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Rooperol tetraacetate decreases cytokine mRNA levels and binding capacity of transcription factors in U937 cells

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WE have previously described inhibition of the synthesis of three acute-phase inflammatory cytokines in human and rat macrophages by acetate esters of rooperol, a dicatechol of plant origin. Analysing the mechanism of anticytokine activity of rooperol, we compared levels of TNFα, IL-1β and IL-6 mRNAs in the human promonocytic U937 cell line pretreated with phorbol myristate acetate (PMA) and incubated with rooperol tetraacetate (RTA) alone or in combination with LPS (500 ng/ml). It was found that 10 μM RTA decreased the levels of cytokine mRNAs both in the presence and absence of LPS, suggesting pretranslational inhibition of cytokine synthesis. Electrophoretic mobility shift analysis (EMSA) showed that RTA may influence cytokine mRNA expression by decreasing the binding activity of transcription factors NF-κB and AP-1.

Key words: U937 cells, Cytokine mRNA, Transcription factors, Rooperol tetraacetate

Introduction

Macrophages, ubiquitous in the body, have the potential to produce several cytokines involved in inflammation, such as TNFα, IL-1β and IL-6.[1–5] Induced synthesis of these cytokines involves multiple cell signalling pathways and can be regulated at the level of gene transcription, mRNA stability and translation, or cytokine precursor processing and secretion.[4,5] Overproduction of cytokines is believed to play a role in the pathogenesis of a wide range of disorders, such as septic shock, rheumatoid arthritis and asthma.[6–9] Compounds which suppress cytokine production may have beneficial effects in the treatment of such diseases. The list of anti-inflammatory drugs affecting cytokine production is growing steadily and involves glucocorticoids, some antioxidants,[6] bicyclic imidazoles,[10] piperazine derivatives,[11] tenidap[7,8] and many others. We have recently described inhibition of the TNFα, IL-1β and IL-6 (at the protein level) in human and rat alveolar macrophages, human blood monocytes and human promonocytic cell line U937 by a plant dicatechol, rooperol, and its acetate esters.[12] Rooperol derived from the plant *Hypoxis rooperi* is a potent inhibitor of 5-lipoxygenase, but in concentrations up to 10 μM has no activity against either isoform of cyclooxygenase.[13] This suggests that the drug might be therapeutically useful in disorders when it is desirable to inhibit the production of leukotrienes but not prostaglandins, such as asthma and inflammatory bowel disease. Evidence is accumulating that pro-inflammatory cytokines also contribute to the pathogenesis of these as well as other inflammatory disorders, so that it would be desirable to inhibit their production as well. Although rooperol exhibit antioxidative properties,[13] and suppresses NO production by rat macrophages[12] and murine endothelial cells,[14] (overproduction of NO by inducible NO synthase may contribute to the pathogenesis of asthma[9]), the mechanism of its anticytokine activity requires elucidation. The study now reported is an analysis of the mechanism by which rooperol may suppress the production of pro-inflammatory cytokines. We describe the effect of rooperol tetraacetate on the TNFα, IL-1β and IL-6 mRNAs, as well as DNA-binding activity of two transcription factors in U937 cells exposed to rooperol in the presence and absence of bacterial endotoxin.

Materials and Methods

Reagents

RPMI medium, fetal bovine serum (FBS) and antibiotics–antimycotics were purchased from Gibco Life Technologies Inc. (Grand Island, NY); [α-^{32}P]dCTP and [γ-^{32}P]ATP were obtained from Amersham International (Amersham, UK). Oligonucleotide probes were synthesized by Dr. A. Okruszek, Macromolecular Research Centre, Polish Academy of Sciences, Łodź, Poland. All other reagents were from Sigma (St Louis, MO, USA). Endotoxin (LPS, lipopolysaccharide from *Escherichia coli* 026:B6, Sigma, St Louis, MO) was dissolved in sterile phosphate-buffered saline (PBS) at

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the concentration of 1 mg/ml. Rooperol ((E)-1,5-bis (3′,4′-dihydroxyphenyl) pent-4-en-1-yne) tetraacetate (RTA) was dissolved in DMSO as described previously[12] to obtain a stock solution (100 mM), and was further diluted in 10%bovine serum albumin in PBS.

Cell culture

U937 cells were grown at 37°C in 75-cm^2 flasks containing RPMI supplemented with 8% FBS and antibiotics under a humidified atmosphere of 95%air and 5% CO_2. Every third day the medium was doubled, and every sixth day cells were centrifuged and suspended (1:4) in the fresh medium. The experiments were carried out on U937 cells differentiated into a macrophage-like cells in the presence of PMA at a concentration of 34 ng/ml for 48 h. During that period viable and differentiated cells adhered to the culture plates. Unattached cells were discarded and tested factors (in RPMI with 2% FBS) added after additional 24 h culturing of the adherent cells without PMA. Cells were cultured in 12-well plates (Costar) (10^6 cells per well) for cytokine estimation by ELISA, in 90 mm Petri dishes (7×10^6cells per dish) for mRNA isolation and in 60 mm Petri dishes (3×10^6 cells per dish) for isolation of nuclear proteins.

Cytokine assay

In order to avoid discrepancies related to cytokine secretion, total (medium + cells) TNFα, IL-1β and IL-6 proteins were determined using commercially available ELISA kits (Genzyme). After 24 h of culture with the tested factors, U937 cells were scraped from the wells directly into the medium, subjected to three cycles of freezing and thawing, and centrifuged (10 000 ***g*** for 3 min) before cytokine determination.

Isolation of RNA and Northern blot analysis

Total U937 RNA was extracted using the phenol extraction method and LiCl precipitation as described by Rose-John *et al.*[15] from the cells treated with 10 μM RTA for 1 h followed by 2 or 18 h incubation with 500 ng/ml LPS up to the moment of RNA extraction. RNA samples (5 μg) were separated by electrophoresis in 1% agarose gel under denaturing conditions.[16] RNA was then transferred to Hybond-N membranes (Amersham, Arlington, IL) according to the manufacturer's instructions. Hybridization with ^{32}P-labelled probes was performed overnight at 65°C in a mixture containing 1.0 M NaCl, 1% SDS and 10% dextran sulphate. Specific mRNAs were detected using the following probes : 1.1-kb *Pst*I-*Pst*I restriction fragment of human TNFα cDNA (ATCC, Rockville, MA), 1.2-kb *Eco*RI-*Hin*dIII fragment of human IL-6 cDNA (a generous gift of Dr T. Kishimoto), pGEMI containing a 570 bp *Sst*I-*Pvu*II cDNA fragment of human IL-1β (kindly provided by Immunex™, Seattle, WA), a cDNA probe specific for the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ATCC, GenBank/EMBL: M17851), and a 1.5-kb P*st*I-PstI fragment of the pSP64 coding for rat β-actin (kindly provided by Dr J. Sipe, Boston, USA). The probes were labelled with Megaprime Labeling Kit (Amersham). As the reference, 18 S and 28 S rRNA were visualized in UV light after ethidium bromide staining, or 18 S rRNA determined by hybridization with the *Eco*RI-*Sal*I fragment of the plasmid containing human 18 S rRNA cDNA (kindly provided by N. Bhownick, Athens, GA). Non-specifically bound radioactivity was removed by washing and the blots were subjected to autoradiography at -70° C using intensifying screens. The relative intensity of the bands was evaluated by densitometry using a computer imaging (MCID) system (Imaging Research Inc., Canada).

Nuclear protein extraction

Nuclear proteins were obtained by the mini extraction procedure described by Suzuki *et al.*[17] PMA-primed U937 cells were cultured for 1 h with 10 μM RTA followed by exposure to 500 ng/ml of LPS up to the moment of cell collection. Cells were collected with a rubber policeman, washed twice with cold PBS and centrifuged for 5 min at 400 × ***g***. The cells, suspended in buffer containing 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM PMSF, were incubated on ice for 15 min. Nonidet P-40 was added and samples were centrifuged for 30 s at 14 000 r.p.m. Pelleted nuclei were resuspended in buffer containing 50 mM HEPES, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT and 0.1 mM PMSF, centrifuged for 5 min at 14 000 r.p.m. at 4°C and the supernatant was frozen in 10% glycerol. The protein concentration was determined by Lowry's method.

EMSA

The following double-stranded oligonucleotides were used for DNA electrophoretic mobility shift assay: the oligonucleotide that contained two binding sites for NF-κB from c-myc oncogene corresponding to bp –1101–1081 (5'-AAGTCCGGGTTTTCCCCAACC-3')[18] and AP-1 oligonucleotide: 5'-TCGACTAGTATGAGT-CAGCCG-3'.[19] These oligonucleotide probes were either end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase (NF-κB) or labelled with [α-^{32}P]dCTP and Klenow polymerase (AP-1) (Megaprime Labeling Kit). For gel shift analysis 5 μg of nuclear proteins were incubated with 0.5 ng (c.10^5cpm) of labelled oligonucleotides. As competitors, a 100-fold excess of the unlabeled oligonucleotide was added to the binding reaction.

Table 1. Total concentrations (supernatants + cell lysates) of cytokines (ng/ml) in U937 cells after 24 h of culture with RTA (10 μM) or LPS (500 ng/ml), or RTA and LPS. All cells were primed with PMA as described in Materials and Methods. Mean of four to five experiments; ±SD. The statistical differences were evaluated by paired Student's *t*-test in pairs: control vs RTA*, or control vs LPS**, or LPS vs LPS+RTA***. NS, not significant ($P > 0.05$)

	TNFα	IL-1β	IL-6
Control	12.11 ± 2.49	17.10 ± 5.38	7.57 ± 3.00
	$P < 0.05$*	$P < 0.05$*	$P < 0.01$*
RTA	9.23 ± 2.72	13.87 ± 5.54	5.37 ± 1.35
LPS	15.75 ± 3.92	23.24 ± 5.42	14.53 ± 5.63
	$P < 0.01$**	$P < 0.02$**	$P < 0.02$**
LPS + RTA	11.73 ± 1.72	18.75 ± 7.60	10.57 ± 3.46
	$P < 0.02$***	NS	$P < 0.05$***

Results

Rooperol tetraacetate inhibition of cytokine mRNAs in PMA-primed U937 cells cultured in the presence and absence of LPS

We found previously that LPS-induced synthesis of TNFα, IL-1β and IL-6 proteins was inhibited in human and rat alveolar macrophages, human blood monocytes/macrophages and U937 cells exposed to rooperol and its acetate esters.[12] This has been confirmed and extended by the data shown in Table 1. The inhibitory effect of rooperol is less pronounced than in blood monocytes[12] because 'control' U937 cells are in fact significantly stimulated by PMA.

Since cytokine gene expression is often regulated at the pretranslational stages[1,4,5] we determined the levels of specific mRNAs in U937 cells primed with PMA and stimulated with LPS. To compare early and late responses, measurements of the cytokine mRNAs were carried out at 2 and 18 h after exposure to LPS. Figure 1A shows that control cells express TNFα mRNA, due to stimulation with PMA, whereas unprimed and unattached cells do not produce cytokines even after stimulation with LPS (Fig. 1B and Ref. 20). As expected, stimulation with LPS increased the abundance of TNF mRNA, especially after 2 h. We also found that pretreatment of cells with RTA decreased levels of TNFα mRNA both in control and LPS-stimulated cells (Fig. 1A and B). Similar pictures were obtained in three consecutive experiments. Exposure of U937 cells to RTA also decreased the amounts of IL-1β and IL-6 mRNAs, but the effects were more pronounced after 18 h culture with LPS or/and RTA (Figs 2 and 3). Six independent experiments were carried out and Figs 2 and 3 show typical results. The inhibitory activity of RTA appears to be rather specific for cytokine mRNAs since the amount of 18 S RNA was unaffected both in the presence and absence of LPS. Moreover, RTA at 10 μM concentration had no significant effect on the expression of

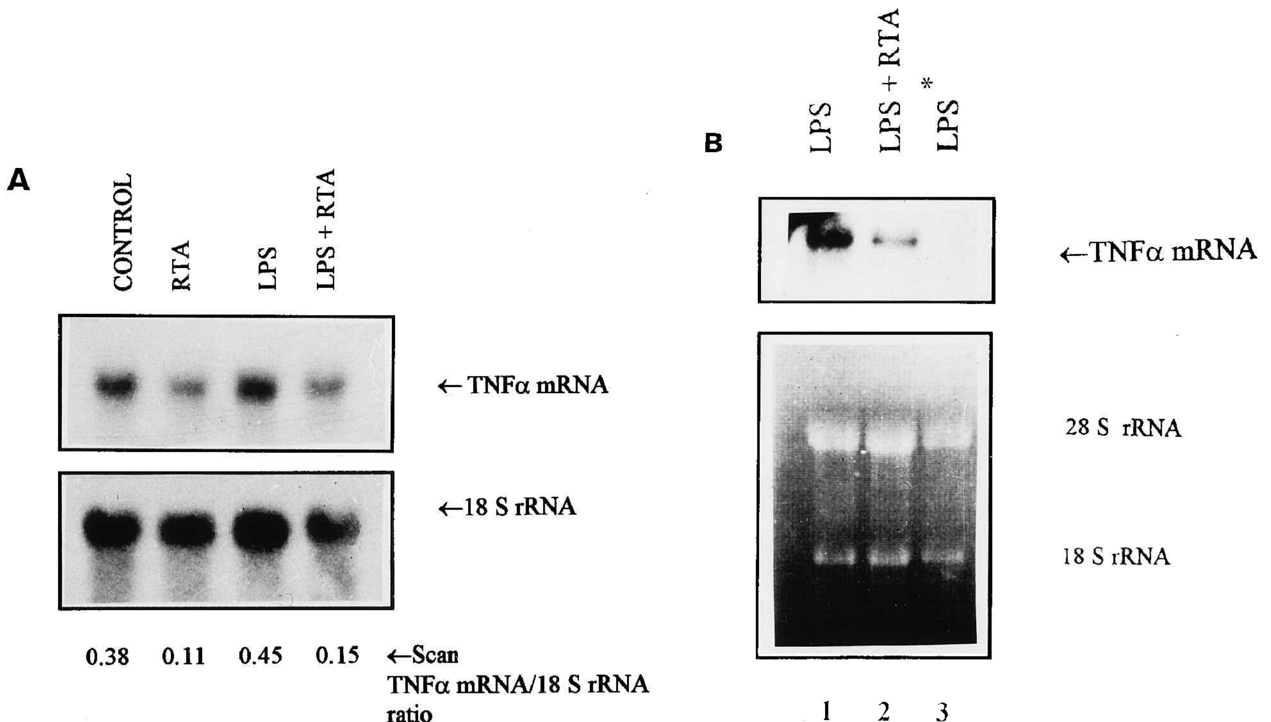

FIG. 1. (A and B) Effect of RTA (10 μM) and/or LPS (500 ng/ml) on relative TNFα mRNA abundance in unprimed* (B, lane 3) U937 cells or in the cells primed for 48 h with PMA (34 ng/ml) and exposed for 1 h to RTA and for the next 2 h to LPS. For comparison blots were rehybridized with a probe for 18 S RNA (A) or UV picture of 18S and 28S rRNA in the ethidium bromide-stained gel is shown (B). For further detail see Materials and Methods. In order to account for differences in RNA load of the gel the ratios of densitometric scans of TNF-α mRNA to 18 S rRNA are reported (A).

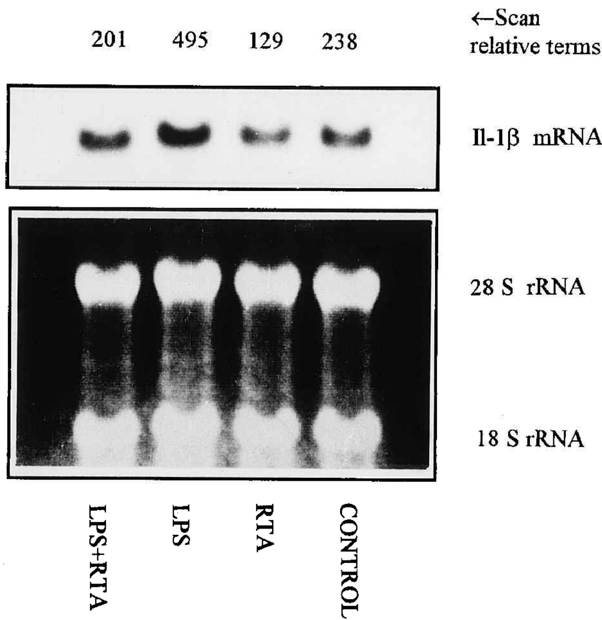

FIG. 2. The effect of RTA (10 μM) and/or LPS (500 ng/ml) on IL-1β mRNA abundance in PMA-primed U937 cells after 18 h incubation with LPS and/or with RTA (cf. Fig. 1). For comparison, the UV picture of 18 S and 28 S rRNA in the ethidium bromide-stained gel is shown. Densitometric scans of IL-1 mRNA are expressed in arbitrary units.

β-actin (a typical structural protein of the cell) and GAPDH (one of the glycolytic pathway enzymes) (Fig. 4). These results suggest that RTA inhibits the synthesis of inflammatory cytokines at the transcriptional level. As shown recently by several authors, translation of cytokine mRNA requires an additional signal provided by stress-responsive kinases and thus in certain cases a dissociation of transcription from translation may occur.[4,21] Our results presented in Table 1 indicate that changes in the total cytokine contents (cell + media) elicited by LPS and RTA in U937 cells roughly correspond to changes in the amount of the specific mRNAs (Figs 1–3), which is consistent with a pretranslational site of action of RTA.

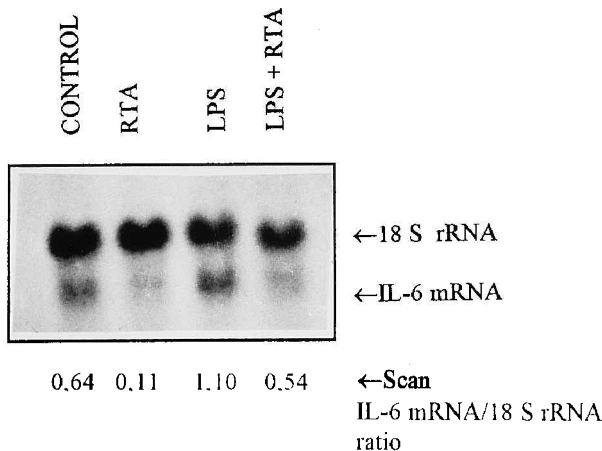

FIG. 3. Effect of RTA (10 μM) and/or LPS (500 ng/ml) on IL-6 mRNA abundance in PMA-primed U937 cells after 18 h incubation (cf. Fig. 2). For comparison, the hybridization pattern of 18 S rRNA is shown. The ratios of densitometric scans of IL-6 mRNA to18 S rRNA are reported.

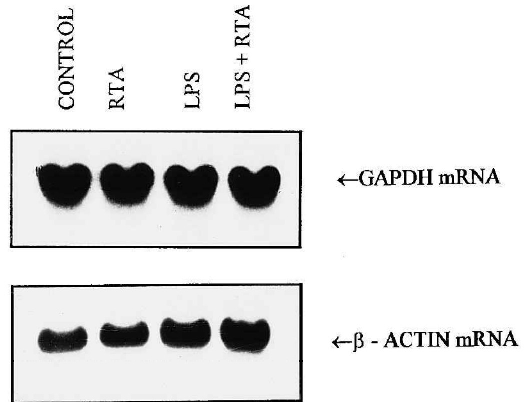

FIG. 4. Abundance of GAPDH mRNA and β-actin mRNA in PMA-primed U937 cells cultured for 18 h with RTA (10 μM) and/or LPS (500 ng/ml).

Inhibition of binding activity of transcription factors NF-κB and AP-1 by RTA in PMA-primed U937 cells cultured in the presence and absence of LPS

Cytokine gene promoters contain multiple regulatory elements recognized by several transcription factors, the most important being NF-κB, AP-1 and C/EBP.[4,5,22] It is known that in U937 cells both PMA and LPS activate NF-κB.[23–25] Figure 5A shows that RTA decreased NF-κB binding activity in PMA-primed (control) and LPS-stimulated U937 cells 30 min. following exposure to LPS. At shorter (15 min) (Fig. 5B) or longer than 4 h incubation times the effect of RTA was less pronounced (data not shown). The inhibitory effect of RTA was comparable in control (PMA-primed) and LPS-stimulated cells (Fig. 5A). When nuclear extracts from U937 cells were analysed by EMSA (three independent experiments) with the AP-1 probe inhibition of binding by RTA was observed after 30 min and after 1 h exposure to LPS (Fig. 6).

Discussion

The U937 cell line, primed toward macrophage differentiation with PMA, is a useful model for studying the regulation of cytokine synthesis.[17,24,25] Although PMA affects protein kinase C and induces synthesis of cytokines (high 'control' values), thereby reducing the relative response to LPS, the U937 cells represent a uniform population that can be easily cultured in amounts sufficient for analysis of RNA and nuclear proteins. Using this model we demonstrated that RTA in 10 μM concentration, which does not

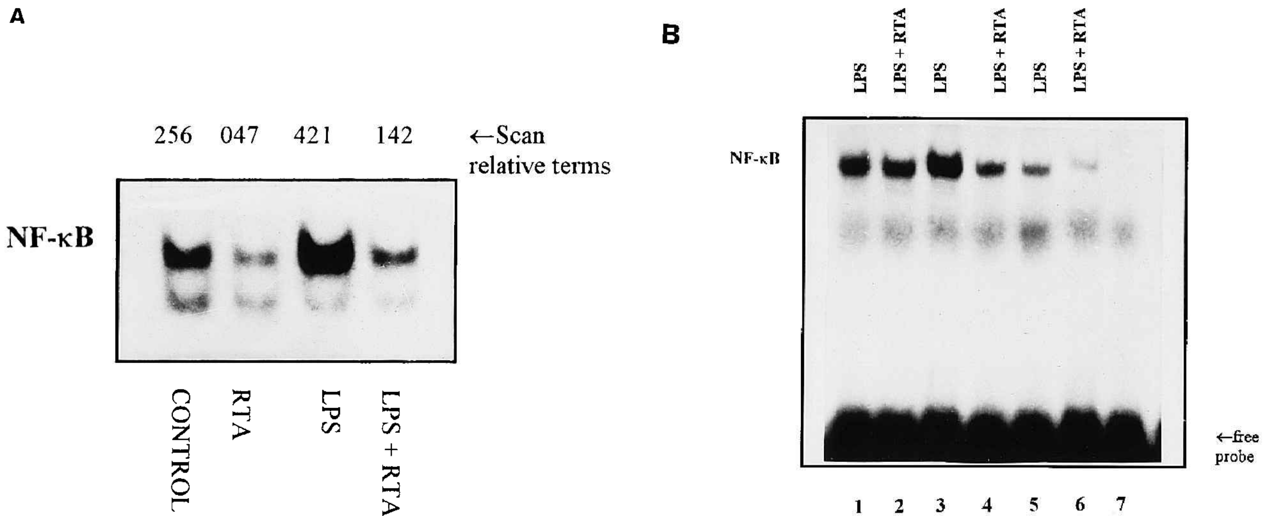

FIG. 5. (A and B) Inhibition by RTA of NF-κB binding to DNA sequences. Electrophoretic mobility shift assay of nuclear extracts isolated from (A) control, RTA- (10 μM), LPS- (500 ng/ml) or RTA + LPS-treated U937 cells primed with PMA; (B) LPS- (500 ng/ml) or LPS+RTA (10 μM)- treated U937 cells primed with PMA. Cells were incubated for 1 h with RTA, followed by LPS addition (A) for 30 min or (B) for 15 min, lanes 1,2; 45 min, lanes 3,4; and 90 min, lanes 5,6. In lane 7 competitor (unlabelled oligo DNA) was run. The nuclear extracts were isolated and processed as described in Materials and Methods. Densitometric scans (A) are expressed in arbitrary units.

show detectable cytotoxic effects such as inhibition of total protein synthesis or leakage of lactate dehydrogenase,[12] decreases abundance of mRNAs coding for TNFα, IL-1β and IL-6 both in control (PMA-primed) and LPS-stimulated U937 cells (Figs 1–3). From comparison of scanning values it is evident that the 'inhibitory index' (control vs. RTA, and LPS vs. LPS+RTA) is very similar suggesting that RTA acts on both PMA-induced and LPS-induced expression of cytokine genes. This may indicate a common target of RTA on the intracellular signalling pathway but the problem requires further studies.

On the other hand, mRNAs coding for other cellular proteins (GAPDH, β-actin) were not detectably affected by RTA. Thus, it can be concluded that RTA inhibits synthesis of the inflammatory cytokines at the transcriptional level, in contrast to other inhibitors such as bicyclic imidazoles[22] or piperazine derivatives[11] which affect translation stages, or tenidap, which acts indirectly by inhibiting the cyclooxygenase pathway.[26] The observed decreases in the amounts of cytokine mRNAs in the presence of RTA could result from corresponding changes in transcription rates or mRNA stability. Cytokine mRNAs contain adenylate/uridylate-rich elements which are held responsible for the short half life of their messages.[27] To exclude the possibility that RTA enhances the rate of cytokine mRNA degradation additional experiments with actinomycin D are required.

On the other hand, the results of our experiments point to the interaction of rooperol with transcription factors, known to be required for cytokine gene transcription: NF-κB and AP-1. The activation of NF-κB

FIG. 6. Inhibition of AP-1 binding to DNA sequences following 60 min RTA treatment or/and 60 min exposure to LPS. Electrophoretic mobility shift assay of nuclear extracts isolated from control, RTA- (10 μM), LPS- (500 ng/ml) or LPS+RTA- treated U937 cells primed with PMA. Cells were incubated for 60 min with RTA, followed by an additional 60 min with LPS and then nuclear extracts were isolated and processed as described in Materials and Methods. Densitometric scans are expressed in arbitrary units.

involves an oxidation step with possible involvement of reactive oxygen species[17,28] and several antioxidants showing anti-inflammatory properties act at this stage.[6,28] As shown by van der Merwe *et al.*[13] rooperol belongs to the group of biologically active antioxidants, and thus by interfering with NF-κB activation may inhibit synthesis of inflammatory cytokines. Redox regulation of the AP-1 transcription factor complex is also well documented in both U937 and HeLa cells[24,29] and may explain the RTA-elicited reduction of AP-1 binding observed in our experiments (Fig. 6). Since EMSA showed only a transient decrease in the binding capacity of NF-κB other possible sites of anticytokine action of RTA may be identified in future.

We can conclude, however, that RTA influences abundance of mRNAs coding for TNFα, IL-1β and IL-6 in human macrophage-like cells and affects binding of transcription factors implicated in cytokine gene expression. This provides a better understanding of the activity of RTA as potential anti-inflammatory and anticytokine drug.

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