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The autocrine TNFα signalling loop in keratinocytes requires atypical PKC species and NF-κB activation but is independent of cholesterol-enriched membrane microdomains

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ABSTRACT

Tumor necrosis factor α (TNFα) is involved in the pathogenesis of many inflammatory skin diseases. Epidermal keratinocytes produce and respond to TNFα via the cognate type 1 receptor (TNFR1). Little is known about regulation of TNFα signalling in this cell type. In this study, we report that in keratinocytes TNFα upregulates its own mRNA synthesis in an autocrine manner. This response peaks at approximately 1 h of stimulation with TNFα but sustained elevated levels of TNFα mRNA are observed for up to 24 h after stimulation and are dependent on the presence of the soluble cytokine. This autocrine response is mediated by the signalling cascade comprising TNFR1, atypical protein kinase C (aPKC) species and the transcription factor NF-κB, but is not dependent on the integrity of cholesterol-enriched membrane microdomains (lipid rafts). TNFα-stimulated keratinocytes produced the membrane-bound form of TNFα. It is conceivable that the described autocrine signalling loop contributes to the proinflammatory TNFα effect in the skin. The discovery of the crucial roles of aPKC and NF-κB might have consequences for the development of more selective anti-TNFα therapies for inflammatory skin diseases.

1. Introduction

Tumor necrosis factor α (TNFα) is a proinflammatory cytokine crucially involved in the pathogenesis of several skin diseases, such as psoriasis and psoriatic arthropathy, pyoderma gangrenosum, cutaneous Crohn's disease and non-melanoma skin cancer [1,2]. The central importance of this cytokine is underscored by the therapeutic effect of the neutralizing agents, including monoclonal antibodies (infliximab and adalimumab) and the decoy receptor fusion proteins (etanercept) [3–6]. TNFα can upregulate several cytokines and cell adhesion molecules, including, but not limited to, interleukin (IL)-1β, IL-6, IL-8 and intercellular adhesion molecule-1 (ICAM-1).

Despite the spectacular success of the anti-TNFα approach in the treatment of skin diseases, the mechanism of action of TNFα in the skin has not yet been elucidated in full. Research is concentrated on the involvement of TNFα in the regulation of immune response, especially on the effect of this cytokine on dendritic cells [1]. Much less is known about the action of TNFα on epidermal keratinocytes. The keratinocyte is likely to be an important constituent of the TNFα signalling network in the epidermis. These cells express type 1 receptor (TNFR1) and can produce substantial amounts of TNFα after different noxious stimuli such as trauma or irritation [7–9].

The cellular signalling pathways triggered by TNFα in keratinocytes have not been investigated extensively. It is assumed that TNFR1 transmits signal via the canonical TRADD–TRAF2–RIP pathway leading to activation of the transcription factor NF-κB and upregulation of anti-apoptotic genes [10]. However, TNFα may signal via different pathways

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depending of the general status of cell metabolism and cell type. Legler et al. showed that TNFR1-dependent activation of NF-κB occurs only when the receptor is associated with lipid rafts [11]. In situations when TNFR1 leaves the raft milieu, it cannot signal efficiently for NF-κB and apoptosis is induced via FADD and caspase-8. In other cell types, the TNFR1-dependent NF-κB induction was not affected by lipid raft integrity [12]. Moreover, in some cell types the TNFR1 signalling may involve atypical PKC species (aPKC), such as PKCλ/ι or PKCζ [13–16]

We have previously described that in murine keratinocytes TNFα can upregulate its own gene expression and TNFα synthesis in an autocrine manner [17]. These results have later been independently confirmed by gene array analysis in TNFα-stimulated cells [18]. In this work, we show that the TNFα autocrine loop is also functional in human keratinocytes in the model of immortalized cell line HaCaT. We present evidence that this positive transcriptional feedback loop is mediated by TNFR1 in a lipid raft-independent mode and involve downstream activation of aPKC species and NF-κB.

2. Materials and methods

2.1. Cell culture

The spontaneously immortalized, human keratinocyte cell line, HaCaT [19] were originally obtained from Dr. Mark Pittelkow. The cells were grown in the Dulbecco's modified growth medium (DMEM) supplemented with 1% glutamine and 10% FCS at 37 °C in a humidified atmosphere with 5% CO_2, and passaged once weekly. For stimulation assays, cells were seeded in 3 cm Petri dishes and allowed to grow to 80–90% confluence for 72 h. Fresh, temperature and CO_2 equilibrated medium was added 2 h before the experiment. For the experiments involving the measurement of NF-κB activation, the HaCaT cells stably expressing the plasmid pBIIX with two copies of an HIV-NF-κB sequence cloned upstream the the *Photinus pyralis* luciferase coding sequence [20] have been used, as described previously [21,22].

2.2. Reagents

All chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA), unless stated otherwise. For stimulation we used recombinant human (rhu) TNF and rhu interleukin (IL)-1β. Blocking studies were performed using anti-TNFα (Infliximab, Centocor, USA). MG132 (Z-Leu-Leu-Leu-CHO) and inhibitory PKCζ pseudopeptide (PKCζPD) [23,24] were obtained from Calbiochem (Merck Biosciences, Darmstadt, Germany). For cholesterol depletion and lipid raft disruption the cells were incubated in 1% (w/v) methyl-β-cyclodextrin, as described previously [25]. Monoclonal murine antibodies HTR9 and MR2-1 (clone HP9003) and polyclonal rabbit anti-human TNFα were purchased from Hycult Biotechnology (Uden, The Netherlands).

2.3. RNA extraction and cDNA synthesis

The cells were washed twice in ice-cold PBS and total RNA was extracted with TRIzol reagent according to the manufacture's instructions (Invitrogen, Taastrup, Denmark). The RNA concentration and purity was measured in triplicate at OD 260/280 using an Ultrospec III spectrophotometer (Pharmacia Biosystems, Allerød, Denmark). One microgram total RNA was retrotranscribed using M-MLV reverse transcriptase and oligo-(dt)15 primers according to the manufacture's instructions (Promega, Mannheim, Germany). The synthesised cDNA was stored at −80 °C until use.

2.4. Real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR)

Primer and probes used in this study were designed using Primer Express software (Applied Biosystems (ABI); Foster City, CA, USA). The sequences were designed to detect human β-actin, TNFα, TNFR1 (CD120A) and TNFR2 (CD120B) and to span an exon–exon boundary. The specificity and potential cross-reactivity of the sequences were checked by NCBI nucleotide BLAST analysis (www.ncbi.nlm.nih.gov/blast). A sequence specific oligonucleotide probe coupled to a FAM (6-carboxyfluorescein) reporter dye located in the 5′-end and a minor groove binding (MGB) moiety on the 3′-end was used.

Real-time, quantitative RT-PCR was conducted using an ABI Prism 7000 sequence detection system (Applied Biosystems). We performed hot-start PCR using 5 μl 1:10 diluted cDNA as template for each PCR reaction. We used 0.5 U/reaction of TaqMan polymerase using the supplied buffer supplemented with 3.5/5 mM $MgCl_2$ (β-actin/TNFα), 300 nM of each primer and 80 nM of each probe. The conditions for the PCR were as follows: 94 °C for 5 min followed by 40 cycles of incubation at 94 °C for 15 s and 60 °C for 60 s. A final extension for 10 min at 60 °C was applied to ensure a complete elongation. Standard curves, produced by serial dilutions of cDNA and negative controls were included in all experiments. Data presented as relative values compared to unstimulated controls.

2.5. Luciferase assay

Stably transfected HaCaT cells were seeded in 24-round multiwell plates (5×10^5 cells/well) and cultured for 2 days until confluent. After treatment, the cells were washed twice with with phosphate-buffered saline and lysed, and luciferase activities were determined using the Luciferase Reporter Assay System (Promega) and Victor II microplate-based luminometer (Perkin-Elmer, Wellesley, MA, USA) equipped with an automatic pipettor, as described by the manufacturer.

2.6. Western blotting

Cells were rinse once with ice-cold PBS and 500 μl of PBS with complete protease inhibitor (Roche Applied Science, Indianapolis, IN, USA), 1 mM phenylmethylsulfonyl fluoride and phosphatase inhibitors Na_3VO_4 (1 mM) and NaF (50 mM) and disrupted by repeated aspiration through a 21-gauge needle. Lysates were transferred to a 1.5 ml centrifuge tube, and centrifuged at 1000 × *g* for 10 min at 4 °C. Membrane fractions were subsequently recovered from the supernatants by ultracentrifugation at 39,000 rpm in a Beckman Coulter Optima™ L-80 XP Ultracentrifuge using a SW41 rotor for 2 h at 2 °C. The lysates were prepared from the pellets as described

previously [26]. Proteins were separated by SDS-PAGE on 10% gels, transferred onto a nitrocellulose membrane (Bio-Rad, Philadelphia, PA, USA) by vertical wet electrotransfer, blocked for 1 h at 4 °C with Li-Cor blocker (Lincoln, NE, USA), and incubated overnight with the primary antibodies at 4 °C. Secondary antibodies labeled with 700 IRDye (anti-rabbit) and 800IRDye (anti-mouse) (both obtained from Li-Cor) were used for the detection with the infrared Odyssey imaging system (Li-Cor).

2.7. Confocal microscopy

Cells were grown on Lab-Tek chamber slides (Nunc, Roskilde, Denmark) and incubated unfixed with the primary antibodies for 60 min at 4 °C. For detection with secondary antibodies, we used Texas Red-labeled goat anti-mouse or anti-rabbit antibodies (Jackson Laboratories, Bar Harbor, ME, USA) for 30 min at 4 °C. The samples were imaged by Olympus IX70 confocal laser scanning microscope using 488 and 568 nm excitation lines from an argon–krypton laser (Olympus Fluo-View Confocal System).

3. Results

3.1. TNFα mRNA synthesis is stimulated by rhu-TNFα in HaCaT cells

To examine whether TNFα is able to stimulate the transcription of its own gene in human keratinocyte cell line HaCaT, we incubated the cells with 10 ng/ml of rhu-TNFα and measured mRNA levels at different time points. The peak stimulation was seen at approximately 60 min of stimulation and mRNA levels declined afterwards (Fig. 1A). The stimulation of mRNA synthesis was also correlated to the concentration of rhu-TNFα. In course of preliminary experiments we discovered

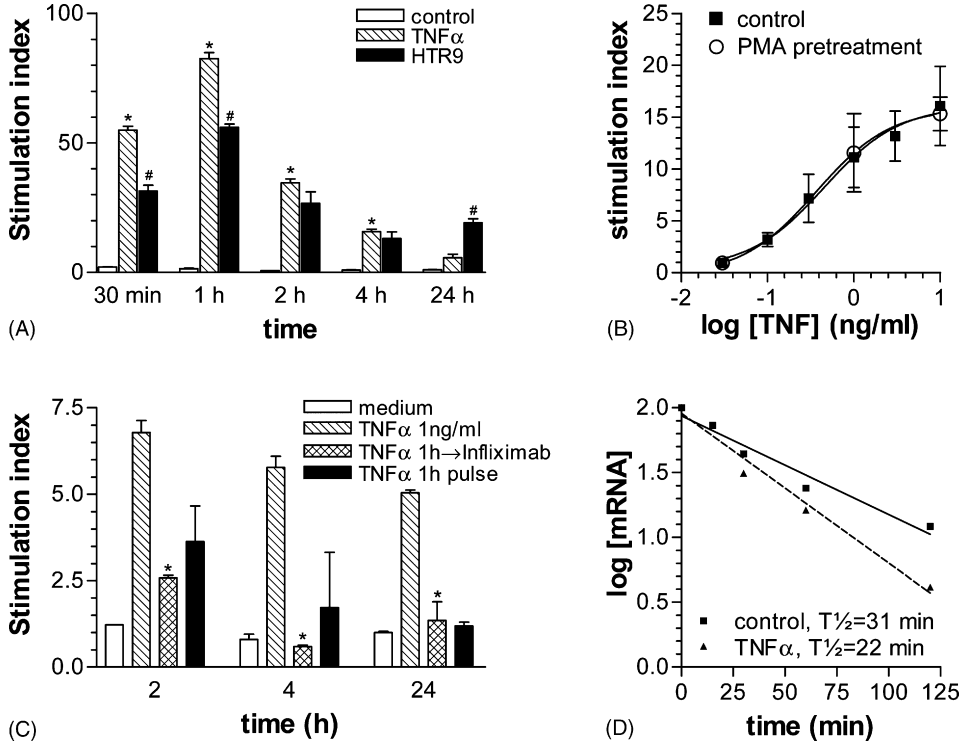

Fig. 1 – rhu-TNFα stimulates TNF mRNA production in cultured HaCaT cells. (A) HaCaT cells were stimulated with 10 ng/ml rhu-TNFα or the TNFR1 agonistic antibody HTR9 (1 μg/ml) for the indicated periods of time. TNFα mRNA expression was analysed by quantitative RT-PCR and shown as mean values with S.D. of three independent experiments. $^{*}p < 0.05$ compared to the control, $^{\#}p < 0.05$, comparison between TNFα and HTR9 treated cells, *t*-test. (B) HaCaT cells were depleted for classical PKC species by the 24 h treatment with 100 nM PMA or left untreated (control). The cells were stimulated with increasing concentrations of TNFα for 60 min followed by TNFα mRNA quantification. Data show mean values with S.D. of three independent experiments. Repeated seven times for the control cells and twice for PMA treated cells with comparable results. $^{*}p < 0.05$ between TNFα and TNFα → infliximab groups, *t*-test. (C) Dependency of long-term TNFα mRNA response on the presence of TNFα. Cells were incubated for up to 24 h in the constant presence of 1 ng/ml TNFα or pulsed with the same concentration of the cytokine for 1 h. To rule out the non-specific action of TNFα, the cells were cultured in the media containing 1 ng/ml TNFα, but in which its biological activity was blocked 1 h after with the neutralizing antibody infliximab (means $n = 3$, bars—S.D.). (D) TNFα mRNA decay in the presence of actinomycin D. The cells were stimulated with 10 ng/ml rhu-TNFα for 1 h and 5 (g/ml actinomycin D was added for the following 120 min. mRNA concentration was determined at the indicated time and normalized to 100% ($t = 0$ min). Half-life ($T_{1/2}$) values were obtained from linear regression equations after logarithmic transformation of data on *y*-axis. The $T_{1/2}$ and regression slopes were not significantly different in the control and TNFα treated cells (analysis of regression, $p > 0.05$).

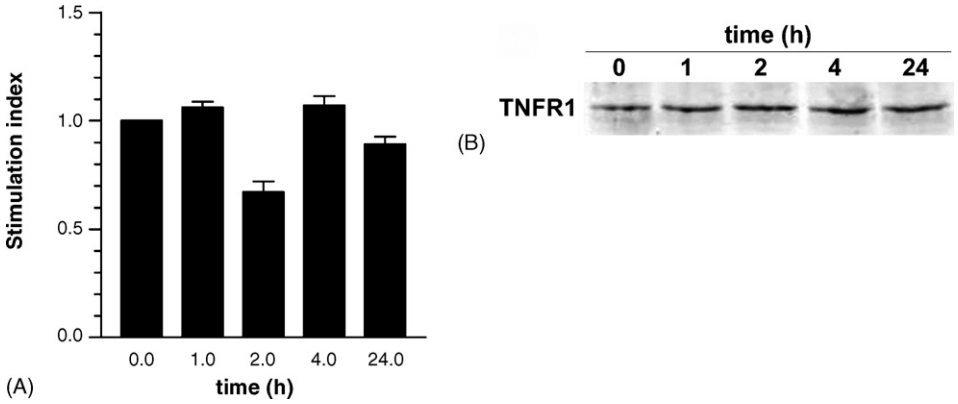

Fig. 2 – TNFR1 expression in HaCaT cells during incubation with rhu-TNFα. Subconfluent HaCaT cells were incubated with 10 ng/ml rhu-TNFα for indicated periods of time. (A) Cells were lysed and TNFR1 mRNA was quantified with RT-PCR. (B) Cells were treated as in (A) and membrane fractions were lysed and analysed by Western blot using the HTR9 antibody.

that peak stimulation (stimulation index) varies from 15 to 85 and depends heavily of the composition of the culture medium (concentration of serum) and the degree of confluence. We have therefore decided to perform the dose–response experiment with subconfluent cells (72 h after plating at 2500 cells/cm^2) cultured in the medium containing 1% of FCS. At these conditions the maximal stimulation was obtained at 5 ng/ml rhu-TNFα and EC_{50} of 1.9 ng/ml (Fig. 1B).

Increase in cellular mRNA amount can occur due to increased transcription, inhibition on RNA decay or combination of both. In the experiment where the TNFα half-life ($T_{1/2}$) was measured (Fig. 1D) we did not detect any significant differences in $T_{1/2}$ between control and TNFα-treated cells. It has been concluded that the observed TNFα-induced TNF mRNA occurred primarily due to increased transcription.

3.2. *TNFα-induced TNFα mRNA depends on the specific stimulation of TNFR1*

In keratinocytes TNFα exerts its effects predominantly via type 1 receptor (TNFR1) that is expressed in much higher quantities than TNFR2, both at the mRNA level and protein level. TNFR1 can be specifically stimulated by an agonistic antibody HTR9 [27]. In the preliminary experiments where a concentration range of 0.01–10 μg/ml HTR9 was included, we determined that the maximal stimulation of TNFα mRNA occurs at 1 μg/ml. The time response for TNFα mRNA induction by HTR9 was identical that that obtained with rhu-TNFα and the levels of stimulation were also comparable (Fig. 1A). The conclusion from these experiments was that TNFα stimulates its own mRNA synthesis via stimulation of TNFR1. This notion was further reinforced by the results of the experiment showing that the TNFR2 agonistic antibody (clone MR2-1) did not produce any TNFα mRNA synthesis, even at the concentrations >10 μg/ml (not shown).

3.3. *Prolonged elevation of TNFα mRNA depends on the presence of the soluble rhu-TNFα*

Although response declined sharply after 1 h stimulation, significantly elevated levels were still seen up to 24 h in the presence of rhu-TNFα (or a stimulating HTR9 antibody). In order to investigate whether the elevated levels of TNFα mRNA seen after the peak response depended on the presence of TNFα or reflected the normal kinetics of mRNA degradation, the cytokine was removed after 1 h stimulation by either washing or addition of a potent blocking antibody (infliximab). In both experiments the levels of TNFα mRNA returned to the background levels at 2 h after stimulation with TNFα (Fig. 1C). The reason for this desensitisation is not clear, but does not seem to be caused by the decline in receptor expression (Fig. 2). However, we cannot exclude that the receptors were removed from the membrane without being proteolysed, e.g. by endocytosis, which would not affect the total level of cellular TNFR1.

3.4. *Autocrine TNFα mRNA synthesis depends on atypical PKC species*

Having shown that the stimulation of TNFR1 leads to the increased TNFα gene transcription we further defined the signalling pathways involved in this process. Our previous experiments in the murine system revealed the role of protein kinase C (PKC) as an intermediary signalling step. We confirmed this finding for HaCaT by showing that a broad inhibitor of PKCs, H7, was able to inhibit the rhu-TNFα-induced TNF-mRNA synthesis ($p < 0.05$ for all tested concentrations, *t*-test in comparison with the solvent-treated control; Fig. 3A). To determine whether the classical PKC species were involved we incubated the cells with PMA, which is a well-known technique for depleting of PKCα, β, γ. The PMA-depleted cells had normal responses to rhu-TNFα (Fig. 1B). We have therefore employed the myristoylated peptide Myr-SIYRRGARRWRKL (PKCζPS) that is a specific pseudosubstrate for atypical PKC species (PKCζ and PKCλ/ι) and blocks these enzymes at EC_{50} 10–20 μM. Fig. 3 shows that PKCζPS blocks rhu-TNFα-induced TNF-mRNA induction at EC_{50} 14–17 μM (95% confidence interval, calculated from the response curve in Fig. 3B) and totally at the concentration of 50 μM. PKCζPS alone did not have any effect on TNFα gene transcription (not shown). An additional proof that the classical PKCs were not involved was that Gö 6976, which does not block the atypical

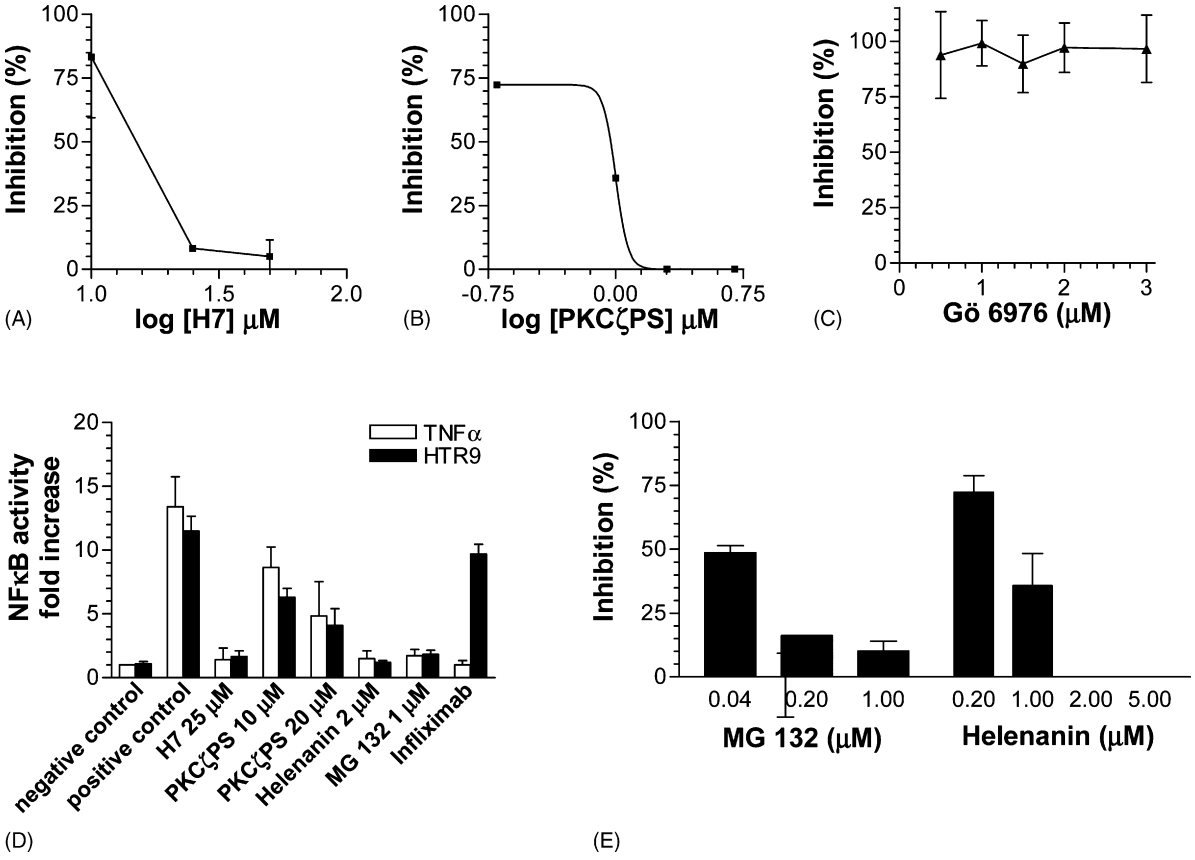

Fig. 3 – Involvement of aPKCs and NFκB in the autocrine TNF signaling. Cells were preincubated for 30 min with different PKC blockers: 10–50 μM H7, 2–50 μM PKCζPS, 0.5–3 μM Gö 6976 and NFκB blockers: MG132 and helenanin or infliximab, as indicated, and stimulated with 1 ng/ml rhu-TNFα or 1 μg/ml HTR9. (A–C) TNFα-mRNA was measured and the degree of inhibition calculated as in Section 2 (experimental − inhibitor alone)(TNFα-medium) × 100%. (D and E) NFκB activity was measured in HaCaT cells stably transfected with the pBIIX plasmid containing luciferase gene under the control of the NFκB responsive promoter. The negative control cells were incubated in media without rhu-TNFα or HTR9, whereas positive controls were treated with rhu-TNFα and HTR without PKC or NFκB blockers. Percentage of inhibition of NFκB in (E) was calculated as for the TNFα-mRNA. Data points represent means ($n = 3$) with S.D.

PKCs at nanomolar concentrations, did not affect TNFα-induced TNF-mRNA transcription at the concentrations up to 3 μM (Fig. 3C).

3.5. *NF-κB is necessary for autocrine TNFα transcription*

As expected, both TNFα and the specific agonistic TNFR1 antibody HTR9 has a strong stimulatory effect on the transcription from the NFκB-dependent promoter, which was measured in the HaCaT cells stably transfected with the HIV-NFκB plasmid (Fig. 3C). PKC antagonists, H7 and PKCζPS efficiently blocked NF-κB induction in this system, suggesting that the NFκB activation step is placed downstream to atypical PKC activation in the signalling cascade. To examine the involvement of the transcription factor NF-κB in the TNFα-induced TNF gene transcription upregulation we measured the levels of TNFα mRNA in HaCaT cells treated with two different blockers of the NF-κB: helenanin [28], which irreversibly alkylates NF-κB, and MG132 (Z-Leu-Leu-Leu-CHO) is a potent, reversible proteasome inhibitor which prevents NF-κB activation at IC_{50} of 3 μM due to blockade of TNFα-induced IκB ubiquitin-proteasome degradation [29,30]. Both in TNFα and HTR9-stimulated cells the treatment with the inhibitors significantly diminished, and in case of helenanin (2–5 μM) completely abolished the TNFR1-dependent TNFα mRNA induction ($p < 0.05$, *t*-test in comparison with the vehicle-treated control; Fig. 3D).

3.6. *TNFR1 signalling to NFκB is independent of cholesterol-enriched membrane microdomains*

Since recent data suggested that TNFR1-dependent signalling might be dependent of the trafficking of the receptor to cholesterol-enriched membrane lipid microdomains (lipid rafts) [11] we examined the putative involvement of lipid rafts in the TNFα-induced TNFα mRNA autocrine loop. The consistent finding in all lipid raft-depending events is their susceptibility to the cholesterol sequestering agents, such as methyl-β-cyclodextrin (MBCD). In HaCaT cells acutely depleted with 30 min treatment with 1% MBCD we noticed a moderate increase of the level of NFκB activity ($p < 0.05$, *t*-test; Fig. 4), which was not blocked by PKCζPS or infliximab. The

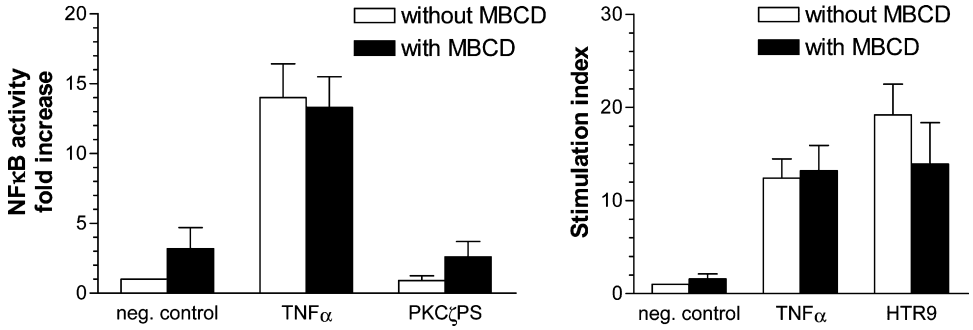

Fig. 4 – TNFR1 stimulates NFκB and TNFα gene transcription in a lipid raft-independent manner. Lipid rafts were disrupted with 1% MBCD for 5 min at 37 °C and subsequently exposed to rhu-TNFα (1 ng/ml), 1 μg/ml HTR9 or PKCζPC, as indicated in the graphs, for 30 min. MBCD remained in the incubation medium throughout the whole experiment. NFκB activity (A) and TNFα mRNA (B) were measured in the pBIIX transfected HaCaT cells as described in Section 2 and the legend for Fig. 3. The negative control cells were incubated in media without rhu-TNFα, HTR9 or PKCζPS. Data points represent means (n = 3) with S.D.

TNFα- or HTR9-induced NFκB activity or TNFα mRNA was comparable in MBCD-treated cells and the controls (non-significant difference, t-test). This suggests that the described above TNFR1–PKC–NFκB signalling cascade does not depend on the association or dissociation of TNFR1 with lipid rafts.

3.7. *TNF-mRNA induced by activation of TNFR1 is translated to membrane-bound TNFα*

The final question we wished to address was the biological significance of the TNFα-dependent increase in *TNFα* gene translation. Measurements of the secreted cytokines by the dedicated Luminex kit did not reveal any significant increase in the level of soluble TNFα, despite the fact that other cytokines, notably IL-6 and IL-8 were markedly upregulated (not shown). However, Western blots of the membrane fractions revealed increased levels of TNFα protein in HTR9-treated cells (Fig. 5A). Confocal microscopy showed that membrane-bound TNFα is increased in the HTR9-stimulated cells (Fig. 5B). Taken together, the data indicate that the autocrine TNFα response results in a moderate increase in the membrane-bound TNFα in HaCaT cells.

4. Discussion

In this study, we show that TNFα upregulates its own mRNA synthesis in an autocrine manner through stimulation of type

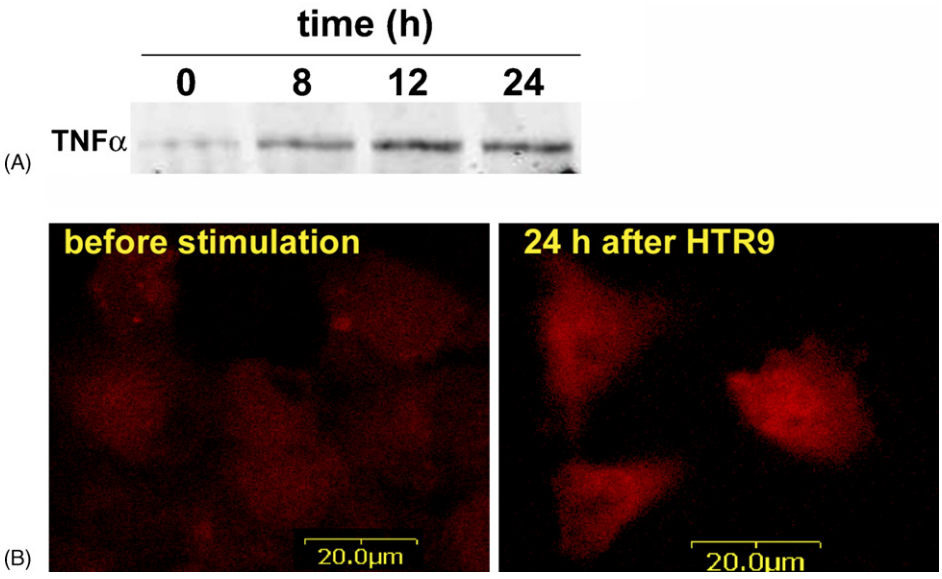

Fig. 5 – Autoregulatory stimulation of membrane-bound TNFα. HaCaT cells were stimulated with 1 μg/ml murine HTR9 antibody for the indicated time and the amount of TNFα determined in the membrane fractions by Western blotting using the rabbit anti-TNFα (A). In (B) the cells were stimulated with 1 μg/ml HTR9 for 24 h, washed and stained with the rabbit anti-TNFα antibody followed by the anti-rabbit Texas Red-conjugated secondary antibody for confocal laser scanning microscopy. The images were obtained at identical scanning conditions and show an increase in the membrane fluorescence in the stimulated cells.

1 receptor (TNFR1) in human keratinocytes. This is in accordance with our findings in murine keratinocytes [17]. Moreover, gene array analysis in TNFα-stimulated keratinocytes has recently confirmed that TNFα rapidly induces its own gene expression [18]. This positive autocrine TNFα loop indicates that keratinocytes are important participants in the initiation of inflammatory skin reactions through their capacity to amplify the immune response. The maximum TNFα response in our study occurred after approximately 1 h of stimulation with TNFα similar to that found by Banno et al. [18]. After the peak response, we observed an elevated level of TNFα mRNA for up to 24 h. This sustained mRNA level was shown to depend on the constant presence of soluble TNFα. Thus, removal of TNFα either by washing or addition of a blocking antibody (infliximab) caused the return of mRNA levels to the background values within 2 h but was apparently not caused by a decline in receptor expression (Fig. 2). This finding might reflect a cellular protection mechanism preventing an exaggerated TNFα response following TNFα stimulation. TNFα may trigger the initial inflammation but the effect rapidly decreases when TNFα is removed or blocked.

Our results furthermore provide evidence for the involvement of atypical protein kinase C (aPKC) species in the TNFR1 signalling pathway leading to increased TNFα transcription. PKC species are critically involved in TNFα mediated regulation of TNFα gene expression in murine keratinocytes [17]. We therefore decided to address this question in human keratinocytes and examine the involved subclasses of PKCs. We found that blocking of aPKCs (ζ, λ/ι) led to a reduced or diminished TNFα response depending on the amount of blocking antibodies used whereas depletion of classical PKCs (α, β, γ) had no impact on TNFα-induced TNF mRNA transcription. The aPKCs were furthermore demonstrated to act upstream in the signalling cascade for NF-κB activation. Interestingly, blocking of NF-κB abolished the TNFα gene expression, which implies that NF-κB activation is essential for TNFα upregulation.

The participation of aPKCs in TNFα induced NF-κB activation is supported by data from studies on several other cell types [13–16,31,32]. Atypical PKCs may play an essential role in NF-κB activation at two different levels. They may bind to p62/RIP (receptor interacting protein) complex and activate IKK (IκB kinase) [15]. Moreover, aPKCs can directly phosphorylate p65 (RelA) subunit of NFκB [33]. To our knowledge, only one study has addressed the role of aPKCs in human keratinocytes [21]. In agreement with our data, aPKCs were identified as regulators of NF-κB activation induced by TNFα. It should be noticed, however, that our data are not necessarily valid for normal keratinocytes, since the overall level of NF-κB in nontransformed cells is lower [34,35]. However, the mechanism of NF-κB activation by TNF-α appears to be similar for the two cell types [34].

The discovery of the essential role of aPKC in the proinflammatory NF-κB pathway might have consequences for the development of future anti-TNFα therapies. The currently available anti-TNFα agents carry the risk of severe side effects including infection and malignancy [36]. It is conceivable that the inhibition of aPKC in the skin may provide a more selective way of the treatment of inflammation.

The final question we addressed was the influence of sphingolipid and cholesterol-enriched microdomains of the cell surface (lipid rafts) on TNFR signalling. In human fibrosarcoma HT1080 cells, it has recently been shown that TNFR1 translocates to lipid rafts within 2 min after TNFα binding [11]. Subsequently, the TRADD–TRAF2–RIP complex forms and initiates the signalling pathway leading to NF-κB activation. Disruption of the lipid rafts by cholesterol depletion inhibits activation of NF-κB and apoptosis is induced. This suggests that lipid rafts serve as platforms for cellular signalling through TNFR1. However, in primary cultures of mouse macrophages the NF-κB activation through TNFR1 does not depend on lipid rafts integrity [12]. The present experiment indicates that in human keratinocytes the TNFR1–PKC–NF-κB signalling pathway does not depend on the association or dissociation of TNFR1 with lipid rafts.

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