Connecting glutathione with immune responses to occupational methylene diphenyl diisocyanate exposure

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Abstract

Methylene diphenyl diisocyanate (MDI) is among the leading chemical causes of occupational asthma world-wide, however, the mechanisms of disease pathogenesis remain unclear. This study tests the hypothesis that glutathione (GSH) reacts with MDI to form quasistable conjugates, capable of mediating the formation of MDI-conjugated “self” protein antigens, which may participate in pathogenic inflammatory responses. To test this hypothesis, an occupationally relevant dose of MDI (0.1% w/v) was reacted with varying concentrations of GSH (10 μM-10 mM), and the reaction products were characterized with regard to mass/structure, and ability to carbamoylate human albumin, a major carrier protein for MDI in vivo. LC-MS/MS analysis of GSH-MDI reaction products identified products possessing the exact mass of previously described S-linked bis(GSH)-MDI and its partial hydrolysis product, as well as novel cyclized GSH-MDI structures. Upon co-incubation of GSH-MDI reaction products with human albumin, MDI was rapidly transferred to specific lysines of albumin, and the protein’s native conformation/charge was altered, based on electrophoretic mobility. Three types of modification were observed, intramolecular MDI cross-linking, addition of partially hydrolyzed MDI, and addition of “MDI-GSH”, where MDI’s 2nd NCO had reacted with GSH’s “N-terminus”. Importantly, human albumin carbamoylated by GSH-MDI was specifically recognized by serum IgG from MDI exposed workers, with binding dependent upon the starting GSH concentration, pH, and NaCl levels. Together, the data define a non-enzymatic, thiol-mediated transcarbamoylating mechanism by which GSH may promote immune responses to MDI exposure, and identify specific factors that might further modulate this process.

Keywords
methylene diphenyl diisocyanate (MDI); albumin; carbamoylation; glutathione (GSH); exposure

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1. Introduction

Methylene-diphenl diisocyanate (MDI) belongs to a class of commercially important low molecular weight (LMW) chemicals known as diisocyanates, which are essential cross-linking agents for making polyurethane [1]. Like other diisocyanates, MDI possesses the potential to sensitize the immune system, leading to asthma, hypersensitivity pneumonitis (HP) and dermatitis [2-5]. Collectively, the diisocyanates used for polyurethane production are a leading chemical cause of occupational asthma in industrialized nations and regulatory agencies around the world have established legal occupational exposure limits [5, 6]. Within the last 20 years, MDI has become the most abundantly produced and consumed diisocyanate, for multiple reasons related to its unique physical properties [1].

The mechanisms that mediate MDI, and other diisocyanates' immunogenicity remain unclear, hampering efforts at prevention, diagnosis, and surveillance of exposure-induced disease [7]. It has been hypothesized that diisocyanates' adverse health affects are caused by chemical reactivity with self-molecules, especially primary amine groups of proteins, which are reactive under physiologic conditions [8, 9]. In vivo, albumin appears to be the major protein target for diisocyanate reactivity, and diisocyanate-albumin is the only known “self” protein reaction product known to trigger innate and adaptive cellular immune responses, associated with airway inflammation and asthma [10-14]. In vitro, diisocyanate-albumin specifically stimulates production of histamine releasing factor, and other inflammatory chemokines, from peripheral blood mononuclear cells of sensitized workers [13, 15, 16]. Diisocyanate-albumin specific IgG is frequently found in the serum of exposed workers, however, IgE isotypes are rarely observed (even in hypersensitive individuals), an important distinction between diisocyanate and common environmental asthma triggered by “high molecular weight” antigens [7, 14, 17-20].

The microenvironment under which albumin reacts with diisocyanate in vivo and subsequently induces adaptive immune responses is uncertain. Albumin molecules in airway surfactant, epithelial fluid, or the extracellular skin compartment may be targets for direct reactivity with diisocyanate [21-27]. However, data from animal studies, demonstrating rapid accumulation of diisocyanate-albumin conjugates in the peripheral circulation, following respiratory tract-only exposure [28], have suggested the possible existence of a “shuttle” mechanism (see below) [29], through which diisocyanate is transported from the airways to the blood, where albumin is the dominate protein (reactant).

In addition to albumin, MDI and other diisocyanates are thought to react in vivo with the tripeptide, γ-g glutamyl-cysteinyl-glycine, also known as GSH [30, 31]. GSH is the principal nonprotein thiol compound in most mammalian cells, where it is present in millimolar concentrations [32]. GSH is also the major free thiol of the lower airway fluid, where it is normally present at relatively high levels (100 - 800 μM) compared with peripheral blood (~1 μM) [33-36]. The epidermal layer of the skin, another potential route of diisocyanate exposure, also contains relatively high levels of GSH, compared with the underlying dermis [37].
The reaction of isocyanate with thiols, such as GSH, is reversible, prompting the theory that GSH serves as a shuttle for systemic distribution of inhaled diisocyanate [29, 38-40]. Evidence supporting a “shuttling” capacity of GSH has been derived largely from in vitro studies of toluene diisocyanate (TDI) and aryl mono-isocyanates, demonstrating that GSH conjugates can carbamoylate other peptides/proteins, including albumin [11, 38, 41, 42]. In contrast, GSH mediated transcarbamoylation of MDI, which possesses unique physical properties compared with other mono- and di-isocyanates, remains relatively under-studied. Furthermore, the hypothetically key pathogenic aspect of GSH-mediated carbamoylation with MDI, or other diisocyanates, i.e. modification of proteins in an antigenic manner, e.g. rendering them recognizable by the host immune system [43, 44], has yet to be demonstrated for humans.

In this study, we investigate the hypothetical mechanism whereby GSH serves as a carbamoylating intermediate (e.g. shuttle) in the conjugation of albumin by MDI, and identify specific reaction conditions that further modulate this process. The experimental strategy builds upon limited published data on GSH reactivity with MDI, and other aryl mono-isocyanates, which largely used NMR to characterize thiol-MDI conjugation and disassociation [41, 45, 46]. The present investigation utilizes a combination of LC-MS/MS and electrophoretic analyses to further characterize GSH-MDI reaction products and their unique modification of human albumin, under physiological exposure conditions (neutral pH, normal saline, temperature, aqueous solvent). The biologic relevance of MDI-albumin conjugates, generated via GSH-mediated transcarbamoylation, was further evaluated based on specific binding of serum IgG from MDI exposed workers. The potential contribution of GSH toward MDI-specific immunologic responses is discussed based on the experimental findings.

2. Materials and Methods

2a. Reagents

The following chemicals and proteins were obtained from Sigma-Aldrich (St. Louis, MO): reduced and oxidized forms of glutathione, GSH (CAS # 70-18-8) and GSSH (CAS # 27025-41-8) respectively, and 4,4’-methylenebis(phenyl isocyanate), or MDI (CAS # 101-68-8), human albumin, transferrin, thioredoxin, and ovalbumin from turkey egg white. GSH, GSSG, MDI and protein reagents were of ≥98.0% purity. Buffer reagents, which included mono- and di-basic sodium phosphate, sodium chloride, citric acid, sodium citrate, bicarbonate, sodium carbonate, and deionized water were also from Sigma.

2b. GSH-MDI reactions

Glutathione solutions of varying concentrations were initially prepared in 20 mM sodium phosphate buffer with or without NaCl, and in later experiments in 0.1 M citrate or carbonate buffer. Freshly prepared stock solutions of 10% MDI (w/v) in acetone (JT Baker; Phillipsburg, NJ) were added to GSH solution drop-wise with stirring, to achieve 0.1% MDI final concentration, or approximately 4mM MDI, well below the starting concentrations of chemical (50%) typically aerosolized to generate spray foam insulation [6]. Reactions were allowed to proceed at 37°C, with end-over-end mixing, for 2 hrs, and then pelleted at 10,000
x g and 0.2 μm syringe filtered. Reaction products were immediately tested for carbamoylating activity, or snap frozen and stored at −80°C until analyzed by mass spectrometry. In all experiments, negative controls included reactions identically performed for 2 hours with (a) MDI in buffer without GSH (MDI-buffer control), and in some experiments (b) substituting GSSG for GSH (GSSG-MDI). Preliminary data (not show) demonstrated that MDI at 0.1% w/v in PBS was completely hydrolyzed/polymerized within 2 hours in the absence of GSH (e.g. MDI-buffer control sample).

2c. LC-MS/MS analysis of GSH-MDI reaction products

Total GSH-MDI reactions were dried via SpeedVac and re-constituted with 50 μL water. Next, 15 μL of the re-constituted sample was diluted with 19 μL water, 1 μL acetonitrile and 5 μL of 1% formic acid. Samples were then desalted using a C18 ZipTip, and eluted into 50ul 60% acetonitrile/0.1% formic acid. Five uL of the de-salted samples were then directly infused via Advion TriVersa NanoMate into a Bruker 9.4T FT-ICR MS [47].

2d. Protein carbamoylation by GSH-MDI

Following sterile filtration (0.2 μM), total GSH-MDI reaction products were mixed 1:2 with a solution of human albumin at 5 mg/ml in phosphate buffered without NaCl, or phosphate buffer with NaCl (PBS), each containing 20 mM phosphate. In some experiments 0.1M citrate, or 0.1M bicarbonate, were used to alter the pH. Albumin and GSH-MDI reaction products were co-incubated at 37°C for 1hr, after which time the solution was chilled to 4°C, dialyzed, and subsequently analyzed for MDI modification by MS/MS and electrophoresis, or antigenicity based on specific recognition by serum IgG from MDI exposed workers. For all experiments control samples were tested to ensure that MDI conjugation of albumin was occurring via GSH reaction products, and not via direct reactivity with MDI. Thus, controls included albumin co-incubated with MDI that was "mock reacted" with GSH-free buffer ν (MDI-buffer control, see above), or GSSG (MDI-GSSG). In some experiments, the proteins transferrin, thioredoxin, or ovalbumin were substituted for human albumin in carbamoylation reactions.

2e. LC-MS/MS analysis of albumin carbamoylated by GSH-MDI

Samples of albumin carbamoylated by GSH-MDI, were reduced, acetylated, and trypsin digested, prior to LC-MS/MS at the Yale University Keck Center, as previously described [47, 48]. Samples were run on an LTQ Orbitrap Elite mass spectrometer and all MS/MS spectra were searched using the automated Mascot algorithm against the NCBI nr database. A 95% confidence level was set within the MASCOT search engine for protein hits based on randomness search. In addition, 2 or more MS/MS spectra must have matched the same protein entry and been derived from trypsin digestion. Peptide scores >20 are likely correct based on past experience, and the higher the score the better the match. In addition to oxidation of methionine and acetylation (carbamidomethyl) of cysteine during workup, the data were further queried for expected mass modifications (see Fig. 3) due to carbamoylation by GSH-MDI reaction product(s) (Figs. 1-3).

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2f. Electrophoretic analysis of albumin carbamoylated by GSH-MDI

For analysis of gel electrophoretic mobility under native conditions (which increases upon MDI conjugation), samples were prepared in a glycerol buffer, and run on 10% polyacrylamide gels. Following electrophoresis, gels were stained with Imperial protein stain from Pierce (Rockford, IL).

2g. Human subjects

The study was approved by Yale University’s Institutional Review Board for human subject investigation. All subjects provided informed written consent, and answered questionnaires related to MDI or other diisocyanate exposures. Study subjects included 3 construction workers who reported spraying MDI-based foam insulation >4 hrs/day, >3 days/week for >6 months, and were diagnosed with occupational asthma by a pulmonologist. An additional 12 individuals, without MDI exposure, were enrolled as control subjects. Ten milliliters of blood were obtained from each subject by venipuncture, using red top tubes with serum separators from Becton-Dickinson (Franklin Lakes, NJ). Following centrifugation at 1000 × g for 10 minutes, serum was separated from clotted blood, 0.2 μm syringe filtered (Millipore, Bedford, MA), aliquoted and stored at −80°C.

2h. Antigenicity of proteins carbamoylated by GSH-MDI

ELISAs were performed initially to test the antigenicity of albumin carbamoylated via GSH, based on specific recognition by serum IgG from MDI exposed workers vs. unexposed individuals [9]. 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 MDI-GSH reaction products, as described above. Plates were coated in 0.1 M carbonate buffer, pH 9.5, washed, and “blocked” with 3% (w/v) dry milk/PBS, before addition of human serum samples diluted 1:200 (v/v) in 3% dry milk/PBS/0.05% Tween 20. Following washes and incubation with secondary reagent, peroxidase-conjugated anti-human IgG Fc (Pharmingen; San Diego, CA), diluted 1:2000 (v/v), ELISA plates were developed with tetramethylbenzidine substrate. Optical density (OD) measurements (absorbance of light at 450 nm, minus absorbance at a reference wavelength) were obtained on a Benchmark microtiter plate reader from Bio-Rad (Hercules, CA). All experiments were repeated three times to obtain mean and standard error values. Statistical differences in IgG binding data were calculated using the Wilcoxon rank-sum test.

In some ELISA experiments, albumin and other proteins, carbamoylated by GSH-MDI, were probed for recognition by human serum IgG (as above), or for the presence of MDI, using MDI-specific mAbs [49], in which case peroxidase conjugated anti-mouse IgG was substituted for anti-human IgG as the secondary reagent.

3. Results

a. Reaction of GSH with MDI in aqueous phase

To begin evaluating the interaction of GSH with MDI, reactions were performed under aqueous phase conditions established in previous studies [9]. When an occupationally-relevant concentration of MDI, 0.1% (w/v) or 4 mM, was reacted with 10 mM GSH, e.g. a
slight (25%) molar excess of SH vs. NCO, LC-MS analysis indicated multiple products, with m/z's ranging from 483.13 to 1314.11 (Fig. 1 and Table 1). Two of the reaction products possessed masses identical to the previously described (bis)GSH-MDI, and its partial hydrolysis product (mono)GSH-MDI*, where the * indicates MDI's unbound N=C=O hydrolyzed to a primary amine [45]. Several additional products were also observed, with m/z's consistent with GSH-MDI containing one or more GSH and/or MDI groups with varying degrees of hydrolysis, as listed in Table 1. Molecular modeling of the products, based on their exact mass and LC-MS/MS (not shown), suggests several different cyclized structures (Figure 2) for those products with the strongest relative intensity. It remains unclear if these novel GSH-MDI products form directly, or if they result from secondary reactions of bis(GSH)-MDI and/or mono(GSH)-MDI*.

3b. Ability of GSH-MDI reaction products to carbamoylate human albumin

The ability of GSH-MDI reaction products to carbamoylate human albumin, a major carrier protein for MDI in vivo, was evaluated through FT-ICR-MS/MS analysis of trypsin-digested albumin samples that had been co-incubated with GSH-MDI. Experiments were performed with GSH starting concentrations of 10 mM, as described above, and 10-fold lower levels of GSH (e.g. 1 mM), which are closer to those present in the airway lining fluid in vivo. Carbamoylated albumin peptides were detected by querying the data for exact masses expected to result from (A) addition of partially hydrolyzed MDI, (B) intra-molecular cross-linking with MDI, or (C) addition of MDI-GSH, as depicted in Figure 3. The data identified eight lysine modifications when albumin was co-incubated with GSH-MDI prepared with 1 mM GSH in PBS (Table 2, and supplemental data), but not control samples. Carbamoylation with partially hydrolyzed MDI (modification A, Figure 3), or intra-molecular cross-linking by MDI (modification B, Figure 3), was most prominent on lysines that comprise di-lysine motifs, which account for 4 of the 8 modification sites, and are also preferred sites for direct MDI-albumin conjugation [9, 50]. In contrast, cyclized GSH-MDI appeared to preferentially carbamoylate sites (K162, and K545), distinct from those preferentially targeted by direct MDI reactivity [9, 50]. When albumin carbamoylation was performed with GSH-MDI prepared with a higher starting concentration of GSH (10 mM) in phosphate buffer without NaCl, an additional 5 sites of modification were observed (Table 2, and supplemental data).

3c. GSH-MDI mediated changes in human albumin's charge/conformation

MDI-albumin formed via GSH-MDI was electrophoresed in acrylamide gels to characterize differences in migration that reflect changes in albumin's charge/conformation. As highlighted in Fig. 4A, carbamoylation via GSH-MDI increased albumin's electrophoretic mobility towards the anode under native conditions, suggesting a net increase in negative charge. Notably, the electrophoretic migration of human albumin carbamoylated by GSH-MDI differed from that of MDI-albumin generated via direct exposure (using same starting dose of MDI for GSH and albumin exposure). Despite changes in electrophoretic migration in native gels, no differences were observed in SDS-PAGE gels comparing control albumin samples vs. MDI-albumin resulting from transcarbamoylation via GSH (data not shown).
3d. Antigenicity of albumin carbamoylated by GSH-MDI reaction products

The antigenicity of MDI-albumin, resulting from carbamoylation via GSH-MDI, was evaluated based on recognition by human antibodies in Western blot and ELISAs, using sera from human subjects with and without MDI exposure, described in Table 3. As shown (Figs 4B and 5A), serum IgG from MDI exposed workers (N=3), but not unexposed individuals (N=12), displayed substantial binding to MDI-albumin, prepared via GSH-MDI.

The antigenicity (i.e. specific recognition by serum IgG from MDI exposed workers) of MDI-albumin, formed via GSH-MDI, was highly depended upon the reaction conditions, especially the starting GSH concentration, pH, and presence/absence of NaCl. When GSH was prepared in phosphate buffered saline (PBS), a dose-dependent relationship was observed between the starting GSH concentration and the antigenicity of the final carbamoylation product (e.g. MDI-albumin). Antigen formation reached maximum levels when the starting GSH concentration was 1mM, but decreased as the GSH concentration was further heightened (Fig 5B). In the absence of NaCl the “dose-response curve” was shifted to the right. When GSH was prepared in unbuffered solution (water), limited MDI reactivity/transcarbamoylation was observed, which may be due to GSH's innately low pH, and the acid-stability of thiol-cyanate reaction products [41, 51]. Consistent with this hypothesis, higher pH levels during the carbamoylation reaction (co-incubation of albumin with GSH-MDI), resulted in MDI-albumin products with relatively increased antigenicity (Fig 5C). Under optimal conditions, MDI-albumin conjugates resulting from GSH-mediated transcarbamoylation exhibited antigenicity (based on workers' serum IgG binding in ELISA) comparable to albumin directly reacted with MDI (Fig 4B and not shown).

In additional studies, the ability of GSH-MDI to carbamoylate human proteins other than human albumin, and the potential antigenicity of such MDI-proteins, were evaluated. ELISA data (Fig. 5D) using MDI-specific mAbs, demonstrated the capacity of GSH-MDI to carbamoylate a number of different proteins including thioredoxin, transferrin, and ovalbumin. However, serum IgG from the MDI exposed workers did not recognize these other MDI-protein conjugates despite the presence of IgG that recognized similarly generated MDI-albumin.

4. Discussion

This study defines a non-enzymatic transcarbamoylating mechanism through which GSH mediates the formation of antigenic diisocyanate-albumin conjugates, which have been implicated in pathogenic responses (cytokine production, specific IgE, oxidative stress, innate immune proteins) to occupational exposure [12, 14, 15, 46, 52-54]. The findings expand upon our understanding of MDI’s potential reactivity with self-molecules present at exposure sites, and describe novel (cyclized) GSH-MDI reaction products that form under physiologic conditions. The transfer of isocyanate groups, from GSH to albumin, as described here, highlight the complex chemical-protein interactions that may underlie MDI's immunogenic capacity in vivo. The data support previous theories implicating GSH as a shuttle for isocyanate, which could explain systemic distribution, and chemical conjugation of proteins distant from the exposure site. The data also describe exposure conditions that modify GSH-MDI reactivity and subsequent carbamoylation of albumin, which may explain
individual differences in exposure responses, and serve as the basis for disease prevention/intervention strategies.

The present data expand upon studies by Reissner et al., which described the selective formation of thiol-linked mono and bis(GSH)-MDI under relatively non-physiologic anhydrous conditions, e.g. at −25°C, in organic solvent, with starting concentrations of 160mM GSH and 10 mM MDI [45]. In this study, mono and bis(GSH)-MDI reaction products were observed under more natural exposure conditions, e.g. aqueous solution, 37°C, neutral pH, isotonic saline, physiological GSH concentration and 2.5-fold lower MDI levels. Additional cyclized GSH-MDI reaction products were observed, with MDI conjugated to both the SH and the “amino terminus” of GSH. It remains unclear if these novel cyclized structures result from direct MDI reactivity or via secondary reactions of mono(GSH)-MDI* and bis(GSH)-MDI.

Multiple exposure variables were shown to influence the formation and stability of GSH-MDI reaction products, and their subsequent transfer of MDI to albumin, including GSH concentration, pH, and ionic composition of the reaction buffer. At the occupationally relevant MDI concentration tested (e.g. 0.1% w/v), the dose-response relationship (between starting GSH concentration and ultimate MDI-albumin antigenicity) was non-linear, increasing with GSH concentration to a maximum point, and then decreasing at higher GSH levels. Notably, under isotonic conditions (e.g. PBS), formation of antigenic MDI-albumin occurred at GSH concentrations within the range (100 μM - 1 mM) found in the lower airway fluid [33, 34]. The pH was also an important factor in GSH-mediated transcarbamoylation of albumin, consistent with classic descriptions of thiol-cyanate linkages [40], and the previously described pH-sensitivity of S-linked MDI-cysteine, TDI-GSH, and other monoisocyanate-GSH conjugates [11, 41, 51]. The effect of pH, as well as NaCl, on GSH carbamoylation of human albumin is intriguing with respect to the differences in composition of exposure sites vs. internal tissues, e.g. airway fluid, (intra)cellular, skin, plasma [55]. Together, the data suggest potential mechanisms by which individual variability in airway fluid GSH concentration, ionic composition, and pH, could influence the immune response to isocyanate exposure, by affecting the formation of antigenic MDI-albumin conjugates.

MDI-albumin conjugates formed via GSH were found to possess important similarities and differences compared to MDI-albumin resulting from direct MDI exposure. GSH-MDI and direct MDI exposure both, induced changes in albumin's electrophoretic migration and targeted specific lysine residues, resulting in covalent intra-molecular cross-linking, and conjugation with partially hydrolyzed MDI. However, MDI-albumin formed via GSH also contained a unique modification apparently resulting from a cyclized GSH-MDI intermediate, in which MDI effectively cross links GSH to albumin via GSH’s γ-glutamine. Further studies will be necessary to determine if/how the unique structural modification with MDI-γ-glu-cys-gly affects albumin's antigenicity compared with MDI cross-linking or conjugation with partially hydrolyzed MDI.

During the course of the present investigation we found that proteins other than human albumin (e.g. transferrin, thioredoxin) also underwent MDI conjugation via GSH, but were
not then recognized by serum IgG from exposed workers. These data are consistent with previously published MDI serology, demonstrating the importance of albumin as a “carrier protein” for humoral immune recognition of isocyanate [9, 14]. However, the data also suggest possible mechanisms by which GSH-mediated carbamoylation with MDI could contribute to pathologic immune responses without necessarily evoking chemical-specific immunoglobulin, for example, by altering the functional activity of local airway proteins. Cell membrane proteins, especially those that normally metabolize GSH-conjugates, might be especially sensitive targets for MDI transcarbamoylation, and their functional modulation could evoke inflammation secondary to redox signaling [36].

Limitations of the present study design should be recognized in considering the possible significance of the data, with regard to MDI exposure in vivo. Attention was focused on the potential influence of “biological” variation (e.g. GSH concentration, pH) given a defined occupationally-relevant exposure dose (e.g. 0.1% w/v MDI), rather than variation in exposure dose. The impact of MDI dose on GSH reactivity and carbamoylating capacity will require further study. The lability of GSH-MDI reaction products, including their susceptibility to hydrolysis and potential secondary reactions, limited quantitative analysis of reaction kinetics and complicated product identification and characterization. Measurement of GSH-mediated, MDI-transfer to albumin, and the different types of lysine modification (e.g. cross-linking, partially hydrolyzed MDI, MDI-γ-glu-cys-gly) were also quantitatively limited, with analysis focused more towards qualitative documentation of antigenicity and associated changes in conformational/charge. One of the major GSH-MDI reaction products previously described, bis(GSH)-MDI, reportedly possesses limited solubility in aqueous solution, which may account for some of the differences in the present data compared with previous studies [45, 46].

The influence of GSH concentration on MDI carbamoylation, as described here, warrants further technical consideration, given clinical reports suggesting therapeutic potential of glutathione supplementation for multiple medical conditions [56-59]. The present findings, that very high GSH levels (Figure 5B and data not show) resulted in decreased amounts of antigenic MDI-albumin formation, could be due to the influence of GSH on the pH of the in vitro reaction. At concentrations above 1 mM, GSH begins to override the buffering capacity of PBS, decreasing the pH, and thus, stabilizing S-linked GSH-isocyanate bonds (i.e. preventing carbamoylation). Furthermore, excessive amounts of unreacted GSH may act as a competitive inhibitor of albumin carbamoylation. Ultimately, in vivo studies should help clarify the potential effect of GSH concentration on MDI reactivity, which may represent a mechanism for modifying biological responses to occupational exposure.

In summary, the present findings describe a non-enzymatic, thiol-mediated transcarbamoylating process through which GSH can mediate the formation of MDI-albumin conjugates under physiologic conditions (neutral pH, isotonic saline). MDI-albumin conjugates, generated via GSH-mediated transcarbamoylation exhibit distinct changes in conformation/charge compared with unexposed albumin, and possess unique structures (e.g. addition of MDI-GSH, as shown in Fig 3C) compared with albumin directly reacted with MDI. Perhaps most importantly, MDI-albumin conjugates generated via GSH-mediated transcarbamoylation are specifically recognized by serum IgG of exposed workers.
Together, the data define a possible mechanistic role for GSH as a “shuttle” for MDI, leading to the formation of antigenic MDI-albumin reaction products, and define specific variables that may modulate this process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

GSH reduced glutathione
GSSG oxidized glutathione
HDI hexamethylene diisocyanate
MDI methylene diphenyl diisocyanate
MDI* partially hydrolyzed methylene diphenyl diisocyanate
TDI toluene diisocyanate
Highlights

Interaction of GSH with an important occupational allergen (MDI) is investigated

GSH-MDI reaction products carbamoylate human albumin, altering conformation/charge

GSH mediated transcarbamoylation of human albumin (with MDI) causes antigenic changes

The data define a potential mechanistic role for GSH in MDI asthma pathogenesis
Figure 1. LC-MS analysis of GSH-MDI reaction products
Ten millimolar GSH was reacted with 0.1% MDI in phosphate buffer without NaCl for 2 hrs, microfuged and 0.2 μM filtered before LC-MS. Panel A shows $m/z$ range 200-1200, while panels B, C and D highlight more limited regions. Arrows highlight major products described further in Table 1 and Figure 2 including the previously described mono(GSH)-MDI and bis(GSH)-MDI, indicated with an * in Panel A.
Figure 2. Proposed chemical structures for GSH-MDI reaction products
The structures of some of the reaction products identified in Figure 1/Table 1 are predicted based on their exact mass.
Figure 3. Expected modifications via GSH-MDI

Addition to albumin (via the isocyanate group shown on the left side), of the structures shown, should increase the mass (by the values shown) of peptides resulting from trypsin digestion. Note additional (non-isocyanate) modification in sub-panel C, which may occur during sample processing (e.g. acetylation as circled).
Figure 4. Changes in albumin's charge/conformation and antigenicity after carbamoylation by GSH-MDI

Panel A. Total protein stain of native gels analyzing human albumin carbamoylated by GSH-MDI reaction products prepared with 1 mM GSH/PBS (lane 1), 10 mM GSH/phosphate w/out NaCl (lane 2), or directly reacted with 0.1% MDI (lane 3). Lanes 4 to 6 contain control albumin samples, mock exposed or co-incubated with control GSH or MDI samples (see Materials and Methods for more information). Panel B. Parallel Western blot probed with pooled serum from (N=3) MDI exposed workers. No binding was observed with sera from unexposed subjects (not shown). Arrows highlight differences in electrophoretic migration, which reflect changes in charge and/or conformation.
Figure 5. Antigenicity of albumin carbamoylated by GSH-MDI

Human albumin carbamoylated by GSH-MDI, or control albumin, were used to coat ELISA plates for studies with human sera. The ELISA optical density (O.D.) readings (Y-axis), which reflect IgG binding, provide a measure of antigenicity. Panel A. Serum (1:100) IgG binding to MDI-albumin, resulting from transcarbamoylation via 1 mM GSH/PBS, is compared for (N=12) unexposed subjects vs. (N=3) MDI exposed workers. Panel B. Albumin carbamoylated by GSH-MDI prepared with varying levels of GSH starting concentration (X-axis) in different buffers (as labeled) was tested for antigenicity using pooled sera (1:100 dilution) from MDI exposed workers. Dashed line shows highest O.D. values observed with control serum from unexposed subjects and * indicates significantly (p < 0.05) increased O.D. vs. pooled sera from unexposed subjects (not shown). Panel C. GSH-mediated carbamoylation of human albumin was performed at different pH levels (X-axis), and subsequently tested for antigenicity with individual serum samples. Panel D. Different proteins, including human albumin (alb), transferrin (trans), thioredoxin (thio), or ovalbumin (ova) were carbamoylated by 1 mM GSH/PBS reaction products, and subsequently tested by ELISA using pooled sera from MDI exposed workers, or anti-MDI monoclonal antibodies.
Table 1
List of GSH-MDI reaction products observed by LC-MS

<table>
<thead>
<tr>
<th>mass</th>
<th>charge</th>
<th>m/z</th>
<th>Predicted Product</th>
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</thead>
<tbody>
<tr>
<td>483.1341</td>
<td>1+</td>
<td>483.1341</td>
<td>GSH-MDI (cy)&lt;sup&gt;b&lt;/sup&gt; without glycine&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>532.1872</td>
<td>1+</td>
<td>532.1872</td>
<td>GSH-MDI&lt;sup&gt;a&lt;/sup&gt;* aka mono(GSH)-MDI</td>
</tr>
<tr>
<td>558.1667</td>
<td>1+</td>
<td>558.1667</td>
<td>GSH-MDI (cy)</td>
</tr>
<tr>
<td>378.6449</td>
<td>2+</td>
<td>756.2831</td>
<td>GSH-MDI&lt;sup&gt;<em>&lt;/sup&gt;-MDI&lt;sup&gt;</em>&lt;/sup&gt; or <em>MDI-GSH-MDI&lt;sup&gt;</em>&lt;/sup&gt;</td>
</tr>
<tr>
<td>756.2831</td>
<td>1+</td>
<td>756.2831</td>
<td><em>MDI-GSH-MDI&lt;sup&gt;</em>&lt;/sup&gt;</td>
</tr>
<tr>
<td>782.2619</td>
<td>1+</td>
<td>782.2619</td>
<td>GSH-MDI&lt;sup&gt;*&lt;/sup&gt;-MDI (cy)</td>
</tr>
<tr>
<td>865.2502</td>
<td>1+</td>
<td>865.2502</td>
<td>GSH-MDI-GSH&lt;sup&gt;a&lt;/sup&gt; aka bis(GSH)-MDI</td>
</tr>
<tr>
<td>507.6608</td>
<td>2+</td>
<td>1014.3143</td>
<td>GSH-MDI-GSH-MDI&lt;sup&gt;*&lt;/sup&gt; without glycine</td>
</tr>
<tr>
<td>1014.3143</td>
<td>1+</td>
<td>1014.3143</td>
<td>GSH-MDI-GSH-MDI&lt;sup&gt;*&lt;/sup&gt; without glycine</td>
</tr>
<tr>
<td>1040.2947</td>
<td>1+</td>
<td>1040.2947</td>
<td>GSH-MDI-GSH-MDI without glycine</td>
</tr>
<tr>
<td>545.1770</td>
<td>2+</td>
<td>1089.3540</td>
<td>GSH-MDI-GSH-MDI&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>1089.3458</td>
<td>1+</td>
<td>1089.3458</td>
<td>GSH-MDI-GSH-MDI&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>1115.3243</td>
<td>1+</td>
<td>1115.3243</td>
<td>GSH-MDI-GSH-MDI (cy)</td>
</tr>
<tr>
<td>657.2248</td>
<td>2+</td>
<td>1314.1124</td>
<td>GSH-MDI-GSH-MDI&lt;sup&gt;-&lt;/sup&gt;-MDI (cy)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Indicates N=C=O group hydrolyzed to NH<sub>2</sub>

<sup>b</sup> (cy) denotes possible cyclic structure as shown in Figure 2

<sup>c</sup> Products without glycine may be contaminant of GSH, but can also fragment from parent compound, as observed in MS/MS spectra (not shown).
### Table 2

Sites and types of human albumin modification by GSH-MDI

<table>
<thead>
<tr>
<th>Exposure Conditions [GSH], buffer</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-MDI* (Δ = 224mu)</td>
</tr>
<tr>
<td>1 mM GSH PBS</td>
<td>K137, K351, K414, K525, K541</td>
</tr>
<tr>
<td>10 mM GSH H$_2$PO$_4$/HPO$_4^{2-}$</td>
<td>K136, K137, K351, K190, K199, K414, K525, K541</td>
</tr>
</tbody>
</table>
Table 3

Basic Demographics & Exposure Information of Study Subjects

<table>
<thead>
<tr>
<th></th>
<th>MDI Exposed Workers</th>
<th>Unexposed Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (N)</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Gender: (M/F)</td>
<td>3/0</td>
<td>10/2</td>
</tr>
<tr>
<td>Age (avg. ± SE)</td>
<td>41 ± 9</td>
<td>37 ± 11</td>
</tr>
<tr>
<td>Total IgE (avg. ± SE)</td>
<td>56 ± 41</td>
<td>94 ± 138</td>
</tr>
<tr>
<td>Current smoker (Y/N)</td>
<td>1/2</td>
<td>4/8</td>
</tr>
<tr>
<td>Occupational MDI use*</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>MDI skin contact**</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Years MDI Exposure***</td>
<td>3.1 ± 1.7</td>
<td>-</td>
</tr>
</tbody>
</table>

* Occupational MDI use according to questionnaire data. (+) = yes, (-) = no in response to “Do you spray polyurethane insulation? > 4hrs/day, >3 days/week, >6 months.”

** MDI skin contact according to questionnaire data (+++ = frequently, (-) = never in response to “Do you get isocyanate product on your skin?”

*** Years MDI exposure according to questionnaire data. “Total number of years spraying polyurethane foam insulation.” (-) = none