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Original Contribution

Respiratory sensitizer hexamethylene diisocyanate inhibits SOD 1 and induces ERK-dependent detoxifying and maturation pathways in dendritic-like cells

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ABSTRACT

Respiratory allergy to low-molecular-weight chemicals is a current concern in the context of occupational health, and a certified method to identify respiratory allergens is still under investigation. The aim of this work was to unveil some of the poorly understood initial molecular events and toxicity pathways underlying respiratory sensitization, which might be crucial to disclosing the key building blocks of new testing strategies and may contribute to the development of a valid in vitro method for the identification of respiratory allergens. Immortalized human dendritic cell (DC)-like THP-1 cells were exposed to the respiratory allergen hexamethylene diisocyanate (HDI) for 6 h, and the activation of several signaling pathways was analyzed. Mitochondrial membrane potential (MMP) alterations, superoxide anion (O_2^-) production, and gene expression modulation in HDI-treated cells were also evaluated. According to our results, HDI induces O_2^- increase ($P < 0.001$) through enzymatic inhibition of cytoplasmic superoxide dismutase 1 ($P < 0.05$), which might reduce MMP, further leading to mitochondrial O_2^- production. Increased O_2^- levels promote ERK phosphorylation (approx sixfold compared to control; $P < 0.001$) and downstream transcriptional increase of several genes: *HMOX1* ($P < 0.05$), involved in the protection of chemical reactive species; *MDR1* ($P < 0.01$), responsible for the efflux of xenobiotics in the cell; and *CD83* ($P < 0.05$), a DC maturation marker. These results raise new perspectives on the action of respiratory allergens in DCs and point out key molecular events that are crucial for the development of the so-called adverse outcome pathways, particularly regarding O_2^- increase through enzymatic inhibition, and important for ERK activation. Furthermore, our results highlight the role of ERK signaling, but not p38 MAPK, in the activation of vital mechanisms in cells exposed to a respiratory allergen, such as cell detoxification, migration, and maturation.

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Currently, a certified method to identify respiratory allergens is of particular interest among the scientific community and is still under investigation. Owing to the legislation instituted by the European Union with regard to animal testing (Directive 86/609/EEC [1]), it is mandatory to develop new in vitro methods to discriminate between allergenic and nonallergenic compounds. Some chemicals known to cause respiratory allergy have been identified and comprise isocyanates and acid anhydrides, reactive dyes, and platinum salts and glutaraldehyde. This work focuses

specifically on diisocyanate-induced sensitization. Diisocyanate exposure in the workplace is one of the leading causes of occupational asthma, but airway irritation and asthma-like symptoms, hypersensitivity pneumonitis, rhinitis, and accelerated lung deterioration are commonly reported. However, diisocyanates can also cause both irritant and allergic contact dermatitis as well as skin and conjunctival irritation [2].

Diisocyanates are low-molecular-weight (LMW) chemicals that act as haptens [3], thus first associating with an endogenous or extracellular protein carrier to induce an immune response. Covalent binding of a hapten to a protein is believed to be a relevant mechanism for immune recognition and further development of antigenic LMW-induced chemical allergies ([4], for review). The haptenated protein is then processed intracellularly

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into short peptides for cell surface expression by major histocompatibility complex (MHC) systems. Moreover, chemical binding to macromolecules seems to be involved in immediate (type 1) hypersensitivity sensitization and cell response, in both the skin and the lung ([4], for review). However, the final protein–isocyanate conjugates that act as allergens in vivo are not yet fully understood, but respiratory tract sensitization by chemicals has been related to the development of T helper 2 cell responses (consistent with the production of IL-4 (IL-5 in the mouse), IL-10, or IL-13) and also the production of IgE antibody, although specific IgE antibody is often impossible to detect, particularly in patients with diisocyanate-associated allergy [5,6]. Nevertheless, other respiratory allergens, as trimellitic anhydride, induced specific IgE production and increased IgE concentration in total serum of BALB/c strain mice, upon topical exposure of mice to the chemical, whereas the contact sensitizer 2,4-dinitrochlorobenzene, which is related to a type 1 cytokine profile (IFN- γ and IL-12 production), did not evoke a similar effect, suggesting that contact and respiratory allergens induce different immune responses in vivo [7,8].

Albumin has been identified as a target of diisocyanate binding in vivo [9,10]. Immunologic studies focused on the hapten diisocyanate attached to human albumin demonstrated the presence of IgE and IgG that bind to diisocyanate–albumin adducts in some patients with diisocyanate-induced asthma [10]. Nonetheless, hexamethylene diisocyanate (HDI) was proven to conjugate with other proteins present in the skin and lungs, both in vitro and in vivo [9]. In general, inhaled antigens adhere to trachea and bronchi epithelium, further triggering receptors of the innate immune system, such as Toll-like receptors (TLRs), on epithelial cells and macrophages, which will lead to chemokine production and recruitment of additional neutrophilic leucocytes ([11], for review). The link between innate and adaptive immunity is made by dendritic cells (DCs), a specialized innate immune antigen-presenting cell. DCs express all the receptors of the innate immune system and are also capable of taking up the antigen, processing it into small peptides, and presenting it associated with MHC I and MHC II to be recognized by T cell receptors. However, the interaction between allergens and DCs is complex, and it is still not completely clear if it primarily occurs as the activation of epithelial cells, which then release cytokines to activate DCs, or rather is a direct DC activation event ([11], for review). Nevertheless, epithelial cells and DCs, as well as macrophages, have pivotal roles in the sensitization process; hence these cells are good tools to study the early molecular events elicited during respiratory sensitization, which could be further used in the development of an in vitro assay to detect respiratory allergens.

In this study we used a human monocytic cell line, THP-1 cells, to evaluate the mechanisms associated with the isocyanate HDI-induced sensitization. THP-1 cells have the capacity to develop into DCs and display sensitization markers such as activation of endocytosis, increase in phosphotyrosine levels, upregulation of cell surface markers, and increase in cytokine and chemokine production ([12]; reviewed in [13]). Thus, THP-1 cells allow one to distinguish between allergens and nonallergens, similar to DCs (e.g., monocyte-derived dendritic cells, CD34-DCs), with the advantage of being more homogeneous and hence a suitable model for the identification of the sensitizing potential of chemicals.

The extracellular signal-regulated kinase (ERK) and the p38 mitogen-activated protein kinase (MAPK) pathways are two of the main signaling cascades in mammals. Upstream MAPK kinases activate ERK or p38 through phosphorylation, triggering different cellular outcomes. ERK activation regulates cell proliferation and differentiation in response to mitogens and growth factors, whereas p38 MAPK is preferentially activated by stress-inducing agents [14]. Activation of p38 MAPK represents a common pathway triggered by contact [15] and respiratory [16] allergens. ERK activation in a skin-derived DC cell line subjected to a contact allergen was also

previously reported by us [17], although with regard to the response to a respiratory allergen it is poorly documented.

MAPK activation induced by allergens is also associated with reactive oxygen species (ROS; superoxide, O_2^- , and hydrogen peroxide, H_2O_2) generation. Our and previous results have demonstrated that ERK and p38 MAPK increase phosphorylation levels induced by contact sensitizers [17–19]. On the other hand, in addition to triggering signaling cascades, excessive levels of ROS can also cause cellular and mitochondrial oxidative damage, resulting in mitochondrial dysfunction [20,21]. Mitochondria are the main intracellular source of O_2^- in the cell under physiological conditions [22]. O_2^- is further dismutated into the nonradical H_2O_2 + O_2 by a nonenzymatic or superoxide dismutase (SOD)-catalyzed reaction ([23,24], for review). The enzyme SOD is ubiquitously present in various organelles within the cell: Cu-ZnSOD (SOD 1) is present in the cytosol, nucleus, and intermembrane space of mitochondria; MnSOD (SOD 2) localizes in the mitochondrial matrix; and EC-(Cu-Zn)SOD is present in the extracellular (EC) space [23,25]. Decreased activity of Cu-ZnSOD has been reported in the lung of asthmatic patients [26–30].

In this work, using the case study of HDI, we highlight the perturbation of intracellular toxicity pathways elicited during respiratory sensitization, showing the new version of Tox-21C in action [31,32]. We analyzed alterations in mitochondrial function, namely, mitochondrial membrane potential (MMP) modifications, and ROS production, specifically O_2^- generation. We also determined MAPK signaling pathway activation (e.g., ERK and p38 MAPK) and gene expression modulation in HDI-treated cells. The findings of this work are expected to contribute to the development of a valid in vitro method for the identification of respiratory allergens.

Materials and methods

Materials

The respiratory sensitizer HDI, lipopolysaccharide (LPS) from *Escherichia coli* (serotype 026:B6), N-acetyl-L-cysteine (NAC), rotenone, SOD, and SOD Determination Kit were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). A tetramethylrhodamine ethyl ester (TMRE) mitochondrial membrane potential assay kit was obtained from Abcam. MitoSOX red mitochondrial superoxide indicator was obtained from Molecular Probes. Inhibitors for ERK (U0126) and p38 MAPK (SB203580) were purchased from Merck Millipore (Billerica, MA, USA). Phospho (P-) and total ERK and p38 MAPK antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Alkaline phosphatase-conjugated secondary antibodies were purchased from GE Healthcare (Chalfont St. Giles, UK). Protease and phosphatase inhibitor cocktails were from Roche (Mannheim, Germany). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore Corp. (Bedford, MA, USA). TRIzol reagent was purchased from Invitrogen (Barcelona, Spain) and RNA Storage Solution was from Ambion (Foster City, CA, USA). The iScript Select cDNA synthesis kit was obtained from Bio-Rad (Hercules, CA, USA).

Cell culture

The THP-1 human monocytic cell line (ATCC TIB-202, American Type Culture Collection, Manassas, VA, USA) was cultured and maintained at a cell density between 0.2×10^6 and 1×10^6 cells/ml in RPMI 1640 supplemented with inactivated fetal bovine serum (10%), 14 mM glucose, 10 mM Hepes, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.05 mM 2-mercaptoethanol. Cells were

subcultured every 3 or 4 days and kept in culture for a maximum of 2 months.

Alamar blue cell viability assay

Cell viability was evaluated using Alamar blue [33]. Briefly, cell duplicates were plated at a density of 0.5×10^6 /ml, in a final volume of 0.2 ml/well, in a 96 well plate and exposed to various concentrations of HDI (100, 200, 300, 400, and 500 μ M) for 24 h. Resazurin solution (50 μ M in sterile phosphate-buffered saline (PBS)) was added to each well after 21 h of HDI exposure. After 3 h (for a total of 24 h HDI treatment) absorbance was read at 570 and 600 nm with a standard spectrophotometer (SLT, Salzburg, Austria). Viable cells are able to reduce resazurin (a nonfluorescent blue dye) into resorufin (pink colored and fluorescent) and, hence, their number correlates with the magnitude of dye reduction. The HDI concentration causing a loss of 30% viability (EC_{30} value) compared to control cells was determined and further used.

Chemical exposure

Cells were plated at a density of 1×10^6 /ml and exposed to HDI (300 μ M) for 6 h. The HDI concentration used was determined considering the EC_{30} value.

Treatment with the specific inhibitors for ERK (U0126, 20 μ M) and p38 MAPK (SB203580, 10 μ M) or with the antioxidants NAC (5 mM) or SOD (300 and 500 U) were performed 90 min before HDI incubation. Cells were also exposed to LPS (1 μ g/ml) as a control. To evaluate ROS-dependent ERK activation THP-1 cells were incubated with H_2O_2 (50 and 100 μ M) and rotenone (Rot; 100 nM and 10 μ M) for 6 h.

For evaluating ERK activation due to the allergen, cells were previously incubated with HDI for 5 min, 30 min, 1 h, or 6 h and Western blot of total cell lysates was performed (see *Cell lysates and Western blot analysis*).

Cell lysates and Western blot analysis

Cells plated at a density of 1×10^6 /ml, in a six-well plate with a final volume of 2 ml, were treated as previously described (see *Chemical exposure*). Six hours after HDI incubation, the cells were further collected, centrifuged (250 g, 5 min at 4 °C), and washed in ice-cold PBS supplemented with phosphatase inhibitor. After a second centrifugation, the pellet was incubated in RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA), freshly supplemented with 1 mM dithiothreitol (DTT) and protease and phosphatase inhibitor cocktails, for 30 min in ice. The nuclei and the insoluble cell debris were removed by centrifugation (12,000 g for 10 min, at 4 °C). The postnuclear extracts were collected and used as total cell lysates. Protein concentration was determined using the bicinchoninic acid method, and the cell lysates were denatured at 95 °C, for 5 min, in sample buffer (0.125 M Tris, pH 6.8; 2% w/v SDS; 100 mM DTT; 10% glycerol; and bromophenol blue) for subsequent use in Western blot analysis. Briefly, 30 μ g of protein was electrophoretically separated on a 10% (v/v) sodium dodecyl sulfate-polyacrylamide gel and transferred to a PVDF membrane. The membranes were blocked with 5% (w/v) fat-free dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T), for 1 h, at room temperature. Blots were then incubated overnight at 4 °C with the primary antibodies against P-ERK1/ERK2 (1:1000) and P-p38 MAPK (1:1000). The membranes were then washed for 30 min with TBS-T and incubated for 1 h at room temperature with alkaline phosphatase-conjugated anti-rabbit antibody (1:20,000). The immune complexes were detected by membrane exposure to the enhanced chemifluorescence reagent for 5 min, followed by

scanning for blue excited fluorescence on the Typhoon imager (GE Healthcare). The generated signals were analyzed using ImageQuant TL software. To test whether similar amounts of protein were loaded for each sample, the membranes were stripped and reprobed with antibodies to total ERK1/2 and p38 MAPK. The blots were then developed with alkaline phosphatase-conjugated secondary antibodies and visualized by enhanced chemifluorescence. Phosphorylated protein levels were calculated relative to total protein levels (P-ERK1/2/total ERK and P-p38/total p38).

Mitochondrial membrane potential integrity

The MMP was evaluated by TMRE according to the manufacturer's instructions. Briefly, 1×10^6 cells/ml were plated in a 48-well plate, exposed to HDI for 6 h, and treated as previously described. Cells were also incubated for 10 min, at 37 °C, with 50 μ M FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone), a protonophore that collapses the MMP and was thus used as a negative control. TMRE (1 μ M) was added for 30 min, at 37 °C, and cells were further collected, centrifuged at 250 g for 5 min, and washed with PBS/0.2% bovine serum albumin (BSA). After another centrifugation, the cells were resuspended in PBS/0.2% BSA and TMRE fluorescence was read (excitation peak 549 nm; emission peak 575 nm).

Mitochondrial superoxide anion measurement

Mitochondrial O_2^- generation was determined using MitoSOX, according to the manufacturer's instructions. Briefly, 1×10^6 cells/ml were plated in a 48 well plate, exposed to HDI for 6 h, and treated as described under *Chemical exposure*. Cells were further washed in PBS and incubated with 2.5 μ M MitoSOX in HBSS (in mM: 1.3 $CaCl_2$, 0.5 $MgCl_2$, 5.3 KCl, 138 NaCl, 0.44 KH_2PO_4 , 4.2 $NaHCO_3$, and 0.34 Na_2HPO_4 , pH 7.4) for 30 min at 37 °C in the dark. Cells were then washed two times with HBSS and MitoSOX fluorescence was read (excitation peak 510 nm; emission peak 580 nm).

Determination of superoxide dismutase activity

THP-1 cells were plated at a density of 1×10^6 /ml, in a six-well plate in a final volume of 2 ml and treated as previously described (see *Chemical exposure*). Cells were further collected, centrifuged (250 g, 5 min at 4 °C), and washed in ice-cold PBS. After a second centrifugation, the pellet was incubated in RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) for 30 min on ice. The nuclei and the insoluble cell debris were removed by centrifugation (1000 g for 15 min, at 4 °C) and the supernatant was collected and used as total cell lysates. Total SOD activity was then determined using the SOD Determination Kit, according to the manufacturer's instructions, with modifications. Cell extract duplicates were also incubated with KCN (2 mM) to inhibit SOD 1, thus allowing the measurement of mitochondrial SOD 2. SOD 1 activity was then calculated by subtraction of SOD 2 from total SOD.

Analysis of gene expression by real-time RT-PCR

Total RNA was isolated from cells with TRIzol reagent, according to the manufacturer's instructions, and the concentration was determined by OD_{260} measurement using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and samples were stored in RNA Storage Solution at -80 °C until use. Briefly, 1 μ g of total RNA was reverse-transcribed using the iScript Select cDNA synthesis kit and real-time RT-PCR reactions were performed, in duplicate for each sample, on a Bio-Rad MyCycler iQ5 as previously described [34]. After amplification, a threshold was

set for each gene and C_t values were calculated for all samples. Gene expression changes were analyzed using the built-in iQ5 Optical system software. The results were normalized using *Hprt-1* as reference gene. Primer sequences were designed using Beacon Designer software version 7.7 (Premier Biosoft International, Palo Alto, CA, USA) (see [Supplementary Table S1](#)) and thoroughly tested.

Calculation of real-time RT-PCR results

Because the real-time RT-PCR results are presented as ratios of chemical-treated samples over untreated (control) cells, a two-base logarithmic transformation was used to make observations symmetric and closer to a normal distribution. If x represents the fold change of a gene in one sample, then the two-base logarithmic transformation ($\log_2(x)$) is $\ln(x)/\ln(2)$. Therefore, fold changes of 2 and 0.5 correspond to mean $\log_2$ values of 1 and -1 , respectively.

Statistical analysis

The results are presented as the mean $\pm$ SEM of the indicated number of experiments and were analyzed with one-way ANOVA using Bonferroni's multiple comparison test (GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego, CA, USA; www.graphpad.com). $P < 0.05$ was considered significant.

Results

HDI-induced ROS trigger sustained ERK phosphorylation

In this study we aimed to better elucidate the mechanisms involved in respiratory allergy sensitization. For that purpose we used as a model THP-1 cells, a human DC-like line, which was further exposed to HDI, an isocyanate associated with respiratory allergy [9,10]. First we determined the HDI concentration corresponding to the EC_{30} value through the Alamar blue assay. Thus, in the subsequent experiments we used HDI at a concentration of 300 μ M, hence reflecting 70% of cell viability (see [Supplementary Fig. S1](#)). We then attempted to decipher which pathway would be activated in DC-like cells upon HDI exposure. Therefore, we incubated THP-1 cells with HDI or with the nonsensitizing but immunogenic LPS (an immunostimulatory molecule with origin in gram-negative bacteria that induces the maturation of DCs by binding to TLR at the cell surface [35,36]) for 5 min, 30 min, 1 h, or 6 h, and ERK and p38 MAPK activation was evaluated by Western blotting. In our study DC-like cells displayed a significant increase in phosphorylated ERK levels ([Fig. 1A](#) and [C](#) and [Supplementary Fig. S2](#)) after 6 h of HDI exposure (the time point that was further used in our study, because the role of ERK in respiratory sensitization has been poorly investigated). Interestingly, upon 6 h of LPS stimulation, the DC-like cells did not show altered P-ERK levels, compared to control ([Fig. 1A](#)).

To relate the increase in P-ERK levels at 6 h with ROS formation, we incubated DC-like cells with H_2O_2 and Rot for the same period ([Fig. 1B](#) and [C](#)). Rot is a mitochondrial complex I inhibitor, which induces mitochondrial ROS generation, including the O_2^- species via mitochondrial complex III [37]. As one can observe, both H_2O_2 (50 μ M) and Rot (10 μ M) induced a significant increase in P-ERK levels after 6 h treatment, compared to control (CTR; [Fig. 1B](#) and [C](#)), suggesting that at this time point ROS might be involved in ERK activation. In addition, we also treated the cells with the antioxidant NAC and with SOD, 90 min before the addition of HDI. In this study we used cytosolic Cu-ZnSOD. As predicted, NAC significantly decreased HDI-induced ERK phosphorylation ([Fig. 1A](#) and [C](#)),

compared to HDI, because NAC is a reduced glutathione precursor and a thiol antioxidant [38]. Surprisingly, SOD did not reduce ERK activation promoted by the allergen; instead, it seemed to exert a synergistic effect with HDI, dependent on SOD concentration, thus significantly increasing P-ERK levels, compared to HDI alone (SOD 500 μ M; [Fig. 1A](#) and [C](#)). To validate our experiment, we also treated the cells with ERK (U0126) and p38 MAPK (SB203580) inhibitors, 90 min before HDI addition. ERK inhibition completely reverted the HDI effect; on the other hand, inactivation of the p38 MAPK pathway led to a significant increase in ERK phosphorylation, compared to HDI ([Fig. 1A](#) and [C](#)), probably reflecting a compensatory mechanism through the p38 MAPK pathway. p38 MAPK activation is usually associated with contact allergy [17]. However, HDI also increased p38 MAPK phosphorylation levels ([Fig. 1D](#) and [F](#)) upon 6 h of exposure. Nevertheless, our data suggest that this increase does not strongly depend on H_2O_2 or O_2^- generation, at the time point studied (6 h), as HDI-induced ERK activation does ([Fig. 1D–F](#) and [Fig. 1A–C](#), respectively).

HDI inhibits cytoplasmic SOD and induces mitochondrial dysfunction

We next wondered why SOD leads to increased ERK activation of HDI-treated cells. Thus we evaluated SOD activity in THP-1 cells incubated with LPS or HDI, for 6 h, using a commercial kit (see *Determination of superoxide dismutase activity* under Materials and methods). Interestingly, the results showed an inhibition of SOD 1 in DC-like cells incubated with either LPS or HDI ([Fig. 2A](#)), whereas SOD 2 activity was significantly increased in LPS-treated cells ([Fig. 2B](#)). Though the activity of the mitochondrial enzyme SOD 2 was not affected by HDI, a fraction of SOD 1 is located in the organelle. Hence, we investigated if mitochondrial function was disturbed by the allergen, through the evaluation of MMP by TMRE and the generation of O_2^- by MitoSOX ([Fig. 3](#)). TMRE is a cell-permeative red-orange positively charged dye, which accumulates in active mitochondria because of their relatively negative charge. Depolarized or inactive mitochondria display reduced membrane potential and do not incorporate TMRE. As one can observe in [Fig. 3A](#), HDI significantly reduced MMP, compared to CTR, which was reverted to CTR levels by NAC ([Fig. 3A](#)). This alteration in MMP is probably related to the significant increase in mitochondrial O_2^- levels promoted by HDI ([Fig. 3B](#)), as determined by MitoSOX. MitoSOX red mitochondrial superoxide indicator is a live-cell permeative fluorogenic dye, which is selectively targeted to the mitochondria where it is specifically oxidized by superoxide, thus exhibiting red fluorescence. A significant reduction in O_2^- induced by HDI was accomplished by the presence of NAC ([Fig. 3B](#)), corroborating the TMRE results ([Fig. 3A](#)). The presence of SOD alone at the two concentrations tested (300 and 500 μ M) did not alter O_2^- levels, compared to CTR ([Fig. 3B](#)). However, in the presence of HDI we observed a tendency to an increase of O_2^- with 500 μ M SOD ([Fig. 3B](#)), which could be partially responsible for the increased ERK phosphorylation ([Fig. 1A](#) and [C](#)). Moreover, ERK or p38 MAPK inhibitors did not alter HDI-induced reduction in MMP ([Fig. 3A](#)) or mitochondrial O_2^- level increase ([Fig. 3B](#)) suggesting that these effects are upstream of the two MAPK pathways activation.

HDI-induced ERK signaling pathway promotes DC-like cell detoxification and maturation

We further investigated potential genes modulated by HDI. Thus, THP-1 cells were incubated with the respiratory allergen for 6 h and real-time RT-PCR was performed. We evaluated the transcription of heme oxygenase (decycling) 1 (*HMOX1*) and NAD (P)H dehydrogenase quinone 1 (*NQO1*), both ARE-dependent

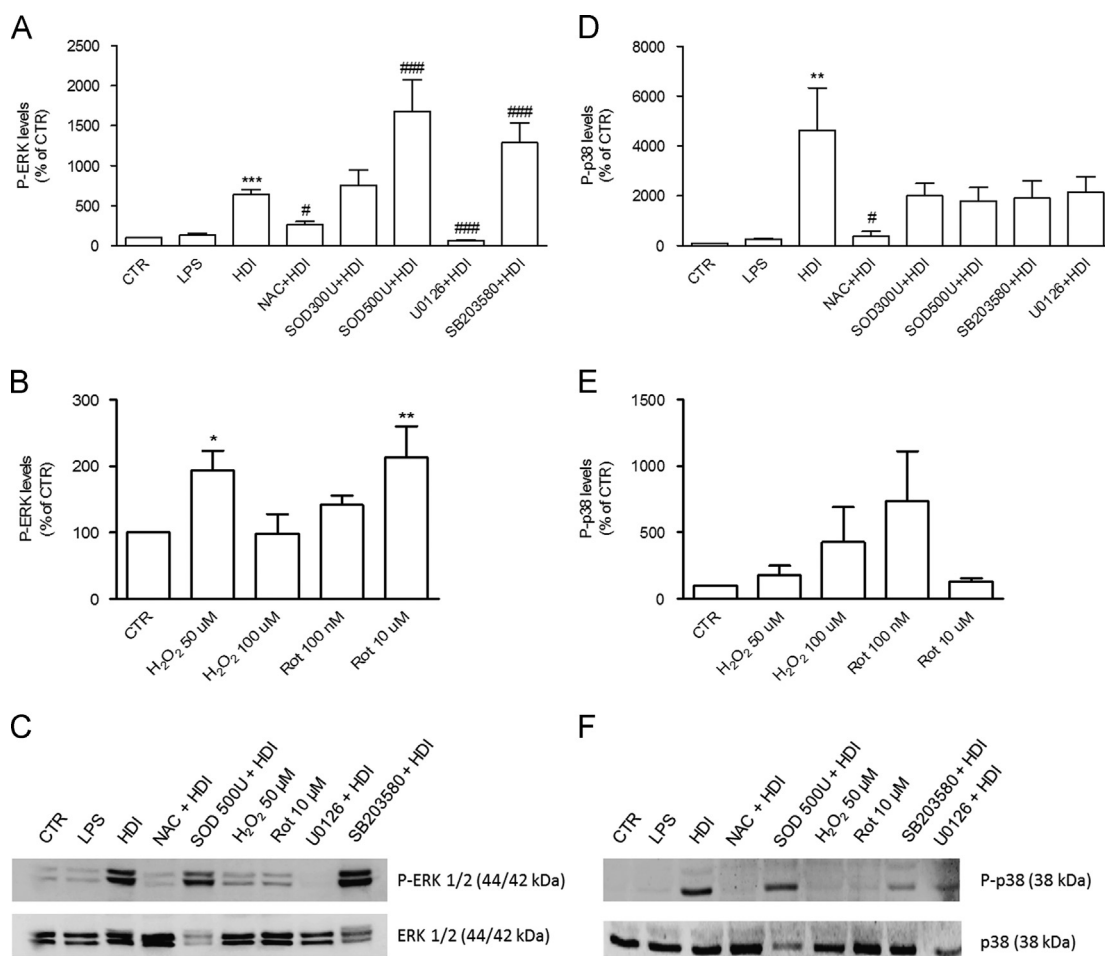


Fig. 1. HDI-induced ERK activation depends on mitochondrial ROS production. (A and D) Immortalized human DC-like THP-1 cells were exposed to hexamethylene diisocyanate and LPS for 6 h. Cells exposed to HDI were also treated with the specific inhibitor of ERK (U0126) and p38 (SB203580), the antioxidant NAC or SOD, 1 h 30 min before HDI exposure. (B and E) THP-1 cells were incubated with H₂O₂ and rotenone (Rot) for 6 h, to evaluate ROS-dependent ERK and p38 activation. ERK and p38 signaling pathway activation was further determined by Western blotting of total cell extracts. HDI induced a significant increase in phosphorylated ERK levels, which was counteracted by NAC (A). Moreover, ERK activation was also observed when cells were incubated with H₂O₂ and Rot alone (B), indicative of ROS-dependent ERK phosphorylation. (C and F) Western blot images showing the bands representative of the treatment concentration that significantly induced ERK activation (e.g., SOD 500 U, H₂O₂ 50 μ M, and Rot 10 μ M). Data correspond to the means $\pm$ SEM of 3 to 16 independent experiments and are represented as % of control cells (CTR). Statistical analysis: one-way ANOVA with Bonferroni's multiple comparison test; * P < 0.05, ** P < 0.01, *** P < 0.001, compared to CTR; # P < 0.05, ### P < 0.001, compared to HDI.

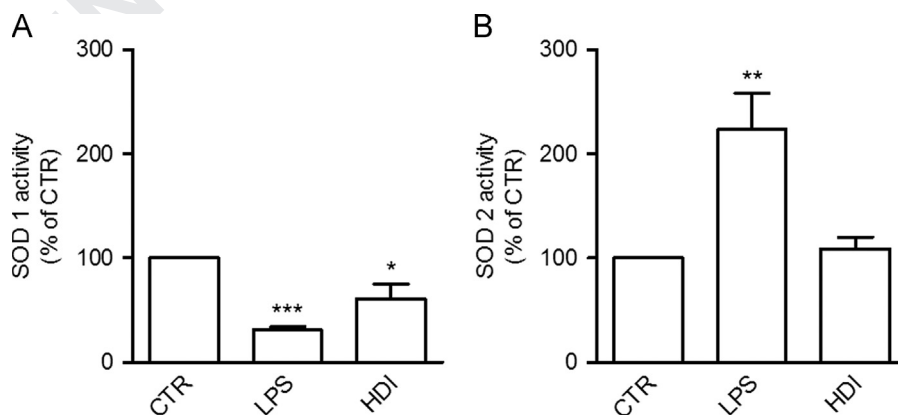


Fig. 2. Superoxide dismutase (SOD) activity upon HDI exposure. (A and B) Human DC-like THP-1 cells were exposed to LPS and HDI for 6 h. Total SOD activity was further evaluated with the SOD Determination Kit. HDI significantly reduced cytoplasmic SOD (SOD 1) activity (A) but did not alter mitochondrial SOD (SOD 2; B) suggesting that HDI interaction with the enzyme might increase superoxide anion levels in the cytoplasm, which will further induce mitochondrial stress. The bars in the graphs correspond to the means $\pm$ SEM of six experiments. Statistical analysis: one-way ANOVA with Bonferroni's multiple comparison test; * P < 0.05, ** P < 0.01, *** P < 0.001, compared to CTR.

genes involved in the protection or neutralization of chemical reactive species; of multidrug resistance protein (*MDR1*), responsible for the transportation of xenobiotics in the cell; and *CD83* and

CD86, related to DC maturation (Fig. 4). According to Fig. 4 HDI significantly induced the expression of *HMOX1*, *MDR1*, and *CD83*, compared to control ($\log_2(1) = 0$); interestingly, it did not affect

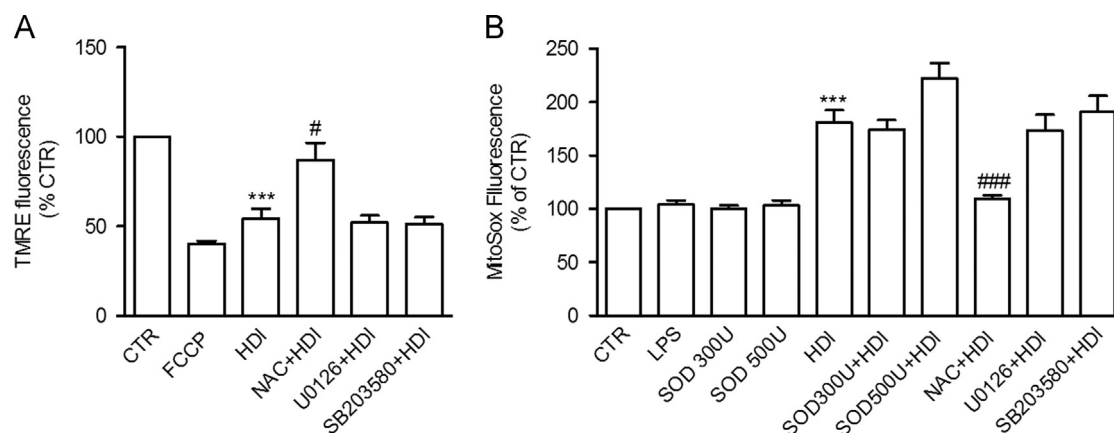


Fig. 3. HDI-induced mitochondrial toxicity. Human DC-like THP-1 cells were exposed to HDI for 6 h. (A) MMP alterations due to HDI exposure were further determined by TMRE fluorescence. FCCP (50 μ M), a protonophore that collapses mitochondrial membrane potential, was used as a negative control. HDI-treated cells were previously incubated with ERK inhibitor (U0126), p38 inhibitor (SB203580), or the antioxidant NAC, 1 h 30 min before HDI. (B) Cells were also treated with SOD, alone (for 6 h) or in the presence of HDI (SOD added 1 h 30 min before HDI). Mitochondrial O_2^- formation was evaluated using the MitoSOX superoxide indicator. The results demonstrate that HDI reduces MMP, which is reverted by the antioxidant NAC (A). This MMP might underlie the increase in mitochondrial O_2^- levels observed in cells exposed to HDI (B). Data depicted in the graphs correspond to the means $\pm$ SEM of 3 to 11 experiments. Statistical analysis: one-way ANOVA with Bonferroni's multiple comparison test; *** P < 0.001, compared to CTR; # P < 0.05 and ### P < 0.001 compared to HDI.

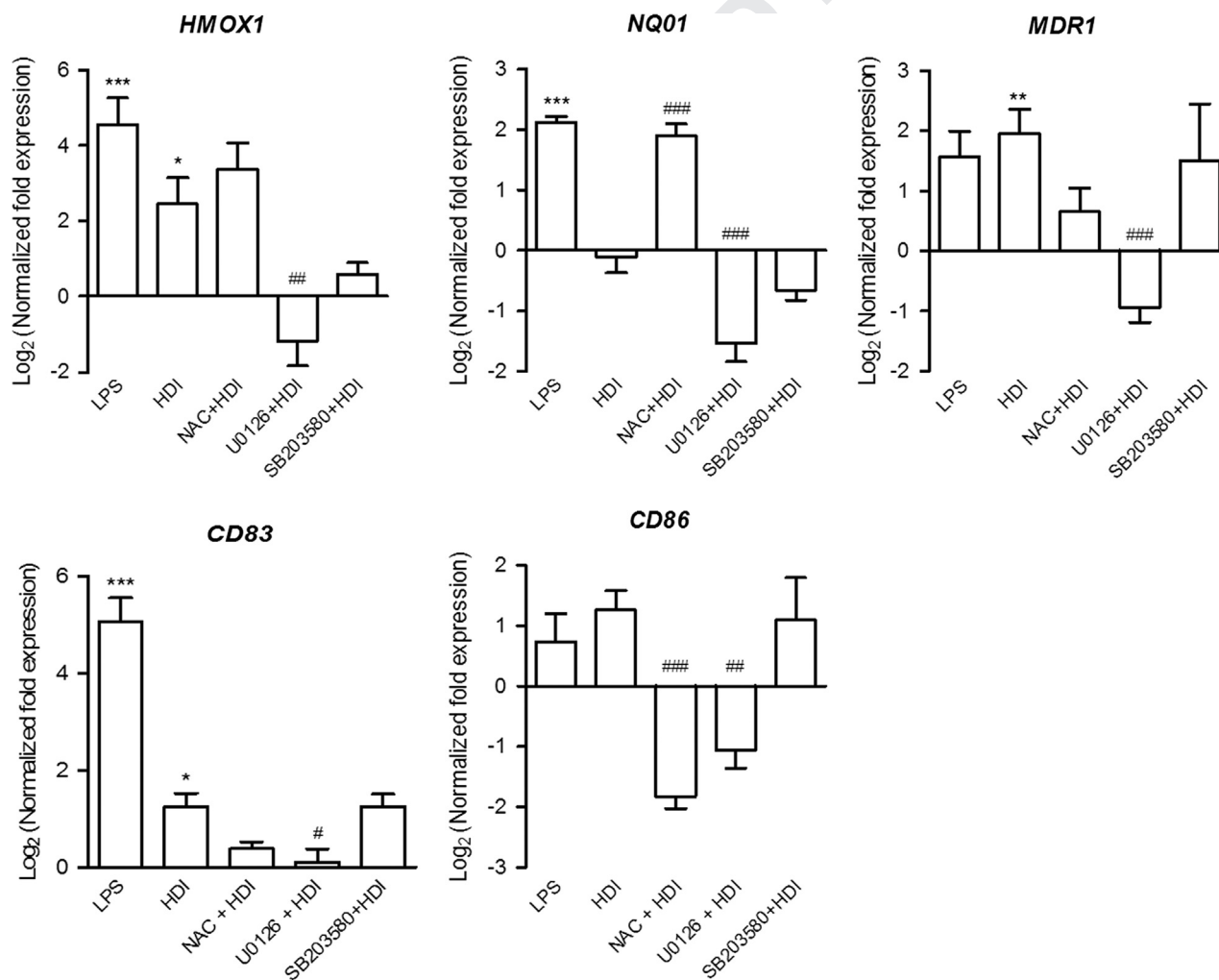


Fig. 4. DC detoxifying and maturation pathways induced by HDI are ERK-dependent. Human THP-1 cells were exposed to HDI and LPS for 6 h. HDI-treated cells were incubated with the specific inhibitors for ERK (U0126) and p38 MAPK (SB203580) and with the antioxidant NAC, 1 h 30 min before HDI exposure. mRNA levels of HMOX1, NQO1, CD83, CD86, and MDR1 were determined by real-time RT-PCR. HDI induced HMOX1, MDR1, and CD83 gene expression, which was completely repressed by ERK inhibitor (U0126), suggesting that cell-detoxifying mechanisms (HMOX1 and MDR1) as well as cell maturation (CD83) triggered by HDI depend on ERK activation. Data are represented as log₂ of fold expression levels normalized to control cells. Means $\pm$ SEM of 3–12 experiments are presented. Statistical analysis: one-way ANOVA with Bonferroni's multiple comparison test; * P < 0.05, ** P < 0.01, *** P < 0.001 compared to CTR (log₂ = 0); # P < 0.05, ### P < 0.01, #### P < 0.001 compared to HDI.

the expression of *NQO1*, which is strongly modulated by contact allergens, similar to *HMOX1* [39]. Surprisingly, NAC did not revert the effect of HDI on *HMOX1* expression and, in addition, it increased the expression of *NQO1* (NAC + HDI; Fig. 4). Another interesting result was that inhibition of the ERK pathway (U0126 + HDI) significantly decreased the expression of all the genes modulated by HDI (e.g., *HMOX1*, *MDR1*, and *CD83*; Fig. 4), which was not observed when using the p38 MAPK inhibitor (SB203580 + HDI), further supporting the hypothesis that the effects of HDI are strongly dependent on ERK activation.

Discussion

Current practice in toxicological risk assessment of health risk associated with chemical exposure is most commonly based on clinical or histopathological endpoints determined in animal models. Apart from ethical objections to the use of animals [40], there is also a scientific motivation for reevaluating these models. Currently, toxicologists are aware of the (re)definition of the paradigm of toxicology; rather than relying on apical endpoints of toxicity as determined in animal models, the toxicity of a compound can be determined using in vitro methods focusing more specifically on mechanisms and modes of action. Therefore, using in vitro systems, early cellular responses can be studied that may help predict toxic responses in vivo. In accordance with these recent changes in the paradigm of toxicity testing, herein we studied the effects of the respiratory allergen HDI on MAPK pathway activation, mitochondrial dysfunction, and gene expression markers, in a DC-like cell line, to disclose cell mechanisms and toxicity pathways attributable to respiratory allergens that could be further used in the development of an in vitro assay for respiratory sensitization evaluation. For that purpose we used a human monocytic cell line, THP-1, the cells of which are phagocytic. When undifferentiated (i.e., in their naïve state) the cells express typical monocytic surface markers (such as *CD86^{low}*), whereas markers such as *CD83* are almost negative. However, upon induction of a DC-like phenotype the cells express high levels of maturation markers, whereas monocytic markers such as *CD86* are decreased ([12,13], for review). Nevertheless, because better results have been obtained with naïve THP-1 cells most studies use undifferentiated cells ([13], for review). In the present study, we were able to demonstrate that the presence of HDI inhibits SOD 1 activity, which might lead to MMP reduction with increased O_2^- levels. This occurs upstream of ERK activation, which is triggered possibly by an accumulation of O_2^- . In turn, HDI-induced ERK activation leads to the expression of detoxifying genes (e.g., *HMOX1*), ATP-binding cassette-related genes (e.g., *MDR1*, an ATP-dependent drug efflux pump for xenobiotic compounds and also involved in DC migration), and maturation markers (e.g., *CD83*). Interestingly, the increased expression of *HMOX1*, *MDR1*, and *CD83* genes due to HDI is impaired when the ERK pathway is inhibited but is not affected by p38 MAPK inhibition, reinforcing the pivotal role of ERK in modulating respiratory allergen-induced cell response.

Isocyanates can promptly react with proteins in vitro and in vivo, and their conjugates represent the major immunogenic form of the chemical. Human albumin is the main protein carrier of isocyanates in vivo [9,10] and Wisniewski et al. [41] have previously reported that monocytes, but not T cells, were able to take up hapten-like HDI-albumin in vitro, suggestive of localized sequestration. This internalization of HDI-albumin conjugates further stimulated marked changes in morphology, phenotype, and gene/protein expression [41], strongly suggesting that human monocytes may have an important function in isocyanate exposure events, which are apparently different from those evoked by

common allergens. Moreover, HDI is an aliphatic isocyanate capable of reacting with thiol molecules (such as in glutathione, GSH). In a recent work Wisniewski and collaborators [42] demonstrated that HDI associates with GSH in vitro and that GSH-HDI conjugates are able to carbamoylate human albumin, i.e., transfer HDI to albumin in a pH-dependent manner. The reactivity of GSH with HDI remains less clear than with aromatic isocyanates. For the aromatic isocyanate toluene diisocyanate (TDI), it was demonstrated that chemical vapors readily cross a liquid-phase barrier to react with GSH, further depleting intracellular GSH in human airway epithelial cells [43]. In our experiments we hypothesize that HDI may associate with serum albumin to enter the cell, in a mechanism similar to that reported by Wisniewski et al. [43] concerning the aromatic isocyanate TDI.

GSH is an important component of the glutathione system for the reduction of organic and inorganic hydroperoxides, particularly when at low concentrations; otherwise, catalase is most effective in the presence of high H_2O_2 concentrations (reviewed in [44]). In healthy cells and tissue circa 90% of the total glutathione is reduced (GSH) to ensure the active reduction of H_2O_2 ; thus an increased GSSG/GSH ratio is considered indicative of oxidative stress, such as exposure to O_2^- or H_2O_2 , which leads to transient GSH depletion in vitro ([44], for review). However, we did not find alterations in the GSH/GSSG ratio induced by HDI (Supplementary Fig. S3), although with DNFB, GSH levels significantly decreased (not shown). ROS generation and protein oxidation plays an important role in MAPK activation induced by allergens. We have previously shown that the contact sensitizer DNFB activates p38 MAPK and ERK1/2 through ROS (possibly related to GSH levels reduction) in a fetal skin-derived DC line [17]. These results corroborate previous studies demonstrating the role of oxidative stress in the activation of MAPKs [18,19,45,46]. In our study, both ERK and p38 MAPK were activated in DC-like cells exposed to the respiratory allergen HDI after 6 h. In both cases this effect was reverted by NAC, an antioxidant and glutathione precursor. NAC was previously shown to restore the GSH/GSSG ratio in DCs exposed to contact sensitizers, thus abolishing MAPK phosphorylation (e.g., p38 MAPK) and *CD86* increased expression [18]. Therefore, our results reinforce the involvement of oxidative stress in allergen-induced MAPK activation. However, in the particular case of the respiratory sensitizer HDI, H_2O_2 and particularly O_2^- seem to be responsible for ERK activation at the time point studied (6 h), but not for p38 MAPK activation. This is supported by the fact that both Rot (10 μ M, the higher concentration tested) and H_2O_2 (50 mM, the lowest concentration tested) increased P-ERK levels but not P-p38 MAPK. Surprisingly, Cu-ZnSOD added exogenously before HDI treatment increased ERK activation. This raised the hypothesis of an altered SOD function, probably due to cytoplasmic HDI interaction with the enzyme, leading to O_2^- accumulation and further ERK activation. In fact, decreased SOD activity, but not protein levels, has been reported in asthmatic humans [26–30].

Therefore, we determined SOD activity in HDI-exposed DCs, which confirmed that the cytoplasmic Cu-ZnSOD activity is reduced, but not mitochondrial MnSOD. Interestingly, although MnSOD activity seems to be unaffected by HDI, in LPS-treated cells we observed a significant increase in the activity of mitochondrial SOD, suggestive of a compensatory-like mechanism. Interestingly and in agreement with our results, in a different research model, LPS-triggered inflammatory conditions decreased SOD 1 but increased SOD 2 mRNA levels in rat renal mesangial cells [47]. SOD 2 increased activity could account for the unaltered MMP or mitochondrial O_2^- levels in cells exposed to LPS, as determined by TMRE and MitoSOX fluorescence, respectively. On the other hand, HDI significantly reduced MMP and increased O_2^- in the mitochondria. These findings disclose differential pathways of toxicity triggered by the respiratory

sensitizer and the immunogenic, but nonsensitizing, LPS. Inhibition of either the ERK or the p38 MAPK pathway through specific inhibitors did not influence HDI's effect on mitochondria, supporting the notion of mitochondrial ROS formation as an upstream event of both signaling cascades. However, ERK (as p38 MAPK) activation in the presence of HDI was counteracted by NAC, further suggesting that dysfunctional antioxidant enzymes and increased H_2O_2 levels have to occur primarily over O_2^- -induced ERK activation. Interestingly, in DCs preincubated with SOD and exposed to HDI we observed a trend to increased mitochondrial O_2^- formation with SOD concentration. These results might explain the significant increase in HDI-induced ERK phosphorylation after SOD incubation at the highest concentration tested (500 U), again reinforcing the role of mitochondrial O_2^- in ERK activation due to respiratory allergen exposure.

Kinase phosphorylation/dephosphorylation regulates cell signal transduction, which will further lead to surface marker expression and cytokine/chemokine secretion. Therefore components of the signal transduction cascade might be promising biomarkers to distinguish sensitizers from nonsensitizers. p38 MAPK has been one of the most extensively tested signaling proteins in DC-based assays ([13], for review). Several studies regarding contact sensitization have reported the role of p38 MAPK activation in human DC maturation induced by contact allergens, such as DNFB and nickel [48–50], whereas the ERK pathway has been described as a negative regulator of DC maturation, as observed in monocyte-derived DCs stimulated with LPS and TNF- α [49]. In contrast, our results show a mild but significant increase in CD83 (a DC lineage-specific marker) gene expression promoted by HDI. This increase was almost completely blocked by an ERK-specific inhibitor (but not p38 MAPK inhibitor), suggesting that ERK activation is needed for DC maturation triggered by a respiratory allergen. In a previous study, a significant increase in CD83 expression was also observed in THP-1 cells exposed to contact allergens [12]. Although CD83 function in the induction phase of skin sensitization is still unknown, the pattern of expression of CD83 suggests that it may have an important role in antigen presentation or cellular interaction [51].

The MAPK activation during DC maturation seems to be specific for (contact) sensitizers because the exposure to an irritant such as SDS did not induce changes in signaling pathways [52]. The authors observed that p38 MAPK and also JNK were markedly activated, whereas ERK1/2 was mainly inhibited, except in nickel-treated human DCs. Interestingly, when the p38 MAPK pathway was inactivated upon incubation with the p38 MAPK inhibitor SB203580, ERK1/2 inhibition induced by contact sensitizers was reverted, suggesting interplay between the p38 MAPK and the ERK1/2 pathways [52]. This could also explain the additional increase in P-ERK levels after incubation of HDI-exposed DCs with p38 MAPK inhibitor, indicative of cell choice between divergent pathways depending on the environment. Nevertheless, other studies reported p38 MAPK and ERK phosphorylation in the THP-1 human monocytic cell line, promoted by DNCB and nickel; moreover, inhibition of these two pathways caused a selective downregulation of CD86, CD54, and/or CD40 expression [15]. We also observed an increase in *HMOX1* gene expression, as similarly reported for contact sensitizers [39]. Ade et al. [39] showed that THP-1 cells exposed to contact sensitizers, but not to irritants, display increased *HMOX1* and *NQO1* mRNA levels at early time points, which was counteracted by NAC. However, in our study NAC failed to revert HDI-induced *HMOX1* mRNA to CTR levels. Moreover, we did not detect changes in *NQO1* gene expression in DCs due to HDI (or to trimellitic anhydride, another respiratory allergen; data not shown). Thus, the *NQO1* gene might be a promising candidate to distinguish contact from respiratory sensitizers. *HMOX1* is also modulated at a lower extent by HDI compared to the contact sensitizer DNFB (DNFB increased *HMOX1* expression in THP-1 cells by sixfold compared to approx twofold

after HDI exposure; our unpublished results). Increased expression of Nrf2-dependent ARE-responsive genes (e.g., *HMOX1* and *NQO1*) is usually related to alterations in GSH/GSSG ratio by contact sensitizers. In contrast, in our study HDI did not significantly alter glutathione status (see Supplementary Fig. S3), which might partially explain the lack of responsive gene expression linked to xenobiotic metabolism (as *NQO1*). Instead, HDI seems to induce cell detoxification through *HMOX1* and *MDR1*. In fact, *MDR1* gene expression was augmented in HDI-exposed DCs. P-glycoprotein (P-gp), the human *MDR1* gene product, is an ATP-dependent drug efflux pump, which gives cellular resistance to cytotoxic xenobiotics by reducing intracellular drug accumulation and further induced drug toxicity [53,54]. P-gp is physiologically expressed by human immune cells. We cannot speculate about the role of *MDR1* in our cells. However, previous studies demonstrated that skin DCs and T cells express *MDR1* on their surface and that *MDR1* antagonists inhibit migration of epidermal DC from the periphery to lymph nodes (which would further initiate T-lymphocyte-mediated immunity) [55]. In addition, Frank and co-workers showed for the first time that P-gp is functional in human alloantigen-dependent T cell activation [56]. Similar to what was described for *HMOX1* and *NQO1*, NAC did not revert *MDR1* gene modulation by HDI, which might support the hypothesis that O_2^- is the main ROS species in respiratory allergen-induced ERK activation. Indeed, HDI itself might inactivate intracellular SOD, thus increasing O_2^- up to the necessary levels for ERK activation, even in the presence of GSH and consequent normal H_2O_2 levels. Nevertheless, ERK inactivation by its specific inhibitor significantly decreased the expression of the three genes, supporting the hypothesis that DCs stimulated by a respiratory allergen trigger the ERK pathway to increase cell antioxidant defenses and survival, migration, and maturation, as suggestive of increased *HMOX1*, *MDR1*, and CD83 transcription, respectively.

Overall our results indicate that HDI inhibits cytoplasmic SOD 1 in DC-like cells, leading to an increase of O_2^- that in turn might account for MMP reduction and further increase in mitochondrial O_2^- levels. The accumulation of O_2^- activates ERK, further inducing the expression of cytoprotective genes (*HMOX1*), genes responsible for the transportation of xenobiotics (*MDR1*), and DC maturation markers (CD83).

These results brought new insights into the action mechanism and cellular response to respiratory allergens in DC, particularly with regard to the formation/accumulation of O_2^- through enzymatic inhibition, as a consequence of respiratory sensitization, and a necessary event for ERK activation is strongly sustained. The role of ERK in DCs challenged by a respiratory allergen, to survive, migrate, and mature, is supported.

Acknowledgments

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Appendix A. Supplementary material

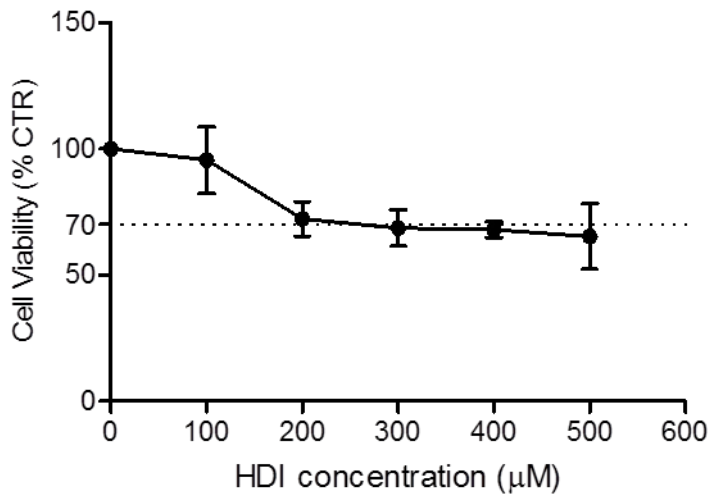
Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.freeradbiomed.2014.04.005>.

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Table S1. Primer sequences used to amplify target cDNAs

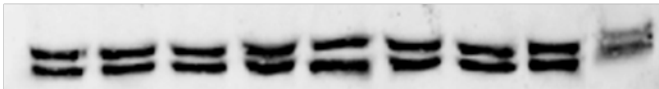
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<i>nq01</i>	GAG TCT GTT CTG GCT TAT	AAC TGG AAT ATC ACA AGG T
<i>mdr1</i>	AGA GAC ATC ATC AAG TGG AGA G	AGC AAG GCA GTC AGT TAC A
<i>cd83</i>	ATT GAG TCA TTA TCC TTG CTA	GCT TCT TGG TAA CCT TCT T
<i>cd86</i>	GAA CCT AAG AAG ATG AGT	TCC AGA ATA CAG AAG ATG



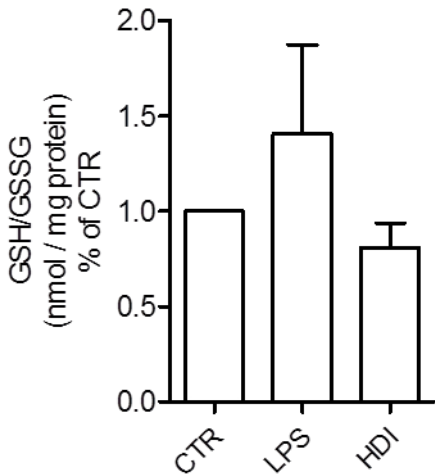
	5 min		30 min		1 h		6 h	
	LPS	HDI	LPS	HDI	LPS	HDI	LPS	HDI
CTR								



P-ERK 1/2 (44/42 kDa)



ERK 1/2 (44/42 kDa)



Quantification of intracellular levels of reduced (GSH) and oxidised (GSSG) glutathione

Intracellular levels of reduced and oxidised glutathione (GSH and GSSG, respectively) were determined using a fluorimetric assay as described previously (Hissin and Hilf, 1976). After the period of incubation, the medium was removed by centrifugation (250 *g*, 5 min at 4°C) and the cells were washed with PBS (4°C) and further centrifuged. The pellet was resuspended in 750 µl of 100 mM phosphate buffer containing 5 mM EDTA, pH 8.0 at 4°C. Then 250 µl of 0.6 M perchloric acid was added. After vigorous vortex, the suspension was centrifuged at 14000 rpm for 5 min at 4° C. For the GSH determination, a volume of 100 µl of supernatant was incubated with 100 µl of ortho-phthaldehyde (0.1% w/v in methanol) and 1.8 ml of 100 mM Na₂HPO₄ for 15 min at room temperature. Fluorescence intensity was then read in a Synergy HT plate reader (Bio-Tek Instruments) using an excitation wavelength of 350 nm and an emission wavelength of 420 nm. For GSSG determination 250 µl of sample was pre-incubated 30 min with 100 µl NEM (0.5% w/v in methanol). Then, 140 µl of this solution was incubated for 15 min in 1.76 ml 100 mM NaOH plus 100 µl OPT, and the fluorescence was measured (λ_{exc} =350 nm; λ_{em} =420 nm). Intracellular GSH and GSSG contents were calculated using the respective standard curves, containing known concentrations of GSH and GSSG (0–2 µg) and expressed as nmoles of GSH or GSSG per milligram of protein. The ratio GSH:GSSG was further calculated and expressed as percentage of control (non-treated; CTR) cells. Cellular protein content was quantified by the Bio-Rad protein assay dye, using bovine serum albumin as the standard.

References

Hissin, P.J.; Hilf, R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem.* 74:214-226; 1976.