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ORIGINAL PAPER

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Differential activation of nuclear factor kappa B subunits in a skin dendritic cell line in response to the strong sensitizer 2,4-dinitrofluorobenzene

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Abstract Dendritic cell (DC) maturation is essential for the initiation of T-dependent immune responses. Nuclear factor kappa B (NF-κB) transcription factors are ubiquitously expressed signalling molecules, known to regulate the transcription of a large number of genes involved in immune responses, including cytokines and cell surface molecules. In this work, we studied the time-dependent activation of five members of the NF-κB family, p50, p52, p65, RelB and cRel, in a mouse skin DC line in response to stimulation with the strong sensitizer, 2,4-dinitrofluorobenzene (DNFB). Western blot assay revealed that exposure of fetal skin DC (FSDC) to DNFB induced the degradation of the inhibitor of NF-κB (IκB). Three out of its five members, i.e. p50, p52, and RelB, were similarly activated upon DNFB stimulation, with subsequent translocation of these subunits from the cytosol to the nucleus, but with different kinetics. In contrast, p65 expression was diminished in both the nucleus and the cytosol. The electrophoretic mobility shift assay (EMSA) showed that exposure of FSDC to DNFB induced DNA binding to NF-κB. Together, these results show that DNFB differentially activates the various members of the NF-κB family in skin DC.

Keywords Skin dendritic cell · DNFB · NF-κB · IκB

Abbreviations *DC* dendritic cell · *DNFB* 2,4-dinitrofluorobenzene · *EMSA* electrophoretic mobility shift assay · *FSDC* fetal skin dendritic cell line · *IκB* inhibitor of NF-κB · *IKK* IκB kinase · *iNOS* inducible nitric oxide synthase · *LPS* lipopolysaccharide · *MAPK* mitogen-activated protein kinase · *NF-κB* transcription nuclear factor kappa B · *PMSF* phenylmethylsulfonylfluoride

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Introduction

Epidermal skin dendritic cells (DC), namely Langerhans cells, are an important cell population involved in allergic contact dermatitis and other cell-mediated immune reactions due to their potent antigen-presenting capacity. They capture the antigen and leave the epidermal environment, migrating to the lymph nodes, where they come into contact with and sensitize the T cells responsible for the adaptive immune response [14]. DC migration is a tightly regulated event, dependent on allergens and epidermal cytokines, which also induce DC maturation with the expression of cell surface markers essential for antigen presentation, such as the major histocompatibility complex class II molecules and costimulatory molecules [5]. However, the intracellular signalling pathways activated by the allergens that induce DC maturation are not completely understood.

The promoter region of genes encoding key proteins of the immune system, such as the major histocompatibility complex class I and II molecules, cytokines and their receptors, or cell adhesion molecules, contain binding sites for the transcription nuclear factor kappa B (NF-κB) [27]. Five members of the mammalian NF-κB/Rel family, p50, p52, p65 (RelA), RelB, and cRel, have been cloned and characterized, and in resting cells, NF-κB proteins are localized in the cytosol in association with inhibitory proteins called IκB [10]. Cell activation by various inducers results in IκB protein phosphorylation and degradation, which in turns leads to NF-κB protein translocation to the nucleus, where it upregulates NF-κB-dependent gene expression [18].

We have previously reported that in a fetal skin DC (FSDC) line, which exhibits functional characteristics of a

DC precursor, lipopolysaccharide (LPS) and granulocyte-macrophage colony-stimulating factor activate the transcription factor NF-κB [12, 13]. The aim of this study was to investigate whether the strong sensitizer, 2,4-dinitrofluorobenzene (DNFB), activates NF-κB in skin DC and to determine which members of this protein family are translocated from the cytosol to the nucleus after exposure of skin DC to DNFB.

Material and methods

Materials

Rabbit anti-human NF-κB p65 was from Serotec (Oxford, UK) and rabbit anti-IκB-α polyclonal antibody was from New England BioLabs (Beverly, Mass.). NF-κB consensus oligonucleotide, goat anti-mouse NF-κB cRel, rabbit anti-human NF-κB RelB, rabbit anti-human NF-κB p50, and rabbit anti-human NF-κB p52 were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.). Mouse monoclonal antibody against actin and protease inhibitor cocktail were purchased from Roche (Carnaxide, Portugal). ^{32}P-Labelled γ-ATP, the ECL Western blotting analysis system, X-ray films, T4 polynucleotide kinase and poly(dI-dC).poly(dI-dC) were from Amersham Pharmacia Biotech (Carnaxide, Portugal). Horseradish peroxidase-conjugated swine anti-rabbit and horseradish peroxidase-conjugated swine anti-goat were from DAKO (Copenhagen, Denmark). Horseradish peroxidase-conjugated goat anti-mouse was from Pierce (Rockford, Ill.). DNFB was purchased from Sigma-Aldrich Química (Madrid, Spain), fetal calf serum was from Biochrom (Berlin, Germany), and trypsin from Gibco (Paisley, UK). All other reagents were from Sigma Chemical Co (St. Louis, Mo.).

Cell culture

The FSDC cell line, which has been shown to have antigen-presenting capacity to T cells, was kindly supplied by Dr. G. Girolomoni [17]. The cells were cultured in endotoxin-free Iscove's medium supplemented with 10% (v/v) fetal calf serum, 1% (w/v) glutamine, 3.02 g/l sodium bicarbonate, 100 μg/ml streptomycin and 100 U/ml penicillin. For Western blotting and the electrophoretic mobility shift assay (EMSA), FSDC were plated at 2×10^6 cells/well in six-well culture dishes for 24 h prior to treatment.

Western blot analysis

For immunodetection of IκB-α, p50, p52, p65, cRel and RelB, FSDC were treated with culture medium (control) or with DNFB (1 μg/ml) for 2, 5, 15 and 30 min. Proteins of the cytosolic fraction were obtained after harvesting the cells in 10 m*M* NaCl, 3 m*M* $MgCl_2$, 0.5% (v/v) Nonidet P-40, 1 m*M* dithiothreitol, 0.1 m*M* PMSF, 10 m*M* Tris-HCl, pH 7.5, and protease inhibitor cocktail. The lysates were incubated on ice for 15 min and the cytosolic proteins were isolated from the supernatant obtained after centrifugation at 2300 *g* for 10 min. In order to obtain proteins of the nuclear fraction the pellet obtained was resuspended in 300 m*M* NaCl, 3 m*M* $MgCl_2$, 20% (v/v) glycerol, 1 m*M* dithiothreitol, 0.1 m*M* PMSF, 0.2 m*M* EDTA, 20 m*M* HEPES buffer (pH 7.5), and protease inhibitor cocktail, incubated on ice for 1 h, and centrifuged at 12,000 *g* for 20 min. The supernatant containing the nuclear proteins was collected, and the protein concentration was determined using the bicinchoninic acid method. Total cell lysates were obtained after harvesting the cells in a sonication buffer containing 10 m*M* Tris-HCl, pH 7.5, 0.32 *M* sucrose, 1 m*M* EDTA, 1 m*M* dithiothreitol, 0.1 m*M* PMSF and protease inhibitor cocktail. The lysates were then incubated on ice for 30 min and sonicated on ice at low amplitude (four times for 4 s at 20 μm peak to peak) to disrupt the cells and release the NF-κB proteins, and the protein concentration was determined using the bicinchoninic acid method. Protein samples were denatured and separated on a 15% (v/v) SDS-PAGE, and transferred to a PVDF membrane. The membranes were blocked with 5% (w/v) dried milk in Tris-buffered saline with 0.1% (v/v) Tween 20 for 1 h. IκB-α, p50, p52, p65, cRel and RelB proteins were detected by incubation with a rabbit polyclonal anti-IκB-α antibody (1:1000), a rabbit polyclonal anti-human p50 antibody (1:100), a rabbit polyclonal anti-human p52 antibody (1:500), a rabbit polyclonal anti-human p65 antibody (1:1000), a goat polyclonal anti-mouse c-Rel antibody (1:500) and a rabbit polyclonal anti-human RelB antibody (1:500) for 1 h, followed by incubation with a horseradish peroxidase-conjugated swine anti-rabbit antibody (1:1000), a horseradish peroxidase-conjugated swine anti-goat antibody (1:5000) or a horseradish peroxidase-conjugated goat anti-mouse antibody (1:25,000). The immunocomplexes were visualized by the ECL chemiluminescence method. To demonstrate equivalent protein loading the membranes were stripped and reprobed with an anti-actin antibody (1:10,000).

EMSA

FSDC were treated for 15 min, 30 min and 1 h with DNFB (1 μg/ml). The cells were then washed with PBS and lysed in 10 m*M* NaCl, 3 m*M* $MgCl_2$, 0.5% (v/v) Nonidet P-40, 1 m*M* dithiothreitol, 0.1 m*M* PMSF, 10 m*M* Tris-HCl, pH 7.5, and protease inhibitor cocktail. The lysates were incubated on ice, for 15 min, and centrifuged at 2300 *g* for 10 min. The resulting pellet was resuspended in 300 m*M* NaCl, 3 m*M* $MgCl_2$, 20% (v/v) glycerol, 1 m*M* dithiothreitol, 0.1 m*M* PMSF, 0.2 m*M* EDTA, 20 m*M* HEPES buffer (pH 7.5), and protease inhibitor cocktail, incubated on ice for 1 h, and centrifuged at 12,000 *g* for 20 min. The supernatant containing the nuclear proteins was collected, and the protein concentration was determined using the bicinchoninic acid method. The EMSA method used was similar to that described previously [18], with slight modifications. The probes consisted of a double-stranded oligonucleotide containing the consensus binding sequence for NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Typical binding reactions consisted of 20 μg nuclear extract, about 100,000 cpm [γ-^{32}P]-labelled oligonucleotide, 100 μg/ml poly(dI-dC).poly(dI-dC) in a buffer containing 20 m*M* HEPES (pH 7.9), 1 m*M* $MgCl_2$, 4% (w/v) Ficoll 400, 0.5 m*M* dithiothreitol, 50 m*M* KCl, and 1 mg/ml bovine serum albumin, and were incubated at room temperature for 45 min. Binding reactions were separated on 7% (v/v) nondenaturing polyacrylamide gels in a buffer system containing 0.044 *M* Tris-base (pH 8.0), 44.5 m*M* boric acid and 1 m*M* EDTA at a constant 150 V for 2 h and 15 min at room temperature. The gels were transferred to Whatman paper, dried, and subjected to autoradiography. In competition experiments, unlabeled oligonucleotide was added to the nuclear extracts for 1 h before the addition of the radiolabeled probe. To detect supershifted bands, anti-p50, anti-p52, anti-p65, anti-cRel and anti-RelB antibodies (2 μg) were incubated with the nuclear extracts for 1 h before the addition of radiolabeled probe.

Data analysis

The results are presented as means±SEM of the indicated number of experiments. The results were analysed using the unpaired Student's *t*-test. The significance level was 0.05.

Results

DNFB induces NF-κB binding to DNA in FSDC

Stimulation of FSDC with DNFB (1 μg/ml) induced a time-dependent increase in NF-κB binding to DNA (not

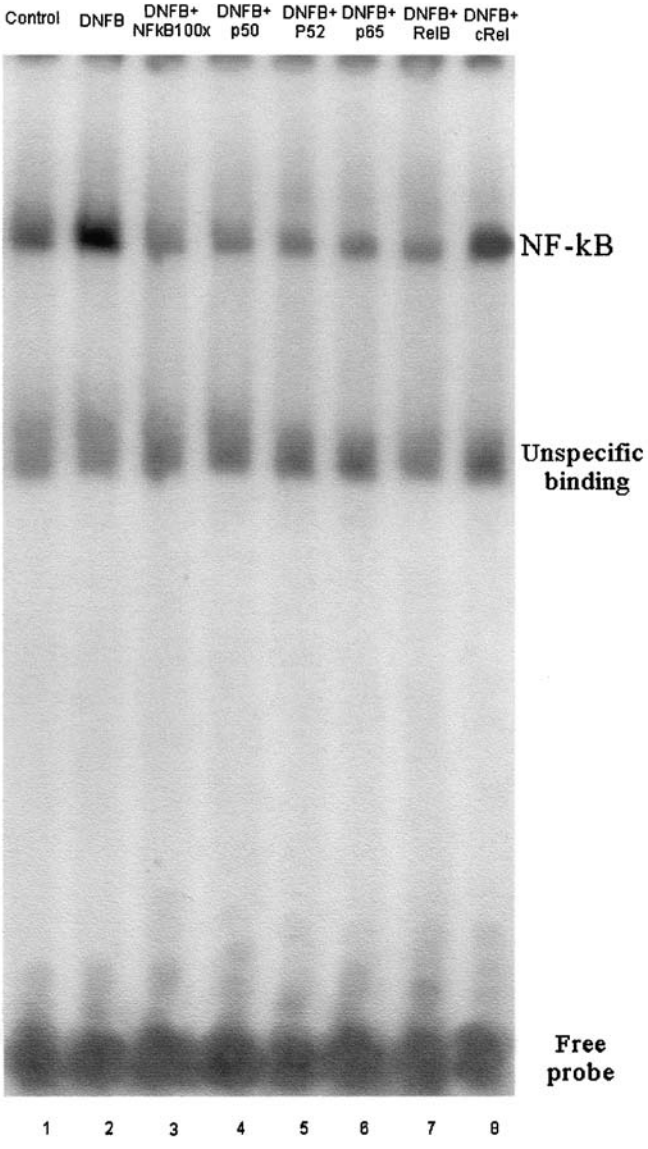

Fig. 1 DNFB induced NF-κB activation in FSDC. FSDC (2×10^6 cells) were incubated in culture medium alone (*Control*, *lane 1*), or in the presence of 1 μg/ml DNFB for 15 min (*lane 2*). Nuclear extracts were subjected to EMSA as described in the experimental procedures. Supershift experiments were done using specific anti-p50, anti-p52, anti-p65, anti-RelB and anti-cRel antibodies (*lanes 4–8*). To demonstrate the specificity of the induced bands, binding was carried out in the presence of a molar excess (100×) of nonradioactive NF-κB consensus oligonucleotide (*lane 3*). The gel shown is representative of three gels yielding similar results

shown). Maximal NF-κB binding to DNA (Fig. 1) was observed within 15 min (lane 2). Supershift experiments using antibodies against the p50 (lane 4), p52 (lane 5), p65 (lane 6) and Rel B (lane 7) NF-κB subunits showed a decrease in NF-κB complex formation in cells stimulated with DNFB. In contrast, supershift experiments using an antibody against the cRel NF-κB subunit was without effect (lane 8). As control for the gel shift assays, unlabeled oligonucleotide (100-fold excess) was used (lane 3) which inhibited NF-κB complex formation.

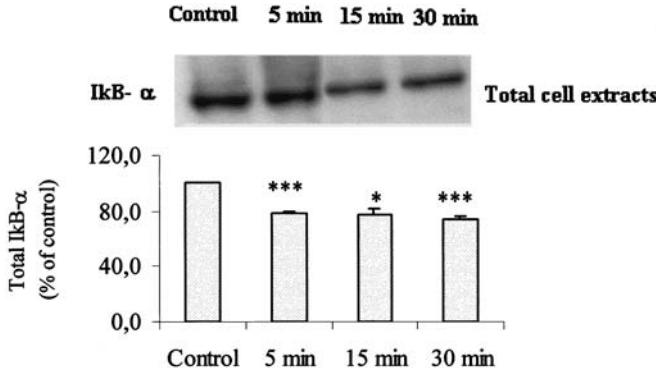

Fig. 2 DNFB induced cytosolic degradation of IκB-α in FSDC. FSDC (2×10^6 cells) were incubated with culture medium alone (*Control*) or with DNFB (1 μg/ml) for the times indicated in the figure. Total cell extracts were electrophoresed through SDS-PAGE and subjected to Western blot analysis using anti-IκB-α antibody as described in the experimental procedures. The blot shown is representative of three blots yielding similar results. Each bar represents the mean±SEM from at least three experiments. **P*<0.05, ****P*<0.001

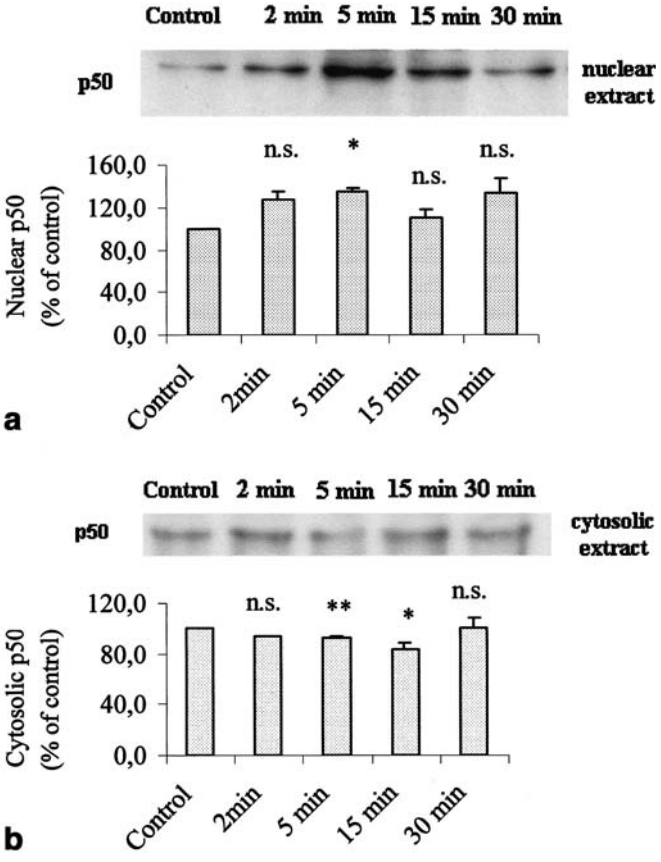

Fig. 3a, b DNFB induced protein NF-κB p50 translocation from the cytosol into the nucleus in FSDC. FSDC (2×10^6 cells) were incubated with culture medium alone (*Control*) or with DNFB (1 μg/ml) for the times indicated in the figure. Cytosolic and nuclear cells extracts were electrophoresed through SDS-PAGE and subjected to Western blot analysis using anti-p50 antibody as described in the experimental procedures. The blots shown are representative of three blots yielding similar results. Each bar represents the mean±SEM from at least three experiments. **P*<0.05, ***P*< 0.01 (*n.s.* not significant)

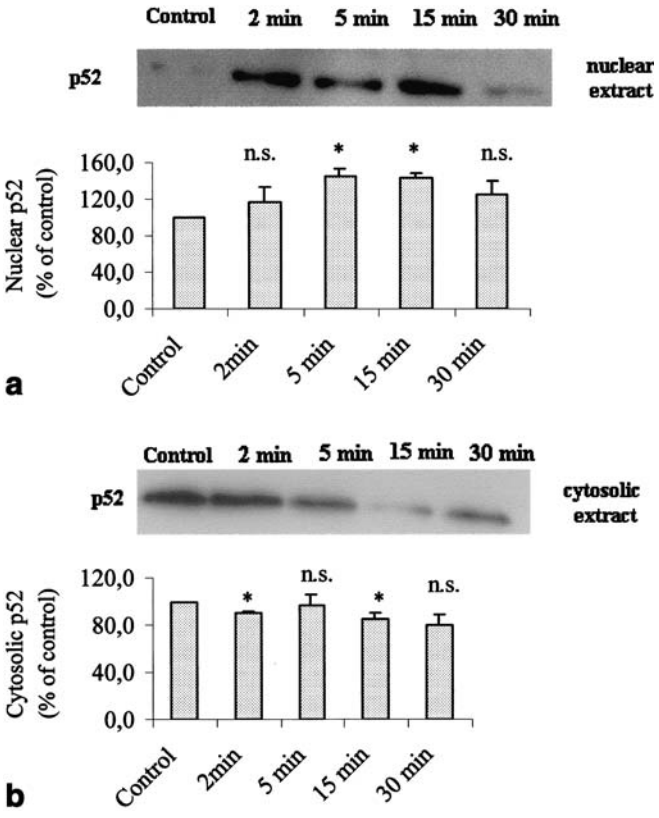

Fig. 4a, b DNFB induced protein NF-κB p52 translocation from the cytosol into the nucleus in FSDC. FSDC (2×10^6 cells) were incubated with culture medium alone (*Control*) or with DNFB (1 μg/ml) for the times indicated in the figure. Cytosolic and nuclear cells extracts were electrophoresed through SDS-PAGE and subjected to Western blot analysis using anti-p52 antibody as described in the experimental procedures. The blots shown are representative of three blots yielding similar results. Each bar represents the mean±SEM from at least three experiments. *P<0.05 (*n.s.* not significant)

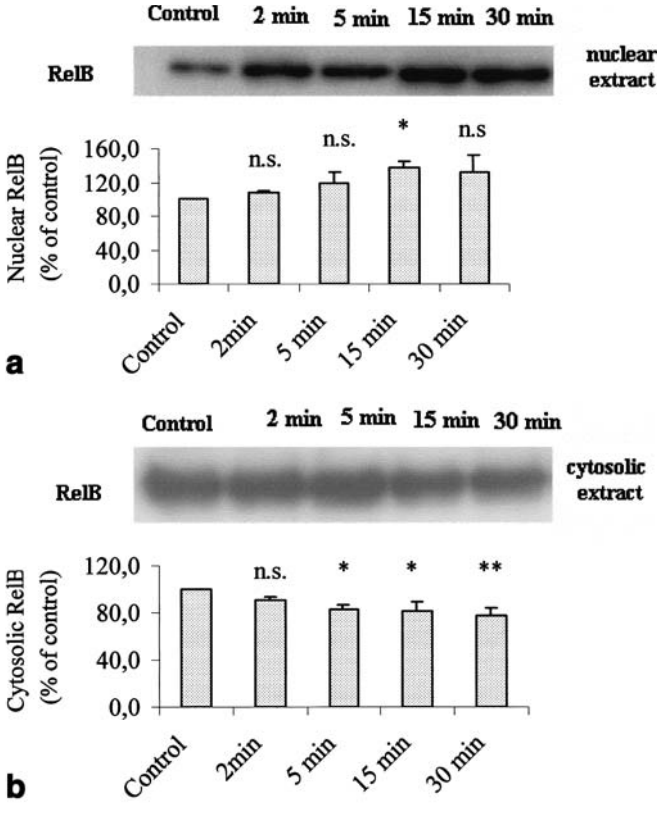

Fig. 5a, b DNFB induced protein NF-κB RelB translocation from the cytosol into the nucleus in FSDC. FSDC (2×10^6 cells) were incubated with culture medium alone (*Control*) or with DNFB (1 μg/ml) for the times indicated in the figure. Cytosolic and nuclear cells extracts were electrophoresed through SDS-PAGE and subjected to Western blot analysis using anti-RelB antibody as described in the experimental procedures. The blots shown are representative of three blots yielding similar results. Each bar represents the mean±SEM from at least three experiments. *P<0.05, **P< 0.01 (*n.s.* not significant)

DNFB induces the degradation of cytosolic IκB-α protein in FSDC

To determine whether DNFB induced IκB-α degradation, the levels of IκB-α protein in total cell extracts were determined by Western blot analysis. As shown in Fig. 2, treatment of cells with 1 μg/ml DNFB for 5, 15 and 30 min caused a reduction in the total amount of the protein in FSDC (from 100% in control cells to 74.6±1.9% after 30 min DNFB incubation).

DNFB induces the translocation of the NF-κB p50, p52 and RelB proteins from the cytosol to the nucleus in FSDC

In order to determine which members of the NF-κB family are activated upon stimulation of FSDC with DNFB, cytosolic and nuclear extracts were subjected to Western blot analysis, using antibodies against the proteins p50, p52, p65, RelB and cRel. As shown in Figs. 3, 4 and 5, stimulation of cells with DNFB (1 μg/ml) resulted in a significant reduction in the cytosolic levels and an increase in the nuclear levels of NF-κB proteins p50, p52 and RelB, but with different kinetics. Indeed, translocation of p50 and p52 was observed after 5 min incubation with DNFB (Fig. 3a and Fig. 4a), whereas translocation of RelB was observed after 15 min incubation with DNFB (Fig. 5a). Treatment of FSDC with DNFB (1 μg/ml) did not significantly modify the levels of cRel protein in the cytosol or in the nucleus (Fig. 6).

DNFB decreases the NF-κB protein p65 expression in total cell lysates of FSDC

In contrast to the results observed for the p50, p52 and RelB proteins, DNFB induced a decrease in p65 protein expression in both the nucleus and the cytosol of FSDC (Fig. 7). We next determined p65 protein expression in total cell lysates obtained from FSDC stimulated with DNFB for 5, 15 and 30 min and 1 h (Fig. 8). A decrease in p65 protein expression was observed mainly after 1 h incubation with DNFB (from 100% to 59.1±2.8%; Fig. 8).

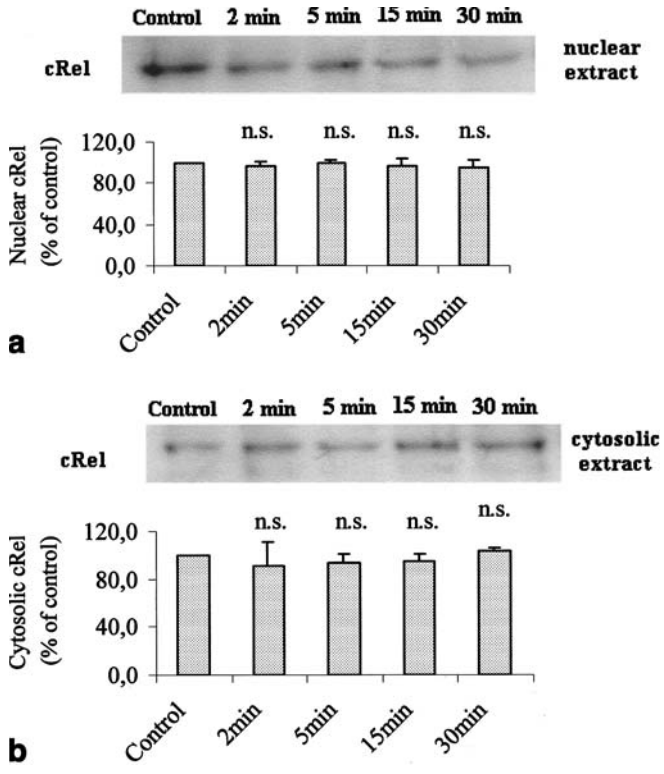

Fig. 6a, b DNFB did not induce protein NF-κB cRel translocation from the cytosol into the nucleus in FSDC. FSDC (2×10^6 cells) were incubated with culture medium alone (*Control*) or with DNFB (1 μg/ml) for the times indicated in the figure. Cytosolic and nuclear cells extracts were electrophoresed through SDS-PAGE and subjected to Western blot analysis using anti-cRel antibody as described in the experimental procedures. The blots shown are representative of three blots yielding similar results. Each bar represents the mean±SEM from at least three experiments (*n.s.* not significant)

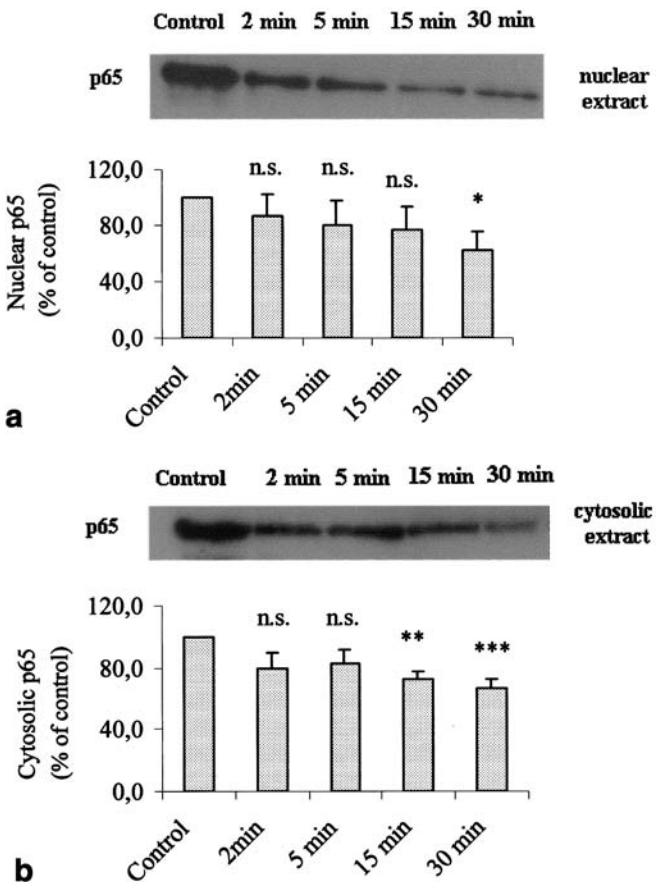

Fig. 7a, b DNFB induced a decrease in protein NF-κB p65 expression in both the nucleus and the cytosol of FSDC. FSDC (2×10^6 cells) were incubated with culture medium alone (*Control*) or with DNFB (1 μg/ml) for the times indicated in the figure. Cytosolic and nuclear cells extracts were electrophoresed through SDS-PAGE and subjected to Western blot analysis using anti-p65 antibody as described in the experimental procedures. The blots shown are representative of three blots yielding similar results. Each bar represents the mean±SEM from at least three experiments. $^*P<0.05$, $^{**}P<0.01$,$^{***}P<0.001$ (*n.s.* not significant)

Discussion

In the present work, we showed that exposure of FSDC to DNFB induced degradation of IκB-α protein with subsequent translocation of the NF-κB proteins (p50, p52 and RelB) into the nucleus (Figs. 2, 3, 4 and 5), although with different kinetics, to bind DNA (Fig. 1). In contrast, NF-κB protein p65 was diminished in both the nucleus and the cytosol of FSDC (Fig. 7) due to a decrease in p65 protein expression observed in total cell lysates after 1 h incubation with DNFB (Fig. 8).

Previous in vitro studies have shown that skin contact sensitizers induce DC maturation [3]. However, the intracellular signalling pathways by which DNFB induces DC maturation are not known. The promoter region of a large number of genes involved in the inflammatory response contains binding sites for NF-κB [27]. More than 150 NF-κB-responsive genes have been identified, including genes coding for cytokines, chemokines, cell adhesion molecules and growth factors [27]. However, depending on the cell and the stimulus, the NF-κB pathway displays the capacity to activate only a subset of the total repertoire of NF-κB-responsive genes [23]. In macrophages and DC the NF-κB pathway responds to different stimuli by activating, in a cell-specific manner, unique signalling pathways and subsets of NF-κB target genes [4]. We have previously reported that in FSDC, LPS and granulocyte-macrophage colony-stimulating factor induce IκB-α degradation, NF-κB translocation into the nucleus and iNOS expression [12, 13], indicating that in skin DC the activation of NF-κB is a crucial step in DC maturation. Accordingly, it has recently been demonstrated that effective antigen presentation by DC is NF-κB-dependent [33].

In normal epidermis, the NF-κB proteins p50 and p52, in addition to p65, are mostly expressed in the cytoplasm of basal cells [7, 19], and the NF-κB proteins have been found to be involved in DC differentiation and maturation [1, 15, 25, 26, 31]. It has also recently been demonstrated that the NF-κB signal transduction pathway is involved in the survival and maturation of LPS-stimulated human

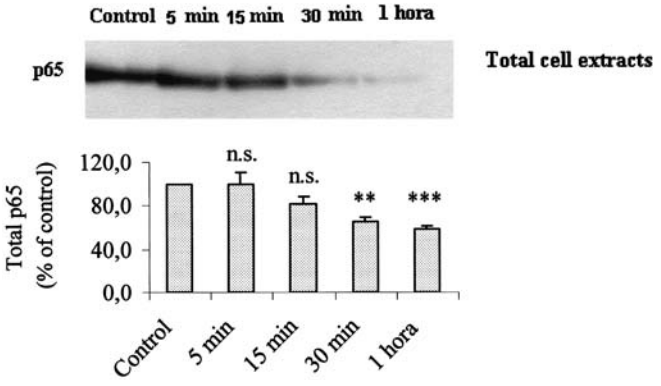

Fig. 8 DNFB induced a decrease in protein NF-κB p65 expression in total cell extracts of FSDC. FSDC (2×10^6 cells) were incubated with culture medium alone (*Control*) or with DNFB (1 μg/ml) for the times indicated in the figure. Total cell extracts were electrophoresed through SDS-PAGE and subjected to Western blot analysis using anti-p65 antibody as described in the experimental procedures. The blot shown is representative of three blots yielding similar results. Each bar represents the mean±SEM from at least three experiments. $^{**}P<0.01$,$^{***}P<0.001$ (*n.s.* not significant)

monocyte-derived DC [2]. The NF-κB member RelB is also involved in DC differentiation [11, 32] and in the transactivation of genes of central importance for functional antigen-presenting cells [8, 21, 29]. Those results demonstrate that the NF-κB signalling pathway is of major importance in DC.

The DNFB-induced signalling pathway(s) responsible for NF-κB activation have not yet been identified. Recent studies have shown that an early molecular event during the activation of antigen-presenting cells by contact sensitizers, namely DNFB, is the induction of tyrosine phosphorylation [20, 24]. Moreover, it has also been demonstrated that upregulation of CD80, CD86 and CD83 induced by DNFB [30] is coupled to the phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) [3]. Almost all signals that lead to activation of NF-κB converge on a high molecular weight complex that contains a serine-specific IκB kinase (IKK). IKK contains two related kinases, IKKα and IKKβ, that are active as a dimer. Activation of IKK leads to the phosphorylation of two specific serines near the N terminus of IκB-α, which targets IκB-α for ubiquitination and degradation by the proteasome. The unmasked NF-κB can then enter the nucleus to activate target gene expression [16]. The p38 MAPK pathway has been shown to contribute to NF-κB-mediated transactivation [6, 9]. These results suggest that in FSDC DNFB may activate protein kinases, namely p38 MAPK, which in turn activate the IκB kinase complex leading to NF-κB activation.

We demonstrated that DNFB selectively translocates, with different kinetics, the NF-κB proteins p50, p52 and RelB into the nucleus of skin DC (Figs. 3, 4 and 5), but induced a decrease in p65 protein expression in total cell lysates after 1 h of incubation (Fig. 8). Furthermore, DNFB was without effect on the expression of cRel (Fig. 6). This differential regulation of the NF-κB subunits probably contributes to the specificity and selectivity of the NF-κB response. In fact, the target genes activated by NF-κB will vary depending upon the cellular context, and this is achieved through a combination of regulatory mechanisms. The selective activation of NF-κB complexes, with subtle differences in DNA-binding specificities, results in the targeting of different promoters and enhancers [28]. Moreover, NF-κB transactivation and DNA binding can also be regulated through phosphorylation of NF-κB itself, namely by p38 MAPK, protein kinase A and casein kinase II [22].

The differential expression and activation of various NF-κB factors in DC by different skin sensitizers should have profound biological consequences because it directly affects the function of NF-κB factors and defines the set of κB-responsive genes under transcription and consequently may affect the antigen-presenting capacity of DC.

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