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# INHIBITION OF *EGR-1* AND NF-κB GENE EXPRESSION BY DEXAMETHASONE DURING PHORBOL ESTER-INDUCED HUMAN MONOCYTIC DIFFERENTIATION

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**Abstract**—The treatment of human myeloid leukemia cells (HL-60, U-937, THP-1) with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is associated with growth arrest and appearance of a differentiated monocytic phenotype. While previous studies have reported that the glucocorticoid dexamethasone blocks phenotypic characteristics of monocytic differentiation, we demonstrated in the present work that dexamethasone delays the effects of TPA on the loss of U-937 cell proliferation. We also demonstrated that this glucocortocoid inhibits TPA-induced increases in expression of the *EGR-1* early response gene. The results of nuclear run-on assays and half-life experiments indicated that this effect of dexamethasone is regulated at the post-transcriptional level. Similar studies were performed for the NF-κB gene. While TPA treatment was associated with transient increases in NF-κB mRNA levels, this induction was blocked by dexamethasone. In contrast, dexamethasone had no significant effect on the activation of pre-existing NF-κB protein as determined in DNA-binding assays. Taken together, these findings suggest that the activated glucocorticoid receptor inhibits signaling pathways which include expression of the *EGR-1* and NF-κB genes and that such effects may contribute to a block in TPA-induced monocytic differentiation.

Transcription factors, such as Jun/AP-1, EGR-1 and nuclear factor κB (NF-κB),† are believed to play an important role in regulating gene expression during phorbol ester-induced monocytic differentiation of myeloid leukemia cells. Previous studies have demonstrated that treatment of human U-937 myeloid leukemia cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is associated with the appearance of the monocytic phenotype and loss of proliferative capacity. These effects are also associated with increased expression of the c-*jun* early response gene [1–4]. The c-*jun* gene codes for the major form of the AP-1 transcription factor. AP-1 binds to a heptameric DNA consensus sequence $TGA^{G}/_{C}TCA$ (TPA-responsive element, TRE) in the promoter region of certain genes [5, 6], such as TNF-α and vimentin, which are expressed during monocytic differentiation. Other studies have demonstrated that the glucocorticoid dexamethasone inhibits TPA-induced monocytic differentiation [3]. This effect may be related in part to the finding that the activated glucocorticoid receptor (GCR) can function as an inhibitor of the transcription factor Jun/AP-1 through direct interaction between these two protein complexes [7–9]. However, it is not known whether the activated GCR inhibits other signaling pathways which include expression of early response genes.

In this context, we were interested in determining whether, like c-jun, other transcription factors including EGR-1 and NF-κB are also sensitive to the inhibitory effects of dexamethasone.

The *EGR-1* gene (*zif/268*, *NGFI-A*, *krox 24*, *TIS-8*) belongs to a family coding for zinc finger transcription factors [10, 11]. *EGR-1* is induced during mitogenic stimulation of a variety of cell types including fibroblasts, epithelial cells and B-cells [12, 13]. Moreover, we have demonstrated recently that the *EGR-1* gene is expressed during TPA-induced monocytic differentiation and that this effect is regulated by post-transcriptional mechanisms [14]. The precise role of *EGR-1* in cellular growth and differentiation, however, remains unclear.

Another transcription factor, NF-κB, recognizes and binds an 11-bp DNA sequence present in the κ immunoglobulin light chain gene enhancer [15]. NF-κB-like binding is associated with regulation of genes coding for the Class I major histocompatibility antigen [16, 17], $\beta_2$-microglobulin [18], granulocyte-macrophage colony-stimulating factor [19], interleukin-2 (IL2) [20, 21] and IL2-receptor α chain [22]. The affinity of NF-κB for DNA binding is activated by TNF, IL1 [23] and phorbol esters [15, 24]. Moreover, recent studies have demonstrated that NF-κB is also inducible in human myeloid leukemia cells by ionizing radiation [25] and the DNA-damaging agent, 1-β-D-arabinofuranosylcytosine (ara-C) [26]. NF-κB is located in the cytoplasm bound to an inhibitor protein complex, IκB, which prevents uptake of NF-κB into the nucleus [27, 28]. Treatment of cells with TPA results in dissociation

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† Abbreviations: NF-κB, nuclear factor κB; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; GCR, glucocorticoid receptor; TRE, TPA-responsive element; and SSC, sodium citrate sodium chloride.

of the NF-κB/IκB complex and thus movement of NF-κB to the nucleus [29].

The present work demonstrates that TPA-induced monocytic differentiation of U-937 cells is associated with transient expression of the transcription factors EGR-1 and NF-κB and that these events are inhibited by dexamethasone. These findings suggest that the activated GCR interferes with multiple signaling pathways associated with TPA-induced monocytic differentiation.

## MATERIALS AND METHODS

*Cell culture.* The U-937 cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. The cells were treated with 5 nM TPA (Sigma Chemical Co., St. Louis, MO) and 1 μM dexamethasone (Sigma).

*Proliferation assay.* The proliferation rate of U-937 cells was measured as described previously [30]. Briefly, U-937 cells were plated in 96-well microtiter plates at a density of $2 \times 10^3$ cells/well and treated with 5 nM TPA alone or 1 μM dexamethasone/5 nM TPA for up to 72 hr. The cells were pulsed with 20 μL of a 1:40 dilution of 6.7 Ci/mmol [$^3$H]-thymidine for 1 hr and the incorporated radioactivity was measured in a β-counter.

*Preparation of RNA and Northern blot hybridization.* Total cellular RNA was isolated by the guanidine isothiocyanate-cesium chloride technique. Total cellular RNA (20 μg) was subjected to electrophoresis in a 1% agarose/2.2 M formaldehyde gel, transferred to nitrocellulose, and hybridized to the following $^{32}$P-labeled DNA probes: (1) the 3.9-kb *NotI* fragment isolated from the NF-κB cDNA [31]; (2) the 0.7-kb non-zinc finger insert of a murine *Egr-1* cDNA [11]; and (3) the 2.0-kb *PstI* insert of a chicken β-actin cDNA purified from the pA1 plasmid [32]. Hybridization reactions were carried out for 16–24 hr at 42° in 50% (v/v) formamide, 2× SSC, 1× Denhardt's solution, 0.1% (w/v) sodium dodecyl sulfate, and 200 μMg/mL salmon sperm DNA. Filters were washed and exposed to Kodak X-Omat XAR film using an intensifying screen.

*Nuclear run-on assays.* Nuclei were isolated and newly elongated transcripts labeled with [α-$^{32}$P]UTP (800 Ci/mmol, DuPont-New England Nuclear) at 26° for 30 min. The labeled RNA was hybridized to the following DNAs: (1) the 2.0-kb *PstI* insert of a chicken β-actin cDNA purified from the pA1 plasmid [32]; (2) the 1.8-kb *Bam*HI/*Eco*RI insert of a human c-*jun* DNA probe purified from a pBluescript SK (+) plasmid [33]; (3) the 0.7-kb non-zinc finger insert of a murine *Egr-1* cDNA [11]; and (4) the 3.1-kb *Xho*I/*Nco*I fragment of a human c-*fos* DNA purified from the pc-*fos*-1 plasmid [34]. The digested DNAs were heated to 65° for 15 min, separated in 1% agarose gels, and transferred to nitrocellulose filters. Conditions for prehybridization, hybridization and washing have been described [3].

*Electrophoretic mobility shift assay (EMSA).* Nuclear proteins were prepared according to previously described methods [25]. A 22-bp synthetic oligomer (5'GATCGAGGGGACTTTCCCTAGC3') containing the 11-bp NF-κB binding sequence (GGGGACTTTCC) was end-labeled with [α-$^{32}$P]-dATP using DNA polymerase I and purified in a 12% polyacrylamide gel. Radiolabeled DNA (1 ng) was incubated with 10 μg nuclear protein for 20 min at 20° in 25 mM Tris–HCl, pH 7.6, 250 ng/mL poly dI/dC, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol. The reaction products were analyzed by 5% polyacrylamide gel electrophoresis and autoradiography.

## RESULTS

Previous studies have demonstrated that TPA-induced monocytic differentiation of human myeloid leukemia cells is associated with growth arrest and $G_0/G_1$ cell cycle exit [35, 36]. In the present studies, TPA reduced the proliferative capacity of U-937 cells by more than 99% after 72 hr (Fig. 1). Although exposure of these cells to both 1 μM dexamethasone and 5 nM TPA exhibited a delay in growth inhibition after 24 hr, similar effects were observed after 72 hr when compared to treatment with 5 nM TPA alone (Fig. 1). In contrast, dexamethasone blocked phenotypic characteristics of TPA-induced differentiation, such as adherence and Mac-1 expression ([3, 37] and data not shown). Taken together, these results suggested that the activated GCR has little if any effect on TPA-induced growth arrest, but inhibits appearance of the differentiated U-937 cell phenotype.

Recent work has demonstrated that the inhibition of TPA-induced monocytic differentiation by dexamethasone is associated with down-regulation of Jun/AP-1 [3]. The present results demonstrate that the activated GCR also inhibits induction of the *EGR-1* gene (Fig. 2). While 5 nM TPA transiently increased the level of *EGR-1* transcripts between 1 and 12 hr, expression of this gene was inhibited

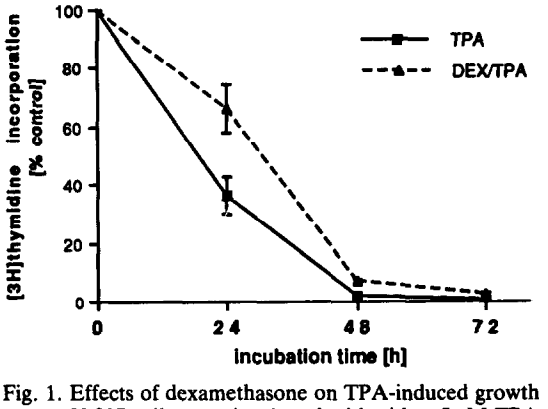


Fig. 1. Effects of dexamethasone on TPA-induced growth arrest. U-937 cells were incubated with either 5 nM TPA (■) or 1 μM dexamethasone/5 nM TPA (▲) for up to 72 hr. Proliferation was measured by incorporation of [$^3$H]-thymidine for 1 hr, and calculated as a percentage of U-937 control cells (U-937 = 100%). Absolute values for control cells were: 24 hr, 62,560 ± 5,100 cpm; 48 hr, 122,350 ± 7,200 cpm; and 72 hr, 201,300 ± 17,800 cpm. Data represent the means ± SD of three independent experiments.

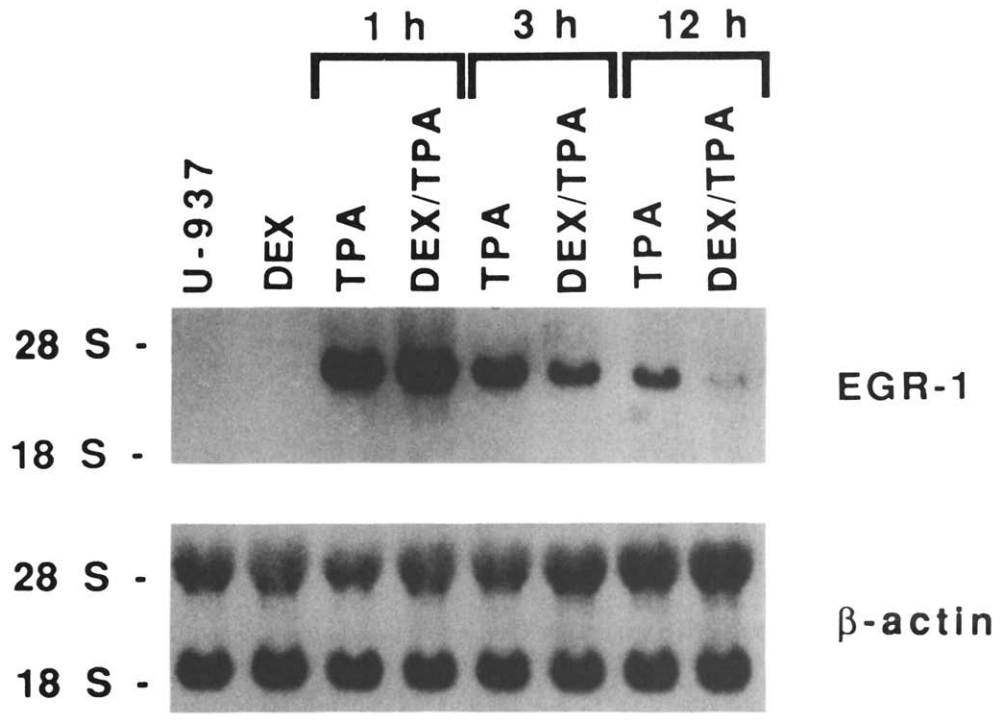


Fig. 2. Inhibition of TPA-induced increases in *EGR-1* mRNA levels by dexamethasone. U-937 cells were treated with 5 nM TPA for 1–12 hr (TPA), 1 μM dexamethasone for 8 hr (DEX) or both agents (DEX/TPA). Total cellular RNA (20 μg/lane) was hybridized to a $^{32}$P-labeled *EGR-1* probe. Hybridization to β-actin probe (18 S signal) demonstrated equal loading of the lanes.

progressively following treatment with both 1 μM dexamethasone and 5 nM TPA (Fig. 2). This finding indicated that, in addition to down-regulating c-*jun* expression [3], dexamethasone also inhibits TPA-induced increases in *EGR-1* transcripts.

We have shown previously that TPA regulates *EGR-1* mRNA levels by a post-transcriptional mechanism [14]. Nuclear run-on assays were therefore performed in order to determine whether the inhibitory effects of dexamethasone are regulated at the transcriptional and/or post-transcriptional level. The β-actin gene (positive control) was constitutively transcribed in control cells, as well as in cells treated with dexamethasone, TPA or the combination of these agents (Fig. 3). While dexamethasone alone enhanced the constitutive expression of the c-*jun* gene and the c-*fos* gene, the levels of the *EGR-1* gene transcription remained nearly unchanged (Fig. 3). Moreover, TPA treatment was associated with a 2-fold increase in the c-*jun* transcription rate and dexamethasone blocked the activation of this gene. In contrast, while there was little if any effect of TPA and dexamethasone on *EGR-1* gene transcription, dexamethasone also inhibited the 3.8-fold induction of the c-*fos* gene in TPA-treated U-937 cells (Fig. 3). These findings indicated that dexamethasone inhibits transcriptional activation of the c-*jun* and c-*fos* genes by TPA, but blocks TPA-induced increases in *EGR-1* expression primarily by post-transcriptional mechanisms. To confirm this hypothesis half-life studies were performed (Fig. 4). While the half-life of TPA-induced *EGR-1* mRNA was 32 min, exposure to 1 μM dexamethasone significantly reduced the stability of *EGR-1* transcripts to 19 min (Fig. 4).

NF-κB represents another transcription factor which may contribute to the induction of differentiation-associated genes after TPA treatment. NF-κB transcripts were increased between 4 and 8 hr following exposure of U-937 cells to 5 nM TPA (Fig. 5). However, treatment of U-937 cells with 1 μM dexamethasone for 8 hr and 5 nM TPA for an additional 8 hr resulted in no detectable expression of the NF-κB gene (Fig. 5). In contrast, inhibition of protein synthesis with 10 μg/mL cycloheximide (CHX) for 2 hr and addition of 5 nM TPA for an additional 6 hr demonstrated expression of NF-κB mRNA. These findings indicated that the TPA-induced increase in NF-κB mRNA levels is a glucocorticoid-sensitive event which is independent of *de novo* protein synthesis.

While the present results demonstrate that dexamethasone inhibits NF-κB expression at the mRNA level, previous work has shown that pre-existing NF-κB protein is located in the cytoplasm in an inactive form. To determine whether dexamethasone also inhibits NF-κB expression at the protein level, we studied interaction of U-937 nuclear proteins with a synthetic oligomer containing an 11-bp NF-κB consensus sequence in EMSAs. There was a low level of nuclear binding to the NF-κB oligomer when using nuclear proteins from

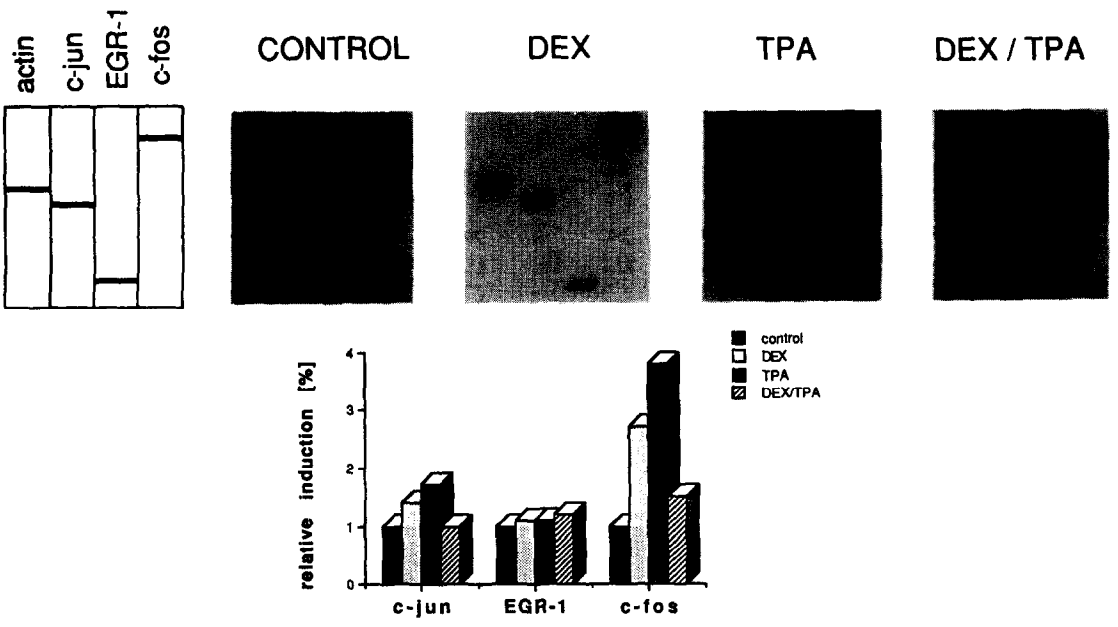


Fig. 3. Top panel: Effects of TPA and dexamethasone on rates of β-actin, c-*jun*, *EGR-1*, and c-*fos* gene transcription. U-937 cells were treated with 1 μM dexamethasone for 8 hr (DEX), 5 nM TPA for 1 hr (TPA), or both agents (DEX/TPA). Nuclei were isolated, and newly synthesized $^{32}$P-labeled RNA was hybridized to 2 μg of β-actin, c-*jun*, *EGR-1*, or c-*fos* DNA inserts after restriction enzyme digestion and Southern blotting. The schematic diagram represents the relative positions of the inserts. Bottom panel: The autoradiograms were scanned using an LKB (Bromma, Sweden) Ultroscan XL laser densitometer and analyzed using the Gelscan XL software package. Intensities of the c-*jun*, *EGR-1* and c-*fos* hybridization signals were normalized against β-actin expression (β-actin = 1). Similar results were obtained from two different experiments.

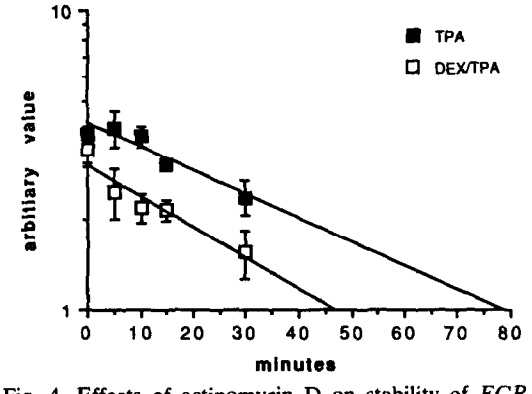


Fig. 4. Effects of actinomycin D on stability of *EGR-1* transcripts. U-937 cells were treated with either 5 nM TPA for 1 hr (TPA) or 1 μM dexamethasone for 8 hr and 5 nM TPA for an additional hour (DEX/TPA). Actinomycin D (5 μg/mL) was then added for 5, 10, 15, and 30 min, respectively. Total cellular RNA (20 μg) was hybridized to the $^{32}$P-labeled *EGR-1* probe (data not shown). Hybridization to the β-actin probe demonstrated equal loading of the lanes (data not shown). The half-life for each treatment was calculated from the autoradiographs following quantification by scanning using an LKB (Bromma, Sweden) Ultroscan XL laser densitometer and the Gelscan XL software package. The results demonstrate the mean ± range of two independent experiments.

untreated cells, while exposure to 5 nM TPA was associated with a progressive increase in NF-κB binding between 1 and 6 hr (Fig. 6). This effect was transient and binding of nuclear proteins to the NF-κB oligomer was reduced significantly by 24–48 hr (Fig. 6). There was little if any effect on this binding of TPA-induced nuclear proteins to the NF-κB oligomer when the cells were exposed to 1 μM dexamethasone for 8 hr and to 5 nM TPA for an additional 6 hr (Fig. 6). Moreover, incubation with 10 μg/mL CHX for 2 hr and an additional 6 hr with 5 nM TPA demonstrated similar binding as compared to TPA treatment alone. These findings indicated that TPA stimulates DNA binding of pre-existing NF-κB protein and that this event is insensitive to the effects of dexamethasone.

## DISCUSSION

The GCR contains a highly conserved domain that mediates receptor binding to specific DNA sequences termed glucocorticoid-responsive elements [38, 39]. The activated GCR also functions as an inhibitor of the transcription factor Jun/AP-1 through a direct interaction between these two protein complexes [7–9]. This regulatory interaction

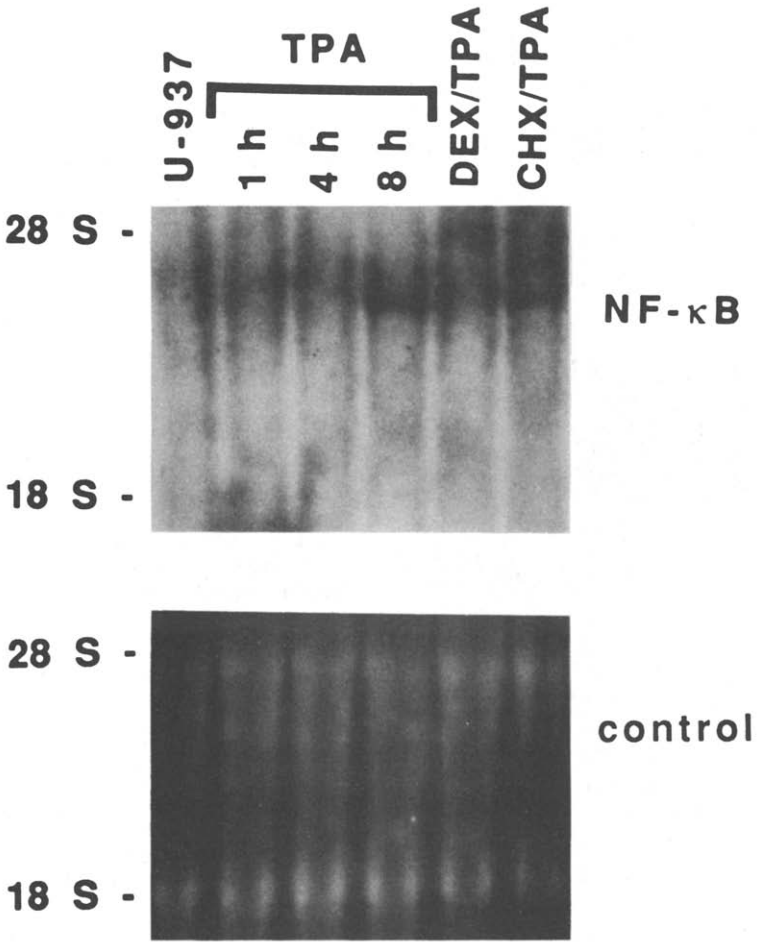


Fig. 5. Effects of dexamethasone and cycloheximide on TPA-induced NF-κB mRNA levels in U-937 cells. U-937 cells were treated with 5 nM TPA for the indicated times. Cells were also incubated with 1 μM dexamethasone for 8 hr and 5 nM TPA for additional 8 hr (DEX/TPA), or with 10 μg/mL cycloheximide for 2 hr and 5 nM TPA for an additional 6 hr (CHX/TPA). Total cellular RNA (100 μg/lane) was isolated and analyzed by hybridization to a $^{32}$P-labeled NF-κB cDNA probe. Ethidiumbromide staining of the 28S and the 18S rRNA (control) demonstrated equal loading of the lanes.

between transcription factors may explain at least in part recent findings that glucocorticoids inhibit TPA-induced monocytic differentiation of U-937 cells [3]. Indeed, previous studies have demonstrated that the c-*jun* gene is induced during TPA treatment of myeloid leukemia cell lines and that this effect is inhibited by dexamethasone [2, 3]. Since Jun/AP-1 autoinduces c-*jun* transcription [40], inhibition of this transcription factor by the activated GCR is associated with a block in expression of the c-*jun* gene. Although the precise role of Jun/AP-1 and c-*jun* gene expression in TPA-induced differentiation remains unclear, it would appear unlikely that this is the only signaling cascade required for the appearance of the monocytic phenotype. Consequently, the present studies have asked whether dexamethasone also inhibits other pathways which involve transcription factors.

In addition to the finding that the *EGR-1* gene is expressed during TPA- and cytokine-induced monocytic differentiation [14], recent studies have demonstrated that *EGR-1* mRNA levels are increased during neural and cardiac cell differentiation [11]. The product of this early response gene may therefore also function in the induction of genes which contribute to the differentiated phenotype. In this context, we were interested in determining whether dexamethasone affects *EGR-1* expression during monocytic differentiation. The results demonstrate that TPA-induced increases in *EGR-1* mRNA levels are blocked by this glucocorticoid. However, in contrast to down-regulation of the c-*jun* gene which occurs at the transcriptional level [3], the present findings show that dexamethasone blocks *EGR-1* expression by a post-transcriptional mechanism.

The present results further demonstrate that TPA treatment of U-937 cells is associated with increased expression of the NK-κB gene. Moreover, the findings indicate that this event is also sensitive to inhibition by dexamethasone. As in the *EGR-1* studies, nuclear run-on assays were performed to determine the mechanism responsible for this effect. However, we were unable to detect sufficient signals

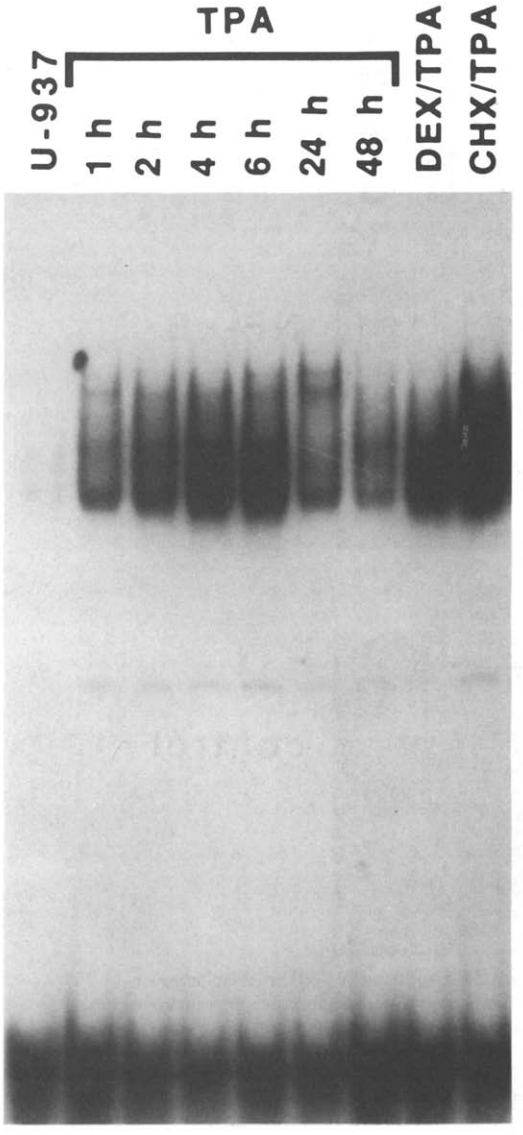


Fig. 6. Effects of dexamethasone and cycloheximide on TPA-induced NF-κB binding activity. Nuclear extracts were isolated from U-937 cells after exposure to 5 nM TPA for the indicated times. Cells were also treated with 1 μM dexamethasone for 8 hr and 5 nM TPA for an additional 6 hr (DEX/TPA) or with 10 μg/mL cycloheximide for 2 hr and 5 nM TPA for 6 hr (CHX/TPA). EMSAs were performed using 10 μg nuclear protein and 1 ng of a $^{32}$P-labeled 22-bp oligonucleotide containing the NF-κB consensus sequence.

to provide a reliable measure of NF-κB gene transcription in control or TPA-treated cells. Consequently, we are unable to determine whether dexamethasone inhibits TPA-induced increases in NF-κB transcripts by a block in transcriptional or post-transcriptional control. Another possibility remained that dexamethasone might inhibit TPA-induced activation of pre-existing NF-κB protein and thereby its binding to DNA. However, the results of gel retardation assays indicate that the activated GCR has little if any effect on DNA binding of NF-κB. While previous studies suggest that protein kinase C (PKC) may contribute to the activation of pre-existing NF-κB protein [29], the present results are in concert with our recent work demonstrating that dexamethasone has no detectable effect on TPA-induced activation of PKC [3]. Taken together, although these findings indicated that dexamethasone inhibits NF-κB signaling by blocking expression of this gene at the mRNA level, interactions of the activated GCR with TPA-induced activation of pre-existing NF-κB protein seem to be of little consequence.

The induction of monocytic differentiation is associated with both growth arrest and appearance of the differentiated phenotype. Dexamethasone treatment appears to dissociate these two events [41]. While this glucocorticoid blocks differentiated characteristics, such as adherence and cell surface monocyte antigen expression, there was only a delayed effect of dexamethasone on TPA-induced loss of proliferative capacity. These findings suggest that distinct signaling pathways control arrest of cell cycle progression and appearance of the differentiated phenotype during TPA treatment. Moreover, the finding that dexamethasone blocks expression of the c-*jun*, *EGR-1*, and NF-κB genes suggests that these events contribute to induction of the monocytic phenotype, while their involvement in the regulation of cell cycle arrest remains unclear.

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