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Vapor conjugation of toluene diisocyanate to specific lysines of human albumin

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ABSTRACT

Exposure to toluene diisocyanate (TDI), an industrially important crosslinking agent used in the production of polyurethane products, can cause asthma in sensitive workers. Albumin has been identified as a major reaction target for TDI in vivo, and TDI–albumin reaction products have been proposed to serve as exposure biomarkers and to act as asthmagens, yet they remain incompletely characterized. In the current study, we used a multiplexed tandem mass spectrometry (MS/MS) approach to identify the sites of albumin conjugation by TDI vapors, modeling the air/liquid interface of the lung. Vapor phase TDI was found to react with human albumin in a dose-dependent manner, with up to 18 potential sites of conjugation, the most susceptible being Lys351 and the dilysine site Lys413–414. Sites of vapor TDI conjugation to albumin were quantitatively limited compared with those recently described for liquid phase TDI, especially in domains IIA and IIIB of albumin. We hypothesize that the orientation of albumin at the air/liquid interface plays an important role in vapor TDI conjugation and, thus, could influence biological responses to exposure and the development of in vitro assays for exposure and immune sensitivity.

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Diisocyanates are highly reactive electrophilic compounds that are industrially useful as crosslinking agents in polyurethane production for diverse products such as flexible and rigid foams, fibers, paints, and varnishes. Global diisocyanate production is dominated by two aromatic diisocyanates, toluene diisocyanate (TDI)[1] and methylene diphenyl diisocyanate (MDI), which together account for more than 90% of the diisocyanate market [1]. Exposure to TDI and other diisocyanates is associated with adverse health effects, including asthma, contact dermatitis, and hypersensitivity pneumonitis [2]. Worldwide, diisocyanates are the most commonly reported cause of occupational asthma, with an estimated 5% to 30% of exposed workers at risk for developing disease [2–5].

The mechanistic connections between diisocyanate exposure and health outcomes remain unclear, in large part due to uncertainty regarding diisocyanate reactivity in vivo. The self-protein albumin has been identified as a major reaction target for inhaled diisocyanate, and diisocyanate–albumin reaction products (e.g., conjugates), which accumulate in the circulating blood, may serve as biomarkers of exposure [6–9]. In some workers, diisocyanate–albumin-specific immunoglobulin E (IgE) can be measured in sera and may participate directly in asthma pathogenesis [10,11]. Thus, a better understanding of diisocyanate–albumin reactivity is central to understanding exposure outcomes and to the development of assays for exposure monitoring and disease surveillance.

Human albumin possesses numerous functional groups that could potentially react with diisocyanate; however, diisocyanate–albumin conjugates that form in vivo in exposed workers remain largely uncharacterized due to technical limitations. Studies of diisocyanate–albumin reactivity to date have relied primarily on in vitro modeling and reveal a marked influence of exposure conditions on the conformation and antigenicity of the resulting diisocyanate–albumin reaction products [12,13]. For example, high concentrations of diisocyanate, relative to albumin, result in excessive amounts of diisocyanate conjugation, protein precipitation, and lack of specific antibody recognition [12–18]. Data to date suggest that under occupational exposure conditions (e.g., low diisocyanate concentrations), individual albumin molecules undergo limited conjugation.

The majority of in vitro studies on diisocyanate–albumin reactivity have been performed with liquid phase chemical; however, for volatile diisocyanates such as TDI, the airway microenvironment is exposed to vapor rather than liquid phase chemical [19]. TDI–albumin conjugates that form under such mixed (vapor/liquid) phase exposure conditions differ structurally and conformationally from those formed in liquid phase and have been hypothesized to more closely reflect those that form in vivo, based on immune recognition by IgE from diisocyanate asthma patients [16].

Tandem mass spectrometry (MS/MS) is well-suited to the structural analysis of modified peptides and proteins [20]. Accurate

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[1] *Abbreviations used:* TDI, toluene diisocyanate; MDI, methylene diphenyl diisocyanate; Ig, immunoglobulin; MS/MS, tandem mass spectrometry; PBS, phosphate-buffered saline; TDA, toluene diamine; mAb, monoclonal antibody; UPLC, ultra-performance liquid chromatography; qTOF, quadrupole time-of-flight; CID, collision-induced dissociation.

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mass measurement of fragment ions can discriminate between isomass peptides produced by the enzymatic cleavage of large proteins [20,21]. The current study undertakes a comprehensive MS/MS approach [14,22,23] to unambiguously map the sites on human albumin conjugated by vapor phase TDI. A mixed (vapor/liquid) phase in vitro exposure system, in which vapor dose was titrated by varying the duration of exposure, was used to identify those sites on albumin most susceptible to vapor phase TDI conjugation. Preferential sites of vapor TDI conjugation were compared with those recently identified for liquid TDI and highlight the influence of exposure biophysics on TDI–albumin reactivity [14]. The data are discussed in the context of disease pathogenesis and the development of assays for exposure monitoring and disease surveillance.

Materials and methods

Preparation of vapor TDI–albumin conjugates

Vapor TDI–albumin conjugates were prepared by using a previously described isocyanate vapor phase exposure system [17]. In brief, TDI vapor concentrations in the range of 0.14 to 1.4 mg/m^3 ($\sim$1–10 μmol/m^3) were passively generated inside an exposure chamber monitored with an Autostep monitor (GMD, Pittsburgh, PA, USA). TDI was an 80:20 mixture of 2,4- and 2,6-TDI isomers obtained from Aldrich (St. Louis, MO, USA). Low endotoxin human albumin in phosphate-buffered saline (PBS, pH 7.2) at a concentration of 5 mg/ml (73 nmol/ml) was exposed in open 60-mm Petri dishes (Becton Dickinson, Franklin Lakes, NJ, USA) for 0 min (control), 20 min, 1 h, 4 h, and 24 h. The exposure unit was cleaned with 70% ethanol, and protein solutions were filtered (0.2 μm) before and after exposure.

Quantitation of dissolved TDI after vapor exposures

TDI from 0-min (control), 20-min, 1-h, 4-h, and 24-h exposures was trapped in open 60-mm Petri dishes containing 0.5% H_2SO_4. Reaction of TDI with dilute sulfuric acid results in rapid hydrolysis of TDI to toluene diamine (TDA), which is stable in solution. Aliquots (1 ml) of the TDA-containing trap solution were selected and buffered back to alkaline pH by adding 1 ml of saturated sodium borate. TDA was subsequently derivatized by adding 50 μl of 1 mg/ml fluorescamine (Fisher Scientific, Pittsburgh, PA, USA) in acetonitrile. Samples were quantified by fluorescence spectroscopy using an LS50B luminescence spectrometer (PerkinElmer, Waltham, MA, USA) controlled by FL WinLab software (version 4.00.02, PerkinElmer) using excitation at 410 nm and observing the emission at 510 nm. A calibration curve was generated using 2,4-TDA (Sigma–Aldrich, St. Louis, MO, USA).

Native gel and anti-TDI Western blot

TDI conjugation to human albumin was detected in native gels based on characteristic changes in electrophoretic mobility, as described previously [17,24]. For native protein analysis, samples were prepared in a 10% glycerol running buffer and then electrophoresed on 10% polyacrylamide gels and stained with Imperial protein stain (Pierce, Rockford, IL, USA). For Western blot analysis, samples were electrophoresed under reducing conditions (for optimal anti-TDI monoclonal antibody [mAb] binding) on precast 4% to 15% gradient gels and transferred to nitrocellulose using an aqueous trans-blot system (Bio-Rad, Hercules, CA, USA). Nitrocellulose membranes were blocked with 3% dry milk in PBS, probed with 1 μg/ml of the anti-TDI mAb 60G2 [25] followed by anti-mouse IgG_1 (Pharmingen, San Diego, CA, USA), and developed with ECL reagent (Thermo Fisher Scientific, Rochester, NY, USA).

Trypsin digestion

Aliquots (100 μl) of each conjugate and control were taken for analysis. Disulfide bonds were reduced by reaction with tributylphosphine (5 mM) for 30 min at room temperature, followed by alkylation with iodoacetamide (15 mM) for 1 h at room temperature. Alkylation was quenched by further addition of tributylphosphine (5 mM) for 15 min at room temperature. Samples were twice dialyzed against 3 L of 25 mM NH_4HCO_3 using 3500-MWCO (molecular weight cutoff) mini dialysis units (Slide-A-Lyzer, Thermo Scientific, Waltham, MA, USA). Porcine trypsin was suspended in 25 mM NH_4HCO_3 and added to each aliquot at a 40:1 (protein/trypsin) ratio. Samples were incubated overnight at 37 °C with shaking (300 rpm). Samples were centrifuged at 14,100*g* in a microcentrifuge (MiniSpin, Eppendorf, Hamburg, Germany) to pellet any insoluble material.

Ultra-performance liquid chromatography

Enzymatic peptides were separated on a Waters (Milford, MA, USA) nanoACQUITY ultra-performance liquid chromatography (UPLC) system. Aliquots (1 μl) of the digest mixture were injected and trapped/desalted on a 5-μm Symmetry C_{18} trapping column (180 μm × 20 mm) 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} analytical column (100 μm × 100 mm) using gradient elution at a flow rate of 400 nl/min and a gradient of 99:1 to 60:40 A/B over 60 min.

Tandem mass spectrometry

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 using 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, Haverhill, MA, USA). [Glu1]-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) was measured as an external calibrant at 30-s intervals. Collision-induced dissociation (CID) was performed using ultra-high-purity (UHP) argon as collision gas. Spectra were acquired in an "MS^e" fashion [22]. Briefly, alternating 1-s mass spectra are acquired. The first spectrum acquired at low (6 eV) collision energy allows high mass accuracy precursor ion mass measurement. The second spectrum acquired at high (15–30 eV ramp) collision energy allows high mass accuracy fragment ion mass measurement. The fragment ion spectra may be temporally correlated with precursor spectra postrun. This method of data acquisition allows all precursor ions to be fragmented and analyzed, as opposed to so-called "data-dependent acquisition" methods that require making real-time decisions on which ions to select for fragmentation, which may miss low-abundance precursor ions.

Data analysis

Data were analyzed with BioPharmaLynx (version 1.2, Waters), a software program for analysis of peptide mass maps and identification of sites of modification on known protein sequences. Default peptide mass map analysis criteria of 30 ppm mass error in both low and high collision energy mode were specified. Trypsin