Various glucocorticoids differ in their ability to induce gene expression, apoptosis and to repress NF-κB-dependent transcription

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Abstract Glucocorticoids (GCs) influence a great variety of cellular functions by at least three important modes of action: the activation (or repression) of genes controlled by binding sites for the glucocorticoid receptor (GR), the induction of apoptosis in lymphocytes and the recently discovered cross-talk to other transcription factors such as NF-κB. In this study we systematically compared various natural and synthetic steroid hormones frequently used as therapeutic agents on their ability to mediate these three modes of action. Betamethasone, triamcinolone, dexamethasone and clobetasol turned out to be the best inducers of gene expression and apoptosis. All GCs including the antagonistic compound RU486 efficiently reduced NF-κB-mediated transactivation to comparable extents, suggesting that ligand-induced nuclear localization of the GR is sufficient for transrepression. Glucocorticoid treatment of cells did not result in elevated IκB-α expression, but impaired the tumor necrosis factor (TNF)-α-induced degradation of IκB-α without affecting DNA binding of NF-κB. The structural requirements for the various functions of glucocorticoids are discussed.

Key words: Glucocorticoid; Apoptosis; NF-κB; Gene expression; Cross-talk

1. Introduction

The physiological and therapeutic effects of steroid hormones and glucocorticoids (GCs) rely on direct posttranscriptional and pharmacological effects and, more importantly, on their ability to influence gene expression. This is achieved by binding of the hormone to transcription factors belonging to the family of steroid hormone receptors. Among those is the glucocorticoid receptor (GR), which in its resting state is associated with heat shock proteins and one p59 immunophilin molecule in the cytoplasm of cells [1]. Heat shock protein 90 acts as a chaperone and allows binding of the hormone, which then leads to the nuclear translocation of the GR complex. Once in the nucleus, the GR can utilize several different mechanisms in order to influence gene expression [2]. Upon binding as a homodimer to the so-called glucocorticoid-response elements (GREs) or negative GREs (nGREs) it can induce or repress the transcription of target genes. Some genes lacking GREs or nGREs in their promoters can still be affected by GCs. This finding is explained by functional interactions of the GR with other transcription factors such as AP1, CREB, C/EBP, octamer binding factors, STAT5 and NF-κB [3]. The interaction of the GR with AP-1 and NF-κB is of special interest, since it provides a further mechanism to explain the clinically relevant anti-inflammatory and immunosuppressive properties of GCs [4]. Similar to the GR, NF-κB is also mainly regulated by compartmentalization. Inactive NF-κB resides in the cytoplasm by binding to one inhibitory IκB protein [5]. A wide range of pro-inflammatory signals including tumor necrosis factor (TNF)-α, IL-1 and reactive oxygen intermediates results in the induced phosphorylation and ubiquitinylation of IκB, which tag it for the degradation by the proteasome. The released DNA-binding subunits are then translocated into the nucleus, bind their cognate DNA and induce transcription of numerous target genes, many of them involved in the acute and acquired immune response [6]. The repressive effect of the GR on NF-κB-dependent gene expression is either explained by the GC-induced upregulation of IκB-α, or alternatively by the mutual interaction and inactivation of both transcription factors [7]. Besides the obvious uses of GCs for the treatment of adrenocortical insufficiencies and as anti-inflammatory drugs [8], their administration causes thymic involution by inducing survival or apoptosis of T-cells. This is physiologically relevant for shaping the T-cell repertoire. However, the apoptosis-inducing property is also clinically important, since GCs are frequent components of drug regimens used for the treatment of lymphomas [9]. It is currently still controversial whether the apoptosis-inducing properties of GCs rely on the repression of survival genes or the induction of apoptosis genes [10,11].

In some cases, transactivation and transrepression can be distinguished by the use of synthetic, so-called dissociating ligands, allowing transactivation without transrepression and vice versa [12]. Such dissociating ligands have been found for the retinoic acid receptor and the GR [11]. This study is the first systematic comparison of a number of natural and synthetic steroids, revealing remarkable differences in their ability to trigger GRE-dependent gene expression, to induce apoptosis and to transrepress NF-κB-dependent transcription. These differences hopefully contribute to the future development of new therapeutic protocols, which allow the combination of optimal therapeutic results with minimal side effects.

2. Materials and methods

2.1. Cell culture and transient transfections

CEM-C7 T-cells were grown in RPMI 1640 medium containing

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Abbreviations: EMSA, electrophoretic mobility shift assay; GCs, glucocorticoids; GR, glucocorticoid receptor; GREs, glucocorticoid-response elements; nGREs, negative GREs; MMTV, mouse mammary tumor virus; TNF, tumor necrosis factor

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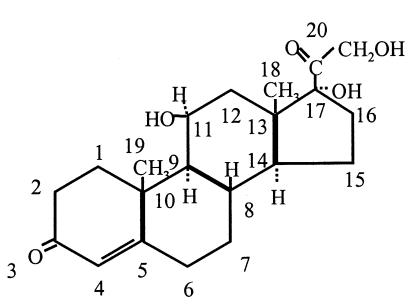
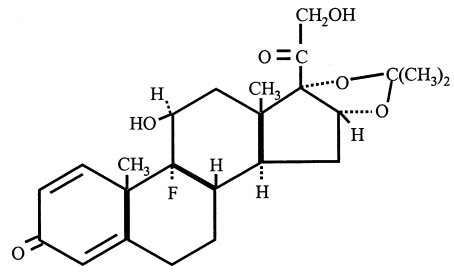
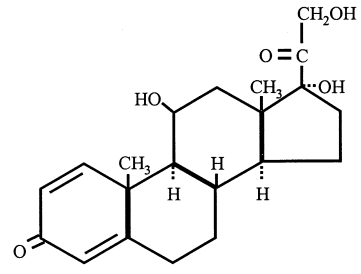


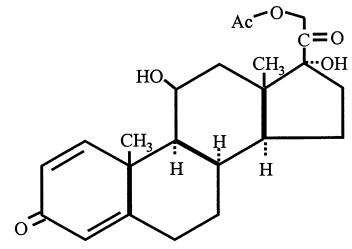
Hydrocortisone



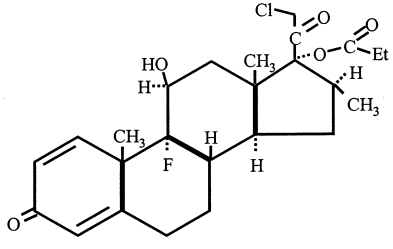
Triamcinolone acetonide



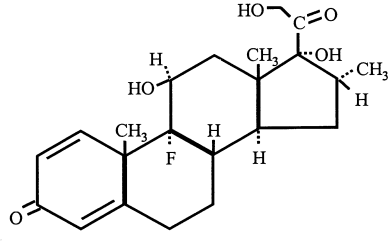
Prednisolone



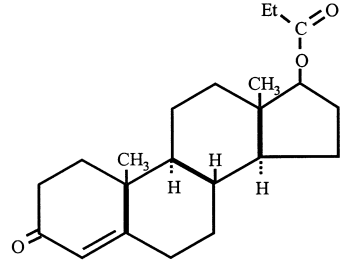
Prednisolone acetate



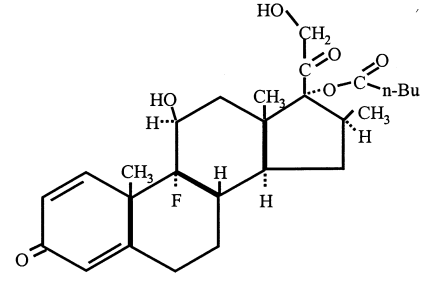
Clobetasol propionate



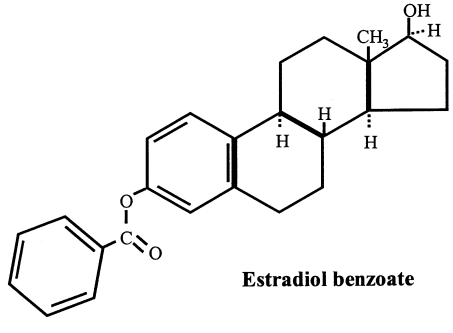
Dexamethasone



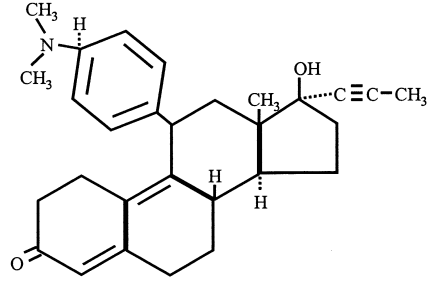
Testosterone propionate



Betamethasone valerate



Estradiol benzoate



RU486

Fig. 1. List of the different steroids tested. The positions of the different C-atoms are indicated for hydrocortisone.

10% (v/v) heat-inactivated fetal calf serum, 10 mM HEPES and 1% (v/v) penicillin/streptomycin (all from Life Technologies, Grand Island, NY, USA). Murine L929sA fibrosarcoma cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal calf serum and 1% (v/v) penicillin/streptomycin. All cells were grown in a humidified incubator at 37°C and 5% CO_2. L929 cells were transiently transfected with lipofectamine (Gibco-BRL, Eggenstein, Germany) according to the manufacturer's instructions.

2.2. DNA fragmentation assay

CEM C7 cells were harvested by centrifugation, washed twice with phosphate-buffered saline and lysed in 0.5× TBE (25 mM Tris, 25 mM boric acid and 0.5 mM EDTA) containing 0.25% (v/v) NP-40 and 0.5 mg/ml RNase H. The samples were incubated for 45 min at 37°C and subsequently 0.5 mg/ml proteinase K (Sigma, St. Louis, MO, USA) was added. After a further incubation for 45 min at 37°C, the cell debris was pelleted upon centrifugation with 14 000 rpm at 4°C for 10 min. The DNA fragments contained in the supernatant were separated on a 1.5% (w/v) agarose gel in 1× TBE buffer at 50 V for 3 h. The DNA was stained with ethidium bromide and analyzed under ultraviolet light.

2.3. Detection of apoptosis by FACS analysis

Apoptosis was measured by determining the percentage of hypoploid cells after lysis in a hypotonic buffer containing 50 μg/ml propidium iodide, 0.1% (w/v) sodium citrate and 0.1% (v/v) Triton X-100 (all from Sigma, St. Louis, MO, USA). Fluorescence was measured in a FACScan and analyzed with CELLQuest software (Becton Dickinson, Heidelberg, Germany) as described earlier [13].

2.4. Electrophoretic mobility shift assays (EMSAs)

Murine L929sA cells were stimulated with 2000 units/ml recombinant TNF-α (Boehringer Mannheim, Mannheim, Germany) for the indicated periods, washed twice with cold phosphate-buffered saline and scraped off with a rubber policeman. After centrifugation for 3 min with 3000 rpm the total cellular proteins were extracted from the pellet by lysis in TOTEX buffer (20 mM HEPES/KOH, pH 7.9, 0.35 M NaCl, 20% (v/v) glycerol, 1% (v/v) NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride). The tubes were incubated on ice for 30 min and vortexed every 10 min. Equal amounts of protein contained in the supernatant were tested for NF-κB DNA binding activity as described [14].

2.5. Western blot analysis and luciferase assays

For Western blotting the proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) using a semi-dry blot apparatus (Biorad, München, Germany). The IκB-α proteins were detected using rabbit polyclonal antibodies and a horseradish peroxidase-coupled secondary antibody essentially as described [14]. For the determination of luciferase activity, the cells were washed with phosphate-buffered saline and lysed in 100 μl of lysis buffer (Tropix, Bedford, MA, USA). The luciferase assays were performed according to the manufacturer's manual (Promega, Mannheim, Germany) and quantified in a Duo Lumat LB 9507 (Berthold, Wildbad, Germany).

3. Results and discussion

The structures of the various steroids used in this study are displayed in Fig. 1. The sex steroids testosterone propionate and estradiol benzoate are included as controls since they have no significant affinity to the GR. RU486 acts as a GR antagonist, all other steroids belong to the family of glucocorticoids. The gene-inductive effects of the various hormones displayed in Fig. 1 were systematically compared. Murine L929 fibrosarcoma cells were transiently transfected with a luciferase reporter gene controlled by the mouse mammary tumor virus (MMTV) long terminal repeat which contains several GREs. The analysis of gene expression induced by the different concentrations of the various hormones revealed no significant transactivation by RU486 and the two sex steroids (Fig. 2). Hydrocortisone, one of the endogenous GCs, prednisolone and prednisolone acetate induced gene expression to an intermediate extent. Full transcriptional activation was reached by betamethasone, triamcinolone, dexamethasone and clobetasol. The strongly transactivating GCs were still active even when applied at 10^{-7} M, the weakly transactivating GCs showed a strongly impaired transactivation at this concentration. The gene-inductive effects of all steroids were almost completely lost in the presence of 1 μM RU486 (data not shown).

The therapeutic effects of GCs in the treatment of leukemias and lymphomas are not fully understood and largely empirical [9]. It is generally believed that most of the cytostatic effects of GCs can be explained by their ability to induce apoptosis in T-cell lymphomas [10]. Therefore we systematically compared the apoptosis-inducing effects of the various steroids listed in Fig. 1. The suitability of CEM C7 T-cells as a model system to study GC-induced apoptosis was characterized by analyzing the GC-induced DNA fragmentation, a characteristic feature of programmed cell death. CEM C7 cells were treated for 48 h with 1 μM of dexamethasone and the genomic DNA was analyzed on agarose gels (Fig. 3A). Dexamethasone induced the typical cleavage of DNA into multimers of domain-sized fragments, thus ensuring that the cells die from apoptosis rather than from necrosis. The various hormones were added to exponentially growing CEM C7 T-cells at different concentrations for two days. Apoptosis was measured in a FACScan by the determination of hypoploid cells (Fig. 3B). Triamcinolone, dexamethasone, betamethasone, clobetasol and prednisolone acetate efficiently induced apoptosis at all concentrations to comparable extents. Prednisolone at a concentration of 10^{-6} M was still apoptosis-inducing, but the concentration of 10^{-7} M killed only a fraction of the T-cells. Hydrocortisone induced apoptosis only very weakly. Both sex steroids and RU486 did not display any cytotoxic effects. To address the question whether GC-induced apoptosis requires activation or repression of target genes, we measured the effects of the antagonist RU486 on GC-induced apoptosis. The addition of RU486 efficiently repressed apoptosis induced by all glucocorticoids tested here

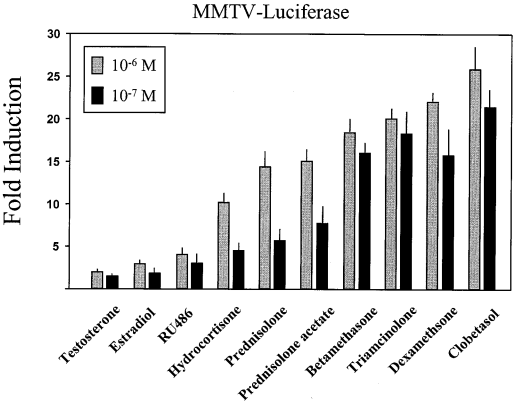


Fig. 2. Effects of various GCs on transactivation. L929 cells were transiently transfected with the MMTV-luciferase reporter construct. One day after transfection, cells were stimulated for 12 h with the various GCs at concentrations of 10^{-6} M (grey bars) and 10^{-7} M (black bars). Luciferase activity contained in equal amounts of cell extracts was determined. The mean of four separate experiments is shown, standard deviations are indicated by bars.

(Fig. 3B), thus highlighting the importance of gene-inductive effects for apoptosis. This idea is supported by the analysis of mice expressing a dominant negative GR mutant (A458T) that abolishes transactivation. Thymocytes derived from these animals are unable to undergo apoptosis induced by the synthetic steroid dexamethasone [15].

We next tested the repressive effects of the various hormones on NF-κB-induced transcription. The influence of GCs on TNF-α-stimulated NF-κB-dependent transcription was measured in L929 cells stably transfected with an NF-κB-dependent luciferase reporter gene [16]. The TNF-α-induced NF-κB activation was significantly reduced by all GCs (with the exception of both sex steroids) to a comparable extent (Fig. 4). Transrepression of NF-κB was even seen for the antagonist RU486 and the relatively weakly transactivating GCs hydrocortisone and prednisolone. Taken together, these findings show that: (i) all GCs tested here can repress NF-κB transcription, suggesting that simply a ligand-induced

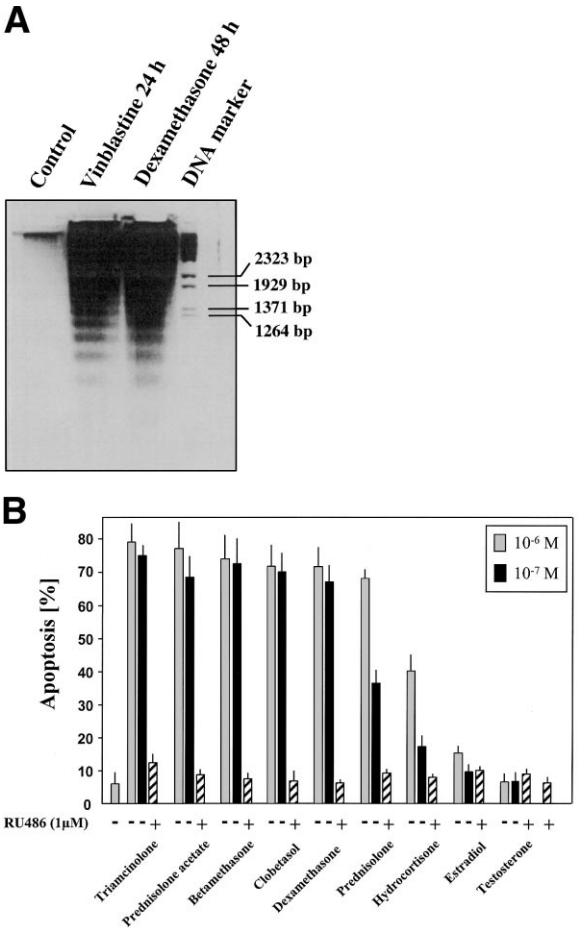


Fig. 3. Analysis of GC-induced apoptosis. A: CEM C7 cells were treated for 48 h with 1 μM dexamethasone or for 24 h with 1 μM vinblastine, which was used as a positive control. The fragmentation of DNA into oligonucleosomes was determined by horizontal agarose gel electrophoresis followed by staining with ethidium bromide. B: Comparative analysis of GC-induced apoptosis. CEM C7 T-cells were incubated for 48 h with 10^{-6} M (grey bars) or 10^{-7} M (black bars) of the indicated GC. RU486 was added at the indicated concentration to 1 μM of the respective GC. Apoptosis was measured by FACS analysis and the percentage of hypoploid cells is given. Mean values from 4 independent experiments are shown, bars show standard deviations.

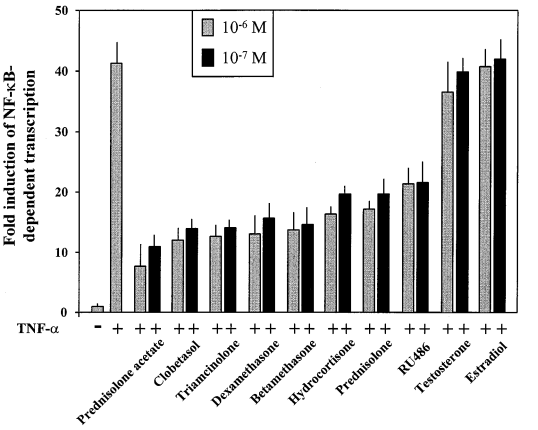


Fig. 4. Comparative analysis of NF-κB transrepression by GCs. L929 cells stably transfected with an NF-κB-controlled luciferase reporter gene were preincubated with 10^{-6} M (grey bars) or 10^{-7} M (black bars) of the indicated GCs for 1 h. Subsequently transcription was induced by the addition of TNF-α (2000 U/ml) for 10 h and luciferase activity was determined. Mean values from 3 independent experiments are displayed, bars represent standard deviations.

nuclear localization is sufficient for transrepression; (ii) there seems to be a correlation between the ability of GCs to induce GRE-dependent transcription and apoptosis. We speculate that the individual transactivating capacity of each GC relies on its ability to induce a specific conformation that enables the transcription activation, e.g. by allowing contacts to coactivators and/or components of the basal transcription machinery [2]. It is imaginable that each bound hormone will allow the formation of an individual hydrogen-bonding network and individual amido-keto interactions with the ligand binding domain of the GR, thus influencing its three-dimensional structure. Along this line, binding of 17β-estradiol or of the selective antagonist raloxifene induced distinct conformations in the transactivation domain within the ligand-binding domain of the estrogen receptor [17]. The same applies to the progesterone receptor, which adopted distinct conformations depending on the binding of progesterone or RU486 [18].

The question whether or not GCs may induce the increased production of the inhibitory IκB-α molecule which then would lead to the dissociation of NF-κB from its cognate DNA is still a matter of debate [7,19]. To address the question whether these conflicting results may be attributed to different stuctures of the GCs, we treated L929 cells with various GCs and looked at the impact on TNF-α-induced DNA binding of NF-κB (Fig. 5A). DNA binding of NF-κB was not affected by any of the GCs tested here. The same extracts were assayed for the relative levels of IκB-α in Western blot experiments (Fig. 5A). Short-time treatment of cells with TNF-α resulted in the induced degradation of IκB-α, but the cells pretreated with betamethasone, triamcinolone, prednisolone acetate, clobetasol, hydrocortisone and dexamethasone showed elevated levels of IκB-α. This may be explained either by an induced synthesis or by an impaired degradation of IκB-α. In order to distinguish between both possibilities, we exposed L929 cells for 8 h to the various steroids and determined the IκB-α protein levels from unstimulated or TNF-α-stimulated cells (Fig. 5B). The unstimulated cells showed no significant increase in the levels of IκB-α protein, excluding the induced synthesis of IκB-α as a possible mechanism. Therefore it

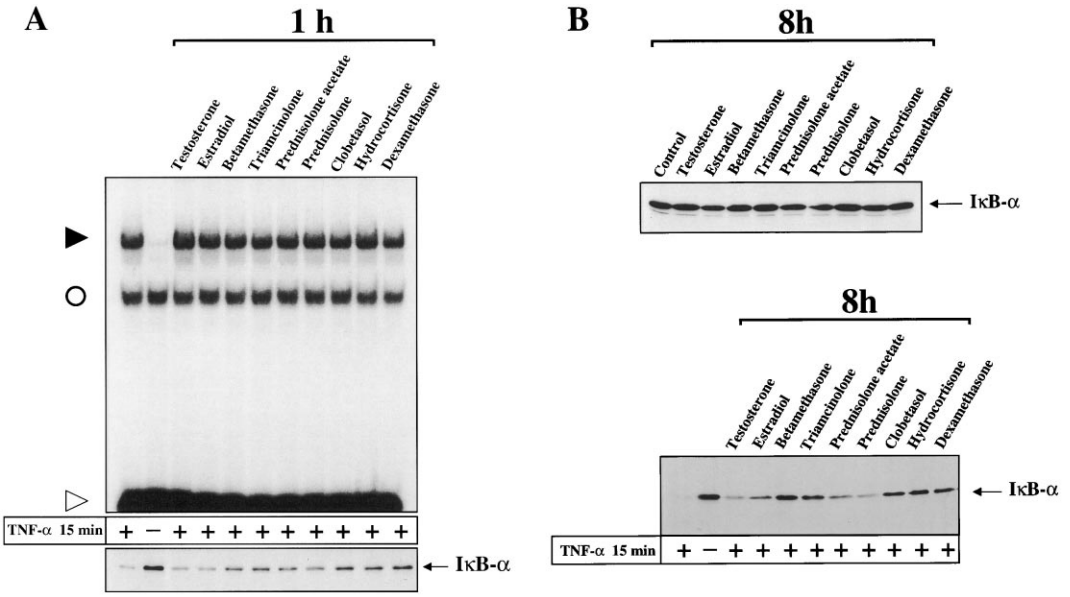


Fig. 5. Impact of various GCs on DNA binding of NF-κB and IκB-α degradation. A: TNF-α (2000 U/ml) was added for 15 min to L929 cells that were preincubated as shown for 1 h with 1 μM of the indicated GCs. Total cell extracts were prepared and DNA binding of activated NF-κB was measured by EMSA (upper). The filled arrowhead indicates the location of the DNA-NF-κB complex, the circle indicates the position of a constitutively DNA-binding protein and the open arrowhead points to the unbound oligonucleotide. The same extracts were tested for the IκB-α protein in a Western blot (lower). The arrow points to the IκB-α protein. B: L929 cells were grown for 8 h in the presence of 1 μM of the various GCs, the indicated cells were subsequently treated for 15 min with TNF-α (2000 U/ml). Equal amounts of total cellular proteins were analyzed by immunoblotting for the occurrence of IκB-α which was detected with a polyclonal α-IκB-α antibody.

seems that the described upregulation of IκB-α protein as seen for instance in lymphoid cells [20,21] is restricted to certain cell types, since many cell lines including mouse fibroblast L929 (this study), human fibroblast 293, monkey COS, human T-cell CEM-C7 and endothelial TC10 and BAEC cells did not show elevated IκB-α protein levels after treatment with GCs [22–25]. Stimulation of cells with TNF-α resulted in a complete degradation of IκB-α in the absence of any hormone or in the presence of both sex steroids. All other GCs prevented the degradation of IκB-α to various extents (Fig. 5B). However, the TNF-α-induced DNA-binding activity of NF-κB contained in the different extracts remained unchanged (data not shown). The uncoupling between both IκB-α upregulation and DNA binding of NF-κB is also evident from the analysis of GC analogues that were still competent for IκB-α synthesis but were unable to repress NF-κB [12]. Similarly, the dexamethasone-induced IκB-α synthesis in human pulmonary epithelial cells did not cause a reduction of NF-κB DNA-binding activity [19]. Therefore the elucidation of the possible consequences and the identification of the signaling steps responsible for the impaired IκB-α degradation discovered here requires future studies.

4. Conclusion

Taken together, the NF-κB-repressing activities of the various steroid hormones tested do not rely on special structural requirements. Triamcinolone, clobetasol, dexamethasone and betamethasone are very effective in the induction of transcription and apoptosis, suggesting a correlation between these two activities. These GCs display a high structural similarity and differ only in the modifications of C atoms C16 and C17. In accordance with the results presented here, a previous study revealed higher antileukemic activities and a five to six times higher cytotoxicity of dexamethasone, when directly compared to prednisolone [26]. Therefore future studies should investigate whether prednisolone, which is frequently contained in chemotherapeutic anti-cancer drug regimens [9], can be replaced by GCs displaying a higher cytotoxicity.

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