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Mass spectrometric identification of isocyanate-induced modifications of keratins in human skin

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ABSTRACT

In the current paper we show that exposure of human callus to isocyanates leads to covalent modifications within keratin proteins. Mass spectrometric analyses of pronase digests of keratin isolated from exposed callus show that both mono- and di-adducts (for di-isocyanates) are predominantly formed on the ε-amino group of lysine. In addition, numerous modified tryptic keratin fragments were identified, demonstrating rather random lysine modification. Interestingly, preliminary experiments demonstrate that in case of MDI a similar lysine di-adduct was formed with lung elastin. Our data support the hypothesis that skin sensitization through antigenic modifications of skin proteins by isocyanates could play a role in occupational isocyanate-induced asthma. It is further envisaged that the elucidated adducts will also have great potential for use as biomarkers to assess skin exposure to isocyanates. Advantageously, the various lysine adducts display the presence of a characteristic daughter fragment at *m/z* 173.1 $[\text{lysine-NCO}]^+$, enabling generic and rapid screening for exposure to isocyanates.

1. Introduction

Isocyanates are a group of chemicals which are widely used in chemical industry, e.g. in the production of polyurethanes and as hardener in coatings and paints. Isocyanates have also been implicated in causing adverse health effects. Probably the most notorious representative of the isocyanate family is methyl isocyanate (MIC). This highly toxic chemical accidentally escaped from a chemical plant during the Bhopal disaster in 1984, resulting in thousands of (fatal) casualties [1,2]. Occupational exposure to isocyanates is known to be an important cause of asthma [3]. In addition, alleged health problems after exposure to di-isocyanates like toluene di-isocyanate (TDI) and methylene diphenyl di-isocyanate (MDI) after application of home insulation have also received much attention [4,5].

It has been hypothesized [6–8] that skin exposure to isocyanates can lead to systemic immune sensitization, eventually causing the typical isocyanate-induced asthma-like inflammatory responses. Indeed, the skin is a highly immunogenic organ, able to elicit serious systemic immune responses such as contact allergy, inter alia caused by skin protein modification [9,10]. Within this framework we reasoned that isocyanate modification of particular skin keratins could play a relevant role in this process, because keratins are so abundant in skin tissue. We here report the detailed mass spectrometric elucidation of adducts of a number of isocyanates with keratins present in the stratum corneum of the human skin. It is envisaged that the present work will also lead to novel biomarkers of exposure to isocyanates, as a useful addition to the already existing biomarkers based on adducts to blood proteins [11,12] and urinary metabolites [13–15]. Various studies dealing with keratin adducts of other xenobiotics have been published, demonstrating the potential utility of keratin adducts as biomarker of exposure [16,17].

2. Materials and methods

2.1. Chemicals

2,6-Toluene diisocyanate (2,6-TDI), methylene diphenyl diisocyanate (MDI), 4-bromophenylisocyanate (4-BrPIC), 2-nitrophenylisocyanate (2-NPIC), phenethylisocyanate (PEIC), 2-methacryloyloxethyl isocyanate (MOI), allylisocyanate (AIC), dithiothreitol (DTT), sodium iodoacetate and TPCK-treated trypsin were purchased from Sigma (St. Louis, MO, USA). Protease mixture of exo- and endonucleases from *Streptomyces Griseus* (pronase) was

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purchased from Fluka (Buchs, Switzerland). For the present study, human callus obtained from a local podotherapist, was used as the source of human keratin. All other chemicals used were of analytical grade and purchased from Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany). All buffers were prepared with Millipore grade water. PD MiniTrap G-25 columns were purchased from GE Healthcare (Buckinghamshire, UK).

2.2. Incubation of human callus with isocyanates

Human callus was mechanically homogenized to a fine powder using a MM 400 ball mixer mill (Retsch, Haan, Germany) to increase the surface area for subsequent exposure to isocyanate compounds. Two different incubation procedures were used for the IC reaction with human callus. Procedure (1) (water-free incubation): the callus (100 mg) was washed twice with DMSO. After centrifugation (30 min, 400 rpm) the supernatant was discarded and the pellet of callus was resuspended in 200 μL DMSO. Subsequently, 4 μL of a stock-solution of IC compound in DMSO was added to a 10 mM final concentration, and the mixture was incubated overnight at 37 °C in a temperature-controlled incubator under continuous shaking. Procedure (2) (incubation in aqueous solution): the callus (100 mg) was washed twice with Tris·HCl buffer (20 mM, pH 7.4) and then allowed to hydrate in a 0.9% NaCl solution. After centrifugation (30 min, 400 rpm) the supernatant was discarded and the pellet of hydrated callus was resuspended in 200 μL 0.9% NaCl. Subsequently, 4 μL of a stock-solution of IC compound in 2-propanol was added to a 10 mM final concentration, and the mixture was incubated overnight at 37 °C in a temperature-controlled incubator under continuous shaking. Subsequent work-up after incubation was similar for both incubation methods, i.e. the suspension was centrifuged briefly and the supernatant containing the excess IC compound was discarded. Next, the callus was resuspended in Milipore water (2 mL), centrifuged briefly (15 min, 400 rpm) and allowed to incubate for some time (2 h) to allow any unreacted IC to hydrolyze. The supernatant was then discarded. This wash step was repeated three times to remove all excess IC compound.

2.3. Isolation of keratins from human callus

2.3.1. Extraction of keratins

Extraction of keratins was performed following a standard protocol adapted from Van der Schans et al. [18]. Briefly, the exposed callus was soaked in Tris·HCl buffer (5 mL, 20 mM, pH 7.4) overnight, under continuous stirring. After centrifugation (30 min, 400 rpm) the supernatant was discarded and the pellet was stirred in a buffer (5 mL, pH 7.4) containing Tris·HCl (20 mM) and urea (8 M) overnight. After centrifugation (30 min, 400 rpm) the supernatant was again discarded and the pellet was resuspended in extraction buffer (5 mL, pH 7.4) containing Tris·HCl (20 mM), urea (7 M), thiourea (2 M) and bis(2-mercaptoethyl)sulfone (BMS; 0.025 M) overnight, under continuous stirring.

2.4. Enzymatic digestion of keratins

2.4.1. Reduction and carboxymethylation

To an aliquot of keratin in extraction buffer (0.5 mL, roughly 5 mg/mL protein), containing Tris·HCl (20 mM), urea (7 M), thiourea (2 M) and BMS (0.02 M), 2.2 mg DTT was added to reduce all cysteine bridges. The mixture was incubated for 60 min at 50 °C in a heating block. Next, sodium iodoacetate (5 mg) was added for carboxymethylation of free cysteine residues and the mixture was incubated for another 60 min at 50 °C in a heating block. Subsequently, the proteins were isolated from the reaction mixture by filtration over a PD MiniTrap G-25 column, pre-equilibrated with aqueous NH_4HCO_3 (50 mM), to remove salts and unreacted reagents.

2.4.2. Pronase digestion

A solution of pretreated keratin (reduced and carboxymethylated; 5 mg/mL) in aqueous NH_4HCO_3 (50 mM; 0.5 mL) was incubated with pronase (5%, w/w) at 37 °C overnight to digest the protein material to individual amino acids. Samples were analyzed with LC-Electrospray (Tandem) MS for identification of modified amino acids.

2.4.3. Tryptic digestion and mass spectrometric analysis for keratin identification

A solution of pretreated keratin (reduced and carboxymethylated; 5 mg/mL) in aqueous NH_4HCO_3 (50 mM; 0.5 mL) was incubated with TPCK treated trypsin (5%, w/w) at 37 °C overnight, to ensure maximum tryptic digestion.

A number of samples were analyzed using the data dependent LC-autoMS/MS mode. The ten most abundant ions (charge states 2^+, 3^+ and 4^+) in the MS spectrum (300–1300 m/z, 2 Hz) of eluting peptides were selected for data dependent MS/MS analysis by collision-induced dissociation using nitrogen as the collision gas. MS/MS scans were acquired over the mass range 100–2000 m/z (2–10 Hz, depending on signal intensities).

Peak lists were generated using DataAnalysis 4.1 software (Bruker) and exported as Mascot Generic (MGF) files. These files were searched against the NCBI database with the Mascot search algorithm (Mascot 2.2.04, Matrix Science, London, UK). An MS tolerance of 50 mmu and a MS/MS tolerance of 50 mmu were used. Trypsin was designated as the enzyme and one missed cleavage was allowed. Carboxymethylcysteine was selected as a fixed modification and oxidation of methionine as a variable modification. The samples contained approx. 80 (exposure in water) and approx. 120 (exposure in DMSO) abundant peptides (MW 600–5000) of keratin proteins and except trypsin no other proteins with more than 2 unique tryptic fragments per protein proved to be present.

2.5. LC-electrospray tandem MS analysis of pronase and trypsin digests for adduct elucidation

LC–ES MS(/MS) experiments were conducted on a Q-TOF™ hybrid instrument equipped with a standard Z-spray electrospray interface (Micromass, Altrincham, U.K.) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consisted of a precolumn splitter (type Acurate; LC Packings, Amsterdam, The Netherlands), a six-port valve (Valco, Schenkon, Switzerland) with a 10 μl injection loop mounted and a PepMap C_{18} column (150 × 1 mm, 3 μm; LC Packings). A gradient of eluents A (H_2O with 0.2% (v/v) formic acid) and B (CH_3CN with 0.2% (v/v) formic acid) was used to achieve separation, following: 100% A (v/v) to 80% (v/v) B in 45 min. (LC-system I) or in 60 min. (LC-system II). The flow delivered by the liquid chromatograph was split pre-column to allow a flow of approx. 40 μl/min through the column. Mass spectra were recorded at a speed of 1–2.4 s. with nitrogen as the nebulizer and desolvation gas (at a flow of 20 and 400 l/h, respectively). MS/MS product ion spectra were recorded using a collision energy of 10–30 eV, with argon as the collision gas.

Alternatively, experiments were conducted on a Bruker maXis Impact Q-Tof instrument (Bruker Daltonics, Bremen, Germany) equipped with an Ultimate 3000 UPLC (Thermo Fisher Scientific, San Jose, USA). The system was provided with a PepMap C_{18} column (150 × 1 mm, 3 μm; Thermo Fischer Scientific). A gradient of eluents A and B (see above) was used: 100% A to 70% B in 30 min. at a flow rate of 80 μl/min (LC-system III) or 100% A to 50% B in 20 min. at a flow rate of 50 μl/min (LC-system IV). As