesis of IκBα was observed over 30 h (results not shown).] The other steroid analogues RU486, ZK97397 and ZK079642 did not alter the level of synthesis of IκBα at 2.5 h (Figure 5A) nor over 30 h (results not shown). The steroids that temporarily enhanced IκBα synthesis also mediated a GR-induced activation of the MMTV indicator gene in a transient transfection assay in the same ΩE cells (Figure 5B). The most decisive results were in the transrepression assay. Only dexamethasone and ZK 091588 showed a clear inhibition of NF-κB activity (Figure 5C). The dissociating ligands ZK57740 and ZK077945 that enhanced IκBα activity did not show any significant repression of NF-κB activity (Figure 5C). RU486, ZK97397 and ZK079642 did not enhance IκBα synthesis nor were they able to repress NF-κB activity (Figure 5C). The fact that ZK57740 and ZK077946 enhanced IκBα synthesis but did not show any significant repression of NF-κB activity demonstrates that hormone-mediated increase in IκBα synthesis is not sufficient for repression of NF-κB activity.

The IκBα promoter is responsive to steroid hormones

The glucocorticoid-mediated increase in IκBα protein synthesis can occur either through IκBα mRNA stabilization or at the level of transcription of the gene coding for IκBα. To find out which of the two possibilities holds true, we first treated HeLa cells with actinomycin D for 15 min to block *de novo* transcription and subsequently measured the decay of the pre-existing IκBα mRNA in the absence and presence of dexamethasone. In the absence of dexamethasone, the half-life of IκBα was ~30 min (Figure 6A). This rate of IκBα mRNA decay was not altered in the presence of dexamethasone (Figure 6A). Pretreatment with actinomycin D prevented dexamethasone-induced synthesis of IκBα mRNA (Figure 6A, compare lanes 9 and 10 with 11 and 12). This indicates that the effect of dexamethasone is mainly at the transcriptional level and does not affect the stability of the IκBα mRNA.

To determine whether the cloned IκBα promoter (Ito *et al.*, 1994) contains the necessary signals for the hormonal regulation, we transiently transfected a luciferase reporter plasmid driven by the IκBα promoter sequence (positions –623 to +11) into HeLa cells and treated the transfected cells with dexamethasone, or as a positive control, with TPA to enhance NF-κB activity. Dexamethasone alone enhanced the activity of the construct by a factor of 2 (Figure 6B), possibly through a GRE in the promoter region since the empty expression vector did not respond to dexamethasone (results not shown). TPA also enhanced the activity of the construct, possibly through the NF-κB sites in the IκBα promoter (Ito *et al.*, 1994) (Figure 6B). Treatment of the transfected cells with a combination of dexamethasone and TPA led to more than an additive effect, indicating a cooperative action of these inducers through their respective response elements on the IκBα promoter. Such a cooperative action of factors that normally antagonize each other's activity has been reported for the GR and AP-1 sites on the hMTIIa gene (Jonat, 1990; Wade *et al.*, 1995).

These results demonstrate that the hormone-induced synthesis of IκBα results from transcriptional activation of the gene encoding this protein. However, the enhanced expression of IκBα is not a prerequisite for the hormone-induced repression of NF-κB regulated genes.

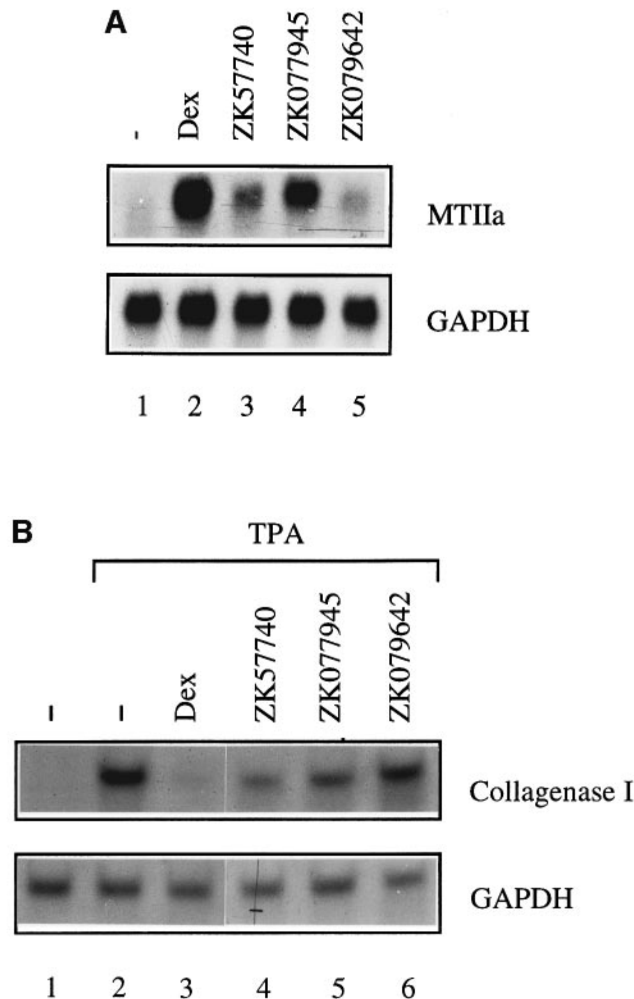


Fig. 4. Identification of glucocorticoid analogues with dissociated transactivating and transrepressing activities. (**A**) Northern blot analysis of RNA isolated from HeLa cells. 5 μg poly(A)$^+$ RNA from HeLa cells treated for 2 h without or with 10^{-7} M dexamethasone, ZK57740, ZK077945 or ZK079642 were separated on a 1% agarose gel and transferred onto nylon membranes. The membranes were hybridized with radioactively labelled DNA fragments specific for human metallothionein IIa and GAPDH. (**B**) Northern blot analysis of RNA isolated from HeLa cells after treatment with TPA and various steroidal ligands. 5 μg poly(A)$^+$ RNA from HeLa cells treated for 4 h without or with 10^{-7} M dexamethasone, ZK57740, ZK077945 or ZK079642 in the presence of the phorbol ester TPA (80 ng/ml) were separated on a 1% agarose gel. The RNA was transferred onto a nylon membrane and hybridized with radioactively labelled DNA fragments specific for human collagenase I and GAPDH. (**C**) Northern blot analysis of RNA isolated from THP-1 human monocytic cells. 5 μg poly(A)$^+$ RNA from THP-1 cells treated for 5 h without or with 10^{-7} M dexamethasone, ZK57740, ZK077945 or ZK079642 in the presence of 10 μg/ml LPS were separated on a 1% agarose gel. The RNA was transferred onto a nylon membrane and hybridized with radioactively labelled DNA fragments specific for human IL-1β and GAPDH.

Discussion

NF-κB controls genes encoding pro-inflammatory cytokines and chemokines, and is therefore an important regulatory factor in inflammatory processes. Downregulation of its activity, as occurs in the presence of glucocorticoids, is of immense scientific and medical interest.

Two types of data have led to suggestions on how NF-κB activity may be downregulated: through protein–protein interaction of NF-κB subunits and the GR (Ray and Prefontaine, 1994; Caldenhoven *et al.*, 1995; Scheinman *et al.*, 1995b) or through GR-induced synthesis of IκBα (Auphan *et al.*, 1995; Scheinman *et al.*, 1995a). Newly synthesized IκBα would inactivate NF-κB function by sequestering it into an inactive cytoplasmic form. For the latter hypothesis, a positive regulatory action of the GR would indirectly exert a repressive action on NF-κB activity.

Our data have shown that glucocorticoid induces synthesis of IκBα, but that the elevated IκBα is not necessary for downregulation of NF-κB activity. This conclusion is based on the following experimental results: a dimerization-defective mutant GR, that does not transactivate glucocorticoid-responsive genes nor enhance IκBα synthesis, is still able to repress NF-κB activity. The failure of the dimerization-defective GR to induce IκBα synthesis indicates that homodimer formation and possibly recognition of one or several classical glucocorticoid response elements (GREs) are needed for the enhanced synthesis of IκBα. This idea is supported by the fact that the progesterone receptor (PR) that recognizes a GRE (von der Ahe *et al.*, 1985) also enhances IκBα synthesis. In contrast, the androgen receptor (AR) that preferentially recognizes a related but different sequence (Adler *et al.*, 1991, 1992; Claessens *et al.*, 1996) and the oestrogen receptor (ER) that does not recognize the GRE at all (Beato, 1989) are unable to increase IκBα synthesis. Our data with oestradiol confirm an earlier report that oestradiol repression of NF-κB regulated expression of the IL-6 gene occurs in the absence of an increased synthesis of IκBα (Stein and Yang, 1995). Recently, another laboratory found that DHT-mediated repression of IL-6 gene expression is accompanied by a 69% increase in IκBα synthesis in LNCaP cells (Keller *et al.*, 1996). This differs from our results as we did not find any increased IκBα synthesis after DHT treatment of the same cell line. The reason for the differences in the two experimental results is at the moment not clear.

We have further shown in our studies that glucocorticoid analogues that do not repress NF-κB regulated genes can still enhance IκBα synthesis. This establishes that a hormone-mediated increase in IκBα levels is not required

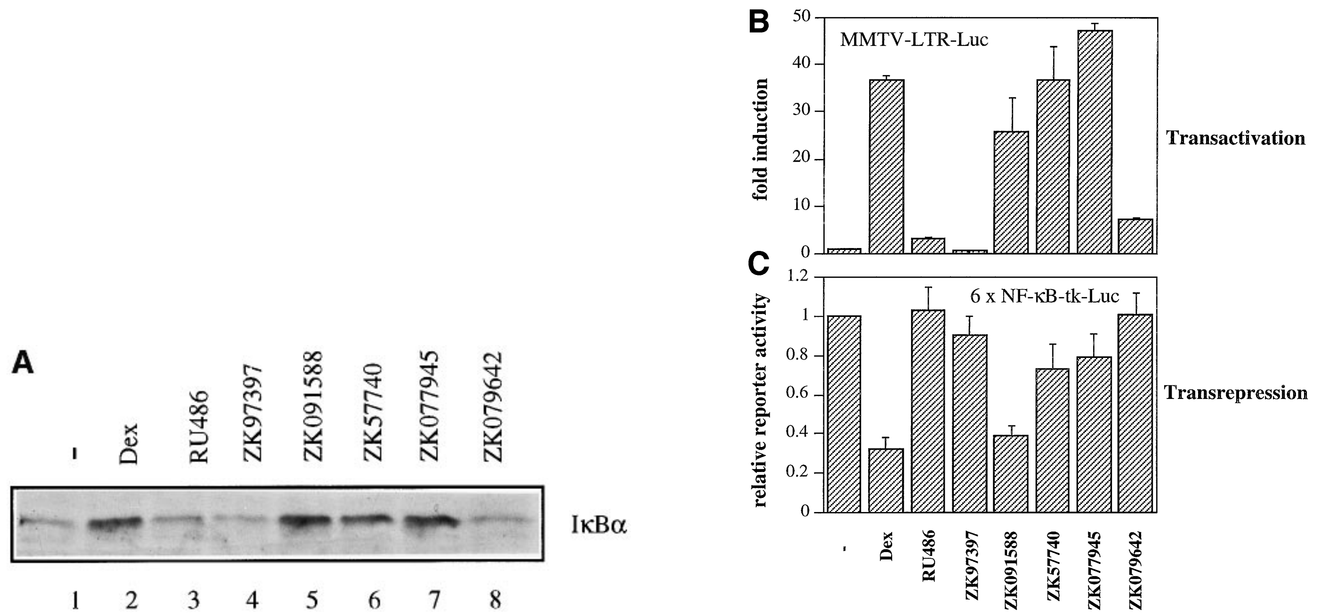


Fig. 5. Increased synthesis of IκBα by various steroid analogues in stably transfected ΩE cells and its effect on transrepression of NF-κB regulated genes. (**A**) Immunoblot analysis with protein extracts from ΩE cells stably transfected with the wild-type rat GR and treated without or with 10^{-7} M dexamethasone, RU486, ZK97397, ZK091588, ZK57740, ZK077945 or ZK079642 for 2.5 h. In the last half-hour of treatment, TNFα was added to a final concentration of 5 ng/ml. The cells were harvested and disrupted, and protein extracts were analysed for the presence of IκBα with the use of an anti-IκBα antibody (1:1000 dilution). (**B**) Transactivation assay in ΩE cells stably transfected with the wild-type rat GR expression vector. The transactivation experiments were carried out transiently with 7×10^5 cells/9 cm culture dish with 9 μg MMTV-LTR-Luciferase reporter plasmid. Immediately after transfection, the cells were treated without or with 10^{-7} M of the indicated glucocorticoid analogues. The transfected cells were harvested 30 h thereafter, disrupted and extracts with the same amount of protein were used for the determination of luciferase activity. The results were presented as the level of induced expression of the reporter gene relative to unity, which was the luciferase activity measured in the absence of hormone. The bar chart represents the mean and standard deviation of four independent experiments. (**C**) Transrepression assay in ΩE cells stably transfected with the wild-type rat GR expression vector. Transrepression experiments were performed by transfecting the same number of cells as above with 5 μg of the 6xNF-κB-tk-luciferase reporter plasmid. The transfected cells were treated with TNFα (5 ng/ml) in the presence or absence of 10^{-7} M of the indicated glucocorticoid analogues. Thirty hours later, the cells were harvested and disrupted, and equal amounts of protein from the cellular extracts were used in the determination of luciferase activity. The results are calculated from the ratio of the level of luciferase activity induced by TNFα in the presence of hormone over the value in the absence of hormone. This ratio was expressed relative to unity, which was the value assigned to the luciferase activity measured in the absence of hormone. The bar charts are the averages of the residual reporter activity with standard deviations from four independent experiments. Cells that received no hormone treatment received the same amount of an 80% ethanol solution into which the hormones had been dissolved.

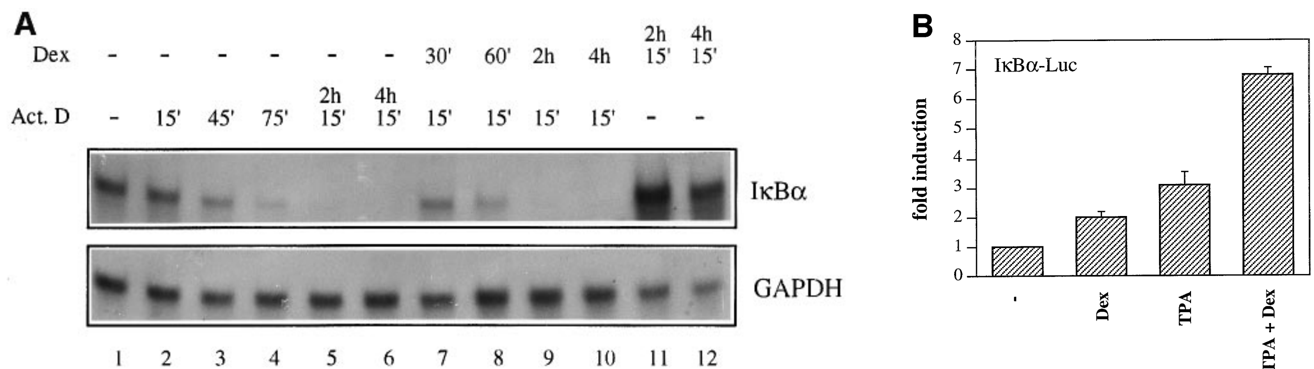


Fig. 6. Hormone-induced synthesis of IκBα occurs at the promoter level. (**A**) Northern blot analysis of RNA from HeLa cells. 7 μg poly(A)$^+$ RNA from HeLa cells treated for the indicated time points without or with 10^{-7} M dexamethasone and actinomycin D (5 μg/ml) were separated on a 1% agarose gel. In lanes 2–6, the cells were treated for 15 min, 45 min, 75 min, 2 h 15 min and 4 h 15 min with actinomycin D before RNA preparation. In lanes 7–10, the cells were pre-treated with actinomycin D for 15 min and then simultaneously with actinomycin D and dexamethasone for an additional 30 min, 60 min, 2 h and 4 h. In lanes 11 and 12, the cells were treated with dexamethasone for 2 h 15 min and 4 h 15 min, respectively. RNAs isolated from cells after the various treatments were transferred onto a nylon filter and hybridized with radioactively labelled DNA fragments specific for IκBα and GAPDH. (**B**) Transient transfection of an IκBα promoter construct. 7×10^5 HeLa cells per 9 cm culture dish were transiently transfected with 5 μg of the IκBα-Luciferase reporter construct pGL2 MAD3-Luc and treated without hormone, with TPA (80 ng/ml) or with 10^{-7} M dexamethasone, or both TPA and dexamethasone for 24 h. Thereafter, the cells were harvested and disrupted, and cellular extracts with equal amounts of protein were used for luciferase assay. The results are expressed as the level of induced expression of the reporter construct relative to unity, which was the nominal value assigned to the luciferase activity in the absence of hormone. The bar chart represents the mean and standard deviation of at least three independent experiments.

for downregulation of NF-κB regulated genes. We have also investigated how hormone-induced expression occurs at the IκBα promoter. Actinomycin D inhibition studies show that the effect of glucocorticoids is at the transcriptional level. The human IκBα promoter carries the motif **G**GACGAAGCC**AGTTCT** at positions –93 to –73 which contains the conserved hexanucleotide motif A/TGTTCT (underlined) and three of the four contact residues (in bold) for GR binding to DNA (Beato, 1989; Scheidereit *et al.*, 1989). This receptor binding motif is possibly responsible for the glucocorticoid response of the IκBα promoter in HeLa cells. On the human IκBα promoter, this putative GRE is localized between two NF-κB sites (Ito *et al.*, 1994) and possibly acccounts for the cooperative effect of TPA and dexamethasone which we measured at this promoter. This would agree with reports of cooperative action of a GRE with binding sites for other transcription factors in several promoters (Cato *et al.*, 1988; Schüle *et al.*, 1988; Strähle *et al.*, 1988).

An important question to be answered concerns the biological significance of the increased synthesis of IκBα by glucocorticoids. At the moment, we cannot associate any biological relevance with the hormonal regulation of IκBα synthesis. Although we can confirm that dexamethasone induces increased synthesis of IκBα protein 2- to 3-fold, as reported by other investigators (Auphan *et al.*, 1995; Scheinman *et al.*, 1995a), in the presence of TNFα no net increase in IκBα synthesis occurs over 30 h. Under these conditions, and within the same time period, NF-κB activity is repressed by the GR. These findings make it difficult to relate hormone-induced IκBα synthesis with the repression of NF-κB activity by glucocorticoids. Could another IκB protein which is similarly regulated by glucocorticoids as IκBα be responsible for downregulation of NF-κB activity? No IκB protein other than IκBα has been reported to be regulated by glucocorticoids (Auphan *et al.*, 1995). Further, our experiments that dissociate increased IκBα synthesis and repression of NF-κB activity make it unlikely that another IκB protein, e.g. IκBε, is involved. Despite the fact that IκBε shares many properties with IκBα and is a potent inhibitor of NF-κB regulated genes (for a review, see Baeuerle and Baltimore, 1996), it could only be considered for NF-κB repression after hormone treatment if its mode of regulation were totally different from what we have discussed here (e.g. if it were transcriptionally upregulated by our GR mutant or by androgen and oestradiol).

In the course of the experiments described here, we noted a number of features of the GR that may be relevant in anti-inflammation. Anti-inflammatory capacity is strongly correlated with the transrepressing activity of the GR on NF-κB and AP-1 regulated genes. Glucocorticoid analogues that did not show anti-inflammatory activities in animal experiments were also incapable of inhibiting NF-κB as well as AP-1 regulated genes. Nonetheless, the same glucocorticoid analogues mediated GR-dependent transactivation, showing that the transrepressing function of the receptor is the decisive parameter in the anti-inflammatory action of the receptor. The transrepressing function of the GR can be separated from its transactivation properties by the use of a dimerization-defective GR mutant that did not transactivate but repress NF-κB and AP-1 activities (this study and Heck *et al.*, 1994). Thus, the molecular features of the receptor responsible for repression of NF-κB and AP-1 activities are linked. This introduces a practical aspect in the anti-inflammatory function of the GR. It appears that only one mechanism of repression by the GR is needed for anti-inflammatory processes and this is what needs to be considered in drug development.

From the above considerations, it is tempting to postulate that while the negative regulatory activity of the GR is essential for its anti-inflammatory response, its positive action may be responsible for the unwarranted side-effects of glucocorticoid therapy. The separation of the positive and negative actions of GR that we have achieved in this work offers itself for further improvement of the pharmacological properties of glucocorticoids.

Materials and methods

Plasmid constructs

Vectors expressing the wild-type rat GR (rGR) and the LS-7 mutant receptor were constructed by ligating the blunt-ended *Xba*I rat GR- and LS7-fragments of the expression vectors pRc/βact-GR-wt and pRc/βact-GR-LS7 (Helmberg *et al.*, 1995) into the *Bam*HI-linearized, blunt-ended pBABE Puro expression vector (Morgenstern and Land, 1990).

Vectors expressing the wild-type human GR and the mutant hGR (D4X) were constructed by first linearizing the plasmids phGRSB and phGR(D4X) (Heck *et al.*, 1994) with Asp718, followed by a filling-in reaction with DNA polymerase. A 3 kb fragment was isolated after a prior cleavage of the linearized vector with *Xho*I. The Asp718/*Xho*I fragment was cloned into the vector pBABE Puro after linearization with *Bam*HI, followed by a fill-in reaction and a second digestion with *Sal*I.

The construct 6xNF-κB-tk-Luciferase (3 Enh-tk-Luc) is described by Israël *et al.* (1992) and the construct IL-6-Luciferase (–158/+11 IL-6-Luc) by Stein and Yang (1995).

The IκBα (–623/+11)-Luciferase construct (pGL2 MAD 3-Luc) was kindly provided by A.Israël (Institut Pasteur, Paris). The MMTV-Luciferase construct (pHC$_{wt}$-Luc) was generated by removal of the 450 bp *Hin*dIII/*Bgl*II MMTV-LTR fragment (Cato *et al.*, 1988) and cloning it into the luciferase cassette in plasmid pXP$_2$ (Nordeen, 1988).

Cell culture

Mouse ΩE fibroblasts, HeLa cells and human MCF-7 breast carcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM). Human T47D mammary tumour cells, LNCaP prostatic carcinoma cells and THP-1 monocytic leukaemia cells were all cultured in RPMI 1640 medium. The medium for T47D cells was supplemented with 0.6 μg/ml insulin and that for the THP-1 cells contained 2 mM glutamine and 0.5 mM β-mercaptoethanol. The media for all the cells were further supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were kept at 37°C in a 5% CO_2 atmosphere.

Transfection of cells

Prior to transfection, MCF-7 cells were cultured in phenol red-free DMEM supplemented with 3% charcoal-stripped FCS for 2 days. The medium for LNCaP cells was changed from RPMI to DMEM before transfection.

The ΩE fibroblasts, MCF-7 and LNCaP cells were transiently transfected with the calcium phosphate procedure, while HeLa cells were transfected with the DEAE–dextran method. After transfection, HeLa, LNCaP and MCF-7 cells were cultured in medium containing 3% charcoal-stripped FCS until ready for harvesting. Unless otherwise stated, the transfected cells were treated with 10^{-7} M dexamethasone and/or 5 ng/ml TNFα (Calbiochem, Bad Soden, Germany) for 30 h. The transfected cells were then harvested, disrupted and cellular extracts were prepared. Extracts with identical protein concentrations were used for luciferase activity determination as previously described (Schneikert *et al.*, 1996).

Stable clones expressing the wild-type and mutant receptors were obtained by transfection of the GR-negative ΩE fibroblasts (Morgenstern and Land, 1990) with 10 μg *Not*I-linearized expression vectors either

empty or containing cDNA sequences for the rGR, rGR (LS-7), hGR and hGR (D4X). Transfection was carried out with the calcium phosphate precipitation method on 5×10^5 cells. One day after transfection, the cells were cultured in medium supplemented with puromycin (1 μg/ml) for 2 weeks. Single puromycin-resistant clones were isolated and checked for the expression of the receptor by immunoblotting.

Immunoblot (Western blot) analysis

For immunoblot analysis, 5 cm dishes of confluent cells were treated with hormone and/or TNFα (5 ng/ml) as indicated. Thereafter, the cells were washed with phosphate-buffered saline and lysed on ice in a buffer consisting of 20% glycerol, 4% SDS, 0.16 M Tris (pH 6.8), 4% β-mercaptoethanol, 0.5% bromophenol blue. The lysate was sonicated to shear the DNA and boiled for 5 min. Extracts with equal amounts of protein were resolved on a 10% SDS–polyacrylamide gel, transferred onto an Immobilon-PVDF membrane (Millipore, Bedford, UK) and then detected with an antibody against the GR (H.H.—an antiserum directed against amino acids 305–428 of the human GR, kindly supplied by M.N.Alexis) or against IκBα (C-21, Santa Cruz, USA). The primary antibody was detected by counterstaining with horseradish peroxidase-linked antibodies and visualized by the ECL detection Kit (Amersham, Braunschweig, Germany) according to the manufacturer's instructions. In all cases, parts of the SDS gels were stained with Coomassie blue to verify whether equal amounts of proteins had been used.

RNA preparation and Northern blot analysis

Cells used for Northern blot analysis were starved in medium containing 0.5% FCS for 24 h before the isolation of RNA. Polyadenylated RNA preparation and Northern blot analysis were performed as described by Nebl *et al.* (1994). Five micrograms of poly$(A)^+$ RNA were denatured and fractionated on a 1% agarose gel and transferred onto a Hybond N^+ membrane (Amersham International, UK). The filter was hybridized with randomly primed radioactively labelled cDNA fragments for collagenase I (Angel *et al.*, 1987), human metallothionein IIa (Karin and Richards, 1982), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fort *et al.*, 1985), IκBα (generously provided by C.Scheidereit) and IL-1β. The IL-1β DNA fragment was first generated by PCR amplification of cDNA from RNA isolated from THP-1 cells treated with LPS (10 mg/ml) for 5 h. The oligonucleotide primers used were 5′-GGAAGACACAAATTGCATGGTGAAGTC-3′ and 5′-ATGGCAGAA-GTACCTGAGCTCGCCAGT-3′. The product of the amplification was cloned into the *Eco*RI site of the vector pCR^{tm} (Invitrogen, San Diego, USA) and checked for its authenticity by DNA sequencing. For the hybridization of the Northern blot filter, the *Eco*RI insert fragment was used.

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Note added

While this work was being completed, we learnt of the results of Brostjan *et al.* (1996; *J. Biol. Chem.*, **271**, 19612–19616), that glucocorticoids repress NF-κB activity in endothelial cells in the absence of enhanced IκBα synthesis. The IκBα-independent repression of NF-κB activity by glucocorticoids that we have reported now applies to several cell types if the data of Brostjan *et al.* are taken together with our present results.