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Gene expression profiling of *in vitro* cultured macrophages after exposure to the respiratory sensitizer hexamethylene diisocyanate.

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Short title: Gene expression profiling of hexamethylene diisocyanate

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Abbreviations

aaUTP: 5-(3-aminoallyl)-UTP

aRNA: amplified RNA

BT: biuret trimer

CCL: chemokine (C-C motif) ligand

DC: dendritic cells

DNCB: 2,4-dinitrochlorobenzene

GO: Gene Ontology

GSH: glutathione

HDI: hexamethylene diisocyanate

HSC: heat shock cognate protein

HSP: heat shock protein

IC: inhibition concentration

Ig: immunoglobulin

IPDI: isophorone diisocyanate

IVT: in vitro transcription

MDI: diphenylmethane diisocyanate

NiSO₄: nickel sulphate

OA: occupational asthma

PMA : phorbol 12-myristate 13-acetate

pPD: p-phenylenediamine

SAM: significance analysis of microarrays

TDI: toluene diisocyanate

Abstract

Occupational exposure to chemicals is one of the main causes of respiratory allergy and asthma. Identification of chemicals that trigger allergic asthma is difficult as underlying processes and specific markers have not yet been clearly defined. Moreover, adequate classification of the respiratory toxicity of chemicals is hampered due to the lack of validated *in vivo* and *in vitro* test methods. The study of differential gene expression profiles in appropriate human *in vitro* cell systems is a promising approach to identify selective markers for respiratory allergy.

As alveolar macrophages display important immunological and inflammatory properties in response to foreign substances in the lung, we aimed at gaining more insight in changes of human macrophages transcriptome and to identify selective genetic markers for respiratory sensitization in response to hexamethylene diisocyanate (HDI).

In vitro cultures of human THP-1 cells were differentiated into macrophages and exposed to 55 µg/ml HDI for 6 and 10 hours. Using human oligonucleotide microarrays, changes were observed in the expression of genes that are involved in diverse biological and molecular processes, including detoxification, oxidative stress, cytokine signaling, and apoptosis, which can lead to the development of asthma. These genes are possible markers for respiratory sensitization caused by isocyanates.

1. Introduction

Occupational asthma (OA) is defined as asthma that is completely or predominantly due to an agent encountered at the workplace. This definition is distinct from work-aggravated asthma, where a pre-existing asthma sufferer has symptoms brought on by workplace exposures (Curran and Fishwick, 2003). Approximately 300 substances are known to cause OA (Bernstein *et al.*, 2002) and it is the most commonly reported occupational lung disease in many industrialized countries (Herrick *et al.*, 2002; Redlich *et al.*, 2002).

Isocyanate-induced asthma is probably the most frequent type of OA (Deschamps *et al.*, 1998; Redlich *et al.*, 2002) of which the first case was reported in 1951 (Plitnick *et al.*, 2005). The term 'isocyanate' should be understood as the collective term used for substances with free $\text{N}=\text{C}=\text{O}$ -groups, thus including monoisocyanates, diisocyanates, polyisocyanates and prepolymers (Deschamps *et al.*, 1998).

Diisocyanates are very reactive low-molecular weight chemicals and excellent cross-linking agents, making them extremely valuable in a variety of chemical manufacturing processes, such as the production of polyurethane foams, adhesives, surface coatings, spray paints, foundry cores, insecticides, and elastomers (Baur *et al.*, 1994; Tee *et al.*, 1998; Redlich and Karol, 2002). Diisocyanates react rapidly with the hydroxyl groups of polyols, water, and with functional groups present on all proteins, such as amines, hydroxyls, and sulfhydryls (Wisnewski *et al.*, 2000). The most commonly used diisocyanates are toluene diisocyanate (TDI), diphenylmethane diisocyanate (MDI), hexamethylene diisocyanate (HDI), and isophorone diisocyanate (IPDI) (Piiirila *et al.*, 2000; Redlich and Karol, 2002). The worldwide production of diisocyanates is estimated to be 1.4 billion kilo annually. Estimates on the prevalence of diisocyanate asthma among exposed workers range from less than 5 % to 15 % (Deschamps *et al.*, 1998; Tee *et al.*, 1998; Redlich and Karol, 2002; Redlich *et al.*, 2002; Jones *et al.*, 2006). One of the most common and commercially important isocyanates is HDI, which is frequently used in spray painting operations in automobile body shops, and decomposes into polyurethanes that are particularly light and weather resistant (Wisnewski *et al.*, 1999; Lemus *et al.*, 2001).

Several allergic and non-allergic mechanisms have been suggested for isocyanate-induced asthma. The latter include reflex and pharmacologic responses, destructive histopathologic changes, and β -adrenergic blockade. However, these do not explain the characteristic allergic features (Tee *et al.*, 1998). The clinical presentation of diisocyanate asthma is variable and difficult to distinguish from adult onset asthma, often complicating its recognition and

diagnosis. Symptoms of diisocyanate asthma typically do not manifest until after an unpredictable latency period of several months to years after initial exposure. They begin insidiously and include cough, chest tightness, shortness of breath, or wheezing, which are temporally related to workplace exposure. Only about 5-30 % of symptomatic workers have detectable levels of diisocyanate-specific immunoglobulin (Ig)E (Lummus *et al.*, 1998; Piirila *et al.*, 2000; Bernstein *et al.*, 2002; Redlich and Karol, 2002; Redlich *et al.*, 2002; Wisnewski *et al.*, 2003). A variety of immune cell types are involved in the development of OA. As alveolar macrophages display important immunological and inflammatory properties in response to foreign substances in the lung, we aimed at gaining more insight in changes of human macrophages transcriptome and to identify selective genetic markers in response to the respiratory sensitizer HDI. *In vitro* cultures of human THP-1 cells were differentiated to macrophages and were exposed to HDI. Global changes in gene expression were evaluated using oligonucleotide arrays containing 30000 human genes.

Materials and Methods

Cell culture

THP-1, a human monocytic leukemia cell line, was obtained from American Type Culture Collection (LGC Promochem, Teddington, UK). THP-1 cells were cultured in complete culture medium containing 89 % RPMI 1640 Medium with GlutaMAXTM-1 and 25 mM HEPES (Invitrogen, Paisley, UK) supplemented with 10 % fetal calf serum (Harlan Sera-Lab LTD, Crawley Down, UK) and 1 % Penicillin (5000 U/ml)-Streptomycin (5000 µg/ml) (Invitrogen). The cells were kept in a humidified atmosphere at 37 °C and 5 % CO₂. They were subcultured every 3-4 days. Cells were seeded in six-well plates at 1.25×10^6 cells / ml complete medium. To allow adherence and differentiation into macrophages, the cells were incubated with 0.25 mg/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 48 hours before exposure to the test substance (Auwerx, 1991).

Chemical exposure of the cells

After 48 hours incubation with PMA, THP-1 cells (5×10^6 cells/4 ml/well from 6-well-plate) were exposed to 55 µg/ml (327 µM) HDI (Sigma-Aldrich Chemie GmbH, CAS 822-06-0) for a selected time period (3, 6, 10 or 24 hours) at 37 °C, 5 % CO₂ and 95 % humidity. This HDI-concentration reduced cell growth with 20 % (inhibition concentration (IC₂₀)) after 24 hours of exposure, as determined by the neutral red cytotoxicity assay in our laboratory (data not shown). The selection of this concentration was based on previous experiments which showed that a weak distortion of the cells could provoke the production of several substances which trigger the process of sensitization (Hulet *et al.*, 2005; Jacobs *et al.*, 2006; Schoeters *et al.*, 2007). Negative control samples were obtained by treating THP-1 cells in parallel with the solvent. The solvent for HDI was dimethylsulphoxide (LabScan Ltd., Dublin, Ireland) at a final concentration of 0.05 % in the tissue culture medium. Four independent exposure experiments were performed. Only the results of 6 and 10 hours of exposure were further analysed for gene expression.

RNA isolation

After exposure, the supernatants of the cells was removed and the cells were lysed in the plates with RNA lysis buffer, consisting of ½ volume of buffer-saturated phenol (Invitrogen),

½ volume of RLT buffer (Qiagen, Hilden, Germany) and 0.25 mg/ml 8-hydroxyquinolin (Merck, Brussels, Belgium). Total RNA was isolated using mini RNeasy RNA isolation kits, according to the manufacturer's specifications (Qiagen). The RNA concentration was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, USA) and purity was visually inspected for absence of degradation products using agarose gel electrophoresis. RNA was stored in RNase-free water (Qiagen) at -80 °C.

RNA amplification and labelling

RNA amplification was performed on all negative control and exposed samples using the Amino Allyl MessageAmp™ II amplified RNA (aRNA) Amplification Kit (Ambion, Texas, USA). For the first strand cDNA synthesis 1 µg of total RNA was mixed with 1 µl of T7 Oligo(dT) Primer supplemented with T7 promoter in a total volume of 12 µl nuclease-free water, and kept at 70 °C for 10 minutes in a QB-96 Gradient Thermal Cycler (Quanta Biotech, Surrey, UK). To this mixture, the reverse transcription master mix was added, consisting of 2 µl 10x First Strand Buffer, 4 µl dNTP mix, 1 µl RNase Inhibitor and 2 µl ArrayScript. The sample was incubated at 42 °C for 2 hours. To this first strand reaction mix, the second strand master mix was added, consisting of 63 µl nuclease-free water, 10 µl 10x Second Strand Buffer, 4 µl dNTP mix, 2 µl DNA Polymerase and 1 µl RNase H. The sample was incubated at 16 °C for 2 hours. The synthesized double-stranded cDNA was purified with cDNA purification columns provided in the kit and eluted two times with 9 µl of preheated (50-55 °C) nuclease-free water. After clean-up of the cDNA, it becomes a template for *in vitro* transcription (IVT) with T7 RNA Polymerase. To maximize aRNA yield, Ambion's proprietary MEGAscript® IVT technology is used to generate hundreds to thousands of antisense RNA copies of each mRNA in a sample. The IVT master mix consists of 3 µl 5-(3-aminoallyl)-UTP (aaUTP, 50 mM), 12 µl ATP, CTP, GTP mix (25 mM), 3 µl UTP solution (50 mM), 4 µl T7 10x Reaction Buffer and 4 µl T7 Enzyme Mix. The IVT reaction is configured to incorporate the modified nucleotide aaUTP into the RNA. 5-(3-aminoallyl)-UTP contains a reactive primary amino group on the C5 position of uracil that can be chemically coupled to N-hydroxysuccinimidyl ester-derivatized reactive dyes, such as Cy3 and Cy5, in a simple, efficient reaction. The sample was incubated overnight at 37 °C in an Eppendorf ThermoStat Plus (Eppendorf, Hamburg, Germany). The aRNA was redissolved in 60 µl nuclease-free water and purified with aRNA purification columns provided in the kit. The sample was eluted with 100 µl of preheated (50-55 °C) nuclease-free water. The

concentration of the aRNA solution was determined by using the NanoDrop spectrophotometer (NanoDrop Technologies). Five μg of amino allyl aRNA was dried in a SpeedVac (Savant Instruments Inc., New York, USA) and redissolved in 4.5 μl Coupling Buffer. Four and a half microliters of Cy-dye ester (Amersham Biosciences, Buckinghamshire, UK) was added and incubated during 30 minutes in the dark at room temperature. Cy5 was used for the chemical-exposed samples, Cy3 for the negative controls. For each combination of a control and exposed sample, a colorflip experiment was performed. To quench the reaction, 4.5 μl Hydroxylamine (4 M) was added. After an incubation period of 15 minutes in the dark at room temperature, each sample was brought to a volume of 30 μl with nuclease-free water. The samples were purified from non-incorporated Cy-dyes using purification columns provided in the kit, and eluted two times with 10 μl of preheated (50-55 $^{\circ}\text{C}$) nuclease-free water. Yield and incorporation of the dyes were estimated by means of a NanoDrop spectrophotometer (NanoDrop Technologies). Equal amounts of the negative control sample (Cy3 labelled) and the chemical-exposed sample (Cy5 labelled) of the same donor and exposure time point were combined, and dried in a SpeedVac (Savant Instruments Inc.). The combined sample was dissolved in 9 μl of nuclease-free water and 1 μl of fragmentation buffer (Ambion) was added. After incubation at 70 $^{\circ}\text{C}$ for 10 minutes, the reaction was stopped by adding 1 μl of stop solution (Ambion).

RNA Microarray hybridisation

Microarray slides were obtained from the Vanderbilt Microarray Shared Resources Centre (Vanderbilt University, Nashville, USA). Human 30K arrays, containing oligonucleotide clones corresponding to approximately 30000 human genes were used. Combined Cy3/Cy5 labelled samples were redissolved in hybridization solution (1 μg /100 μl , DIG Easy Hyb Granules, Roche, Mannheim, Germany). Samples were applied to the microarray slide and hybridized for 20 hours at 46 $^{\circ}\text{C}$. A HS 400 hybridization station from Tecan (Grödig/Salzburg, Austria) was used for prehybridization, hybridization and subsequent washing and drying of the slides, all according to the instructions of the manufacturer.

Microarray setup

Two-colour slides were used, each hybridized with a mixture of one exposed and one control sample. A full colour-flip setup was applied: each slide has a technical replicate of the same

sample with colour labels inversed. For each control-exposed experiment four biological replicates were used, resulting in eight hybridizations per time point.

Microarray data analysis

Slides were scanned at 543 nm and 633 nm using a Tecan LS200 laser scanner. Grid placing was performed with ArrayPro Analyzer software (MediaCybernetics, Silver Spring, USA) and subsequent quality control and normalization was performed using MatLab tools (The MathWorks, Inc., Natick, MA). Local background was subtracted and the data were printtip-normalized using a Lowess-procedure (Smyth and Speed, 2003). For each spot, the intensities of the colour-flip pairs were combined (using the geometric mean) to one value for the exposed and control sample. The resulting data set contains per exposure condition, the expression signal of each spotted gene in four biological replicates of the exposed and control sample. After quality control of the hybridized slides, only the results of three independent experiments with colorflip transition were used for time point 10 h. For 6 h exposure time, all four independent experiments with colorflip were used for data analysis.

Statistical analysis

We used the Significance Analysis of Microarrays (SAM) technique to find genes with a significant difference in expression between control and exposed cells in our set of microarray experiments (Tusher *et al.*, 2001). The input to SAM is gene expression measurements from a set of microarray experiments, as well as a response variable from each experiment. In our case the response variable is a grouping of exposed and control THP-1 cells. For each gene, SAM quantifies the strength of the relationship between gene expression and the response variable in a statistic d score. Next, it uses repeated permutations of the data to estimate the significance of this relation. The cut-off for significance is user-determined and based on the false positive rate. The q -value of a gene is defined as the lowest false discovery rate at which the gene is called significant (Storey, 2002), and can serve as a selection criterion. As the d score increases, the corresponding q -value decreases.

It is customary to present microarray results as fold changes: ratios of the gene expression levels of treated samples over negative control samples. Since the distribution of fold changes is more closely related to a lognormal than to a normal, we also present the $\log_2$ transformation of this variable, which is consequently more normally distributed. For a set of

significant genes, the mean and standard deviation of all the biological replicates of this logarithmic fold change is presented in the first column of each exposure time in the table below. In the column next to it, the geometric mean of the original fold changes is presented, but without the standard deviation since it is a non-normal variable. A (mean) $\log_2$ value of 1 and -1 corresponds to a (geometric mean) fold change of 2 and 0.5 respectively.

Gene function annotation

Functional information concerning the genes was obtained from Onto-Express (<http://vortex.cs.wayne.edu/projects.htm>) and through database scanning on the internet, in particular the Source ([http://genome-www5.stanford.edu/cgi-bin/source/sourceBatch Search](http://genome-www5.stanford.edu/cgi-bin/source/sourceBatchSearch)), LocusLink (<http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=2213>), GenAtlas (<http://www.dsi.univ-paris5.fr/genatlas/fiche.php?symbol=FCGR2B>) and OMIM (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>) databases were used.

3. Results

Microarray analysis was performed in order to study a large number of genes whose expression may potentially be altered by HDI exposure. Oligonucleotide arrays were hybridized with RNA samples from *in vitro* cultured human macrophages collected 6 and 10 h after exposure to 55 µg/ml HDI or to vehicle-treated cells. Data from the hybridizations for each time point were filtered using the SAM technique. Genes with a q-value <1 % whose expression was changed by HDI after 6 and 10 h exposure, are indicated in table 1 with bold numbers. This means that these genes are significantly influenced by exposure to HDI and the likelihood of false positive results is below the predetermined statistical limit of 1 %. After 6 h, 13 genes (*FCGR2B*, *PIR*, *NQO1*, *GCLM*, *GLRX*, *KITLG*, *RIT1*, *FLJ21908*, *CKS2*, *MGC11257*, *TUBA1*, *BAG3*, *SNRPN*) exhibited significantly increased expression by HDI and the expression of 2 genes (*CCL20* and *YIPF2*) was significantly decreased. After 10 h of exposure to HDI, 19 genes (*FCGR2B*, *PIR*, *NQO1*, *GLRX*, *MGC11257*, *TUBA1*, *BAG3*, *SNRPN*, *HSPA1A*, *TALDO1*, *PGD*, *C20orf24*, *SPTA1*, *POPDC3*, *THRAP5*, *HMOX1*, *HSPB8*, and two unknown genes) were significantly upregulated. Eight genes (*FCGR2B*, *PIR*, *NQO1*, *GLRX*, *MGC11257*, *TUBA1*, *BAG3*, and *SNRPN*) showed differential gene expression changes after 6 and 10 h exposure to HDI. For these genes, except *GLRX*, an obvious time trend was observed with increased expression at 10 h after exposure. The *NQO1* gene is represented by two different accession numbers.

The Onto-Express software tool (<http://vortex.cs.wayne.edu/projects.htm>) was used to categorize the 26 (34 individual genes – 8 overlapping genes) selected genes into functional groups. Onto-Express relates Gene Ontology (GO) terms to specific genes, which provide information on the cellular component, biological process, and molecular function. To classify the genes into functional groups, the molecular and biological GO terms were analyzed. The genes could be arranged into eight different molecular functional groups (figure 1) and six different biological functional groups (figure 2). Some genes were members of more than one group. The largest functional groups contained genes related to binding and catalytic activity for the molecular process, and cellular and physiological processes for the biological function. For two genes (*YIPF2* and *C20orf24*) no assigned GO terms were found.

4. Discussion

The objective of this study was to gain more insight in gene expression changes of the human macrophage transcriptome and to identify selective genetic markers for respiratory sensitization in response to the model chemical HDI. Therefore, global changes in gene expression were evaluated in *in vitro* cultures of human THP-1 macrophages after exposure to HDI or vehicle. After treatment with PMA, THP-1 cells differentiate into a macrophage-like cell which resembles native monocyte-derived macrophages with regard to morphology, expression of membrane antigens and receptors, oncogene expression, and secretory products. We therefore selected the THP-1 cell line as a representative and valuable model for studying gene expression changes in macrophages (Tsuchiya *et al.*, 1980; Auwerx, 1991).

Isocyanates, as well as other low-molecular weight compounds that cause occupational asthma, act as haptens that are presented to the immune system after conjugation to a carrier protein (Wisnewski *et al.*, 1999). The covalent binding of one or several diisocyanate functionalities with NH_2 , OH and SH functional groups of certain amino acids is believed to be the initial step in diisocyanate sensitization (Redlich and Karol, 2002). *In vivo* exposure has identified a number of diisocyanate-conjugated proteins and peptides, including albumin, keratin, tubulin, and glutathione (GSH), which are all abundantly present in the respiratory tract and/or skin (Redlich *et al.*, 2002). In this study, the macrophages were exposed to HDI in RPMI 1640 medium with 10% FCS. The latter contains different proteins (e.g. albumin) and several of these are also present in the cell culture medium (e.g. GSH). Therefore, it is highly likely that HDI binds to these proteins in the medium, as observed by Redlich *et al.* (2002).

In vivo and *in vitro* models have been used to study the pulmonary toxicology of isocyanates and mechanisms by which they contribute to lung disease (Pauluhn and Mohr, 2001; Herrick *et al.*, 2002). Up to now, two studies have been published that used microarrays to analyze gene expression changes after HDI exposure. Lee and coworkers (2005) examined RNA extracts of lung tissue of HDI-biuret trimer (BT)-exposed (10.3 mg/m^3) C57BL/6J mice after different time points (6, 18, and 90 h) using microarrays of approximately 10000 genes (10K) (Lee *et al.*, 2005). The effects of HDI on human airway epithelial cells *in vitro* at the cellular and molecular level were investigated by Wisnewski *et al.* (2002). Microarray analysis of 4608 transcripts (4.6K) was performed on lung mucoepidermoid (NCI-H292) cells after (acute) 20-min exposure to $30 \text{ }\mu\text{M}$ HDI or vehicle.

Using high-density oligonucleotide arrays in the present study, a number of genes were observed to be influenced by exposure of human THP-1-derived macrophages to 55 µg/ml HDI. The genes glutamate-cysteine ligase, modifier subunit (*GCLM*) and glutaredoxin (*GLRX*) play an important role in GSH metabolism. Both genes were upregulated in this study after respectively 6 h, and 6 and 10 h exposure to HDI in GSH containing medium. One important initial target for diisocyanates is GSH, which functions as a major intracellular and extracellular buffer against oxidative stress in the lungs (Day *et al.*, 1997; Lange *et al.*, 1999a). Conjugation of inhaled isocyanates with GSH in the airways results in depletion of free GSH. This depletion is likely to cause oxidative stress by a diminished antioxidant defence, which can lead to a variety of airway diseases, such as asthma (Lantz *et al.*, 2001). In this context and with the results obtained in our study, a hypothetical model for HDI toxicity was developed (figure 3). *GCLM* is the first rate-limiting enzyme of GSH synthesis. *GLRX* is a GSH-dependent hydrogen donor for ribonucleotide reductase and also catalyzes GSH-disulfide oxidoreduction reactions in the presence of NADPH and GSH reductase. *GLRX* is part of the antioxidant defence of the lungs that is upregulated during allergic airway disease. Through its deglutathiolating activity, *GLRX* can be responsible for liberating GSH from HDI to increase GSH levels to combat the oxidant stress associated with the inflammatory response. A previous study reported that human *GLRX* was mainly localized in alveolar macrophages, and only weakly in bronchial epithelium (Peltoniemi *et al.*, 2004). The latter can be confirmed by our study, where *GLRX* was found to be expressed in *in vitro* cultured macrophages.

Besides *GCLM* and *GLRX*, we observed an increased expression in THP-1 macrophages of six genes (*NQO1*, *HMOX1*, *PGD*, *TALDO1*, *HSPA1A*, and *HSPB8*) encoding proteins that regulate stress responses. These results suggest that oxidoreductase activity in the lung may be an important mechanism of HDI toxicity. Four of these genes (*GLRX*, *GCLM*, *HMOX1*, and *HSPA1A*) were also significantly upregulated in the lungs of C57BL/6J mice after 6 h exposure of mice to HDI-BT (Lee *et al.*, 2005).

Heme oxygenase 1 (*HMOX1*) is part of the integrated response to oxidative stress and it has been implicated in the regulation of pulmonary inflammation. It catalyzes the rate-limiting step of heme oxidation to biliverdin, carbon monoxide, and iron (Donnelly and Barnes, 2001; Carter *et al.*, 2004). Expression of *HMOX1* is induced by various stress stimuli, such as UV light, oxidation, heat shock (Keyse and Tyrrell, 1989), and thiol-reactive substances (Carter *et al.*, 2004), as well as inflammatory cytokines, such as tumor necrosis factor- α , interleukin-1 β and interferon- γ (Rizzardini *et al.*, 1993), and oxidants, such as nitric oxide (Donnelly and

Barnes, 2001). HMOX1 is induced in the airways during asthma and has anti-inflammatory effects, probably via enhancement of the secretion of IL-10 and promotion of the percentage of CD4+CD25 regulatory T cells (Carter *et al.*, 2004; Xia *et al.*, 2006). Overexpression of the HMOX1 gene in cells has revealed a role for its protein in protection against oxidative stress (Lee *et al.*, 1996). The distribution and expression of *HMOX1* in various human lung cells in acute and stable asthma was studied by Harju and coworkers (2002). They found prominent HMOX1 immunoreactivity only in alveolar macrophages (Harju *et al.*, 2002). Hirota and Moro (2006) studied changes in gene expression profile of THP-1 monocytes after exposure to skin sensitizers 2,4-dinitrochlorobenzene (DNCB), *p*-phenylenediamine (*p*PD) and nickel sulfate (NiSO₄) using oligo-DNA microarrays. Increased expression of *HMOX1* was shown after exposure to DNCB, *p*PD and NiSO₄ (Hirota and Moro, 2006).

The gene phosphogluconate dehydrogenase (*PGD*) together with transaldolase1 (*TALDO1*) plays an important role in the pentose phosphate pathway, which is responsible for generation of reducing equivalents to protect cellular integrity from reactive oxygen intermediates. In the THP-1 cell line, *PGD* was upregulated after *p*PD and NiSO₄ exposure (Hirota and Moro, 2006).

The gene NAD(P)H dehydrogenase, quinone 1 (*NQO1*) codes for a protein that serves as a quinone reductase in connection with conjugation reactions of hydroquinones involved in detoxification pathways, with NADH and NADPH as electron donor. The enzyme protects the cells against oxidative stress and is involved in biosynthetic processes, such as the vitamin k-dependent γ -carboxylation of glutamate residues in prothrombin synthesis. In case-control studies of candidate genes for asthma, Millstein and colleagues (2006) identified a significant multilocus effect between the *NQO1* gene, the myeloperoxidase gene, and the catalase gene, three genes that are involved in the oxidative stress pathway (Millstein *et al.*, 2006).

The expression of heat shock 70kDa protein 1A (*HSPA1A*), an isoform of heat shock protein (HSP) 70 kDa, was greatly increased by HDI and appears to be particularly susceptible to upregulation by oxidative stress, as all members of the family of heat shock or stress-induced proteins (Morimoto, 1993). In cooperation with other chaperones, HSP70s stabilize pre-existent proteins against aggregation and mediate the folding of newly translated polypeptides in the cytosol, as well as within organelles. They complex with peptides, and bind to dendritic cells (DC) and macrophages before being internalized in a receptor-dependent manner during translation and membrane translocation, or following stress-induced damage. Vignola and coworkers (1995) studied the expression of *HSP70s* on alveolar macrophages from patients with asthma, chronic bronchitis, and control subjects. They demonstrated that bronchial

inflammation in asthma, but not in chronic bronchitis, may be linked to the production of HSP70 (Vignola *et al.*, 1995). In our study, the expression of another HSP with chaperone activity, heat shock 22kDa protein 8 (*HSPB8*), was also significantly enhanced after 10 h exposure to HDI.

In this study, we also found some genes involved in signal transduction and inflammation, namely Fc fragment of IgG, low affinity IIb, receptor (*FCGR2B*) and KIT ligand (*KITLG*), that were upregulated after 6 and 10 h of exposure to HDI. *FCGR2B* codes for a receptor for the Fc region of complexed or aggregated gamma immunoglobulins. This gene is involved in a variety of effector and regulatory functions and plays a role in the cascade of an allergic response. Two studies demonstrated the presence of several activation markers, such as *FCGR2B* (CD32), on alveolar macrophages in asthmatic subjects (Viksman *et al.*, 1997; Viksman *et al.*, 2002). *KITLG* encodes the ligand of the tyrosine-kinase receptor encoded by the KIT locus. *KITLG*, also known as stem cell factor, is a major chemotactic factor for mast cells and their progenitors. It stimulates the proliferation, and sustains the survival, differentiation, and maturation of mast cells, which are important cells in allergic reactions through high-affinity IgE receptor mediated responses. It also mediates cell-cell and cell-substratum adhesion and acts synergistically with other cytokines, probably interleukins (Okayama and Kawakami, 2006). Besides *KITLG*, the *CKS2* (CDC28 protein kinase regulatory subunit 2) gene is also increased in this study and is involved in regulation of cell proliferation. *CKS2* protein binds to the catalytic subunit of cyclin-dependent kinases and is essential for their biological function. *CKS2* was significantly upregulated in lungs of C57BL/6J mice after 90 h exposure to HDI-BT (Lee *et al.*, 2005), which indicates same agreement of our *in vitro* model with *in vivo* work.

Exposure to HDI induced the expression of Bcl-2-associated athanogene 3 (*BAG3*), which promotes both apoptosis and anti-apoptosis. The BAG family of proteins affects diverse cellular processes that include cell differentiation, migration, division and even apoptosis. These proteins are co-chaperones that are negative regulators of HSC/HSP70. All family members possess a BAG domain at the COOH-terminus that binds to the ATPase domain of Hsc70 and stimulates nucleotide release. *BAG3* can also bind to the anti-apoptotic factor Bcl-2 and prevent Fas-induced and Bax-mediated apoptosis, suggesting a role in regulating apoptotic pathways (Youker, 2005), as described in our hypothetical model (figure 3).

Transcripts of spectrin $\alpha 1$ (*SPTA1*) and tubulin α (*TUBA1*) were increased after 6 and 10 h exposure to HDI. Both proteins are constituents of the cytoskeletal network. Tubulin α forms

together with tubulin β a heterodimer which is a constituent of microtubules of the eukaryotic cytoskeleton. Lange and coworkers (1999) have shown that TDI co-localizes with tubulin present in the cilia of differentiated human airway epithelial cells. Thus, airway epithelial cell proteins may serve as biologically relevant carriers that present HDI to the human immune system after inhalation, thereby mediating diisocyanate-induced airway inflammation (Lange *et al.*, 1999b).

The expression of two genes pirin (*PIR*) and thyroid hormone receptor associated protein 5 (*THRAP5*) encoding transcription factors was increased after HDI exposure. *THRAP5* was only upregulated after 10 h exposure time, *PIR* was upregulated at both time points. The latter one also showed increased expression in THP-1 monocytes after exposure to DNCB, *p*PD and NiSO₄ (Hirota and Moro, 2006).

The genes popeye domain containing 3 (*POPDC3*) and small nuclear ribonucleoprotein polypeptide N (*SNRPN*) showed increased expression after respectively 6 h and after both 6 and 10 h exposure. SNRPN plays a role in pre-mRNA processing, possibly tissue-specific alternative splicing events. Although individual SNRPNs are believed to recognize specific nucleic acid sequences through RNA-RNA base pairing, the specific role of this family member is unknown. *POPDC3* encodes a member of the POP family of proteins containing three putative transmembrane domains. This gene is expressed in cardiac and skeletal muscle, and may play an important role in these tissues during development.

No assigned GO terms were found for chromosome 20 open reading frame 24 (*C20orf24*), two unknown genes (*D17184* and *BC029472*) and the genes, *FJL21908* and *MGC11257*, coding for two hypothetical proteins. All these genes were upregulated in our study.

Two genes chemokine (C-C motif) ligand (*CCL20*) and Yip1 domain family, member 2 (*YIPF2*) were downregulated after 6 h exposure to HDI. *YIPF2* has no assigned GO terms. *CCL20* encodes for a chemotactic factor that attracts lymphocytes and to a lesser extent neutrophils, but not monocytes. It inhibits proliferation of myeloid progenitors in colony formation assays, and may be involved in formation and function of the mucosal lymphoid tissues by attracting lymphocytes and DC towards epithelial cells. Marcet and coworkers (2007) demonstrate that nucleotides may modulate CCL20 release and leukocyte recruitment in inflamed airways by acting on both epithelial and immune cells (Marcet *et al.*, 2007).

This study demonstrates the feasibility and utility of microarray technology in an attempt to understand the complex biologic and molecular mechanisms, which may contribute to respiratory effects in human after HDI exposure. Significant changes in the transcriptome of

the human macrophage cell line, as proposed *in vitro* model in the pathway of lung sensitization, points towards the induction of oxidative stress mechanisms. The function of the differentially expressed genes could be linked to biological processes including detoxification, oxidative stress, inflammation, cytokine signaling, and apoptosis. Further studies will be necessary to determine the mechanisms that control gene transcription of the HDI-responsive genes and support these findings with results on protein levels to be measured. This experimental approach can be extended to other diisocyanates or respiratory sensitizers in order to clarify underlying mechanisms and pathways to explain observed effects.

Acknowledgement

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Table 1: Differential gene expression analysis using microarray data from *in vitro* cultured macrophages after 6 and 10 h exposure to the respiratory sensitizer HDI.

Accession number	Gene symbol	Gene description	6 h			10 h		
			Mean log ₂ (S.D.)	Geomean	Score (d)	Mean log ₂ (S.D.)	Geomean	Score (d)
NM_002155	FCGR2B	Fc fragment of IgG, low affinity IIb, receptor (CD32)	2.30 (0.41)	4.91	7.44	2.52 (0.40)	5.73	5.93
NM_003662	PIR	Pirin (iron-binding nuclear protein)	0.87 (0.11)	1.82	5.52	1.08 (0.15)	2.11	3.78
BC007659	NQO1	NAD(P)H dehydrogenase, quinone 1	0.67 (0.06)	1.59	5.09	1.07 (0.10)	2.10	4.23
NM_002061	GCLM	Glutamate-cysteine ligase, modifier subunit	0.69 (0.10)	1.61	4.58	0.32 (0.08)	1.25	1.30
NM_002064	GLRX	Glutaredoxin (thioltransferase)	0.52 (0.08)	1.44	3.74	0.44 (0.01)	1.36	2.17
AK055903	KITLG	KIT ligand	0.76 (0.21)	1.70	3.65	0.61 (0.25)	1.52	1.77
NM_006912	RIT1	Ras-like without CAAX 1	0.47 (0.05)	1.39	3.62	0.48 (0.11)	1.39	1.84
NM_024604	FLJ21908	Hypothetical protein	0.51 (0.08)	1.42	3.56	0.52 (0.24)	1.43	1.55
NM_000903	NQO1	NAD(P)H dehydrogenase, quinone 1	0.66 (0.17)	1.58	3.52	1.11 (0.17)	2.16	3.76
NM_001827	CKS2	CDC28 protein kinase regulatory subunit 2	0.46 (0.08)	1.37	3.18	0.36 (0.02)	1.29	1.75
BC029558	MGC11257	Hypothetical protein	0.54 (0.14)	1.46	3.11	0.87 (0.24)	1.83	2.60
NM_006000	TUBA1	Tubulin, alpha 1	0.41 (0.06)	1.32	3.03	0.80 (0.05)	1.74	3.51
NM_004281	BAG3	BCL2-associated athanogene 3	0.68 (0.27)	1.60	2.86	1.21 (0.35)	2.31	3.05
AF400501	SNRPN	Small nuclear ribonucleoprotein polypeptide N	0.36 (0.05)	1.28	2.78	0.56 (0.08)	1.47	2.29
NM_005345	HSPA1A	Heat shock 70kDa protein 1A	0.15 (0.18)	1.11	0.76	1.98 (0.23)	3.94	6.05
NM_006755	TALDO1	Transaldolase 1	0.41 (0.17)	1.32	2.14	0.74 (0.10)	1.67	2.92
NM_002631	PGD	Phosphogluconate dehydrogenase	0.76 (1.08)	1.70	1.19	0.89 (0.20)	1.85	2.82
NM_018840	C20orf24	Chromosome 20 open reading frame 24	0.20 (0.23)	1.15	1.04	0.59 (0.04)	1.50	2.67
NM_003126	SPTA1	Spectrin, alpha, erythrocytic 1 (elliptocytosis 2)	0.25 (0.27)	1.19	1.05	0.60 (0.08)	1.52	2.48
NM_022361	POPDC3	Popeye domain	0.37 (0.26)	1.29	1.70	0.81 (0.24)	1.76	2.44

NM_005481	THRAP5	containing 3 thyroid hormone receptor associated protein 5	-0.08 (0.14)	0.95	-0.44	0.53 (0.04)	1.44	2.40
D17184	Unknown		0.01 (0.08)	1.01	-	0.74 (0.20)	1.67	2.35
NM_002133	HMOX1	Heme oxygenase (decycling) 1	0.96 (0.69)	1.94	2.13	0.81 (0.27)	1.75	2.30
NM_014365	HSPB8	Heat shock 22kDa protein 8	0.31 (0.17)	1.24	1.64	0.50 (0.07)	1.41	2.13
BC029472	Unknown		0.23 (0.22)	1.18	1.09	0.54 (0.11)	1.45	2.07
NM_004591	CCL20	Chemokine (C-C motif) ligand 20	-0.63 (0.11)	0.64	-4.01	-0.58 (0.25)	0.67	-1.69
NM_024029	YIPF2	Yip1 domain family, member 2	-0.63 (0.12)	0.64	-3.86	-0.48 (0.39)	0.72	-1.14

Figure 1: Functional classification with regard to molecular processes of 26 selected genes (table I) that are upregulated after HDI exposure of THP-1 macrophages using GO terms.

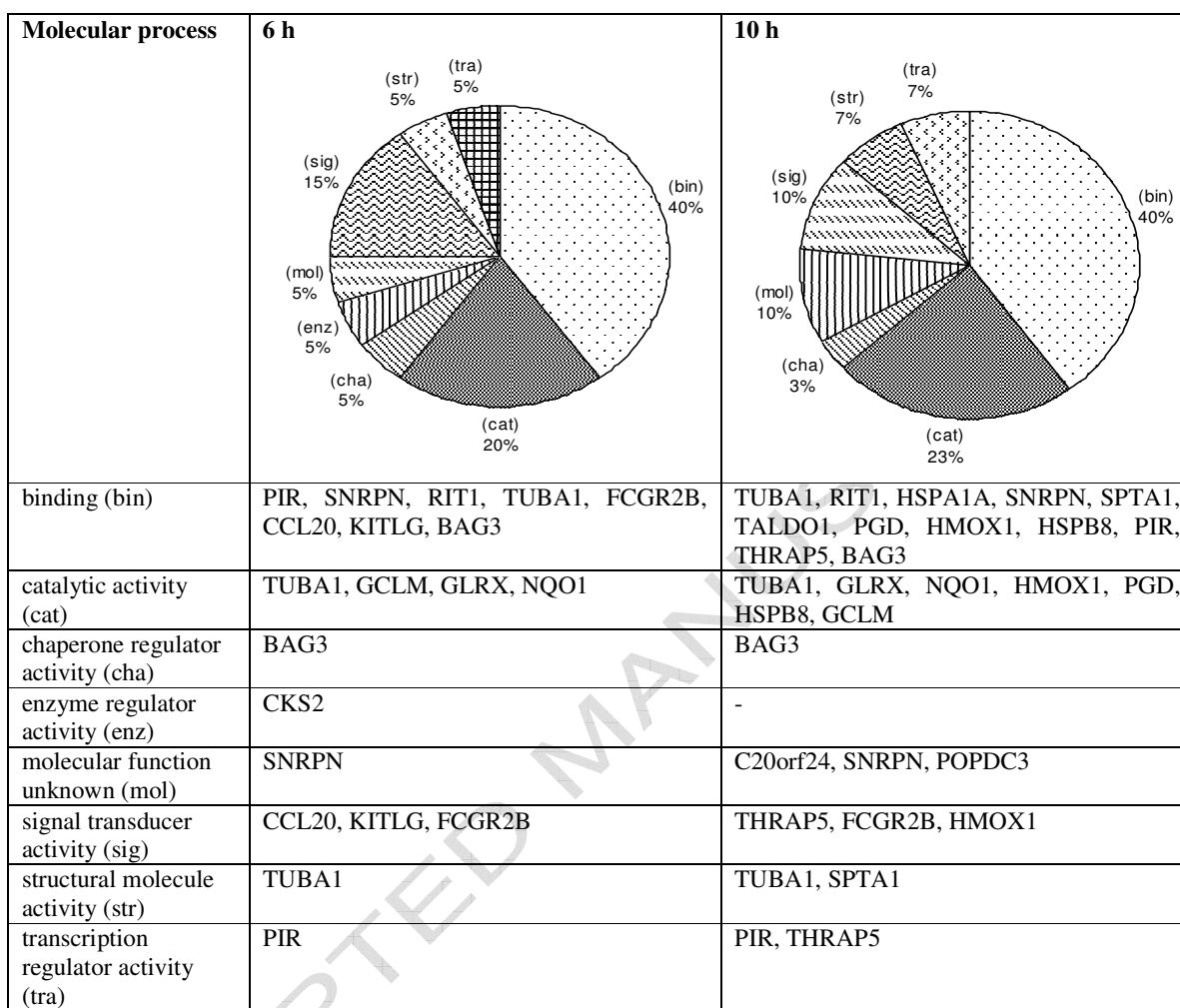


Figure 2: Functional classification according to biological processes of 26 selected genes (table I) that are upregulated after HDI exposure of THP-1 macrophages using GO terms.

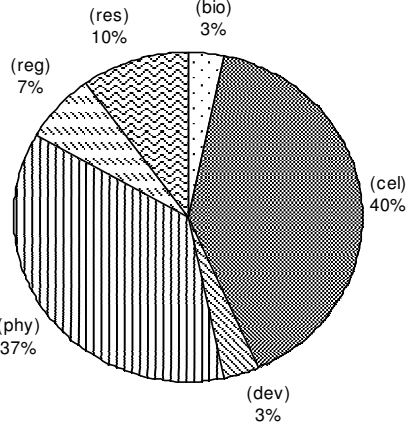
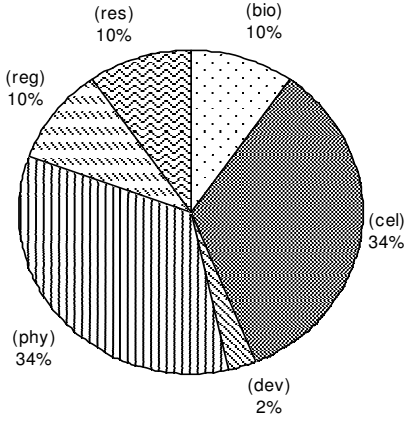
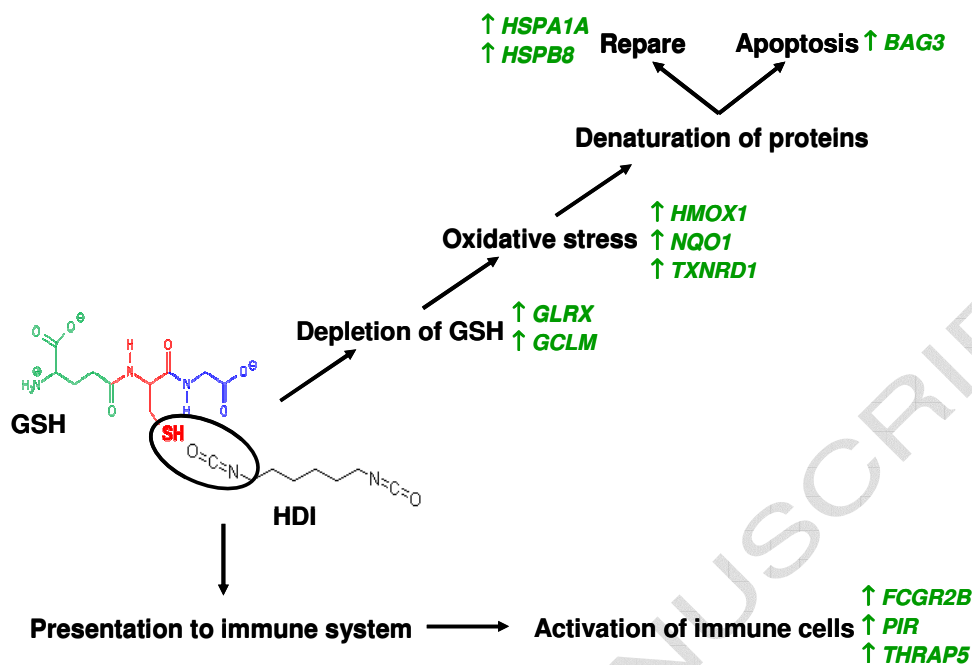
Biological process	6 h	10 h
		
biological process unknown (bio)	SNRPN	C20orf24, SNRPN, POPDC3, HSPB8
cellular process (cel)	KITLG, NQO1, GLRX, PIR, SNRPN, BAG3, GCLM, TUBA1, CKS2, FCGR2B, RIT1, CCL20	HSPA1A, NQO1, GLRX, PIR, SNRPN, BAG3, HSPB8, TUBA1, HMOX1, FCGR2B, THRAP5, TALDO1, SPTA1, PGD
development (dev)	KITLG	SPTA1
physiological process (phy)	CKS2, NQO1, BAG3, FCGR2B, GLRX, TUBA1, GCLM, CCL20, SNRPN, PIR, KITLG	NQO1, BAG3, FCGR2B, GLRX, THRAP5, SPTA1, TALDO1, PGD, HMOX1, SNRPN, TUBA1, PIR, HSPB8, HSPA1A
regulation of biological process (reg)	CKS2, BAG3	HMOX1, BAG3, SPTA1, THRAP5
response to stimulus (res)	CCL20, NQO1, FCGR2B	NQO1, FCGR2B, HSPA1A, HSPB8

Figure 3: Hypothetic model of HDI toxicity derived from current gene expression data and literature.



Legends to the figures

Table 1: Differential gene expression analysis using microarrays data from *in vitro* cultured macrophages after 6 and 10 h exposure to the respiratory sensitizer HDI. Legend: Differential gene expression changes for 26 selected genes are shown by a mean $\log_2$ value (with standard deviation) and the geomean value (representing the corresponding fold change). Data of genes with a q-value < 1 % are indicated in bold. The statistical d score is calculated for all genes. Downregulated genes are indicated with a negative mean $\log_2$ value and are listed under the dashed line.

Figure 1: Functional classification with regard to molecular processes of 26 selected genes (table 1) that are upregulated after HDI exposure of THP-1 macrophages using GO terms. Legend: The pie charts display the contribution of the different functional groups at time point 6 h and 10 h. Functional molecular processes (with their abbreviation) are listed in the table, together with the corresponding genes.

Figure 2: Functional classification according to biological processes of 26 selected genes (table I) that are upregulated after HDI exposure of THP-1 macrophages using GO terms. Legend: The pie charts display the contribution of the different functional groups at time point 6 h and 10 h. Functional biological processes (with their abbreviation) are listed in the table, together with the corresponding genes.

Figure 3 Hypothetic model of HDI toxicity derived from current gene expression data and literature. The most important genes with an increased expression ($\uparrow$) involved in HDI toxicity are shown.

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