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Reactive Oxygen Species in Tumor Necrosis Factor- α -Activated Primary Human Keratinocytes: Implications for Psoriasis and Inflammatory Skin Disease

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Abstract

The multifunctional cytokine tumor necrosis factor- α (TNF- α) is known to play an important role in inflammatory and immunological responses in human skin. Although it has been documented that reactive oxygen species (ROS) are involved in TNF- α -induced signaling pathways associated with certain inflammatory diseases, their role in TNF- α signaling cascades has not been examined in primary human keratinocytes used as a model of inflammatory skin disease and psoriasis. Employing a series of in vitro and in cellulo approaches, we have demonstrated that in primary human keratinocytes (i) TNF- α rapidly induces ROS generation, I κ B degradation, NF- κ B p65 nuclear translocation, and ultimately production of inflammatory cytokines; (ii) TNF- α -induced cytokine production is mediated both by the mammalian target of rapamycin signaling pathway via NF- κ B activation and by ROS; (iii) TNF- α -dependent NF- κ B activation (that is, I κ B degradation and NF- κ B p65 nuclear translocation) is not mediated by ROS; and (iv) a cell-penetrating derivative of the antioxidant enzyme, catalase, as well as taurine and N-acetyl-cysteine attenuate the TNF- α -induced production of cytokines. These latter results suggest that catalase and perhaps other antioxidants should be considered as part of a more specific and effective therapy for the treatment of inflammatory skin diseases, including psoriasis.

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CONFLICT OF INTEREST

SRT is a cofounder of EXT Life Sciences Inc., a Michigan-based biotechnology company owned, in part, by the Wayne State University. SRT retains an equity interest in the company. Transducible catalase-SKL, one of the antioxidants examined in this report, is covered by a United States patent pending, which is owned by the Wayne State University. Other authors state no conflict of interest.

INTRODUCTION

Skin, the outermost layer of the body, is the largest human organ. It plays an important protective role by providing an interactive boundary between the body and the environment. Skin is repeatedly exposed to ionizing and UV radiation and systemically introduced to potentially toxic dietary and drug metabolites, all of which may influence its health and appearance (Sander *et al.*, 2004). Many of these environmental agents and endogenous metabolites are natural oxidants. They can directly or indirectly drive the production of a variety of reactive oxygen species (ROS) through a number of physiological processes (Kohen, 1999; Trouba *et al.*, 2002). The surface of the skin is especially sensitive to ROS. Many studies have documented their role in skin aging, the development of inflammatory skin disorders, and carcinogenesis (Briganti and Picardo, 2003; Sander *et al.*, 2004; Okayama, 2005).

One particular skin pathology, psoriasis, is an incurable disorder that has been defined as a chronic inflammatory disease largely mediated by immune cells (Nickoloff and Nestle, 2004). Although it affects 2–4% of the general population worldwide, its etiology remains largely unknown. However, it can be triggered by stress or trauma, and by bacterial, fungal, or viral infection of the skin (Krueger, 2002; Lebwohl, 2003). Psoriasis is generally characterized by hyperproliferation, abnormal differentiation, and altered maturation of the epidermis, as well as inflammation in the epidermis and dermis (Banno *et al.*, 2004; Chamian and Krueger, 2004). The pathogenesis of psoriasis has been reported to be driven by activated T cells or antigen-presenting cells, which release a number of cytokines and chemokines. These then bind to surface receptors and signal keratinocytes to hyperproliferate and abnormally differentiate. This, in turn, causes the accumulation of additional cytokines and inflammatory mediators in the lesional skin (Barker, 1991; Nickoloff, 2001; Gudjonsson *et al.*, 2004).

Tumor necrosis factor- α (TNF- α) is a major mediator in the pathogenesis of psoriasis. It is a multifunctional cytokine that is believed to play an important role in mediating inflammation, immune responses, and apoptosis (Tracey and Cerami, 1994; Aggarwal and Natarajan, 1996; Locksley *et al.*, 2001). It has been reported that there are much higher levels of TNF- α found in the skin of patients with psoriatic lesions as compared to that of normal subjects (Mease, 2004; De Rycke *et al.*, 2005; Veale *et al.*, 2005). In addition to TNF- α , other inflammatory cytokines and a transcription factor, NF- κ B, are found to be highly expressed in psoriatic patients (Bachelez, 2005). One of the molecular mechanisms proposed is that TNF- α produced locally within psoriatic lesions causes NF- κ B activation, subsequently leading to increased expression of other cytokines as well as of TNF- α itself. Thus, the view is that a TNF- α →NF- κ B activation→increased TNF- α /inflammatory cytokine expression positive feedback loop exists that maintains and may enhance the inflammatory process within psoriatic plaques (Barnes and Karin, 1997; Bachelez, 2005; Punzi *et al.*, 2007). A number of studies support the proposed importance of TNF- α -positive feedback loops in the pathophysiology of psoriasis (Mease, 2005; Nash and Florin, 2005; Saripalli and Gaspari, 2005). However, there is also the speculation that increased ROS production and deficient function of cellular antioxidant systems play a critical role in the etiology of the disease (Antille *et al.*, 2002; Briganti and Picardo, 2003). Importantly, these

points have yet to be examined in a cell model of inflammatory skin disease, specifically primary human keratinocytes treated with TNF- α .

Although the extent to which keratinocytes themselves contribute to the TNF- α load seen in psoriatic skin remains a matter of some debate, these cells can clearly produce the cytokine and other inflammatory mediators (for example, see Johansen *et al.*, 2006), and therefore a molecular analysis of the underlying mechanisms of such production is clearly warranted. The overall aim of this study was to investigate, in primary human keratinocytes, the role of ROS in psoriasis-associated TNF- α signaling cascades. We show that treatment with TNF- α (i) stimulates ROS generation; (ii) activates NF- κ B signaling through I κ B degradation and NF- κ B p65 nuclear translocation; and (iii) increases mRNA expression of the cytokines, IL-8, IL-6, and TNF- α itself. We implicate the serine/threonine protein kinase, mammalian target of rapamycin (mTOR), in these processes and also show that quenching hydrogen peroxide and downstream ROS with a cell-penetrating catalase derivative or other antioxidants is an effective means of reducing inflammatory cytokine gene expression. Taken together, these results shed light on the biochemistry and pathophysiology of inflammatory skin diseases, including psoriasis, at the level of the keratinocyte, and may provide previously unknown targets for therapeutic intervention.

RESULTS

TNF- α treatment induces hydrogen peroxide/ROS generation

To evaluate whether or not cellular hydrogen peroxide production was involved in TNF- α -mediated signaling, we measured levels of the ROS produced in primary human keratinocytes. First, we found that treatment with TNF- α caused a time-dependent increase in hydrogen peroxide production; this occurred within 5 minutes of TNF- α stimulation and reached a maximum at approximately 20 minutes (Figure 1). Having identified a “threshold time” (~20 minutes), we addressed ROS production in a dose–response analysis (Figure 2). We found that TNF- α increases ROS production in a dose-dependent (0–100 ng ml⁻¹) manner. These findings correlate well with results showing TNF- α -induced ROS generation in other human cell types (Schulze-Osthoff *et al.*, 1992; Goossens *et al.*, 1995) as well as in canine keratinocytes (Kohler *et al.*, 2001). However, to the best of our knowledge, we are the first group to demonstrate these phenomena in primary human keratinocytes.

TNF- α stimulates NF- κ B activation in human primary keratinocytes

The NF- κ B is a ubiquitous transcription factor for genes encoding proinflammatory and inflammatory cytokines, chemokines, and growth factors (Liou and Baltimore, 1993; Baldwin, 1996; Ghosh and Karin, 2002; Takao *et al.*, 2003). In resting cells, I κ B forms a complex with NF- κ B that masks the nuclear localization sequences of NF- κ B and thus prevents it from entering the nucleus. Upon cell stimulation, for example after TNF- α treatment, I κ B proteins are rapidly phosphorylated and degraded by the proteasome pathway, and the newly freed NF- κ B molecule translocates into the nucleus to activate expression of the multiple target genes noted above. The phosphorylation and degradation of I κ B are thus the important steps in the pathway leading to NF- κ B activation.

To assess activation of NF- κ B following TNF- α exposure, we examined I κ B degradation and NF- κ B nuclear translocation as the relevant parameters. I κ B degradation was evaluated by western blot analysis using an anti-I κ B α antibody. Our results show that degradation of I κ B α induced by TNF- α occurs very rapidly, with some 70% of the molecule being lost in the first 15 minutes (Figure 3). Next, we studied the effect of TNF- α on nuclear translocation of the NF- κ B p65 subunit. To accomplish this, we performed laser scanning indirect immunofluorescence confocal microscopy using an antibody to the p65 molecule. As shown in Figure 4, the immunofluorescence staining pattern showed quite clearly the translocation of p65 into the nucleus of human keratinocytes after treatment with TNF- α . As expected, this molecule remained in the cytoplasm of untreated control cells.

Induction of TNF- α , IL-8, and IL-6 mRNA expression following TNF- α stimulation

Although it has been well documented that high concentrations of TNF- α are present in skin lesions of patients with psoriasis (Mease, 2004; De Rycke *et al.*, 2005; Veale *et al.*, 2005), to date the effect of TNF- α on cytokine expression in primary human keratinocytes has not been determined. To address this point, we examined whether or not TNF- α is able to increase the (mRNA) expression of itself or other potent inflammatory cytokines in primary human (cultured) keratinocytes. We performed real-time reverse transcriptase-PCR (RT-PCR) (Figure 5) and found that in response to TNF- α , the message levels for TNF- α , IL-8, and IL-6 were all significantly increased. For IL-6, this induction was nearly 40-fold as compared to untreated controls (Figure 5).

Role of the mTOR pathway in TNF- α -induced cytokine upregulation

mTOR is, as mentioned above, a conserved serine/threonine protein kinase and a member of the cellular phosphatidylinositol 3-kinase/AKT pathway. We investigated its role in NF- κ B activation and ROS production induced by TNF- α by using rapamycin (sirolimus), a well-characterized immuno-suppressant and potent inhibitor of the signaling cascade. As described above, the phosphorylation and subsequent degradation of I κ B play a critical role in regulating NF- κ B translocation to the nucleus and subsequent transcriptional regulation. In Figure 6, we asked if rapamycin has any effect on TNF- α -induced degradation of I κ B α ; clearly, it did. I κ B α is largely stabilized by the pretreatment.

Furthermore, we showed that rapamycin inhibits mRNA expression of TNF- α , IL-8, and IL-6 (Figure 5). These results support the notion that there is a connection between the mTOR signaling cascade, NF- κ B activation, and inflammatory cytokine expression. Importantly, such a link between mTOR and NF- κ B has already been put forth (Ghosh *et al.*, 2006), and is supported by our observations.

Finally, our analysis of the mTOR pathway showed that rapamycin has only modest effects on TNF- α -induced ROS production in these cells (Figure 7). One interpretation of these results is that ROS production lies upstream of the mTOR to NF- κ B signaling cascades in TNF- α -activated primary human keratinocytes.

Involvement of ROS in TNF- α signaling in primary human keratinocytes

The results presented thus far have demonstrated that in primary human keratinocytes, treatment with TNF- α rapidly induces ROS generation, activation of the mTOR and NF- κ B signaling and transcription pathways, and mRNA expression of the proinflammatory cytokines TNF- α , IL-8, and IL-6. We next asked what the effects of TNF- α would be on cells pretreated with a targeted antioxidant. To analyze this point, we employed a transducible form of catalase as a specific ROS scavenger that efficiently decomposes hydrogen peroxide. This genetically engineered catalase molecule (hereafter called Cat*) is modified with a cell-penetrating peptide (specifically, an arginine nonamer) at its amino terminus and a high efficiency peroxisomal targeting signal 1 (specifically, serine-lysine-leucine) at its carboxy terminus. This enzyme rapidly enters cells and efficiently traffics to the peroxisome to metabolize hydrogen peroxide. The strategy of restoring or supplementing peroxisomal catalase in cells has been shown to re-establish oxidative balance, to renew mitochondrial integrity (by repolarizing the organelle), and to delay senescence (Wood *et al.*, 2006; Koepke *et al.*, 2007; Terlecky and Koepke, 2007). Our results indicate that Cat* prophylaxis significantly reduces the amount of hydrogen peroxide and related ROS present in TNF- α -treated keratinocytes (Figure 7). Furthermore, these cells are unable to induce expression of TNF- α mRNA to the level seen in non-antioxidant pretreated cells (Figure 8).

Importantly, similar results were seen with taurine, a sulfur-containing β -amino acid, and *N*-acetyl cysteine, the acetylated precursor of both L-cysteine and reduced glutathione (Figures 7 and 8). Both molecules possess antioxidant properties and elicit a variety of effects on cells (Petty *et al.*, 1990; Huxtable, 1992; Atmaca, 1998). Taurine's demonstrated ability to control release of proinflammatory cytokines in a number of organisms is consistent with the effects seen in Figure 8.

The obvious suggestion is that hydrogen peroxide and related ROS are stimulating expression of inflammatory cytokines in this model system. To further test this, cells were pretreated with hydrogen peroxide directly or with 3-aminotriazole, a specific inhibitor of catalase. In both cases, the resultant oxidative load present in cells enhanced TNF- α 's ability to induce cytokine expression (Figure 8).

We next asked if activation of NF- κ B in response to TNF- α was mediated by ROS. This does not appear to be the case, as cells pretreated with Cat*, taurine, or *N*-acetyl-cysteine still degrade I κ B(α) in response to TNF- α (Figure 9). Therefore, even though ROS certainly plays a role in the expression of inflammatory cytokines induced by TNF- α , it apparently is not through I κ B degradation and accompanying NF- κ B activation/nuclear translocation. Rather, it appears that there exists a redox-independent I κ B degradation/NF- κ B activation step involved in TNF- α -activated primary human keratinocytes.

DISCUSSION

The pathogenesis of psoriasis is still not completely understood. It is certainly a disease of immune cells with some, as yet to be clearly defined, contribution by skin cells including keratinocytes. Collectively, these cells conspire to potentiate the development of chronic skin inflammation through the production of cytokines (Bhalerao and Bowcock, 1998;

Krueger *et al.*, 2001; Krueger, 2002; Lebwohl, 2003; Nickoloff and Nestle, 2004; Albanesi *et al.*, 2005). Among these is TNF- α , the major proinflammatory cytokine of psoriasis, which activates cells in a positive feedback loop producing other inflammatory mediators, including ROS and various other cytokines (Gottlieb *et al.*, 2005; Lowes *et al.*, 2005; Pelle *et al.*, 2005). It is also thought that an insufficient antioxidant system, together with increased levels of ROS, contributes to the pathogenesis (Antille *et al.*, 2002; Briganti and Picardo, 2003; Baz *et al.*, 2003; Bickers and Athar, 2006).

One aim of this work was to gain a better understanding of the interactions between TNF- α , ROS, and antioxidants in the initiation and progression of the disease. Our studies employing primary human keratinocytes clearly support a role for ROS in TNF- α -induced cytokine expression. We demonstrate rapid TNF- α -dependent ROS generation (Figures 1 and 2). In addition, we show that quenching the resultant oxidative stress with either a cell-penetrating, peroxisomally targeted, catalase derivative or the antioxidants taurine or *N*-acetyl-cysteine significantly reduces the accompanying elevated cytokine expression (Figure 8). The suggestion is that disturbances in the oxidant-antioxidant system in human skin may play an important role in the pathogenesis of psoriasis. As current systemic medications for psoriasis have been reported to have significant side effects, we propose that targeted antioxidant prophylaxis, akin to the strategy employed here with a transducible catalase derivative, is worth considering as a potential treatment modality.

Our results also implicate the critical cellular kinase, mTOR, in response to TNF- α . This enzyme participates in, and often regulates, a variety of cellular functions, including protein synthesis and autophagy, as well as (cell) growth, survival, and proliferation (Hay and Sonenberg, 2004; Inoki *et al.*, 2005; Ghosh *et al.*, 2006). The molecule and associated complex partners integrate a variety of upstream signals and modulate a number of downstream effectors. Perhaps best underscoring the importance of mTOR and interaction complex members is the now well-established role of these molecules, when dysregulated, in cancer (Tokunaga *et al.*, 2004; Tsang *et al.*, 2007). In primary human keratinocytes treated with TNF- α , mTOR appears to regulate NF- κ B activation (Figure 6). Interestingly, (inhibiting) mTOR has little effect on TNF- α 's ability to elicit ROS formation (Figure 7), suggesting that distinct mechanisms are at work. Therefore, TNF- α 's stimulation of inflammatory cytokine expression employs both mTOR-dependent and mTOR-independent reactions.

Considering NF- κ B activation further, it was unexpected that quenching TNF- α -induced ROS did not inhibit the process (Figure 9). Indeed, I κ B α was degraded (and thus NF- κ B activated) as well or better in the presence of the antioxidant. At first glance, this seemed inconsistent with a number of observations in other cellular contexts, suggesting a role for ROS in NF- κ B activation (Schreck *et al.*, 1991; Bubici *et al.*, 2006; Gloire *et al.*, 2006; Nakano *et al.*, 2006). Although the answer is not completely clear, we speculate that perhaps NF- κ B-induced transcription of relevant genes is redox sensitive. Therefore, the ROS-requiring step lies downstream of NF- κ B activation. Further experimentation will be required to confirm this conjecture.

In summary, our findings suggest a rather straightforward series of biochemical relationships. In primary human keratinocytes, TNF- α induces ROS, stimulates mTOR, and activates NF- κ B, resulting in expression of inflammatory cytokines. What should not be lost is that the pathways may interact or may function independently. The intricacy presumably reflects the complexity of psoriasis and related inflammatory skin diseases. Having identified some of the relevant pathways in the overall response of human primary keratinocytes to TNF- α , a more careful dissection of specific components and underlying molecular mechanisms may now begin.

MATERIALS AND METHODS

Reagents and antibodies

TNF- α was obtained from R&D Systems (Minneapolis, MN). DMEM/F12, fetal bovine serum, trypsin–EDTA, phosphate-buffered saline (PBS), and other products for cell culture were supplied by Invitrogen (Carlsbad, CA). Serum-free culture EpiLife Medium and EpiLife Defined Growth Supplement for human primary keratinocytes culture were purchased from Cascade Biologics Inc. (Portland, OR). The Amplex Red reagent kit for measuring hydrogen peroxide was purchased from Invitrogen (Carlsbad, CA). *N*-acetylcysteine, rapamycin, taurine, and 2',7'-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma-Aldrich (St Louis, MO). Antibodies to I κ B α and NF- κ B p65 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-catalase antibodies were generated under contract by our laboratory. Anti-tubulin antibodies were kindly provided by Dr Raymond Mattingly (Department of Pharmacology, Wayne State University School of Medicine).

Transducible catalase-SKL: plasmid preparation and enzyme purification

A transducible form of the human antioxidant enzyme, catalase, was created by adding a cell-penetrating peptide, specifically nine arginine residues, to the protein's amino terminus. To accomplish this, a double-stranded oligonucleotide encoding nine arginine residues was produced by annealing a pair of synthesized complementary oligonucleotides (Operon Biotechnologies Inc., Huntsville, AL) flanked by restriction enzyme sites. The oligonucleotides were (top strand) 5'-tatgCGCCGTCGCCGTCGCCGTCGCCGTCGCctgca-3' and (bottom strand) 5'-gGCGACGGCGACGGCGACGGCGACGGCGca-3'. An *Nde*I restriction site (lowercase letters) is encoded at the upstream end, whereas a *Pst*I site is at the downstream end of the double-stranded oligonucleotides. The double-stranded oligonucleotide was directly ligated into *Nde*I–*Pst*I-digested pQE-2 (Qiagen, Chatsworth, CA).

In addition, the enzyme's carboxy-terminal peroxisomal targeting signal 1 was altered from the naturally occurring alanine–asparagine–leucine to the more efficient serine–lysine–leucine (SKL). To generate this plasmid, the human *catalase* gene was PCR amplified from a full-length cDNA clone (Invitrogen, Paisley, UK). The forward primer, 5'-AAActgcagATGGCTGACAGCCGGGATCCCGCC-3', complemented the amino-terminal sequence of human catalase along with a *Pst*I restriction site (lowercase letters). The reverse primer was designed to produce the SKL amino-acid sequence (5'-

GGGCGCaagcttTCACAGTTTCGATTTCTCCCTTGCCGCCAAGT-3'). A *Hind*III restriction site (lowercase letters) was also incorporated downstream of the stop codon.

The *catalase-SKL* gene amplified by PCR was digested appropriately, and ligated into the pQE-2 vector containing the sequence encoding the nine arginine residues. Ligation products were transformed into the *E. coli* strain DH5 α , and the recovered plasmids were confirmed to be correct by restriction analysis and DNA sequencing. The sequence-verified (His)₆-tagged cell-penetrating catalase-SKL construct was then expressed in the *E. coli* strain Rosetta 2 (DE3)pLysS (Novagen, Madison, WI), according to the manufacturer's instructions.

Recombinant catalase protein was purified using a modified denaturing protocol. To prepare denatured fusion proteins, the induced cells were harvested and lysed by freeze/thawing in a binding buffer (5mM imidazole, 500mM NaCl, and 20mM Tris-HCl, pH 7.9) containing 2 M urea. After the removal of the cell debris by centrifugation, the clarified cell extracts were loaded onto a Ni-NTA column. The column was washed first with the binding buffer containing 2 M urea and then with a wash buffer (30mM imidazole, 500mM NaCl, and 20mM Tris-HCl, pH 7.9). The proteins were released by an elution buffer (500mM imidazole, 500mM NaCl, and 20mM Tris-HCl, pH 7.9), followed by desalting with a PD10 column. The purified transducible recombinant catalase fusion protein (in PBS containing 20% glycerol) was then aliquoted and stored at -80 °C.

Primary human keratinocyte isolation from neonatal foreskin

Neonatal foreskins from healthy males were the source of primary human keratinocytes. They were obtained through an Internal Review Board protocol approved by the Human Investigative Committee at the Wayne State University School of Medicine. Skin samples were obtained and pooled together from three or more surgically circumcised foreskins. Isolation of keratinocytes was performed essentially as described by Liu and Karasek (1978). Briefly, skin samples were washed, cut, scraped, and treated with a dispase solution to prepare a largely epidermal layer. The tissue was then trypsinized, filtered, centrifuged, and the resultant keratinocytes cultured and seeded as required. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in a serum-free low-Ca²⁺ (0.06mM) EpiLife keratinocyte growth medium (Cascade Biologics Inc.) supplemented with bovine hydrocortisone, insulin, pituitary extract, and transferrin, as well as recombinant human epidermal growth factor (Cascade Biologics Inc.). The medium was changed every other day. For all experiments, cells were seeded at passage numbers 3–5 into 60mm dishes (2 × 10⁶ cells per well), 24-well plates (5 × 10⁵ cells per well), or 96-well plates (1 × 10⁵ cells per well), and were treated upon reaching 80–90% confluence. In experiments designed to examine effects of TNF- α , cells were grown in standard medium without growth factors for 24 hours to avoid the effects of supplements in the growth medium and to obtain quiescent cells with low levels of activated NF- κ B.

Measurement of intracellular hydrogen peroxide/ROS

Production of intracellular hydrogen peroxide was determined using either DCF-DA (Bass *et al.*, 1983; LeBel *et al.*, 1992; Ohba *et al.*, 1994) or the Amplex Red reagent (Molecular

Probes/Invitrogen, Carlsbad, CA). The method employed in a given experiment is indicated in the accompanying figure legend.

With the DCF-DA assay, the medium was removed from cells, which were then washed and incubated with 50 μ M DCF-DA in keratinocyte growth medium at 37 °C for 30 minutes. The DCF-DA was then removed and the cells were washed once again. A fluorescence microplate reader was used to take a time zero (F_{t_0}) point at an emission wavelength of 535nm and an excitation wavelength of 490 nm. Immediately thereafter, the cells were subjected to TNF- α treatment with or without pretreatment with rapamycin, catalase, taurine, or *N*-acetyl-cysteine in keratinocyte growth medium, and a time course was initiated. At each time point, the fluorescence intensity was again determined. For data analysis, the percentage increase in fluorescence per well was calculated by the formula $[(F_{t_x}-F_{t_0})/F_{t_0}100]$, where F_{t_x} is fluorescence at time x minutes and F_{t_0} is fluorescence at time 0 minute.

Measurement of intracellular hydrogen peroxide was also directly determined using Amplex Red hydrogen peroxide/peroxidase assay kit. The principle behind this assay is that Amplex Red is a derivative of 10-acetyl-3,7-dihydroxyphenoxazine, which becomes highly fluorescent upon oxidation by hydrogen peroxide in the presence of horseradish peroxidase. Experimentally, after a given treatment, equal numbers of cells were washed twice with PBS (pH 7.4) and harvested into reaction buffer containing the Amplex Red reagent for 30 minutes, according to the manufacturer's protocol. Fluorescence was recorded in a microplate reader (Molecular Devices, Sunnyvale, CA) set at 530nm excitation and 590nm emission wavelengths.

Cytokine mRNA expression determined by real-time quantitative RT-PCR

At the end of a particular treatment, human primary keratinocyte samples were harvested, treated with lysis buffer, and total RNA was extracted according to the RNeasy Mini Kit manual (Qiagen). RNA concentrations and 260/280 ratios were assessed using a DU530 spectrophotometer (Beckman, Fullerton, CA). Reverse transcription of cDNA was performed using random hexamers as primers according to the SuperScript II Reverse Transcriptase System Reagents kit manual (Invitrogen, Carlsbad, CA).

To assess the relative transcript levels of specific genes, the cDNA products were subjected to real-time SYBR-green-based quantitative PCR as described by Lee *et al.* (2006). The primers used for these studies were generated from GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) with Gene Runner software (Hastings Software, Hastings, NY). Primer sequences used were as indicated in Table 1. We confirmed that primers amplified the appropriate products by testing under real-time PCR conditions. Each assay typically was repeated twice, with each sample run in triplicate. Signals from each sample were normalized to values obtained for the *18S rRNA* gene, which was run as a "housekeeping gene" simultaneously with the experimental samples. Cycle threshold numbers (C_t) were recorded from the exponential phase of PCR amplification. The relative expression of cytokine mRNA was calculated using the comparative 2^{-C_t} method, as previously

described (Livak and Schmittgen, 2001). The results from each run for each sample were averaged and expressed as the relative level of the mRNA transcript.

Detection of NF- κ B activation by western blot analysis

After human primary keratinocytes were incubated under the appropriate experimental conditions, cells were harvested in 2 \times SDS-PAGE sample buffer and subjected to western blot analysis. Briefly, equal amounts of whole-cell extracts were resolved by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. After nonspecific binding sites were quenched using blocking buffer (5% non-fat dry milk, 0.5% Tween-20 in 20mM Tris-buffered saline, pH 7.6), the membrane was incubated overnight with primary antibodies specific for I κ B α or tubulin. The anti-I κ B α antibodies were used at a 1:500 dilution, and the anti-tubulin antibodies were used at a 1:200 dilution. The blot was washed and incubated again with horseradish peroxidase-conjugated anti-goat or anti-rabbit IgG secondary antibodies. After washing once again, protein levels were determined by chemiluminescence using an ECL detection system. The intensities of the bands were measured using a Fujifilm LAS1000plus luminescent image analyzer.

NF- κ B p65 subunit translocation detected by indirect immunofluorescence confocal microscopy

Human keratinocytes grown on glass coverslips were fixed with 4% (w/v) formaldehyde for 10 minutes and permeabilized in 1% (v/v) Triton X-100 for 5 minutes. The cells were blocked with 4% (w/v) BSA for 1 hour and then incubated with primary antibodies for 1 hour and secondary antibodies for 45 minutes. All reactions were conducted in PBS; rabbit anti-p65 antibodies were used at a 1:500 dilution and CY3-conjugated goat anti-rabbit secondary antibodies were used at a 1:250 dilution. Coverslips were mounted in PBS containing anti-fade mounting media and examined using a confocal fluorescence microscope (Zeiss LSM510) at the Core Facility, Department of Pharmacology, Wayne State University School of Medicine.

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Abbreviations

| | |
|--------------------------------|-------------------------------------|
| DCF-DA | 2',7'-dichlorofluorescein diacetate |
| mTOR | mammalian target of rapamycin |
| ROS | reactive oxygen species |
| RT | reverse transcriptase |
| TNF-α | tumor necrosis factor- α |

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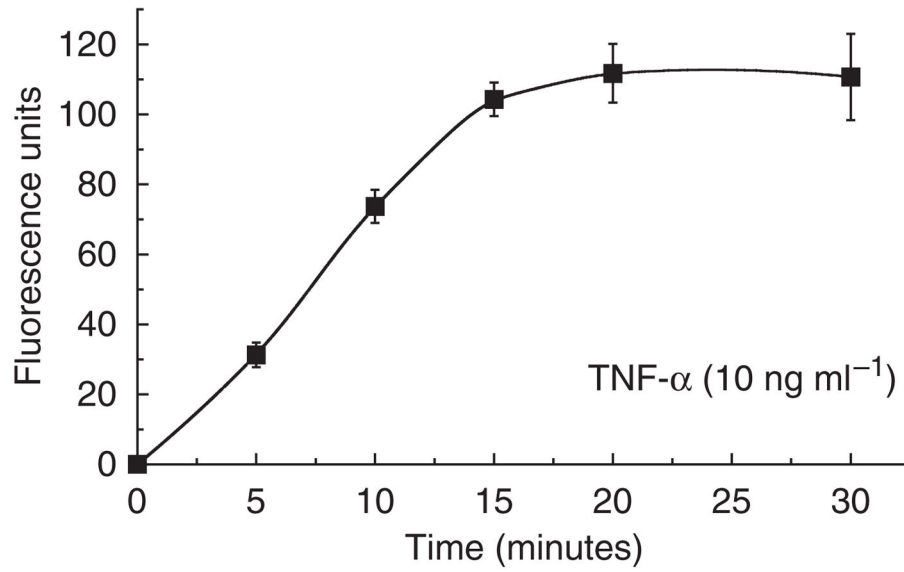


Figure 1. Time course of hydrogen peroxide production induced by TNF- α in primary human keratinocytes

Cells (2×10^5 ml⁻¹) were treated with 10 ng ml⁻¹ TNF- α for the indicated times. The amount of hydrogen peroxide produced in cells was determined using oxidation of the fluorogenic indicator Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine). Fluorescence units data presented reflect subtraction of untreated control cell values and are the means \pm SEM of triplicate samples. Similar results were seen in two additional experiments.

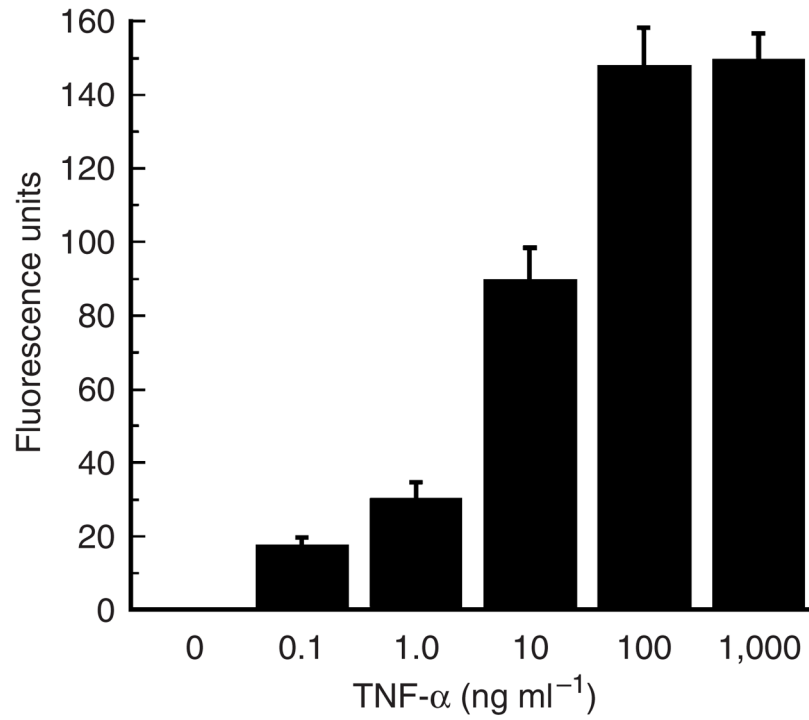


Figure 2. Dose response of hydrogen peroxide production induced by TNF- α in primary human keratinocytes
Cells (2×10^5 ml⁻¹) were treated with varying amounts of TNF- α (from 0 to 1,000 ng ml⁻¹) for 20 minutes, and the amount of hydrogen peroxide produced in cells was determined as in Figure 1. Results presented are the means \pm SEM of triplicate samples. Similar results were seen in two additional experiments.

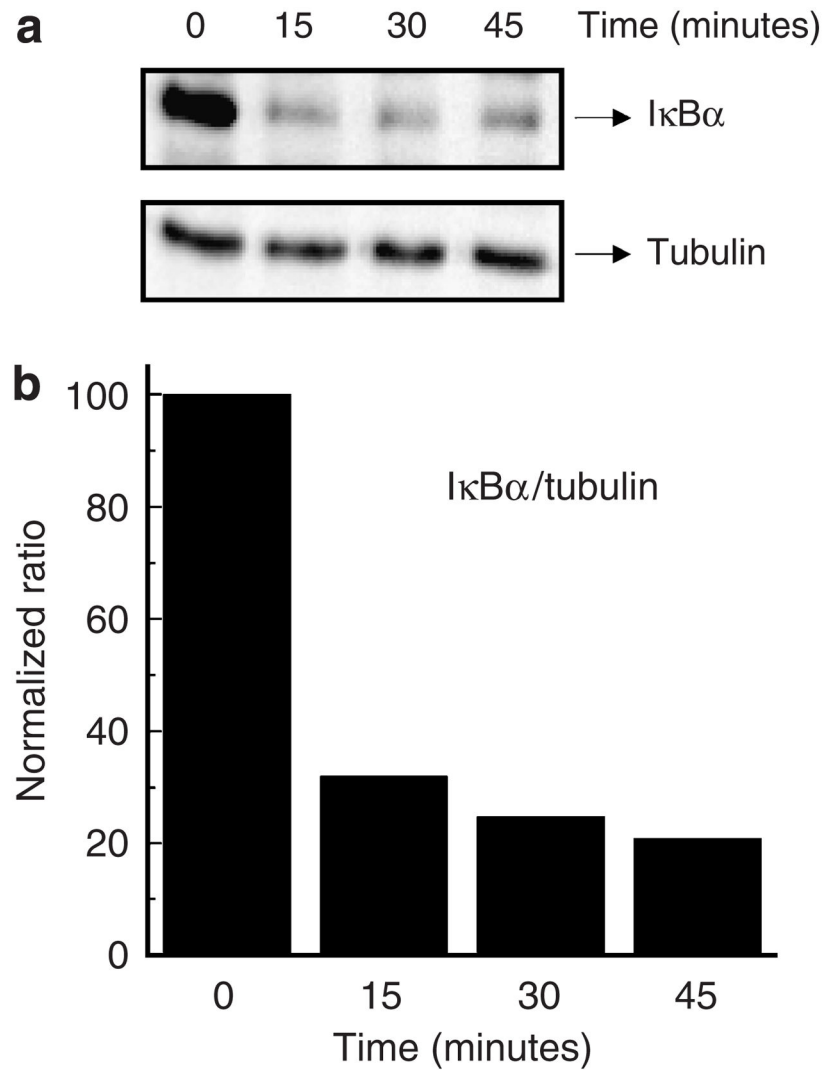


Figure 3. Degradation of IκB(α) induced by TNF-α in primary human keratinocytes
Cells ($2 \times 10^6 \text{ ml}^{-1}$) were treated with 10 ng ml^{-1} TNF-α for the indicated times, and cell lysates were prepared as described in Materials and Methods. (a) Western blot analysis of IκBα or tubulin using appropriate polyclonal antibodies. (b) Densitometric analysis of the immunoreactive bands obtained after western blotting. Results are presented as IκBα/tubulin normalized ratios. Similar results were seen in duplicate experiments.

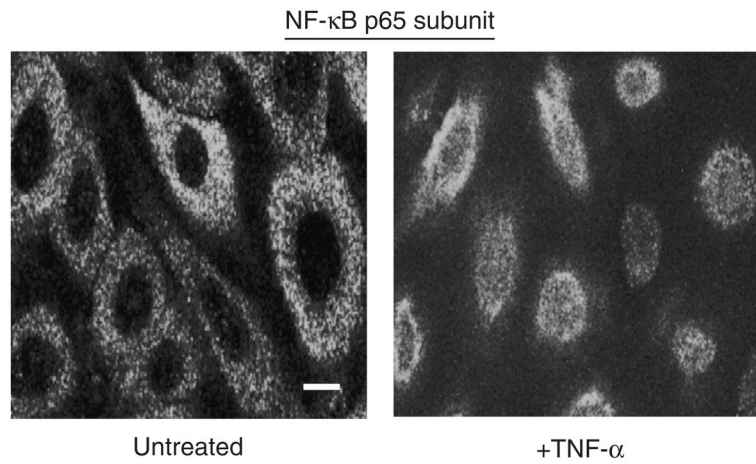


Figure 4. Nuclear translocation of the NF- κ B p65 subunit induced by TNF- α in primary human keratinocytes

Cells were stimulated with TNF- α (10 ng ml^{-1}) for 1 hour. Nuclear translocation of the NF- κ B p65 subunit was assessed by indirect immunofluorescence confocal microscopy using anti-p65 subunit antibodies and appropriate fluorescently tagged secondary antibodies. A representative image of several obtained is included here. Bar=10 μm . Similar results were seen in duplicate experiments.

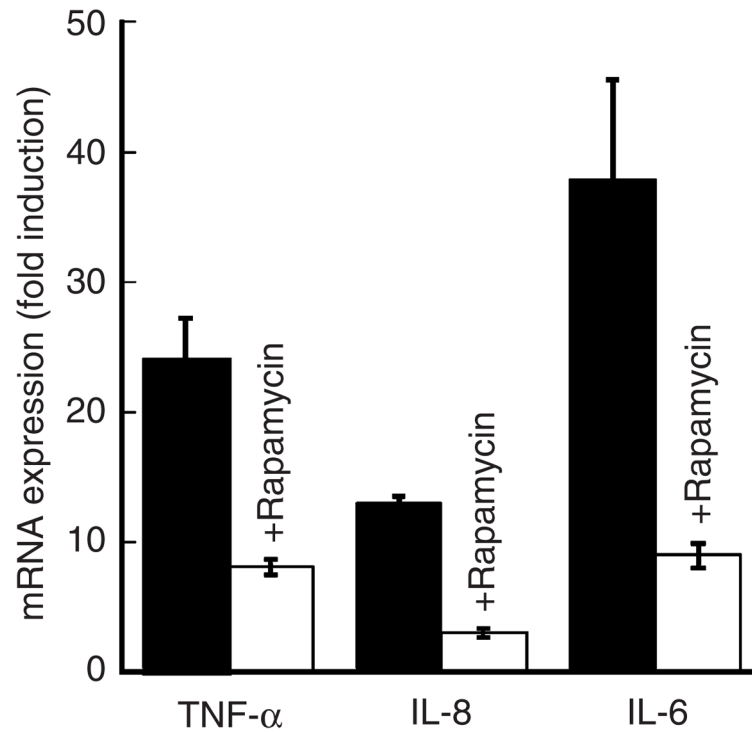


Figure 5. Effects of TNF- α and rapamycin on expression of TNF- α , IL-8, and IL-6
Primary human keratinocytes were treated with TNF- α (10 ng ml^{-1}) for 6 hours. Total RNA was then isolated and mRNA analysis performed by real-time RT-PCR. Where indicated, cells were pretreated with rapamycin (20 nM) for 1 hour and then challenged with TNF- α . Data are expressed as fold induction of relevant mRNAs as compared to untreated controls. Representative results (means \pm SEM) from one of three independent experiments are shown.

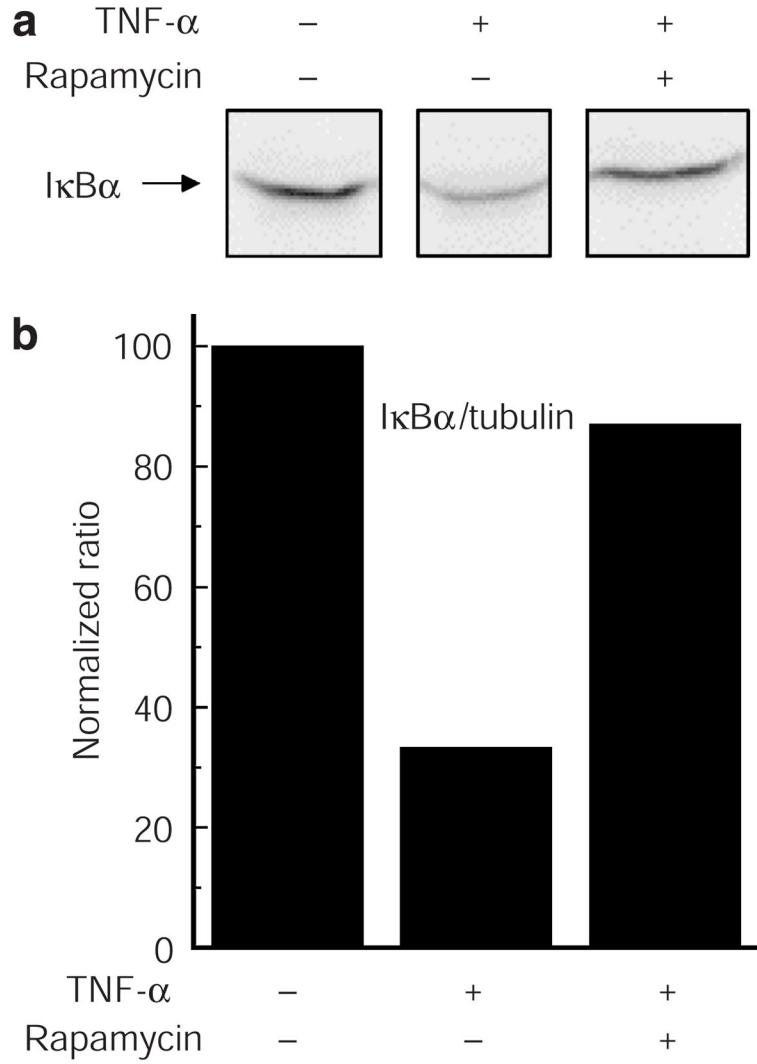


Figure 6. Effects of rapamycin on TNF- α -induced I κ B degradation in primary human keratinocytes

Cells ($2 \times 10^6 \text{ ml}^{-1}$) were pretreated or not with rapamycin (20 nM) for 1 hour and then challenged with 10 ng ml^{-1} TNF- α for 30 minutes. (a) Western blot analysis, and (b) densitometric measurements and normalization of I κ B α and tubulin levels were performed as outlined in Figure 3. Similar results were seen in duplicate experiments.

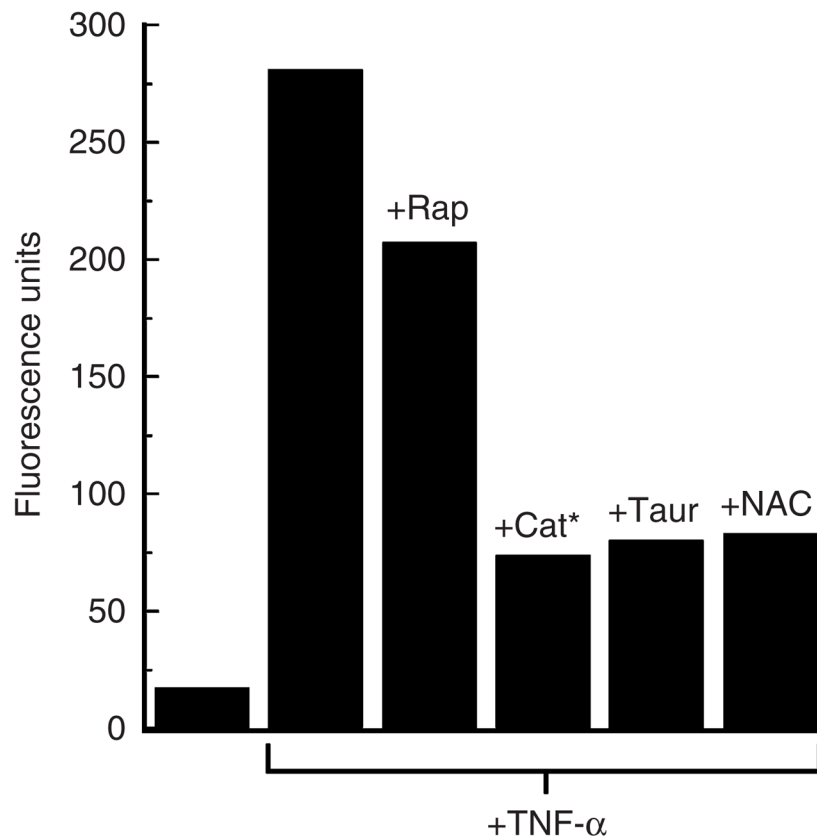


Figure 7. Effects of rapamycin and antioxidants on hydrogen peroxide production induced by TNF- α in primary human keratinocytes

Cells ($2 \times 10^5 \text{ ml}^{-1}$) were pretreated or not with rapamycin (Rap) (20 nM), catalase (Cat*) (1 μM), taurine (Taur) (40mM), or *N*-acetyl-cysteine (NAC) (5mM) for 1 hour and then challenged with TNF- α (10 ng ml^{-1}) for 30 minutes. Hydrogen peroxide production was measured using DCF-DA as outlined in Materials and Methods. Fluorescence unit data presented reflect subtraction of untreated control cells values and are the means of triplicate samples. Similar results were seen in two additional experiments.

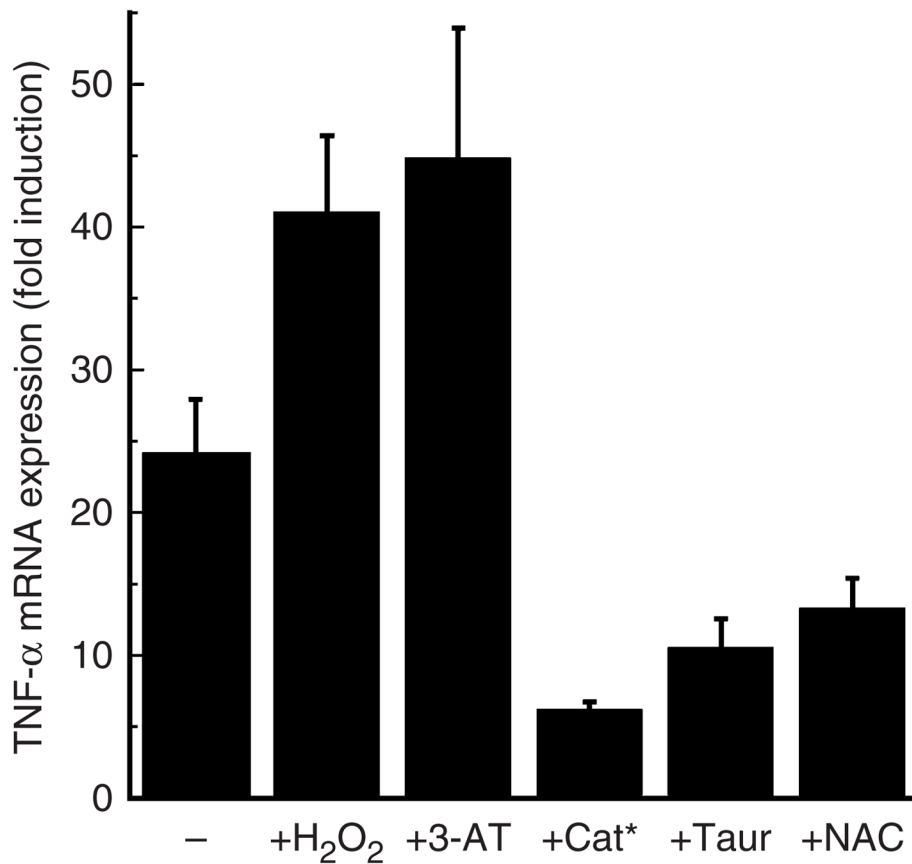


Figure 8. Effect of hydrogen peroxide on TNF- α -induced gene expression in primary human keratinocytes

Cells were pretreated or not with 3-aminotriazole (3-AT) (1 μ M), hydrogen peroxide (2.5 μ M), catalase (Cat*) (1 μ M), taurine (Taur) (40mM), or *N*-acetyl-cysteine (NAC) (5mM) for 1 hour and then challenged with TNF- α (10 ng ml⁻¹) for 6 hours. Total RNA was then isolated and TNF- α mRNA analysis performed by real-time RT-PCR. Data are expressed as fold induction of relevant mRNAs as compared to untreated controls. Representative results (means \pm SEM) from one of three independent experiments are shown.

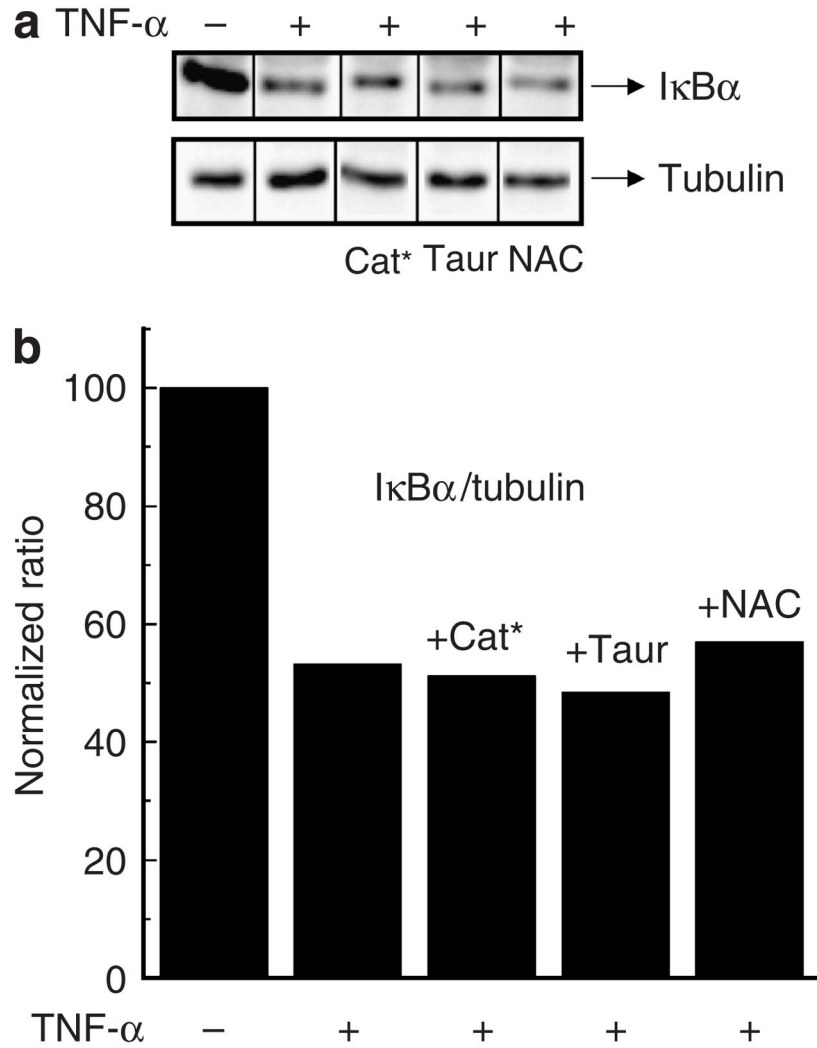


Figure 9. Effects of antioxidants on TNF- α -induced I κ B degradation in primary human keratinocytes

Cells ($2 \times 10^6 \text{ ml}^{-1}$) were pretreated or not with catalase (Cat*) ($1 \mu\text{M}$), taurine (Taur) (40mM), *N*-acetyl-cysteine (NAC) (5mM) for 1 hour and subsequently challenged with TNF- α (10 ng ml^{-1}) for 30 minutes. (a) Western blot analysis, and (b) densitometric measurements and normalization of I κ B α and tubulin levels were performed as outlined in Figure 3. Similar results were seen in duplicate experiments.

Table 1

Primer sequences for real-time PCR

| Gene | Primer | Sequence | Product size (bp) |
|---------------|-----------|-------------------------------|-------------------|
| TNF- α | Sense | 5'-CTACTCCCAGGTTCTCTTCAA-3' | 137 |
| TNF- α | Antisense | 5'-GCAGAGAGGAGGTTGACTTTC-3' | |
| IL-6 | Sense | 5'-ATGAACTCCTTCTCCACAAGC-3' | 156 |
| IL-6 | Antisense | 5'-CTACATTTGCCGAAGAGCCCCTG-3' | |
| IL-8 | Sense | 5'-ATCAAGGCGCATGTGAACTC-3' | 140 |
| IL-8 | Antisense | 5'-AGAGCCCCAGATCCGATTTT-3' | |
| 18S rRNA | Sense | 5'-CGGCTACCACATCCAAGGAA-3' | 178 |
| 18S rRNA | Antisense | 5'-GCTGGAATTACCGCGGCT-3' | |

TNF- α , tumor necrosis factor- α .