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RESEARCH ARTICLE

Determination of albumin adducts of 4,4'-methylenediphenyl diisocyanate in workers of a 4,4'-methylenedianiline factory

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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 have been found in subjects classified as 4,4'-methylenedianiline (MDA) workers. The mean adduct levels in these MDA-workers were 1.5 times higher than in MDI-workers of the same company. MDA-specific hemoglobin adducts, were present ten times more in the MDA-workers than in the MDI-workers. MDA-workers with specific work task had significantly higher albumin adduct levels.

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Introduction

4,4'-Methylenediphenyl diisocyanate (MDI) is used for several applications, for example: polyurethane foam, paints, adhesives, elastomers, coatings and consolidation of loose rock zones in coal mining or tunneling (Munn et al., 2005). Inhalation of MDI vapors is associated with various pulmonary ailments, such as eosinophilic airway inflammation, airway hyper-reactivity, early and late-onset asthma, exogenous allergic alveolitis and direct toxic responses (Baur, 1990; Karol, 1986; Mapp et al., 1994; Redlich & Karol, 2002). 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).

Arylisocyanates react directly with biomolecules and/or hydrolyze to arylamines (Figure 1). Arylisocyanates (Bolognesi et al., 2001; Vock & Lutz, 1997) and arylamines (Delclos & Kadlubar, 1997; Sabbioni & Jones, 2002) can bind with proteins and/or DNA (Figure 1) and lead to cytotoxic and genotoxic effects. Protein adducts of arylisocyanates are believed to be involved in the etiology of sensitization reactions (Raulf-Heimsoth & Baur, 1998; Wisniewski et al., 2000). Arylamine-specific adducts are of the sulfonamide type (Kazanis & McClelland, 1992; Ringe et al., 1988), which can be cleaved with mild base hydrolysis.

Isocyanates do not need any further activation to react with biomolecules (Figure 1). Important vehicles for isocyanates are their reaction products with glutathione (Pearson et al., 1991; Sabbioni et al., 2012; Slatter et al., 1991;

Wisniewski 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.

Arylamine-specific adducts (Figure 1) with hemoglobin (Hb) and urinary metabolites of 4,4'-methylenedianiline (MDA) were present in rats exposed chronically to MDI (Sepai et al., 1995). Isocyanate-specific adducts of MDI with the N-terminal valine of Hb (Sabbioni et al., 2000) and/or with N^ε-lysine of albumin were determined in rats and in humans (Gries & Leng, 2013; Sabbioni et al., 2010). In rats chronically exposed to MDI, the Hb-adducts (Sepai et al., 1995) and the DNA adducts (Vock et al., 1996) of the nasal epithelial cell correlate with the dose (Bolognesi et al., 2001). In rats, Hb-adducts have been found to correlate with the administered dose of either MDI, given by inhalation (Sepai et al., 1995) or the corresponding arylamine MDA, given by gavage (Sabbioni & Schutze, 1998). MDA binds to Hb (Sabbioni & Schutze, 1998) and to DNA (Schutze et al., 1996) (Figure 1).

Urinary metabolites of xenobiotics are usually detectable up to 48 h after exposure (Henderson et al., 1989). Reaction products with blood proteins such as Hb and albumin are present over a larger time-frame if the adducts are stable. Hb has a lifetime of 120 days and albumin a half-life of 20–25 days (Skipper & Tannenbaum, 1990; Tornqvist et al., 2002). Therefore, Hb-adducts can be found over a longer period. Albumin is one of the potential targets involved in the etiology of sensitization reactions of isocyanates (Raulf-Heimsoth & Baur, 1998;

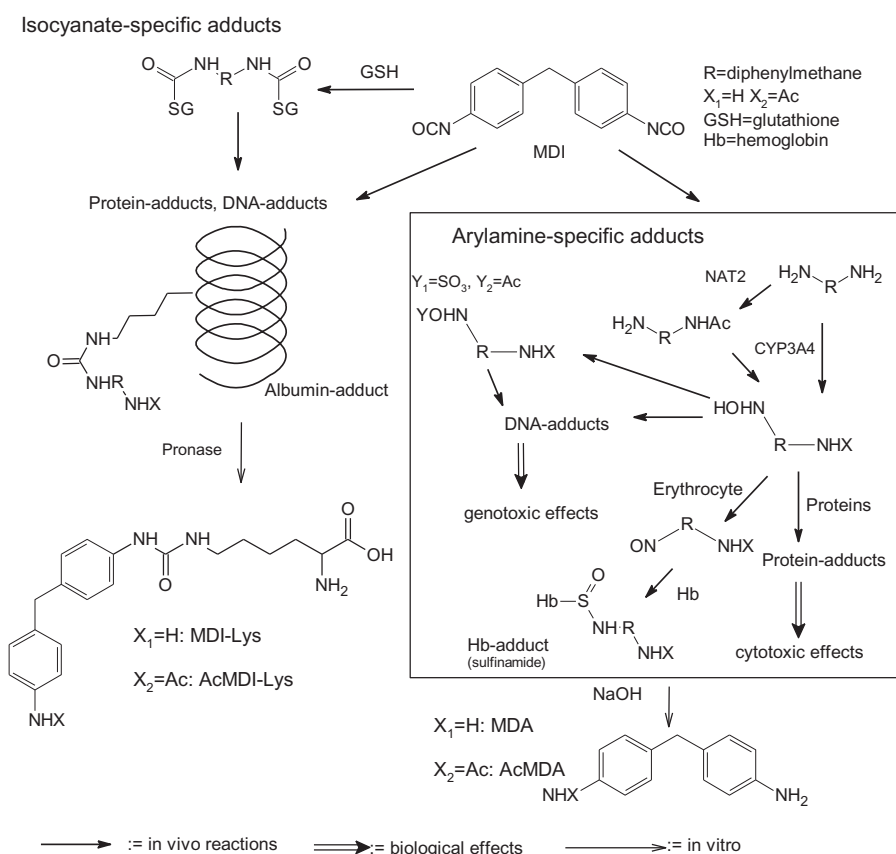


Figure 1. Adduct formation of MDI and MDA with biomolecules.

Wisniewski et al., 2000). Albumin adducts are a marker of exposure and a potential surrogate marker for the pathological effect.

For the present project, we investigated the presence of isocyanate specific adducts in workers which were classified as MDA-workers. These are the same samples used for the analysis of MDA-specific adducts with Hb (Sepai et al., 1995). With the former approach it was not possible to distinguish between exposures to MDA or MDI. The results of this study will increase the awareness of occupational hygienist for unexpected pathways (Henriks-Eckerman et al., 2015) of exposures to MDI products.

Materials and methods

Workers

Blood and urine specimens were collected from a chemical factory (MDA-workers) (Schutze et al., 1995). All biological samples were obtained at the end of the work-shift on Thursdays at 13:30 on the 2nd, 9th, 16th, 23th and 30th of April 1992. The MDA-workers (35 males) from the chemical industry were exposed to MDA. The mean age was 42.0 ± 9.9 (range 23–57), the median age was 41.0. Seventeen workers were smokers and 18 were non-smokers.

Chemicals

N^6 -[({4-[4-aminobenzyl]phenyl}amino)carbonyl]lysine (MDI-Lys), N^6 -[({4-[4-aminobenzyl]phenyl}amino)carbonyl] [$^{13}\text{C}_6^{15}\text{N}_2$]lysine (MDI- $^{13}\text{C}_6^{15}\text{N}_2$ Lys), N^6 -[({4-[4-(acetylamino)

benzyl]phenyl}amino)carbonyl] lysine (AcMDI-Lys) and N^6 -[({4-[4-(acetylamino)-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) (Figure 1). Amicon ultra centrifugal filter tube (30K MWC; 4 mL), ammonium bicarbonate, methanol for sample preparation (A452-4, HPLC-grade) and methanol for LC-MS/MS (Optima, A454-4) were obtained from Fisher Scientific (Bridgewater, NJ). 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) 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 AllegraTM 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 (Schutze et al., 1995). The erythrocytes were washed thrice with an equal volume of 0.9% sodium chloride. All samples were stored at -25°C . Albumin was isolated the same time (2009) as the samples from MDI-workers published recently (Sabbioni et al., 2010). Albumin was purified with HiTrap Blue HP columns 1.0 mL ($0.7 \times 2.5\text{ cm}$) from GE-Life sciences, Inc (Pittsburgh, PA). The HiTrap Blue HP column was equilibrated with six volumes of binding buffer (20 mM NaH_2PO_4 , pH 7.0). Human plasma (0.5 mL) was diluted with binding buffer (0.5 mL). After centrifugation the supernatant was loaded on the column. After eluting with 6 mL of binding buffer, albumin was eluted with 6 mL of elution buffer (20 mM $\text{NaH}_2\text{PO}_4 + 2\text{ M NaCl}$, pH 7.0). The columns were then rinsed with washing buffer (10 mL, 50 mM Tris buffer + 0.2 M NaSCN, pH 7.5). Purified fractions were concentrated in Amicon ultra centrifugal filter tube (30 k MWC; 4 mL) by centrifuging with 4000 rpm at 4°C for 10–15 min, and washed with water ($3 \times 4\text{ mL}$). Samples were reconstituted in 10 mM sodium phosphate buffer (pH 7.0). 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- $[\text{C}_6^{15}\text{N}_2]\text{Lys}$ (13.23 pmol) and $\text{Ac}[\text{H}_4]\text{MDI-Lys}$ (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, Foster City, CA). 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- $[\text{C}_6^{15}\text{N}_2]\text{Lys}$ and $\text{Ac}[\text{H}_4]\text{MDI-Lys}$ 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, $150 \times 2.0\text{ mm}$, $3\text{ }\mu\text{m}$) (Phenomenex Inc., Torrance, CA) protected by a C18 guard column (AJO-4287; $4\text{ mm L} \times 3.0\text{ mm ID}$), 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%) and 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- $[\text{C}_6^{15}\text{N}_2]\text{Lys}$ (13.23 pmol) and $\text{Ac}[\text{H}_4]\text{MDI-Lys}$ (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- $[\text{C}_6^{15}\text{N}_2]\text{Lys}$). 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 & Gupta, 2011).

Statistical analyses

The statistical analyses were performed with IBM SPSS for Windows Version 19.0 (IBM Corp., Armonk, NY). The human data were not normally distributed (one-sample Kolmogorov–Smirnov test, $p < 0.05$) except for MDA-Hb and MDI-Lys. Root square transformation of the data yielded normal distribution for all data except for urinary levels of MDA obtained after acid hydrolysis (=MDA-U-acid). Therefore, non-parametric tests are more relevant for MDA-U-acid data analyses than parametric tests. All the independent samples t-test analyses were performed with the square rooted data.

N-Acetyltransferase phenotype

The phenotype was determined by Lewalter et al. (Blaszewicz et al., 2004; Lewalter & Korallus, 1985; Lewalter & Miksche, 1991) using the urinary ratio of 5-acetylamino-6-formylamino-3-methyluracil to 1-methylxanthine obtained with the caffeine test (Grant et al., 1984). (Table 2).

Results

Plasma was collected from workers in the chemical industry (Schutze et al., 1995). In the previous study (Schutze et al., 1995), other biomarkers had been determined in this group of workers: analysis of urinary metabolites after acid treatment (MDA-U-acid), urinary metabolites after base extraction (MDA-U-base, AcMDA-U-base) and arylamine-specific (Figure 1) Hb-adducts (MDA-Hb, AcMDA-Hb) released after mild basic extraction (Table 1). These workers were specifically classified as MDA-workers by the occupational hygienists of the factory. For the present work, isocyanate specific adducts with albumin were analyzed (MDI-Lys, AcMDI-Lys) (Figure 1). Albumin was isolated from plasma with Cibachron blue affinity

Table 1. Comparison of biomarkers found in workers.

	MDA-workers ^a	Mean (±SD) Median (Range)	MDI-workers ^b	Mean (±SD) Median (Range)	Rats ^c
MDI-Lys	76.5%	50.5 (±44.7)	62.5%	36.1 (±38.6)	10.6 (±1.71)
[fmol/mg]	(n = 34)	51.5 (0–221)	(n = 24)	30.5 (0–138)	pmol/mg
AcMDI-Lys	47.1%	9.82 (±18.3)	4.2%	25.6 ^d	0.486 (±0.118)
[fmol/mg]	(n = 34)	0.0 (0–97.6)	(n = 24)		pmol/mg
MDA-U-acid	94.3%	9.4 (±31.8)	95.7%	2.07 (±1.99)	65.5 (±18.5)
[pmol/mL]	(n = 35)	1.89 (0–188)	(n = 23)	1.70 (0–10.2)	pmol/mL
MDA-U-base	97.0%	0.187 (±0.473)	95.7%	0.0537 (±0.0565)	0.214 (±0.0263)
[pmol/mL]	(n = 33)	0.071 (0–2.76)	(n = 23)	0.0565 (0–0.139)	pmol/mL
AcMDA-U-base	81.8%	1.60 (±4.29)	78.3%	0.605 (±0.658)	8.28 (±3.20)
[pmol/mL]	(n = 33)	0.370 (0–23.4)	(n = 23)	0.54 (0–3.0)	pmol/mL
MDA-Hb	97.1%	398 (±454)	38.5%	40.5 (±60.4)	15.3 (±5.39)
[fmol/g]	(n = 34)	284 (0–2614)	(n = 26)	0 (0–219)	pmol/g
AcMDA-Hb	61.7%	647 (±992)	0%	0	24.3 (±8.88)
[fmol/g]	(n = 34)	425 (0–4214)	(n = 26)		pmol/g

MDI-Lys and AcMDI-Lys are the isocyanate specific adducts with albumin. MDI-Lys and AcMDI-Lys for MDA-workers are new, all the other data have been published previously (Sabbioni et al., 2007; Schutze et al., 1995). MDA-U-acid corresponds to MDA found after acid treatment of urine. MDA-U-base and AcMDA-U-base correspond to MDA and AcMDA found after base extraction of urine. MDA-Hb and AcMDA-Hb correspond to MDA and AcMDA found after mild base hydrolysis of Hb.

^a34 blood samples and 35 urine samples were available (Schutze et al., 1995). The amount of 2 urine samples was not enough to determine MDA-base and AcMDA-base.

^bWorkers exposed to MDI in a chemical factory (=MDI-workers) (Sabbioni et al., 2010; Sepai et al., 1995).

^cBiomarker levels found in rats exposed to 0.26 mg/m³ MDI (Kumar et al., 2009; Sepai et al., 1995).

^dValue of the only positive sample.

Table 2. Comparison of biomarkers in workers with different duties.

	Σ-MDI-Alb MDI- + AcMDI-Lys [fmol/mg]	Σ-MDA-Hb MDA + AcMDA [fmol/g]	Σ-MDA-U MDA + AcMDA [pmol/mL]	MDA-U-acid MDA [pmol/mL]	Acetylator phenotype Slow/intermediate/fast
Group A) System control at the measuring station (n = 13)					
Mean (±SD)	78.2 (±78.9) ¹	1597 (±1798) ⁴	0.616 (±0.853) ^{5,21}	2.31 (±1.90) ⁶	7/3/20
Median	67.1 ^{8,11}	1144 ⁹	0.25 ¹³	1.46 ^{10,12,14}	
Range	0.00–319	128–6189	0.02–2.82	0.44–5.83	
Group B) Sample drawing (n = 10)					
Mean (±SD)	59.2 (±33.9) ³	1075 (±1052)	4.63 (±8.13) ⁵	27.6 (±57.5) ^{6,7}	5/3/2
Median	63.3 ^{15,18}	829	1.68 ¹³	6.09 ^{14,19}	
Range	33.1–127	64–3067	0.04–26.2	1.08–188	
Group C) Round tour and sample taking (n = 4)					
Mean (±SD)	96.7 (±15.7) ^{2,22}	581 (±577) ²²	0.733 (±0.500)	4.44 (±2.50)	0/0/4
Median	101 ^{11,15,16}	292	0.64	3.74 ^{12,17}	
Range	79.4–110	206–1245	0.09–1.28	1.89–7.86	
Group D) Shift supervisor (n = 6)					
Mean (±SD)	17.9 (±30.1) ^{1,2,3}	334 (±268) ⁴	0.394 (±0.322) ²³	0.707 (±0.661) ⁷	1/1/4
Median	0.00 ^{8,16,18}	268 ⁹	0.26	0.76 ^{10,17,19}	
Range	0.00–73.7	112–716	0.12–0.91	0.00–1.63	
Group E) Occupational medical doctor (n = 2)					
Mean	26.2	140	0.247	1.19	0/0/2
Median	26.2	140	0.247	1.19	
range	0.00–52.4	125–156	0.01–0.48	0.42–1.96	

For the independent samples t-test t-test analysis, root-squared transformed data were used. The t-test yielded significant data for the pairs 1–7 ($p < 0.05$). The non-parametric test (Mann–Whitney) yielded significant differences for the pairs, 8–10, 14, 16, 17, 18 and 19 ($p < 0.05$). The pairs 11–13, 15 and yields a p value < 0.1 . 20: Acetylator phenotype measured with the caffeine test. 21: $n = 12$. 22: $n = 3$. 23: $n = 5$.

chromatography. 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.

The isocyanate specific adduct with albumin, MDI-Lys, could be found in 76.5%, of the MDA-workers. The results were summarized in Table 1. The levels of the acetylated metabolite AcMDI-Lys were found in 47.1% of the MDA-workers. The mean levels of the isocyanate specific adduct (MDI-Lys) was about 125 times higher than the mean levels of the arylamine-specific adduct (MDA-Hb).

In order to find the source of MDI for the MDA-workers we analyzed the biomarker levels of the workers in relation to their job description (Table 2). The following five groups of workers were compiled: group (A) system control at the measuring station ($n = 13$), group (B) sample drawing ($n = 10$), group (C) round tour and sample taking ($n = 4$), group (D) shift supervisor ($n = 6$) and group (E) occupational medical doctor. Due to the small number, the two medical doctors were not included in the statistical analyses. The Σ-MDI-Alb (=MDI-Lys + AcMDI-Lys) levels were the highest in the group C workers and the lowest in the group D workers. The group D workers had significantly lower mean levels than the other groups (Table 2). Group C workers had significantly higher median values than the other group of workers. The Σ-MDA-Hb (=MDA-Hb + AcMDA-Hb) mean and median levels

Table 3. Comparison of the biomarker levels with the acetylator phenotype.

<i>N</i> -Acetylator phenotype	Σ -MDI-Alb MDI- + AcMDI-Lys [fmol/mg]	Σ -MDA-Hb MDA + AcMDA [fmol/g]	Σ -MDA-U MDA + AcMDA [pmol/mL]	MDA-U-acid MDA acid [pmol/mL]
Slow (<i>n</i> = 13)				
Mean ($\pm$ SD)	76.2 ($\pm$ 79.9) ^a	1513 ($\pm$ 1649) ^{b,d}	3.17 $\pm$ 7.44	19.6 $\pm$ 51.6
Median	56.1 ^a	724 ^{c,d}	0.261	1.73
Range	0.00–319	287–6189	0.035–26.2	0.58–187
Intermediate (<i>n</i> = 7)				
Mean ($\pm$ SD)	58.7 ($\pm$ 38.3)	1060 $\pm$ 1680	0.840 $\pm$ 1.13 ^e	4.04 $\pm$ 5.73
Median	69.9	445	0.325	1.63
Range	0.00–98.5	64–4721	0.035–2.95	1.08–16.8
Fast (<i>n</i> = 15)				
Mean ($\pm$ SD)	47.0 ($\pm$ 41.2) ^{a,f}	1045 $\pm$ 1338 ^{b,d,f}	0.920 $\pm$ 0.843 ^f	3.15 $\pm$ 3.03
Median	56.6 ^a	672 ^{c,d}	0.775	1.89
Range	0.00–110	77–1431	0.013–2.82	0.00–9.89

^aOnly the mean and median levels of the single biomarker AcMDI-Lys were significantly ($p < 0.05$ and $p < 0.1$, respectively) higher in the slow acetylators.

^bIndependent samples *t*-test $p < 0.05$.

^cMann-Whitney $p < 0.05$.

^dThe mean and median levels of AcMDA were significantly ($p < 0.05$ and $p < 0.1$, respectively) higher in the slow acetylators.

^e $n = 6$.

^f $n = 14$.

decrease in the order group A > B > C > D. However, only the mean and the median levels of group A were significantly different from the group D. The highest mean and median levels of the urinary metabolites Σ -MDA-U (=MDA-U-base + AcMDA-U-base) and MDA-U-acid were in workers taking samples from the reaction pots (group B and C). Σ -MDA-U was significantly higher in group B than in group A. The mean and median levels of MDA-U-acid were significantly higher in group B compared to group A and group D. Urinary metabolites are short-term markers. Therefore, it appears that the exposure of the workers from group B and group C was not chronic as for the other workers; otherwise, the Σ -MDI-Alb and Σ -MDA-Hb levels would be higher in relation to the urinary metabolites.

For the present set of workers, the *N*-acetylator phenotype determinations were performed using the caffeine test (Table 2, 3). The biomarker levels were compared between the slow and fast acetylators. Σ -MDI-Alb, Σ -MDA-Hb, Σ -MDA-U, and the levels of MDA released from acid treated urine (=MDA-U-acid) were compared using the independent samples *t*-test, and/or the non-parametric two-independent samples test. Only the mean and the median levels of Σ -MDA-Hb were significantly higher in slow acetylator than in fast acetylators. In addition, the mean and median levels of the single biomarkers (AcMDA-Hb and AcMDI-Lys) were significantly higher in slow acetylator than in fast acetylators. In fast acetylators it is expected that MDA is metabolized to *N'*-acetyl-*N*-acetyl-4,4'-methylenedianiline (DiAcMDA). DiAcMDA cannot form adducts with Hb. Therefore, the presence of higher levels of AcMDA in slow acetylators compared to fast acetylators is not a contradiction.

Discussion

4,4'-Methylenediphenyl diisocyanate (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 presence of such adducts in workers, which were classified as MDA workers. For these

workers, exposure to MDA was monitored using personal air samplers (Sepai et al., 1995). The values found for most workers were below the detection limit of 20 μ g MDA/m³ air. One worker was positive on the first day and 3 workers on the second day. Therefore, air measurements were not effective to monitor the exposure of MDA. The MDI-air-levels were not measured.

Isocyanates such as MDI form adducts with the epsilon amino group of lysine yielding disubstituted 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) with 0.3M NaOH and/or 6M HCl. Therefore, such adducts present in proteins are very stable. The same biomarkers described in this paper were also measured in samples from rats stored for many years. The rats were chronically exposed to MDI in 1992/1993 (Hoymann et al., 1995; Sepai et al., 1995). The urinary metabolites and the Hb-adducts were determined in 1993/1994 (Sepai et al., 1995). The isocyanate specific adducts with albumin were determined in 2009 (Kumar et al., 2009). The dose-response relationship remained intact. The adduct levels increased with the dose. The correlation of the biomarkers determined in 1993–1994 (urinary metabolites, Hb-adducts) correlate 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 published recently (Scholl & Groopman, 2008). Scholl et al (Scholl & Groopman, 2008) showed with plasma stored for 20 years in the freezer, that the same albumin-adduct levels of aflatoxin were obtained as in the first measurements. The reaction product of aflatoxin with albumin is with lysine (=AFB-Lys). The adducts resulting from isocyanates with lysine (Sabbioni et al., 1997) are certainly 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.

Table 4. Pearson and Spearman rank correlations of the biomarkers from MDA-workers and rats exposed to MDI.

	MDA-Hb	Σ-MDA-Hb ^a	MDA-U-base ^b	AcMDA-U-base ^b	MDA-U acid ^c	Σ-MDI-Alb ^d
MDI-Lys	0.502 ^{e,g**}	0.456**	0.228	0.157	0.218	0.978**
	0.437 ^{f**}	0.334	0.271	0.126	0.336	0.968**
	0.940 ^{i**}	0.952 ^{i**}	0.967 ^{i**}	0.900 ^{i**}	0.963 ^{i**}	0.991 ^{i**}
Σ-MDI-Alb	0.615**	0.592**	0.253	0.176	0.251	
	0.557**	0.481**	0.319	0.178	0.382 ^{h*}	
	0.926 ^{i**}	0.944 ^{i**}	0.944 ^{i**}	0.881 ^{i**}	0.944 ^{i**}	
MDA-Hb		0.820**	0.272	0.266	0.251	
		0.876**	0.489**	0.310	0.391*	
		0.991 ^{i**}	0.942 ^{i**}	0.886 ^{i**}	0.862 ^{i**}	
Σ-MDA-Hb			0.408*	0.392*	0.416*	
			0.414*	0.236	0.366*	
			0.944 ^{i**}	0.902 ^{i**}	0.881 ^{i**}	

^aMDA + AcMDA released after mild base hydrolysis of Hb (Schutze et al., 1995).
^bMDA and AcMDA obtained after extraction from urine. (Schutze et al., 1995).
^cMDA obtained after acid hydrolysis of urine (Schutze et al., 1995).
^dThe sum of MDI-Lys + AcMDI-Lys obtained from pronase hydrolysis of albumin.
^ePearson correlation of root squared transformed data.
^fSpearman rank correlations.
^g^{**} $p < 0.01$.
^h^{*} $p < 0.05$.
ⁱSpearman rank correlation ($p < 0.01$) of the biomarkers obtained from rats exposed to (0–2.06 mg/m³) MDI (Kumar et al., 2009; Sepai et al., 1995).

Thus, although all the isocyanate analyses listed in Table 1 were performed many years after the other measurements it is unlikely that the isocyanate biomarkers are an under-estimation. The samples of the MDA-workers were obtained the same time as the MDI-workers (Schutze et al., 1995). The MDA specific adducts were analyzed at the same time (Schutze et al., 1995). Albumin of the MDA-workers was isolated at the same time as the albumin of the MDI-workers (Sabbioni et al., 2010).

The adduct levels found in the MDA-workers were compared to the adduct levels present in MDI-workers from the same company (Sabbioni et al., 2010) (Table 1). The MDI-Lys levels and the MDA-Hb adduct levels are higher in the MDA workers than in the MDI-workers. The mean Hb-adduct levels are 10 times higher in the MDA workers than in the MDI-workers. In addition, 2.5 times more samples are positive for MDA-Hb adducts in MDA-workers than in MDI-workers. The MDA-workers were exposed also to higher levels of MDI than the MDI workers. This might be a consequence of an unknown source of MDI in the factory. Recently unusual *N*-carbonyl-*O*-glucuronides have been found for drugs with an amino-group (Argikar, 2012; Bunnelle, 2011). It is unknown whether *N*-carbonyl-*O*-glucuronide formation is a sequential reaction of substrate with CO₂ yielding unstable carbamic acids, followed by glucuronidation. Therefore, such products might be possible as metabolites in workers exposed to MDA in urine and feces. However, adducts of MDA with albumin through such a pathway is very unlikely.

Higher levels of MDA-specific Hb-adducts were found in slow acetylators. Higher Hb-adduct levels were found previously in slow acetylator exposed to aniline (Lewalter & Korallus, 1985; Lewalter & Miksche, 1991).

How does the isocyanate-specific marker relate to the other markers measured previously? The data were compared to biomarkers presented in a previous publication (Schutze et al., 1995) for the same group of workers: analysis of urinary metabolites after acid treatment (MDA-U-acid),

urinary metabolites after base extraction (MDA-U-base, AcMDA-U-base) and the arylamine-specific (Figure 1) Hb-adducts (MDA-Hb, AcMDA-Hb) released after mild basic extraction. The results are summarized in Tables 1–4. The sum of the MDI-albumin adducts (Σ-MDI-Alb) (Table 4) correlate with the MDA-adduct levels ($r = 0.557$, $p < 0.01$) and with urinary MDA released after acid hydrolysis (MDA-U-acid), but not with the urinary metabolites found after base extraction. The sum of the MDA-Hb adducts (Σ-MDA-Hb) correlate with all urinary biomarkers. In comparison, the biomarkers in rats correlate with >0.86 (Spearman rank). These rats were exposed to 0–2.06 mg/m³ MDI for 17 h per day and 5 days per week (Sepai et al., 1995). In the rats exposed to 0.26 mg/m³, first pathological effects were seen in the lung. Interstitial fibrosis was found in 79% of this exposure group and 10% bronchiolar type hyperplasia was found in this exposure group in comparison to 0% in the control group (Hoymann et al., 1995). The urinary levels were for both the MDA-workers and rats in the same order of magnitude. However, the Hb-adduct and MDI-adduct levels were 1000 times higher in the rats, than in the MDA-workers. The rats were exposed chronically to MDI, but the workers were not constantly exposed to the same levels of MDA or MDI. This is reflected by the poor correlation of the urinary metabolites to the protein adducts, and by the varying ratio of protein-adducts to urinary metabolites (Table 2, compare group A and group D).

Conclusions

4,4'-Methylenedianiline (MDA)-workers have had 10-times more MDA-specific adducts than MDI-workers. Surprisingly, isocyanate specific adducts with albumin were found also in MDA-workers. Therefore, unknown exposure sources were present in the factory. In the future air monitoring and skin monitoring has to be performed in order to avoid the exposure of the workers.

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