Differential modulation of IL-8 and TNF-α expression in human keratinocytes by buflomedil chlorhydrate and pentoxifylline

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ABSTRACT

Pentoxifylline (PTX) is a methylxanthine derivative used in a wide range of dermatoses. As well as its hemorrhheologic activity, PTX has anti-inflammatory properties. Buflomedil chlorhydrate (BC) is another hemorrhheological drug with peripheral vasodilatory action, whose clinical uses are similar to those of PTX. Both drugs increase intracellular levels of cAMP, either secondary to phosphodiesterase inhibition (PTX) or adenyl-cyclase stimulation (BC). Long-term cultures of normal human keratinocytes were prepared in a free-serum medium, and stimulated with 1 mg/ml of phorbol 12-myristate 13-acetate (TPA) and PTX or BC (100-1000 µg/ml). Levels of TNF-α, IL-1α, IL-1β, IL-8 and TGF-β1 using ELISA and Northern blot or RT-PCR techniques were measured. TPA-induced TNF-α and IL-8 release from keratinocytes. TPA did not induce IL-1α or IL-1β release of keratinocytes. TPA increased RNA expression of the TNF-α, IL-1α, IL-1β, IL-8 and TGF-β1. BC diminished TPA-induced TNF-α and IL-8 release from keratinocytes; in the case of IL-8 it is possible that this inhibition occur to transcriptional level. Moreover PTX was unable to inhibit TNF-α and IL-8 synthesis and expression. PTX and BC reduced TPA-induced IL-1α and β expression. It is possible that BC action is specifically exerted on keratinocytes, because we did not find similar results with TNF-α and IL-8 synthesis in mononuclear peripheral blood cells.

KEY WORDS: pentoxitylline - butlomedil - phorbol - esters - cytokines

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INTRODUCTION

The keratinocyte, the main constituent cell in the epidermis, may respond to numerous external stimuli by producing certain cytokines which are responsible for the initial phase of various inflammatory skin diseases; it may therefore be regarded as an immune cell capable of triggering an inflammatory response (1-4). It is known that keratinocytes are capable of secreting the following cytokines, among others: TNF-α, IL-1α, IL-1β, IL-3, IL-5, IL-6, IL-7 (5), IL-8, IL-10 (6), IL-11, IL-12, GM-CSF, G-CSF, M-CSF (7, 8), TGF-α, TGF-β and PDGE. Complex interaction between these cytokines occurs in the epidermis; each one may have diverse inflammatory effects, and at very low concentrations they may facilitate and improve the transendothelial migration of other inflammatory cells (9). Pentoxifylline (PTX) is a methylxanthine derivative that has been used therapeutically for quite a long time in a huge number of patients suffering from vascular disorders (10). Its hemorrheological, antiaggregant and anti-inflammatory properties, and its ability to protect the vascular endothelium has led to its use in a wide range of dermatoses (11, 12). Additionally to its hemorrheologic activity, PTX has recently been found to suppress the production of TNF-α by murine and human leucocytes (13). The majority of studies involving PTX reported in the literature were performed on LPS-stimulated monocytes, and there are no specific studies using keratinocyte cultures. We are unaware of any studies of the action of PTX on cell lines stimulated by using phorbol derivatives (TPA).

Bufomedil chlorhydrate (BC) is another hemorrheological drug with peripheral vasodilatory action, whose clinical applications are similar to those of PTX. Of particular interest among its effects are the increase in erythrocyte deformability, the encouragement of platelet antiaggregation, and the increase in tissue oxygenation and collateral circulation (14). There is wide clinical experience in the use of BC in the treatment of peripheral vascular disease, and its pharmacokinetics and tolerance are well known (15), even though acute BC intoxication has been published (16). Some studies have also shown the efficacy of this drug in diabetic microangiopathy, Raynaud's disease (17) and in the treatment of trophic skin lesions associated with vascular insufficiency. In vitro studies on human neutrophils showed that BC reduces aggregation, formation of pseudopods and, therefore, adhesion and degranulation of lisosomal enzymes (18). BC can play a role in adhesive molecule expression on the leukocyte and endothelial cells (19). Increase in cAMP, secondary to the stimulation of adenyl cyclase by BC, has been reported. In the same way as PTX, BC may exert a modulatory effect on cytokine synthesis, although there are no in vitro or in vivo studies about this action.

Phorbol esters are well-known to induce the transcription of several genes, among them those coding for the inflammatory cytokines TNF-α, IL-1α, IL-1β, IL-6 and IL-8. In this study these data are verified in a culture of keratinocytes, and then the possible modulatory effects of PTX and BC with respect to the expression and synthesis of these cytokines are tested.

MATERIALS AND METHODS

Cell culture

Long-term cultures of normal human KC were prepared from human female breast skin as described previously (20) with some modifications. Briefly, skin samples were washed in phosphatebuffered salive (PBS) and connective tissue was trimmed. The skin
samples were then treated by 1% dispase (Boehringer Mannheim, Madrid, Spain) in PBS with gentamycin (20 mg/ml) at 37°C overnight. Epidermal sheets were peeled from the dermis and stirred in 0.05% trypsin and 0.53 mM ethylenediamine tetra-acetic acid (EDTA) solution (trypsin-EDTA solution) for 10 min at 37°C. The trypsin-EDTA solution was inactivated with Eagle's minimum essential medium (MEM) with 10% fetal bovine serum (FBS) (Gibco BRL, Zaragoza, Spain), and cells centrifuged at 1000 x g for 10 min. Cell pellets were resuspended in 10 ml of complete keratinocyte serum free medium (K-SFM) containing bovine pituitary extract (BPE) and epidermal growth factor (EGF; Gibco BRL, Zaragoza, Spain) and cultured at 37°C in a humidified atmosphere of 5% CO₂, in tissue culture 75 cm² (Nunclon, Denmark). Cultures were fed every third day and after confluency they were subcultured by trypsin-EDTA solution followed by addiction of Eagle's MEM with 10% FBS and centrifugation, and replated at a split ratio of 1:3. Testing of third-passage cells was carried out at 70-80% confluency. Cell viability was determined by trypan blue exclusion and by flow cytometry using propidium iodide. Similar levels of viability were detected before and after incubation with the different drugs and concentrations used (>85%). No significant changes in keratinocytes viability was found for any of the PTX or BC concentrations evaluated. We changed the complete K-SFM to basal medium without BPE and EGF 72 h before testing to avoid a medium-changing effect on cytokine production (21, 22). Data were generated using KC from three different donors.

**Phorbol 12-myristate 13-acetate, buflomedil and pentoxifylline preparation**

Phorbol 12-myristate 13-acetate (TPA, Sigma, Madrid, Spain) was dissolved in DMSO (1000 mg/ml). Bufomedil and Pentoxifylline (Sigma, Madrid, Spain) were dissolved in saline and utilized at concentrations of 100 and 1000 µg/ml.

**TNF-α, IL-1α, IL-1β, IL-6 and IL-8 Enzvinelinked innnunosorbeizt assay (ELISA)**

Commercially available ELISA kits (R&D Systems, Minneapolis, MN) were used to measure human TNF-α, IL-1α, IL-1β, IL-6 and IL-8 proteins levels in cell-free supernatants. These kits are based on the use of a mouse anti-cytokine monoclonal antibody and a polyclonal antibody against cytokine, conjugated to horseradish peroxidase. These kits are sensitive from 0.3-5 pg/ml, and do not crossreact with any other known cytokines at concentrations between 50-100 ng/ml. Each supernatant was analyzed in duplicate.

**Northern blot analysis**

Northern blot analysis of cytokine RNA expression was performed (23). Cultured KC were gently detached, resuspended in guanidinium isothiocyanate (Sigma, Madrid, Spain), and extracted with an equal volume of acetate/EDTA-equilibrated phenol (60°C for 25 min with frequent vortexing). The aqueous phase was recovered after centrifugation and extracted once with an equal volume of phenol/chloroform and twice with chloroform. The resulting RNA was precipitated overnight at —20°C with 2.5 volumes of ethanol.

After extraction, the amount of total RNA was measured by a spectrophotometer Lambda Bio UVNIS (Perkin Elmer, Madrid, Spain). The purified RNA was quantified by optical density at 260 nm. Five mg of RNA from each sample was then electrophoresed on a 1% agarose gel containing 20 mM sodium borate, pH 8.3, 0.5 mM EDTA, and 3% formaldehyde. Ribosomal RNA 18S and 28S were determined by UV visualization after ethidium bromide staining and the RNA was transferred to
nitrocellulose paper (Schleicher and Schuell, Dasel, Germany) in 10x (SSC) (1.5 M sodium chloride and 150 mM sodium citrate) using capillary blotting overnight. The blots were baked at 80°C under vacuum for 2 h, and prehybridized at 55°C in Rapid-hyb buffer (RPN 1635, Amersham) for at least 45 min. TNF-α, IL-1α, IL-1β and TGF-β1 were obtained of the pAW737, phL1 - AcDNA, YEpsecl and phTGFβ-2 plasmids (American Type Culture Collection) respectively. For control hybridization a cDNA probe encoding for the house-keeping protein β-actin was used (HHCl89 plasmid, American Type Culture Collection). cDNA fragments were radiolabeled by random priming with a 32P-dCTP (>6000 Ci/mmol) (Stratagene, La Jolla, California). Hybridization was performed over 2 h at 55°C. The blots were then washed twice in 2X SSC and 0.1% sodium dodecylsulfate (SDS) at room temperature followed by a 2x high-stringency wash in 0.1x SSC at 65°C. Autoradiography was carried out at —80°C by using Kodak X-omat film (Eastman Kodak, Rochester, NY) with an intensifying screen. The developed autoradiographs were scanned by a BioRad Model 620 Video Densitometer (BioRad, Bilbao, Spain).

**Polymerase chain reaction analysis**

Total RNA from keratinocytes was extracted by the guanidinium thiocyanate/phenol-chloroform procedure (23). The total amount of RNA was quantitated by spectrophotometry. Using equal amounts of RNA, cDNA was synthesized by reverse transcription using GeneAmp RNA PCR kit (Perkin-Elmer) following the manufacturer's instructions. Primers for amplifying IL-8 (US: GAG AAG TTT TTG AAG AGG CTG AGA A, and DS: CTG TGA GGT AAG ATG GTG GCT AAT A) were designed using Oligo 4.0 software and synthesized and high performance liquid chromatography-purified by Bio-Synthesis (Texas, USA). β-Actin control primers were obtained from Clontech (California, USA). A hot-start PCR protocol with cDNA templates after reverse transcriptase was used. The PCR master mix contained 1.25 U Taq polymerase (Perkin-Elmer), 0.1 mM dNTPs (Perkin-Elmer), 10x PCR buffer (100 mM TrisHC1, pH 8.3; 500 mM KCl; 15 mM MgCl2; 0.01% wt/vol gelatin; autoclaved, Perkin-Elmer), 20-50 pmol each of 5'-3' primers and deionized water in a total volume of 50 ml. The reaction was carried out in a thermocycler 2400 (Perkin-Elmer) and consisted of 30 cycles of denaturing at 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 90 s. A second round of PCR was performed using internal primers according to confirm the PCR product. The PCR cycle and primer concentration dependency of signal strength were preliminary optimized. For each experiment, internal controls lacking template were performed and verified to be negative. PCR using total RNA without reverse transcriptase reaction as template to check for DNA contamination revealed no specific amplification. PCR products were analyzed by electrophoresis on 2.5% agarose gels and visualized by ethidium bromide staining and the size of amplified products was compared with molecular weight markers run in parallel. Densitometric analysis was performed using the BIO1D software (Vilber Lourmat).

**Mononuclear cells**

Mononuclear cells were isolated from heparinized blood samples of four normal adult donors by Ficoll-Hypaque density gradient centrifugation and adjusted at 2x10^6 cells/ml in RPMI 1640 (with 10% FBS and 100 mg/ml Gentamycin). The mono-nuclear cells were cultured in a 12-well microtiter plate (2 million cells per well) and were stimulated with 10 µg/ml of LPS (Sigma, Madrid, Spain), 1 µg/ml of TPA and various concentrations of Buflomedil or saline. After 24 h culture supernatants were harvested
for IL-1β measurement and after 48 h for TNF-α, IL-6 and IL-8 measurements. The quantifications of these cytokines were estimated in duplicate by ELISA (R&D Systems, Minneapolis, MN) and the results expressed in pg/million cells.

RESULTS

In vitro effects of PTX on cytokine production by TPA-induced keratinocytes

Tumor necrosis factor-α. As shown in Fig. 1, TPA induced TNF-α production by keratinocytes. Following PTX (100 µg/ml) stimulus, no effects on TNF-α synthesis were observed (Fig. 1). At a dose of 1000 µg/ml, protein synthesis was slightly increased. The addition of PTX to 1 µg/ml TPA cultures slightly decreased TNF-α synthesis (Fig 1). After 24 h, however, the addition of PTX produced a slight increase in TNF-α in culture supernatants (media from 210 to 223 pg/ml) (data not shown). Concerning RNA expression, there was no observable change measured after either 4 (Fig. 2a) or 8 h, nor was there any tendency of reduction in gene expression between specimens stimulated with 1 µg/ml of TPA plus PTX at different concentrations and those stimulated only with TPA. When PTX (100 µg/ml) was added 2 h prior to stimulation with TPA, no change was observed.

Interleukin-1α. Neither PTX alone in comparison with the control, nor the combination of TPA and PTX at the doses studied in comparison with TPA 1 µg/ml, affected the protein translation of IL-1α in culture supernatants, measured after 8 (Fig. 1) or 24 h (data not shown). Regarding RNA synthesis, PTX (1000 pg/ml) did not influence IL-1α expression. TPA (1 µg/ml) plus PTX (100-1000 pg/ml) produced no variation in the IL-1α RNA concentration measured after 4 h, but after 8 h this expression fell by a half (Fig 2b).

Interleukin-1β. Neither PTX alone in comparison with control, nor the combination of TPA and PTX at the doses studied in comparison to TPA 1 µg/ml affected the protein translation of IL-1β in culture supernatants, either after 8 (Fig. 1) or 24 h (data not shown). As for RNA, PTX (1000 µg/ml) by itself neither stimulated nor inhibited IL-1β synthesis in comparison with control. Unlike IL-1α, the combination of TPA (1 µg/ml) plus PTX (100 or 1000 µg/ml) after 4 h induced a non dose-dependent reduction by half — with respect to TPA (1 µg/ml) — in the expression of the IL-1β gene (Fig. 2c). No observable changes were found after 8 h.

Interleukin 8. TPA induced IL-8 production by keratinocytes. The addition of PTX to TPA 1 µg/ml or control cultures, did not affect IL-8 protein levels after 8 h (Fig. 1) or 24 h (data not shown).

In vitro effects of BC on cytokine production by TPA-induced keratinocytes

Tumor necrosis factor-α. As shown in Fig. 3, TPA induced TNF-α production by keratinocytes. Following BC (100 and 1000 pg/ml) stimulus no effects on TNF-α synthesis were observed. The combination of TPA and BC at the studied doses, showed a significant dose-dependent inhibition of protein synthesis after 8 h, with staggered inhibition of the production of this cytokine, proportional to the doses of BC from 100 µg/ml (Fig. 3) to 2000 µg/ml (data not shown). Regarding TNF-α RNA analysis, expression remained constant with TPA plus BC (100 µg/ml) with respect to TPA 1 mg/ml; however, a dose of 1000 µg/ml BC decreased the expression of this cytokine measured after 8 h (Fig. 4a). When BC (10, 50 or 100 µg/ml) was added 2 h prior to stimulation with TPA, keratinocytes-derived TNF-α expression remained constant.
Interleukin-la. As with PTX, BC added to control or TPA induced cultures had no influence on IL-la protein measured after 8 (Fig. 3) or 24 h (data not shown). Regarding RNA, BC (1000 pg/ml) did not stimulate nor inhibit IL-la expression. After TPA (1 µg/ml) stimulation, low doses of BC (100 µg/ml) produced no effect on IL-la RNA. Higher doses (1000 pg/ml) produced a 50 or 75% reduction of IL-la RNA expression measured after 4 (Fig. 4b) and 8 h.

Interleukin-1β. BC added to control or TPA 1 µg/ml cultures, had no influence on IL-1β protein translation measured after 8 (Fig. 3) or 24 h (data not shown). Concerning RNA, the addition of BC (1000 µg/ml) increased by 2 to 2.5 times the expression of the IL-1β gene; however, after stimulation with 1 µg/ml of TPA, BC at doses of 100-1000 µg/ml reduced by as much as 50% and 15% this cytokine's expression measured after 4 and 8 h, this inhibition being most evident after 4 h (Fig. 4c).

Interleukin 8. BC (100 to 1000 µg/ml) slightly increased the synthesis of IL-8. Addition of BC to TPA keratinocyte cultures induced a dose-dependent inhibition of protein synthesis after 8 h. After both 8 and 24 h, addition of 1000 µg/ml of BC made IL-8 levels fall almost to half with respect to TPA 1 µg/ml (Fig. 3). Unstimulated keratinocytes did not express IL-8 RNA. Treatment with 100 ng/ml TPA resulted in a marked increase of IL-8 RNA after 8 h (Fig. 5). Preincubation with BC for 2 h prior to stimulation with TPA resulted in a dosedependent reduction of keratinocytes-derived IL-8 expression (Fig. 5).

Transforming Growth factor-β1. We did not perform assays for TGF-β1 in culture supernatants. BC or PTX (100 and 1000 µm/ml) did not inhibit nor stimulate RNA expression of TGF-β1 induced by TPA 1 mg/ml measured after 4 and 8 h (data not shown).

**In vitro effects of BC on cytokine production by TPA or LPS-induced mononuclear peripheral blood cells**

As shown in Fig. 6, TPA induced TNF-α, IL-1β, IL-6 and IL-8 production by mononuclear peripheral blood cells. BC at doses of 50, 100 and 1000 pg/ml did not inhibit nor stimulate TNF-α, IL-1β, IL-6 and IL-8 protein synthesis, measured after 24 h, in TPA 1 pg/ml (Fig. 6) or LPS 10 µg/ml (data not shown) induced cultures. In contrast similar experiments with dexamethasone decreased cytokine levels in culture supernatants (data not shown).

**DISCUSSION**

PTX is a methylxanthine derivative that functions as a phosphodiesterase inhibitor (24). PTX is well known for its hemorrheologic and anti-inflammatory properties (10, 13, 25-27). Recent studies in animals have shown the effects of PTX in inhibiting the afferent phase of contact eczema. This effect has been attributed to inhibition of TNF-α synthesis (28). The same research team has demonstrated in vitro how PTX suppresses UVB-induced TNF-α, IL-1 and IL-6 release by keratinocytes, while not after stimulation with a phorbol derivate (29). In our trials PTX was found not to have a clear and significant inhibitory action on the synthesis of TNF-α (Fig. 1). Regarding RNA, there was no significant difference between the levels found in the specimens stimulated with TPA plus PTX and the control samples stimulated with TPA alone (Fig. 2a). These data show that PTX does not suppress TPA-induced TNF-α release from keratinocytes; whereas in other immune cells, PTX reduced LPS or anti-CD3-induced TNF-α expression, probably via the generation of intracellular cAMP (13, 25, 30).
Theoretically there are two possible explanations for this last fact. On one hand, we may attribute this lack of immunomodulatory capacity to the degree of differentiation reached by these keratinocytes, or it may be specific to the studied cell line. On the other hand, a more plausible hypothesis can be proposed, in which TPA increases TNF-α via PKC, with PTX not being able to modify this pathway. PTX does not suppress TPA-induced IL-8 release by keratinocytes (Fig. 1), data that agrees with that obtained in vivo by Martich et al. (31).

However, in our cultures BC caused a dose-dependent reduction in TNF-α and IL-8 synthesis (Fig. 3). BC (1000 µg/ml) reduced TPA-induced TNF-α expression (Fig. 4a). BC inhibition of TPA-induced IL-8 upregulation could occur at transcriptional level (Fig. 5). It may happen, also, that the inhibiting effect of BC on TNF-α synthesis is exerted during transcription. Nevertheless, in cultures of different cell lines such as mononuclear blood cells, and lower doses such as 100 µg/ml, different results were obtained.

IL-1α and IL-1β RNA expression were increased after TPA stimulation. We did not perform nuclear run-on assays, and so were unable to distinguish between an increase in transcription and an increase in the stability of the RNA of the two cytokines. PTX reduced TPA-induced IL-1α and IL-1β RNA (Fig. 2b, 2c). These findings were more obvious in the case of BC (Fig. 4b, 4c). No changes in IL-1α or IL-1β levels, after TPA stimulation or different combinations with PTX and BC, were detected, being similar to those found in the control in almost all assays (Figs 1 and 3). Although we did not perform cell lysis in order to measure the intracellular protein production, probably these results suggest a secretion deficiency rather than an abnormality in the synthesis of these two cytokines. Although TPA is a significant inducer of IL-1, also it has been demonstrated that TPA can induce surface IL-1 receptor (R) in keratinocyte cultures. Some studies in human keratinocytes demonstrated that at 24 h after TPA treatment there was only a slight increase in detectable IL-1 released to the supernatants, and there was an increase of 9- to 20-fold in surface IL-1R levels per cell in postconfluent cultures (32). Low levels of supernatant IL-1 activity in our keratinocyte cultures after TPA treatment may be partly due to interaction between IL-1 and IL-1R. In this way it is difficult to explain the theoretical downregulator effect of PTX or BC about these cytokines. The mechanism by which BC acts is not totally understood. It acts as an adrenergic antagonist (mainly a-2), stimulating adenyl cyclase, therefore increasing intracellular cAMP (33). BC at high doses seems to have a non-specific calcium and sodium antagonist channel action.

There are different opinions in the literature with regard to the modulation of IL-1 by substances which increase cAMP. In this way, some authors (34, 35) show that PTX or other xanthines suppress LPS-induced IL-1 release by monocytes. On the other hand, both myeloid leukemia cells and human monocytes show an increase in IL-1β production when stimulated simultaneously by phorbol derivates and cAMP analogs (dibutryl cAMP) or substances, such as PGE2, which raises the endogenous levels of cAMP (35, 36). Other authors (37) found that an inhibition of this cytokine was produced by substances which increase the cGMP, but not by those which raise the cAMP. In any case, these studies were not carried out on keratinocytes, and the results may therefore vary according to the cell line studied. Lastly, PTX and BC did not induce any effect on TPA-induced TGF-β1 RNA expression. In the literature we have been unable to find any reference to the action of PTX on this cytokine, either in keratinocytes or in other cell lines.

From the above data, we can conclude that BC in vitro, under our experimental conditions can inhibit, in a dose-dependent way, TPA-induced cytokines by
keratinocytes, specifically TNF-α and IL-8, and probably IL-1β and IL-1α as well. Modulation of BC on TPA-induced IL-1α and expression is similar to that obtained with PTX and therefore could be related to the rise in cAMP produced by both drugs by different means. Unlike PTX, BC alone raises the IL-10 RNA expression, but does not affect the transcription of IL-1α. This action may also be attributable to the intracellular rise in cAMP, although this mechanism would not entirely account for the inhibiting action of BC on TPA-induced TNF-α and IL-8 synthesis, as such an inhibition is not produced in the case of PTX.

Further studies should be performed on keratinocytes and other immune cells in order to confirm these findings and determine the mechanism by which they act. It may be that BC, by increasing the cAMP and exerting a calcium antagonist effect, or by some unknown mechanism, can inhibit PKC, act on some stage of the complex cascade of protein synthesis, or interfere with cell transcription. It may be that BC's action is specifically exerted on keratinocytes, as we found no similar results concerning the synthesis of TNF-α and IL-8 in peripheral blood mononuclear cells (Fig. 6). These findings may help us find new topical or systemic applications for a drug that is widely used in everyday clinical practice.

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Figure 1. Determination of TNF-α, IL-1α, IL-1β and IL-8 concentration in cultured supernatants. The KC were cultured in basal K-SFM without BPE and EGF for 72 h before induction. Human KC were cultured for 8 h with PTX 1000 (1000 µg/ml), TPA (1 µg/ml), TPA + PTX 100 (1 µg/ml +100 µg/ml), TPA + PTX 1000 (1 µg + 1000 ng/ml) and under basal conditions (control). Supernatants were tested for cytokines in triplicate using ELISA kits. Data teptesent the mean±SD of three experiments.
Figure 2. Regulation of cytokine RNA extracted from KC following TPA and PTX exposure. Lane 1: control; lane 2: PTX 1000 µg/ml; lane 3: TPA 1 µg/ml; lane 4: TPA (1 µg/ml) + PTX (100 µg/ml); lane 5: TPA (1 ng/ml) + PTX (1000 µg/ml). Northern blot analysis was performed on KC treated with the indicated concentrations of TPA and PTX, and total cellular RNA was analyzed (5 µg per lane) for cytokine RNA expression. β-actin probing was performed to ensure uniformity of loading and transfer of RNA.

A) TNF-α RNA expression 4 h after exposure. Densitometry results are plotted in arbitrary units of TNF-α/β-actin densitometry values, with control (lane 1) TNF-α/β-actin = 1. B) IL-1α RNA expression 8 h after exposure. Densitometry results were calculated and plotted as in A). C) IL-1β RNA expression 4 h after exposure. Densitometry results were calculated and plotted as in A).
Figure 3. Determination of TNF-α, IL-1α, IL-1β and IL-8 concentration in cultured supernatants. The KC were cultured in basal K-SFM without BPE and EGF for 72 h before induction. Human KC were cultured for 8 h with BC 1000 (1000 µg/ml), TPA (1 µg/ml), TPA + BC 100 (1 µg/ml + 100 µg/ml), TPA + BC 1000 (1 µg + 1000 µg/ml) and under basal conditions (control). Supernatants were tested for cytokines in triplicate using ELI- SA kits. Data represent the mean±SD of three experiments.
Figure 4. Regulation of cytokine RNA extracted from KC following TPA and BC exposure. Lane 1: control; lane 2: BC 1000 µg/ml; lane 3: TPA 1 µg/ml; lane 4: TPA (1 µg/ml)+BC (100 µg/ml); lane 5: TPA (1 µg/ml) + BC (1000 µg/ml). Northern blot analysis was performed on KC treated with the indicated concentrations of TPA and BC, and total cellular RNA was analyzed (5 µg per lane) for cytokine RNA expression. B actin probing was performed to ensure uniformity of loading and transfer of RNA. Densitometry results were calculated and plotted as in Fig. 2. A) TNF-α RNA expression 8 h after exposure. B) IL-1α RNA expression 4 h after exposure. C) IL-1β RNA expression 4 h after exposure.
Figure 5. Regulation of IL-8 RNA extracted from KC following TPA and BC exposure (after 8 h). Lane 1: control; lane 2: BC 100 µg/ml; lane 3: TPA 100 ng/ml; lane 4: TPA (100 ng/ml) + BC (100 µg/ml); lane 5: TPA (100 ng/ml) + BC (50 µg/ml); lane 6: TPA (100 ng/ml) + BC (10 µg/ml). RT-PCR analysis was performed on KC treated with the indicated concentrations of TPA and BC, and total cellular RNA was analyzed for IL-8 RNA expression. β-actin probing was performed to ensure uniformity of loading and transfer of RNA. Densitometry results were calculated and plotted as in Fig. 2.
Figure 6. Determination of TNF-α, IL-1β, IL-6 and IL-8 concentration in cultured supernatants. Mononuclear cells were cultured for 24 h with TPA (1 µg/ml), TPA + BC (50) (1 µg/ml + 50 µg/ml), TPA + BC (100) (1 µg + l00 µg/ml), TPA + BC (1000) (1 µg/ml+1000 µg/ml) and under basal conditions (control). Supernatants were tested for cytokines in triplicate using ELISA kits. Data represent the mean±SD of four experiments.