

Jette L. Riis · Claus Johansen · Borbala Gesser
Kristine Møller · Christian G. Larsen · Knud Kragballe
Lars Iversen

1 α ,25(OH) $_2$ D $_3$ regulates NF- κ B DNA binding activity in cultured normal human keratinocytes through an increase in I κ B α expression

Received: 15 May 2004 / Revised: 5 August 2004 / Accepted: 11 August 2004 / Published online: 15 September 2004
© Springer-Verlag 2004

Abstract NF- κ B is a dimeric transcription factor which regulates transcription of a number of different genes including IL-8 and p53. In resting cells NF- κ B is usually retained in an inactive state in the cytoplasm through binding to a member of the inhibitory κ B (I κ B) protein family. The purpose of this study was to determine the effect of 1 α ,25(OH) $_2$ D $_3$ on NF- κ B activation in both unstimulated and stimulated (IL-1 α) cultured normal human keratinocytes. NF- κ B DNA binding activity was determined by EMSA using two different oligonucleotides containing the κ B sequence from either the IL-8 or the p53 promoter. I κ B α and p53 expression was determined by Western blotting and IL-8 expression by ELISA. In unstimulated keratinocytes no NF- κ B binding to the IL-8 κ B binding sequence was detectable, whereas stimulation with IL-1 α (10 ng/ml) led to a significant ($P < 0.05$) induction of NF- κ B binding. In contrast NF- κ B binding to the p53 κ B binding sequence was detectable in unstimulated cells, although it was significantly increased after IL-1 α (10 ng/ml) stimulation. Incubation with 1 α ,25(OH) $_2$ D $_3$ (10^{-8} – 10^{-7} M) was shown to significantly ($P < 0.05$) stimulate the expression of I κ B α and in parallel experiments with normal human keratinocytes stimulated with IL-1 α (10 ng/ml) a significant ($P < 0.05$) time and dose-dependent decrease in NF- κ B binding to the IL-8 κ B binding sequence and in IL-8 expression were seen. A less-pronounced decrease in NF- κ B binding to the p53 κ B response element was seen after preincubation with 1 α ,25(OH) $_2$ D $_3$ and IL-1 α stimulation, and it did not result in any change in p53 expression. These results demonstrate that 1 α ,25(OH) $_2$ D $_3$ inhibits NF- κ B binding to the IL-8 κ B binding sequence more potently than binding to the p53 κ B binding sequence. We propose that this selectivity

may be mediated through an increased expression of I κ B α which leads to an inhibition of specific NF- κ B subunits resulting in a selective regulation of NF- κ B-induced gene transcription.

Keywords NF- κ B · I κ B · 1 α ,25(OH) $_2$ D $_3$ · IL-8 · p53 · Keratinocytes

Introduction

Nuclear factor- κ B (NF- κ B) is a dimeric transcription factor formed by hetero- or homodimerization of the five Rel family proteins which comprise RelA (p65), RelB, cRel, p52 and p50 (Foo and Nolan 1999). NF- κ B is believed to play a pivotal role in immune and inflammatory responses and in the regulation of cell proliferation and apoptosis. It regulates the transcription of genes encoding proinflammatory cytokines (e.g. IL-1, IL-2, TNF α and GM-CSF), chemokines (e.g. IL-8 and RANTES), adhesion molecules (e.g. ICAM, VCAM and E-selectin), inducible enzymes (e.g. COX2 and iNOS) and also the MHC proteins important for the adaptive immune response (Baldwin 2001a). Furthermore, the gene coding for the tumor suppressor protein p53 as well as other regulators of apoptosis and cell proliferation (e.g. c-IAP-1, c-IAP-1 Bcl-X $_L$ and c-myc) are regulated by NF- κ B (Baldwin 2001b).

In resting cells NF- κ B is usually retained in an inactive state in the cytoplasm through binding to a member of the NF- κ B inhibitor protein family, inhibitory κ B (I κ B), of which I κ B α and I κ B β are the major inhibitors (Thompson et al. 1995). A wide variety of agonists including the proinflammatory cytokine IL-1 have been shown to activate NF- κ B. NF- κ B activation is mediated through activation of a specific I κ B kinase (IKK) and subsequent phosphorylation of I κ B molecules. Once phosphorylated, I κ B is targeted for ubiquitination and degradation by proteasomes allowing translocation of the NF- κ B dimer to the nucleus where it binds to specific

J. L. Riis · C. Johansen · B. Gesser · K. Møller · C. G. Larsen
K. Kragballe · L. Iversen (✉)
Department of Dermatology, Aarhus Sygehus,
Aarhus University Hospital, P.P. Orumsgade 11,
8000 Aarhus C, Denmark
E-mail: lars.iversen@ki.au.dk
Fax: +45-8949-1870

response elements in the promoter region of the various target genes (Rothwarf and Karin 1999). A 10-bp κ B response element serves as the recognition site for NF- κ B and the κ B response element differs from gene to gene (Yamamoto et al. 1992).

NF- κ B has recently been shown to play different roles in different phases of inflammation (Lawrence et al. 2001). During the onset of inflammation NF- κ B activation is associated with proinflammatory gene expression whereas NF- κ B activation during the resolution of inflammation is associated with the expression of anti-inflammatory genes and the induction of apoptosis.

IL-8 is a proinflammatory chemokine which activates and attracts neutrophils and T-lymphocytes from the blood into sites of infection and inflammation. IL-8 is produced by keratinocytes, fibroblasts, peripheral blood monocytes and endothelial cells by an inflammatory stimulus and is believed to play an important role in the development of inflammation (Larsen et al. 1991). Furthermore, IL-8 expression has been demonstrated to be markedly upregulated in psoriatic skin (Jiang et al. 2001). Regulation of IL-8 gene transcription occurs mainly through sequences -94 to -71 of the 5' flanking region of the IL-8 gene. This part of the IL-8 promoter region contains binding sites for the transcription factor NF- κ B (Harant et al. 1997) and the κ B binding site has been demonstrated to be essential for IL-1-induced IL-8 gene expression.

p53 is a regulator of cellular apoptosis and cell proliferation but does not exhibit any proinflammatory properties (Lotem and Sachs 1998). p53 also contains a κ B binding site in the promoter region and NF- κ B has been shown to regulate p53 gene expression (Lawrence et al. 2001). Interestingly, the κ B binding site in the p53 promoter region is different from the κ B binding site in the IL-8 promoter region (Pei et al. 1999; Kim et al. 2000).

An imbalance in NF- κ B signaling has previously been suggested to play an important, albeit controversial role, in the pathogenesis of psoriasis (Danning et al. 2000; McKenzie and Sabin 2003). Recently, we have shown that in involved psoriatic skin the expression of p53 is decreased compared to that in uninvolved psoriatic skin (own unpublished results) whereas it is well known that IL-8 expression is increased in involved psoriatic skin compared to that in uninvolved skin.

Vitamin D and different synthetic vitamin D analogues have been widely used in the treatment of psoriasis (Kragballe et al. 1991). The cellular actions of $1\alpha,25$ -dihydroxyvitamin D₃ ($1\alpha,25$ (OH)₂D₃), the bioactive form of vitamin D, are not fully understood, but its effects have traditionally been ascribed to its binding to the vitamin D receptor (VDR) (Haussler 1986). However, recently $1\alpha,25$ (OH)₂D₃ has also been demonstrated to activate other signal transduction pathways resulting in regulation of transcription factors such as activator protein 1 (AP-1) (Johansen et al. 2000, 2003). Furthermore, $1\alpha,25$ (OH)₂D₃ has been shown to inhibit IL-1 α -induced IL-8 synthesis in human keratinocytes in vitro

(Larsen et al. 1991) and in a melanoma cell line TNF α -induced IL-8 expression has been shown to be inhibited by $1\alpha,25$ (OH)₂D₃ through inhibition of NF- κ B DNA binding (Harant et al. 1997). Thus, the mechanism behind this modulation of NF- κ B activity has not been shown.

The purpose of the present study was to determine the effect of $1\alpha,25$ (OH)₂D₃ on NF- κ B activation in cultured normal human keratinocytes and to determine whether or not $1\alpha,25$ (OH)₂D₃ leads to a selective and differentiated regulation of NF- κ B/DNA binding activity to proinflammatory (IL-8) and proapoptotic (p53) genes.

Materials and methods

Materials

Keratinocyte growth medium, human recombinant epidermal growth factor, bovine pituitary extract, gentamicin and 8–16% Tris-glycine gels were obtained from Invitrogen (Carlsberg, Calif.). $1\alpha,25$ (OH)₂D₃ dissolved in 2-propanol was kindly provided by Leo Pharmaceuticals Products (Ballerup, Denmark). Gel shift binding 5 \times buffer, T4 polynucleotide kinase, T4 polynucleotide kinase 10 \times buffer and goat anti-mouse HRP-conjugated antibodies were obtained from Promega (Madison, Wis.). IL-8 NF- κ B response element (5'-AAT CGT GGA ATT TCC TCT GAC A-3') and p53 NF- κ B response element (5'-GGG ATT GGG GTT TTC CCC TCC C-3') were purchased from DNA Technology (Aarhus, Denmark). [γ -³²P]ATP, enhanced chemiluminescence detection system (ECL), microspin G-25 columns and nick spin columns Sephadex G-50 fine DNA-grade were obtained from Amersham/Pharmacia Biotech (Uppsala, Sweden). Complete medium was purchased from Boehringer Mannheim, Germany. Trypsin, EDTA, Tris-base, Tris-HCl, DTT, *O*-phenylenediamine dihydrochloride tablets and boric acid were obtained from Sigma Chemical Company (St. Louis, Mo.). The I κ B α , p53, p50 and p65 antibodies for Western blotting and supershifting experiments were from Santa Cruz Biotechnology (Santa Cruz, Calif.). The secondary antibody for Western blotting was purchased from DAKO, Denmark. IL-1 α was obtained from RD System (Abingdon, UK). The monoclonal recombinant human IL-8 (rIL-8) antibody for the IL-8 ELISA was kindly provided by Kouji Matsushima (Dainippon Pharmaceuticals, Osaka, Japan). Bio-Rad protein assay and acrylamide were purchased from Bio-Rad (Copenhagen, Denmark).

Cell cultures

Normal adult human keratinocytes were obtained by trypsinization of skin samples from patients undergoing plastic surgery as previously described (Kragballe et al.

1985). First-passage keratinocytes were grown in serum-free low-calcium (0.09 mM) keratinocyte growth medium supplemented with 5 ng/ml human recombinant epidermal growth factor, 50 µg/ml bovine pituitary extract, and 5 µg/ml gentamicin. Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂. At 70–90% confluence (judged by light microscopy) keratinocyte growth medium supplemented only with 5 µg/ml gentamicin was added for 24 h. 1 α ,25(OH)₂D₃ at various concentrations (10⁻⁷–10⁻¹¹ M final concentration) was added and cells were incubated from 10 min to 48 h. In separate experiments cells were further stimulated with IL-1 α for 15 min. The 1 α ,25(OH)₂D₃ stock solution was diluted in 2-propanol, IL-1 α was diluted in PBS, pH 7.0, and 0.15% of bovine serum albumin added. In all experiments, vehicle was added to controls. All incubations were carried out in duplicate.

Whole cell extracts

Keratinocytes were washed twice with ice-cold PBS, lysed with cold lysis buffer (50 mM Tris-HCl (pH 6.8), 10 mM DTT, 10 mM β -glycerophosphate, 10 mM NaF, 0.1 mM Na orthovanadate, 10% glycerol, 2.5% SDS, 25 mM PMSF and 50 \times complete), pelleted into tubes and boiled for 3 min. The lysates were centrifuged at 13,600 g for 3 min and the supernatants were assayed for protein concentration as previously described by Bradford (1976).

Nuclear and cytosolic extraction

Nuclear extracts were prepared as previously described (Johansen et al. 2000; Rosette and Karin 1995). Briefly a hypotonic buffer (10 mM HEPES, 10 mM KCl, 0.2 mM EDTA, 0.1 mM EGTA, 1 mM DTT, pH 7.9) containing 0.5% detergent was added to the cultured cells. Cells were pelleted into tubes. The suspension was passed through a 27-gauge needle six times and centrifuged at 13,600 g at 4°C for 1.5 min. The supernatant was considered the cytosolic extract. The pelleted nuclei were resuspended in a hypertonic buffer (20 mM HEPES, 0.4 mM NaCl, 1 mM EDTA and 1 mM DTT), rotated at 4°C for 30 min and centrifuged at 13,600 g at 4°C for 5 min. The supernatant was considered the nuclear extract. Protein concentration was determined as previously described by Bradford (1976) with bovine serum albumin as standard.

Labeling of consensus oligonucleotides for NF- κ B

NF- κ B consensus oligonucleotide (4 µl, 1.75 pmol/µl), 2 µl T4 polynucleotide kinase 10 \times buffer (700 mM Tris-HCl, 100 mM MgCl₂ and 50 mM DTT), 4 µl [γ -³²P]ATP (3000 Ci/mmol, 10 mCi/ml), 8 µl nuclease-free water and 2 µl T4 polynucleotide kinase were added

to an Eppendorf tube and incubated at 37°C for 10 min. To stop the reaction, 1 µl 0.5 M EDTA was added. Finally, 200 µl TE buffer (10 mM Tris-HCl, pH 8, and 1 mM EDTA) was added. The suspension was centrifuged at 300 g for 5 min on a Nick spin column in order to remove the unlabelled oligonucleotides.

Electrophoretic mobility shift assay

Nuclear protein (3–8 µg) was preincubated for 10 min at room temperature in binding buffer (5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), 20% glycerol, 0.25 mg/ml poly(dI-dC)) and hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 \times protease inhibitors, and 0.5 mM PMSF). ³²P-labelled NF- κ B probe (1 µl) was added and the suspension was incubated for an additional 20 min before loading on a 4% polyacrylamide gel in 0.5 \times TBE buffer (22.5 mM Tris-base, 4 mM boric acid, and 1 mM EDTA). In control experiments a specific competitor (unlabelled NF- κ B oligo) or a nonspecific competitor (unlabelled SP-1 oligo) was added 10 min before addition of labeled NF- κ B oligo. Supershifting experiments with p50 and p65 antibodies were also carried out in order to confirm the identity of NF- κ B in the various experiments with an electrophoretic mobility shift assay (Fig. 1). The gels were dried and exposed to X-ray film at -80°C.

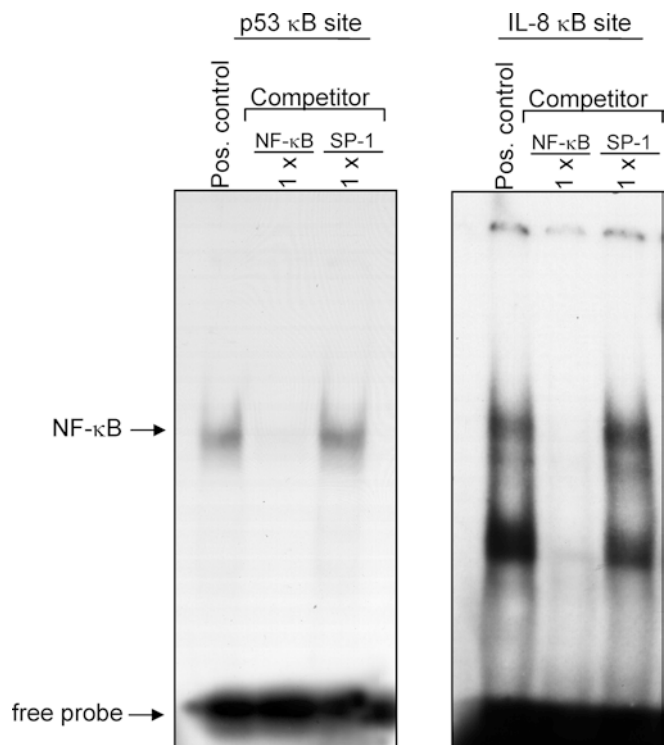


Fig. 1 Control experiments using a specific competitor (unlabelled NF- κ B oligo) and a nonspecific competitor (unlabelled SP-1 oligo)

Western blotting

Cytosolic protein (20 μg), freeze-dried at -50°C was added to 20 μl loading buffer (100 μl Tris-glycine SDS sample buffer, 20 μl 0.5 M DTT, 80 μl dH₂O). The suspension was boiled, cooled and centrifuged then separated by SDS-PAGE (8–16% Tris-glycine) and blotted onto nitrocellulose membranes. The gels were stained and dried and the membranes incubated overnight at 4°C in skimmed milk powder diluted in TBS (20 mM Tris-base, pH 7.6, 135 mM NaCl). Primary antibody (I κ B α , 1:1000) in TBS was added and incubated for 1.5 h at room temperature. Secondary antibody (rabbit immunoglobulins/HRP, swine; 1:1000) in TBS was added and the mixture incubated for 1 h at room temperature. The membrane was washed in TBST (TBS + 0.05% Tween-20), and the reaction revealed by the ECL method.

IL-8 ELISA

A 96-well plate was coated with monoclonal IL-8 antibody in coating buffer, 1.5 $\mu\text{g}/\text{ml}$, and kept overnight at 4°C . After washing in PBS the plate was blocked for 1 h at 20°C before the samples and the standards were added and left overnight at 4°C . Then the secondary antibody was added for 1 h at 20°C , followed by the addition of peroxidase-labeled antibody diluted in assay buffer for 1 h at 20°C . After washing, the reaction was developed with *O*-phenylenediamine, and 30 min later the reaction was stopped by adding 1.6 N H₂SO₄. Optical density (OD) was measured in an ELISA reader adjusted to 490 nm.

Statistics

Results are expressed as means \pm SD. Statistical significance ($P < 0.05$) was assessed by Student's *t*-test. To test for normal distribution, a probability test was carried out.

Results

Effects of $1\alpha,25(\text{OH})_2\text{D}_3$ on NF- κ B DNA binding activity in cultured normal human keratinocytes

Studies were performed to determine the effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on NF- κ B DNA binding activity to the p53 and the IL-8 κ B response element in cultured normal human keratinocytes. Because the keratinocytes were not stimulated, only the basal NF- κ B DNA binding activity was measured. When human keratinocytes were incubated with $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-7} M) a time-dependent decrease in NF- κ B DNA binding activity to the p53 κ B response element was seen (Fig. 2a). It became significant after 6 h and reached a maximal

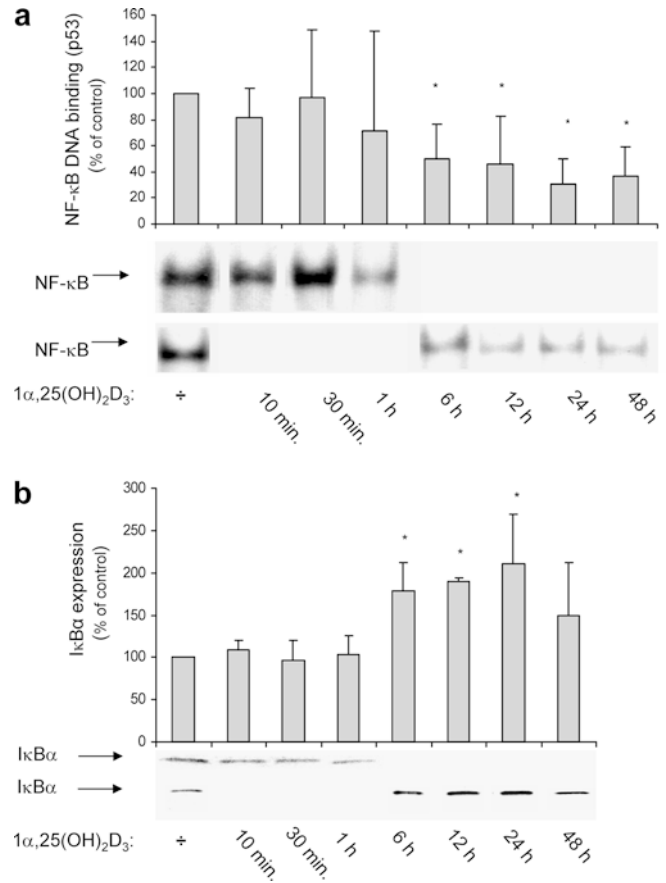


Fig. 2a, b Human keratinocytes were incubated with $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-7} M) for the indicated times. Control represents keratinocytes cultured with vehicle only. NF- κ B DNA binding activity was determined by EMSA using a synthetic p53 NF- κ B target sequence. **a** The ^{32}P -bands were analyzed densitometrically. NF- κ B DNA binding activity is expressed as percentage of control. Each bar represents the mean \pm SD from four separate experiments assayed in duplicate. **b** I κ B α expression was determined by Western blotting using a specific antibody. The intensity of the bands was analyzed densitometrically. I κ B α expression is expressed as percentage of control. Each bar represents the mean \pm SD from at least four separate experiments assayed in duplicate. Equal loading was confirmed by incubation with an anti-TFIIB antibody ($*P < 0.05$). Due to the number of lanes the results shown are the combined results from two different gels. A control lane was included on each gel.

inhibition of 69% compared to controls after 24 h (Fig. 2a). No detectable NF- κ B binding activity to the IL-8 κ B response element was found in cultured normal human keratinocytes incubated with either vehicle or $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-7} M) from 10 min to 48 h (data not shown).

The expression of I κ B α was determined by Western blotting and a significant time-dependent increase in the expression of I κ B α was seen when human keratinocytes were incubated with $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-7} M) (Fig. 2b). This increase became significant after 6 h and reached a maximum after 24 h of incubation. At 6, 12 and 24 h of incubation the levels of I κ B α were 178%, 189% and 211%, respectively, compared to control levels (Fig. 2b).

Human keratinocytes were also incubated with different concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-11} – 10^{-7} M final concentrations) for 12 h and a dose-dependent decrease in NF- κ B binding activity to the p53 κ B response element was seen (Fig. 3a). These findings were paralleled by a similar dose-dependent increase in cytoplasmic I κ B α expression (Fig. 3b) which became significant at a concentration of 10^{-8} M $1\alpha,25(\text{OH})_2\text{D}_3$.

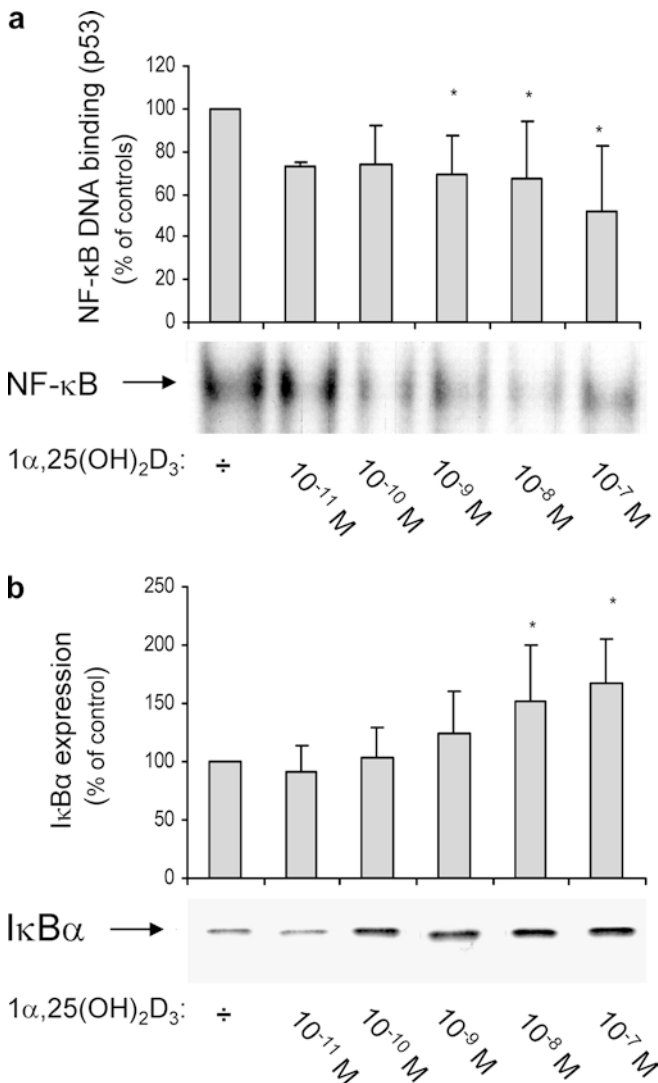


Fig. 3a, b Human keratinocytes were incubated with $1\alpha,25(\text{OH})_2\text{D}_3$ at different concentrations for 12 h. Control represents keratinocytes incubated with vehicle only. NF- κ B DNA binding activity was determined by EMSA using a synthetic p53 NF- κ B target sequence. **a** The ^{32}P -bands were analyzed densitometrically. NF- κ B DNA binding activity is expressed as percentage of control. Each bar represents the mean \pm SD from five separate experiments assayed in duplicate. **b** I κ B α expression was determined by Western blotting using a specific antibody. The intensity of the bands was analyzed densitometrically. I κ B α expression is expressed as percentage of control. Each bar represents the mean \pm SD from at least five separate experiments assayed in duplicate. Equal loading was confirmed by incubation with an anti-TFIIB antibody (* P < 0.05)

$1\alpha,25(\text{OH})_2\text{D}_3$ modulates IL-1 α -induced NF- κ B DNA binding activity in cultured normal human keratinocytes

When cultured normal human keratinocytes were stimulated with IL-1 α (10 ng/ml) for 15 min a significant increase in NF- κ B DNA binding activity to both the p53 and the IL-8 κ B response element was seen compared to stimulation with vehicle only (Fig. 4a).

In order to determine whether $1\alpha,25(\text{OH})_2\text{D}_3$ could modulate the IL-1 α -induced increase in NF- κ B DNA binding activity normal human keratinocytes were preincubated with $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-8} and 10^{-7} M) for 12 or 24 h before stimulation with IL-1 α (10 ng/ml) for 15 min. NF- κ B DNA binding activity to the p53 κ B response element showed a slight but significant (P < 0.05) decrease in the NF- κ B DNA binding activity when the keratinocytes were preincubated with $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-7} M) for 12 and 24 h before stimulation with IL-1 α (Fig. 4b). Preincubation for 12 and 24 h resulted in a 50% and a 25% decrease, respectively, compared to controls. Preincubation with $1\alpha,25(\text{OH})_2\text{D}_3$ at 10^{-8} M resulted in only insignificant changes. When NF- κ B DNA binding activity to the IL-8 promoter region was determined (Fig. 4c) a significant decrease was seen after 24 h of preincubation with $1\alpha,25(\text{OH})_2\text{D}_3$ at both 10^{-7} and 10^{-8} M . The mean levels of inhibition at 10^{-7} and 10^{-8} M were 72% and 65%, respectively. Only insignificant changes were seen when keratinocytes were preincubated with $1\alpha,25(\text{OH})_2\text{D}_3$ for 12 h.

In order to determine whether the $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated downregulation in IL-1 α -induced NF- κ B DNA binding activity was accompanied by changes in I κ B α levels, the expression of I κ B α was determined in the above-mentioned IL-1 α -stimulated cell samples. Preincubation for 24 h with $1\alpha,25(\text{OH})_2\text{D}_3$ at 10^{-7} M resulted in a 50% increase in the I κ B α level after IL-1 α stimulation as compared to controls (P < 0.05; Fig. 5). Preincubation for 12 h with 10^{-7} M $1\alpha,25(\text{OH})_2\text{D}_3$ and preincubation with 10^{-8} M $1\alpha,25(\text{OH})_2\text{D}_3$ for both 12 and 24 h before stimulation with IL-1 α led only to insignificant increases in the I κ B α levels when compared to controls. The P -values were 0.057, 0.076 and 0.116, respectively.

$1\alpha,25(\text{OH})_2\text{D}_3$ regulates IL-1 α -induced IL-8 expression in cultured normal human keratinocytes

p53 and IL-8 expression were also measured in cultured normal human keratinocytes in order to determine whether the $1\alpha,25(\text{OH})_2\text{D}_3$ -modulated changes in IL-1 α -induced NF- κ B DNA binding activity were reflected by similar changes in protein expression. p53 expression was determined by Western blotting and IL-8 expression was determined by ELISA.

Cultured keratinocytes were preincubated with $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-7} M) for various times (6–48 h) before stimulation with IL-1 α , and then 12 h later protein expression was measured. Interestingly, no significant

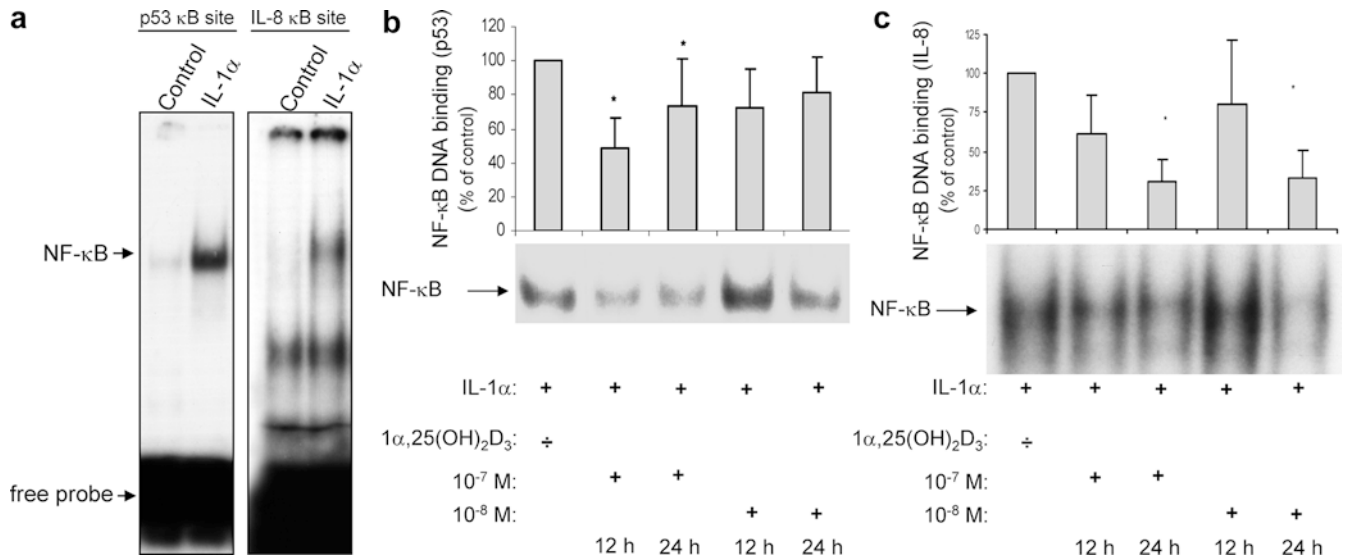


Fig. 4a-c Human keratinocytes stimulated with or without IL-1 (10 ng/ml) for 15 min in order to demonstrate increased NF-κB DNA binding to both the p53 and the IL-8 NF-κB target sequence (a). Human keratinocytes were also incubated with 1α,25(OH)₂D₃ (10⁻⁷ or 10⁻⁸ M) for 12 or 24 h before stimulation with IL-1α (10 ng/ml) for 15 min. NF-κB DNA binding activity was assayed by EMSA using a synthetic p53 NF-κB target sequence (b) or a synthetic IL-8 NF-κB target sequence (c). The ³²P-bands were analyzed densitometrically. NF-κB DNA binding activity is expressed as percentage of control. Control represents keratinocytes incubated with IL-1α only. Each bar represents the mean ± SD from three separate experiments (**P* < 0.05)

changes in p53 expression were seen after preincubation with 1α,25(OH)₂D₃ (10⁻⁷ M) from 6 to 48 h before stimulation with IL-1α (Fig. 6). In contrast a significant

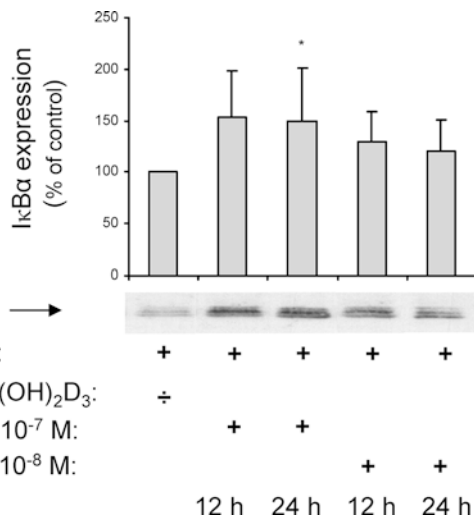


Fig. 5 Keratinocytes were incubated with 1α,25(OH)₂D₃ (10⁻⁷ or 10⁻⁸ M) for 12 or 24 h followed by stimulation with IL-1α (10 ng/ml) for 15 min. Then the cytosolic level of IκBα was determined by Western blotting. The bands were analyzed densitometrically. The cytosolic level of IκBα is expressed as percentage of controls. Control represents keratinocytes incubated with IL-1α only. Each bar represents the mean ± SD from five separate experiments assayed in duplicate. Equal loading was confirmed by incubation with an anti-TFIIB antibody (**P* < 0.05)

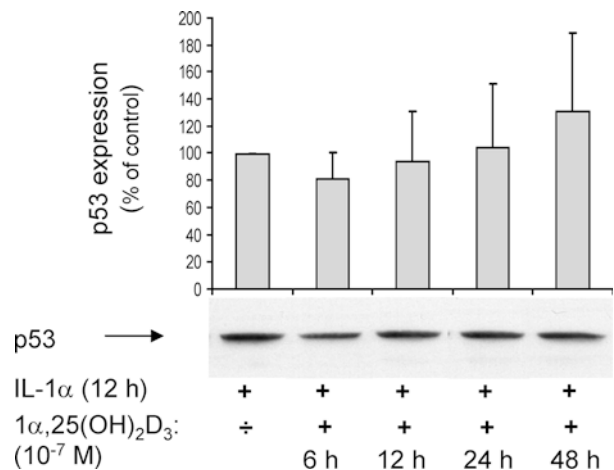


Fig. 6 Whole cell extracts from human keratinocytes preincubated with 1α,25(OH)₂D₃ (10⁻⁷ M) or vehicle for the indicated times and stimulated with IL-1α (10 ng/ml) for 12 h were isolated and the p53 expression levels determined by Western blotting. The bands were analyzed densitometrically. Control represents keratinocytes stimulated with IL-1α only. The expression levels of p53 are expressed as a percentage of control. Each bar represents the mean ± SD from five separate experiments assayed in duplicate. Equal loading was confirmed by incubation with an anti-TFIIB antibody

decrease in IL-8 expression was seen after preincubation with 1α,25(OH)₂D₃ (10⁻⁷ M) from 6 to 48 h before stimulation (Fig. 7). After 24 and 48 h of preincubation a 47% and 52% inhibition of IL-8 expression were seen, respectively.

Discussion

In this study we found a difference in NF-κB binding activity to the κB sequence from the IL-8 and the p53 promoter in normal human keratinocytes in vitro. In unstimulated keratinocytes we could not detect any NF-κB binding to the IL-8 κB binding sequence whereas stimulation with IL-1α led to a significant induction of

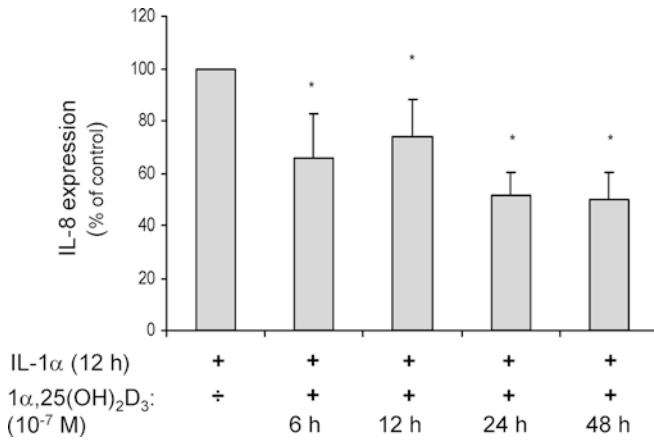


Fig. 7 Normal human keratinocytes were incubated with $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-7} M) or vehicle for the indicated times and stimulated with IL-1 α (10 ng/ml) for 12 h. ELISA was used to determine the expression levels of IL-8. IL-8 expression is expressed as percentage of control. Control represents keratinocytes stimulated with IL-1 α only. Each bar represents the mean \pm SD from five separate experiments assayed in duplicate (* $P < 0.05$)

NF- κ B binding. NF- κ B binding to the p53 κ B binding sequence was detectable even in unstimulated cells, but it was significantly increased after IL-1 α stimulation. Furthermore, $1\alpha,25(\text{OH})_2\text{D}_3$ stimulated the expression of I κ B α . In parallel with this increase in I κ B α expression, we found a time- and dose-dependent decrease in NF- κ B binding to the IL-8 κ B binding sequence and in IL-8 expression after preincubation of normal human keratinocytes with $1\alpha,25(\text{OH})_2\text{D}_3$ and stimulation with IL-1 α . A less-pronounced decrease in NF- κ B binding to the p53 κ B response element was seen after preincubation with $1\alpha,25(\text{OH})_2\text{D}_3$ and stimulation with IL-1 α , and it did not result in any changes in p53 expression.

The inhibitory effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on NF- κ B activation has previously been demonstrated in different cell types such as keratinocytes (Komine et al. 1999), lymphocytes (Komine et al. 1999) and fibroblasts (Harant et al. 1998), but the mechanism involved has not been fully elucidated. Harant et al. (1998) have shown that $1\alpha,25(\text{OH})_2\text{D}_3$ inhibits NF- κ B DNA binding without affecting the translocation of NF- κ B to the nucleus. Komine et al. (1999) only observed a $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated decrease in NF- κ B controlled luciferase activity in keratinocytes but did not study the mechanism. To our knowledge, our results are the first to demonstrate a $1\alpha,25(\text{OH})_2\text{D}_3$ -induced specific and selective regulation of NF- κ B binding to the p53 and IL-8 κ B response element as well as a specific regulation of the expression of IL-8 and p53.

The observed $1\alpha,25(\text{OH})_2\text{D}_3$ -induced selective regulation of NF- κ B DNA binding activity can be explained by a specific modulation of some of the NF- κ B subunits. p65 has been shown by other groups to interact with a subset of κ B DNA sequences not recognized by p50 and vice versa (Kunsch et al. 1992) and interestingly the IL-8 κ B response element used in their study was shown to

bind p65 (Kunsch et al. 1992). This is in accordance with the findings of another study showing that p65 but not p50 is important for the NF- κ B-induced IL-8 gene transcription (Kunsch and Rosen 1993) whereas the NF- κ B-dependent regulation of p53 expression has been shown to be mediated through the p50/p50 homodimer (Wu and Lozano 1994; Lawrence et al. 2001). I κ B α is also selective in its mode of action. It predominantly binds to p65 with only weak binding to p50 (Phelps et al. 2000). It is therefore possible that the $1\alpha,25(\text{OH})_2\text{D}_3$ -induced increase in I κ B α expression leads to a selective inhibition of p65 resulting in the observed decrease in NF- κ B binding to the IL-8 κ B response element and the subsequent decrease in IL-8 expression. The decrease in NF- κ B binding to the p53 κ B response element may be due a decrease in other NF- κ B dimers than the p50/p50 homodimer, e.g. the p50/p65 heterodimer which is inhibited by the increased expression of I κ B α . However, because p53 expression is regulated by the p50/p50 homodimer this decrease in NF- κ B DNA binding did not result in any changes in p53 expression.

One intriguing hypothesis may therefore be that $1\alpha,25(\text{OH})_2\text{D}_3$ mediates its immunomodulatory and antiinflammatory effects by selectively inhibiting NF- κ B-mediated transcription of proinflammatory genes without affecting NF- κ B-induced transcription of genes such as p53.

An inhibition of NF- κ B activation has been suggested as part of the mechanism of action of several antiinflammatory drugs. $1\alpha,25(\text{OH})_2\text{D}_3$ exerts some of its effects through binding to the VDR. The VDR belongs to the nuclear receptor superfamily which includes more than 60 different nuclear receptors including the glucocorticoid receptor (GR) (Carlberg and Polly 1999). Interestingly, inhibition of NF- κ B activation has been suggested as part of the immunomodulatory effect of the glucocorticoids (Scheinman et al. 1995; Auphan et al. 1995; De Bosscher et al. 1997), and it has been shown that glucocorticoids increases I κ B α transcription leading to enhanced expression of cytosolic I κ B α (Scheinman et al. 1995; Auphan et al. 1995). This is similar to what was demonstrated with $1\alpha,25(\text{OH})_2\text{D}_3$ in this study.

IL-1 α was chosen to stimulate the keratinocytes because it is an important proinflammatory cytokine in the skin and it is expressed in keratinocytes (Wood et al. 1996). Furthermore, IL-1 α has been suggested to play a key role in hyperproliferative and inflammatory skin diseases (Haase et al. 2001) and it is known to contribute to the activation of NF- κ B (Wolf et al. 2001).

In the present study, we demonstrated that $1\alpha,25(\text{OH})_2\text{D}_3$ inhibits NF- κ B binding to the IL-8 κ B binding sequence more potently than binding to the p53 κ B binding sequence which leads to a significant decrease in IL-8 expression, whereas no significant changes in p53 expression were seen. We therefore propose that this selectivity at least in part may be mediated through an increased expression of I κ B α . Increased expression of I κ B α and selective inhibition of the expression of NF- κ B-regulated proinflammatory cytokines and chemo-

kines may be one of the mechanisms by which vitamin D analogues exert their immunomodulatory and beneficial effects in the treatment of psoriasis.

Acknowledgements This work was supported by the Danish Medical Research Council.

References

- Auphan N, DiDonato JA, Rosette C, Helmsberg A, Karin M (1995) Immunosuppression by glucocorticoids: inhibition of NF- κ B activity through induction of I κ B synthesis. *Science* 270:286–290
- Baldwin AS (2001a) Control of oncogenesis and cancer therapy resistance by the transcription factor NF- κ B. *J Clin Invest* 107:241–246
- Baldwin AS (2001b) The transcription factor NF- κ B and human disease. *J Clin Invest* 107:3–6
- Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Carlberg C, Polly P (1999) The nuclear receptor superfamily. *Retinoids* 15:71–74
- Danning CL, Illie GG, Hitchon C, Greer MR, Boumpas DT, McInnes IB (2000) Macrophage-derived cytokine and nuclear factor κ B p65 expression in synovial membrane and skin of patients with psoriatic arthritis. *Arthritis Rheum* 43:1244–1256
- De Bosscher K, Schmitz ML, Vanden Berghe W, Plaisance S, Fiers W, Haegeman G (1997) Glucocorticoid-mediated repression of nuclear factor- κ B-dependent transcription involves direct interference with transactivation. *Proc Natl Acad Sci U S A* 94:13504–13509
- Foo SY, Nolan GP (1999) NF- κ B to the rescue: RELs, apoptosis and cellular transformation. *Trends Genet* 15:229–235
- Haase I, Hobbs RM, Romero MR, Broad S, Watt FM (2001) A role for mitogen-activated protein kinase activation by integrins in the pathogenesis of psoriasis. *J Clin Invest* 108:527–536
- Harant H, Andrew PJ, Reddy GS, Foglar E, Lindley IJ (1997) 1 α ,25-dihydroxyvitamin D₃ and a variety of its natural metabolites transcriptionally repress nuclear-factor- κ B-mediated interleukin-8 gene expression. *Eur J Biochem* 250:63–71
- Harant H, Wolff B, Lindley IJ (1998) 1 α ,25-dihydroxyvitamin D₃ decreases DNA binding of nuclear factor- κ B in human fibroblasts. *FEBS Lett* 436:329–334
- Haussler MR (1986) Vitamin D receptors: nature and function. *Annu Rev Nutr* 6:527–562
- Jiang WY, Chattedee AD, Raychaudhuri SP, Raychaudhuri SK, Farber EM (2001) Mast cell density and IL-8 expression in nonlesional and lesional psoriatic skin. *Int J Dermatol* 40:699–703
- Johansen C, Iversen L, Ryborg A, Kragballe K (2000) 1 α ,25-Dihydroxyvitamin D₃ induced differentiation of cultured human keratinocytes is accompanied by a PKC-independent regulation of AP-1 DNA binding activity. *J Invest Dermatol* 114:1174–1179
- Johansen C, Kragballe K, Henningsen J, Westergaard M, Kristiansen K, Iversen L (2003) 1 α ,25(OH)₂D₃ stimulates AP-1 DNA binding activity by a PI3-kinase/Ras/MEK/ERK1/2 and JNK1 dependent increase in c-Fos, Fra1 and cJun expression in human keratinocytes. *J Invest Dermatol* 120:561–570
- Kim J, Sanders SP, Siekierski ES, Casolaro V, Proud D (2000) Role of NF- κ B in cytokine production induced from human airway epithelial cells by rhinovirus infection. *J Immunol* 165:3384–3392
- Komine M, Watabe Y, Shimaoka S, Sato F, Kake K, Nishina H, Ohtsuki M, Nakagawa H, Tamaki K (1999) The action of a novel vitamin D₃ analogue, OCT, on immunomodulatory function of keratinocytes and lymphocytes. *Arch Dermatol Res* 291:500–506
- Kragballe K, Desjarlais L, Marcelo CL (1985) Increased DNA synthesis of uninvolved psoriatic epidermis is maintained in vitro. *Br J Dermatol* 112:263–270
- Kragballe K, Gjertsen BT, De Hoop D, Karlsmark T, van de Kerkhof PC, Larko O, Nieboer C, Roed-Petersen J, Strand A, Tikjob G (1991) Double-blind, right/left comparison of calcipotriol and betamethasone valerate in treatment of psoriasis vulgaris. *Lancet* 337:193–196
- Kunsch C, Rosen CA (1993) NF- κ B subunit-specific regulation of the interleukin-8 promoter. *Mol Cell Biol* 13:6137–6146
- Kunsch C, Ruben SM, Rosen CA (1992) Selection of optimal κ B/Rel DNA-binding motifs: interaction of both subunits of NF- κ B with DNA is required for transcriptional activation. *Mol Cell Biol* 12:4412–4421
- Larsen CG, Kristensen M, Paludan K, Deleuran B, Thomsen MK, Zachariae C, Kragballe K, Matsushima K, Thestrup-Pedersen K (1991) 1,25(OH)₂-D₃ is a potent regulator of interleukin-1 induced interleukin-8 expression and production. *Biochem Biophys Res Commun* 176:1020–1026
- Lawrence T, Gilroy DW, Colville-Nash PR, Willoughby DA (2001) Possible new role for NF- κ B in the resolution of inflammation. *Nat Med* 7:1291–1297
- Lotem J, Sachs L (1998) Cytokine suppression of protease activation in wild-type p53-dependent and p53-independent apoptosis. *Proc Natl Acad Sci U S A* 95:1330–1337
- McKenzie RC, Sabin E (2003) Aberrant signaling and transcription factor activation as an explanation for the defective growth control and differentiation of keratinocytes in psoriasis: a hypothesis. *Exp Dermatol* 12:337–345
- Pei XH, Nakanishi Y, Takayama K, Bai F, Hara N (1999) Benzo[a]pyrene activates human p53 gene through induction of nuclear factor κ B activity. *J Biol Chem* 274:35240–35246
- Phelps CB, Sengchanthalangsy LL, Huxford T, Ghosh G (2000) Mechanism of I κ B α binding to NF- κ B dimers. *J Biol Chem* 275:29840–29846
- Rosette C, Karin M (1995) Cytoskeletal control of gene expression: depolymerization of microtubules activates NF- κ B. *J Cell Biol* 128:1111–1119
- Rothwarf DM, Karin M (1999) The NF- κ B activation pathway: a paradigm in information transfer from membrane to nucleus. *Sci STKE* 1999:RE1
- Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS Jr (1995) Role of transcriptional activation of I κ B alpha in mediation of immunosuppression by glucocorticoids. *Science* 270:283–286
- Thompson JE, Phillips RJ, Erdjument-Bromage H, Tempst P, Ghosh S (1995) I κ B-beta regulates the persistent response in a biphasic activation of NF- κ B. *Cell* 80:573–582
- Wolf JS, Chen Z, Dong G, Sunwoo JB, Bancroft CC, Capo DE, Yeh NT, Mukaida N, Van Waes C (2001) IL (interleukin)-1 α promotes nuclear factor- κ B and AP-1-induced IL-8 expression, cell survival and proliferation in head and neck squamous cell carcinomas. *Clin Cancer Res* 7:1812–1820
- Wood LC, Elias PM, Calhoun C, Tsai JC, Grunfeld C, Feingold KR (1996) Barrier disruption stimulates interleukin-1 alpha expression and release from a pre-formed pool in murine epidermis. *J Invest Dermatol* 106:397–403
- Wu H, Lozano G (1994) NF- κ B activation of p53. *J Biol Chem* 269:20067–20074
- Yamamoto K, Nakayama K, Shimizu H, Mitomo K, Fijimoto K (1992) Molecular mechanisms for the regulation of inflammation. *Prog Immunol* 8:369–376