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HMOX1 and NQO1 Genes are Upregulated in Response to Contact Sensitizers in Dendritic Cells and THP-1 Cell Line: Role of the Keap1/Nrf2 Pathway

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Electrophilicity is one of the most common features of skin contact sensitizers and is necessary for protein haptenation. The Keap1 (Kelch-like ECH-associated protein 1)/Nrf2 -signaling pathway is dedicated to the detection of electrophilic stress in cells leading to the upregulation of genes involved in protection or neutralization of chemical reactive species. Signals provided by chemical stress could play an important role in dendritic cell activation and the aim of this work was to test whether contact sensitizers were specific activators of the Keap1/Nrf2 pathway. CD34-derived dendritic cells (CD34-DC) and the THP-1 myeloid cell line were treated by a panel of sensitizers (Ni, 1-chloro 2,4-dinitrobenzene, cinnamaldehyde, 7-hydroxycitronellal, 1,4-dihydroquinone, α-methyl-*trans*-cinnamaldehyde, 2-4-tert-(butyl-benzyl)propionaldehyde or Lilial, and 1,4-phenylenediamine), irritants (sodium dodecyl sulfate, benzalkonium chloride), and a nonsensitizer molecule (chlorobenzene). Three well-known Nrf2 activators (tert-butylhydroquinone, lipoic acid, sulforaphane) were also tested. Expression of *hmox1* and *nqo1* was measured using real-time PCR and cellular accumulation of Nrf2 was assessed by Western blot. Our results showed an increased expression at early time points of *hmox1* and *nqo1* mRNAs in response to sensitizers but not to irritants. Accumulation of the Nrf2 protein was also observed only with chemical sensitizers. A significant inhibition of the expression of *hmox1* and *nqo1* mRNAs and CD86 expression was found in 1-chloro 2,4-dinitrobenzene–treated THP-1 cells preincubated with N-acetyl cysteine, a glutathione precursor. Altogether, these data suggested that the Keap1/Nrf2-signaling pathway was activated by electrophilic molecules including sensitizers in dendritic cells and in the THP-1 cell line. Monitoring of this pathway may provide new biomarkers (e.g., *Nrf2*, *hmox1*) for the detection of the sensitization potential of chemicals.

Key Words: skin sensitization; *Nrf2*; *hmox1*; oxidative stress.

Allergic contact dermatitis (ACD) is a complex skin pathology occurring in reaction against environmental substances found in the workplace (cements, hair dyes, textile dyes) or in the private environment (e.g., household products, cosmetic ingredients). Dendritic cells (DCs) are playing a key role in ACD. Indeed, in the presence of chemical sensitizers, DCs migrate from the skin to the draining lymph nodes and present the hapten to T cells leading to T lymphocyte activation and proliferation of specific hapten T-cell clones (Ryan *et al.*, 2007). This profound modification of DC function is performed by numerous biomolecular changes including the downregulation of CCR6 and E-cadherin, and the upregulation of molecules such as CCR7, major histocompatibility complex class II, CD86, CD54, CD80, CD40, interleukin (IL)-1β, and IL-12 (Krasteva *et al.*, 1999).

Skin chemical sensitizers compose a wide family of structurally unrelated low molecular weight compounds (Divkovic *et al.*, 2005). However, they share two common features: hydrophobicity and electrophilicity. Electrophilic properties of chemical allergens are at the basis of their reactivity against nucleophilic groups leading to protein haptenation and immunogenicity of the protein-hapten complex. Mizuashi *et al.* (2005) recently showed in human monocyte-derived dendritic cells (Mo-DC) that chemical sensitizers induced oxidative stress using the glutathione GSH/GSSG ratio as a redox marker. Moreover, these authors also showed that reduction of the glutathione GSH/GSSG ratio was accompanied by CD86 upregulation and p38 mitogen-activated protein kinase (p38 MAPK) phosphorylation, suggesting that the electrophilic properties of chemical sensitizers may be perceived by DCs as a danger signal leading to DC maturation (Sasaki and Aiba, 2007).

Many signalling pathways are known to be redox-sensitive, including proteins affected by the redox potential (nuclear factor-kappa B [NF-κB], activator protein-1, signal transducer and activator of transcription, p38 MAPK) or proteins more directly involved in oxidative stress detection (Keap-1 [Kelch-like ECH-associated protein 1]/Nrf2, hypoxia inducible factor-1, thioredoxin). Such pathways are affected by redox potential through various mechanisms including direct protein modification,

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alteration of protein expression and/or nuclear accumulation, phosphorylation status and transcriptional activity (Kim and Surh, 2006). Among them, the Keap1/Nrf2 pathway is a dedicated cellular signalling pathway for the detection of endogenous or exogenous electrophiles and is therefore implicated in the regulated response of the cell to pro-oxidant and electrophilic aggressions (for reviews, see Dinkova-Kostova *et al.*, 2005; Lee *et al.*, 2005). Briefly, in the absence of electrophilic stress, Keap1 is associated with Nrf2 mostly in the cell cytoplasm targeting Nrf2 to proteosomal degradation (Furukawa and Xiong, 2005). In the presence of an electrophilic compound, Keap1 conformation is modified probably through electrophilic attack of its cysteine-rich domain leading to Nrf2 release and translocation to the nucleus. In the nucleus, Nrf2 acts as a transcription factor and binds to antioxidant response element/electrophile response elements participating to the transcription of target genes mainly coding for phase II detoxication enzymes (catalase, heme oxygenase-1, glutathione S-transferase α, nicotinamide adenine dinucleotide phosphate quinone oxidoreductase 1).

Recently, a number of reports suggested that chemical sensitizers may activate the Nrf2 pathway. Ryan et al. (2004) showed upregulation of ARE-inducible genes in microarray studies using peripheral blood-derived DC treated with dinitrobenzenesulfonic acid for 24 h. DNCB (1-chloro 2,4-dinitrobenzene), a well-described strong sensitizer, is classically used as a Keap1 ligand (Dinkova-Kostova *et al.*, 2002). Other sensitizers such as nickel or eugenol have been shown to activate Nrf2 in MCF-7 cells or human monocytic cells (Han *et al.*, 2007; Lewis *et al.*, 2006).

The main objective of this work was to address the hypothesis that contact sensitizers are potent inducers of the Keap1/Nrf2 pathway. Cord blood CD34+-derived dendritic cells (CD34-DC) and THP-1 cells were used because they are widely used in the field of contact sensitization. In both models, it has been shown that chemical sensitizers provoked phenotypic modification with upregulation of membrane molecules such as CD86. Expression of Nrf2-dependent ARE-responsive genes (*hmox1* and *nqo1*) was measured using real-time PCR in response to a panel of molecules including chemical sensitizers, irritants, well-described activators of the Nrf2 pathway, and a nonsensitizer. Cellular accumulation of Nrf2 was also assessed in both cell types as a marker of Keap1/Nrf2 modifications. Finally, the impact of an antioxidant (N-acetyl cysteine [NAC]) was assessed in the THP-1 cell line on both mRNA expression and CD86 expression. Our results suggest that the Keap1/Nrf2-signalling pathway is specifically activated by skin sensitizers.

MATERIAL AND METHODS

Chemicals. Dimethyl sulfoxide (DMSO) (endotoxin-tested, hybridoma tested, cell culture tested) was from Sigma-Aldrich (St Quentin-Fallavier, France). Chemical sensitizers used were the following: extreme/strong, DNCB (cas 97-00-7), 1,4-dihydroquinone (HQ, cas 123-31-9), 1,4-phenylenediamine (pPD, cas 106-50-3); moderate, cinnamaldehyde (CIN, cas 104-55-2), α-methyl-*trans*-cinnamaldehyde (MCIN, cas 101-39-3), nickel sulfate (Ni, cas 10101-97-0) and weak, 2-4-tert-(butylbenzyl)propionaldehyde or Lilial (Lili, cas 80-54-6), 7-hydroxycitronellal (HCIT, cas 107-75-5), irritant molecules employed in this study were sodium dodecyl sulfate (SDS, cas 151-21-3), benzalkonium chloride (BZK, cas 8001-54-5). The nonsensitizer employed in this study was chlorobenzene (ChlB, cas 108-90-7) well-known Nrf2 activators were also tested: tert-butylhydroquinone (tBHQ, cas 1948-33-0), lipoic acid (Lipa, cas 1077-28-7), sulforaphane (SUL, cas 142825-10-3). All chemicals were from Sigma-Aldrich at the highest possible purity (often > 98%).

For the THP-1 model, all chemicals were dissolved in DMSO at a 1000× stock except BZK and Ni that were solubilized in saline. All vehicles were used at a 0.05% final concentration in culture.

Preparing human DC from cord blood. Human umbilical cord blood was obtained from Biopredic International (Rennes, France) and processed within 24 h. Cord blood samples were diluted 1:3 in phosphate-buffered saline (PBS). After separation on Ficoll-Hypaque gradient (lymphocyte separation medium LSM 1077, PAA, Les Mureaux, France), mononuclear cells were collected and washed three times in PBS supplemented with 2% of heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, Saint-Quentin, France). $CD34^+$ hematopoietic cells were isolated using MiniMACS separation columns (Miltenyi Biotec, Bergish, Germany) through magnetic positive selection using the direct CD34 progenitor cell isolation kit (Miltenyi Biotec). After purification, the isolated cells were 80%–95% $CD34^+$ cells. $CD34^+$ cells were adjusted to the concentration of 1.5×10^5 cells/ml and cultured at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 Glutamax I medium (Gibco, Invitrogen, Paisley, UK), 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1mM sodium pyruvate (Gibco Invitrogen), and supplemented with 200 U/ml granulocyte macrophage-colony-stimulating factor (GM-CSF) (Abcys SA, Paris, France), 50 U/ml recombinant human tumor necrosis factor (rhTNF)-α (R&D Systems, Lille, France), 50 ng/ml stem cell factor (SCF) (Abcys SA), and 50 ng/ml Flt3 ligand (Flt3-L, Peprotech, Tebu, Le Perray-en Yvelines, France). From day 4 to day 7, cells were diluted 1:2 each day by adding complete RPMI medium. At day 4, the added volume was supplemented with GM-CSF (200 U/ml), rhTNF-α (50 U/ml), and IL-4 (2000 U/ml).

Cell culture and chemical treatment. THP-1 cells (ATCC ref: TIB-202, American Tissue Culture Collection, Manassas, VA) were cultured and maintained at a cell density between 2×10^5 cells/ml and 1×10^6 cells/ml in RPMI 1640 with Glutamax complemented with 10% FCS under classical cell culture protocols and facilities. Frozen stocks were regularly thawed to avoid high passages subcultures. For induction studies, cells were grown for at least 24 h and collected, counted, and seeded in fresh medium at a density of 8×10^5 cells/ml. Chemicals were added to fresh medium at a 2× the final concentration with an equal volume of cell suspension. The final concentration of cells and chemicals was therefore 4×10^5 cells/ml and 1×, respectively. Cells were treated at different time points depending on the experiments. Cell viability after treatment with the different molecules was never less than 70% as assessed by propidium iodine (CD34-DC) or 7-AAD (7-amino-actinomycin D) staining (THP-1). In the case of CD34-DC and DNCB treatment, cells were washed after a 30 min treatment and left for the remaining time of the experiment.

Flow cytometry. For the THP-1 model, after 24 h incubation with chemicals, cells were centrifuged and washed with PBS complemented with 1% FCS and 0.1% sodium azide. For labeling, 2.5×10^5 cells were incubated 30 min on ice in the presence of 5 μl of the antibody. CD86-PE and the corresponding isotype control were from BD Biosciences (San Jose, CA). 7-AAD (Beckman Coulter, Villepinte, France) was added to each tube (5 μl) for viability determination. Cytofluorometry acquisitions were performed on a FC500 cytometer (Beckman Coulter) and data analysis was performed only on 7-AAD negative cells (viable cells) using the CXP software.

For the CD34-DC model, cell staining was performed using mouse mAb PE-anti-human-CD86 (B-T7, Diaclone, Besançon, France) and the isotype control PE-IgG1 (B-Z1, Diaclone). Cell labeling procedures were identical to the one used for THP-1 but cell viability was determined using propidium

iodide (10 μg/ml) (Invitrogen, Eugene, OR). Results were then analyzed using the CellQuest Software (Becton Dickinson, San Jose, CA) based on a collection of 1×10^4 cells with a FACScalibur flow cytometer (Becton Dickinson).

Results were expressed using RFI (relative fluorescence intensity) calculated with the following formula or cMFI (corrected mean fluorescence intensity [MFI]):

$RFI = (MFI\ spe - MFI\ iso)^{treated}/(MFI\ spe - MFI\ iso)^{vehicle}$ where MFI is the total mean fluorescence intensity of samples labeled with isotype (iso) or antigen-specific antibody (spe).

mRNA expression analysis using semi-quantitative RT-PCR. For the CD34-DC model, cells were prepared in TRIzol Reagent (Invitrogen) and total RNA was isolated as described by Chomczynski and Sacchi (1987). Reverse transcription was performed in a total 25-μl reaction mixture containing: 2 μg of total RNA, 1× avian myeloblastosis virus (AMV) reverse transcriptase Buffer (Promega, San Luis Obispo, CA), 2mM of each deoxy-nucleotide triphosphate (dNTP) (Promega), 4μM of oligo d(T) (MWG Biotech, Ebersberg, Germany), 20 U of RNase Inhibitor (RNasin, Promega), and 2 U of AMV reverse transcriptase (Promega). After the reverse transcription reaction, 2.5 μl of first strand cDNA were transferred to the following PCR mix: 1× PCR Buffer (Qbiogen, Montreal, Canada), 2mM of each dNTP, 1mM of each specific primer, and 1.25 U of Taq Polymerase (Qbiogen). Specific primers were used in the PCR reaction mixture (forward and reverse primers, respectively): *hmox*: 5′-CCC ACG CCT ACA CCC GCT AC-3′ and 5′-GGT GGC ACT GGC AAT GTT GG-3′/*nqo1*: 5′-GGG CAA GTC CAT CCC AAC TG-3′ and 5′-GCA AGT CAG GGA AGC CTG GA-3′/β-*actin*: 5′-GGG TCA GAA GGA TTC CTA TG-3′ and 5′-GGT CTC AAA CAT GAT CTG GG-3′. The number of cycles and the hybridization temperature used for the PCR were optimized for all the genes studied: *hmox* (30 cycles, 66.6°C), *nqo1* (27 cycles, 64.5°C), and β-*actin* (25 cycles, 55°C). Sixteen microliters of the PCR product was mixed with 4 μl of loading buffer and visualized after migration on a 2% agarose gel in 1× Tris-acetate-EDTA containing 0.3 μg/ml ethidium bromide under short-wavelength ultraviolet.

mRNA expression using real-time PCR. For the CD34-DC model, real-time PCR analysis was performed using the SYBR Green technology on a LightCycler rapid thermal cycler (Roche Diagnostics, Meylan, France). Expressions of *hmox1*, *nqo1*, and β-*actin* mRNAs were measured using the Fast start DNA Master Plus SYBRGreen Kit (Roche Diagnostics) and specific primers: *hmox1*: 5′-GGC CTG GCC TTC TTC ACC TT-3′ and 5′-GAG GGG CTC TGG TCC TTG GT-3′/*nqo1*: 5′-GGG CAA GTC CAT CCC AAC TG-3′ and 5′-GCA AGT CAG GGA AGC CTG GA-3′/β-*actin*: 5′-GGC ATC CTC ACC CTG AAG TA-3′ and 5′-GCA CAC GCA GCT CAT TGT AG-3′. Each sample was monitored in duplicate.

For the THP-1 model, $3–4 \times 10^6$ cells were collected per samples and cells were lysed at 4°C (cytoplasmic RNA preparation protocol, Qiagen, Courtaboeuf, France). RNA extraction was performed using a column-based RNA extraction kit (RNeasy kit, Qiagen). Total RNA content was measured at 230, 260, 280 nm by spectrometry for quantification and quality assessment. Then, 1 μg total RNA was reverse-transcribed using the Superscript II reverse-transcription kit (Invitrogen, Paisley, UK). 1/50th of each reverse transcription was then used for real-time PCR analysis. Real-time PCR was performed on a LightCycler apparatus using the Fast Start DNA Master plus SybrGreen kit (Roche, Mannheim, Germany). Expression of *gapdh*, *hmox1*, and *nqo1* mRNAs were monitored on each sample ran in duplicate. The primer sequences were identical to the one used for the CD34-DC model. For THP-1 cells, *gapdh* was used as the house-keeping gene, the primer sequence was (forward and reverse primer, respectively): 5′-ACTGGCGCTGCCAAGGCTGT-3′ and 5′-GCCCCAGCGTCAAAGGTGGA-3′).

For both models, the results were expressed as fold factor calculated by comparing the Ct values obtained from treated and untreated (vehicle) samples and corrected for *gapdh* or β-*actin* expression. To note, *gapdh* and β-*actin* expressions were not significantly affected by any of the compounds used in this study.

Immunoblotting. For the CD34-DC model, western blot analysis was performed according to a standard procedure previously described (Ade *et al.*, 2007). Briefly, cells were washed with cold PBS and cell lysates were prepared by resuspending the cell pellet containing 2.5×10^6 cells in 150 μl of Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA) supplemented with β-mercaptoethanol (Sigma) and incubated for 4 min at 100°C followed by centrifugation at 17,600 g for 20 min. Thirty microliters was then subjected to 10% SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto polyvinylene difluoride membranes (Amersham Biosciences, Les Ulis, France) and the membranes were probed with a rabbit anti-Nrf2 monoclonal Ab (H-300, Santa Cruz Biotechnology, Santa Cruz, CA) followed by a goat anti-rabbit polyclonal Ab conjugated to horseradish peroxidase (Cell Signaling Technology, Ozyme, St-Quentin-en Yveline, France). Membranes were stripped for the primary antibodies and reprobed with a mouse anti-β-tubulin antibody (TUB 2.1, Sigma) as a loading control. Immunoblots were visualized using enhanced chemiluminescence (Amersham Biosciences).

For the THP-1 model, a similar protocol was performed except for the use of a nitrocellulose membrane for protein transfer (Amersham Biosciences) and the use of mouse anti-β-actin antibody (clone AC-14, Sigma) as a loading control.

Data analysis. Results are shown as mean ± SEM. Statistical differences between two groups (treated and control group) were evaluated using the Student's *t*-test. Differences were considered statistically significant when $p < 0.05$ and indicated by an asterisk (*).

RESULTS

Phenotypical Changes by Contact Sensitizers in CD34-DC

In CD34-DC, Ni has been previously described to induce the expression of CD86, CD83, HLA-DR, and CD40 whereas DNCB induced mainly CD86 and CD83 (Ade *et al.*, 2007). In this work, we focused our investigations on CD86 expression in response to the following chemicals sensitizers: Ni, DNCB, CIN, and pPD. Two irritants, SDS and BZK were also tested. CD34-DCs were differentiated for 7 days using GM-CSF, TNF-α, SCF, and Flt3-L. IL-4 was added at day 4 to reduce the number of residual $CD14^+$ cells (data not shown). At day 7, CD34-DCs were incubated for an additional 24-h period in the presence or in the absence of the chemicals. The highest chemical concentration used was selected based on a cell viability above 70% (Table 1). Results showed that the expression of CD86 was significantly increased in CD34-DC treated with Ni, DNCB, and pPD ($p < 0.05$). CIN slightly augmented the expression of CD86. In contrast, SDS and BZK did not modify CD86 expression.

Contact Sensitizers Induce hmox1 and nqo1 mRNA Expression in CD34-DC

Contact sensitizers are believed to possess an intrinsic chemical reactivity leading to cellular stress. The Keap1/Nrf2 pathway is known to play a major role in cellular stress. To investigate the role of the Keap1/Nrf2 pathway, we chose two target genes of Nrf2: *hmox1* and *nqo1*. CD34-DCs were treated for 8 h with different chemical concentrations and mRNA expression was measured using semi-quantitative RT-PCR. As shown in Figure 1, expression of *hmox1* and *nqo1* mRNAs was induced following treatment with all the contact sensitizers tested: Ni, pPD, DNCB, and CIN (Fig. 1A). SDS and BZK did not modify the expression of *hmox1* and *nqo1*mRNAs (Fig. 1A).

TABLE 1
Expression of CD86 in CD34-DC after Treatment with Chemicals Sensitizers

	Viability[a] %	CD86[b] %	CD86[b] cMFI	CD86[b] Fold increase
V	87.4 ± 1.9	31.0 ± 2.0	5.2 ± 1.1	
DNCB, 25μM	72.1 ± 2.1	45.0 ± 4.6*	15.0 ± 4.0*	4.18
V	90.7 ± 1.8	22.8 ± 7.7	4.2 ± 0.7	
CIN, 100μM	68.4 ± 4.8	33.0 ± 6.4	7.1 ± 1.3	2.45
V	82.6 ± 0.1	23.9 ± 8.6	4.3 ± 1.5	
$NiSO_4$, 500μM	74.1 ± 3.8	58.1 ± 6.6*	20.6 ± 7.9*	11.65
V	83.6 ± 2.9	36.4 ± 1.1	5.9 ± 0.8	
PPD, 75μM	72.9 ± 5.2	42.5 ± 1.6*	9.8 ± 2.5*	1.94
V	92.8 ± 2.2	35.1 ± 3.6	5.4 ± 0.4	
SDS, 250μM	77.0 ± 0.1	31.9 ± 6.2	5.2 ± 1.0	0.88
V	81.9 ± 1.7	30.2 ± 5.0	5.8 ± 0.7	
BZK, 2 μg/ml	72.6 ± 0.3	30.5 ± 13.0	5.0 ± 1.7	0.87

Note. *$p < 0.05$.

[a]Viability: Percentage of viable cells was determined using propidium iodide. %: percentage of propidium iodide negative cells.

[b]CD86 expression was analyzed by flow cytometry. %: percentage of CD86 positive cells. cMFI: mean fluorescence intensity corrected for the background intensity of isotype control antibodies. Fold increase: calculated according the formula: $FI = (\%CD86 \times cMFI)^{treated}/(\%CD86 \times cMFI)^{vehicle}$. Results are means of three independent experiments ± SD.

These results were confirmed using real-time PCR showing that all contact sensitizers were able to induce a concentration-dependent increase of *hmox1* and *nqo1* mRNAs (Figs. 1B and 1C, respectively). CIN strongly upregulated *hmox1* mRNA with a 94-fold induction at 100μM, whereas Ni augmented *hmox1* expression by a 2.8-fold at 500μM. Neither SDS nor BZK induced *hmox1* mRNA expression. *Nqo1* mRNA level was also significantly augmented in response to Ni, pPD, and CIN in a concentration-dependent manner (Fig. 1C). As for the *hmox1* gene, *nqo1* mRNA expression was not affected by irritants whatever was the concentration used. These results clearly showed that chemical sensitizers but not irritants were able to induce the transcription of Nrf2 target genes.

Contact Sensitizers Augment the Nrf2 Protein Level in CD34-DC

In the absence of any electrophilic stress, Keap1 allows a rapid ubiquitin-mediated proteasomal degradation of Nrf2. Upon electrophilic stress, Keap1 detaches from Nrf2 leading to Nrf2 accumulation, nuclear translocation and activation of Nrf2 specific target genes. CD34-DC were treated for 5 h with optimal concentrations of chemicals and Nrf2 protein level was measured by Western blotting. As expected, there was no detectable Nrf2 protein in cells treated with the vehicle (Fig. 2). Following treatment with tBHQ or with the contact sensitizers (pPD, DNCB, Ni, CIN), Nrf2 protein expression was detected suggesting an accumulation of Nrf2 in CD34-DC. In contrast, irritants such as SDS and BZK had no effect on the protein level of Nrf2. These results indicated that chemical sensitizers were able to augment specifically the level of Nrf2.

The Nrf2-Dependent Genes, hmox1 and nqo1, are Upregulated by Chemical Sensitizers in the THP-1 Cell Line

To test whether the observations made in CD34-DC were applicable to a cell line model currently under evaluation for the prediction of the sensitizing properties of chemicals, we performed a similar study in THP-1 cells. Expression of *hmox1* and *nqo1* mRNAs were investigated in response to a panel of chemicals including well-known Nrf2 activators, weak to extreme sensitizers, irritants, and nonsensitizers (see Chemicals in Material and Methods for detailed description). As for CD34-DC, the highest chemical concentration used was selected to ensure a cell viability above 70% (data not shown). Due to differences in their kinetic of expression (data not shown), *hmox1* mRNA and *nqo1* mRNAs were measured after 6 h or after 24 h of treatment, respectively (Fig. 3). Well-described Nrf2 activators (tBHQ, Lipa, and SUL) induced *hmox1* mRNA overexpression with tBHQ >> Lipa > SUL (Fig. 3A). All the sensitizers tested upregulated *hmox1* although the effects mediated by HQ and HCIT were found not statistically significant due to experimental variability. Irritants (SDS, BZK) and nonsensitizer (ChlB) did not modify *hmox1* mRNA expression. Similar results are presented in Figure 3B showing *nqo1* mRNA expression after chemical exposure. Irritants (particularly SDS) slightly upregulated *nqo1* mRNA although it did not reach statistical significance. This effect of chemical irritants on *nqo1* mRNA expression, as compared with *hmox1* mRNA expression, may be the consequence of the longer time of exposure (24 vs. 6 h). CD86 expression was measured at 24 h for each experiment using flow cytometry to validate the concentrations used above. As shown in Figure 3C, all chemical sensitizers augmented CD86 expression. Interestingly, among the three Nrf2 activators used, tBHQ and Lipa augmented CD86 expression, whereas SUL did not.

Chemical Sensitizers Provoke the Accumulation of Nrf2 in THP-1 Cells

To define if the upregulation of *hmox1* and *nqo1* mRNAs was linked to Nrf2 activation, intracellular accumulation of Nrf2 was evaluated by Western blotting. In preliminary experiments using a specific anti-Nrf2 antibody and Western blotting, we found Nrf2 at 90 kDa in THP-1 cells (total cellular extracts) treated with tBHQ or MG132, a proteosomal inhibitor known to provoke Nrf2 accumulation (data not shown). We then measured Nrf2 protein level in THP-1 cells treated for 5 h with the chemical sensitizers, irritants, or nonsensitizers used above (Fig. 4). In all experiments, nontreated (NT) samples

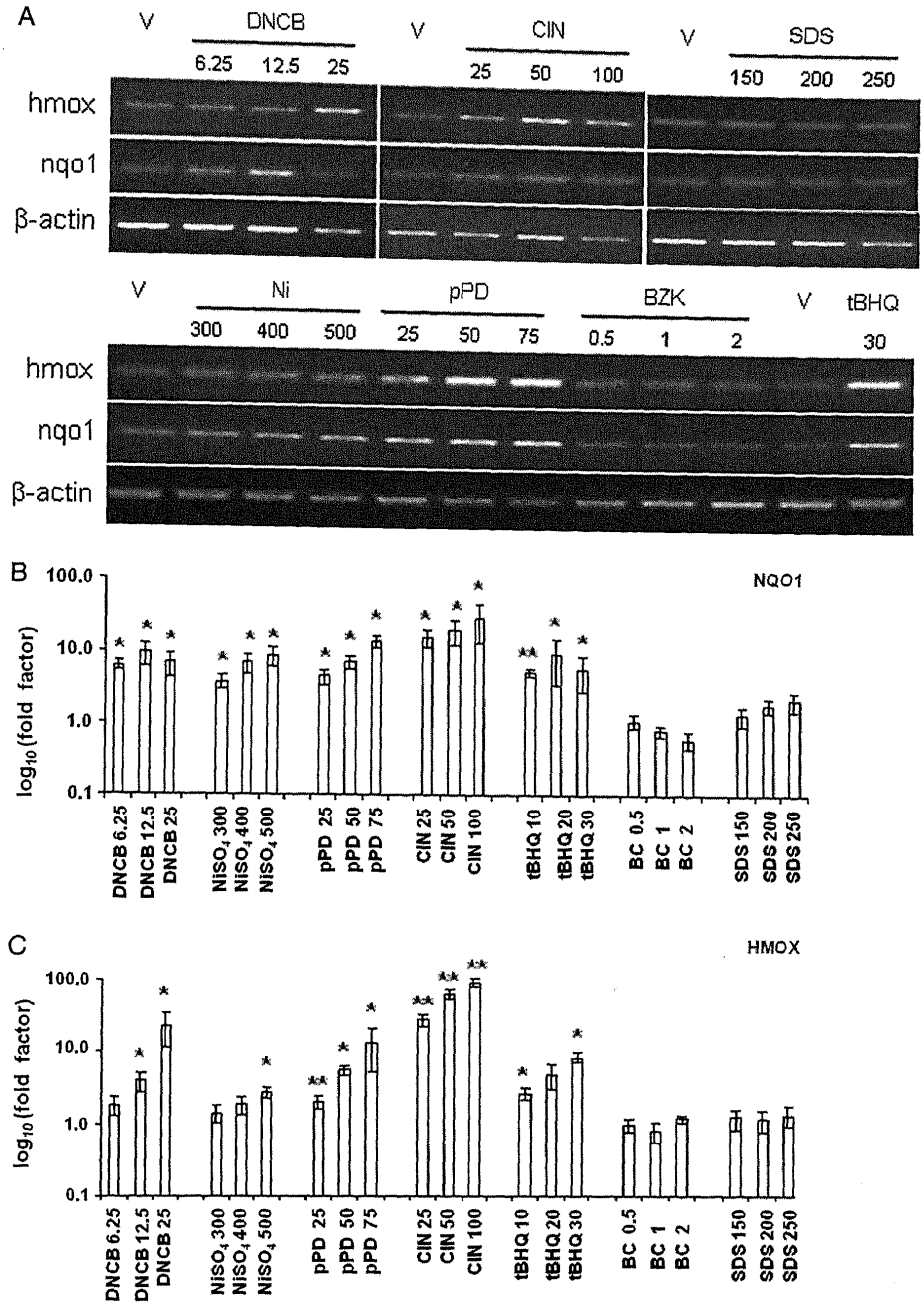

FIG. 1. Expression of *hmox1* and *nqo1* mRNAs in human CD34-DC in response to various concentrations of chemicals. Human CD34-DCs were treated for 8 h with various concentrations of chemicals ($NiSO_4$, DNCB, pPD, CIN, SDS, and BZK). tBHQ was used as a positive control. Concentrations were expressed in μM; only BZK concentrations were expressed in μg/ml. *hmox1* and *nqo1* mRNAs expression were evaluated using semi-quantitative RT-PCR. (A) mRNA expression of *hmox1* and *nqo1* was visualized on a 2% agarose gel. *Hmox1* (B) and *nqo1* (C) mRNA expressions were analyzed by real-time PCR. Results were expressed as fold induction compared with control samples and corrected by the expression of the house-keeping gene β-actin as described in materials and methods. Mean ± standard deviation of at least three independent experiments. $*p < 0.05$, $**p < 0.01$.

showed no accumulation of Nrf2. In contrast, high levels of the Nrf2 protein were found after treatment with the positive control tBHQ indicating a massive accumulation of Nrf2. Nrf2 protein level was also strongly augmented upon treatment with chemical sensitizers tested, except for HCIT and Lilial which generated a weak but significant Nrf2 increase. Interestingly, irritants used in similar conditions (same toxicity levels compared with sensitizers) did not generate any signal in our experiments. The nonsensitizer, ChlB, did not augment Nrf2 protein level. These results suggested that Nrf2 accumulation was a specific response to electrophilic molecules including chemical sensitizers.

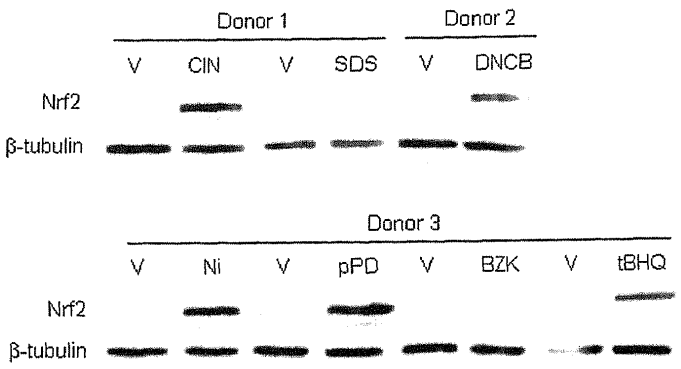

FIG. 2. Nrf2 protein level in human CD34-DC in response to chemical sensitizers. CD34-DC were treated for 5 h with $NiSO_4$ (500μM), DNCB (25μM), pPD (75μM), CIN (100μM), SDS (250μM), BZK (2 μg/ml), and tBHQ (30μM). Nrf2 protein level was determined by Western blotting. β-Tubulin was used as a loading control. V: Vehicle control cells incubated with 0.05% DMSO for DNCB and CIN, with 0.05% Ethanol for tBHQ and with culture media for $NiSO_4$, pPD, SDS, and BZK. Data are from a representative experiment out of two.

Nrf2 Activation by Chemical Sensitizers is Reversed by NAC Treatment

The observation that the Nrf2 protein accumulated in cells treated with chemical sensitizers suggested that these molecules induced an electrophilic stress leading to thiol depletion. Preincubation of cells with NAC (a precursor of glutathione) is often used to reinforce the redox potential of cells. We then tested if NAC was able to reverse the effects of DNCB on the Nrf2 pathway. Our results showed that 2-h pretreatment with NAC inhibited *hmox1* and *nqo1* mRNA expressions in response to DNCB (Fig. 5). NAC also significantly inhibited CD86 expression and cytotoxicity due to DNCB treatment. Interestingly, at a high DNCB concentration, NAC effects were no more observed suggesting saturation of thiol functions by DNCB. These observations were confirmed at the Nrf2 protein level suggesting a strong correlation between cell phenotype modifications and activation of the Nrf2 pathway (Fig. 6).

DISCUSSION

One of the well-accepted properties of chemical sensitizers is their ability to bind covalently to proteins enabling haptens to be immunogenic. This property of chemical sensitizers is mainly due to their electrophilicity although the mechanism of chemical sensitization is more complex because not all electrophilic molecules are chemical sensitizers. It is also now well-accepted that DCs need to receive signals from their environment to migrate and to present antigens to T lymphocytes located in the lymph nodes. These signals are mainly provided through Toll-like receptors that recognize specific structures of microbes or through pro-inflammatory cytokines. In the case of chemical sensitizers, the current hypothesis is that the chemical itself could provide a specific signal to the DC allowing its maturation (Casati *et al.*, 2005; Ryan *et al.*, 2007). Previous works have described the activation of MAPK and NF-κB by chemical sensitizers and their respective roles in DC maturation (Ade *et al.*, 2007; Trompezinski *et al.*, 2008). Activation of these pathways may represent the consequence of stress induced by chemicals. To manage this stress induced by chemical sensitizers, detoxication pathways may also be specifically activated in the DC. In this work, this question was addressed by looking at the activation of Nrf2 in DC by chemical sensitizers with the idea that the Keap1/Nrf2 pathway may be specifically activated by this category of chemicals.

Two different cellular models were used in this study: human CD34-DC obtained from human CD34+ progenitor cells and the human THP-1 myeloid cell line. Both CD34-DC and THP-1 have been shown to respond to chemical sensitizers. We used the CD34-DC model with a limited number of chemicals to test whether our hypothesis was valid in human primary cells. THP-1 cells were chosen because they are currently in the process for validation as an *in vitro* model for chemical sensitizer detection (Sakaguchi *et al.*, 2006). For this reason we decided to use a broader range of molecules (including those tested in CD34-DC) to test if genes regulated by the Nrf2 pathway could be used as new parameters for chemical sensitizer detection in *in vitro* models. Due to the use of these two models, time points for the measurement of *hmox1* and *nqo1* mRNAs expression were different for CD34-DC and THP-1 cells. The molecule tBHQ, a well-known activator of the Nrf2 pathway, was used to set up the optimal time for measuring mRNA expression in both models.

Our results showed that all the chemical sensitizers tested augmented the expression of mRNAs for *hmox1* and *nqo1* in both models. This increased expression was dose-dependent (e.g., in CD34-DC) and statistically significant. Moreover, in THP-1, additional molecules including weak haptens were also able to induce *hmox1* and/or *nqo1* mRNAs expression with the exception of HCIT. In response to chemical sensitizers, *hmox1* mRNA expression was strongly upregulated compared with *nqo1* mRNA without notable differences found in the quantity of Nrf2 protein in cell extracts suggesting that other pathways were mobilized by chemical sensitizers. Cinnamaldehyde, a moderate sensitizer, was the most potent inducer of *hmox1* and *nqo1* mRNAs in both models and we do not have yet an explanation for this observation. Future studies will try to elucidate the mechanism underlying these differences and if it could be related to the electrophilic potency of the molecule. However, with this set of molecules, there were no obvious correlation between the potency of the chemical sensitizers tested and the level of gene expression. Concerning the specificity of the response, *hmox1* mRNA was never induced by nonsensitizers or chemical irritants in both models. However, a weak but significant induction of the *nqo1* mRNA was observed in the THP-1 model in response to SDS. SDS has been shown to be a false positive in the local lymph

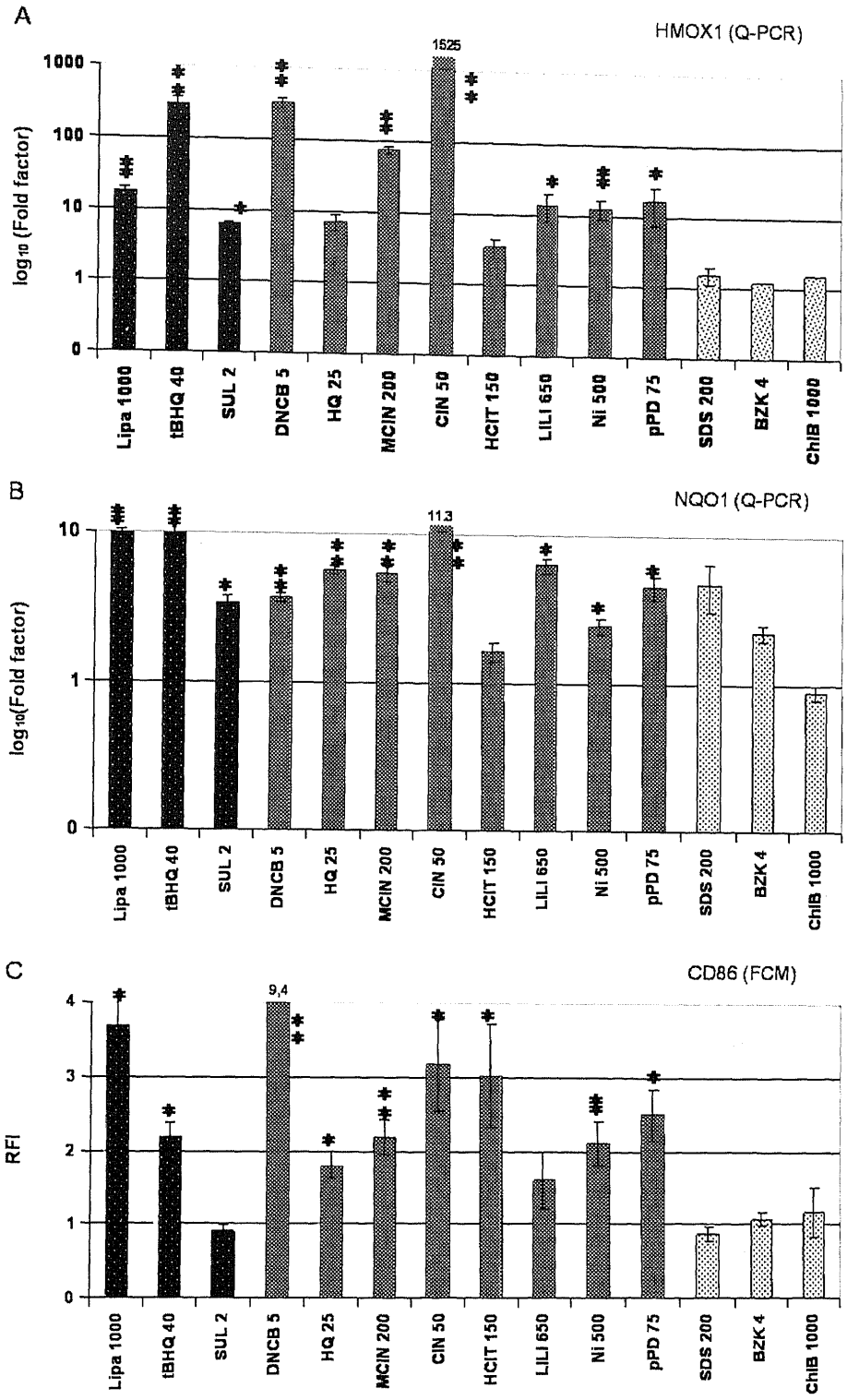

FIG. 3. Expression of *hmox1* and *nqo1* mRNAs by real-time PCR and CD86 protein by flow cytometry in THP-1 cells in response to a panel of chemicals. THP-1 cells were treated with a panel of Nrf2 activators (black bars), chemical sensitizers (gray bars), irritants, and nonsensitizer (white bars) at the indicated concentrations (in μM). Relative expression of *hmox1* and *nqo1* genes at the mRNA level was measured by real-time PCR after 6 h (A) or 24 h (B) of chemical treatment. Results were expressed as fold factor by comparison with untreated samples and corrected by the expression of the house-keeping gene *gapdh* as described in material and methods. As a control for cell activation, expression of the CD86 protein was measured in each experiment at 24 h using flow cytometry (C). Data presented in (C) were expressed using RFI (relative fluorescence intensity) as explained in material and methods. For all graphs, data were calculated as the mean ± standard deviation of at least three independent experiments. $*p < 0.05$, $**p < 0.01$. To note: Fold factor values for CIN (A, B) and DNCB (C) over the scale of the graph are indicated on top of the figure.

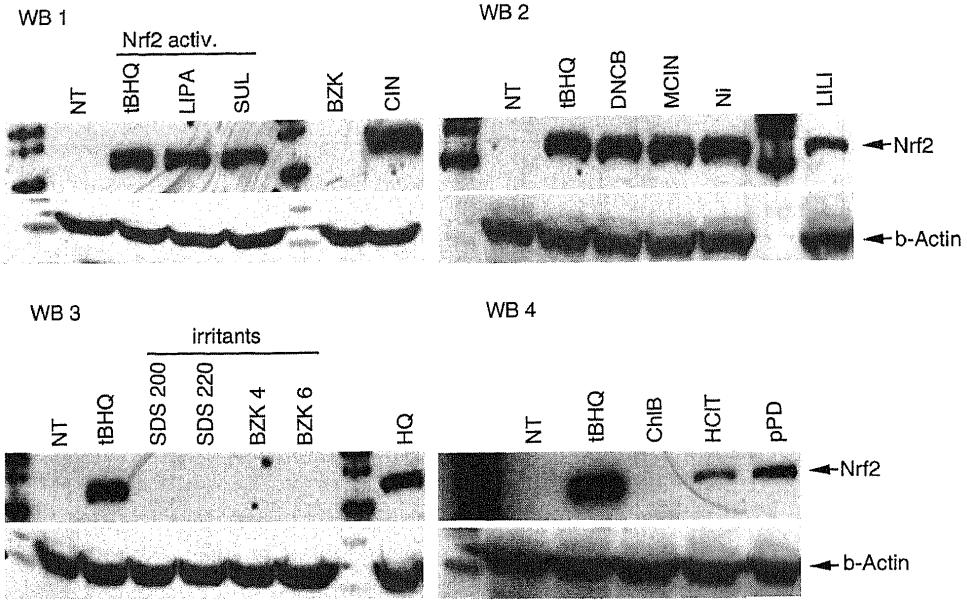

FIG. 4. Intracellular accumulation of the Nrf2 protein in THP-1 cells in response to a panel of chemicals. THP-1 cells were treated for 5 h with a panel of Nrf2 activators, sensitizers, irritants, and nonsensitizer at the same concentrations as in Figure 3. Total cellular lysates were analyzed by Western blotting as described in material and methods for the expression of Nrf2 (top) and β-actin (bottom). Four different Western blotting experiments are presented in this figure covering the full panel of chemicals used in this study. These experiments are representative of at least two independent experiments performed for each chemical.

node assay model but the mechanism of this effect is still unknown (Basketter *et al.*, 1998).

We then measured the level of the Nrf2 protein in cells treated with chemicals. At steady state, Nrf2 is associated with Keap1 a protein actively involved in Nrf2 ubiquitination leading to subsequent proteosomal degradation (Furukawa and Xiong, 2005; Kobayashi and Yamamoto, 2006). As a consequence, proteasome inhibitors and/or electrophilic molecules provoke a cytoplasmic accumulation of Nrf2 allowing its detection using Western blotting techniques. In unstressed conditions, there is no detectable level of the Nrf2 protein in most cell types (Itoh *et al.*, 2003). Our results were in agreement with these observations. No detectable level of the Nrf2 protein was found in non treated samples but high levels of the Nrf2 protein was observed on samples treated with MG132 (a proteosomal inhibitor, data not shown) or with known Nrf2 activators such as lipoic acid, tBHQ and sulforaphane. In these conditions, we showed that all sensitizers tested in both models induced Nrf2 protein accumulation. In addition, Nrf2 was not detected when cells were treated with irritants or nonsensitizer molecules. In contrast to what was observed for mRNA expression of *hmox1* and *nqo1*, little variations of the Nrf2 protein expression were observed between chemical sensitizers. These results suggested that additional mechanisms may play a role in the expression of these Nrf2 target genes by chemical sensitizers.

Interestingly, the apparent molecular weight of Nrf2 was different in CD34-DC and THP-1 cells, around 110 kDa in CD34-DC and 85 kDa in THP-1 cells. Such differences cannot be explained by protocol variations because the same antibody used for Western blotting experiments was utilized in both laboratories. Nrf2 molecular weight variations have been already observed by others and discussed by Li *et al.* (2005). In unstressed conditions, Keap1 functions as an adaptor for cullin 3–based E3 ligase, a complex responsible for ubiquitination of Nrf2 with the consequence that various forms of ubiquitinated Nrf2 can exist with different molecular weights. The predominance of a cell-type dependent poly-ubiquitinated form may explain our observations. However, it is important to note that for all chemical sensitizers tested the Nrf2 molecular weight was comparable in each model (CD34-DC or THP-1) suggesting common mechanisms of activation.

Accumulation of the Nrf2 protein was dose-dependently inhibited in THP-1 cells pretreated with NAC. NAC is classically used as a reducing agent and as a precursor of glutathione. Our data suggested that chemical sensitizers were able to alter the level of SH functions in DCs. Inhibition of DC activation by chemical sensitizers using NAC has already been observed and a relationship between redox imbalance and signalling pathway such as MAPKs has been established (Bruchhausen *et al.*, 2003; Mizuashi *et al.*, 2005; Trompezinski *et al.*, 2008). In this work, we extended these observations to the Keap1/Nrf2 pathway.

Taken together, our study points out the role of oxidative/electrophilic stress in the initiation of the sensitization phase of ACD and may provide a molecular basis in the case of chemical allergy to the formerly danger signal hypothesis formulated by Matzinger (1994). This concept has been evoked in the context of ACD but, at that time, it was the irritant properties of chemical sensitizers that were proposed to provide

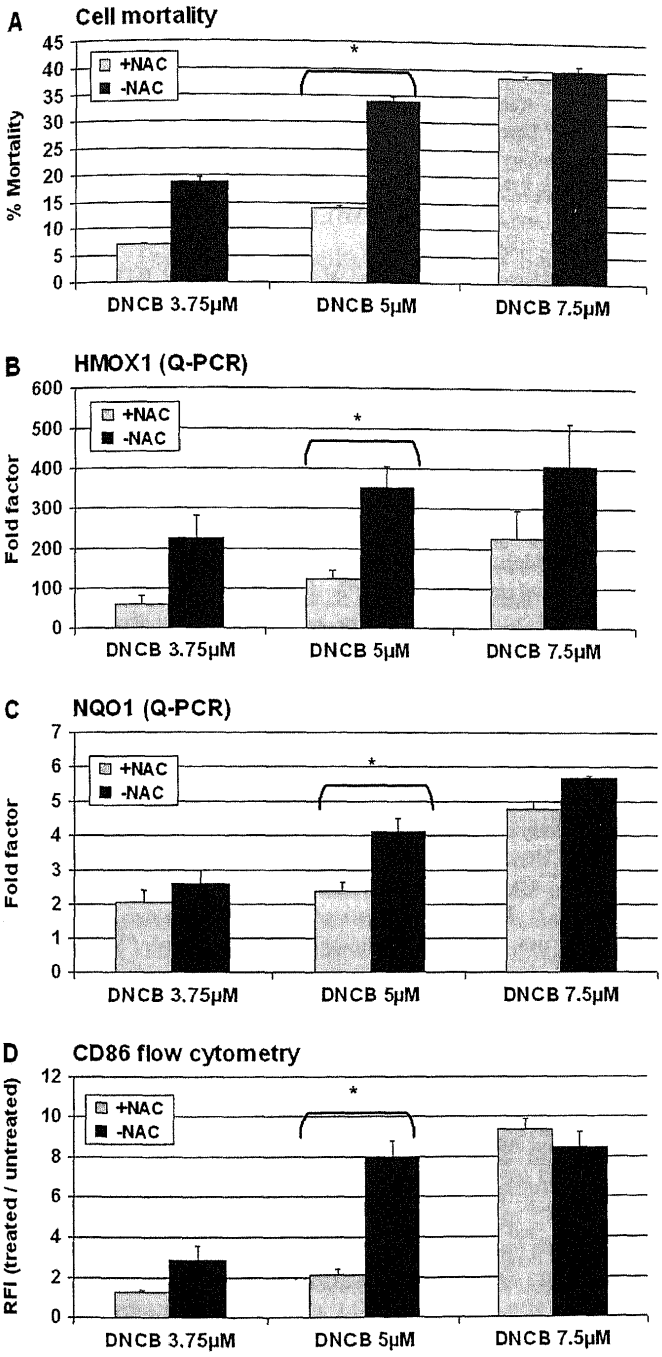

FIG. 5. Effect of NAC preincubation on *hmox1* and *nqo1* mRNAs and CD86 protein expression on THP-1 cells in response to DNCB. THP-1 cells were pretreated with (gray bars) or without (black bars) 25mM NAC for 2 h. After removal of NAC-containing medium, cells were incubated with 0, 3.75, 5, 7.5µM of DNCB for 6 or 24 h. *Hmox1* (B) mRNA expression was measured after 6 h; cell viability (A), *nqo1* mRNA (C) and CD86 protein (D) levels were measured after 24 h. Data were expressed as % of cell mortality compared with untreated samples (A), as fold factor (B and C), as RFI (D). Results represent the mean ± standard deviation of two independent experiments. $*p < 0.05$.

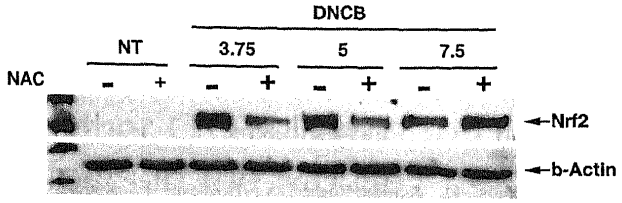

FIG. 6. Effect of NAC preincubation on Nrf2 protein accumulation induced by DNCB on THP-1. THP-1 cells were pretreated with (+) or without (−) 25mM NAC for 2 h. After removal of NAC-containing medium, cells were incubated with 0, 3.75, 5, 7.5µM of DNCB for 5 h (NT). Total cell lysates were analyzed for the expression of Nrf2 (top) and β-actin (bottom) by Western blotting. The Western blot is representative of two independent experiments.

the danger signal (McFadden and Basketter, 2000). More recently, Sasaki and Aiba (2007) re-examined this concept in the light of their observations concerning the putative role of redox imbalance provoked by sensitizers in generating this stress-related signal (Sasaki and Aiba, 2007). They proposed that one of the biological roles of ACD is a defense reaction against chemicals having the potential to alter the redox balance. This hypothesis suggested that the common electrophilic properties of chemical sensitizers, a family of structurally unrelated compounds, lead to the same biological response in DC. Our observations showing that Nrf2 is activated specifically by chemical sensitizers and not by irritants in DCs, are in favor of an electrophilic-based cell stress as one of the component of the danger signal leading to DC activation by chemical sensitizers.

One of the objectives of our work was also to identify if the Nrf2 pathway represented a new biomarker for the detection of chemical sensitizers using *in vitro* models. According to our data, intracellular Nrf2 accumulation and *hmox1* gene expression were good candidates for the detection of chemical sensitizers and allowed the discrimination between sensitizers and irritants, a major drawback in many sensitization tests. However, non-sensitizing electrophile may generate false positive response as shown with sulforaphane. Very recently, Natsch and Emter (2007) used the AREc32 cell line, a MCF7-derived cell line stably transfected with eight ARE sequences upstream of a luciferase gene reporter, to screen for 100 molecules selected from skin sensitization testing. They found good sensitivity (81.4%) and the overall accuracy was 83%. Limitations of the assay were found in the detection of some weak sensitizers and of skin-specific pro-haptens. However, this study confirms that the Keap1/Nrf2 pathway may represent a source of complementary biomarkers to refine the present tests in development.

In conclusion, our study identified the Nrf2 pathway as a new signalling pathway activated by chemical sensitizers. It is our opinion that activation of this pathway may provide the possibility for DC to handle the electrophilic stress induced by chemical sensitizers allowing DC maturation by activation of MAPK and NF-κB. Future studies will be conducted to address this hypothesis. In addition, genes regulated by Nrf2 such as *hmox1* and measurement of Nrf2 protein accumulation are good candidates for new biomarkers for chemical sensitizer detection in cell-based models.

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