Effects of Hexamethylene Diisocyanate Exposure on Human Airway Epithelial Cells: In Vitro Cellular and Molecular Studies

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In this study we developed an in vitro exposure model to investigate the effects of hexamethylene diisocyanate (HDI) on human airway epithelial cells at the cellular and molecular level. We used immunofluorescence analysis (IFA) to visualize the binding and uptake of HDI by airway epithelial cell lines (A549 and NCI-NCI-H292) and microarray technology to identify HDI sensitive genes. By IFA, we observed that subcytotoxic concentrations of HDI form microscopic micelles that appear to be taken up by cells over a 3-hr period postexposure. Microarray analysis (4.6K genes) of parallel cultures identified four genes (thioredoxin reductase, dihydriodiol dehydrogenase, TG interacting factor, and stanniocalcin) whose mRNA levels were up-regulated after HDI exposure. Northern analysis was used to confirm that HDI increased message levels of these four genes and to further explore the dose dependence and kinetics of the response. The finding that HDI exposure increases thioredoxin reductase expression supports previous studies suggesting that HDI alters thiol-redox homeostasis, an important sensor of cellular stress. Another of the HDI-increased genes, a dihydriodiol dehydrogenase, encodes a protein previously shown to be specifically susceptible to HDI conjugation, and known to detoxify other hydrocarbons. Together, the data describe a novel approach for investigating the effects of HDI binding and uptake by human airway epithelial cells and begin to identify genes that may be involved in the acute response to exposure. Key words: exposure, hexamethylene diisocyanate, human airway epithelial cell, redox, thiol, thioredoxin reductase. Environ Health Perspect 110:901–907 (2002). [Online 25 July 2002] http://ehpnet1.niehs.nih.gov/docs/2002/110p901-907/wisnewski/abstract.html

Disocyanates are commercially important, toxic, human-made chemicals used in the manufacture of polyurethane (1,2). Although strict standards have been mandated by the U.S. Occupational Safety and Health Administration to limit respiratory tract exposure to these chemicals, disocyanates remain a leading cause of occupational asthma worldwide (1,2). Environmental exposures to disocyanates have been documented in individuals living near production facilities and may also occur in proximity to end-use settings in residential neighborhoods (3). Hexamethylene diisocyanate (HDI), the focus of this study, is one of the most commonly used diisocyanates and is employed in autobody spray paints, the manufacture of spinnable fibers, and the preservation of animal hides (4).

The effects of HDI on airway epithelial cells remain poorly described despite the documented susceptibility of airway epithelial cell proteins to diisocyanate conjugation (5,6) and their anatomical location at the air–liquid interface in the lung, a primary site of exposure. Our laboratory has recently identified specific epithelial cell proteins that selectively become conjugated with HDI after exposure, including keratin 18, dihydriodiol dehydrogenase, actin, and the 78-kDa glucose-regulated stress response protein (6). In addition, Lange et al. (7) have shown that toluene diisocyanate (TDI) colocalizes 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 (5,8).

The functional consequences of HDI exposure on airway epithelial cells, including protein conjugation and potential effects on cellular homeostasis, remain unclear. Limited studies to date have suggested that cellular thiol levels may be especially sensitive to diisocyanate exposure under physiologic conditions. Occupationally relevant doses of TDI have been shown to cause a rapid reduction of glutathione levels in normal human bronchial epithelial cells in vitro (9–11). It remains unclear whether TDI-induced changes in glutathione levels are a direct effect of exposure or secondary to protective mechanisms such as oxidant injury-induced protein glutathionylation (12). However, these data clearly demonstrate the potential for diisocyanates to alter airway epithelial cell thiol-redox homeostasis, an important modulator of numerous genes under the control of redox-sensitive transcription factors such as NFκB, Rel-1, and AP-1 (13,14).

Thus, studies to date suggest that HDI and other diisocyanates react with specific proteins in human airway epithelial cells and may modulate cellular thiol-redox homeostasis through unknown mechanisms. However, the physical interactions of HDI with airway cells and the potential effects of HDI on gene expression remain poorly characterized. In this study, we developed an in vitro model to further characterize the binding and uptake of HDI as well as the molecular effects of exposure on human airway epithelial cells. The studies were made possible by using recently developed reagents and methodologies, including an HDI-specific polyclonal antiserum generated by our laboratory and microarray technology. The sensitivity of immunocytocchemical approaches and the broad-scope molecular analysis achievable with microarrays are uniquely applicable to such exploratory investigations. The present studies are the first we are aware of to exploit microarray technology to investigate the effects of HDI and illustrate the potential utility of this novel technique in studying the response of human airway epithelial cells to exposure. The results of the in vitro cellular and molecular studies provide new data on the effects of HDI on human airway epithelial cells and identify specific genes that may be important in the acute response to exposure.

Materials and Methods

Reagents and cell lines. We purchased HDI, CaCl2, MgCl2; endotoxin-free water, and streptomycin/penicillin from Sigma Chemical Company (St. Louis, MO). Northern Max-Gly kit, ULTRAhyb Buffer, Brightstar Psoralen-Biotin Nonisotopic Labeling Kit, and CDP-Star/Brightstar Biodekt kit were from Ambion (Austin, TX). Fetal bovine serum (FBS), RPMI 1640, gentamicin, l-glutamine, phosphate-buffered saline (PBS), Trizol, trypsin blue, trypsin-EDTA, and nonessential amino acids (NEAA) were from Gibco BRL Life Technologies (Grand Island, NY). Hybond-N nylon membrane was from Amersham Pharmacia Biotech (Piscataway, NJ); triton X-100, from Calbiochem (La Jolla, CA); rhamdine anti-rabbit immunoglobulin γ, from Santa Cruz (Santa Cruz, CA); acetone, from JT Baker Company (Phillipsburg, NJ); prolong antifade mounting media, from Molecular Probes (Eugene, OR); RNAeasy Minikit, from Qiagen (Valencia, CA).

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Human airway epithelial cell lines A549 and NCI-H292 were obtained from the American Type Culture Collection (Rockville, MD).

**Cell culture.** Human airway epithelial cell lines A549 and NCI-H292 were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 1 mM NEAA, 10 U/mL streptomycin/penicillin, and 10 µg/mL gentamicin. Cells were grown in a 75-cm² tissue culture flask and passaged as needed by trypsin-EDTA treatment.

**Dose toxicity studies.** Exposure of human lung epithelial cells to HDI in vitro was performed as described previously (5,6). Briefly, cells were grown to confluence in 75-cm² tissue culture flask, washed three times with PBS, and exposed to HDI in PBS (containing 1 mM CaCl₂ and 1 mM MgCl₂ to prevent detachment) at a concentration of 0.5% (volume/volume) and then washed again with PBS and immediately lysed in 8 mL of Trizol. For dose titration, HDI was serially diluted in 10% goat serum/PBS for 30 min at room temperature. Preimmune serum was added to the cultures for additional periods of time in complete growth media, and then washed again with PBS and lysed in Trizol.

**Cytotoxicity of HDI to human airway epithelial cells in vitro.** Monolayers of A549 (circles) and NCI-H292 (squares) cells in tissue culture flasks were exposed to titrated doses of HDI, and percentage viability (y-axis) was assessed immediately or 24 hr after a 20-min exposure.

**Table 1. IMAGE clones.**

| IMAGE clone no. | Gene Bank accession no. | Corresponding gene |
|-----------------|-------------------------|--------------------|
| 789376          | AA453335                | Thiorodoxin reductase |
| 196992          | R993124                 | Dihydrodiol dehydrogenase (AKRC1) |
| 547247          | AA085318                | Stanniocalcin |
| 194214          | R93270                  | TG-interacting factor |

**Table 2. PCR primers for probe generation.**

| Gene name | Forward [5’ to 3’] | Reverse [5’ to 3’] | PCR (bp) |
|-----------|--------------------|--------------------|----------|
| Thiorodoxin reductase | TGAACAACTGTGCTTGTGG | GCTTAAAGAGATGAGGAC | 380 |
| Dihydrodiol dehydro. | CAGTGCTCTGTAAGGCGAG | GTGTGAAAGAGATGAGAC | 240 |
| TG-interacting factor | AGACTAGTGTGCGATCATC | AAATGTCAATGTTGCCGT | 360 |
| Stanniocalcin | ACATCTGGCAAGCGAGGAC | TCGGTGACTGCTGGS | 220 |

**Microarray.** Microarray was performed by the Yale University School of Medicine Microarray Center. RNA from exposed cells was double purified using Trizol reagent and RNAeasy columns. RNA (50 µg) obtained from NCI-H292 cells 2 hr after a 20-min exposure to HDI or vehicle alone was directly labeled with cyanine 3 (Cy3) and cyanine 5 (Cy5) and hybridized to the “in-house” HU-4.6K glass slide, a broad-spectrum array. The HU-4.6K arrays contain 4,608 cDNAs (48 × 96-well plates) from the Research Genetics 40K list (15). The cDNAs are arrayed in duplicate in a 4 × 4 array with 24 × 24 spots in each of 16 subarrays. Fluorescence hybridization data were acquired on an Axon GenePix 4000A scanner using GenePix Pro3 software as detailed at the Yale University School of Medicine Microarray Center web site (15). Quality control studies with the HU-4.6K slide and the “in-house” HU-4.6K glass slide testing the microarray were further studied by Northern analysis (Table 1).

**Northern blot analyses.** To validate microarray results and to further study the kinetics of HDI-induced changes in gene expression patterns, we undertook Northern blot analyses. RNA was double purified from HDI-exposed cells using Trizol followed by RNAeasy columns. Total RNA (15 µg/lane) was electrophoresed on a 1.2% agarose gel using the Northern Max-Gly kit according to the manufacturer’s instructions. RNA was transferred to Hybond-N nylon membrane in 20 x saline sodium citrate (SSC) overnight and immobilized by ultraviolet cross-linking. Membranes were prehybridized in ULTRAhyb Buffer at 42°C for 2 hr. Biotin-labeled probes (see below) were denatured by heating at 90°C for 10 min, added to the 10 mL of ULTRAhyb Buffer, and hybridized over night (~16 hr). After hybridization, the blots were washed twice in 2 x SSC/0.1% SDS for 10 min at room temperature and in 0.1 x SSC/0.1% SDS at 42°C for 15 min. Hybridized probes were detected with the CDP-Star detection system according to the manufacturer’s instructions. Briefly, blots were blocked, incubated with a 1:1,000 dilution of alkaline phosphatase-conjugated streptavidin for 30 min, and extensively washed before development with chemiluminescence CDP-Star substrate and exposure to X-ray film. The blots were stripped once in 0.1 x SSC/0.1% SDS and reanalyzed with a β-actin probe. Quantification of RNA signals by densitometry scanning of the final X-ray film was achieved using the AlphaImager 2000 documentation and analysis system by Alpha Innotech Corporation (San Leandro, CA). The level of each mRNA was calculated based on the integrated density value of a constant area and standardized to the β-actin signal. Three different experiments were completed with 0, 3, 17, and 30 µM exposure doses. Student’s t-test was used to evaluate the statistical significance of the change in mRNA levels of HDI-exposed cells compared with “mock” exposed cells.

**Probes.** The probes were prepared by biotin labeling polymerase chain reaction (PCR) products, except for β-actin, which was purchased from Ambion. For PCR amplification of gene fragments, cDNA was prepared from the airway epithelial cells using the SuperScript first-strand synthesis system for reverse transcription–PCR by Gibco BRL Life Technologies, according to the manufacturer’s instructions. Briefly, blots were blocked, incubated with a 1:1,000 dilution of alkaline phosphatase-conjugated streptavidin for 30 min, and extensively washed before development with chemiluminescence CDP-Star substrate and exposure to X-ray film. The blots were stripped once in 0.1 x SSC/0.1% SDS and reanalyzed with a β-actin probe. Quantification of RNA signals by densitometry scanning of the final X-ray film was achieved using the AlphaImager 2000 documentation and analysis system by Alpha Innotech Corporation (San Leandro, CA). The level of each mRNA was calculated based on the integrated density value of a constant area and standardized to the β-actin signal. Three different experiments were completed with 0, 3, 17, and 30 µM exposure doses. Student’s t-test was used to evaluate the statistical significance of the change in mRNA levels of HDI-exposed cells compared with “mock” exposed cells.

**Figure 1. Cytotoxicity of HDI to human airway epithelial cells in vitro.** Monolayers of A549 (circles) and NCI-H292 (squares) cells in tissue culture flasks were exposed to titrated doses of HDI, and percentage viability (y-axis) was assessed immediately or 24 hr after a 20-min exposure.
the manufacturer’s instructions, using oligo(dT) primer and 5 µg of RNA/cDNA reaction. Table 2 lists the primers used.

For PCR reactions, 2 µL cDNA, 15 pmol primers, and 45 µL platinum PCR supermix from Gibco BRL were amplified for 35 cycles at 94°C, 58°C, and 72°C. PCR products were purified from low-melting-point gels and sequence validated. Next, 100 ng of purified PCR products in 10 µL was labeled with the Brightstar Psoralen-Biotin Nonisotopic Labeling Kit from Ambion, according to the manufacturer’s instructions. Labeling and specificity were verified by dot blot as the manufacturer suggested and by Southern blot against different PCR products. For Northern analysis, probes were used at 0.1–1 ng/mL.

Results

Development of an in vitro HDI exposure system. To facilitate investigations on the dose dependence and kinetics of HDI’s effects on human airway epithelial cells, we developed and characterized an in vitro exposure system. Two different cell lines, A549 and NCI-H292, were chosen for these studies; both are hearty immortalized cell types commonly employed for in vitro studies of human airway epithelial cell types. A549 cells, a type II-like alveolar epithelial cell, are one of the most widely used cell lines for studying human airway epithelial cell biology. NCI-H292 cells are mucoepidermoid type I-like alveolar epithelial cells. Analysis of the effects of HDI encompassed toxicity studies, IFA, microarray, and Northern blot analyses. Toxicity was based on trypan blue dye exclusion as evidence of cell viability after exposure. IFA studies were analyzed microscopically for evidence of HDI binding and uptake, which was detected with an HDI-specific antiserum. Expression profiling was accomplished based on cDNA hybridization to a broad-spectrum 4.6K human gene chip and further defined based on quantitative measurements of mRNA levels by Northern blot analysis.

In vitro toxicity studies. Initially we determined the in vitro toxicity of HDI to A549 and NCI-H292 cells grown as monolayers in flasks, through dose titration studies. Cells were exposed to HDI concentrations ranging from 300 nM to 30 mM and viability was assessed. A 20-min exposure to doses > 3 mM was cytotoxic, whereas cells exposed to doses < 300 µM remained viable when analyzed immediately after exposure (Figure 1). However, when cells were analyzed 24 hr after HDI exposure, substantial cell death (< 10% viability) was noted in cultures exposed to concentrations > 300 µM, whereas cultures exposed to doses < 30 µM remained > 90% viable. The toxicity of HDI to A549 and NCI-H292 cells was comparable over this broad exposure dose range.

Figure 2. Binding of HDI to human airway epithelial cells: IFA of A549 cells immediately after a 20-min exposure to a subcytotoxic dose (30 µM) of HDI. Phase contrast (A,C) and immunofluorescent (B,D) micrographs were probed with α-HDI rabbit serum (A,B) or α-ovalbumin rabbit serum (C,D). Arrows point to foci of HDI staining.

Figure 3. Time course of binding and uptake of HDI by human airway epithelial cells: IFA (using α-HDI rabbit serum) of A549 cells at different times after a 20-min exposure to a subcytotoxic dose (30 µM) of HDI. The four horizontal rows (A–D) show different microscopic fields from one experiment to provide a sense of the range of staining patterns observed at different time points and the unique morphology and subcellular localization of HDI in individual cells.
**Binding of HDI to human airway epithelial cells.** To visualize the binding of HDI to human airway epithelial cells in vitro, we performed IFA using an HDI-specific antiserum developed by our laboratory (5,6). Immediately after a 20-min exposure to 30 µM HDI, airway epithelial cells exhibited foci of HDI-specific staining along the cell surface. Microscopic comparison of fluorescence and phase-contrast images revealed that these concentrated areas of cell-associated HDI corresponded to "droplets," structurally consistent with micelles of HDI (Figure 2). The observed foci of cell-associated HDI appeared to be taken up and distributed within the cell during a 3-hr period after exposure (Figure 3).

**Molecular changes induced by HDI exposure.** We screened for genes that may be selectively induced by HDI exposure using microarray analysis and assumed a priori that any potential effects of HDI would be time and dose dependent. As a starting point for these studies, we analyzed cells that were exposed to the highest nontoxic dose of HDI and then cultured them for additional periods of time to permit changes in cellular mRNA levels to occur. When this approach was used to compare mRNA profiles from NCI-H292 cells 2 hr after a 20-min exposure to 30 µM HDI (relative to control cultures identically exposed to vehicle), increases (> 2.0-fold) were noted in just 4 of 4,608 transcripts analyzed. Figure 4 shows fluorescent images and corresponding false color overlay for 4 of 16 quadrants of a representative 4.6K gene microarray. The readily apparent differentially expressed cDNA hybridization signals are circled and correspond to thioredoxin reductase, a dihydrodiol dehydrogenase, aldo-keto reductase C1 (AKRC1), stanniocalcin, and TG-interacting factor (Figure 4A–D, respectively). Similar results were obtained with A549 cells, although the relative changes in gene expression were not as great as those observed with NCI-H292 cells because baseline levels of each of these mRNA were higher in A549 cells (data not shown).

To validate the results of our microarray studies and further analyze the kinetics and dose dependence of HDI-induced gene expression, we performed Northern blot analysis with NCI-H292 cells exposed to titrated doses of HDI and cultured for varying amounts of time after exposure. For the selected genes, mRNA levels were quantitated as described, and changes induced by different doses were compared with "mock exposed" cultures. As shown in Figures 5 and 6, thioredoxin reductase, dihydrodiol dehydrogenase, stanniocalcin, and TG-interacting factor mRNA levels were increased in a dose-dependent manner. A statistically significant increase in mRNA levels for all four of these proteins was observed as early as 2 hr after exposure to doses ≤ 30 µM (Figure 6). The mRNA levels for these different genes remained increased for up to 4 hr after exposure and returned to baseline by 24 hr (data not shown).

**Discussion**

This report characterizes previously undescribed effects of HDI exposure on human airway epithelial cells. The study also demonstrates the potential advantages of microarray technology as an aid to understanding the effects of chemical exposures on biological systems. The experiments were based on previous investigations that have demonstrated that specific human airway epithelial cell proteins become conjugated with HDI after exposure (5–7), and the hypothesis that HDI–epithelial cell protein conjugation may induce pathogenic functional changes. In the present studies, we coupled microarray (> 4K genes) technology with an in vitro HDI exposure system using airway epithelial cell lines to screen for genes whose message levels are significantly increased by HDI. The kinetics and HDI dose dependence of increases in mRNA levels...
levels for four up-regulated genes, one of which (AKRC1) encodes a protein previously shown to be specifically susceptible to HDI conjugation (6), were verified by Northern blot studies. The in vitro exposure model was also used to visualize the interaction of HDI with living airway epithelial cells by IFA and provides new evidence for the rapid binding and uptake of HDI by exposed cells. Together with previously published reports, the data yield insights into pathways through which HDI may exert its toxic effects on airway epithelial cells.

The initial studies of this report established an in vitro system to permit titrated HDI exposure of human airway epithelial cell lines. First, we determined the toxicity of HDI to two different types of human airway epithelial cells, A549 and NCI-H292, which had not been previously established. We demonstrated that a 20-min exposure to doses < 30 µM HDI did not cause measurable cytotoxicity over a 24-hr period. These concentrations are at least 2,000-fold less than the concentration of HDI present in aerosol droplets of autobody shop paints, which typically contain 1% HDI (~ 60 mM). Given that HDI aerosols have been shown to deposit in the airways of exposed workers, the concentrations of HDI used in the present studies are relevant to current common occupational exposures and possibly also to environmental exposures (16,17).

The ability of HDI to bind and be taken up by airway epithelial cells over time was further tested by IFA using an HDI-specific polyclonal antiserum generated in our laboratory (5,6). These IFA studies provide, for the first time, visual evidence of the affinity of HDI for live cells. The fluorescence staining pattern observed suggests that HDI initially interacts with the cell membrane and is taken up within 3 hr after exposure. The cell-associated HDI foci observed in these studies resemble droplet-like structures that are consistent with HDI micelles and the tendency for HDI to form emulsions in aqueous solutions (4), but might also reflect localized HDI–protein aggregates.

On the basis of current IFA studies and previous reports suggesting that diisocyanates can bind and be taken up by airway epithelial cells (5,6,11), we investigated the possibility that HDI exposure may also result in specific changes in gene expression. To screen for HDI-sensitive airway epithelial cell genes, we used microarrays to expression profile in vitro exposed cells. HDI induced differential expression in only a limited number of 4,608 genes tested. We subsequently used Northern blot analyses to confirm the microarray results for the four genes whose message levels were up-regulated > 2-fold by microarray analysis. The limited number of genes (≤ 8) whose message levels were decreased or less strongly increased represent other potentially HDI-sensitive genes that may be investigated in future studies.

Two of the up-regulated genes AKRC1 and thioredoxin reductase are relevant to cellular thiol-redox homeostasis. One of these, AKRC1, encodes the same dihydrodiol dehydrogenase protein previously shown to be susceptible to HDI conjugation (6) and belongs to a family of oxidant stress-induced enzymes that detoxify hydrocarbons (18–20). AKRC1 is one of just four HDI-conjugated proteins previously identified in exposed human airway epithelial cells and appears to be especially sensitive to low exposure concentrations (6). The conjugation of AKRC1 protein with HDI and the up-regulation of its mRNA after exposure suggest that this protein may be important in the metabolism of HDI and/or the repair of oxidants resulting from exposure.

Thioredoxin reductase is an essential enzyme important in maintaining cellular thiol-redox homeostasis and defense against oxidant stress and also mediates important cytokine activities via its substrate thioredoxin, which can act as a potent T-cell growth factor (21–30). Increases in thioredoxin reductase may thus not only signal HDI-induced thiol-redox imbalance but also provide an “antigen-receptor–independent” mechanism by which epithelial cell responses to exposure may promote immunologic sensitization to HDI via either a) epithelial cell–derived cytokines/chemokines under redox-dependent transcriptional regulation (13,14) or b) immunologically active thioredoxin (21–25). Interestingly, thioredoxin reductase contains selenium, which is thought to be crucial to its role as a cellular sensor of redox potential and which, based on its biochemical properties, should be highly susceptible to nucleophilic addition reactions with diisocyanates (10,28,31).

Of the other two genes we have documented to be up-regulated by HDI exposure, one, TG-interacting factor, possesses important regulatory capacity, through its ability to block retinoic acid signaling (32,33). The
In summary, the present study develops an in vitro exposure model to characterize the effects of HDI on human airway epithelial cell lines and defines specific molecular changes that result from exposure. Importantly, the data support previous studies suggesting that disocyanates can enter airway epithelial cells and upset cellular thiol-redox homeostasis. Given that many pro-inflammatory cytokines/chemokines are under the control of redox-sensitive transcription factors, the present results suggest an "antigen-receptor-independent" mechanism through which HDI may promote airway inflammation and asthma. Future studies should determine whether individual differences exist in airway epithelial cell sensitivity to HDI and whether these potential differences help explain the widely disparate responses of exposed workers.

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This study also demonstrates the potential utility and feasibility of microarray technology in beginning to understand the complex biologic effects of human exposure to chemicals such as HDI. The results demonstrate the selectivity and validity of microarray analysis under well-controlled experimental conditions. Only a select number of genes were found to be up-regulated by HDI using microarray analysis, and these effects were confirmed using Northern blot analysis. Although there has been rapidly growing interest in microarray technology, this is one of the first reports demonstrating its utility in studying the effects of exposure to occupationally relevant chemicals.

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