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Effect of Staurosporine on Transcription Factor NF-κB in Human Keratinocytes

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ABSTRACT. Activation of the transcription factor NF-κB is known to be important in the regulated expression of a large number of pro-inflammatory genes including interleukin-8 (IL-8). Previously, we showed that the protein kinase inhibitor staurosporine potentiates IL-1-stimulated IL-8 production in human keratinocytes. Moreover, recent studies by other investigators demonstrated that staurosporine treatment alone results in a concentration-dependent increase in IL-8 mRNA and protein production. Therefore, in order to understand the mechanism underlying this observation, the effect of staurosporine on the activation of NF-κB was investigated. Electrophoretic mobility shift assays using an oligonucleotide containing the NF-κB consensus motif demonstrated that staurosporine treatment resulted in the activation of NF-κB by 15 min post-treatment. The ability of staurosporine to activate NF-κB was investigated further, using luciferase reporters under the control of the HIV-LTR or IL-8 core promoter transfected into human U937 cells. Stimulation with staurosporine resulted in a concentration-dependent induction of luciferase activity. In contrast, the very selective protein kinase C inhibitor 3-[8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido-[1,2-*a*]indol-10-yl]-4-(1-methyl-3-indolyl)-1*H*-pyrrole-2,5-dione hydrochloride (Ro32-0432) did not stimulate the activation of NF-κB, as measured in the luciferase reporter assay. The mechanism underlying NF-κB activation does not appear to involve the classical activation pathways in that staurosporine does not induce the disappearance of IκB family members. In conclusion, staurosporine appears to stimulate the activation of NF-κB in at least two cell types, and this effect appears to be independent of protein kinase C. BIOCHEM PHARMACOL **56**;1:71–78, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. NF-κB; interleukin-8; protein kinase C; keratinocytes; staurosporine; reporter genes

Staurosporine is a *Streptomyces* microbial alkaloid reported to inhibit PKC† *in vitro* with a K_i of 2.7 nM [1]. As such, staurosporine is widely used to study the role of PKC in a variety of cellular processes, although the compound is fairly nonselective for this enzyme versus others such as phosphorylase kinase [2]. In most instances, staurosporine potently inhibits cellular responses induced by PMA, a potent activator of PKC. However, several studies reveal staurosporine effects on cytokine-induced responses that are in direct contrast to its effects on phorbol ester-induced responses. Taylor and coworkers [3] reported that staurosporine enhances IL-1α-induced increases in fructose 2,6-bisphosphate and prostaglandin E production by subcultured rheumatoid synovial cells, whereas phorbol ester-induced increases in these products is inhibited by the drug. Furthermore, nanomolar concentrations of staurosporine strongly potentiate IL-1-induced production of IL-2 by EL4 murine thymoma cells but inhibit the production of this cytokine in response to phorbol ester stimulation [4, 5]. The protein kinase inhibitors auranofin and staurosporine have contrasting effects on cytokine- versus phorbol ester-stimulated IL-8 production in human keratinocyte cultures [6]. Whereas these compounds are potent inhibitors of phorbol ester-stimulated IL-8 production, both potentiate IL-8 synthesis induced by IL-1β. This effect appears to be mediated at the level of gene transcription in that both compounds potentiate the levels of IL-1β-induced IL-8 mRNA [6]. Thus, such observations have led to the speculation that staurosporine may exert its effects through a number of different mechanisms, depending on the cellular signal transduction pathway activated.

Although the mechanism underlying the ability of staurosporine to potentiate IL-1-induced responses is unclear, various studies suggest that staurosporine may induce certain cellular functions in the absence of additional stimulation. Staurosporine is reported to induce keratinocyte differentiation [7], neurite growth and differentiation [8, 9],

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† *Abbreviations:* DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; IL, interleukin; KGM, keratinocyte growth medium; PKC, protein kinase C; PMA, phorbol myristate acetate; RLU, relative light unit; RT–PCR, reverse transcriptase–polymerase chain reaction; and TNF, tumor necrosis factor.

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collagenase mRNA expression in normal and *ras*-oncogene-transformed rat fibroblasts [10], and the expression of urokinase-type plasminogen activator in a porcine epithelial cell line [11]. With respect to IL-8 production, staurosporine induces IL-8 mRNA levels with concomitant increases in IL-8 protein levels [12]. Similarly, preliminary results in our laboratory suggest that staurosporine induces the production of IL-8 by human keratinocytes.

The 5′-flanking region of the IL-8 gene contains several potential binding sites for known nuclear factors. Included among these is the transcription factor NF-κB [13, 14]. NF-κB belongs to a family of closely related dimeric transcription factor complexes composed of various combinations of the Rel/NF-κB family of polypeptides [for review, see Ref. 15]. The family consists of five individual gene products in mammals, RelA (p65), NF-κB1 (p50/p105), NF-κB2 (p49/p100), c-Rel, and RelB, all of which can form hetero- or homodimers. The activity of NF-κB is regulated by its interaction with a member of the inhibitor IκB family of proteins [15]. A wide variety of stimuli activate NF-κB through the phosphorylation and subsequent degradation of IκB. Once free from IκB, the active NF-κB complexes migrate to the nucleus where they bind in a selective manner to preferred gene-specific enhancer sequences such as those in the IL-8 promoter.

The potential regulation of NF-κB activity by staurosporine is suggested from studies in which HL-60 and EL4 cells show an increase in the levels of NF-κB activity induced by TNF or IL-1 in the presence of staurosporine compared with levels in its absence [5, 16]. Furthermore, similar amounts of NF-κB are activated after incubation with either TNFα or staurosporine in the EL4 cell line [5]. Therefore, the role of NF-κB in staurosporine-induced IL-8 production and its relation to PKC in human keratinocytes were investigated. The treatment of cultured human keratinocytes with staurosporine resulted in the production of IL-8, which is mediated at the level of gene transcription. Associated with staurosporine treatment was the activation of NF-κB in these cells. The effects of staurosporine on IL-8 production and NF-κB activation were independent of its effects on PKC in that the highly selective PKC inhibitor 3-[8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido-[1,2-*a*]indol-10-yl]-4-(1-methyl-3-indolyl)-1*H*-pyrrole-2,5-dione hydrochloride (Ro32-0432) was inactive. Thus, our findings suggest that staurosporine effects on gene transcription may be mediated through the PKC-independent activation of NF-κB.

MATERIALS AND METHODS

Materials

Staurosporine was obtained from the Sigma Chemical Co. The selective PKC inhibitor Ro32-0432 was synthesized by the Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, according to the reported procedure [17]. IL-1β and TNFα were prepared at SmithKline Beecham Pharmaceuticals as described previously [18, 19].

Cell Culture

Human foreskin keratinocytes were purchased from the Clonetics Corp. and grown as monolayers at 37°, 5% CO_2 in KGM (Clonetics Corp.). KGM is based on the MCDB 153 formulation supplemented with epidermal growth factor (0.1 ng/mL), hydrocortisone (0.5 μg/mL), insulin (5.0 μg/mL), gentamicin (50 μg/mL), amphotericin-B (50 ng/mL), and Bovine Pituitary Extract (30 μg/mL). Tertiary cultures were used upon reaching 80–90% confluency.

IL-8 Production from Human Keratinocytes In Vitro

To investigate the effect of staurosporine on IL-8 production, keratinocytes were incubated in KGM alone or with staurosporine. After 18 h of incubation, the supernatants were collected and stored frozen until assayed for IL-8 content. IL-8 in the culture supernatants was determined using an IL-8 immunoassay kit, purchased from R & D Systems, following the manufacturer's instructions.

Analysis of IL-8 mRNA Levels

The levels of IL-8 mRNA were determined using RT-PCR as follows. The keratinocyte cultures were stimulated with staurosporine (10 nM) in fresh medium and incubated at 37° for 0, 3, or 18 hr. Total RNA was extracted from the samples using TRIzol® reagent (Life Technologies, Inc.). All of the RNA samples were treated with Dnase (Deoxyribonuclease I, amplification grade, Life Technologies, Inc.) before use. The RT portion of the reaction was carried out using the Reverse Transcription System (Promega Corp.) and the DNA amplification with *Taq* DNA polymerase (Fisher Scientific) according to the manufacturer's instructions. A human IL-8 amplifier set yielding an approximately 300-bp product (Clontech Laboratories, Inc.) was used according to the manufacturer's instructions. Duplicate samples were run through the entire assay with no reverse transcriptase. The full-length cDNA for IL-8 (1 ng) (Clontech) was included in the PCR portion of the reaction. Aliquots (15 μL) of the reaction products were electrophoresed in 1.0% agarose gels in Tris acetate/EDTA buffer. The bands were visualized by ethidium bromide staining.

Preparation of Cellular and Nuclear Extracts

Human keratinocytes were cultured as described above to 80% confluence. The cells were harvested by trypsinization and centrifugation, washed in PBS without Ca^{2+} and Mg^{2+}, and resuspended in PBS with Ca^{2+} and Mg^{2+} at 1×10^7 cells/mL. To examine the effect of staurosporine on the activation of NF-κB, the cell suspensions were treated with various concentrations of drug or vehicle (DMSO, 0.1%) for various lengths of time at 37°. Cellular and nuclear extracts were prepared as previously described [20, 21].

Briefly, at the end of the incubation period, the cells (1×10^7 cells) were washed twice in PBS without Ca^{2+} and Mg^{2+}. The resulting cell pellets were resuspended in 20 μL of Buffer A [10 mM of HEPES (pH 7.9), 10 mM of KCl, 1.5 mM of $MgCl_2$, 0.5 mM of DTT and 0.1% NP-40] and incubated on ice for 10 min. The nuclei were pelleted by centrifugation in an Eppendorf microcentrifuge at 3500 rpm for 10 min at 4°. The resulting supernatant was collected as the cellular extract, and the nuclear pellet was resuspended in 15 μL of Buffer C [20 mM of HEPES (pH 7.9), 0.42 M of NaCl, 1.5 mM of $MgCl_2$, 25% glycerol, 0.2 mM of EDTA, 0.5 mM of DTT, and 0.5 mM of phenylmethylsulfonyl fluoride (PMSF)]. The suspensions were mixed gently for 20 min at 4° and then microcentrifuged at 14,000 rpm for 10 min at 4°. The supernatant was collected and diluted to 60 μL with Buffer D [20 mM of HEPES (pH 7.9), 50 mM of KCl, 20% glycerol, 0.2 mM of EDTA, 0.5 mM of DTT, and 0.5 mM of PMSF]. All samples were stored frozen at −80° until analyzed. The protein concentration of the extracts was determined according to the method of Bradford [22] with BioRad reagents.

EMSA

The effect of staurosporine on the activation of NF-κB was assessed in the electrophoretic mobility shift assay using nuclear extracts prepared from treated cells as described above. The double-stranded NF-κB consensus oligonucleotide (Santa Cruz Biotechnology) (5′-AGTTGAGGG GACTTTCCCAGGC-3′) was labeled with T_4 polynucleotide kinase and [γ-^{32}P]ATP. The binding mixture (25 μL) contained 10 mM of HEPES-NaOH (pH 7.9), 4 mM of Tris–HCl (pH 7.9), 60 mM of KCl, 1 mM of EDTA, 1 mM of DTT, 10% glycerol, 0.3 mg/mL of bovine serum albumin, and 1 μg of poly(dI-dC) · poly(dI-dC). The binding mixtures (10 μg of nuclear extract protein) were incubated for 20 min at room temperature with 0.5 ng of ^{32}P-labeled oligonucleotide (50,000–100,000 cpm) in the presence or absence of unlabeled competitor after which the mixture was loaded on to a 4% polyacrylamide gel prepared in 1× Tris borate/EDTA and electrophoresed at 200 V for 2 h. Following electrophoresis the gels were dried and exposed to film for detection of the binding reaction.

IκB Immunoblot Analysis

Cellular extracts were subjected to SDS–PAGE on 10% gels (BioRad) and the proteins were transferred to nitrocellulose sheets (Hybond™-ECL, Amersham Corp.). Immunoblot assays were performed using polyclonal rabbit antibodies directed against IκB-α, IκB-β, or IκB-γ (p105) (Santa Cruz Biotechnology, Inc.) at a 1:500 dilution for 1 h followed with a peroxidase-conjugated goat anti-rabbit secondary antibody. Immunoreactive bands were detected using the Enhanced Chemiluminescence (ECL) assay system (Amersham).

NF-κB-driven Luciferase Reporter Assay

U-937 cells that had been stably transfected with an NF-κB-driven luciferase reporter were centrifuged twice at 300 *g* for 5 min and resuspended in RPMI 1640 with 10% fetal bovine serum to a density of 1×10^6 cells/mL. Two reporter plasmids were investigated. The pHIVlucneo luciferase reporter plasmid contains two classical κB sites, whereas the pIL8lucneo reporter contains an alternative κB site. Aliquots (1 mL) of the transfected cells were treated with staurosporine or DMSO carrier (1 μL), and the samples were incubated for 5 h at 37°, 5% CO_2. The samples were transferred to 1.9-mL polypropylene tubes and centrifuged at 200 *g* for 5 min. The cell pellets were washed twice in 1 mL PBS without Ca^{2+} and Mg^{2+} and centrifuged as indicated above. The resulting cell pellets were lysed in 50 μL of 1× lysis buffer (Promega Corp.), vortexed, and incubated for 15 min at room temperature. A 20-μL aliquot of each lysate was transferred to an opaque white 96-well plate (Wallac Inc.), and assayed for luciferase production in a MicroLumat LB 96 P luminometer (EG&G Berthold). The luminometer dispensed 100 μL of luciferase assay reagent (Promega Corp.) into each well and recorded the integrated light output for 20 sec. Light output was measured in RLUs.

RESULTS

Stimulation of IL-8 Production in Human Keratinocytes by Staurosporine Treatment

Studies aimed at investigating the mechanism(s) whereby IL-8 synthesis may be regulated in human keratinocytes revealed that production of this cytokine in response to stimulation with IL-1β was potentiated in response to treatment of the cells with the PKC inhibitor staurosporine [6]. Therefore, this phenomenon was investigated further by treating human keratinocytes with staurosporine in the absence of other inducers, such as IL-1β. Cultured human keratinocytes responded to treatment with staurosporine with a concentration-dependent increase in the production of IL-8 (Fig. 1). Interestingly, the highly specific PKC inhibitor Ro32-0432 was inactive (Fig. 1). Ro32-0432, although somewhat less potent than staurosporine against rat brain PKC (IC_{50} = 42 vs 9 nM), is considerably more selective against a wide variety of serine/threonine and tyrosine kinases [16]. Thus, these findings suggest that the ability of staurosporine to stimulate IL-8 production is not mediated through its effects on PKC. The effect of staurosporine on IL-8 production was mediated at the level of gene transcription, since IL-8 mRNA levels are undetectable in unstimulated cells (Fig. 2). Treatment of the keratinocyte cultures with 10 nM of staurosporine resulted in the appearance of the IL-8 mRNA after 3 hr and its subsequent decline, such that IL-8 mRNA was no longer evident after 18 hr of stimulation (Fig. 2).

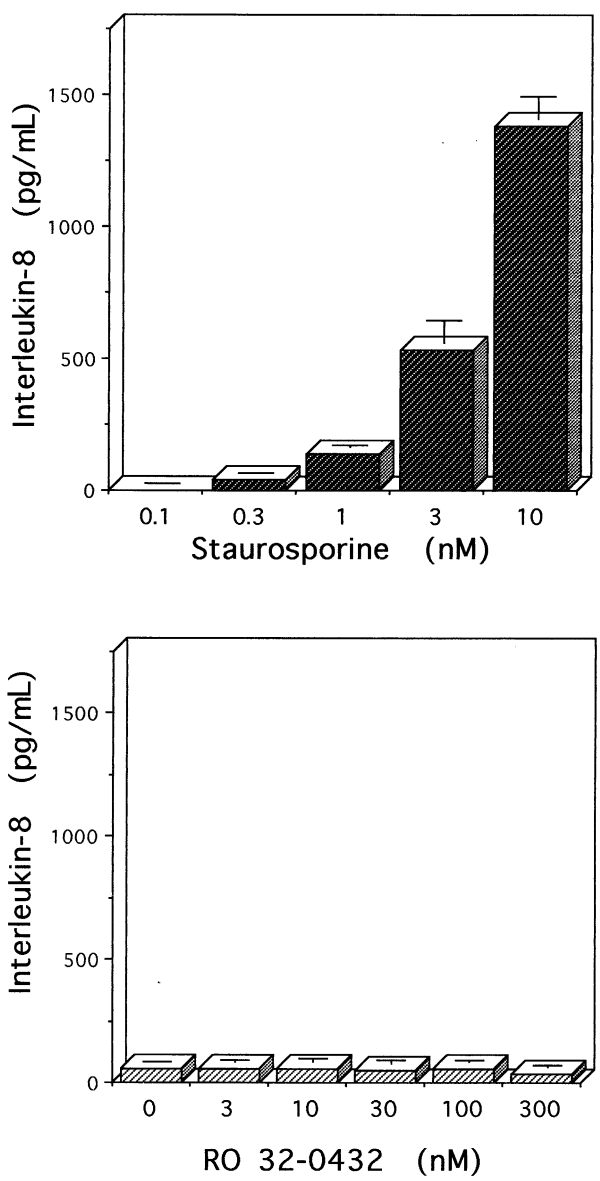

FIG. 1. IL-8 production in staurosporine-stimulated keratinocytes. Human keratinocytes *in vitro* were stimulated with various concentrations of staurosporine (top panel) or the selective PKC inhibitor Ro32-0432 (bottom panel), as described in Materials and Methods. After 18 hr of incubation, the supernatants were collected, and the IL-8 content was determined by immunoassay as described. Each point represents the mean ± SD of four individual samples from a representative of at least two experiments.

Staurosporine Activation of NF-κB in Human Keratinocytes

In light of the ability of staurosporine to induce the transcription and translation of the IL-8 gene, the effect of this compound on the activation of the transcription factor NF-κB was investigated. NF-κB has been shown to play a critical role in the regulated expression of IL-8. Therefore, it served as a likely target for staurosporine action. To address this question, human keratinocytes were harvested and stimulated with staurosporine for various lengths of time, after which nuclear extracts were prepared to monitor the activation of NF-κB. EMSAs revealed that resting keratinocytes contain significant levels of activated NF-κB complexes (Fig. 3, lane 1). Three separate bands, suggestive of three different NF-κB complexes, were apparent in the cells. As expected, stimulation with PMA (0.1 μM) resulted in a marked increase in protein complexes binding to the NF-κB consensus oligonucleotide within 30 min (Fig. 3, lane 2). Particularly striking was an increase in complex III in the PMA-stimulated cells. Similarly, stimulation of human keratinocytes with staurosporine resulted in a time-dependent increase in activated NF-κB complexes (band II and at later times band III) in the nuclear extracts evident

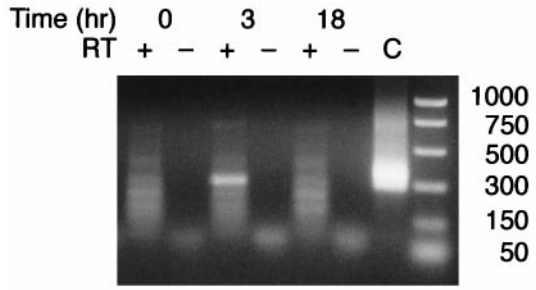

FIG. 2. Induction of IL-8 mRNA. Total RNA was prepared from human keratinocytes *in vitro* treated with 10 mM staurosporine for 0, 3, or 18 hr as described. RNA samples (1 μg) were subjected to RT–PCR using a human IL-8 amplimer set as described. Duplicate samples were run through the assay minus RT. Samples were electrophoresed in a 1.0% agarose gel and visualized by ethidium bromide staining.

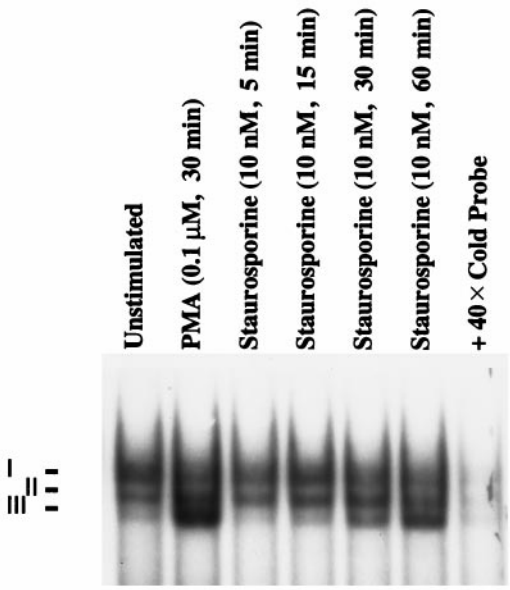

FIG. 3. NF-κB DNA binding activity in staurosporine-treated keratinocytes. Nuclear extracts were prepared from human keratinocytes *in vitro* before or after treatment with PMA (0.1 μM, 30 min) or staurosporine (10 nM) for the indicated times. The extracts (10 μg) were tested for binding activity to a ^{32}P-labeled NF-κB consensus oligonucleotide, as described in Materials and Methods.

within 15 min after stimulation (Fig. 3, lanes 3–6). The shifted complexes were specific for the NF-κB/Rel proteins in that they were competed almost completely in the presence of 40-fold excess unlabeled probe (Fig. 3, lane 7). In addition, supershift analysis of the staurosporine-activated complexes revealed that complex I was shifted completely with an antibody directed against p65 (Rel A), whereas anti-p50 shifted complex III (data not shown). The activation of NF-κB in response to staurosporine stimulation displayed a bell-shaped concentration dependence (data not shown), mirroring its effect on IL-8 production.

Effect of Staurosporine on NF-κB Activity Measured in Luciferase Reporter Assay

EMSA of nuclear extracts from staurosporine-treated human keratinocytes indicated that the transcription factor NF-κB was activated in these cells. Therefore, the ability of staurosporine to activate NF-κB-driven gene transcription was investigated. To this end, human U937 cells that had been stably transfected with an NF-κB-driven luciferase reporter were stimulated with various concentrations of staurosporine. Consistent with the effects on the activation of NF-κB evident in the EMSAs, staurosporine treatment resulted in a concentration-dependent increase in luciferase activity (Fig. 4), which was maximal at 30 nM. Activation of NF-κB-driven gene transcription was evident using a luciferase reporter gene driven by either the HIV-LTR (consensus NF-κB motif, top panel) or the IL-8 core promoter (alternative κB motif, middle panel), suggesting that the activation was not promoter specific. As expected, the specific PKC inhibitor Ro32-0432 did not result in the activation of NF-κB as measured in the luciferase reporter assay (Fig. 4, bottom panel).

Effect of Staurosporine on the Disappearance of IκB-α, IκB-β, or p105(Iκβ-γ)

The activation of NF-κB in response to a variety of stimuli involves its dissociation from the inhibitor IκB, allowing the active NF-κB dimers to migrate to the nucleus. This activation is known to involve the phosphorylation of IκB on two N-terminal serines followed by degradation of the protein via the ubiquitin-proteasome pathway [15]. Thus, the effect of staurosporine on IκB family members was investigated. Western blot analysis of keratinocyte IκB before and after stimulation with staurosporine suggested that the activation of NF-κB in response to this stimulus was not mediated through an effect on the levels of the inhibitory IκB proteins (Fig. 5). Staurosporine stimulation failed to induce the detectable degradation of IκB-α, IκB-β, or p105(IκB-γ) within the time frame of the observed NF-κB activation. Thus, staurosporine does not appear to be stimulating NF-κB through a classical activation pathway.

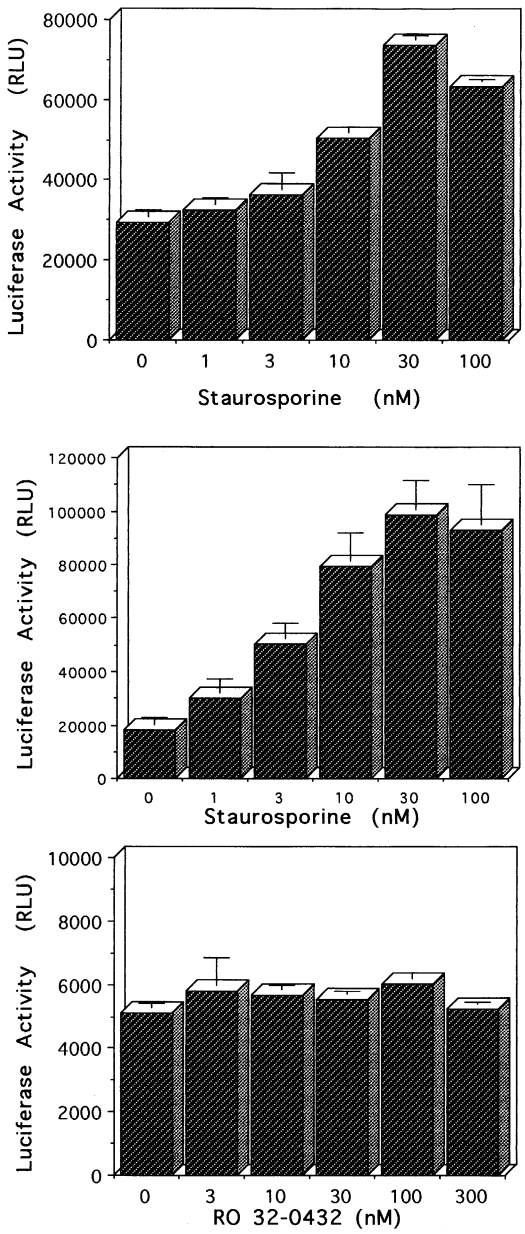

FIG. 4. NF-κB-driven reporter gene expression. U937/pHIV-lucneo (top panel) and U937/pIL8lucneo (middle and bottom panels) (1×10^6 cells/sample) were left untreated or stimulated with various concentrations of either staurosporine or Ro32-0432 for 5 hr as indicated. Cellular extracts were prepared and measured for luciferase activity. Each bar represents the mean ± SD of three determinations in a representative of at least two experiments.

DISCUSSION

The microbial alkaloid staurosporine is widely used as an inhibitor of PKC where it is proven to be a potent inhibitor of PMA-stimulated events, although staurosporine is known to be a fairly non-selective inhibitor [2]. Several recent studies report on the ability of staurosporine to potentiate a variety of cellular responses induced by the cytokines IL-1 and TNF [3–6]. Such observations have led investigators to speculate on the role of PKC in the signal

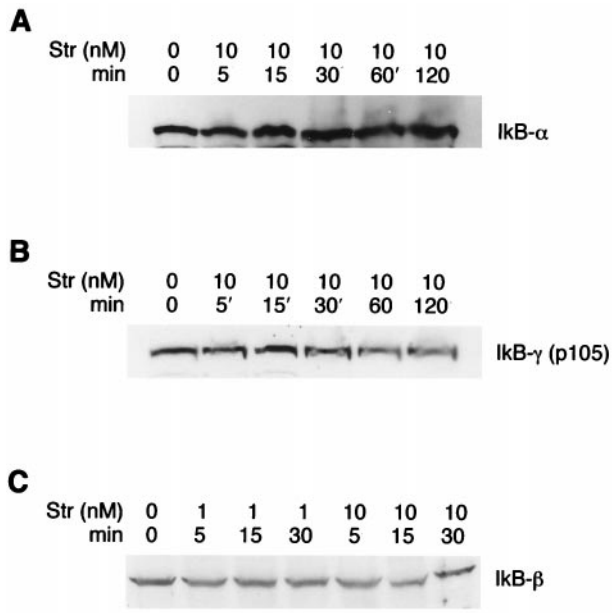

FIG. 5. Analysis of IκB family members in staurosporine-treated keratinocytes. Cellular extracts were prepared from human keratinocytes *in vitro* treated with staurosporine for various lengths of time as indicated above the panels. Samples (50 μg) were separated on a 10% SDS–PAGE gel, followed by electrophoretic transfer to a nitrocellulose membrane. The proteins were immunoblotted with anti IκBα (top panel), anti-IκBγ (middle), or anti-IκBβ (bottom panel) as shown, and the bands were visualized by ECL, as described in Materials and Methods.

transduction pathways induced by these cytokines versus PMA. Furthermore, several studies now indicate that staurosporine may induce a number of cellular events that are independent of PKC. Hedberg *et al.* [23] reported that staurosporine dramatically alters the actin microfilament cytoskeleton of a variety of cultured cells whether or not they are PKC deficient. In addition, the ability of staurosporine to induce keratinocyte differentiation and raise intracellular calcium is apparently independent of PKC in that the specific PKC inhibitor Ro31-8220 is inactive in these model systems [7]. Similarly, R031-8220 did not potentiate IL-2 production in IL-1-stimulated EL4 thymoma cells. In contrast, staurosporine not only potentiated IL-1-induced IL-2 production, but was able to stimulate IL-2 production independently [5]. As such, it has become evident that staurosporine is able to induce a variety of cellular functions independent of its effects on PKC.

Consistent with the findings related to IL-2 production in the murine EL4 thymoma system, we and others [6, 12] have shown that the production of the pro-inflammatory cytokine IL-8 is stimulated by staurosporine. Staurosporine was found to strongly potentiate IL-1-induced IL-8 production by human keratinocytes [6]. Furthermore, we now show that staurosporine is able to induce IL-8 production, independent of IL-1 co-stimulation in human keratinocytes. The studies described herein investigate the mechanism underlying the induction of IL-8 production by staurosporine in human keratinocytes. Consistent with the findings of Cassatella *et al.* [12] in human neutrophils, the induction of IL-8 in human keratinocytes is mediated at the level of gene transcription as is evidenced by the appearance of IL-8 mRNA in response to staurosporine stimulation. This effect is clearly independent of PKC in that the specific PKC inhibitor Ro32-0432 is unable to induce IL-8 production in these cells.

In light of the finding that staurosporine induction of IL-8 production is mediated at the level of gene transcription, a possible mechanism underlying this effect is the activation of transcription factors regulating the expression of the IL-8 gene. As discussed previously, the 5′-flanking region of the IL-8 gene contains several potential binding sites for known nuclear factors. Previous studies have identified a minimal region in the IL-8 promoter, between -94 and -71 bp from the start of the first exon, which is essential and sufficient for the induction of IL-8 by either IL-1, TNF, or PMA [13, 14]. This region is characterized by binding sites for the transcription factors NF-κB and C/EBPβ (NF-IL6), which have been shown to be essential for activation [14]. Of these, the studies of Kunsch and co-workers [24] would argue that the NF-κB binding site is more critical in that mutation of the NF-κB site completely abolishes inducible expression. In contrast, deletion of the NF-IL6 sites reduces inducible expression but does not eliminate it [24]. As such, we were interested in determining whether staurosporine-induced IL-8 production is mediated through an activation of the transcription factor NF-κB.

First identified in B cells as a protein that bound to a decameric oligonucleotide present in the κ-light chain gene intronic enhancer [25, 26], NF-κB transcription factor complexes have since been demonstrated to be present in an inactive form in the cytoplasm of all cells studied [27]. The activity of NF-κB is regulated by its interaction with a member of the inhibitor IκB family of proteins. This interaction effectively blocks the nuclear localization sequence on the NF-κB proteins, preventing migration of the dimer to the nucleus [28, 29]. A wide variety of stimuli activate NF-κB through what are likely to be multiple signal transduction pathways. Common to most stimuli, however, is the phosphorylation of IκB by a recently identified kinase complex [30–34] followed by its subsequent degradation. Once free from IκB, the active NF-κB complexes are able to translocate to the nucleus where they bind in a selective manner to preferred gene-specific enhancer sequences. Straurosporine was found to induce the activation of NF-κB in human keratinocytes as evidenced by both EMSAs using nuclear extracts from stimulated cells and in a U937 cell-based luciferase reporter assay. The ability of staurosporine to activate NF-κB is consistent with the findings of Hohmann *et al.* [16] in human promyelocytic HL-60 cells and with the recent report of the ability of staurosporine to activate NF-κB and increase the expression of an NF-κB-linked reporter gene in the EL4 thymoma cell system [5]. Interestingly, both the work of Mahon *et al.*

[5] and the studies described herein suggest that this activation is independent of PKC in that specific PKC inhibitors do not induce the activation of NF-κB.

Although we and others clearly [5, 16] demonstrate the activation of NF-κB in response to cell stimulation with staurosporine, the mechanism underlying this activation in unclear. As discussed above, the classical pathway leading to the activation of NF-κB involves the phosphorylation and subsequent degradation of IκB. Surprisingly, we were unable to detect a change in the cellular levels of IκBα, IκBβ, or IκBγ in response to staurosporine stimulation. This absence of IκB family degradation, concomitant with the activation of NF-κB binding and function, leads to the conclusion that staurosporine does not activate NF-κB through the classical activation pathway. However, one cannot rule out the possibility of a slight degradation of these proteins that is not detectable by Western analysis. Furthermore, the possibility that staurosporine activation of NF-κB is mediated through the breakdown of another IκB family member must be considered.

Recently, alternative pathways of NF-κB activation have been suggested, including pathways that do not involve the degradation of IκB. Imbert and coworkers [35] have reported that, in Jurkat T cells, tyrosine phosphorylation of IκBα activates NF-κB without proteolytic degradation of IκBα. Staurosporine has been reported to increase the activity of several protein kinases isolated from platelets [36]. Thus, the possibility exists that staurosporine induces the tyrosine phosphorylation of IκBα and the subsequent activation of NF-κB in the absence of IκB degradation in human keratinocytes. However, we have been unable to detect such an effect using phosphotyrosine immunoblot analysis of IκBα immunoprecipitated from staurosporine-stimulated cells (data not shown).

Although NF-κB is a key regulator in the inducible expression of the IL-8 gene, one cannot discount the contribution played by C/EBPβ (NF-IL6). Many studies clearly demonstrate the synergistic interaction of NF-κB and NF-IL6 in the regulated expression of a number of genes including IL-8 [24]. In addition, recent studies suggest that staurosporine may induce the expression of inducible nitric oxide synthase through an increase in the activity of C/EBPβ [37]. Clearly, a role for C/EBPβ in the activation of IL-8 expression by staurosporine in keratinocytes cannot be ruled out in the present studies. However, it is unlikely that the observed increase in IL-8 production is mediated solely through C/EBPβ in that staurosporine activated a luciferase reporter gene under the control of the HIV-LTR which lacks a C/EBPβ binding site, although it does contain a related motif [38, 39]. As such, staurosporine may exert its stimulatory effects by increasing the synergistic interaction between NF-κB and C/EBP family members. In conclusion, the studies described herein clearly demonstrate a role for the transcription factor NF-κB in the staurosporine-induced expression of IL-8 by human keratinocytes. The exact mechanism underlying the activation of NF-κB by staurosporine in these cells is unclear but may be related to the ability of the drug to increase the activity of novel kinases involved in this regulatory pathway and thereby modulate transcription factor activity.

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