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Hexamethylene diisocyanate (HDI) vapor reactivity with glutathione and subsequent transfer to human albumin

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ABSTRACT

Introduction: Airway fluid glutathione (GSH) reactivity with inhaled vapors of diisocyanate, a common occupational allergen, is postulated to be a key step in exposure-induced asthma pathogenesis.
Methods: A mixed (vapor/liquid) phase exposure system was used to model the *in vivo* reactivity of inhaled HDI vapor with GSH in the airway fluid. HDI–GSH reaction products, and their capacity to transfer HDI to human albumin, were characterized through mass spectrometry and serologic assays, using HDI-specific polyclonal rabbit serum.
Results: HDI vapor exposure of 10 mM GSH solutions resulted in primarily S-linked, bis(GSH)–HDI reaction products. In contrast, lower GSH concentrations (100 μM) resulted in mainly mono(GSH)–HDI conjugates, with varying degrees of HDI hydrolysis, dimerization and/or intra-molecular cyclization, depending upon the presence/absence of $H_2PO_4^-$/HPO_4^{2-} and Na^+/Cl^- ions. The ion composition and GSH concentration of the fluid phase, during HDI vapor exposure, strongly influenced the transfer of HDI from GSH to albumin, as did the pH and duration of the carbamoylating reaction. When carbamoylation was performed overnight at pH 7, 25 of albumin's lysines were identified as potential sites of conjugation with partially hydrolyzed HDI. When carbamoylation was performed at pH 9, more rapid (within 3 h) and extensive modification was observed, including additional lysine sites, intra-molecular cross-linkage with HDI, and novel HDI–GSH conjugation.
Conclusions: The data define potential mechanisms by which the levels of GSH, $H_2PO_4^-$/HPO_4^{2-}, and/or other ions (e.g. H^+/OH^-, Na^+, Cl^-) affect the reactivity of HDI vapor with self-molecules in solution (e.g. airway fluid), and thus, might influence the clinical response to HDI respiratory tract exposure.

1. Introduction

Isocyanate (N=C=O) chemicals used to make polyurethane are a well-recognized cause of occupational asthma (Bernstein, 1999; Redlich and Karol, 2002; Tarlo and Liss, 2002). Two classes of isocyanates, aromatic and aliphatic, possess different chemical properties and are typically used for distinct applications (http://www.cdc.gov/niosh/topics/isocyanates/; Klees and Ott, 1999; Ulrich, 1996). Aromatic isocyanates, such as toluene diisocyanate (TDI) and methylene diphenyl diisocyanate (MDI) are excellent for foam production, elastomers and durable coatings (e.g. truck-bed liners), but are sensitive to photo-oxidation (Allport et al., 2003; Davis and Sims, 1983; Ulrich, 1996). Aliphatic isocyanates, such as HDI, possess better UV light resistance, and are typically used for exterior coatings, such as protective finishes on automobiles and aircrafts (Davis and Sims, 1983; http://www.alipa.org/; Ulrich, 1996).

The pathogenic mechanisms through which isocyanates cause asthma remain uncertain but, are believed to depend upon N=C=O reactivity with "self" molecules at exposure sites (Day et al., 1996, 1997; Lange et al., 1999; Lantz et al., 2001; Wisnewski and Jones, 2010). The fluid lining the human lower respiratory tract contains a relatively high level (>100 μM) of an essential tri-peptide, GSH, postulated to be a primary reactant for inhaled isocyanates (Cantin et al., 1987; Day et al., 1997; Lange et al., 1999; Lantz et al., 2001). Airway fluid GSH levels are normally maintained within a limited range, by complex genetically-defined homeostatic feedback mechanisms (Day et al., 2004; Rahman and MacNee, 2000). However, substantial individual variability may occur secondary to environmental exposures (e.g. smoking, infection), or genetic mutations/polymorphisms, and has been suggested to influence the pathogenesis of several different diseases (Day, 2005, 2009; Pacht et al., 1991; Rahman and MacNee, 1999, 2002; Roum et al.,

Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; HDI, hexamethylene diisocyanate; TDI, toluene diisocyanate.

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1999; Smith et al., 1993; van der Vliet et al., 1999). In animal studies, inhalation of isocyanate vapors acutely alters airway fluid GSH levels, while GSH depletion exacerbates MDI's respiratory tract toxicity (Pauluhn, 2000a,b). Among occupationally exposed workers, the development of isocyanate asthma, and rate of chemical excretion have been associated with genetic polymorphisms in GSH-dependent enzymes (Broberg et al., 2010; Littorin et al., 2008; Mapp et al., 2002; Piirila et al., 2001; Wikman et al., 2002).

In vitro studies have begun to dissect possible mechanisms through which isocyanate reactivity with GSH might influence immunologic and/or other biological responses to exposure. For the aromatic diisocyanate, TDI, it has been demonstrated that chemical vapors readily cross a liquid phase barrier to react with GSH, and cause acute depletion of intracellular GSH in human airway epithelial cells (Lantz et al., 2001; Wisnewski et al., 2011). Furthermore, thiol-linked vapor TDI–GSH reaction products are capable of carbamoylating (transferring TDI to) peptide/protein molecules, including human albumin, the major "carrier" protein for TDI *in vivo*, and essential element of allergenic (IgE) recognition by the human immune system (Day et al., 1997; Lange et al., 1999; Wass and Belin, 1989; Wisnewski et al., 2011).

The reactivity of GSH with HDI, and other aliphatic isocyanates used in polyurethane manufacturing, remains less clear and may differ from aromatic isocyanates, given inherent differences in their chemical reactivity. The hydrolysis of aliphatic isocyanates is much slower than aromatic isocyanates, which may provide greater opportunity to react with GSH in solution (Brown et al., 1987). Thiol-linked (e.g. GSH) reaction products with aliphatic isocyanates are likely more stable than reaction products with aromatic isocyanates, based on prior studies with cysteine-methyl-ester (Chipinda et al., 2006). In short-term (1 h) vapor exposure studies *in vitro,* GSH has been reported to prevent HDI protein conjugation, rather than mediate transcarbamoylation as observed with TDI (Day et al., 1997; Wisnewski et al., 2005, 2011). A better understanding of the GSH reactivity with HDI vapor should provide insight into the basic mechanisms underlying the biological effects of HDI exposure.

The present study used a mixed (vapor/liquid) phase exposure system to model the biophysics of HDI reactivity in the airway microenvironment, where vapor phase chemical contacts the airway lining fluid, which contains high levels of GSH (Wisnewski et al., 2004, 2011). A variety of physiologically relevant, interdependent exposure variables (pH, [GSH], and ion/buffer content of the solution) were evaluated for their potential influence on HDI vapor phase reactivity with GSH, and subsequent carbamoylation of human albumin, the major carrier protein for isocyanates *in vivo*. The potential biological relevance of the *in vitro* data is discussed.

2. Experimental procedures

2.1. HDI vapor exposure

Solutions (100 μM–10 mM) of reduced or oxidized glutathione (GSH or GSSG respectively) from Sigma (St. Louis, MO) were prepared in 20 mM phosphate buffered saline (PBS, pH 7.4) from Gibco (Grand Island, NY), 140 mM NaCl, or de-ionized water (Sigma). All GSH solutions and reaction products were 0.2 μm filtered (Millipore; Billerica, MA) to ensure sterility. Solutions were exposed as previously described, to room air or HDI vapor, for 18 h, in 35 × 10 mm Petri dishes obtained through VWR International from Bridgeport, NJ (Wisnewski et al., 2004, 2011). HDI vapors were obtained by passive diffusion from puriss grade HDI (PubChem Substance ID: 24874557, CAS Number: 822-06-0) solution (Sigma–Aldrich), ⩾99% purity by gas chromatography, with a refractive index of *n*20/D 1.453, and a density of 1.047 g/mL at 20 °C. HDI vapor concentration was monitored with an Autostep toxic gas monitor (GMD Systems, Pittsburgh, PA, USA), and maintained at 180 ± 20 ppb, by adjusting the intake air flow rate. The mixed phase exposure conditions were empirically developed in prior studies, based on specific recognition of (albumin) reaction products by serum IgG from HDI exposed workers. Each exposure condition was replicated in four independent experiments to determine biological variability of the experimental results.

2.2. LC–MS

Experiments were performed on a Waters (Milford, MA) nanoACQUITY ultra-performance liquid chromatography (UPLC) system. Aliquots (1 μL) of each GSH–HDI mixture were injected and trapped/desalted on a 5 μm SymmetryC_{18} (180 μm × 20 mm) trapping column with 99.5/0.5 A/B (A:0.1% formic acid; B:0.1% formic acid in acetonitrile) at a flow rate of 15 μL/min for 1 min. Separation was performed on a 1.7 μm BEH130 C_{18} (100 μm × 100 mm) analytical column utilizing gradient elution at a flow rate of 400 nL/min and a gradient of 99/1 to 60/40 A/B over 60 min. The eluent from the UPLC system was directed to the nanoelectrospray source of a Waters SYNAPT MS quadrupole time-of-flight (qTOF) mass spectrometer. Positive ion nanoelectrospray was performed utilizing 10 μm PicoTip (Waters) emitters held at a potential of +3.5 kV. The cone voltage was held constant at +40 V for all experiments. Dry N_2 desolvation gas was supplied to the instrument via a nitrogen generator (NitroFlowLab, Parker Hannifin Corp., Haverhill, MA). $[Glu]^1$-Fibrinopeptide B (100 fmol/μL in 75/25 A/B) was supplied to an orthogonal reference probe and the $[M + 2H]^{2+}$ ion (m/z = 785.84265 u) measured as an external calibrant at 30 s intervals. Ultra-high purity (UHP) argon was used as collision gas. Data were analyzed with MassLynx v. 4.1 (Waters). Samples from four independent experiments were analyzed to assess reproducibility of the experimental findings.

2.3. GSH–HDI mediated carbamoylation of human albumin

Solutions of HDI vapor exposed GSH were co-incubated 1:2 (v/v) with a 5 mg/mL solution of human albumin (Sigma) at 37 °C. Initial studies, including those analyzed by ELISA and Western blot (Fig. 3A and B), were performed overnight in 0.1 M carbonate, pH 9.0. Subsequent carbamoylation reactions were performed for varying time periods (1 h–3 days) in carbonate buffer, or at pH 7.0 using 0.1 M (mono/dibasic) phosphate buffer. Four different experiments were performed in triplicate using GSH–HDI reaction products from four independent experiments.

2.4. Anti-HDI ELISA

Maxisorp® microtiter plates from Nunc (VWR International) were incubated overnight at 4 °C with 5 μg/well of human albumin that had been co-incubated with solutions of HDI vapor-exposed GSH. Plates were coated in 0.1 M carbonate buffer, pH 9.5, washed, and "blocked" with 3% (w/v) dry milk, before addition of anti-HDI rabbit serum, diluted 1:200 (v/v). As previously described, the HDI-specific rabbit polyclonal antiserum was raised against HDI–KLH, depleted of KLH binding activity by affinity chromatography, and specifically recognizes HDI when bound to a larger carrier protein, such as human albumin (Wisnewski et al., 1999, 2000, 2004, 2008, 2010). ELISA plates were developed with peroxidase-conjugated anti-rabbit IgG (Pharmingen; San Diego, CA), diluted 1:2000 (v/v), and TMB substrate. Optical density (OD) measurements (absorbance of light at 450 nm, minus absorbance at a reference wavelength), reflecting detection of HDI, were obtained on a Benchmark microtiter plate reader from Bio-Rad. Samples from