*Free Rad. Res.*, Vol. 27(3), pp. 301–309
Reprints available directly from the publisher
Photocopying permitted by license only



# Involvement of Different Transduction Pathways in NF-κB Activation by Several Inducers

SYLVIE LEGRAND-POELS, LAURENT ZECCHINON, BERNARD PIRET, SONIA SCHOONBROODT and JACQUES PIETTE*

*Laboratory of Virology, Institute of Pathology B23, University of Liège, B-4000 LIEGE, Belgium*

Accepted by Prof. H. Sies

*(Received 19 December 1996; In revised form 20 May 1997)*

Double-stimulation was used to demonstrate that, in a T lymphocytic cell line (CEM), phorbol myristate acetate (PMA) rapidly induced NF-κB through a signaling pathway which did not involve reactive oxygen species (ROS) and was different from the activation triggered by either $H_2O_2$ or tumor necrosis factor-α (TNF-α). Since these latter compounds were known to activate NF-κB translocation in a redox-sensitive way, we have demonstrated that NF-κB activation by PMA was resistant to antioxidant N-acetyl-L-cysteine (NAC) and sensitive to kinase inhibitors staurosporine and H7 while activation by $H_2O_2$ or TNF-α were not.

*Keywords:* NF-κB, CEM cell line, PMA, TNF-α, oxidative stress

## INTRODUCTION

The transcription factor NF-κB has been first identified as a lymphoid-specific protein that binds to a decameric oligonucleotide found in the κ-light chain gene intronic enhancer.[1] Its biological significance has been demonstrated by its involvement in the expression of a wide variety of genes controling immune and inflammatory responses, cell cycle, differentiation and pathogenesis of AIDS and cancer (for review, 2). NF-κB is a dimer of Rel family proteins but the most ubiquitous complex is the heterodimer p50/RelA. Its activity is inducible in the majority of cells by various agents such as pro-inflammatory cytokines (TNF-α, IL-1), phorbol esters, bacterial lipopolysaccharides (LPS),.. (for review, 2).

In resting cells, NF-κB is maintained inactive in the cytoplasm through its association with inhibitor proteins belonging to the IκB family[2–4]. The members of the IκB family are IκB-α, IκB-β, IκB-ε, p100 and p105.[4] Bcl-3 also belongs to this family but, at least in some cellular types, is nuclear and participates in NF-κB transactivating activity.[5] In response to various stimuli, including the interaction of the proinflammatory cytokines TNF-α and IL-1β with their receptors, IκB-α is first phosphorylated on serines 32 and 36 and then ubiquitinated on lysines 21 and 22, to be rapidly degraded by the 26S proteasome, allowing NF-κB

* Corresponding author. Tel.: 32-4-366.24.42. Fax: 32-4-366.24.33. E-mail: jpiette@vm.1.ulg.ac.be.

nuclear translocation and gene activation[3]. In mammals, several different signal transduction pathways have been implicated in NF-κB activation, all of which culminate in the degradation of IκB-α. Unfortunately, none of the pathways has been fully elucidated.[4] Reactive oxygen species (ROS) are involved as second messengers in the NF-κB activation pathways, as demonstrated by the inhibitor effect of several antioxidants.[6] But, recently, a ROS-independent pathway has been underlined after stimulation with TNF-α and IL-1[7,8].

Even if a significant body of data has been accumulated recently about NF-κB activation pathways, many questions remain unanswered: which kinases or molecules are involved, what is the role of ROS and what are the signaling pathways initiated by the other agents such as phorbol esters, LPS or ds RNA.

In this paper, we attempted to identify signaling pathways shared between different NF-κB inducers, by using subsequent inductions[9]. This method is based on a typical property of many signaling cascades: their down-modulation and transient nonresponsiveness subsequent to primary stimulation. After stimulation of cells with the first inducer (pretreatment), a restimulation (second treatment) with the same inducer is blocked due to down-modulation of one or several components of signal transduction. Restimulation within this period of refractoriness by another inducer that does not use the same pathway permits a normal response. Using this rationale, it should be possible to easily detect whether two stimuli use identical signal transduction pathways with respect to the down-regulated component(s).

## MATERIALS AND METHODS

### Cell Culture and Reagents

The human T lymphocytic cell line CEM was obtained from the NIH AIDS Reagent Program (Rockville, MD, USA) and was grown in RPMI 1640 medium (Gibco-BRL, Bethesda, MD, USA) supplemented with 2% glutamax I (L-alanyl-L-glutamine, Gibco-BRL, Bethesda, MD, USA) and 10% Fetal bovine serum (FBS).

PMA (Sigma, Saint-Louis, MO, USA), TNF-α (Boerhinger, Germany) and $H_2O_2$ (UCB, Brussels, Belgium) stimulation were performed by direct addition to the cell suspension. Before treatment with PMA, CEM cells were grown for one day in the medium up to a density of $1 \times 10^6$ cells/ml. *N*-acetyl-L-cysteine (NAC), $H_7$ and Staurosporine (Sigma, St-Louis, USA) were added to the cells 60 min before stimulation with PMA, TNF-α or $H_2O_2$.

### Nuclear and Cytoplasmic Protein Extraction

Nuclear and cytoplasmic protein extracts were prepared as described[10]. Cytoplasmic buffer contained 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES pH 7.9), 1 mM EDTA, 2 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 500 μM PMSF and protease inhibitors (Boehringer, Germany). The pelleted nuclei were resuspended in nuclear buffer (50 mM HEPES pH 7.9, 2 mM $MgCl_2$, 0.05 M KCl, 1 mM EDTA, 0.3 M NaCl, 10% (v/v) glycerol, 1 mM DTT, 500 μM PMSF, and protease inhibitors), incubated for 20 min on ice and centrifuged for 5 min at 14,000 rpm. Protein amounts were quantified with the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA).

### Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSA) and supershifting analysis were performed on a non-denaturing 6% (w/v) polyacrylamide gel run for 4 h at 180 V[10]. The gel was then dried and autoradiographed on a Fuji X-Ray film (General Electrics, Antwerp, Belgium).
The wild-type NF-κB probe (wt) was:

5′-GGTTACAAGGGACTTTCCGCTG
TGTTCCCTGAAAGGCGACG
GTT-5′

The mutated NF-κB probe (mut) was:

5′-GGTTACAACTCACTTTCCGCTG
TGTTGAGTGAAAGGCGACG
GTT-5′

For supershifting experiments, 1μl of the antibody was preincubated with the extracts for 20 min on ice before addition of the labelled κB probe.

The amount of NF-κB bound to the probe was quantified by phosphorimaging (Molecular Dynamics Corporation, CA, USA) using an image quantification analysis program.

### Western Blot Analysis

Cytoplasmic protein extracts (15 μg) were run on a 10% SDS-PAGE gel. After transfer onto a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA) and blocking overnight at 4°C with Tris Buffered Saline-Tween (20mM Tris-HCl pH 7.5, 500 mM NaCl, 0.2% Tween-20) plus 5% dry milk, the membranes were incubated for 120 min with the first antibody, washed and then incubated with the second peroxidase-conjugated antibody. The reaction was revealed with the enhanced chemoluminescence detection method (ECL kit, Amersham, UK).

### Antibodies

The polyclonal IκB-α antibody was from Euromedex (Strasbourg, France), the anti-peptide antibody directed against an amino-terminal peptide of p50 and the anti-peptide antibody directed against the N-terminal 14 amino acids following the initiator methionine of p65 were kindly provided by Dr Siebenlist (National Institute of Health, Bethesda, USA).

## RESULTS

Treatment of CEM cells with PMA at 0.1 μM led to a very transient NF-κB induction (Fig. 1A). Indeed, a κB DNA-protein complex visualized in EMSA by a band migrating slower than the free probe (Fig. 1A), appeared as shortly as 20 min after induction, reached its maximal level after around 30 min and disappeared after 120 min (Fig. 1A). The DNA-binding activity was specific to the κB probe since this retarded band disappeared after a competition with a 50-fold molar excess of the unlabeled probe (Fig. 1B, lane 3) but could not be competed out by a probe encompassing a mutated κB site (Fig. 1B, lane 4). The NF-κB complex induced in CEM cells by PMA treatment was constituted of the p50 and p65 subunits as shown by supershifting experiments (Fig. 1B). Antibodies raised against p50 (Fig. 1B, lane 6) and p65 (Fig. 1B, lane 7) both retarded the κB-specific complex. From these experiments, it turned out that the heterodimer p50/p65 is rapidly translocated into the nucleus after PMA treatment and this induction is quite transient since the nuclear complex started to disappear from the nucleus after 70 min. When the cytoplasmic IκB-α was followed by western blot, it could be shown that its degradation was concommitant with the NF-κB translocation in the nucleus (Fig. 1C). Its degradation was almost completed 30 min after induction but it was rather rapidly resynthesized to recapture NF-κB within the cytoplasm.

The kinetics of NF-κB translocation in CEM cells were also followed after induction with TNF-α and $H_2O_2$. As shown in Fig. 2B, NF-κB translocation after induction with 100 U/ml TNF-α was also transient, being maximal around after 60 min before going back to basal level after 300 min. On the other hand, treatment of CEM cells with 250 μM $H_2O_2$ gave rise to a sustained NF-κB translocation which culminated after two hours but which did not go back to basal level even after 400 min (Fig. 2C). From these experiments, CEM cells induction with PMA and TNF-α appeared to be transient while exhibiting different kinetic characteristics (Fig. 2 A,B), whereas $H_2O_2$ led to a sustained NF-κB induction (Fig. 2C).

In order to validate the strategy of the down-modulation and transient nonresponsiveness, CEM cells were first pretreated with PMA at 0.1 μM and restimulated with the same PMA

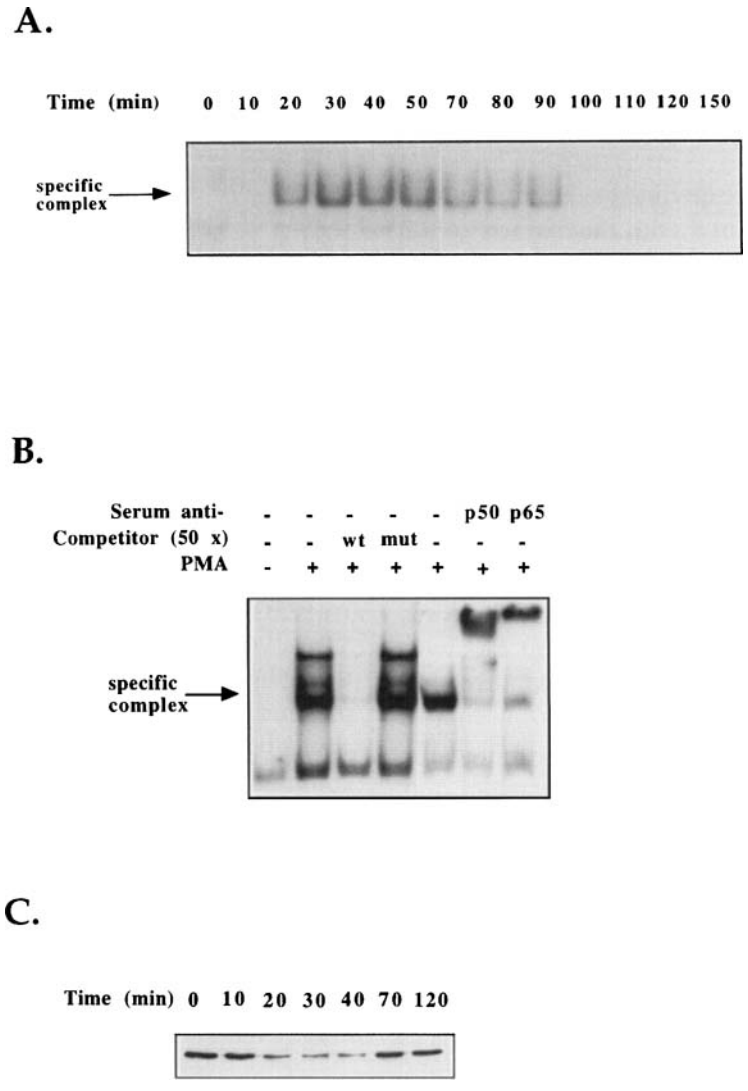


FIGURE 1 NF-κB translocation in the nucleus of CEM cells after induction with PMA (0.1 μM) **A.** Time course NF-κB DNA binding activity after treatment with PMA (0.1 μM). Nuclear extracts were prepared at various times after induction and an equal amount of proteins was mixed with a $^{32}$P-labelled probe encompassing the κB element. Samples were loaded on 6% native polyacrylamide gels and electrophoresed at 180 V. The upper part of the autoradiogram of the gel is shown. Arrow indicates the position of the specific complex. **B.** NF-κB DNA-binding specificity was assessed by competition with a 50-fold molar excess of unlabeled wild-type probe (wt) or mutated (mut) probe. Supershift experiments were carried out by addition of antibodies raised against p50 or p65. **C.** IκB-α proteolysis in PMA-induced CEM cells. Cells were taken at various times after PMA treatment and used to prepare cytoplasmic extracts. Cytoplasmic proteins (15 μg) were analyzed by SDS PAGE and transferred on nylon membranes followed by Western blot analysis using polyclonal IκB-α antibody.

concentration 30 min after the pretreatment, i.e. when the nuclear appearance of NF-κB is maximal (Fig. 2A). As shown in Fig. 3A, the second addition of PMA to PMA-pretreated CEM cells does not lead to a subsequent appearance of NF-κB in the nucleus demonstrating that CEM cells became unresponsive to the second addition of PMA. These experiments demonstrated that the signaling events are transient in CEM cells and one or several kinases activated by a pretreatment with PMA are down-modulated for at least 30 min.

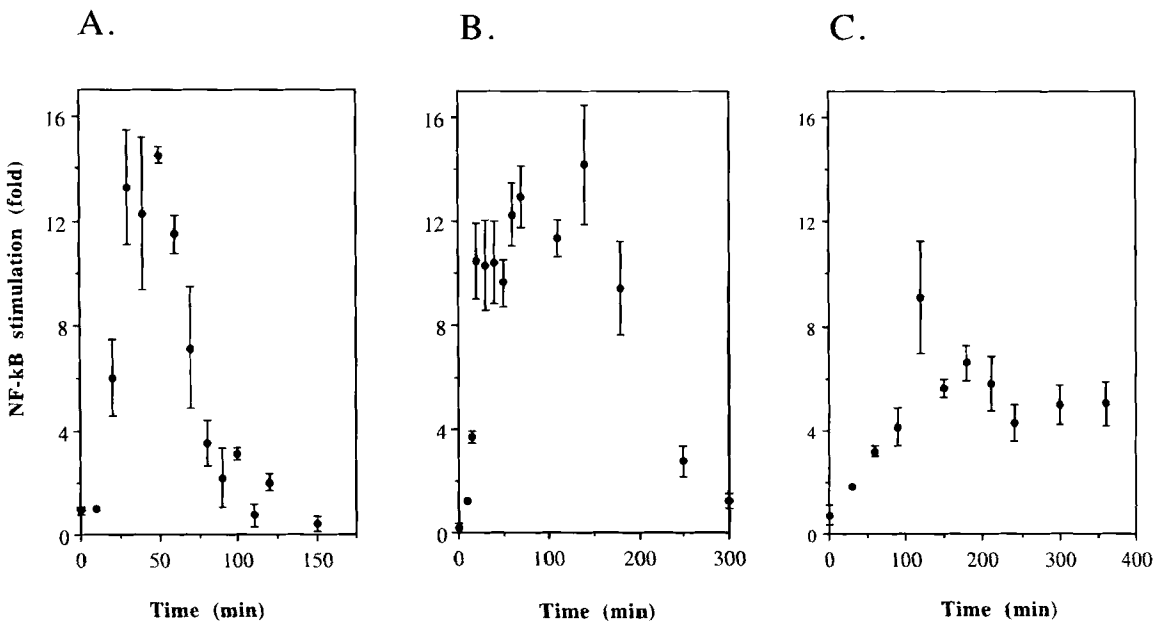


FIGURE 2 Time-course translocation of NF-κB in the nucleus of CEM cells after treatment with 0.1μM PMA **(A)**, 100 U/ml TNF-α **(B)** or 250 μM $H_2O_2$. NF-κB DNA-binding activities were measured by EMSA and the specific complexes were quantified by phosphoroimaging. DNA binding activities (expressed in fold-stimulating compared to untreated cells) were plotted *vs* incubation times. These curves are the mean of at least three independent experiments.



### Pretreatment by PMA Does Not Prevent Response to $H_2O_2$ or the TNF-α

When a pretreatment carried out with 0.1 μM PMA was followed by a treatment with 250 μM $H_2O_2$, a subsequent NF-κB translocation could be visualized. As shown in Fig. 3B, NF-κB appearance in the nuclei of CEM cells occurred in two successive waves: a first one due to PMA addition and a second one which could be detected 100 min after $H_2O_2$ addition, culminating at 150 min and sustained for at least 360 min. This experiment clearly demonstrated that NF-κB could still be inducible by $H_2O_2$ after a pretreatment with PMA. This result allowed us to postulate that the signaling pathways triggered by PMA and $H_2O_2$ did not share intermediates.

A similar behaviour could be observed when CEM cells pretreated with PMA were induced with TNF-α (100 U/ml). As shown in Fig. 3C, NF-κB translocation also occurred in two successive waves: a first one induced by the pretreatment with PMA, the second one due to TNF-α induction which was maximal 60 min after TNF-α addition. These data showed that, again, PMA and TNF-α did not share components of signal transduction. From these results, we could suspect that signaling pathway(s) induced by TNF-α or $H_2O_2$ in CEM cells did not use PKC which is a well-known and widely used example of down-modulated activity after pretreatment with phorbol esters.[11] Since NF-κB induction by TNF-α was shown to use ROS as second messengers,[6] the different behaviour observed above could well be explained by the stimulation of a transduction pathway by PMA which did not involve ROS as intermediates.

### NF-κB Activation by PMA is ROS-Independent

CEM-lymphocytes have been treated with various NAC concentrations (from 10 to 50 mM) before being stimulated by PMA (0.1 μM) or TNF-α

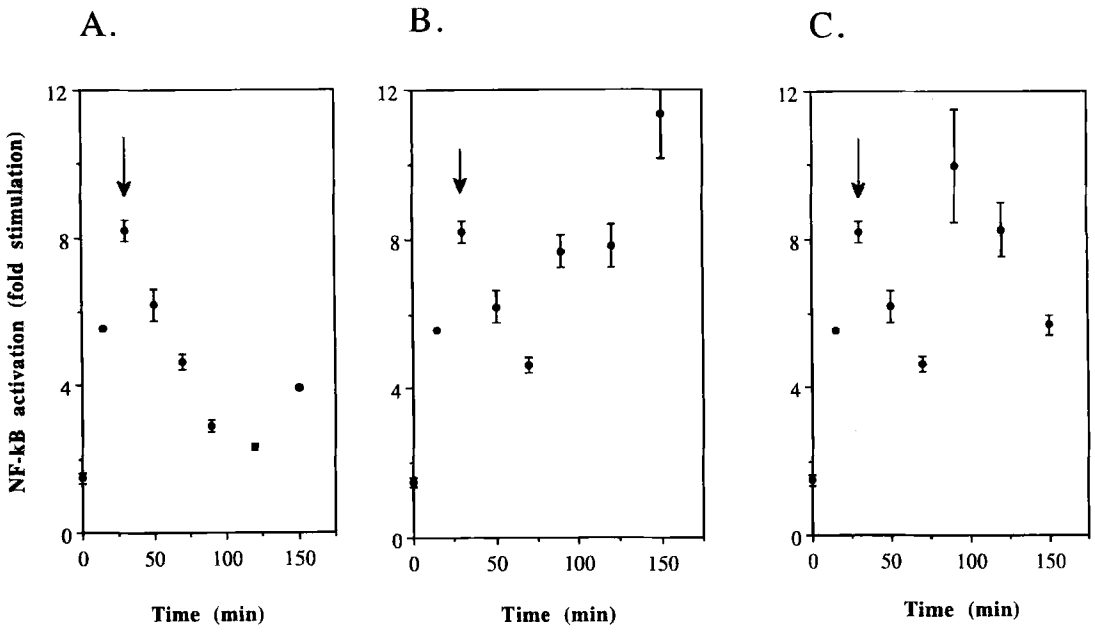


FIGURE 3 Pretreatment of CEM cells with PMA inhibits a subsequent PMA response **(A)**, but does not inhibit NF-κB translocation after a second treatment induced by $H_2O_2$ (250 μM) **(B)** or by TNF-α (100 U/ml) **(C)**. NF-κB DNA-binding activities were measured by EMSA and the specific complexes were quantified by phosphorimaging. DNA binding activities (expressed in fold-stimulation compared to untreated cells) were plotted *vs* incubation times. These curves are the mean of at least independent experiments. The arrows indicate when the addition of the second inducer was done.

(100 U). Fig. 4A shows that NF-κB activation by PMA is only weakly affected by the addition of NAC, suggesting that PMA signaling did not involve ROS while NF-κB induction by TNF-α was strongly repressed by NAC even at 10 mM. This absence of inhibition recorded when CEM cells were pretreated by NAC contrasted with the strong inhibitory effects of NAC when NF-κB translocation was promoted by TNF-α which was shown to use ROS as second messenger.[6] Thus, from all these data, it turned out that PMA induced NF-κB translocation through a pathway very distinct from those initiated by TNF-α and $H_2O_2$.

### Kinase Inhibitors Exhibit Differential Effects on NF-κB Induction

Since PMA and oxidative stress mediated by $H_2O_2$ clearly involved different transduction pathways leading to NF-κB induction, we tested the effects of protein kinase inhibitors H7[12] and staurosporin[13] on CEM cells induced either by PMA or $H_2O_2$. As shown in Fig. 4B, NF-κB translocation can be strongly inhibited by increasing concentrations of both H7 and staurosporin when cells are induced by PMA while these compounds did not exert inhibiting effects when cells were induced by $H_2O_2$. By themselves these inhibitors did not show any effects on NF-κB induction in this cell line (data not shown). These results reinforce the idea that the transduction pathway triggered by PMA primarily involved protein kinase C (PKC) whereas oxidative stress-inducing agents did not use this kinase or other kinases sensitive to staurosporine.

## DISCUSSION

The characterization of the signal transduction pathways leading to NF-κB activation after the

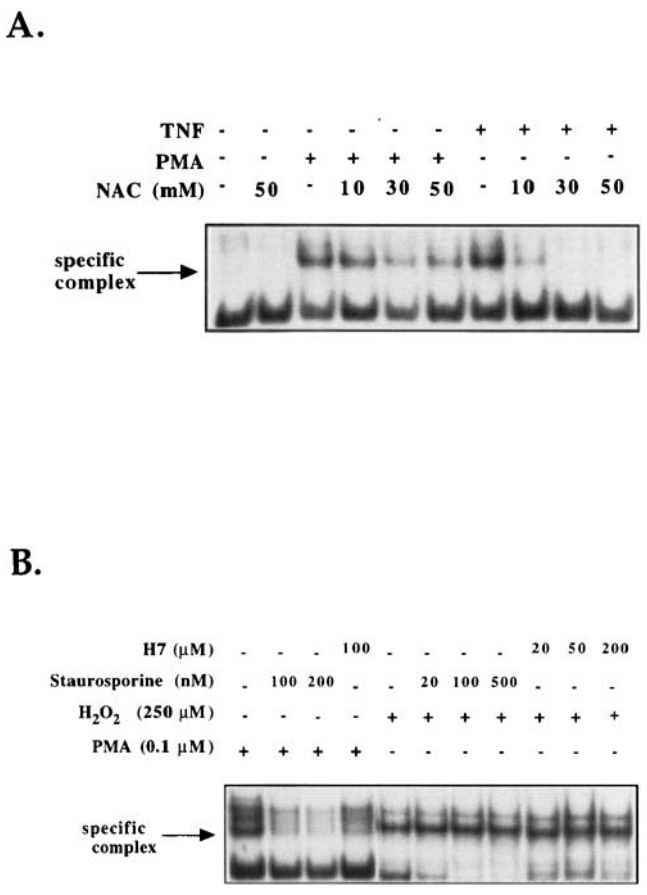


FIGURE 4 **A.** Effects of N-acetyl-L-cysteine on NF-κB induction in PMA- or TNF-α-induced CEM cells. CEM cells were incubated with various concentrations of NAC (in mM) for 60 min before being induced with PMA (0.1 μM) or TNF-α (100 U/ml). Nuclear proteins (5 μg) were prepared 30 min after induction and analyzed by EMSA. NF-κB specific complex is shown by an arrow. **B.** Effects of kinase inhibitors staurosporine and H7 in PMA- or H2O2-induced CEM cells. Cells were incubated with various concentrations of inhibitors for 1 hour before being induced by PMA (0.1 μM) or H2O2 (250 μM). Nuclear proteins (5 μg) were prepared either 30 min after induction with PMA or 120 min after induction by H2O2 and analyzed by EMSA. The arrow indicates specific complex.

interaction of pro-inflammatory cytokines with their receptors is of the highest importance. There is some evidence in the literature that more than one pathway can account for TNF-α- or IL-1β-mediated NF-κB activation.[14] Several reports have claimed that NF-κB activation is dependent, in lymphoid cells, on oxidative stress and activation of 5-lipoxygenase[6,15] while others have demonstrated that the acidic sphingomyelinase pathway is necessary and sufficient for NF-κB induction after TNF-α stimulation.[16] Similarly, different authors have reported that ceramide is a major second messenger for TNF-α-induced NF-κB activation[16-18] while others have denied any role for ceramide in IκB-α degradation.[19] However, it remains unclear whether different pathways can coexist or prevail in some cell types. Here, we report that phorbol ester induces NF-κB in a T cell line via a transduction pathway different from those triggered by TNF-α and $H_2O_2$. This conclusion is based on the use of three different experimental tools: (i) kinetics of NF-κB translocation, (ii) cross-refractoriness of signaling cascades and (iii) sensitivities to antioxidant and inhibitory treatments.

While ROS are largely used as secondary messengers for NF-κB activation in lymphoids cells, they are not released intracellularly after PMA treatment of CEM cells. This situation is reminiscent to what happened in epithelial cells activated with pro-inflammatory cytokines such as IL-1β or TNF-α.[7] Indeed, in an ovarian carcinoma cell line, the antioxidants NAC and PDTC failed to block NF-κB activation following IL-1β stimulation. Other authors had previously reported that some NF-κB inducing signals, including IL-1β and TNF-α, still work in the presence of antioxidants.[8]

This study demonstrates that the activation of NF-κB can occur through different pathways. Stimulation of CEM cells by $H_2O_2$ leads to a slow activation of NF-κB. Stimulation of the same cells by PMA activates NF-κB with a much faster kinetics and proceeds exclusively through a degradation of IκB-α which does not involve intracellular oxidative stress. TNF-α treatment of CEM cells also induces a rapid degradation of IκB-α and activation of NF-κB but here through the production of ROS. While TNF-α is one of the most potent activators of NF-κB, the mechanism by which the activation occurs is not understood. Roles for ceramide, hydrogen peroxide, mitochondrial respiratory chain, proteases and kinases have been suggested (for rev. 20). A recent report showed that tyrosine phosphatase plays in the TNF-dependent activation of NF-κB but neither okadaic acid nor calyculin A, inhibitors of serine-threonine phosphatase, had any effect.[21] In this system, it was found that okadaic acid by itself could activate NF-κB but that this activation could not be blocked by pervanadate, suggesting a difference in the pathway leading to NF-κB activation by TNF-α and okadaic acid.[21] These differences in the kinetics and pathways of NF-κB activation might translate into specific functions. The array of genes activated by NF-κB is specific for a given cell type at a given time. Indeed, the transactivation of any promoter depends on the binding of several proteins exerting activating or repressing functions, and NF-κB can induce transcription only if the promoter context is favorable. Every signal transduction pathway activates several transcription factors, which transactivate a specific set of genes. In other words, different transduction pathways might allow NF-κB to be activated simultaneously with other transcription factors leading to the transcription of distinct genes.

Since the pretreatment of CEM cells by PMA did not prevent restimulation by neither TNF-α nor $H_2O_2$, it could be suspected that the transduction pathways utilized by these compounds are different in terms of intermediates but also in the nature of the final kinases which phosphorylate IκB-α. Recently, a kinase which phosphorylates IκB-α on serine residues 32 and 36 was isolated and characterized.[22] In HeLa cells, it belongs to a large multisubunit complex exhibiting kinase activity upon treatment by okadaic acid. On the other hand, it has also been shown that the mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK1) induces the site-specific phosphorylation of IκB-α *in vivo* and, most strinkingly can directly activate the IκB-α kinase complex *in vitro*.[23] Thus, MEKK1 which is a critical component of both the c-jun and NF-κB stress response pathways could well be activated by TNF-α and $H_2O_2$ but not by PMA. From the data presented in this paper, it could be postulated that there are several kinases capable of phosphorylating IκB-α; some of them are very likely redox-sensitive.

### *Acknowledgments*

This work was supported by grants from the Belgian National Fund for Scientific Research (Brussels, Belgium), by Télévie, by a Concerted Action Program from the "Communauté Française de Belgique" and SIDACTION (Paris, France). JP is Research Director from the NFSR (Brussels, Belgium), S.L-P. is Scientific Collaborator from the NFSR (Brussels, Belgium), SS is supported by an ARC program and BP is a fellow from the Belgian FRIA (Brussels, Belgium).

## References


[1] Sen, R. and Baltimore, D. (1986). Multiple Nuclear Factors Interact with the Immunoglobulin Enhancer Sequences, *Cell*, **46**, 705–716.

[2] Grilli, M., Chiu, J. J.-S. and Lenardo, M. J. (1993). NF-κB and Rel: Participants in a Multiform Transcriptional Regulatory System, *International Review of Cytology*, **143**, 1–61.

[3] Palombella, V. J., Rando, O. J., Goldberg, A. L. and Maniatis, T. (1994). The Ubiquitin-Proteasome Pathway is required for Processing the NF-κB1 Precursor Protein and the Activation of NF-κB, *Cell*, **78**, 773–785.

[4] Baldwin, A. S. (1996). The NF-κB and IκB proteins: new discoveries and insights, *Annual Review of Immunology*, **14**, 649–681.

[5] Franzoso, G., Bours, V., Park, S., Tomita-Yamaguchi, M., Kelly, K. and Siebenlist, U. (1992). The candidate oncoprotein Bcl-3 is an antagonist of p50/NF-κB-mediated inhibition, *Nature*, **359**, 339–342.

[6] Schreck, R., Rieber, P. and Baeuerle, P. A. (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1, *EMBO Journal*, **10**, 2247–2258.

[7] Bonizzi, G., Dejardin, E., Piret, B., Piette, J., Merville, M. P. and Bours, V. (1996). Interleukin-1β induces nuclear factor κB in epithelial cells independently of the production of reactive oxygen intermediates, *European Journal of Biochemistry*, **242**, 544–549.

[8] Brennan, P. and O'Neill, L. A. J. (1995). Effects of oxidants and antioxidants on Nuclear Factor κB activation in three different cell lines: evidence against an universal hypothesis involving oxygen radicals, *Biochimica Biophysica Acta*, **1260**, 167–175.

[9] Sachsenmaier, C., Radler-Pohl, A., Zinck, R., Nordheim, A., Herrlich, P. and Rahmsdorf, H. J. (1994). Involvement of Growth Factor Receptors in the Mammalian UVC Response, *Cell*, **78**, 963–972.

[10] Legrand-Poels, S., Bours, V., Piret, B., Pflaum, M., Epe, B., Rentier, B. and Piette, J. (1995). Transcription Factor NF-κB is activated by Photosensitization generating Oxidative DNA Damages, *Journal of Biological Chemistry*, **270**, 6925–6934.

[11] Mosior, M. and Newton, A. C. (1995). Mechanism of Interaction of Protein Kinase C with Phorbol Esters, *Journal of Biological Chemistry*, **270**, 25526–25533.

[12] Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984). Isoquinolinesulfonamides, Novel and Potent Inhibitors of Cyclic Nucleotide Dependent Protein Kinase C, *Biochemistry*, **23**, 5036–5041.

[13] Tamaoki, T., Nomoto, H., Yakahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986). Staurosporine, a potent inhibitor of phospholipid/$Ca^{++}$ dependent protein kinase *Biochemical Biophysical Research Communication*, **135**, 397–402.

[14] Schütze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K. and Krönke, M. (1992). TNF Activates NF-κB by Phosphatidylcholine Specific Phospholipase C Induced Acidic Sphingomyeline Breakdown, *Cell*, **71**, 765–776.

[15] Schreck, R., Meier, B., Mannel, D. N., Droge, W. and Baeuerle, P. A. (1992). Dithiocarbamates as Potent Inhibitors of Nuclear Factor κB Activation in Intact Cells, *Journal of Experimental Medecine*, **175**, 1181–1194.

[16] Wiegmann, K., Schütze, S., Machleidt, T., Witte, D. and Krönke, M. (1994). Functional Dichotomy of Neutral and Acidic Sphingomyelinases in Tumor Necrosis Factor Signaling, *Cell*, **78**, 1005–1015.

[17] Mathias, S., Younes, A., Kan, C-C, Orlow, I., Joseph, C. and Kolesnick, R. N. (1993). Activation of the Sphingomyelin Signaling Pathway in Intact Cells and in a cell-free system by IL-1β, *Science*, **259**, 519–522.

[18] Dbaibo, G. S., Obeid, L. M. and Hannun, Y. A. (1993). Tumor Necrosis Factor-α (TNF-α) Signal Transduction Through Ceramide, *Journal of Biological Chemistry*, **268**, 17762–17766.

[19] Betts, J. C., Agranoff, A. B., Nabel, G. J. and Shayman, J. A. (1994). Dissociation of Endogenous Cellular Ceramide from NF-κB Activation, *Journal of Biological Chemistry*, **269**, 8455–8458.

[20] Baeuerle, P. A. and Henkel, T. (1994). Function and activation of NF-κB in the immune system. *Annual Review Immunology*, **12**, 141–179.

[21] Singh, S. and Aggarwal, B. B. (1995). Protein-tyrosine phosphatase inhibitors block tumor-necrosis factor-dependent activation of the nuclear transcription factor NF-κB. *Journal of Biological Chemistry*, **270**, 10631–10639.

[22] Chen, Z. J., Parent, L. and Maniatis, T. (1996). Site-Specific Phosphorylation of IκBα by a Novel Ubiquitination-Dependent Protein Kinase Activity, *Cell*, **84**, 853–862.

[23] Lee, F. S., Hagler, J., Chen, Z. J. and Maniatis, T. (1997). Activation of the IκBα kinase complex by MEKK1, a kinase of the JNK pathway, *Cell*, **88**, 213–222.