

Glutathione protects human airway proteins and epithelial cells from isocyanates

A. V. Wisnewski, Q. Liu, J. Liu and C. A. Redlich

Department of Internal Medicine, Yale School of Medicine, New Haven, CT, USA

Summary

Background Glutathione (GSH), one of the major anti-oxidants of the lung, has been linked to the human response to isocyanate exposure. However, the ability of GSH to modulate key chemical reactions, thought to be central to the development of human isocyanate allergy, has not been directly analyzed under biologic exposure conditions.

Objective To better understand the potential role of GSH in the response to occupational isocyanate exposure, we evaluated its effects on two processes thought to be involved in the development of isocyanate allergy, isocyanate–protein conjugation and epithelial cell toxicity.

Methods The effects of GSH on (1) isocyanate conjugation with albumin, its major target in the airway fluid and (2) isocyanate-induced toxicity to human airway epithelial cell lines, A549 and NCI-H292, were tested using two different *in vitro* models. For protein conjugation studies, a newly described vapour exposure system was used to model the air/liquid interface at the surface of the epithelial fluid in the airways. Epithelial cell exposures were performed in fluid phase to mimic the *in vivo* exposure of airway cells covered by epithelial lining fluid.

Results Reduced GSH prevented hexamethylene diisocyanate (HDI) conjugation to albumin in a dose-dependent manner, while oxidized GSH (GSSG) conversely increased conjugation rates. GSH levels equivalent to those found in normal human airway fluid (100 µM) provided >90% protection against HDI–protein conjugation when albumin was exposed to HDI vapour levels 10-fold above permissible occupational limits. Physiologic levels of GSH, but not GSSG, also reduced HDI toxicity to human airway epithelial cells in a dose-dependent manner, when present extracellularly, however, drugs that modulate intra-cellular GSH levels did not significantly alter isocyanate toxicity.

Conclusions Together with previously reported genetic and toxicity studies, the data suggest that airway GSH plays an important role in protection against HDI exposure and may help prevent the development of allergic sensitization and asthma.

Keywords airway epithelial cell, albumin, conjugation, glutathione, GSH, GSSG, HDI, hexamethylene diisocyanate, oxidized, reduced, toxicity

Submitted 29 June 2004; revised 20 October 2004; accepted 10 December 2004

Introduction

Isocyanates are low molecular weight chemicals used in commercial production of polyurethane and are a leading cause of occupational asthma world-wide [1, 2]. Industrially common isocyanates include hexamethylene diisocyanate (HDI), toluene diisocyanate (TDI) and diphenylmethane diisocyanate (MDI), each of which possess two reactive N = C = O groups. In the absence of appropriate respiratory tract protection, up to 20% of exposed individuals may develop immunologic sensitivity and/or occupational asthma; however, the majority of workers appear to be 'resistant' to the chemical [1, 3, 4]. A better understanding of the natural biologic mechanism(s) that protects against the development

of isocyanate asthma may lead to novel strategies for disease prevention, diagnosis and treatment.

The reactivity of isocyanate with proteins and cells along the epithelial layer of the respiratory tract is thought to be a key step in the pathogenic asthmatic response to exposure [5–8]. Physiologic conditions in human airways favour spontaneous nucleophilic addition reactions between N = C = O and NH₂, OH and SH functional groups of certain amino acids [9]. Isocyanate conjugated albumin and keratin have been identified *in vivo* in the airway fluid and tissue of exposed human subjects, and have been suggested to represent the major antigens that trigger airway inflammation [8, 10]. Glutathione (GSH), a major airway anti-oxidant, has also been shown to react with isocyanate *in vivo* in mice, and to transfer isocyanate to an major histocompatibility complex-binding murine peptide *in vitro* [11, 12]. Recently, associations between certain human GSH-S-transferase polymorphisms and isocyanate asthma have also been described [13, 14]. Together, these findings have suggested a potentially

Correspondence: Adam V. Wisnewski, Yale School of Medicine, 1 Gilbert Street; TAC-S415, New Haven, CT 06510, USA.

E-mail: adam.wisnewski@yale.edu

important role for GSH in the human response to isocyanate exposure, including the possibility that GSH may promote the development of allergic chemical sensitization [15, 16].

As one of the major anti-oxidants in the airway, GSH participates in a variety of essential biochemical reactions, as a co-enzyme, as part of the γ -glutamyl cycle, and in the detoxification of xenobiotics [17]. GSH levels of human airway fluid are quite high, 100-fold greater than those of peripheral blood or other bodily fluids, presumably reflective of the high oxidative burden at this epithelial surface [18, 19]. Individual variations in airway GSH concentrations can be attributed to both environmental (e.g. smoke exposure) and genetic factors (e.g. DNA mutations in chloride channels), which influence a complex, highly regulated feedback-system of homeostasis [18, 20]. The level of GSH and the balance between reduced and oxidized forms may be significantly altered in certain diseases and is thought to modulate a variety of lung inflammatory responses via pathways triggered by oxidative stress [21–27].

In this study, we investigate the potential role of GSH in the human response to isocyanate exposure by evaluating its effects on two putatively key steps in isocyanate asthma pathogenesis, isocyanate–protein conjugation and epithelial cell toxicity. Our results define the previously unappreciated potential for reduced, but not oxidized, GSH to protect against both, protein conjugation and cytotoxicity, and suggest that airway GSH homeostasis may be important in preventing isocyanate allergy. This novel hypothesis is discussed along with potential theoretical and practical implications.

Materials and methods

Vapour exposure system

To model the reactivity of HDI vapours in the airway space with proteins in the epithelial fluid lining the airways, we employed the mixed phase (vapour/liquid) exposure system recently developed in our laboratory [28]. Solutions of endotoxin-free human albumin, and reduced or oxidized GSH (Sigma, St Louis, MO, USA) were dissolved in phosphate-buffered saline (PBS), pH 7.4. Albumin was tested at 2.5 mg/mL, a concentration believed to represent that found in human airway epithelial lining fluid [29], and GSH concentrations were based on molar equivalents of monomeric GSH, i.e. 1 mol oxidized GSH (GSSG) = 2 mol GSH, and varied as indicated. Exposure conditions involved passive generation of HDI vapours in a closed circuit system, monitored in real-time with an Autostep toxic gas monitor (GMD Systems, Pittsburgh, PA, USA). Solutions of albumin, GSH, GSSG or mixtures were exposed to >0.2 parts per million (ppm) vapours of HDI for variable time periods in 35 mm diameter polystyrene Petri dishes in a 3 mL volume. All solutions were filter sterilized (0.2 μ m) and the exposure unit was sterilized with 70% ethanol.

Western blot analysis of hexamethylene diisocyanate conjugation to albumin

Conjugation of HDI to albumin was evaluated by Western blotting with a previously described HDI-specific polyclonal antiserum, and quantitated by densitometric scanning [10, 30,

31]. The antiserum was raised in rabbits by immunization with HDI–KLH, exhibits specificity for HDI when bound to a carrier protein, and has been depleted of KLH binding activity by affinity chromatography. Established blotting procedures [32] were followed except that samples were not heated prior to SDS-PAGE, to avoid heat-induced aggregation. Nitrocellulose blots were probed with processed antiserum diluted 1:200, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:1000 (Pharmingen San Diego, CA, USA), and developed by chemiluminescence using SuperSignal[®] West Femto Maximum Sensitivity Substrate by Pierce (Rockford, IL, USA). No binding of the HDI antiserum to unexposed albumin was noted and equal protein loading levels/lane were verified in parallel coomassie gels (not shown). All experiments were performed six times.

Cell toxicity studies

Human airway epithelial cell lines, A549 and NCI-H292, were obtained from the ATCC (Rockville, MD, USA) and cultured in RPMI 1640 media supplemented with 10% FBS and gentamicin (Gibco BRL, Gainsburgh, MD, USA). Cell lines (>90% confluency) were exposed in T75 flasks (Becton Dickinson; Franklin Lakes, NJ, USA) to varying doses of HDI (diluted in acetone) for 20 min at room temperature, as previously described [30, 31]. HDI concentrations in cell culture studies are expressed as molar equivalents of reactive isocyanate groups (i.e. 1 mol of HDI = 2 mol of reactive isocyanate groups). After HDI exposure, cells were washed once, and grown for an additional 18–24 h in 5 mL of complete media, in some experiments supplemented with 2 μ Ci/mL ³H-thymidine. Cell survival was quantitated based on ³H-thymidine uptake and trypan blue exclusion. Extracellular GSH and GSSGs effects on HDI toxicity were tested by adding varying concentrations of GSH or GSSG to the cultures immediately prior to HDI exposure. The effects of intracellular GSH levels were tested on cultures pre-incubated, overnight with 10 mM buthionine sulfoximine (BSO) to deplete GSH, or 2 h with 2 mM GSH monoethyl ester to raise GSH, as previously described [33, 34]. Intracellular GSH levels were determined based on the method of Tietze [35], which verified an 80% decrease in GSH levels with BSO and a two-fold increase with GSH monoethyl ester, as previously reported [33, 34]. Isocyanate conjugation to airway epithelial cell proteins was evaluated by Western blot analysis as described above for studies with albumin.

Statistical analyses

Statistical significance was determined by using a Student's *t*-test or by means of analysis of covariance when data from more than one experiment were pooled.

Results

Glutathione protects human proteins from isocyanate conjugation

We used a recently described mixed phase (vapour/liquid) *in vitro* exposure system to model the air/liquid interface at the

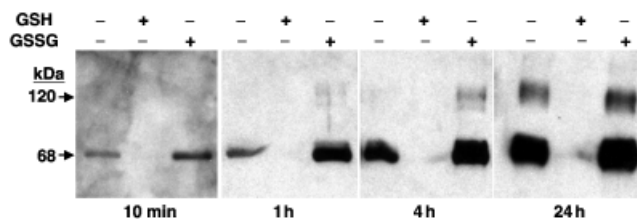


Fig. 1. Glutathione (GSH) protects albumin from hexamethylene diisocyanate (HDI) conjugation. Human albumin was exposed to ≥ 0.2 ppm HDI vapours in the absence or presence of GSH or oxidized GSH (GSSG) for the time periods indicated. HDI conjugation was detected by Western blot with HDI-specific polyclonal antiserum. The Western blot shown is representative of six different experiments. The ECL detection step for the 10 min exposure was 10-fold longer than the other time points.

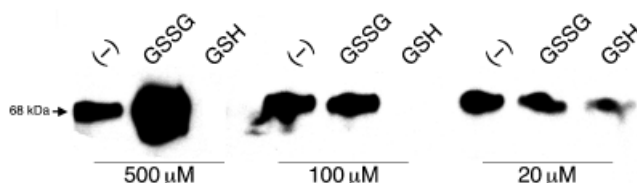


Fig. 2. Physiologic levels of glutathione (GSH) protect albumin against occupational levels of hexamethylene diisocyanate (HDI) vapours. Human albumin was exposed to ≥ 0.2 ppm HDI vapours for 2 h in the absence (–) or presence of oxidized GSH (GSSG) or GSH, and Western blotted with HDI-specific antiserum. Three concentrations of GSH were tested as shown (bottom). The Western blot shown is representative of six different experiments.

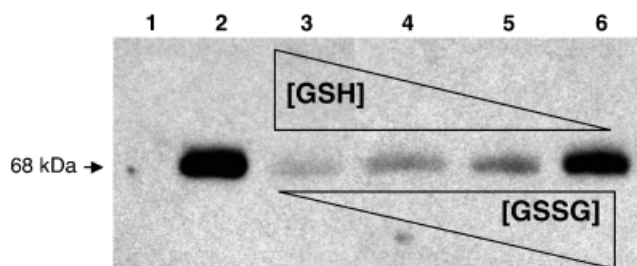


Fig. 3. Dose-dependent protection of albumin against hexamethylene diisocyanate (HDI) conjugation by glutathione (GSH). Albumin was exposed to ≥ 0.2 ppm HDI vapours in the presence of 100 μ M total GSH, where the ratio of reduced:oxidized GSH (GSH:GSSG) was varied from 90:10 (lane 3), 50:50 (lane 4), 25:75 (lane 5) or 10:90 (lane 6). Control lanes are shown without GSH (lane 2) or without HDI exposure (lane 1). The Western blot shown is representative of six different experiments.

surface of the airway epithelial lining fluid [28]. Human albumin in liquid phase was exposed to ≥ 0.2 ppm HDI vapour in the presence or absence of GSH or GSSG, and HDI-conjugation to albumin was detected by Western blot with HDI-specific antiserum as previously described [10, 30, 31]. In the absence of GSH or GSSG, detectable HDI conjugation to human albumin occurs within 10 min, and increases in a time-dependent manner, as evidenced by increased intensity of HDI specific staining (Fig. 1). The majority of HDI conjugated-albumin exists in the 68 kDa monomeric form, while a minor fraction can be found at ~ 120 kDa, which likely reflects dimerized conjugates, consistent with previous studies [28, 36]. Importantly, in the presence of 5 mM GSH, HDI conjugation to albumin was inhibited to levels below detection. In contrast, equivalent

concentrations of GSSG increased HDI conjugation, especially during short exposure times (10 min–4 h).

We further defined the protective capacity of physiologic levels of GSH, against isocyanate protein conjugation, through exposure studies with titrated doses of reduced and oxidized GSH. In the presence of 500, 100 or 20 μ M GSH, HDI vapour conjugation to albumin was reduced $>99\%$, $>98\%$ and $>64\%$, respectively, when analyzed by anti-HDI Western blot (Fig. 2), followed by densitometry (not shown). Oxidized GSH conversely increased HDI conjugation to albumin when present at 0.5 and 5 mM, but had no statistically significant effect at concentrations of 100 or 20 μ M (Figs 1 and 2). The dose-dependent protective effects of reduced GSH were not antagonized by oxidized GSH when the total GSH concentration was fixed at 100 μ M and the ratio of GSH:GSSG was varied (Fig. 3).

Glutathione protects human airway epithelial cells from hexamethylene diisocyanate-induced toxicity

We also tested the ability of GSH to protect human airway epithelial cell lines, A549 and NCI-H292, from the toxic effects of HDI exposure. Initially, cells were exposed to the lowest dose of reactive isocyanate, 400 μ M, that kills $>95\%$ of cells within 24 h of exposure, based on previous experiments [10, 30, 31]. At 400 μ M isocyanate, an equimolar concentration of GSH, but not GSSG, prevented cell death $>90\%$ and when present extracellularly during exposure (Fig. 4). Under these conditions, HDI conjugation to epithelial cell proteins was also inhibited (Fig. 4, panel E). In further studies, the dose dependency of GSH-mediated protection from isocyanate toxicity was evaluated. Physiologic concentrations of GSH present in normal human airway epithelial lining fluid (100 and 500 μ M) were shown to significantly protect A549 cells against HDI-induced cell death and shift the toxicity curve to the right (Fig. 5). The level of GSH protection from toxicity was largely dependent upon the HDI exposure dose. At 100 μ M GSH, the maximum level of protection from HDI was 56%, when equimolar concentrations of reactive isocyanate were present, but protection levels were less than 20% with a 2:1 M excess of

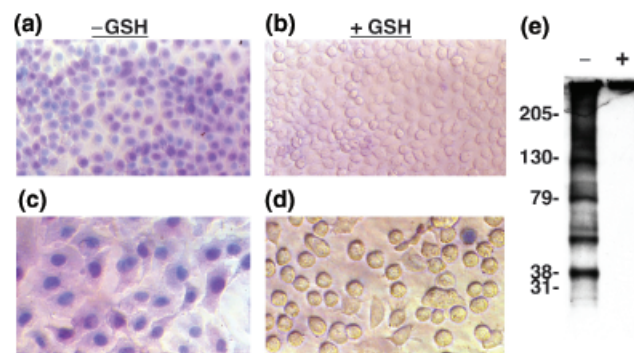


Fig. 4. Glutathione (GSH) protects human airway epithelial cells from hexamethylene diisocyanate (HDI) toxicity. Human airway epithelial cell lines, NCI-H292 (top) and A549 (bottom), were exposed for 20 min to 400 μ M isocyanate, a toxic concentration that cross-links the cells to the tissue culture flasks [27]. Micrographs of trypan blue stained cells were taken 18 h after exposure for control cultures (a and c) or cultures exposed in the presence of 400 μ M GSH (b and d). Panel e shows an HDI-specific Western blot of proteins from A549 cells immediately after exposure to HDI in the absence (–) or presence (+) of GSH.

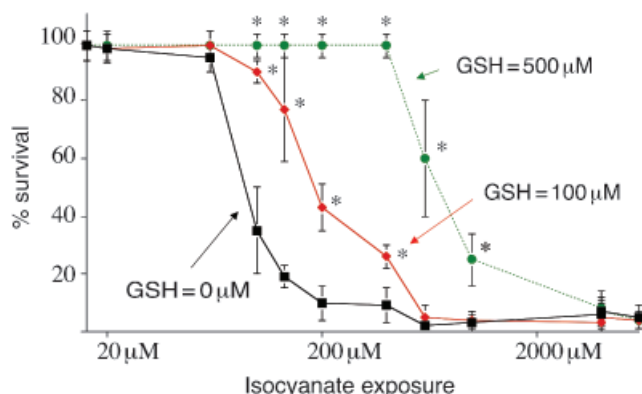


Fig. 5. Glutathione (GSH) shifts the toxicity curve of hexamethylene diisocyanate (HDI) for human airway epithelial cells. A549 cells were exposed for 20 min to varying doses of isocyanate (μM expressed on the x-axis) in the absence (black squares) or presence of 100 μM (red diamonds) or 500 μM (green circles) GSH. The percentage of cell survival (y-axis) was based on ^3H -thymidine uptake in the 18 h post exposure period. Data shown are the mean and standard error of six experiments. Similar findings, and statistically significant differences between GSH treated and control cultures, were observed with NCI-H292 cells. * $P \leq 0.05$ compared with control cultures exposed without GSH.

isocyanate. At 500 μM GSH, cells were >90% resistant to cytotoxicity induced by isocyanate exposure doses up to 500 μM . In contrast, neither equivalent concentrations of extra-cellular GSSG, nor modulation of intra-cellular GSH, with BSO or GSH monoethyl esters, had detectable effects on HDI toxicity (data not shown). Together these *in vitro* cellular studies demonstrate the protective capacity of GSH against HDI cytotoxicity. While the mechanism of this process remains unclear, the difference in the activity of extra- and intra-cellular GSH suggests protection of extra-cellular protein domains against HDI conjugation may be important in preventing HDI-induced toxicity.

Discussion

The capacity of reduced GSH to protect human proteins from isocyanate conjugation and human airway epithelial cells from isocyanate toxicity is described for the first time in this report. Physiologic levels of GSH protected human albumin against occupationally relevant concentrations of HDI vapours, and significantly reduced A549 and NCI-H292 cell toxicity *in vitro*. Together, the data suggest that airway GSH may play an essential role in natural protection against occupational isocyanate exposure, a novel concept that may impact preventive, diagnostic and therapeutic intervention strategies.

The present data extend recent animal toxicology and *in vitro* human cellular studies, which also suggest a potential role for GSH in the physiologic response to isocyanate exposure. One of the first effects of respiratory tract isocyanate exposure that can be measured *in vivo* in animals is increased GSH levels in the fluid lining the airways. This increase in extracellular GSH occurs in conjunction with decreased GSH in the airway tissue, an exposure effect also observed in human airway epithelial cells *in vitro* [12, 16, 37]. Depletion of total GSH levels in rats *in vivo* increases acute respiratory tract toxicity of isocyanates [38]. With chronic

exposure, the ability to adequately maintain GSH homeostasis may be crucial in determining outcomes, given the ability of GSH to modulate redox homeostasis and inflammatory signalling intracellularly [23, 39–43], as well as protect against isocyanate exposure, as demonstrated here.

The finding that GSH can protect against HDI protein conjugation and cytotoxicity is also intriguing in the context of recent genetic studies linking isocyanate asthma with polymorphisms in GSH S-transferase enzymes, which utilize GSH as an essential co-factor [13, 14]. Null genotypes of the GST-M1 class of enzymes confer a two-fold risk for isocyanate asthma, while homozygosity for a common GST-P1 allele, is associated with protection from isocyanate asthma. The mechanism(s) by which these polymorphisms effect sensitivity to isocyanates remains unclear, but based on the present findings may involve GSTs co-factor usage of GSH.

The protective effect of GSH against isocyanate-induced protein modification and cellular toxicity is similar to that reported for other chemicals and oxidants, such as hypochlorous acid and hydrogen peroxide [18, 44]. For isocyanates, the chemical mechanism(s) by which this process might occur remains uncertain, but is likely related to the scavenging ability of GSH. Previous reports suggest that GSH undergoes nucleophilic addition reactions with isocyanate, via its free thiol, but that this reaction is unstable [11, 45] and subsequently the isocyanate moiety may be released. We hypothesize that in the present exposure model, as well as the physiologic microenvironment, this process may result in hydrolysis of the isocyanate moieties to amines, or result in polyureas as shown *in vitro* for monomeric isocyanate [11, 45]. An equally likely, alternative explanation may be that GSH stabilizes proteins, or a particular conformation (i.e. glutathionylation) that is resistant to isocyanate conjugation under physiological exposure conditions [11, 45]. Further investigations will be necessary to determine the chemical mechanisms by which GSH mediates its protective effects against HDI.

If GSH protects against isocyanate exposure *in vivo* in human airways, as it does *in vitro*, then airway GSH levels may be an important determinant of susceptibility to isocyanate asthma. GSH levels in human airway fluid are challenging to quantitate, but limited data available to date suggest substantial variability in innate levels, as well as the ability to modulate GSH in response to environmental stimuli [18, 19]. Interestingly, augmentation of airway GSH levels, via inhaled GSH, has been proposed as an adjunct therapy to protect against oxidative stress and damage in pulmonary diseases, such as cystic fibrosis and idiopathic pulmonary fibrosis [20, 46]. Using inhaled, aerosolized GSH, it is possible to increase normal airway epithelial lining fluid concentrations ≥ 7 -fold in large animals [47]. In limited published clinical studies, inhaled GSH has been shown to reduce oxide release by airway cells, to undergo conversion to GSSG (presumably reflecting adequate delivery and usage) and to exhibit no clinically detectable toxicity [20, 46, 48, 49]. Together with these prior studies, the present findings support future clinical studies to evaluate the potential protective effects of GSH against isocyanate exposure *in vivo*.

In summary, we have shown that biologic levels of GSH, but not GSSG, protect human airway proteins and epithelial

cells against HDI conjugation and toxicity that can result from exposure to occupationally relevant doses. Together with previous studies linking isocyanates, GSH and GSTs, and the fact that GSH is one of the major anti-oxidants of airway fluid, the data suggest an important protective role for GSH against occupational isocyanate exposure. Future studies should determine if the current results extend to other commercially important isocyanates such as MDI and TDI, and if, GSH-mediated protection against isocyanates might modulate the development of asthma *in vivo* in humans.

Acknowledgements

We would like to thank Dr Zhou Zhu for helpful discussions. This work was supported by grants from the NIH (HL62622 and M01RR00125) and NIEHS (ES00355).

References

- Brugsch HG, Elkins HB. Toluene di-isocyanate (TDI) toxicity. *N Engl J Med* 1963; 268:353–7.
- Liu Q, Wisniewski AV. Recent developments in diisocyanate asthma. *Ann Allergy Asthma Immunol* 2003; 90:35–41.
- Swenson A, Holmquist CE, Lundgren KD. Injury to the respiratory tract by isocyanates used in making lacquers. *Br J Ind Med* 1955; 12:50–3.
- Pepys J, Pickering CA, Breslin AB, Terry DJ. Asthma due to inhaled chemical agents, tolylene di-isocyanate. *Clin Allergy* 1972; 2:225–36.
- Scheel LD, Killens R, Josephson A. Immunochemical Aspects of Toluene Diisocyanate (TDI) Toxicity. *Am Ind Hyg Assoc J* 1964; 25:179–84.
- Avery SB, Stetson DM, Pan PM, Mathews KP. Immunological investigation of individuals with toluene diisocyanate asthma. *Clin Exp Immunol* 1969; 4:585–96.
- Redlich CA, Karol MH. Diisocyanate asthma: clinical aspects and immunopathogenesis. *Int Immunopharmacol* 2002; 2:213–24.
- Wisniewski AV, Redlich CA. Recent developments in diisocyanate asthma. *Curr Opin Allergy Clin Immunol* 2001; 1:169–75.
- Tse CS, Pesce AJ. Chemical characterization of isocyanate-protein conjugates. *Toxicol Appl Pharmacol* 1979; 51:39–46.
- Wisniewski AV, Srivastava R, Herick C et al. Identification of human lung and skin proteins conjugated with hexamethylene diisocyanate in vitro and in vivo. *Am J Respir Crit Care Med* 2000; 162:2330–6.
- Day BW, Jin R, Basalyga DM, Kramarik JA, Karol MH. Formation, solvolysis, and transcarbamoylation reactions of bis(S-glutathionyl) adducts of 2,4- and 2,6-diisocyanatotoluene. *Chem Res Toxicol* 1997; 10:424–31.
- Lange RW, Day BW, Lemus R, Tyurin VA, Kagan VE, Karol MH. Intracellular S-glutathionyl adducts in murine lung and human bronchoepithelial cells after exposure to diisocyanatotoluene. *Chem Res Toxicol* 1999; 12:931–6.
- Mapp CE, Fryer AA, De Marzo N et al. Glutathione S-transferase GSTP1 is a susceptibility gene for occupational asthma induced by isocyanates. *J Allergy Clin Immunol* 2002; 109:867–72.
- Piirila P, Wikman H, Luukkonen R et al. Glutathione S-transferase genotypes and allergic responses to diisocyanate exposure. *Pharmacogenetics* 2001; 11:437–45.
- Karol MH. Bonding and transfer: do epithelial conjugates have a role in chemical asthma? *Clin Exp Allergy* 2001; 31:357–60.
- Lantz RC, Lemus R, Lange RW, Karol MH. Rapid reduction of intracellular glutathione in human bronchial epithelial cells exposed to occupational levels of toluene diisocyanate. *Toxicol Sci* 2001; 60:348–55.
- Meister A. Glutathione and the gamma-glutamyl cycle. In: Arias IM, Jakoby WB, eds. *Glutathione: Metabolism and Function*. New York: Raven Press, 1976; 35–41.
- Cantin AM, North SL, Hubbard RC, Crystal RG. Normal alveolar epithelial lining fluid contains high levels of glutathione. *J Appl Physiol* 1987; 63:152–7.
- van der Vliet A, O'Neill CA, Cross CE et al. Determination of low-molecular-mass antioxidant concentrations in human respiratory tract lining fluids. *Am J Physiol* 1999; 276:L289–96.
- Roum JH, Borok Z, McElvaney NG et al. Glutathione aerosol suppresses lung epithelial surface inflammatory cell-derived oxidants in cystic fibrosis. *J Appl Physiol* 1999; 87:438–43.
- Rahman I, van Schadewijk AA, Hiemstra PS et al. Localization of gamma-glutamylcysteine synthetase messenger rna expression in lungs of smokers and patients with chronic obstructive pulmonary disease. *Free Radical Biol Med* 2000; 28:920–5.
- Rahman I, Swarska E, Henry M, Stolk J, MacNee W. Is there any relationship between plasma antioxidant capacity and lung function in smokers and in patients with chronic obstructive pulmonary disease? *Thorax* 2000; 55:189–93.
- Rahman I, MacNee W. Oxidative stress and regulation of glutathione in lung inflammation. *Eur Respir J* 2000; 16:534–54.
- Smith LJ, Houston M, Anderson J. Increased levels of glutathione in bronchoalveolar lavage fluid from patients with asthma. *Am Rev Respir Dis* 1993; 147:1461–4.
- Bunnell E, Pacht ER. Oxidized glutathione is increased in the alveolar fluid of patients with the adult respiratory distress syndrome. *Am Rev Respir Dis* 1993; 148:1174–8.
- Pacht ER, Timerman AP, Lykens MG, Merola AJ. Deficiency of alveolar fluid glutathione in patients with sepsis and the adult respiratory distress syndrome. *Chest* 1991; 100:1397–403.
- Bowler RP, Crapo JD. Oxidative stress in allergic respiratory diseases. *J Allergy Clin Immunol* 2002; 110:349–56.
- Wisniewski AV, Cartier A, Liu Q et al. Isocyanate vapor-induced antigenicity of albumin. *J Allergy Clin Immunol* 2004; 113:1178–84.
- Vandenplas O, Delwiche JP, Staquet P et al. Pulmonary effects of short-term exposure to low levels of toluene diisocyanate in asymptomatic subjects. *Eur Respir J* 1999; 13:1144–50.
- Wisniewski AV, Lemus R, Karol MH, Redlich CA. Isocyanate-conjugated human lung epithelial cell proteins: a link between exposure and asthma? *J Allergy Clin Immunol* 1999; 104:341–7.
- Wisniewski AV, Liu Q, Miller JJ, Magoski N, Redlich CA. Effects of hexamethylene diisocyanate exposure on human airway epithelial cells: in vitro cellular and molecular studies. *Environ Health Perspect* 2002; 110:901–7.
- Coligan J, Kruisbeck A, Marguiles D, Sevacch E, Stober W, eds. *Current Protocols in Immunology*. West Sussex: Wiley and Sons, Inc., 1998.
- Kang YJ, Enger MD. Glutathione content and growth in A549 human lung carcinoma cells. *Exp Cell Res* 1990; 187:177–9.
- Rahman I, Mulier B, Gilmour PS et al. Oxidant-mediated lung epithelial cell tolerance: the role of intracellular glutathione and nuclear factor-kappa B. *Biochem Pharmacol* 2001; 62:787–94.
- Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 1969; 27:502–22.
- Karol MH, Hauth BA. Use of hexyl isocyanate antigen to detect antibodies to hexamethylene diisocyanate (HDI) in sensitized guinea pigs and in a sensitized worker. *Fund Appl Toxicol* 1982; 2:108–13.
- Pauluhn J. Inhalation toxicity of 1,6-hexamethylene diisocyanate homopolymer (HDI-IC) aerosol: results of single inhalation exposure studies. *Toxicol Sci* 2000; 58:173–81.

- 38 Pauluhn J. Acute inhalation toxicity of polymeric diphenylmethane 4,4'-diisocyanate in rats: time course of changes in bronchoalveolar lavage. *Arch Toxicol* 2000; 74:257–69.
- 39 Rahman I. Regulation of nuclear factor-kappa B, activator protein-1, and glutathione levels by tumor necrosis factor-alpha and dexamethasone in alveolar epithelial cells. *Biochem Pharmacol* 2000; 60:1041–9.
- 40 Rahman I, MacNee W. Regulation of redox glutathione levels and gene transcription in lung inflammation: therapeutic approaches. *Free Radical Biol Med* 2000; 28:1405–20.
- 41 Hashimoto S, Gon Y, Matsumoto K, Takeshita I, MacHino T, Horie T. Intracellular glutathione regulates tumour necrosis factor-alpha-induced p38 MAP kinase activation and RANTES production by human bronchial epithelial cells. *Clin Exp Allergy* 2001; 31:144–51.
- 42 Allen RG, Tresini M. Oxidative stress and gene regulation. *Free Radical Biol Med* 2000; 28:463–99.
- 43 Peterson JD, Herzenberg LA, Vasquez K, Waltenbaugh C. Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns. *Proc Natl Acad Sci USA* 1998; 95:3071–6.
- 44 Zhu BZ, Carr AC, Frei B. Pyrrolidine dithiocarbamate is a potent antioxidant against hypochlorous acid-induced protein damage. *FEBS Lett* 2002; 532:80–4.
- 45 Reisser M, Schmidt BF, Brown WE. Synthesis, characterization, and solvolysis of mono- and bis-S-(glutathionyl) adducts of methylene-bis-(phenylisocyanate) (MDI). *Chem Res Toxicol* 2002; 15:1235–41.
- 46 Borok Z, Buhl R, Grimes GJ et al. Effect of glutathione aerosol on oxidant-antioxidant imbalance in idiopathic pulmonary fibrosis. *Lancet* 1991; 338:215–6.
- 47 Buhl R, Vogelmeier C, Critenden M et al. Augmentation of glutathione in the fluid lining the epithelium of the lower respiratory tract by directly administering glutathione aerosol. *Proc Natl Acad Sci USA* 1990; 87:4063–7.
- 48 Holroyd KJ, Buhl R, Borok Z et al. Correction of glutathione deficiency in the lower respiratory tract of HIV seropositive individuals by glutathione aerosol treatment. *Thorax* 1993; 48: 985–9.
- 49 Marrades RM, Roca J, Barbera JA, de Jover L, MacNee W, Rodriguez-Roisin R. Nebulized glutathione induces bronchoconstriction in patients with mild asthma. *Am J Respir Crit Care Med* 1997; 156:425–30.