

Comparison of biological effects with albumin adducts of 4,4'-methylenediphenyl diisocyanate in workers

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Abstract Lung sensitization and asthma are the main health effects of 4,4'-methylenediphenyl diisocyanate (MDI). Albumin adducts (isocyanate-specific adducts) of MDI might be involved in the etiology of sensitization reactions. Albumin adducts of MDI were analyzed in sera of diisocyanate-exposed worker with and without diisocyanate occupational asthma (DA), as well as in exposed workers with and without diisocyanate-specific IgG antibodies. In DA-positive workers and IgG-positive workers, albumin adducts were significantly higher versus workers without DA and those who were specific IgG negative. The odds ratio to be DA-positive was 57 times larger for workers with adduct levels above 230 fmol/mg. The odds ratio to be IgG-positive was 10 times larger for workers with adduct levels above 113 fmol/mg. Therefore, albumin adducts appear to be a good predictor of the biological effects. The albumin-adduct levels in workers without biological effects were in the range of the adduct levels found in previous studies of healthy MDI-factory and construction site workers.

Keywords Biomonitoring · Albumin adducts · Isocyanates · Occupational asthma

Introduction

4,4'-Methylenediphenyl diisocyanate (MDI) is used for several applications including: polyurethane foam, paints, adhesives, elastomers, coatings, and consolidation of loose rock zones in coal mining or tunneling (Munn et al. 2005). Diisocyanates are one of the main causes of occupational asthma (Baur et al. 1994; Bernstein 1996; Karol 1986). The predominant routes of occupational exposure are through inhalation and dermal absorption (Liljelind et al. 2010).

Isocyanates do not need any further activation to react with biomolecules (Bolognesi et al. 2001) (Fig. 1). Important vehicles for isocyanates are their reaction products with glutathione (Pearson et al. 1991; Sabbioni et al. 2012; Slatter et al. 1991; Wisnewski et al. 2013). The glutathione adducts release the isocyanate moiety to react with other nucleophiles, e.g., proteins. Therefore, glutathione adducts are thought to be responsible for the transport of isocyanate to reactive sites away from the site of isocyanate uptake.

Isocyanate-specific adducts of MDI bind with the *N*-terminal valine of hemoglobin (Gries and Leng 2013; Sabbioni et al. 2000) and/or with *N*^ε-lysine of albumin (Sabbioni et al. 2010). Urinary metabolites of xenobiotics are usually detectable up to 48 h after exposure (Henderson et al. 1989). Reaction products with blood proteins such as hemoglobin and albumin are present in vivo over a larger time-frame provided the adducts are stable. Albumin adducts have a half-life of 20–25 days (Skipper and Tannenbaum 1990; Tornqvist et al. 2002). Diisocyanate-albumin conjugates are putative antigens that have been extensively investigated as potential sensitizers in workers diagnosed with DA

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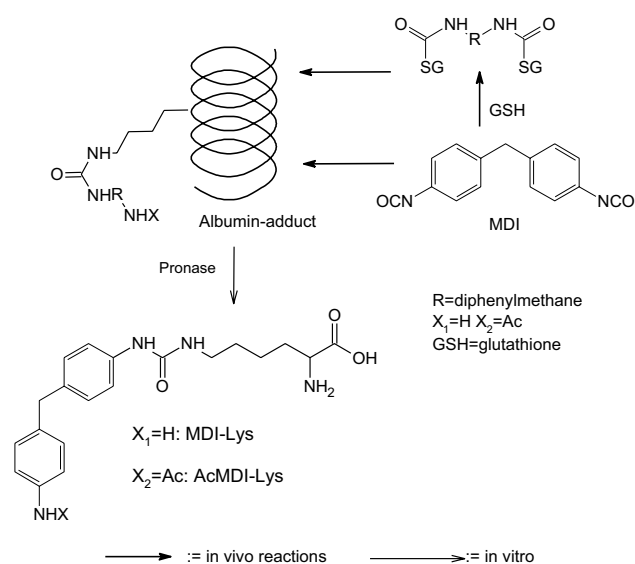


Fig. 1 Adduct formation of MDI with biomolecules

(Bernstein et al. 2002; Raulf-Heimsoth and Baur 1998; Wisniewski et al. 2000). Serologic albumin-diisocyanate adducts have been assessed as potential markers of exposure and a potential surrogate marker of DA.

Specific diisocyanate antibodies have been studied in exposed workers as well as in general unexposed populations as potential biological indicators of environmental diisocyanate exposure (Bernstein et al. 2011; Lushniak et al. 1998; Ott et al. 2007; Wilder et al. 2011). For some chemicals known to cause occupational asthma, the presence of specific antibodies correlates well with clinical disease. In contrast to high-molecular-weight protein allergens that readily elicited serum-specific IgE responses, there is far less concordance between serum-specific antibodies to diisocyanate–protein conjugates and confirmed DA (Ott et al. 2007; Wisniewski 2007; Darcey et al. 2002; Wisniewski and Jones 2010). A recent literature review by Ott et al. (2007) indicates that there is considerable heterogeneity in the prevalence of specific IgE and IgG binding to diisocyanate conjugates across studies. Potential explanations for the observed heterogeneity are evident in the differences in assay methodology and antigen preparation methods (Bernstein et al. 2002; Campo et al. 2007; Wisniewski et al. 2004).

The objectives of this present pilot study are to determine relationships between albumin-adduct levels of isocyanate, isocyanate-specific antibodies, and clinical sensitization defined by confirmed DA. The following working hypotheses were studied: (1) Albumin-adduct levels of isocyanates are significantly higher in workers with isocyanate-specific antibody responses; and (2) Albumin-adduct

levels of isocyanates are associated with a greater likelihood of DA.

Materials and methods

Workers

The serum samples collected from 73 workers exposed to MDI were provided by David I. Bernstein MD (University of Cincinnati, USA). The workers were engaged in urethane mold production employing MDI. Among the workers were 31.5 % non-smokers, 24.7 % ex-smokers, and 43.8 % smokers. The workers had been working for mean of 101.5 (± 110.4) months with MDI. Most workers (93 %) were exposed up to the day of the blood collection. All of the subjects ($n = 73$) were tested for the presence of MDI-specific-antibodies. Thirty-seven workers (51 %) underwent specific inhalation challenge (SIC) testing to confirm or exclude diisocyanate-induced asthma (DA).

Chemicals

N^6 -[(4-[4-aminobenzyl]phenyl)amino]carbonyl]lysine (MDI-Lys) (Fig. 1), N^6 -[(4-[4-aminobenzyl]phenyl)amino]carbonyl [$^{13}\text{C}^{15}\text{N}_2$]lysine (MDI- $^{13}\text{C}^{15}\text{N}_2$ Lys), N^6 -[(4-[4-(acetilamino)benzyl]phenyl)amino]carbonyl]lysine (AcMDI-Lys), and N^6 -[(4-[4-(acetilamino)-3,5-dideuteriobenzyl]-2,6-dideuteriophenyl)amino]carbonyl]lysine (Ac $^{2}\text{H}_4$ MDI-Lys) were synthesized as described previously (Kumar et al. 2009) (Fig. 1). Ammonium bicarbonate, methanol for sample preparation (A452-4, HPLC-grade), and methanol for LC-MS/MS (Optima, A454-4) were obtained from Fisher Scientific (New Jersey, USA). Pronase E from *streptomyces griseus*, ammonium formate, formic acid (#94318, for mass spectrometry), hydrochloric acid, and sodium hydroxide were purchased from Fluka (Buchs, Switzerland). Sodium phosphate monobasic monohydrate, sodium thiocyanate, human serum albumin, and water for LC-MS/MS (Chromasolv; #39253) were purchased from Sigma-Aldrich (St. Louis, MO). Coomassie plus protein assay reagent for protein determination was acquired from Thermo Scientific (Rockford, IL). Strata-X-33u polymeric reversed-phase columns (200 mg/3 mL) were obtained from Phenomenex (Torrance CA).

Instrumentation

An API 4000Q Trap (Applied Biosystems, Foster City, CA) mass spectrometer interfaced to a Prominence 20AD HPLC from Shimadzu (Columbia, MD, USA) was used for the LC-MS/MS analyses as described in detail previously (Sabbioni

et al. 2010). A UV-1800 Spectrophotometer from Shimadzu was used for protein determination. Centrifugations were performed on a Beckman Coulter Allegra™ X-22R centrifuge equipped with a SX4250 swing out bucket rotor.

Isolation of albumin

Plasma was separated from the erythrocytes on the day of collection. All samples were stored at -25°C . Albumin was isolated from thawed pure plasma (0.4 mL) by dropwise addition of an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ (Troester et al. 2000). This mixture was centrifuged at 9000 g to remove immunoglobulins. The supernatant was dialysed using Eppendorf cups with an integrated 10 kDa molecular cut-off filter (Pall Life Sciences (Ann Arbor, MI, USA) centrifugal devices for biomolecular separations, P/N 0D010C35). The tubes were centrifuged for 40 min at 9500 g. The residue was taken up in 500 μL of water and centrifuged again for 30 min. Samples were redissolved in 10 mM sodium phosphate buffer (pH 7.4). The concentrations of the isolated albumin solutions were determined with a Coomassie protein Assay kit for total protein quantitation from Pierce.

Digestion of albumin

Albumin (9 mg) in 50 mM ammonium bicarbonate, pH 8.9 (0.9 mL) was spiked with MDI- $[\text{}^{13}\text{C}_6^{15}\text{N}_2]\text{Lys}$ (13.23 pmol) and $\text{Ac}[\text{}^2\text{H}_4]\text{MDI-Lys}$ (12.02 pmol) (Sabbioni et al. 2010). Samples were digested and incubated with 500 μL of freshly prepared pronase E solution (6 mg/mL; 50 mM ammonium bicarbonate, pH 8.9) for 15 h at pH 8.9 and 37°C . The digests were acidified to pH 4.0 with 2 M hydrochloric acid and purified with solid-phase extraction (SPE) columns (Strata-X-33u, polymeric reversed-phase columns, 200 mg). The SPE columns were first activated with 3 mL of methanol and then equilibrated with 3 mL of 0.1 % formic acid (pH 4.0). The samples were loaded on the SPE column and subsequently washed with 3 mL fraction of 0, 10, 20 % methanol in 0.1 % formic acid. MDI-Lys and AcMDI-Lys were eluted with 6 mL of 80 % methanol in 0.1 % formic acid. The eluate was concentrated to approximately 1 mL in a speed evaporator.

Quantification of albumin adducts, MDI-Lys and AcMDI-Lys, using LC–MS/MS

All data were processed using Analyst software 1.4.2 (Applied Biosystems/MDS Sciex). MDI-Lys and AcMDI-Lys were detected with negative multiple reaction monitoring (MRM) m/z 369.1/145.1 and 411.1/145.1, respectively. The corresponding internal standards MDI- $[\text{}^{13}\text{C}_6^{15}\text{N}_2]\text{Lys}$ and $\text{Ac}[\text{}^2\text{H}_4]\text{MDI-Lys}$ were monitored with the transitions m/z 377.1/153.0 and 415.1/145.1, respectively.

Chromatographic separation was achieved on a Luna C18(2) (100 Å, 150×2.0 mm, 3 μm) (Phenomenex Inc., Torrance CA) protected by a C18 guard column(AJO-4287; 4 mm L $\times$ 3.0 mm ID), using a gradient system with solvent A (10 mM ammonium formate) and solvent B (methanol) at a flow rate of 0.2 mL/min: 0 min (B 20 %), 3 min (B 20 %), 16 min (B 90 %), 20 min (B 90 %). The retention times of MDI-Lys and AcMDI-Lys were 15.2 and 15.7 min, respectively. The column flow was diverted away from the ESI ion source except for the time period from 5 to 18 min. To generate the calibration line, solutions of human serum albumin (9 mg) in ammonium carbonate (50 mM, 2 mL) were spiked with different amounts of MDI-Lys (0.00, 0.54, 2.70, and 8.11 pmol) and AcMDI-Lys (0.00, 0.49, 2.43, and 7.28 pmol) along with MDI- $[\text{}^{13}\text{C}_6^{15}\text{N}_2]\text{Lys}$ (13.23 pmol) and $\text{Ac}[\text{}^2\text{H}_4]\text{MDI-Lys}$ (12.02 pmol), and worked up as described in the paragraph “digestion of albumin.” The concentration levels were plotted against the peak area ratio of the analyte against the peak area of the internal standard (e.g., peak area ratio MDI-Lys/MDI- $[\text{}^{13}\text{C}_6^{15}\text{N}_2]\text{Lys}$). The limit of the quantification (LOQ) following this work up and analysis procedure was 7.5 and 6.7 fmol/mg albumin for MDI-Lys and AcMDI-Lys, respectively. Since this analytical procedure exhibits baseline noise, the LOQ was determined at a signal/noise ratio of >10 according to the guidelines of the International Conference on Harmonization (ICH) of technical requirements for the registration of pharmaceuticals for human use (ICH 1996; Shrivastava and Gupta 2011).

Measurement of diisocyanate-specific antibodies

The serum diisocyanate-specific antibodies were determined in the Allergy Diagnostic Laboratory in Cincinnati (Prof. Dr. David I. Bernstein, University of Cincinnati, USA) following procedures published previously (Bernstein and Jolly 1999; Bernstein et al. 2006). Serum samples were assayed by means of enzyme-linked immunosorbent assay for specific-IgG or specific-IgE binding with MDI-human serum albumin. Results were expressed as mean OD at 405 nm (at 1:10 serum dilution) with a positive result defined as 3 standard deviations above the mean of eight negative reference control sera, with no binding to human serum albumin alone.

Statistical analyses

The statistical analyses were performed with SPSS 19.0. The data were not normally distributed (one-sample Kolmogorov–Smirnov test, $p < 0.05$). Root square transformation of the MDI-Lys values for workers with a measured DA status ($n = 37$) yielded normal distribution of the data ($p = 0.19$). The original non-transformed values were

Table 1 Comparison of the albumin-adduct levels of MDI in workers with asthma and workers with a positive immunological test

		DA pos (<i>n</i> = 9)	DA neg (<i>n</i> = 28)	IgG pos ^e (<i>n</i> = 10)	IgG neg ^e (<i>n</i> = 27)	IgG neg ^f (<i>n</i> = 36)
MDI-Lys (fmol/mg)	Mean (SD)	501 (864) ^a	52.0 (62.6) ^a	414 (842) ^c	67.9 (86.8) ^c	15.3 (9.3)
	Median (25th, 75th)	230 (114, 404) ^b	30.8 (0, 73.8) ^b	148 (26.0, 296) ^d	34.3 (0, 95.0) ^d	12.2 (9.4, 19.8) ^d

^a The mean MDI-Lys levels (see “Statistical analyses” section in “Materials and methods” section) were significantly higher in DA-positive workers according to the independent *t* test, *p* < 0.05

^b The median MDI-Lys levels were significantly higher in the DA-positive workers, Mann–Whitney test, *p* < 0.001

^c The mean MDI-Lys level was higher in the IgG-positive workers according to the independent *t* test, *p* = 0.11

^d The median MDI-Lys levels were significantly higher in the IgG-positive workers, Mann–Whitney test, *p* < 0.001

^e Workers with measured DA status

^f Workers without measured DA status

reported in the text and in Table 1. Including the data of the workers without measured DA status (*n* = 36), no normal distribution was reached for all data (*n* = 73). The odds ratios were calculated with the crosstab function in SPSS 19. One cell was zero in the table with the dichotomized adduct levels and the DA status. In order to calculate the odds ratio or its standard error, 0.5 was added to all cells (https://www.medcalc.org/calc/odds_ratio.php).

Results

Serum samples of workers exposed to MDI were assayed for specific antibodies to MDI-albumin. For the present work, diisocyanate-specific adducts with albumin were analyzed (MDI-Lys, AcMDI-Lys) (Fig. 1). Albumin was isolated from plasma using a precipitation procedure with ammonium sulfate. Albumin was digested with pronase in the presence of the internal standards, MDI-[¹³C₆¹⁵N₂]Lys and Ac[²H₄]MDA-Lys. The digests were purified using solid-phase extraction. The concentrated extracts were analyzed with LC–MS/MS using electrospray ionization in the negative ion mode. Quantification was performed using isotope-labeled internal standards of MDI-Lys and AcMDI-Lys. Three times more samples were positive for MDI-Lys adducts than for AcMDI-Lys adducts. Therefore, the AcMDI-Lys data were not shown and not included in the present analyses. Thirty-seven samples with immunological test results and specific challenge results including nine cases of occupational asthma cases of workers exposed to isocyanates were analyzed (Table 1). Four workers with asthma reported that their last activity with MDI was more than 3 months ago. The mean MDI-Lys level of these workers was 191 fmol/mg, which is much lower than the mean level (750 fmol/mg) of the other five workers with asthma. All the other workers were exposed to MDI at the time of sample collection.

In a first analysis, 37 workers with SIC confirmed DA status, IgG status and albumin-adduct levels were compared

(Table 1). The MDI-Lys levels were transformed using the square root function in order to obtain normal distributed data. The workers with confirmed asthma had significantly higher adduct levels than healthy worker (independent *t* test, and Mann–Whitney test). The workers with a positive MDI-antibody test had significantly higher adduct levels than the worker with no MDI-antibodies (Mann–Whitney test). In a further statistical analysis, the adduct levels were split into two categories: above and below the 50th percentile. This dichotomized exposure variable was compared to the DA status of the workers. The odds ratio to be DA-positive was 57 (confidence interval 95 % (CI), 3.0–1075) for workers with albumin adducts above the median compared to the workers with albumin-adduct levels below the median level. The odds ratio to be IgG-positive was 10.3 (CI, 1.9–54.2) for the workers in the upper third (*n* = 12) of the exposure group (>113 fmol/mg) in comparison with the rest of the workers (*n* = 27). The asthma cases were compared to the results of the immunological tests using the crosstab calculations. In the workers with asthma, 5 of 9 workers had a positive immunological result. For the worker with a negative asthma outcome, 5 of 28 workers had a positive immunological test. The difference is significant according to the Fisher’s exact test (2-tailed, *p* = 0.041). The odds ratio for workers with a positive immunological test to be DA-positive is 5.8 (CI 1.1–29.4) in comparison with workers with a negative immunological test.

In addition, workers (*n* = 36) without DA data but with both IgG data and adduct data were compared to the other workers (Table 1). The adduct levels of the 36 IgG-negative workers were significantly lower in comparison with the DA-positive and IgG-positive workers.

Discussion

MDI-specific albumin adducts have been found previously in workers (Sabbioni et al. 2010) or rats exposed to MDI (Kumar et al. 2009). This is the first report about the

comparison of workers who were screened for the presence of isocyanate antibodies in serum. The samples were collected and stored in the previous 15 years in Cincinnati. MDI forms stable adducts with the epsilon amino group of lysine yielding di-substituted ureas, which are very stable. Lysine adducts with 4-methylphenylisocyanate could be cleaved only after treating the compound for several hours at 100 °C (Sabbioni et al. 1997) in base or acid. Therefore, such adducts present in proteins are very stable. In rats chronically exposed to MDI (Sepai et al. 1995), the urinary metabolites and the Hb-adducts were determined in 1993–1994 (Sepai et al. 1995) and the isocyanate-specific adducts with albumin were determined in 2008 (Kumar et al. 2009). The dose–response relationship remained intact. The biomarkers determined in 1993–1994 (urinary metabolites, Hb-adducts) correlated very well with the biomarkers measured (albumin adducts) 16 years later (Kumar et al. 2009). Further evidence for the stability of lysine adducts in albumin was shown with plasma stored for 20 years in the freezer (Scholl and Groopman 2008). The same albumin-adduct levels of aflatoxin (AFB-Lys) were obtained as in the first measurements. The adducts resulting from isocyanates with lysine (Sabbioni et al. 1997) are more stable than the lysine adducts of aflatoxin (Sabbioni 1990). Therefore, there are no major concerns about the data obtained with the present samples. We do not expect significant loss of the stable urea amino acid adducts of isocyanates over the years.

In a recent paper, Luna et al. (2014) analyzed the albumin-adduct levels in asthma cases using our method. No comparison with immunological tests was included in the study. The authors did not find significant relationships between asthma cases and adducts levels. In contrast, in our present work significant relationships were found between adduct levels and asthma and/or MDI-antibodies present in vivo. The mean adduct levels in the asthma cases ($n = 9$) were higher (501 fmol/mg) than in MDI-workers (36.1 fmol/mg) or construction site workers (102 fmol/mg) using MDI-products (Sabbioni et al. 2010). The mean adduct level in the workers without asthma was 52 fmol/mg and therefore in the range of the former studies (Sabbioni et al. 2010). In comparison, the adduct levels in workers with a positive immunological test were 414 fmol/mg ($n = 10$) and with a negative immunological test 67.9 fmol/mg ($n = 27$), respectively.

Therefore, the starting hypothesis of the present study that workers with a positive biological response have higher adduct levels of MDI was confirmed. Therefore, we suggest to measure albumin adducts of MDI for industrial biomonitoring.

Conclusions

The odds ratio to be DA-positive was 57 times larger for workers with adduct levels above 230 fmol/mg. The odds ratio to be IgG-positive was 10 times larger with adduct levels above 113 fmol/mg. Therefore, albumin adducts appear to be a good predictor of the biological effects.

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