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Nickel and skin irritants up-regulate tumor necrosis factor-α mRNA in keratinocytes by different but potentially synergistic mechanisms

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Abstract

A critical role of tumor necrosis factor (TNF)-α in irritant contact dermatitis and in the challenge phase of allergic contact dermatitis has recently been demonstrated *in vivo*. As *in situ* hybridization studies have indicated that keratinocytes were the cellular source of TNF-α in these reactions, we studied the mechanisms of TNF-α mRNA induction in keratinocytes by agents that induce contact dermatitis. Murine $Ia^-/CD3^-$ epidermal cells were stimulated with phorbol myristate acetate (PMA), dimethylsulfoxide (DMSO), sodium dodecyl sulfate (SDS) and $NiSO_4$, all of which up-regulated epidermal cell TNF-α mRNA production. In contrast, trinitrobenzenesulfonic acid and trinitrochlorobenzene did not significantly up-regulate TNF-α mRNA. These results were confirmed with murine keratinocyte cell lines. In keratinocytes transfected with a chloramphenicol acetyltransferase construct containing the −1059 to +138 base pair TNF-α promoter, increased promoter activity was observed upon stimulation with PMA and DMSO. In addition, PMA stimulation did not affect the stability of TNF-α mRNA. The PMA- but also the DMSO- and SDS-induced up-regulation of TNF-α mRNA was abolished by an inhibitor of protein kinase C (PKC). In contrast, $NiSO_4$ up-regulated TNF-α mRNA by a PKC-independent mechanism, did not increase TNF-α promoter activity, but markedly increased the stability of the TNF-α mRNA. Co-stimulation with PMA and $NiSO_4$ induced a marked increase in TNF-α mRNA over that obtained with each agent alone. Thus, whereas PKC-dependent irritants act by up-regulating TNF-α promoter activity, nickel acts via post-transcriptional regulation. Our results also establish that some irritants and irritant sensitizers directly induce TNF-α in keratinocytes without intermediate Langerhans cell-derived signals.

Introduction

Contact of the skin with chemicals may result in a large variety of harmful short-term effects at the site of contact. The two most frequent manifestations are irritant contact dermatitis and allergic contact dermatitis. Whereas allergic contact dermatitis represents a lymphocyte-mediated delayed type hypersensitivity reaction that requires previous sensitization by the same chemical, irritant contact dermatitis is a form of toxic skin inflammation induced by primary contact with chemicals and is thought not to be mediated by lymphocytes. Although irritant contact dermatitis is the major cause of occupational skin diseases (1), the mechanisms underlying this reaction are poorly understood (2–4). Recently, several studies have drawn attention to cytokines, a class of endogenous mediators that plays a critical role in the induction of inflammation following the epicutaneous application of irritants. Studies in mice have demonstrated that primary irritant contact dermatitis to trinitrochlorobenzene (TNCB) as well as the elicitation phase of allergic contact dermatitis could be inhibited by administration of antibodies directed to tumor necrosis factor (TNF) or by recombinant soluble TNF

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receptors (5,6). In addition, *in vivo* skin painting with TNCB was found to be associated with an up-regulation of TNF-α mRNA in the treated skin areas. TNF-α mRNA was increased within band-shaped zones of the epidermis, suggesting that keratinocytes might be the cellular source (5). These findings were extended by a study demonstrating that *in vivo* painting with a number of irritant sensitizers and non-sensitizing irritants including dimethylsulfoxide (DMSO) increased TNF-α mRNA in Ia^-/Thy-1^- epidermal cells 6 h after epicutaneous application. In addition, when the cells were stimulated *in vitro*, biologically active TNF-α protein was secreted into the supernatant (7). Furthermore, it has recently been shown that perturbation of the cutaneous barrier by *in vivo* application of acetone led to increased TNF-α mRNA and protein within the epidermis (8). These studies have clearly shown that several non-sensitizing skin irritants and some irritant sensitizers up-regulate epidermal TNF-α mRNA and protein that may be responsible for at least some features of the cutaneous inflammatory phenomenon referred to as irritant contact dermatitis.

Cultured keratinocytes have been shown to produce and release TNF-α *in vitro* (5,9), and therefore constitute a useful model to demonstrate any direct effect of irritants on the keratinocyte and to describe the regulation of TNF-α gene expression by irritants and sensitizers. We therefore studied the regulation of TNF-α mRNA in freshly isolated murine keratinocytes and in cultured murine keratinocyte cell lines.

Here we show that the non-sensitizing irritant phorbol myristate acetate (PMA) up-regulates TNF-α mRNA and protein in Ia^-/$CD3^-$ epidermal cells. In addition, DMSO and sodium dodecyl sulfate (SDS) also induce TNF-α mRNA both in Ia^-/$CD3^-$ epidermal cells and in transformed keratinocyte cell lines, via a protein kinase C (PKC)-dependent increase in promoter activity. We also show that nickel, the most frequent contact sensitizer, directly up-regulates TNF-α mRNA and protein in Ia^-/$CD3^-$ epidermal cells. This nickel-induced up-regulation of TNF-α mRNA was PKC-independent and associated with a pronounced increase in mRNA stability rather than an enhanced promoter activity. Thus, whereas the non-sensitizing irritants tested up-regulate TNF-α mRNA via an increase in TNF-α promoter activity, nickel salts act via post-transcriptional modulation of the TNF-α mRNA.

Methods

Epidermal cell cultures

Epidermal cells were obtained from BALB/c mice as described (10) and incubated with mAb 145-2C11 (anti-CD3) and M5/114.15.2 (anti-Ia) followed by MAR 18.5 and complement (Cedar Lane, Hornby, Ontario). The remaining cells were washed and cultured in RPMI 1640 supplemented with penicillin, streptomycin, glutamine, 2-mercaptoethanol and 10% FCS. On day 3, all non-adherent cells were removed by vigorous pipetting. After one additional day of culture, the adherent cells were used for *in vitro* assays. FACS analysis of the adherent cells demonstrated <0.2% Ia^+ or $CD3^+$ cells.

Keratinocyte cell lines

We used the BALB/c-derived PAM212 (11), the C_3H-derived HD_{II} and HEL-30, as well as the C57BL/6-derived BDVIIa keratinocyte cell lines. The cell lines were cultured in DMEM supplemented with antibiotics, glutamine and 5% FCS (PAM212) or 10% FCS (HD_{II}, HEL-30, BDVIIa). All experiments were performed on sub-confluent cell cultures.

Reagents

DMSO and $CoCl_2$ were from Merck (Darmstadt, Germany), SDS was from Serva (Heidelberg, Germany), trinitrobenzenesulfonic acid (TNBS) was from Kodak (Rochester, NY), TNCB was from Siegfried (Zofingen, Switzerland), PMA was from Sigma (St Louis, MO), $NiSO_4$, $NiCl_2$, $Cr_2Na_2O_7$ and Na_2SO_4 were from Fluka (Buchs, Switzerland), pentoxyifylline was from Hoechst (Frankfurt, Germany), and H7, HA1004, actinomycin D (act-D) and cycloheximide (chx) were from Calbiochem (Läufelfingen, Switzerland). Murine rTNF-α was obtained from Genzyme (Cambridge, MA) and rabbit anti-mouse TNF serum was kindly provided by G. E. Grau (Department of Pathology, University of Geneva, Switzerland).

In vitro stimulation of transformed keratinocytes

PAM212 keratinocytes were pulsed for 15 min at 37°C with DMSO (20–1% v/v), SDS (0.01–0.001% w/v), TNCB (10^{-3} M) and TNBS (10^{-2} to 10^{-3} M) under serum-free conditions, followed by washings and incubation in complete media. In addition, PAM212 cells were incubated with PMA (100–1 ng/ml), lipopolysaccharide (LPS; 10 μg/ml) in complete media or with $NiSO_4$, $NiCl_2$ and Na_2SO_4 (10^{-2} to 10^{-4} M) or $CoCl_2$ and $Cr_2Na_2O_7$ (10^{-3} to 10^{-4} M) under serum-free conditions. Total RNA was extracted after 30, 60 and 180 min (PMA, LPS, DMSO, SDS, TNCB and TNBS) or after 60, 120 and 240 min ($NiSO_4$, $NiCl_2$, Na_2SO_4, $CoCl_2$ and $Cr_2Na_2O_7$). Optimal times of extraction and concentrations of stimulants to induce TNF-α mRNA are shown in Table 1 and these conditions were used in all experiments unless otherwise stated.

TNF bioassay

Cell culture supernatants were desalted over Amicon 10 columns (Beverly, MA). The amount of TNF in the supernatants was determined by its inhibitory action on the growth of WEHI 164 subclone 13 fibrosarcoma cells as described (12). Briefly,

Table 1. Optimal induction of TNF-α mRNA in murine PAM212 keratinocytes

Stimulus	TNF-α mRNA (fold induction)[a]	Time optimum (h)	Concentration
PMA	10.5[b]	1	10 ng/ml
DMSO	11.2[b]	1	20% v/v
SDS	15.3[b]	1	0.0075% w/v
$NiSO_4$	7.3[b]	4	10^{-2} M
TNCB	<2		
TNBS	<2		
Na_2SO_4	<2		
$Cr_2Na_2O_7$	<2		
$CoCl_2$	<2		

[a]Ratio between stimulated and non-stimulated samples after normalization to the housekeeping gene.

[b]Determined by computing densitometric analysis of the Northern blots.

2500 WEHI 164/13 cells were incubated for 40 h in the presence of serial dilutions of the cell supernatants to be tested. Proliferation was assessed by [^{3}H]thymidine incorporation (1 μCi for the final 16 h). The specificity of the assay was verified by blocking with rabbit anti-mouse TNF serum (12).

Northern blotting

Total RNA was extracted as described by Chomczynski and Sacci (13). Total RNA (20 μg) was separated on 1.2% agarose gels and transferred to Biodyne A transfer membranes (Pall, Portsmouth, UK) followed by UV cross-linking. To detect specific mRNA, we used the 696 bp (*Taq*I–*Eco*RI) fragment of the mouse TNF-α gene subcloned into the pSP65 vector (14) and the 1100 bp (*Pst*I–*Pst*I) fragment of the glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) gene subcloned into the pSP64 vector (15). The probes were labeled with [^{32}P]UTP and hybridizations were performed at 62°C for 14 h as described (5). The samples were analyzed by computing densitometer analysis of the X-ray films (Molecular Dynamics, Sunnyvale, CA) and results are expressed as relative densitometric values normalized for variations in the house-keeping gene (G3PDH) from the same filter.

Reverse transcription polymerase chain reaction (RT-PCR)

Three μg of total RNA were treated with 3 U DNase I (Boehringer-Mannheim, Mannheim, Germany) for 40 min at 37°C to remove any contaminating genomic DNA. The RNA was retro-transcribed as described (16) and stored at −20°C until use.

RT-PCR was performed with 8 ng cDNA in 1.5 mM $MgCl_2$, containing 250 μM of each deoxynucleotide, 1 U Taq polymerase (Life Technologies, Basel, Switzerland) diluted in the buffer supplied by the manufacturer and 1 μM of each primer. The total reaction volume was overlaid with mineral oil and amplified for 30 or 32 cycles. The amplified PCR products were visualized by ethidium bromide staining after separation on 2% agarose gels. The intensity of the PCR bands was measured by computing densitometry and corrected for variations in amplification of the housekeeping gene; dihydrofolate reductase (DHFR). The primers for TNF-α and DHFR were obtained from Stratagene (La Jolla, CA); and amplified a 276 and 447 bp nucleotide fragment respectively. For each set of primers, serial dilutions of cDNA were amplified for 26, 28, 30 and 32 cycles to define the optimal conditions for linear PCR amplification. The negative controls comprised in each RT-PCR included (i) omitting cDNA, (ii) performing PCR on non-retrotranscribed RNA to exclude the presence of contaminating genomic DNA and (iii) performing PCR using cDNA synthesized on RNA lysis buffer containing no RNA. All controls were negative and only those of category (i) are shown. The specificity of the PCR bands was confirmed by restriction site analysis of the amplified cDNA, which generated restriction fragments of the expected size (not shown).

Transient transfections

HD_{II} keratinocytes were grown to 50% confluence and transfected with a construct containing the −1059 to +138 bp TNF-α promoter fragment cloned into the PBLCAT3 vector (17). In order to transfect the cells, liposomes were formed by incubating 5 μg of the reporter plasmid with 40 μg *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammoniummethyl-sulfate (Boehringer-Mannheim) for 15 min at room temperature in a total volume of 100 μl. After dilution in serum-free media, the liposomes were added to the cells. Following 5 h of incubation, complete media was added to the cultures and the cells were rested 12 h before a 24 h stimulation with PMA, DMSO or $NiSO_4$. The cells were harvested, washed two times in PBS, and lysed in 200 μl lysis buffer (18) by three cycles of freezing and thawing. For CAT assays, 180 μl of the lysates were incubated with acetyl-coenzyme A and [^{14}C]chloramphenicol for 14 h. The acetylation products were separated by thin layer chromatography and quantified by scanning with a phosphoimager (Molecular Dynamics). Protein concentrations in the samples were determined by the Bradford method (Bio-Rad, Glattbrugg, Switzerland) and used for normalization of the CAT activity. In addition, a CAT-PCR technique was established. Briefly, the cells were rested for 12 h after transfection, then stimulated with 10 ng/ml PMA for 1 h or 10^{-2} M $NiSO_4$ for 3 h. The cells were lysed, total RNA was extracted, treated with DNase to remove the plasmid DNA, retrotranscribed and CAT mRNA was amplified for 30 cycles.

Results were calculated as stimulation indexes between unstimulated and stimulated samples.

Results

TNF-α mRNA expression in freshly isolated murine keratinocytes

To investigate the induction of TNF-α in murine keratinocytes, we stimulated Ia$^-$/CD3$^-$ epidermal cells with PMA, LPS, $NiSO_4$, Na_2SO_4, SDS, DMSO and TNBS as described in Methods. LPS is a classical inducer of TNF-α mRNA in monocytes (19) and PMA is the active component of croton oil, a potent skin irritant which has been used for many years in experimental studies. Nickel is the sensitizer with the highest sensitization rate in humans as well as an irritant. TNBS is a well established irritant and potent sensitizer. DMSO and SDS are both irritants.

As shown in Fig. 1, stimulation of the keratinocytes with the irritant PMA for 1 h resulted in a 4-fold up-regulation of TNF-α mRNA expression. In contrast, LPS stimulation resulted in only a slight increase of the background TNF-α mRNA level. Interestingly, we found that the irritants SDS and DMSO, and the irritant contact sensitizer $NiSO_4$, but not TNBS, were capable of up-regulating TNF-α mRNA in epidermal cells (Fig. 1).

To elucidate whether the induced mRNA resulted in increased TNF protein, epidermal cells were grown to monolayer and stimulated with media and PMA or pulsed with 10^{-2} M $NiSO_4$ for 2 h and media or 10^{-2} M $NiSO_4$ for 4 h followed by incubation in complete media. Supernatants were obtained at 24 h. Using a WEHI 164 subclone 13-based bioassay, we found that both PMA and $NiSO_4$ stimulation resulted in a 2.9- and 3.8-fold increase in TNF protein in Ia$^-$/CD3$^-$ epidermal cells respectively (Table 2). The bioactivities induced by PMA and $NiSO_4$ were consistently neutralized by the addition of antiserum to TNF. In addition, increased TNF bioactivity was found in the cell lysates following PMA and $NiSO_4$ stimulation (Table 2); this observation

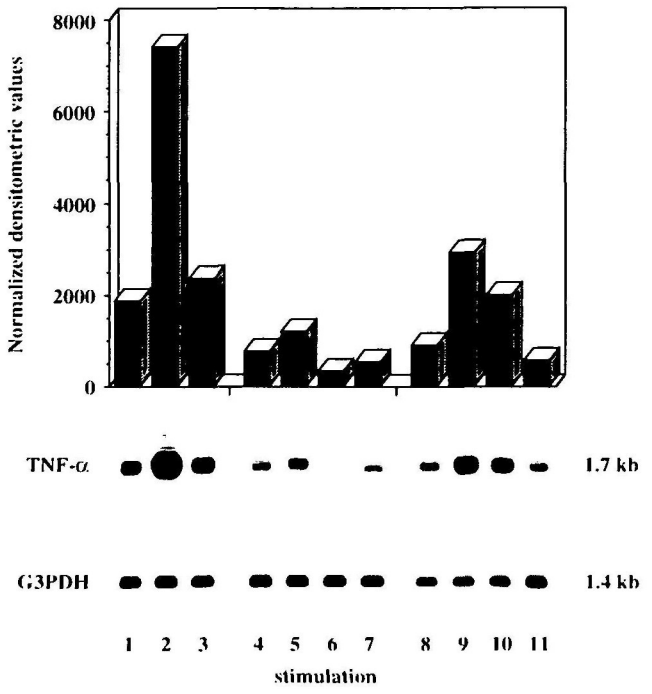

Fig. 1. TNF-α mRNA induction in Ia⁻/CD3⁻ epidermal cells. Epidermal cells were prepared as described in Methods. The cultures were stimulated for 1 h with media (lane 1), PMA (10 ng/ml, lane 2), LPS (10 μg/ml, lane 3) in the presence of complete media; or with $NiSO_4$ (10^{-2} M for 2 h, lane 4), $NiSO_4$ (10^{-2} M for 4 h, lane 5), Na_2SO_4 (10^{-2} M for 2 h, lane 6) and media (lane 7) in the absence of serum; or were pulsed for 15 min at 37°C with media (lane 8), SDS (0.0075% w/v, lane 9), DMSO (20% v/v, lane 10) and TNBS (10^{-2} M, lane 11) followed by 1 h incubation in complete media. Total RNA was extracted and 20 μg total RNA was loaded on each lane. Upon electrophoretic separation and hybridization with probes specific for TNF-α and G3PDH, the intensity of the bands was measured by computing laser densitometry. Data are expressed as relative densitometric values normalized to variations in G3PDH from the same blot.

Table 2. PMA and $NiSO_4$ up-regulate TNF protein in keratinocytes

	Stimulation	Cell lysates (U/ml TNF)[a]	Culture supernatant (U/ml TNF[a])
Experiment I	media	11.7 ± 2.7	13.7 ± 0.9
	PMA	66.2 ± 0.9	43.4 ± 0.1
Experiment II	media, serum-free	16.9 ± 2.5	11.5 ± 1.1
	$NiSO_4$, 2 h	28.4 ± 6.2	23.9 ± 1.8
	$NiSO_4$, 4 h	279.0 ± 1.7	41.7 ± 1.2

All TNF bioactivity could be inhibited >95% by addition of anti-TNF serum.

[a]Data represent mean±SD of triplicate cultures.

speaks against a simple release of TNF from preformed intracellular stores and strongly suggests newly synthesized TNF protein.

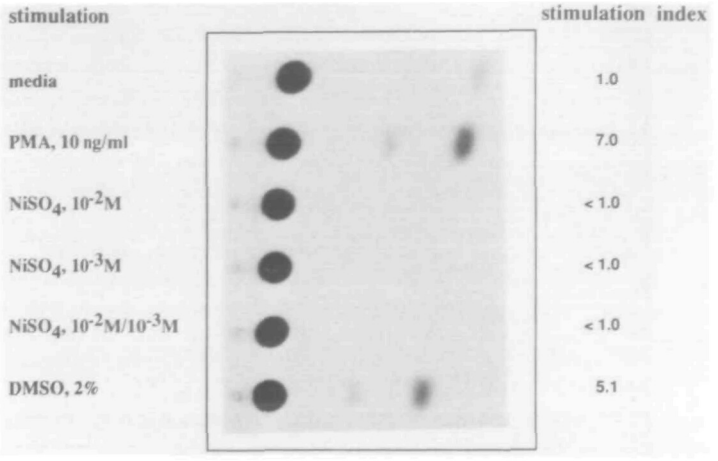

Fig. 2. PMA and DMSO, but not $NiSO_4$, increase TNF-α promoter activity in transiently transfected keratinocytes. HD_{II} keratinocytes were transfected with the −1059 to +138 bp TNF-α CAT construct and stimulated for 24 h with media, PMA (10 ng/ml), DMSO (2% v/v) or $NiSO_4$ at 10^{-2} M, or 10^{-3} M for 24 h, or at 10^{-2} M for 3 h followed by 21 h at 10^{-3} M (10^{-2} M/10^{-3} M). The cells were lysed and incubated with acetyl-coenzyme A and [^{14}C]chloramphenicol. The acetylation products were separated by thin layer chromatography and quantified by scanning with a phosphoimager. Stimulation indexes were calculated as radioactivity incorporation into the acetylated products (stimulated samples/non-stimulated samples) after normalization to total protein in the samples.

TNF-α mRNA expression in keratinocyte cell lines

To determine whether the up-regulation of TNF-α mRNA was a direct effect in keratinocytes, we stimulated the keratinocyte cell lines PAM212, HD_{II}, HEL-30 and BDVIIa with LPS and PMA, and total RNA extracted after 1, 2 and 6 h. Due to a stronger TNF-α mRNA induction in response to PMA, we selected PAM212 and HD_{II} cell lines for further analyses. The induction of TNF-α mRNA was rapid with a maximum occurring 1 h after stimulation as assessed by Northern blotting (Table 1). We next investigated whether irritants or sensitizers that up-regulated TNF-α mRNA *in vivo* and/or in primary epidermal cell cultures *in vitro* were capable of directly inducing TNF-α mRNA in keratinocyte cell lines. Northern blotting as well as RT-PCR revealed that DMSO, SDS and nickel salts up-regulated TNF-α mRNA in keratinocytes (Table 1). In contrast, exposure to TNCB, TNBS, Na_2SO_4, $Cr_2Na_2O_7$ and $CoCl_2$ failed to induce any measurable up-regulation of TNF-α mRNA, as assessed by RT-PCR (Table 1).

PMA and DMSO, but not nickel, induce increased transcription of TNF-α in keratinocytes

To determine whether the increase in TNF-α mRNA observed upon stimulation with PMA, DMSO and nickel was due to an increased promoter activity of the TNF-α gene, HD_{II} cells were transiently transfected with a CAT construct containing the −1059 to +138 bp TNF-α promoter fragment. We found that stimulation with both PMA and DMSO resulted in increased TNF-α promoter activity (Fig. 2). Although the absolute CAT activity varied between separate experiments, similar results were obtained from seven (PMA) and four

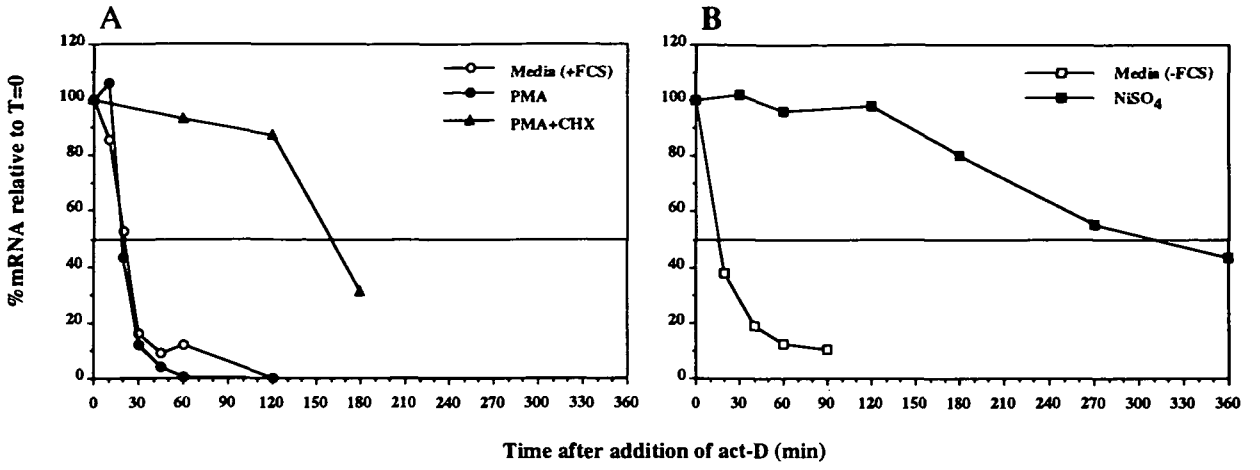

Fig. 3. $NiSO_4$, but not PMA, increases the stability of TNF-α mRNA in keratinocytes. PAM212 cells were incubated for 1 h with PMA (10 ng/ml, panel A) in the presence of FCS-containing culture media, or for 3 h with $NiSO_4$ (10^{-2} M, panel B) under serum-free culture conditions. act-D was added to the cultures (5 μg/ml, t = 0) and total RNA was extracted at the indicated time points. Northern blots were scanned by densitometry and data are expressed as a percentage of the TNF-α mRNA level determined at t = 0.

(DMSO) independent experiments. In sharp contrast, no increase in CAT activity was observed when the cells were stimulated with $NiSO_4$ at concentrations of 10^{-2} M, 10^{-3} M or when they were incubated with 10^{-2} M $NiSO_4$ for 3 h followed by 10^{-3} M for the remaining 21 h of culture (Fig. 2). Similar results were obtained from three independent experiments. In another approach, CAT mRNA was retrotranscribed and amplified by RT-PCR. Again, PMA but not $NiSO_4$ up-regulated CAT mRNA (data not shown), confirming that stimulation with nickel did not increase TNF-α promoter activity.

Nickel, but not PMA, up-regulates TNF-α mRNA by increasing its stability

To determine whether the observed increase in TNF-α mRNA following irritant/hapten stimulation of keratinocytes was the consequence of an increase in mRNA stability, PAM212 keratinocytes were incubated for 1 h with PMA (10 ng/ml) or for 3 h with $NiSO_4$ (10^{-2} M) and then treated with 5 μg/ml act-D to inhibit further RNA synthesis. Total RNA was extracted at different time points and quantified by Northern blot hybridization. The $t_{1/2}$ of TNF-α mRNA was 21 min in unstimulated cultures. In the PMA-stimulated cultures, the $t_{1/2}$ was ~20 min (Fig. 3A). Thus, the increased TNF-α mRNA expression following PMA stimulation of keratinocytes was not due to an increased stability of the mRNA. Furthermore, the addition of the protein synthesis inhibitor chx to PMA-stimulated cultures resulted in a pronounced increase in the TNF-α mRNA stability ($t_{1/2}$ estimated at 160 min; Fig. 3A), indicating that protein synthesis was involved in the degradation of TNF-α mRNA in keratinocytes. In contrast to our findings with PMA, a 15-fold increase in stability of TNF-α mRNA was observed upon stimulation with $NiSO_4$ ($t_{1/2}$ ~5 h; Fig. 3B). Thus, the increased level of TNF-α mRNA observed following exposure to nickel was chiefly the consequence of post-transcriptional regulation.

Involvement of PKC in irritant- but not nickel-induced TNF-α mRNA up-regulation

PMA is known to exert many of its cellular effects via the activation of PKC (20). We therefore studied the involvement of PKC and/or cGMP-/cAMP-dependent kinases in intracellular signaling controlling the induction of TNF-α mRNA in keratinocytes. To this end, PAM212 cells were stimulated with either PMA, DMSO or $NiSO_4$ in the presence of a selective inhibitor of PKC (H7) or a more selective inhibitor of cGMP-/cAMP-dependent protein kinases (HA1004) at graded concentrations from 50 to 5 μM. When added to the cultures 30 min before the stimuli, H7 completely blocked up-regulation of TNF-α mRNA by PMA and DMSO as assessed by RT-PCR (Fig. 4A and B, one representative experiment out of three with similar results). When the keratinocytes were stimulated with SDS comparable results were obtained (Fig. 4C). In contrast, no effect of H7 was observed when the cells were stimulated with $NiSO_4$. Quantitative analysis by Northern blotting confirmed the absence of any significant effect of H7 on the TNF-α mRNA up-regulation induced by nickel (Fig. 5).

In contrast to H7, the inhibitor HA1004 had no effect on TNF-α mRNA up-regulation (Figs 4 and 5). In addition, pentoxifylline, a phosphodiesterase inhibitor that increases the intracellular levels of cAMP, did not affect TNF-α mRNA up-regulation when used at concentrations from 100 to 1 μM (data not shown). Thus intracellular signalling by cAMP or cGMP does not seem to be critically involved in the irritant- or nickel-induced TNF-α mRNA up-regulation observed in keratinocytes.

H7-mediated inhibition of PKC abolishes the PMA-induced increased transcription of TNF-α mRNA

It has been reported that virus-mediated induction of TNF-α mRNA in astrocytes required PKC activity and that the addition

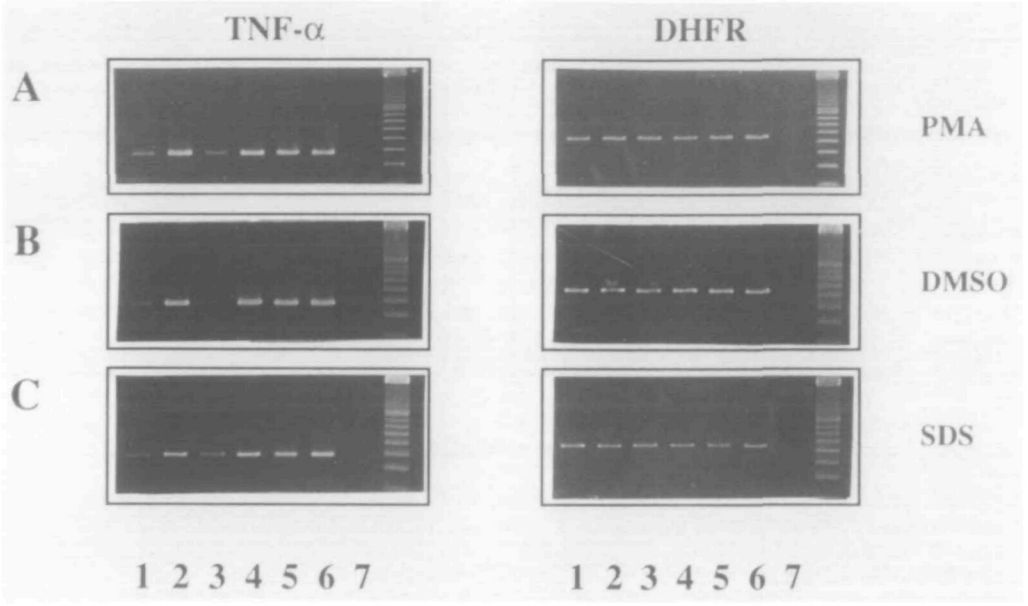

Fig. 4. PMA, DMSO and SDS up-regulate TNF-α mRNA in keratinocytes via a PKC-sensitive pathway. PAM212 cells were incubated for 1 h with PMA (10 ng/ml, panel A) or pulsed for 15 min with DMSO (20%, panel B) or SDS (0.0075%, panel C) followed by washings and culture for an additional 1 h. All cultures were performed either in the absence or in the presence of H7 or HA1004. Total RNA was extracted, retrotranscribed and amplified for 30 cycles by PCR. Unstimulated controls (lane 1), stimulated cultures in the absence of inhibitors (lane 2), stimulated cultures in the presence of either 50 μM H7 (lane 3), 5 μM H7 (lane 4), 50 μM HA1004 (lane 5) or 5 μM HA1004 (lane 6). Lane 7 represents negative controls for PCR (omitting cDNA).

of inhibitors of PKC seemed to destabilize TNF-α mRNA in this system, rather than to inhibit transcription (21). We therefore investigated whether the decrease in TNF-α mRNA accumulation observed following irritant-mediated stimulation in the presence of H7 could be explained by a destabilization of already transcribed TNF-α mRNA. PAM212 cells were thus stimulated with PMA (10 ng/ml). After 1 h of stimulation, act-D (5 μg/ml) was added to the cultures together with media, 50 μM H7 or 50 μM HA1004. As shown in Fig. 6(A), no significant decrease in TNF-α mRNA stability was observed when H7 or HA1004 were added to the act-D treated cultures. To assess the involvement of PKC in transcriptional control, keratinocytes were transiently transfected with the −1059 to +138 bp TNF-α promoter–CAT construct and subsequently stimulated with PMA in the presence of 50 μM H7, 50 μM HA1004 or media (control). As shown in Fig. 6(B), stimulation of the keratinocytes in the presence of H7, but not in the presence of HA1004, abrogated the increase of TNF-α promoter activity (one of three experiments yielding similar results). Thus, H7 inhibited the PMA-induced up-regulation of TNF-α mRNA by decreasing TNF-α promoter activity and not by blocking a PKC-dependent stabilization of the mRNA.

PMA and $NiSO_4$ collaborate to induce TNF-α mRNA accumulation in keratinocytes

To further verify that nickel and the investigated irritants up-regulated TNF-α mRNA via different mechanisms, HD_{II} keratinocytes were stimulated with either PMA, $NiSO_4$ or with PMA plus $NiSO_4$. As shown in Fig. 7, both PMA and $NiSO_4$ up-regulated TNF-α mRNA. Of particular interest was the finding that, when the keratinocytes were incubated in the

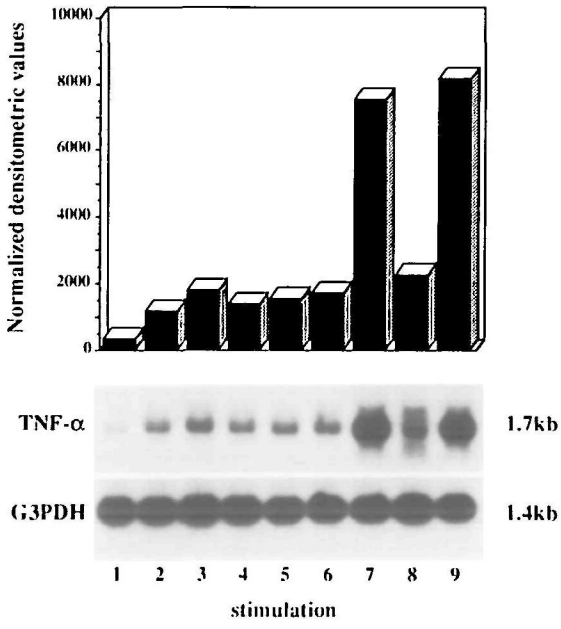

Fig. 5. $NiSO_4$ up-regulates TNF-α mRNA via a PKC-independent mechanism. PAM212 cells were incubated with media (lane 1) or 10^{-2} M $NiSO_4$ (lanes 2–6) either in the absence of inhibitors (lane 2) or in the presence of 50 μM H7 (lane 3), 5 μM H7 (lane 4), 50 μM HA1004 (lane 5) or 5 μM HA1004 (lane 6). As a control, PAM212 cells were stimulated in parallel for 1 h with 10 ng/ml PMA in the absence (lane 7) or in the presence of 50 μM H7 (lane 8) or 50 μM HA1004 (lane 9). Total RNA (20 μg) was loaded on each lane and hybridized with probes specific for TNF-α and G3PDH. Northern blots were scanned by densitometry and the data are expressed as relative densitometric values normalized to variations in G3PDH.

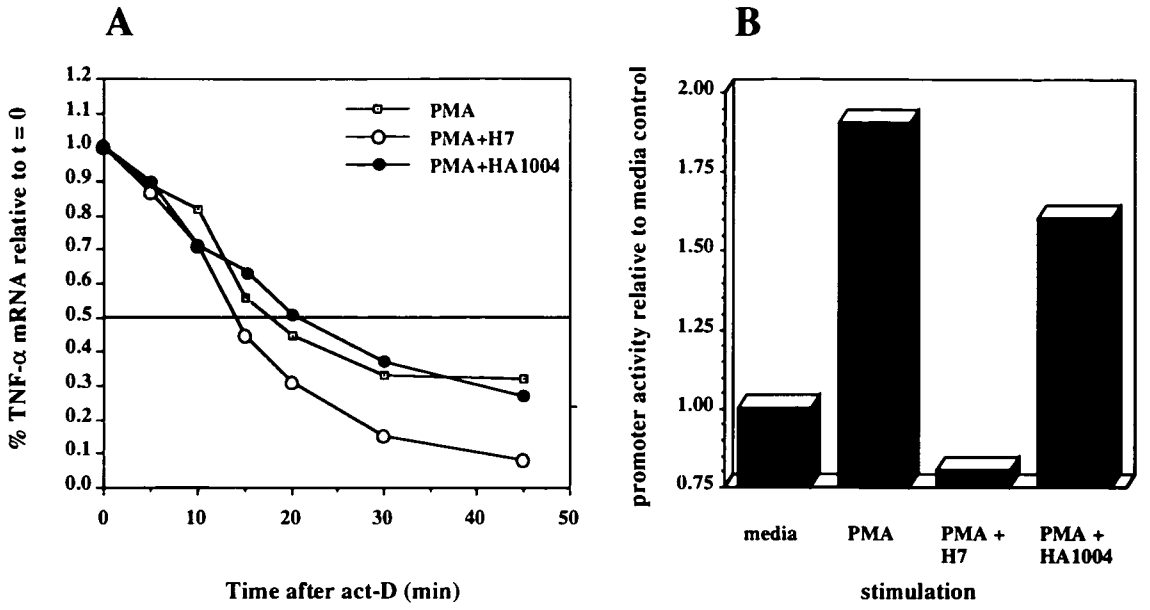

Fig. 6. H7 inhibits the PMA-induced increase in TNF-α mRNA via down-regulation of TNF-α promoter activity. (A) Keratinocytes were stimulated with PMA (10 ng/ml). After 1 h of incubation, 5 μg/ml act-D was added to the cultures either alone (PMA) or together with 50 μM H7 (PMA + H7) or 50 μM HA1004 (PMA + HA1004). Total RNA was extracted at the indicated time points. Northern blots were scanned by densitometry and data are expressed as a percentage of the TNF-α mRNA level determined at $t = 0$. (B) Keratinocytes were transfected with a TNF-α promoter–CAT construct and stimulated with media (media) or PMA (10 ng/ml) in the absence (PMA) or in the presence of 50 μM H7 (PMA + H7) or 50 μM HA1004 (PMA + HA1004). Data represent relative up-regulation of TNF-α promoter activity as compared with media control.

presence of both PMA and $NiSO_4$, a marked increase of TNF-α mRNA over the levels observed with either stimulus alone was observed (Fig. 7, lanes 4, 8 and 12). These results strongly support the concept that PMA, a model for PKC-dependent skin irritants, and nickel salts induce TNF-α mRNA expression in keratinocytes via different but potentially synergistic mechanisms.

Discussion

In this report, we show that PMA, DMSO and SDS as well as $NiSO_4$ directly up-regulate TNF-α mRNA in $Ia^-/CD3^-$ epidermal cells and in transformed keratinocyte cell lines. Using PMA and $NiSO_4$ as models for irritants and contact sensitizers respectively, we found that TNF protein was up-regulated both in cell culture supernatants and in cell lysates following stimulation. Other investigators have reported that TNF-α can be induced in epithelial and keratinocyte cell lines (9,22). Furthermore, studies by Piguet *et al.* and Enk *et al.* demonstrated up-regulation of epidermal TNF-α mRNA expression following *in vivo* painting with both allergens and irritants (5,23). In these reports, the TNF production was attributed to keratinocytes by *in situ* hybridization or by *in vitro* cell depletion analyses. However, because the primary stimulations were performed on intact epidermis, these studies could not determine whether irritants had a direct effect on keratinocytes or whether the up-regulation of TNF-α was a result of indirect stimulation due to factors released from Langerhans cells, T lymphocytes or Thy-1^+ cells within the epidermis. In contrast to the *in vivo* findings, we could not demonstrate an increase

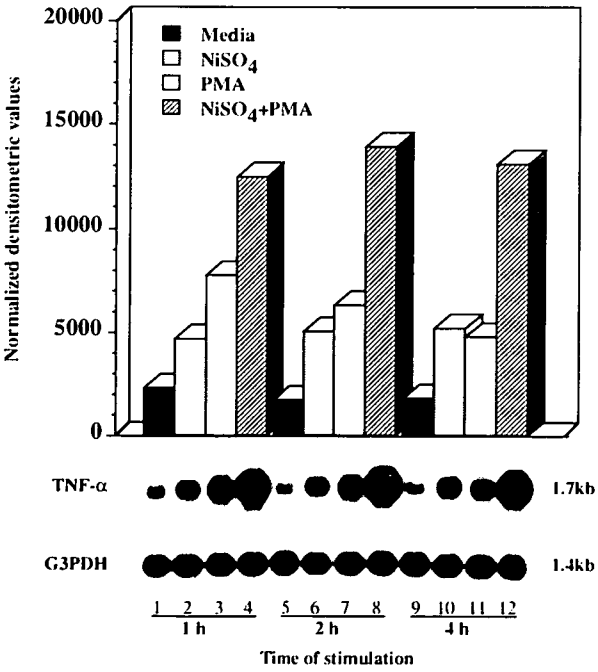

Fig. 7. PMA and $NiSO_4$ up-regulate TNF-α mRNA via different but complementary mechanisms. HD_{II} cells were stimulated with media (lanes 1, 5 and 9), $NiSO_4$ (10^{-2} M; lanes 2, 6 and 10), PMA (10 ng/ml; lanes 3, 7 and 11) or with $NiSO_4$ + PMA in combination (lanes 4, 8 and 12). Total RNA was extracted after 1 h (lanes 1–4), 2 h (lanes 5–8) and 4 h (lanes 9–12) of culture, and 20 μg total RNA was loaded on each lane. Northern blots were scanned by densitometry and the data are expressed as relative densitometric values normalized to variations in G3PDH.

in TNF-α mRNA when we incubated Ia⁻/CD3⁻ epidermal cells or keratinocyte cell lines with TNCB or TNBS *in vitro*. It has been reported that the earliest event in the epidermis following skin painting with the irritative sensitizer TNCB was an up-regulation of IL-1β mRNA in the epidermal Langerhans cells (23–25). The finding of increased epidermal TNF-α following *in vivo* injection of IL-1β (24) strongly supports the hypothesis that Langerhans cell-derived IL-1β induced the increase in keratinocyte TNF-α mRNA expression following *in vivo* skin painting with TNCB (24,26). This could explain our inability to induce TNF-α with TNCB or TNBS in Langerhans cell-free keratinocyte cultures. Thus, there may be two principal pathways by which chemicals can induce TNF-α mRNA in keratinocytes: the indirect pathway used by TNCB via the induction of IL-1β in Langerhans cells, and the direct pathway used by PMA, DMSO and SDS, all of which directly act on keratinocytes. It has been reported that the LPS-induced increase in TNF-α mRNA in macrophages is both transcriptionally and translationally regulated (27). To study whether transcriptional regulation was involved in the induction of TNF-α mRNA, we transfected HD_{II} keratinocytes with a CAT reporter construct containing the −1059 to +138 bp TNF-α promoter. Increased promoter activity was indeed observed following exposure to PMA and DMSO (Fig. 2), strongly suggesting that the PMA- and DMSO-induced up-regulation of TNF-α mRNA in keratinocytes was due to increased transcription. For technical reasons, SDS could not be tested in the CAT assay. On the basis of these findings, we are presently in the process of identifying the PMA-responsive element in the 5′ promoter of murine TNF-α.

Using PMA as a model for skin irritants, we next determined whether post-transcriptional regulation was also involved in the up-regulation of TNF-α mRNA in keratinocytes. The stability of the mRNA was therefore determined both in unstimulated and PMA-stimulated cultures treated with act-D to block RNA synthesis. No significant difference in mRNA stability was observed between unstimulated and stimulated cells (Fig. 3A). This indicated that the increase in TNF-α mRNA observed upon stimulation with PMA was not due to enhanced stability of the mRNA, supporting the view that PMA induces TNF-α in keratinocytes by increasing transcription of the TNF-α gene. It is generally accepted that PMA mediates most of its effects via PKC-dependent signal transduction pathways (21). Accordingly, we found that the PKC inhibitor H7, but not the cyclic nucleoside-dependent protein kinase inhibitor HA1004, could block the PMA-induced up-regulation of TNF-α mRNA in keratinocytes when used at concentrations previously shown to inhibit the induction of IL-1α by PMA (28). The effect of H7 was associated with the inhibition of the PMA-induced activation of the TNF-α promoter and not with a destabilization of the mRNA as described in astrocytes (20). The involvement of PKC in the induction of TNF-α parallels observations with PMA-stimulated murine monocytes (29). Interestingly, DMSO and SDS also induced an increase in TNF-α mRNA via an H7-sensitive signaling pathway (Fig. 4). These results indicate that three chemically different skin irritants all up-regulate keratinocyte TNF-α mRNA transcription via activation of PKC. In addition, this is the first report demonstrating that the most frequent contact sensitizer, nickel, directly up-regulates TNF-α mRNA and protein in purified murine keratinocytes. The induction of TNF-α mRNA following stimulation with $NiSO_4$ and $NiCl_2$ (data not shown) had a slightly different time-course in the used keratinocyte cell lines with maximal mRNA levels detected at 4 h in PAM212 cells (Table 1) and at 1–2 h in HD_{II} cells (Fig. 7). Neither H7 nor HA1004 inhibited the increase in TNF-α mRNA seen following incubation with nickel salts (Fig. 5). It thus appears that, in contrast to the irritants PMA, DMSO and SDS, nickel up-regulates TNF-α mRNA expression in keratinocytes via a PKC-independent mechanism.

No increase in TNF-α promoter activity was observed following stimulation with nickel, as revealed by conventional CAT assay. However, when the transfected keratinocytes were incubated with 10^{-3} M to 10^{-2} M $NiSO_4$ (the optimal concentrations for TNF-α mRNA induction), a decrease in cell viability was observed during the 24 h incubation period necessary to accumulate CAT proteins. Using a CAT-PCR technique to detect CAT mRNA, we confirmed that PMA, but not $NiSO_4$, up-regulated CAT mRNA in keratinocytes. Furthermore, when RNA synthesis was blocked with act-D, a pronounced increase in the stability of TNF-α mRNA was observed after stimulation with nickel as compared with unstimulated control cultures. The concentration of nickel used in our study (10^{-2} M) is within the physiologically relevant range because it represents only ~1/20 of the concentration used to elicit allergic reactions in sensitized humans by epicutaneous patch testing. It is novel that $NiSO_4$ up-regulates TNF-α mRNA in keratinocytes and, to our knowledge, this is the first known environmental agent that significantly up-regulates the stability of TNF-α mRNA. The other metal ions that were tested and that are sensitizers as well, did not induce TNF-α. Thus, the effect of nickel is unique among the tested sensitizers. The target of nickel within the keratinocyte responsible for the induction of TNF-α mRNA and protein seems to have a certain degree of specificity for nickel, and may thus bind it. The molecular target of nickel is presently unknown but may represent enzymes involved in the degradation of the mRNA or interaction with factors binding to AUUUA sequences in the 3′-untranslated region of TNF-α, a region that has previously been shown to influence mRNA stability (30). This hypothesis is supported by our findings that nickel also up-regulates IL-α mRNA in keratinocytes (S. Lisby and C. Hauser, unpublished results). Like TNF-α, IL-1 possesses AUUUA sequences in the 3′-untranslated region. Alternatively, as reported for chx, $NiSO_4$ may act by sequestering TNF-α mRNA in a ribosome-bound complex (31). Since total protein synthesis was not blocked by exposure to $NiSO_4$ (unpublished results) and because TNF protein synthesis was found to be increased after $NiSO_4$ stimulation, the mechanism used by $NiSO_4$ seems to be different from that of chx, which blocks protein synthesis. However, the molecular mechanism underlying the nickel-induced increase in TNF-α mRNA stability awaits further clarification.

The TNF-α inducing capacity of nickel within keratinocytes may be related to the fact that this allergen has a high sensitization rate in humans. Other contact allergens such as $Cr_2Na_2O_7$, $CoCl_2$ and TNBS that do not directly induce TNF-α in keratinocytes may induce contact sensitivity via induction

in Langerhans cells of IL-1β that in turn induces TNF-α in keratinocytes as discussed above for TNCB or, alternatively, utilize different mechanisms to induce sensitization. In contrast to the elicitation phase of allergic contact dermatitis and primary irritant contact dermatitis, the role of TNF-α in the sensitization phase has not been clarified.

Our findings indicate that TNF-α, a highly pleiotropic cytokine, can be directly induced/accumulated in keratinocytes by irritants and the hapten nickel. Besides its direct cytotoxic effects on epidermal cells *in vitro* (32,33), TNF-α has been implicated in the development of allergic contact dermatitis *in vivo* (5,34). TNF-α possesses proinflammatory properties that may contribute to skin inflammation not only in allergic but also in irritant contact dermatitis and may explain why these two conditions very often cannot be distinguished by light microscopic analysis. On endothelial cells, TNF-α can induce the expression of cell adhesion molecules such as E-selectin, vascular cell adhesion molecule-1 and the up-regulation of the constitutive intercellular adhesion molecule-1 expression (35–38). Up-regulation of cell adhesion molecules has been reported to occur very early in the induction of contact hypersensitivity reactions *in vivo* (39) and is thought to be implicated in the recruitment of immunocompetent cells to the skin. There is good evidence that TNF-α-activated endothelium permits the extravasation of polymorphonuclear leukocytes and monocytes/macrophages, which then become activated to release reactive oxygen intermediates and other proinflammatory mediators (40,41). TNF-α may furthermore contribute to skin inflammation by increasing the expression of other proinflammatory cytokines such as IL-8 (42–44).

Further studies on the up-regulation of TNF-α expression by irritants and sensitizers in keratinocytes may not only deepen our understanding of the molecular mechanisms of TNF-α regulation in these cells, but also identify potential sites of therapeutic intervention.

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Abbreviations

act-D	actinomycin-D
CAT	*chloramphenicol acetyltransferase*
chx	cycloheximide
DHFR	dihydrofolate reductase
DMSO	dimethylsulfoxide
G3PDH	glyceraldehyde-3-phosphate-dehydrogenase
LPS	lipopolysaccharide
PCR	polymerase chain reaction
PKC	protein kinase C
PMA	phorbol myristate acetate
RT	reverse transcription
SDS	sodium dodecyl sulfate
TNBS	trinitrobenzenesulfonic acid
TNCB	trinitrochlorobenzene

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