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Research Article

Role of oxidative stress in ERK and p38 MAPK activation induced by the chemical sensitizer DNFB in a fetal skin dendritic cell line

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Summary The intracellular mechanisms involved in the early phase of dendritic cell (DC) activation upon contact with chemical sensitizers are not well known. The strong skin sensitizer 2,4-dinitrofluorobenzene (DNFB) was shown to induce the activation of mitogen-activated protein kinases (MAPK) in DC. In the present study, we investigated a putative role for oxidative stress in DNFB-induced MAPK activation and upregulation of the costimulatory molecule CD40. In a DC line generated from fetal mouse skin, DNFB induced a significant increase in protein oxidation, measured by the formation of carbonyl groups, while it had almost no effect on lipid peroxidation. The antioxidants glutathione and vitamin E, which inhibit protein and lipid oxidation, respectively, were used to assess the role of oxidative stress in DNFB-induced MAPK activation. Glutathione, but not vitamin E, inhibited DNFB-induced p38 MAPK and ERK1/2 phosphorylation, whereas none of the antioxidants interfered significantly with the DNFB-induced upregulation of CD40 protein levels. Taken together, these results indicate that DNFB activates p38 MAPK and ERK1/2 via production of reactive oxygen species, and that protein oxidation plays an important role in MAPK activation.

Key words: dendritic cell, DNFB, intracellular signalling, reactive oxygen species, skin sensitizer.

Introduction

When activated by haptens, DC in the skin lose their antigen uptake and processing capacity and become capable of antigen presentation.[1] In this process, DC switch chemokine receptors, alter cytokine production, and upregulate the expression of MHC molecules and costimulatory molecules (CD80, CD86, CD83 and CD40).[2–6] This also occurs *in vitro*, in cultures of DC, which in the presence of skin sensitizers mature and modify their phenotypic expression,[7,8] as we have shown in the fetal skin derived dendritic cell (FSDC) line exposed to 2,4-dinitrofluorobenzene (DNFB), Ni and *p*-phenylenediamine (PPD).[9–12] CD40, a member of the TNF receptor (TNFR) family, is a 45–50 kDa receptor expressed on a wide range of cell types, including DC.[13,14] During antigen presentation, the interaction of CD40 in DC with its ligand (CD40L) on activated T cells is critical for DC maturation, and it induces IL-12 production, leading to the differentiation of T cells into Th1.[15,16] Several skin sensitizers have been shown to upregulate CD40 protein expression on DC, such as 2,4,6-trinitrobenzene sulphonic acid (TNBS), aminophenol, chlorpromazine hydrochloride, dinitrochlorobenzene (DNCB) and the metal allergen nickel sulphate.[8,17]

The exact cellular and molecular mechanisms of DC activation by haptens remain poorly understood. Reactive oxygen species (ROS) are normally produced by phagocytic cells, such as macrophages, when NADPH oxidase is activated during phagocytosis of invading cells,[18] and increasing evidence suggests that they may play an essential role in signal transduction. In addition to apoptosis, free radicals and derived species regulate many cellular events, including transcription factor activation, gene expression and cell proliferation. ROS have also been described to activate DC.[19–21] Several reports have shown, in different cell types, that oxidants activate intracellular signalling pathways (e.g. mitogen-activated protein kinases [MAPK]),[22–24] suggesting a role for ROS as second messengers. Activation of MAPK has been detected in DC upon stimulation with contact sensitizers: DNFB[7] and DNCB[25] activate p38 high osmolarity glycerol protein kinase (p38 MAPK) and $NiCl_2$ activates both p38 MAPK and ERK1/2 in human monocyte-derived DC.[25] DNCB was also shown to activate p38 MAPK in the immature DC cell line BC1.[26]

In the present work, we found that the strong sensitizer DNFB also activates ERK1/2 and p38 MAPK in FSDC. To clarify some of the signalling events involved in skin DC activation upon contact with chemical sensitizers, we investigated whether MAPK activation was dependent on ROS formation. The role of ROS in the regulation of CD40 expression in sensitized cells was also investigated.

Materials and methods

Reagents

Trypsin was purchased from Gibco-Invitrogen (Life Technologies, Paisley, UK). FCS was from Cambrex (Walkersville, MD, USA). DNFB and 2,4-dichloronitrobenzene (DCNB) were from Aldrich

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(Madrid, Spain). The antibody against phospho-ERK1/2 was from Promega (Madison, WI, USA). The anti-phospho-p38 MAPK antibody was from Cell Signalling Technology (Beverly, MA, USA), and the antibody against dinitrophenyl (DNP) was from Dako (Glostrup, Denmark). The anti-CD40 antibody was purchased from R&D Systems (Minneapolis, MN, USA). The alkaline phosphatase-linked goat antirabbit IgG (H + L) antibody and the enhanced chemifluorescence (ECF) substrate were purchased from Amersham Biosciences (Carnaxide, Portugal), and the alkaline phosphatase-linked goat antirat IgG (H + L) antibody was purchased from Chemicon (Temecula, CA, USA). All other reagents were obtained from Sigma (Madrid, Spain) or from Merck (Darmstadt, Germany).

Cell culture

The FSDC line, kindly supplied by Dr G. Girolomoni, was established from fetal mouse skin suspensions and is representative of an early Langerhans cells (LC) precursor.[27] This cell line is activated when stimulated by skin sensitizers or LPS,[10–12,28] and upon activation by cytokines efficiently presents haptens to primed T cells *in vitro* and induces contact sensitivity in naive syngeneic animals.[27] The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2, in Iscove modified Dulbecco's medium (IMDM; Sigma) supplemented with 10% heat inactivated FCS, 36 mmol/L sodium bicarbonate, 64.4 mmol/L glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were fed with fresh medium every 2–3 days.

Stimulation with chemicals

Cells were plated at 0.69×10^6 cells/well in six-well microplates for western blot analysis, and at 2×10^6 cells/well in 12-well microplates for lipid peroxidation assays, and they were grown for 2 days or 1 day, respectively. Cells were then maintained in serum-free IMDM for 10–30 min, before addition of the strong sensitizer DNFB or of its inactive analogue 2,4-dichloronitrobenzene (DCNB), at 5 μg/mL. At this concentration, DNFB activates MAPK and CD40,[29] and does not reduce cell viability under the experimental conditions used, as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1-3-benzene disulfonate (WST-1) assays (data not shown). Cells were then kept at 37°C for different periods of time (as indicated in the figure captions). Inhibition of protein and lipid oxidation was performed by adding 1 mmol/L glutathione (GSH) or 1 mmol/L vitamin E, respectively, to the stimulation medium. In this case, the cells were preincubated with the antioxidants for 90 min before stimulation with the sensitizer.

Cell lysate preparation

After stimulation, cells were washed with ice-cold PBS and scraped into ice-cold lysis buffer containing protease and phosphatase inhibitors (50 mmol/L HEPES [pH 7.5], 1% Triton X-100, 100 mmol/L NaCl, 2 mmol/L EGTA, 2 mmol/L EDTA, 2 mmol/L Na_3VO_4, and freshly added 1 mmol/L PMSF, 2.5 μg/mL pepstatin, 1 mmol/L DTT, 10 μmol/L E64, 20 μg/mL benzamidin and 10 mmol/L NaF). Cell lysates were clarified by centrifugation at 9700 *g* for 30 min at 4°C. The supernatants were stored at –80°C until protein determination using the bicinchoninic acid (BCA) protein assay method (Pierce, Rockford, IL, USA). Cell lysates were then boiled for 5 min in 6× sample buffer (187.5 mmol/L Tris-HCl [pH 6.8], 12% (w/v) SDS, 60% sucrose, 0.06% bromophenol blue and 37.5% β-mercaptoethanol) and either used immediately for SDS–PAGE electrophoresis or frozen at –20°C until use. Cell lysates for anti-CD40 western blots were prepared with the same solutions but without the reducing agent DTT.

Western blot analysis

Western blotting was performed for the analysis of ERK, JNK and p38 MAPK activation (using antibodies specific for the phosphorylated form of the three kinases) and for the analysis of CD40 protein levels. Briefly, equivalent amounts of protein (25–40 μg) were loaded onto a 10% SDS–polyacrylamide gel, subjected to electrophoresis, and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). The membranes were saturated with 5% (w/v) fat-free dry milk in Tris-buffered saline (50 mmol/L Tris [pH 8.0], 150 mmol/L NaCl) with 0.1% Tween 20 (TBS-T) for 90 min at room temperature. Blots were then incubated overnight with primary antibodies at 1:1000 or 1:5000 dilution for phospho-p38 MAPK and phospho-ERK, respectively. Incubation with the antibody against CD40 (1:500) was performed for 2 h at room temperature. The solutions of primary antibodies were prepared in 1% fat-free dry milk in TBS-T. After extensive washing with 0.5% fat-free dry milk in TBS-T, blots were further incubated for 1 h at room temperature with goat antirabbit or antirat IgG antibodies coupled to alkaline phosphatase, at 1:20 000 or 1:10 000 dilution, respectively, in 1% fat-free dry milk in TBS-T. Blots were then washed in 0.5% fat-free dry milk in TBS-T. The immune complexes were detected using the ECF system (Amersham Biosciences), and the membranes were then scanned for blue excited fluorescence on the Storm 860 (Amersham Biosciences), according to the manufacturer's instructions. The signals were analyzed using ImageQuant software (Amersham Biosciences).

Derivatization of protein carbonyls and dot blot analysis

The FSDC, stimulated as described above, were washed with ice-cold phosphate buffer (PBS) and scraped into ice-cold lysis buffer containing protease and phosphatase inhibitors (300 mmol/L sucrose, 2 mmol/L EGTA, 10 mmol/L HEPES [pH 7.4], and freshly added 1 mmol/L DTT, 1 mmol/L PMSF and complete mini-tablet protease inhibitor cocktail). Cell lysates were clarified by centrifugation at 600 *g* for 10 min at 4°C. The supernatants were stored at –80°C until protein determination using the BCA protein assay method (Pierce), and protein carbonyl derivatization. Protein (2 μg) was derivatized by adding one volume of 12% SDS and two volumes of 20 mmol/L DNPH/10% trifluoroacetic acid (TFA), followed by a 30 min incubation at room temperature in the dark. Then 1.5 volumes of 2 mol/L Tris, 30% glycerol and 18.3% β-mercaptoetanol was added to each sample. Negative control experiments were done by processing one sample as described, except for the omission of the 20 mmol/L DNPH solution, which was replaced by 10% TFA alone. Protein (1 μg) was then applied to a PVDF membrane. The membranes were saturated with 5% (w/v) fat-free dry milk in TBS-T for 90 min at room temperature. Blots were then incubated overnight with anti-DNP primary antibodies at 1:500 000 dilution in 5% fat-free dry milk in TBS-T. After extensive washing with 0.5% fat-free dry milk in TBS-T solution, blots were further incubated for 1 h at room temperature with goat anti-rabbit IgG antibodies coupled to alkaline phosphatase (1:20 000 dilution) in 1% fat-free dry milk in TBS-T. Blots were then washed in 0.5% fat-free dry milk in TBS-T. The immune complexes were detected using the ECF system, as described for western blots. The signals were analyzed using the ImageQuant software (Amersham Biosciences).

Lipid peroxidation assessment

The extent of peroxidation was determined by the thiobarbituric acid (TBA) method. After stimulation, cells were washed with ice-cold PBS and scraped in ice-cold 15 mmol/L Tris (pH 7.4). The samples were then diluted three times with 15% trichloroacetic acid (TCA), 0.375% TBA, 0.015% 2,6-di-*tert*-butyl-4-methylphenol (BHT) and 0.25 mol/L HCl, and incubated for 15 min at 100°C. The samples were centrifuged at 3000 *g* for 10 min at 4°C, the supernatants were collected and the absorbance was measured at 530 nm on the UV/VIS spectrophotometer Lambda 3B (Perkin-Elmer, Wellesley, MA, USA). The amount of thiobarbituric acid-reactive substances (TBARS) formed was calculated using a molar extinction coefficient of 1.56×10^5 mol/L per cm and expressed as nmol TBARS produced by milligram of protein. The results were then expressed as fold increase relative to the control. Protein was measured by the BCA protein assay method (Pierce). A positive control was done using 500 μmol/L H_2O_2 to induce lipid peroxidation.

Data analysis

The results are expressed as mean ± SEM of the indicated number of independent experiments, and statistical analysis was performed using the one-way ANOVA test, with a Dunnett's post-test, or the repeated measures ANOVA with a Bonferroni's post-test, as indicated. A difference with P-value < 0.05 was considered statistically significant.

Results

DNFB induces protein oxidation but not lipid peroxidation

To assess whether DNFB induces any type of oxidative stress in FSDC, we examined the content of oxidized proteins and lipid peroxidation in cells stimulated with the chemical sensitizer (5 μg/mL). The formation of carbonyl groups was assessed to investigate protein oxidation, which could play a role in signal transduction, and lipid peroxidation was evaluated using the TBARS method.

FSDC cells were stimulated with DNFB for different periods of time and the formation of carbonyl groups was examined after derivatization, followed by dot blot analysis of the derivatized groups (Fig. 1A). Protein oxidation increased as soon as 5 min after exposure to DNFB, and further increased 30 min after exposure to the sensitizer (Fig. 1A), suggesting that DNFB induces the formation of ROS that can mediate cellular signalling. The specificity of the sensitizing effect of DNFB was tested using its inactive analogue, 2,4-dichloronitrobenzene (DCNB). In contrast with the results observed with DNFB, DCNB did not affect the oxidation of proteins (Fig. 1A).

In contrast to what we observed for protein oxidation, DNFB did not induce significant lipid peroxidation (Fig. 1B), suggesting that lipid peroxides are not the major free radicals formed. As expected, exposure of FSDC to 500 μmol/L H_2O_2 for 15 min significantly increased lipid peroxidation.

Gluthathione inhibits the DNFB-induced phosphorylation of ERK1/2 and p38 MAPK

Because activation of MAPK has been detected in DC upon stimulation with contact sensitizers, including DNFB,[7,25,26] we investigated whether protein oxidation plays a role in the activation of these signalling pathways. As shown in Figure 2, stimulation of FSDC with DNFB induced the phosphorylation (activation) of ERK1/2 and p38 MAPK, and this effect was almost completely inhibited by adding reduced GSH, which prevents protein oxidation. These results suggest that protein oxidation plays an important role in the activation of MAPK.

Vitamin E does not affect DNFB-induced phosphorylation of ERK1/2 and p38 MAPK

We further investigated whether lipid peroxidation could be involved in MAPK activation. For this purpose, cells were stimulated with DNFB, in the presence or in the absence of vitamin E, a lipid-soluble antioxidant. Vitamin E did not affect the phosphorylation of the MAPK induced by DNFB (Fig. 3), in agreement with the results showing that DNFB does not interfere with lipid peroxidation.

Role of protein and lipid oxidation in DNFB-induced upregulation of CD40 protein levels

To investigate the role of protein and lipid oxidation on the activation and maturation of FSDC, we studied the effect of gluthatione and vitamin E on the DNFB-induced upregulation of CD40 protein levels. This maturation marker is involved in the interaction of DC with T cells and is upregulated under DC maturation induced by contact sensitizers.[8] Both antioxidants partly reduced the effect of the sensitizer on CD40 protein levels after 2 h of stimulation (Fig. 4), but although it was consistent in all of the experiments performed, the inhibition of DNFB-induced CD40 upregulation did not reach statistical significance.

Discussion

The major cellular events involved in DC activation and maturation during the process of skin sensitization are still unknown. Previous studies demonstrated that chemical sensitizers activate p38 MAPK and ERK1/2 in DC.[7,25,26,29] Here, we show that ROS are involved in p38 MAPK and ERK1/2 activation in a skin derived DC line (FSDC) exposed to subtoxic concentrations of the chemical sensitizer DNFB. Furthermore, the DNFB-induced CD40 upregulation in FSDC is largely independent of the ROS-induced signalling pathways.

A growing amount of evidence suggests that ROS can trigger activation of DC.[19–21] In this study, we investigated whether DNFB induces oxidative stress, particularly protein oxidation and/or lipid peroxidation. We found that the sensitizer significantly increased the oxidized protein content (Fig. 1A), whereas a minor increase of lipid peroxides was observed (Fig. 1B). Therefore, the observed increase in the cellular content of ROS suggests a role for a redox-sensitive pathway in DC activation and maturation induced by chemical sensitizers.

Our results are supported by previous reports showing a role for ROS in DC activation and maturation, in studies where human DC activation was induced with LPS (an immunostimulatory molecule that also induces the maturation of DC by binding to receptor molecules at the cell surface)[30]

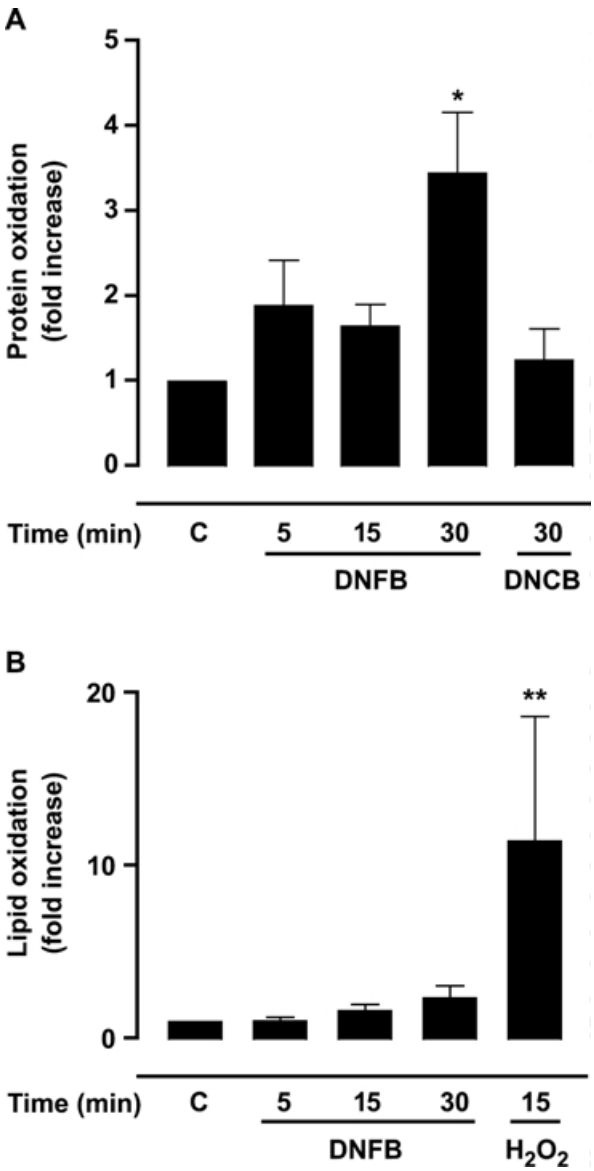

Figure 1 2,4-dinitrofluorobenzene (DNFB) induces oxidation of proteins but not of lipids. Cells were stimulated or not with 5 μg/mL of DNFB. (A) Cell lysates were collected at 5, 15 and 30 min after stimulation, derivatized with dinitrophenylhydrazine and analyzed by western blot using the specific antibody for dinitrophenyl groups, as described in the Materials and methods. The specificity of the sensitizing effect of DNFB was tested by incubation with its inactive analogue 2,4-dichloronitrobenzene (DCNB) (5 μg/mL for 30 min). The results were quantified by scanning the membrane with a fluorescence scanner and analyzed using the ImageQuant software. Results are expressed as the fold increase relative to the control (C) in each experiment. Data are expressed as the mean ± SEM of four independent experiments. Statistical significance was calculated by the one-way ANOVA test with a Dunnett's post-test (*$P < 0.05$). (B) Cell lysates were collected at 5, 15 and 30 min after stimulation and analyzed by the thiobarbituric acid (TBA) colourimetric assay for lipid oxidation, as described in the Materials and methods. Lipid oxidation induced by incubation with H_2O_2 (500 μmol/L) for 15 min is shown as a positive control. Results are expressed as fold increase relative to the control (C) in each experiment. Data are expressed as the mean ± SEM of four independent experiments. Statistical significance was calculated by the one-way ANOVA test with Dunnett's post-test (**$P < 0.01$).

or by stimulation of BC1 cells with TNF-α.[26,31] However, in both cases, the role of ROS was inferred based on the use of antioxidants, and the presence of ROS or derivatives was not analyzed. A recent report demonstrating that upon antigen-specific interaction of DC with T cells, the intracellular oxidation state of DC was elevated, further supports our findings.[32] However, this increase in ROS was detected in the process of antigen presentation and not during activation of DC. Furthermore, hydrogen peroxide also activates DC,[20,21] indicating that the formation of free radicals is important for DC function.

We previously reported that DNFB activates ERK1/2 and p38 MAPK in FSDC.[29] Our findings are in agreement with recent reports showing p38 MAPK phosphorylation in human monocyte-derived DC stimulated with DNFB[7] or DNCB (which has a similar sensitizing potential to DNFB).[25] Furthermore, we also detected a strong activation of ERK1/2 by DNFB, which was not observed by Arrighi *et al*.[7] In experiments using human monocyte-derived DC, these two signalling pathways were also shown to be activated by another skin sensitizer, $NiCl_2$,[25] which, as we have shown for DNFB,[28] also activates the nuclear factor NF-κB transcription factor.[25] The activation of p38 MAPK by DNCB was also recently observed using an immature murine DC line (BC1 cells).[26]

Although MAPK participate in different signalling pathways, they may be functionally related, and may be coactivated in response to certain stimuli. For example, ERK, JNK and p38 MAPK are all activated by LPS in macrophages.[33] In some cases they can be coactivated but may exert opposite effects. In monocyte-derived DC stimulated with LPS and TNF-α, the ERK pathway has been described as a negative regulator of DC maturation, opposing the p38 MAPK pathway.[34] In human $CD34^+$-derived DC stimulated with nickel, ERK, JNK and p38 MAPK are activated and cooperate in the upregulation of CD83, CD86 and CCR7 expression.[35,36] However, when the cells are stimulated with TNF-α only, p38 MAPK is involved in CD83 and CCR7 upregulation,[35,36] while ERK inhibits DC maturation and JNK has no effect.[35] When p38 MAPK is activated by TNF-α, ERK activation may be partially suppressed and, as a consequence, abrogates the inhibition of DC maturation induced by ERK.[35] Accordingly, we observed previously that p38 MAPK inhibits ERK activation in FSDC, an effect that was suppressed by an inhibitor of p38 MAPK (SB203580; data not shown). This suggests that there is a cross-talk between the p38 MAPK and the ERK pathways, which prevents ERK activity in FSDC stimulated with DNFB.

Several reports demonstrate that oxidants can activate MAPK in different cell types,[22–24,37,38] which prompted us to investigate whether ROS could mediate MAPK activation in FSDC. Inhibition of protein oxidation with GSH almost completely inhibited DNFB-induced p38 MAPK and ERK1/2 activation (Fig. 2). In contrast, inhibition of lipid peroxidation with vitamin E did not affect MAPK activation (Fig. 3).

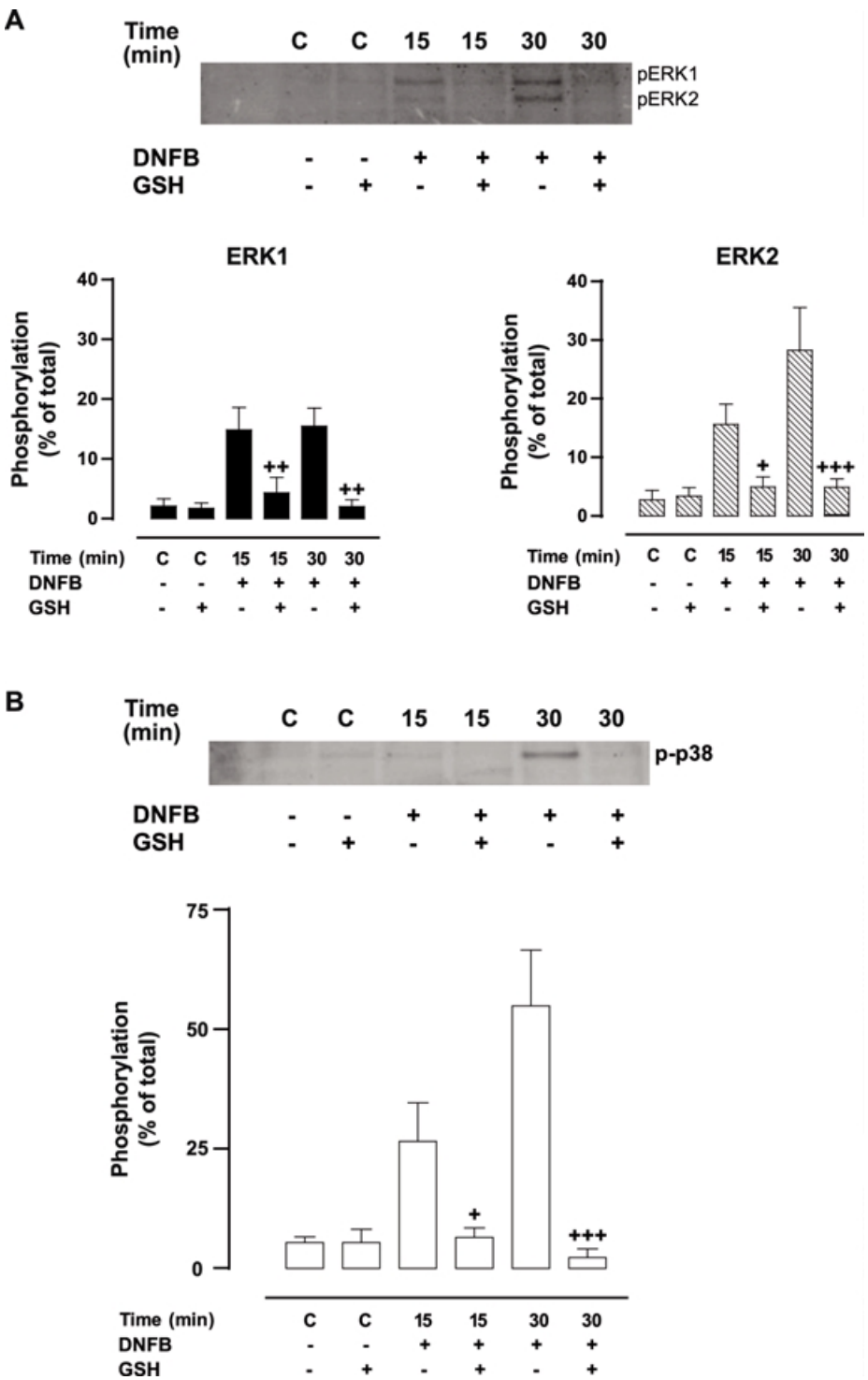

Figure 2 Reduced glutathione (GSH) prevents the phosphorylation of ERK1/2 (A) and p38 mitogen-activated protein kinases (MAPK) (B) induced by 2,4-dinitrofluorobenzene (DNFB). Cells were stimulated or not with 5 μg/mL of DNFB for 15 and 30 min, in the presence or not of 1 mmol/L GSH, and the cell lysates were collected at the indicated times after stimulation. Equal amounts of protein were loaded on 10% SDS–polyacrilamide gels, subjected to electrophoresis and electrotransferred to polyvinylidene difluoride (PVDF) membranes, before probing with a phospho-specific anti-ERK1/2 antibody (A) or anti-p38 MAPK antibody (B), as described in the Materials and methods. The results were quantified by scanning the membrane with a fluorescence scanner and analyzed using the ImageQuant software. The results were expressed as the percentage of phosphorylation relative to total in each experiment. Data are expressed as the mean ± SEM of four independent experiments. Statistical significance was calculated by the one-way anova test with Bonferroni's post-test ($^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ compared to the stimulus). C, control.

Therefore, the increase in ROS induced by DNFB in FSDC plays an important role in the activation of p38 MAPK and ERK1/2.

The effect of GSH on MAPK activation is in agreement with another study where the effect of antioxidants on tyrosine phosphorylation induced by several sensitizers was analyzed, using human monocyte-derived DC. In this study, *N*-acetyl-L-cysteine (NAC; an antioxidant that can act as precursor for reduced GSH) and GSH inhibited tyrosine phosphorylation induced by stimulation of the cells with the sensitizer methylchloroisothiazolinone/methylisothiazolinone (MCI/MI), whereas vitamin E had no effect.[39] Another recent study also suggested that, as we demonstrated, p38 MAPK activity could be increased by ROS, which mediated the DNCB-induced phenotypic changes in a BC1 cell line.[26] However, in this study the direct effect of ROS on p38 MAPK activation was not investigated. Furthermore, only NAC was used as an antioxidant.

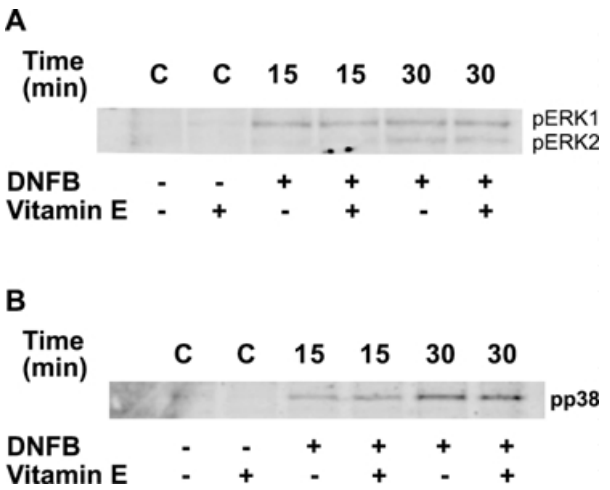

Figure 3 Vitamin E does not affect significantly the phosphorylation of ERK1/2 (A) and p38 MAPK (B) induced by 2,4-dinitrofluorobenzene (DNFB). Cells were stimulated or not with 5 μg/mL of DNFB in the presence or not of 1 mmol/L vitamin E, and cell lysates were collected at the indicated times after stimulation. Equal amounts of protein were loaded on 10% SDS–polyacrylamide gels, subjected to electrophoresis and electrotransferred to polyvinylidene difluoride (PVDF) membranes, before probing with a phospho-specific anti-ERK1/2 antibody (A) or anti-p38 mitogen-activated protein kinase (MAPK) antibody (B), as described in the Materials and methods. Data are representative of three independent experiments. C, control.

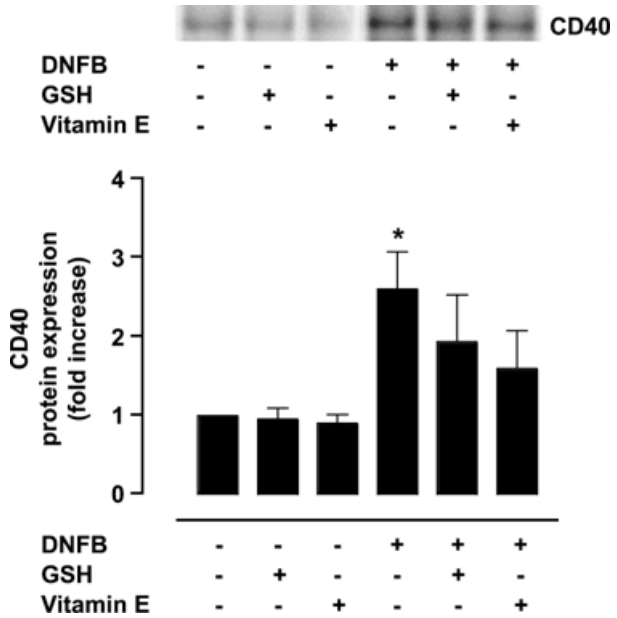

Figure 4 Vitamin E and glutathione (GSH) partially affect 2,4-dinitrofluorobenzene (DNFB)-induced increase of CD40 protein levels in the fetal skin derived dendritic cell (FSDC) line. Cells were stimulated or not with 5 μg/mL of DNFB in the presence of vitamin E (1 mmol/L) or GSH (1 mmol/L), and collected after 2 h of stimulation. Equal amounts of protein were loaded on 10% SDS–polyacrylamide gels, subjected to electrophoresis and electrotransferred to polyvinylidene difluoride (PVDF) membranes, before probing with an anti-CD40 antibody. The results were quantified by scanning the membrane with a fluorescence scanner and analyzed using the ImageQuant software. The results were expressed as fold increase relative to the control in each experiment. Data are expressed as the mean ± SEM of four independent experiments. Statistical significance was calculated by the one-way ANOVA test with Bonferroni's post-test (*$P < 0.05$ compared to the control).

CD40 is a membrane-associated receptor that is upregulated in DC during maturation induced by several stimuli, including skin sensitizers.[8,17] In the present work, we found that the upregulation of CD40 protein levels by DNFB was only slightly changed in the presence of antioxidants (Fig. 4), suggesting that other signalling pathways must be involved. In contrast to our results, one report described that NAC and GSH reduce CD40 expression on murine DC stimulated with TNF-α.[31] Previous studies showed that CD40 protein levels are upregulated in FSDC by a mechanism partially dependent of p38 MAPK, while activation of ERK had no effect.[29] This correlates with previous studies describing no effect of ERK on DC maturation.[7,40,41] However, other studies described a role for ERK in cytokine release,[25] while others described a role in the downregulation of monocyte-derived DC maturation.[34] In FSDC, the role of ERK activation induced by DNFB remains unclear.

The proinflammatory cytokine IL-1β is one of the first to be produced by DC[42] upon challenge with chemical sensitizers. This cytokine induces the production of ROS[43] that act as an intracellular second messenger mediating the biological response to IL-1β.[44] In addition, ROS are involved in LPS-mediated regulation of IL-1 expression in macrophages.[37] Taken together, these studies suggest that ROS may act to induce IL-1 production and to mediate its effect. In fact, DNFB increases production of IL-1β and upregulates IL-1 receptor protein levels in FSDC,[45] suggesting that ROS may be involved in the regulation of both IL-1β production and the response to the cytokine.

We have previously found that DNFB activates the transcription factor NF-κB in FSDC.[28] Because NF-κB activation may be regulated by the production of ROS[46] in the immune response, it can be hypothesized that ROS may be involved in DNFB-induced NF-κB activation in FSDC.

In conclusion, we have shown that DNFB induces protein oxidation in FSDC, upstream of the activation of p38 MAPK and ERK1/2. In contrast, ROS do not seem to play an important role in DNFB-induced upregulation of CD40 expression. This indicates that the intracellular redox status of the cell is important in mediating the activation of the MAPK signalling pathways.

Acknowledgements

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