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Dexamethasone prevents interleukin-1β-induced nuclear factor-κB activation by upregulating IκB-α synthesis, in lymphoblastic cells

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Aims: **Glucocorticoids (GCs) exert some of their anti-inflammatory actions by preventing the activation of the transcription factor nuclear factor (NF)-κB. The GC-dependent inhibition of NF-κB may occur at different levels, but the mechanisms involved are still incompletely understood. In this work, we investigated whether the synthetic GC, dexamethasone (Dex), modulates the activity of NF-κB in the lymphoblastic CCRF-CEM cell line. We also evaluated the ability of Dex to prevent the activation of NF-κB in response to the potent proinflammatory cytokine, interleukin (IL)-1β.**

Results: **Exposure of the cells to Dex (1 μM) induced the rapid degradation of IκB-α, leading to the transient translocation of the NF-κB family members p65 and p50 from the cytoplasm to the nucleus, as evaluated by western blot. Electrophoretic mobility shift assays revealed that, in the nucleus, these NF-κB proteins formed protein–DNA complexes, indicating a transient activation of NF-κB. Additionally, Dex also induced *de novo* synthesis of IκB-α, following its degradation. Finally, when the cells were exposed to Dex (1 μM) prior to stimulation with IL-1β (20 ng/ml), Dex was efficient in preventing IL-1β-induced NF-κB activation. The GC antagonist, RU 486 (10 μM), did not prevent any of the effects of Dex reported here.**

Conclusion: **Our results indicate that, in CCRF-CEM cells, Dex prevents NF-κB activation, induced by IL-1β, by a mechanism that involves the upregulation of IκB-α synthesis, and that depends on the early and transient activation of NF-κB.**

Key words: Nuclear factor-κB, Dexamethasone, IκB-α, Interleukin-1β, Lymphoblastic cells

Introduction

The transcription factor nuclear factor (NF)-κB is composed of several structurally related (Rel) proteins, which include p50 (and its precursor p105), p52 (and its precursor p100), p65 (RelA), c-Rel and RelB.[1–3] The Rel proteins can form either homodimers or heterodimers with each other, except RelB, which can only form heterodimers.[4–6]

In resting cells, the NF-κB dimers are sequestered in the cytoplasm in a latent inactive state, as the result of binding to inhibitor proteins, namely IκB-α, which is the major NF-κB inhibitor.[1,3] Upon stimulation by a variety of extracellular signals, such as the proinflammatory cytokines, ultraviolet light, ionizing radiation, bacterial lipopolysaccharide and phorbol esters,[7–9] IκB-α undergoes phosphorylation-dependent degradation, thus releasing the active NF-κB dimers.[1,8] The freed NF-κB dimers migrate into the nucleus, and bind to specific κB DNA binding sites in the promoter regions of susceptible genes, thereby regulating the expression of a large number of genes coding for proteins mainly involved in immune and inflammatory responses.[1,9,10] Additionally, NF-κB has also been implicated in the transcriptional regulation of the RelB[11] and IκB-α[1,2,12] genes.

After stimulation, the activity of NF-κB is usually transient,[1,3] as a consequence of the rapid re-synthesis of IκB-α following its degradation. Newly synthesized IκB-α replenishes its cytoplasmic levels and simultaneously enters the nucleus, where it binds to the NF-κB dimers, thereby blocking their DNA binding ability and restoring the cytoplasmic pool of latent NF-κB.[1,2]

Glucocorticoids (GCs) are potent immunosupressive agents that, on one hand, may induce the synthesis of anti-inflammatory mediators like annexin 1[13] and, on the other hand, may inhibit the expression of several cytokines and adhesion molecules.[14–16] The anti-inflammatory effects of GCs mediated by inhibition of gene expression have been attributed to the capability of these hormones to interfere with the activity of transcription factors, namely NF-κB.[14,17,18] At least two distinct

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mechanisms may be involved in the inhibition of NF-κB by GCs: (i) a cross-coupling mechanism, in which the activated GC receptor directly interacts with and inhibits the activated NF-κB dimers;[19–21] and (ii) a mechanism involving the transcriptional activation of the IκB-α gene, that leads to the rapid replenishment of its intracellular levels with the consequent blockade of NF-κB activity.[17,18,22,23] The physiological relevance of these mechanisms has not been clearly established, and it still remains unclear whether both mechanisms participate in the inhibition of NF-κB by GCs.

In this work, we used the lymphoblastic human CCRF-CEM cell line to study the mechanisms involved in the inhibition of NF-κB by the synthetic GC dexamethasone (Dex). We investigated the ability of Dex alone to modulate NF-κB activity, by evaluating the cytoplasmic levels of I κB-α as well as the nuclear levels of NF-κB proteins and their DNA-binding ability, in cells treated with Dex. Moreover, we studied the involvement of the GC receptors on these processes, using the GC receptor antagonist RU486. Finally, we investigated whether interleukin (IL)-1β, a proinflammatory cytokine with a potent NF-κB-inducing activity in several cell types,[21,24,25] was able to activate NF-κB in CCRF-CEM cells, and whether and how Dex was able to prevent the effect of IL-1β.

Materials and methods

Chemicals

The human CCRF-CEM acute T-lymphoblastic leukaemia cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Foetal calf serum (FCS) was obtained from Biochrom (Berlin, Germany). Recombinant human interleukin-1β (IL-1β) was from R&D Systems (Abingdon, UK). The protease inhibitor cocktail was obtained from Roche (Manheim, Germany). Oligonucleotide probes were from Santa Cruz (Santa Cruz, CA, USA), whereas [γ-^{32}P]adenosine triphosphate, T4 polynucleotide kinase, and poly(dI-dC) were obtained from Amersham Biosciences (Piscataway, NJ, USA). The rabbit polyclonal antibody against IκB-α was purchased from Cell Signalling Tech. (Beverly, MA, USA), whereas the rabbit polyclonal antibodies against p65, p50, p52 and RelB, and the goat polyclonal antibody against c-Rel were obtained from Santa Cruz (Santa Cruz, CA, USA). The anti-actin monoclonal antibody was purchased from Roche (Manheim, Germany). The horseradish peroxidase-conjugated anti-rabbit, anti-goat and anti-mouse antibodies were obtained from Amersham Biosciences (Piscataway, NJ, USA), Zymed (S. Francisco, CA, USA) and Pierce (Rockford, IL, USA), respectively. The Enhanced Chemiluminescence (ECL) western blotting detection reagents were purchased from Amersham Biosciences (Piscataway, NJ, USA). All other reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

Culture conditions

The human CCRF-CEM acute T-lymphoblastic leukaemia cell line was maintained in RPMI medium supplemented with 10% (v/v) FCS, 100 μg/ml of streptomycin and 100 U/ml of penicillin, at 37°C, in an atmosphere of 5% CO_2/95% air. Prior to any treatments, CCRF-CEM cells were plated at 5×10^6 cells/well, in six-well culture dishes, in FCS-free RPMI supplemented with antibiotics, for 6 h, and maintained in these conditions thereafter. In each experiment, controls were always included, and consisted of incubating the cells in the corresponding volume of culture medium plus the appropriate vehicle.

Cell viability

Viability of CCRF-CEM cells under the various experimental conditions was always assessed by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction method. Assessment of MTT reduction by metabolically active cells was made by a colourimetric assay, as previously described.[26]

Preparation of cytoplasmic and nuclear extracts

After washing with ice-cold phosphate buffered saline, the cells were lysed in buffer 1 (10 mM of NaCl, 3 mM of $MgCl_2$, 0.5% (v/v) Nonidet P-40, 1 mM of dithiothreitol, 0.1 mM of phenylmethylsulfonylfluoride, 10 mM of Tris–HCl, pH 7.5, and the protease inhibitor cocktail) and incubated on ice for 15 min. After brief vortexing, the lysates were centrifuged at $2300 \times g$ for 10 min at 4°C. The supernatants (cytoplasmic extracts) were collected and stored at −70°C until further use. The pellets obtained were resuspended in buffer 2 (300 mM of NaCl, 3 mM of $MgCl_2$, 20% (v/v) glycerol, 1 mM of dithiothreitol, 0.1 mM of phenylmethylsulfonylfluoride, 0.2 mM of ethylenediamine tetraacetic acid, 20 mM of HEPES buffer, pH 7.5, and the protease inhibitor cocktail), incubated on ice for 45 min and centrifuged at $12{,}000 \times g$ for 20 min at 4°C. The supernatants (nuclear extracts) were collected and stored at −70°C until further use. The protein concentration of the extracts was determined using the bicinchoninic acid/copper (II) sulfate protein assay kit.

Western blot analysis

Samples of cytoplasmic and nuclear extracts (25 μg) were diluted (1:1) in sodium dodecylsulfate denaturating buffer (200 mM of Tris, 200 mM of bicine, 8 M of urea, 4% sodium dodecylsulfate, 10% β-mercaptoethanol and 0.2% bromophenol blue), boiled, subjected to 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis, and electrotransferred to a polyvinylidene difluoride membrane. The membrane was then blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20. IκB-α and NF-κB proteins (p65, p50, p52, RelB and c-Rel) were detected by incubation with their respective primary antibodies, followed by incubation with a horseradish peroxidase-conjugated anti-rabbit or anti-goat antibody. The immunocomplexes were visualized by the ECL chemiluminescent method. The membranes were subsequently stripped and reprobed with an anti-actin antibody, to ensure an equal protein loading.

Electrophoretic mobility shift assay

The double-stranded oligonucleotide probe specific for NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was end-labelled with [γ-^{32}P]adenosine triphosphate, by the T4 polynucleotide kinase, and purified through Sephadex G-50 spin columns. Nuclear extracts (12.5 μg protein) were incubated for 40 min at 4°C, with 100,000 cpm of [γ-^{32}P]-labelled oligonucleotide, in a buffer containing 100 μg/ml of poly(dI-dC).poly(dI-dC), 20 mM of HEPES (pH 7.9), 1 mM of $MgCl_2$, 4% (w/v) Ficoll 400, 0.5 mM of dithiothreitol, 50 mM of KCl, and 1 mg/ml of bovine serum albumin. The DNA–protein complexes were resolved by electrophoresis on 7% non-denaturating polyacrilamide gels, in a buffer system containing 0.044 M of Tris–Base (pH 8.0), 4.45 mM of boric acid and 1 mM of ethylenediamine tetraacetic acid, at a constant voltage of 150 V, for 3 h at room temperature. The gels were dried and subjected to autoradiography. In competition experiments, a 100-fold excess of unlabelled oligonucleotide was added to the nuclear extracts immediately before the addition of the radiolabelled probe. For supershift analysis, anti-p65, anti-p50 or anti-RelB antibodies (2 μg/reaction) were incubated with the nuclear extracts from Dex-treated cells, for 2 h before the addition of the radiolabelled probe.

Statistical analysis

Results are presented as average ± SD of at least three different experiments, as indicated. Comparison of data from more than two treatment groups was made by the one-way analysis of variance with Dunnett's post test.

Results

Dex induces the degradation followed by *de novo* synthesis of IκB-α

To evaluate the ability of Dex to modulate the levels of IκB-α, western blot analysis was performed using cytoplasmic extracts obtained from CCRF-CEM cells treated with Dex in concentrations ranging from 50 nM to 1 μM, for 5 min–1 h. The results obtained showed that the amount of IκB-α present in the cytoplasmic extracts obtained from cells treated with 1 μM of Dex, for 5 min, was significantly lower than that found in untreated cells. The other concentrations of Dex did not affect the IκB-α levels at any of the incubation times used (data not shown). Therefore, 1 μM of Dex was the concentration used in all the subsequent experiments performed in this work.

As shown in Fig. 1, treatment of the cells with Dex for 5 min decreased, by 61.8%, the amount of IκB-α found in the cytoplasm, whereas in cells treated for 15 min the cytoplasmic level of the protein increased, approaching the amount found in control cells. After a 30 min treatment, IκB-α reached a level significantly higher than that of the control. The protein synthesis inhibitor, cycloheximide (CHX), was used to investigate whether the time-dependent accumulation of IκB-α was due to *de novo* protein synthesis. CHX (20 μg/ml) did not reduce the viability of the CCRF-CEM cells, as assessed by the MTT assay (data not shown). Moreover, control experiments showed that CHX alone did not affect the cytoplasmic level of IκB-α under resting conditions (data not shown). Pretreatment of the cells with CHX, for 1 h, completely abrogated the appearance of IκB-α in cytoplasmic extracts from cells treated with Dex, for 1 h, thus showing that *de novo* protein synthesis is required for the Dex-induced IκB-α accumulation in the cytoplasm that follows its initial degradation (Fig. 1).

The GC antagonist, RU486, was used to determine whether the effects of Dex on the cytoplasmic level of IκB-α could be attributed to activation of intracellular GC receptors. Control experiments showed that RU486, at concentrations up to 10 μM, did not reduce the viability of CCRF-CEM cells, as assessed by the MTT assay (data not shown). The results obtained show that at concentrations ranging from 1 to 10 μM, RU486 did not affect the cytoplasmic level of IκB-α under resting conditions. Furthermore, RU486 did not revert, at any of the concentrations used, the Dex-induced degradation and re-synthesis of IκB-α in CCRF-CEM cells (data not shown).

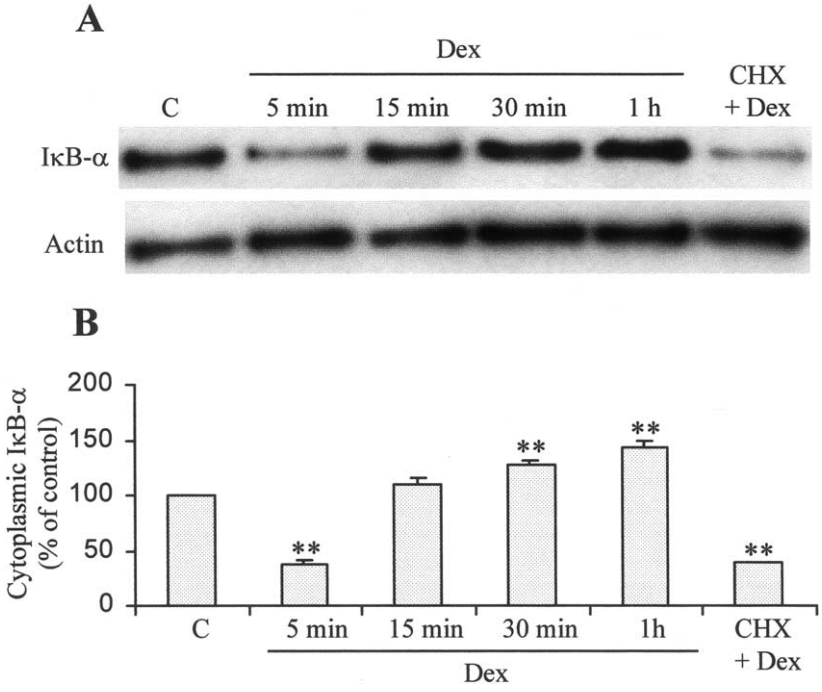

FIG. 1. Dex induces the degradation followed by the re-synthesis of the cytoplasmic protein IκB-α. CCRF-CEM cells were treated with Dex (1 μM), for the indicated periods, in the absence or in the presence of CHX (20 μg/ml) for 1 h. The cytoplasmic extracts were prepared as described in Materials and methods. The cytoplasmic IκB-α content was determined by western blot analysis, using a rabbit polyclonal anti-IκB-α antibody (A), and the bands were quantified with an image analyser (B). The results presented in the upper panel (A) are representative of three independent experiments. The data shown in the lower panel (B) are the average ±SD of three independent experiments. ** $p < 0.01$, as determined by one-way analysis of variance with Dunnett's post test.

Dex induces the translocation of NF-κB proteins p65 and p50 from the cytoplasm to the nucleus and increases the synthesis of RelB

To study the ability of Dex to modulate NF-κB activity in CCRF-CEM cells, we evaluated the cytoplasmic and nuclear levels of the individual members of the NF-κB family. Matched cytoplasmic and nuclear extracts were subjected to western blot analysis, with antibodies against p65, p50, p52, RelB and c-Rel. As shown in Figs. 2 and 3, treatment of the cells with Dex (1 μM), for 15–30 min, resulted in significant reductions of the cytoplasmic levels (Fig. 2A and Fig. 3A) accompanied by corresponding increases in the nuclear levels (Fig. 2B and Fig. 3B) of both p65 and p50. Those changes were transient, since in cells treated with Dex for 1 h the levels of both proteins in nuclear and cytoplasmic extracts were similar to those found under resting conditions. Therefore, these results show that Dex alone is sufficient to induce p65 and p50 translocation to the nucleus, although both proteins rapidly returned to their original cytoplasmic location.

On the other hand, the cytoplasmic and nuclear levels of RelB increased continuously over the time periods tested (Fig. 4). Nevertheless, accumulation of RelB in the nucleus occurred more rapidly than in the cytoplasm, where the increase was only observed in cells treated with Dex for 30 min or longer. Since the level of RelB increased in both subcellular compartments, we used CHX to find whether *de novo* protein synthesis accounted for those increases. The results presented in Fig. 4A show that the level of RelB in cytoplasmic extracts prepared from cells pretreated with CHX for 1 h before addition of Dex, for 1 h, was significantly lower than that found in extracts from control cells. The nuclear level of RelB in cells incubated with Dex was not affected by pretreatment of the cells with CHX (data not shown). Taken together, these results show that the accumulation of RelB in the cytoplasm is dependent on *de novo* protein synthesis.

Finally, treatment of CCRF-CEM cells with Dex did not significantly modify the protein level and the subcellular localization of p52 and c-Rel, which were found to be constitutively expressed in the cytoplasm and nucleus, or exclusively in the nucleus, respectively (data not shown).

Dex induces a time-dependent NF-κB activation

To study NF-κB activation, we detected the presence of active NF-κB proteins in nuclear extracts from CCRF-CEM cells, by electrophoretic mobility shift assay analysis, with an oligonucleotide probe containing the specific consensus binding sequence for this transcription factor. In nuclear extracts from cells treated with Dex (1 μM) for 30 min, the intensity of the band corresponding to the binding of nuclear

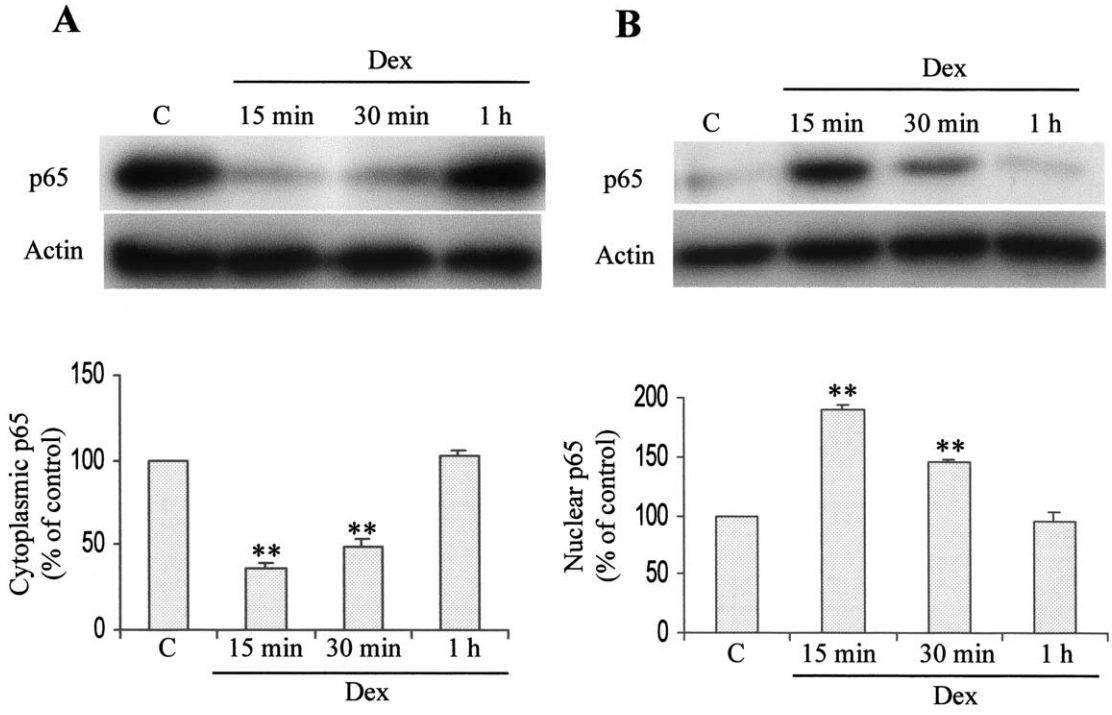

FIG. 2. Dex induces the translocation of the NF-κB protein p65 from the cytoplasm to the nucleus. CCRF-CEM cells were treated with Dex (1 μM) for the indicated periods. The cytoplasmic and nuclear extracts were prepared as described in Materials and methods. The p65 cytoplasmic (A) and nuclear (B) content were determined by western blot analysis, using a rabbit polyclonal anti-p65 antibody, and the bands were quantified with an image analyser. The results presented in the upper panels are representative of four independent experiments. The data shown in the lower panels are the average ±SD of four independent experiments. ** $p < 0.01$, as determined by one-way analysis of variance with Dunnett's post test.

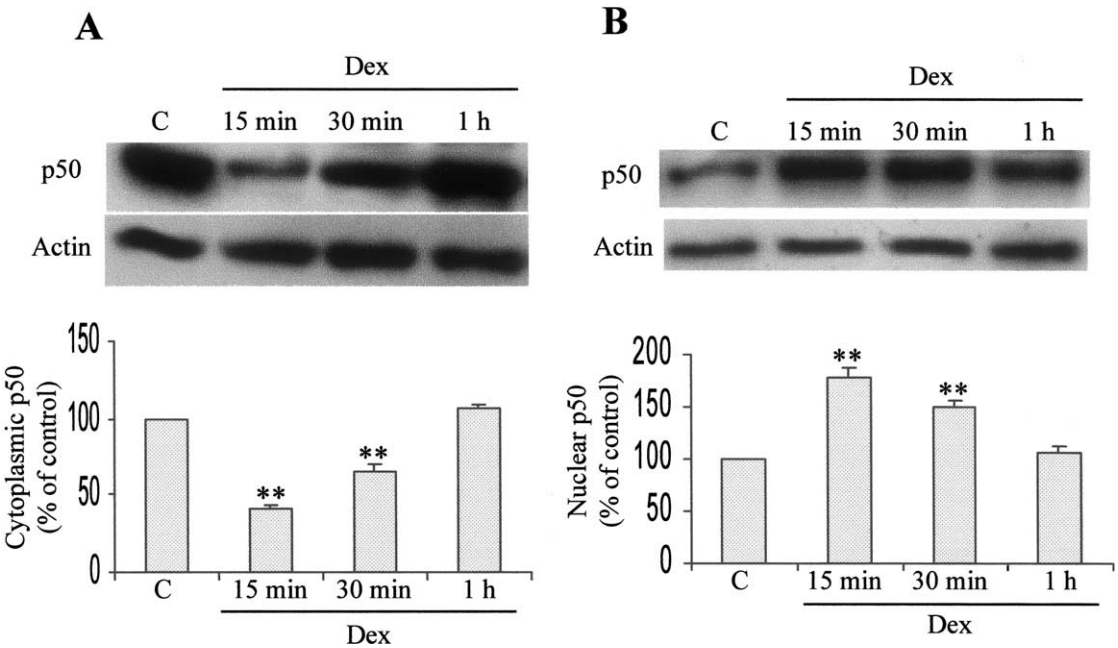

FIG. 3. Dex induces the translocation of the NF-κB protein p50 from the cytoplasm to the nucleus. CCRF-CEM cells were treated with Dex (1 μM) for the indicated periods. The cytoplasmic and nuclear extracts were prepared as described in Materials and methods. The p50 cytoplasmic (A) and nuclear (B) content were determined by western blot analysis, using a rabbit polyclonal anti-p50 antibody, and the bands were quantified with an image analyser. The results presented in the upper panels are representative of four independent experiments. The data shown in the lower panels are the average ±SD of four independent experiments. * $p < 0.05$ and ** $p < 0.01$, as determined by one-way analysis of variance with Dunnett's post test.

proteins to the [^{32}P]-labelled NF-κB probe was significantly higher than that obtained with nuclear extracts from control cells (Fig. 5, lanes 1–3). The effect of Dex on NF-κB binding to DNA was time dependent; that is, the maximal intensity was observed in nuclear extracts from cells treated with Dex

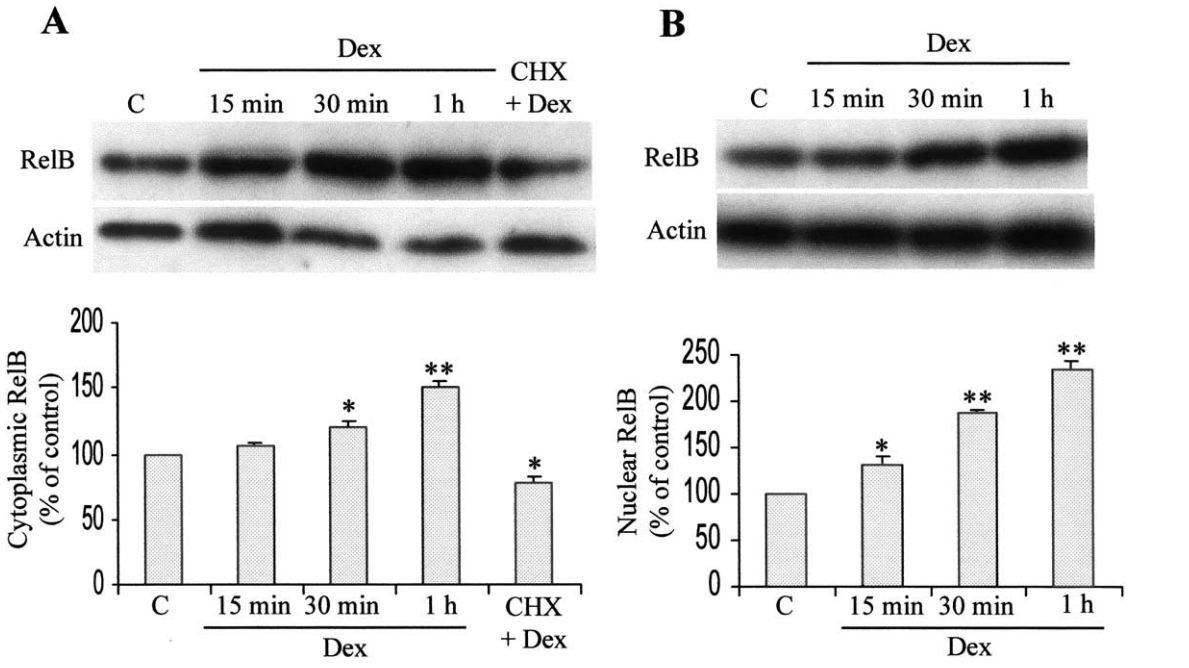

FIG. 4. Dex induces the translocation of the NF-κB protein RelB from the cytoplasm to the nucleus. CCRF-CEM cells were treated with Dex (1 μM), for the indicated periods, in the absence or in the presence of CHX (20 μg/ml) for 1 h. The cytoplasmic and nuclear extracts were prepared, as described in Materials and methods. The RelB cytoplasmic (A) and nuclear (B) content were determined by western blot analysis, using a rabbit polyclonal anti-RelB antibody, and the bands were quantified with an image analyser. The results presented in the upper panels are representative of four independent experiments. The data shown in the lower panels are the average ± SD of four independent experiments. * $p < 0.05$ and ** $p < 0.01$, as determined by one-way analysis of variance with Dunnett's post test.

for 30 min (lane 3), whereas after 1 h of stimulation with Dex (lane 4) the intensity of the bands decreased, approaching the DNA binding activity found in control cells.

To evaluate the specificity of the complexes formed, competition assays were performed by incubation of nuclear extracts from Dex-treated (30 min) cells with the [^{32}P]-labelled NF-κB oligonucleotide probe and with a 100-fold excess of unlabelled oligonucleotide probes, specific for either NF-κB (lane 9) or an unrelated transcription factor, octamer-1 (lane 10). Only the unlabelled NF-κB probe was effective in preventing the formation of specific complexes between the proteins present in the nuclear extracts and the labelled NF-κB probe. This indicates that the bands detected in the autoradiograms represent the formation of specific complexes between NF-κB proteins and the NF-κB-specific oligonucleotide probe.

Supershift analysis performed by incubation of nuclear extracts prepared from Dex-treated cells, for 30 min, with an antibody against p65, prior to the addition of the labelled NF-κB probe, showed the formation of slower migrating complexes that appeared in the autoradiography close to the origin of the gel (lane 7). This indicates that the supershifted band contains the protein p65. In identical assays using antibodies against p50 and RelB, no supershifted bands were detected, but the intensity of the NF-κB–oligonucleotide probe complexes was greatly reduced (lanes 6 and 8, respectively). This indicates that binding of each antibody to NF-κB dimers, containing each of those proteins, prevented their binding to the NF-κB probe. These results indicate that the NF-κB dimers that bind to the specific NF-κB consensus sequence, in response to Dex, contain p65, p50 and RelB, probably arranged in various types of dimers that migrate differently, producing the two bands seen on the autoradiograms.

RU486 (1–10 μM) did not affect NF-κB activation in the absence of Dex, and did not prevent the Dex-induced NF-κB activation (data not shown), indicating that this response of the CCRF-CEM cells to Dex is not mediated by the intracellular GC receptors.

Dex prevents IL-1β-induced NF-κB activation

To determine whether Dex affects the activation of NF-κB elicited by other stimuli, we investigated the effect of this synthetic GC on the IL-1β-induced NF-κB activation. Treatment of the cells with IL-1β alone for 30 min resulted in the disappearance of IκB-α from the corresponding cytoplasmic extracts. In contrast, when the cells were pretreated with Dex (1 μM) before the addition of IL-1β (20 ng/ml), for 30 min, the cytoplasmic level of IκB-α remained identical to that found in the control (Fig. 6A). Moreover, the results presented in Fig. 6B show that IL-1β significantly increased the intensity of the bands corresponding to the NF-κB–DNA complexes rela-

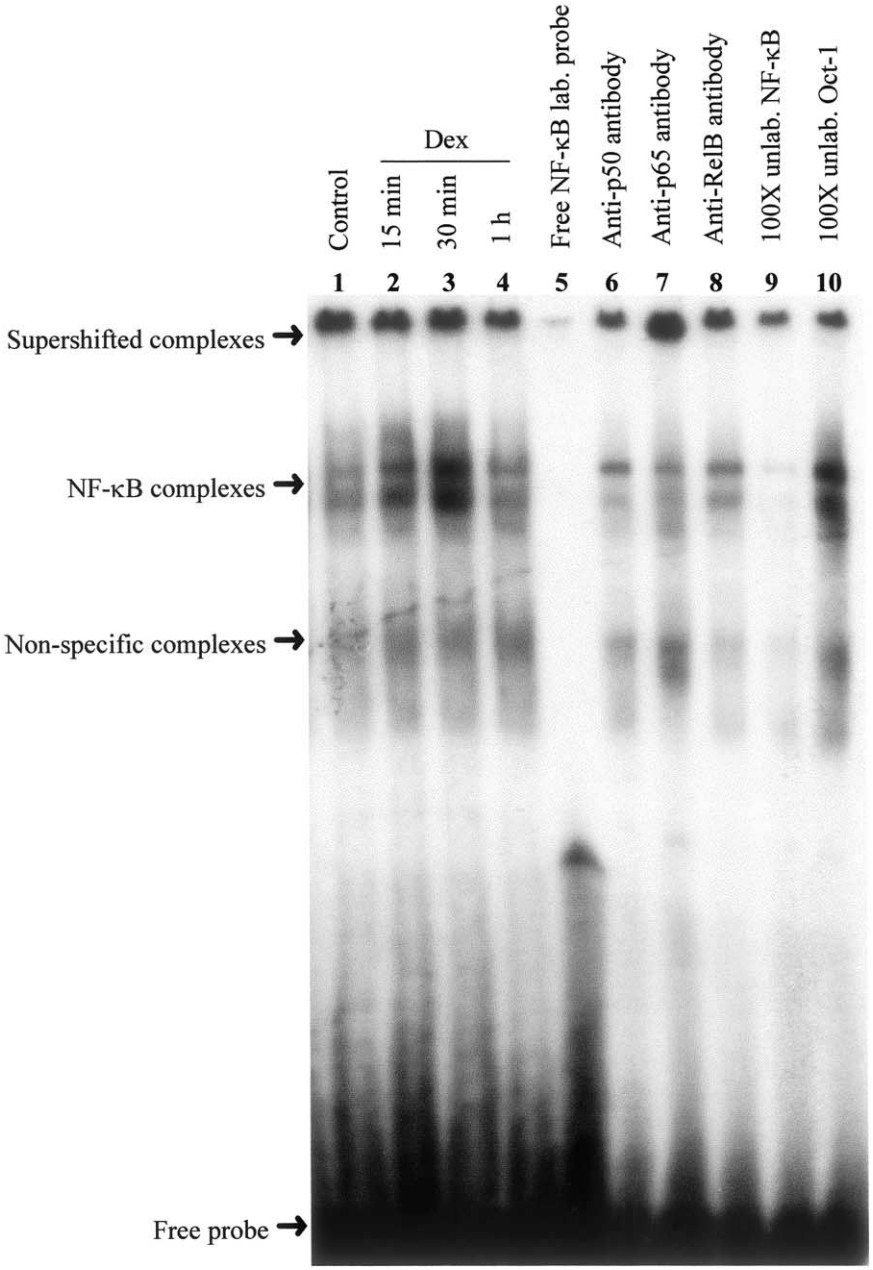

FIG. 5. Activation of NF-κB by Dex. CCRF-CEM cells were treated (lanes 2–4) or not (control, lane 1) with Dex (1 μM), for the indicated periods. Nuclear extracts were prepared and subjected to electrophoretic mobility shift assay analysis as described in Materials and methods. Supershift experiments were performed using antibodies against p50 (lane 6), p65 (lane 7) or RelB (lane 8) in nuclear extracts from cells treated with Dex, for 30 min. Competition experiments with a 100-fold excess of unlabelled competitor probe relatively to the labelled NF-κB probe were also performed using nuclear extracts from cells treated with Dex, for 30 min (lanes 9 and 10). The results are representative of three independent experiments.

tive to control extracts (lanes 1 and 2), whereas pretreatment of the cells with Dex effectively prevented IL-1β-induced NF-κB activation (lane 3).

Discussion

Inhibition of transcription factors, like NF-κB, is considered a major mechanism by which GCs block inflammation and suppress the activation of the immune system.[14,15,27] Although many studies have focused on the ability of GCs to prevent NF-κB activation in response to a wide variety of stimuli,[19,23,24] the mechanisms involved in this inhibition are still incompletely understood. In the present work, we investigated whether and how Dex modulates NF-κB activity in CCRF-CEM cells. The results obtained show that Dex alone induced a transient NF-κB activation, as revealed by its ability to induce IκB-α degradation (Fig. 1), the translocation to the nucleus of p65 and p50 (Fig. 2 and Fig. 3) and NF-κB–DNA binding (Fig. 5). Furthermore, we found

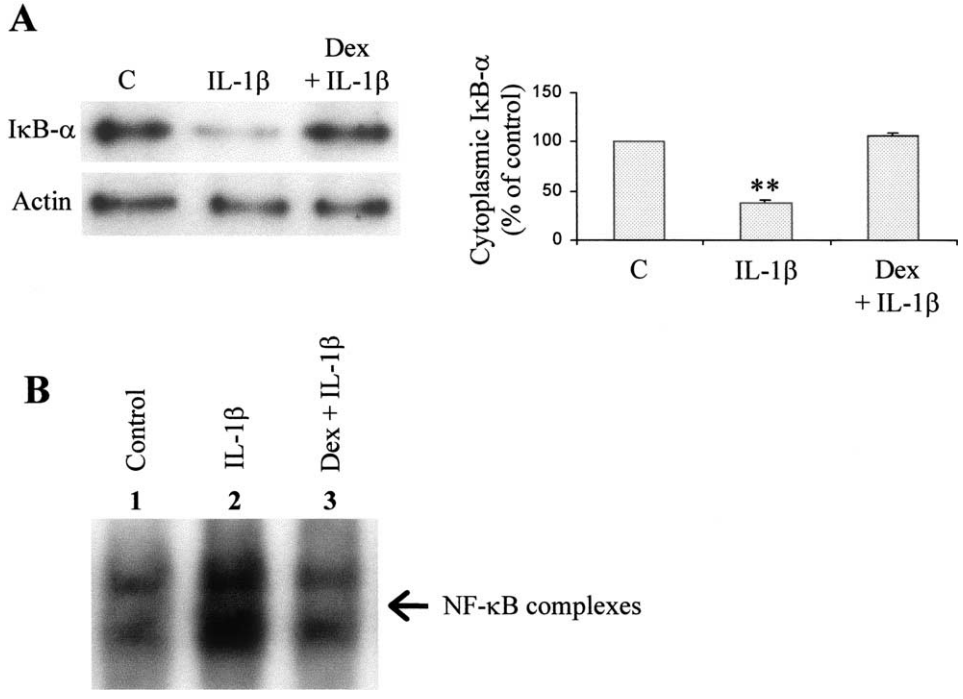

FIG. 6. Dex prevents IL-1β-induced NF-κB activation. CCRF-CEM cells were treated with IL-1β (20 ng/ml) for 30 min in the absence (lane 2) or in the presence of Dex (1 μM for 1 h; lane 3), or left untreated (control, lane 1). Cytoplasmic and nuclear extracts were prepared as described in Materials and methods. (A) The cytoplasmic IκB-α content was determined by western blot analysis, using a rabbit polyclonal anti-IκB-α antibody, and the bands were quantified with an image analyser. The results presented in the left panel are representative of four independent experiments. The data shown in the right panel are an average ±SD of four independent experiments. ** $p < 0.01$, as determined by one-way analysis of variance with Dunnett's post test. (B) Nuclear extracts from cells subjected to the same experimental conditions were analysed by electrophoretic mobility shift assay. The results are representative of three independent experiments.

that Dex prevents NF-κB activation by the potent proinflammatory cytokine IL-1β (Fig. 6).

The effect of Dex on the cytoplasmic levels of IκB-α was biphasic. After the rapid decrease of the protein levels within the first 5 min of stimulation with Dex, the levels of the protein increased in a time-dependent manner, becoming even higher then those found in control cells (Fig. 1). These results, together with the ability of CHX to prevent the increase of the IκB-α levels elicited by Dex (Fig. 1), suggest that this synthetic GC induced *de novo* synthesis of IκB-α protein.

The initial translocation of p65 and p50 to the nucleus was quickly followed by their return to the cytoplasm, where these proteins reached levels identical to those found in control cells, after 1 h treatment with Dex (Figs. 2 and 3). The rapid decrease in the IκB-α levels in the cytoplasm, most probably due to a degradation of the protein,[1,8] was correlated with a fast translocation of p65 and p50 to the nucleus. Furthermore as observed for IκB-α, the cytoplasmic levels of p65 (Fig. 2) and p50 (Fig. 3) showed a secondary increase in cells incubated with Dex for 1 h. Gel shift analysis showed that the intensity of the NF-κB–DNA complexes, which contained p65, p50 and RelB, increased over time, reaching the maximum intensity in cells treated with Dex for 30 min. After the initial increase, the intensity of the NF-κB–DNA complexes decreased in cells treated with Dex for 1 h (Fig. 5), which correlates with the observed increase in the amount of p65 and p50 present in the cytoplasm. These results suggest that these NF-κB subunits migrate back to the cytoplasm after exerting their effect in the nucleus.

Induction of IκB-α expression is one of the mechanisms by which GCs can inhibit NF-κB activation in several cells.[17,18,22,23] Increased synthesis of the IκB-α protein, following its loss, functions as a negative feedback control mechanism since, once re-synthesized, IκB-α enters the nucleus, binds to the NF-κB proteins and thus inhibits NF-κB–DNA binding.[1,2] Furthermore, once bound to the NF-κB proteins, IκB-α may transport them back to the cytoplasm, hence re-establishing their cytoplasmic pool.[7,8,10] In agreement with other reports, our results also suggest that initial activation of NF-κB by Dex was terminated due to the quick replenishment of the cytoplasmic IκB-α levels, which resulted from induction of the synthesis of this inhibitory protein in response to Dex.

NF-κB, particularly p65, has been shown to upregulate the transcription of the IκB-α gene, which contains multiple NF-κB binding sites that are functional in upregulating IκB-α gene expression in response to stimuli that activate NF-κB.[1,2,12] Therefore, our results suggest that the observed increase in

the expression of the inhibitory protein IκB-α was a result of the Dex-induced NF-κB activation.

In human T cells (Jurkat cells)[18] and in brain cells,[22] the induction of IκB-α synthesis by GCs has been reported to be mediated by the GC receptor. Therefore, we used the potent GC receptor antagonist, RU486, to investigate whether the effects of Dex on IκB-α synthesis and NF-κB activation were mediated through intracellular GC receptors. The results obtained showed that pretreatment of the cells with RU486 (1–10 μM) did not prevent the early Dex-induced IκB-α degradation and NF-κB–DNA binding (data not shown), thus suggesting that the effects of Dex on NF-κB reported in this work are independent of the activation of the intracellular GC receptors.

Additionally, treatment of CCRF-CEM cells with Dex induced a parallel increase of both the cytoplasmic and the nuclear levels of RelB, which was prevented by CHX, indicating that, in these cells, this protein is also newly synthesized in response to Dex (Fig. 4A). These results are in agreement with others which showed that Dex also induced the expression and nuclear localization of RelB in T cells.[11] Moreover, while most NF-κB proteins are sequestered in the cytoplasm by IκB-α, RelB is not.[6,11,28] Instead, the translocation of RelB from the cytoplasm to the nucleus appears to be a function of its synthesis[6,11] (i.e. occurring in response to increased synthesis). This may explain the parallel augmentation of both cytoplasmic and nuclear levels of RelB in cells exposed to Dex (Fig. 4).

Like the IκB-α gene, the RelB gene is also susceptible to transcriptional induction by NF-κB.[11] Therefore, it is possible that *de novo* RelB synthesis as well as *de novo* IκB-α synthesis are both mediated by the early and transient activation of NF-κB dimers during the initial response to Dex.

Taken together, the results presented in Figs. 1–5 show that, in an early phase, Dex activates NF-κB in CCRF-CEM cells, quickly followed by repression of the activity of this transcription factor. Nevertheless, these results might argue against a NF-κB-mediated anti-inflammatory action of Dex in these cells, in contrast to what has been described.[14–16,18] To elucidate this question, we investigated the effect of Dex on the activation of NF-κB by IL-1β, a known proinflammatory cytokine. IL-1β is a potent NF-κB activator, which induces the rapid degradation of IκB-α, thereby promoting the translocation of the NF-κB dimers to the nucleus.[21,24,25] IL-1β was also able to rapidly induce IκB-α degradation and the consequent NF-κB activation in the CCRF-CEM cell line (Fig. 6), indicating that these cells respond to inflammatory stimuli, such as IL-1β, by activating NF-κB. Therefore, we evaluated the ability of Dex to counteract that inflammatory stimulus and found that pretreatment of the cells with Dex for 1 h before stimulation with IL-1β prevented the loss of IκB-α (Fig. 6A), as well as the formation of NF-κB–DNA complexes (Fig. 6B). Hence, these results show that Dex efficiently prevents NF-κB activation in response to inflammatory stimuli, in CCRF-CEM cells, as previously reported for other cell systems.[14,18,23]

Since the IκB-α protein is intrinsically unstable and rapidly degraded unless it associates with NF-κB dimers,[1,22] it seems probable that the increased IκB-α levels, in response to Dex, retain the NF-κB proteins in the cytoplasm, even in the presence of a strong inducer like IL-1β. This is in agreement with studies in HeLa cells,[17] T lymphocytes[18] and rat brain[22] showing that the mechanism by which GCs prevent NF-κB activation, in response to various stimuli, is through the induction of the expression of IκB-α.

In summary, this study indicates that, in CCRF-CEM cells, Dex prevents NF-kB activation induced by a proinflammatory stimulus like IL-1β, by a negative feedback mechanism that involves the upregulation of IκB-α synthesis. This effect seems to depend on the early and transient activation of NF-κB, and is not mediated by the intracellular GC receptors.

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