Glutathione Depletion Is a Major Determinant of Inhaled Naphthalene Respiratory Toxicity and Naphthalene Metabolism in Mice

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Naphthalene (NA) is metabolized to highly reactive intermediates that are primarily detoxified by conjugation to glutathione (GSH). Intraperitoneal administration of naphthalene causes substantial loss of both hepatic and respiratory GSH, yet only respiratory tissues are injured in mice. The liver supplies GSH to other organs via the circulation, making it unclear whether respiratory GSH losses reflect in situ respiratory depletion or decreased hepatic supply. To address this concern, mice were exposed to naphthalene by inhalation (1.5–15 ppm; 2–4 h), thereby bypassing first-pass hepatic involvement. GSH levels and histopathology were monitored during the first 24 h after exposure. Half of the mice were given the GSH depletor diethylmaleate (DEM) 1 hour before naphthalene exposure. Lung and nasal GSH levels rapidly decreased (50–90%) in mice exposed to 15 ppm naphthalene, with cell necrosis throughout the respiratory tract becoming evident several hours later. Conversely, 1.5 ppm naphthalene caused moderate GSH loss and only injured the nasal olfactory epithelium. Neither naphthalene concentration depleted hepatic GSH. Animals pretreated with DEM showed significant GSH loss and injury in nasal and intrapulmonary airway epithelium at both naphthalene concentrations. DEM treatment, perhaps by causing significant GSH loss, decreased water-soluble naphthalene metabolite formation by 48% yet increased NA-protein adducts 193%. We conclude that (1) GSH depletion occurs in airways independent of hepatic function; (2) sufficient GSH is not supplied by the liver to maintain respiratory GSH pools, or to prevent injury from inhaled naphthalene; and (3) GSH loss precedes injury and increases protein adduct formation.

Key Words: glutathione (GSH); respiratory toxicity; naphthalene; P450 metabolism; high-performance liquid chromatography; HPLC.

INTRODUCTION

Glutathione (GSH) loss has long been associated with the toxicity of reactive electrophilic chemicals. The respiratory toxicant naphthalene (NA) is metabolized by cytochrome P450 monoxygenase enzymes into reactive intermediates (e.g., epoxides) capable of depleting GSH and adducting cellular proteins (Buckpitt et al., 2002). NA selectively injures the non-ciliated airway epithelial cell (i.e., Clara cell) of the mouse lung and the nasal olfactory epithelium of most rodents, regardless of the route of exposure (Plopper et al., 1992; West et al., 2001). These cell types are rich in P450 enzymes, particularly the CYP2F2 isoform, and rapidly metabolize NA (Harkema, 1992; Plopper et al., 1997; Shultz et al., 1999). GSH loss, protein adduct formation, and epithelium injury, have been demonstrated to occur in a dose-dependent fashion in the lung (Warren et al., 1982), suggesting a link between NA metabolism, GSH loss, protein adduct formation, and NA cytotoxicity. However, these and other studies relied on intraperitoneal dosing (Buckpitt et al., 1992; O’Brien et al., 1985; Plopper et al., 2001; Warren et al., 1982), where injected NA must first pass through the liver. Since the lung turns over GSH rapidly, taking it up from the circulation (Martensson et al., 1989), and the liver, which exports GSH to other organs (Meister, 1981), loses a significant amount of GSH in response to intraperitoneal doses of NA, then pulmonary GSH losses might reflect decreased hepatic supply of GSH more than direct depletion within the respiratory tract. If true, then this would not support the idea that NA respiratory toxicity is dependent on GSH loss within the respiratory tract, i.e., these facts would be casually associated. Therefore, an approach was needed to determine if NA was causing GSH loss directly within respiratory tissues and if that loss was linked to toxicity.

We hypothesized that NA acts directly on respiratory tissues to cause the loss of GSH in animals exposed to NA. To test this hypothesis, we exposed mice to NA vapor, which when inhaled reaches the respiratory tract directly, avoiding first-pass hepatic metabolism. Following a single NA inhalation exposure (2–4 h duration), GSH levels and cytotoxicity were monitored 8–24 h post-exposure. In addition to measuring liver and lung GSH levels, we surgically isolated defined regions of the respiratory tract (i.e., intrapulmonary conducting airways and nasal ethmoid turbinates) by microdissection, for GSH analysis. The respiratory architecture is complex, with nearly 50 cellular phenotypes organized into intricate branching patterns (Harkema, 1992;...
GSH LOSS IN NAPHTHALENE RESPIRATORY TOXICITY

Plopper et al., 1998). Isolating intrapulmonary airways and olfactory epithelium allowed us to measure GSH levels within samples enriched for target cells, e.g., Clara cells represent 4% of the lung by mass but 50% of the airway epithelium (Plopper et al., 1997). We utilized two concentrations of NA vapor (1.5 and 15 ppm) anticipated to show different degrees of injury based on a prior inhalation dose-response study (West et al., 2001). These concentrations bracket the current human workplace exposure limit of 10 ppm for 8 h (OSHA, 2003).

Half of the mice in each treatment group were given the GSH depleting agent diethylmaleate (DEM) 1 hour prior to the start of NA exposure. DEM produces a rapid, yet transient, loss of GSH through enzymatic conjugation of DEM to GSH (Boyland and Chasseaud, 1967). With less GSH available, more of the reactive NA metabolites would persist within target cells, potentially increasing severity and onset of toxicity. Earlier studies had demonstrated that depletion of GSH with DEM can increase the severity of lung injury and increase protein adduct formation 6-fold in mice given intraperitoneal doses of NA (Warren et al., 1982). However, reactive NA metabolites formed in the liver are capable of reaching the lungs via the bloodstream (Buckpitt and Warren, 1983), raising the possibility that increased protein adducts reflect changes in hepatic metabolism and detoxification of NA. Inhalation delivers NA directly to the lung, minimizing the role of the liver as a source of circulating metabolites targeting the lung. In order to better understand how GSH loss affects NA metabolism and adduct formation within the respiratory tract, intrapulmonary airway segments were isolated from DEM treated animals by microdissection and then incubated with [14C]-labeled naphthalene in vitro.

Using NA inhalation and isolated airways, this study addressed the following questions: (1) does exposure to inhaled NA deplete respiratory tract GSH directly; (2) can the liver supply sufficient GSH to maintain respiratory GSH pools during inhaled toxicant exposure; and (3) does GSH loss increase NA covalent binding within airways exposed to NA in culture?

METHODS

Chemicals. Naphthalene (99%+) and diethylmaleate (98.4%) were purchased from Aldrich Chemicals (Milwaukee, WI). [14C]-naphthalene (50 mCi/mmol) obtained from American Radiolabeled Chemicals (St Louis, MO) as a 20 mM methanolic stock, was 99.8% pure by reverse phase HPLC. Waymouth MB 752/1 media was purchased from Sigma (St. Louis, MO) or Gibco (Grand Island, NY). Glycol methacrylate resin was obtained from Electron Microscopy Sciences (Fort Washington, PA). All other chemicals were reagent grade or better.

Animals and treatment. Outbred male NIH Swiss mice, aged 7–10 weeks, were purchased from Harlan Laboratories (San Diego, CA). Animals were allowed free access to food and water and were not fasted before treatments. They were housed in cages on sterile paper fiber bedding in HEPA-filtered isolation chambers at the University of California, Davis, for at least 5 days before use. Mice were exposed to filtered air (control) or naphthalene vapor in glass metabolism cages (BioServ, Inc., Frenchtown, NJ) as described previously (West et al., 2001). Air volume through the chamber was maintained at 1.0 liter min⁻¹ resulting in a complete change of air every 5 min. Samples of chamber air were extracted into acetonitrile (80% extraction efficiency), and the naphthalene chamber concentration was determined by absorbance at 219 nm (extinction coefficient of 8,896 M⁻¹ cm⁻¹). Exposures were conducted between 8:00 a.m. and noon to minimize diurnal fluctuations in GSH levels and lasted 2 or 4 h. Samples were collected for GSH analysis and histopathology immediately after exposure to naphthalene vapor or after allowing the mice to recover in fresh air for 4–24 h as indicated. Half of the mice were given the GSH depleting agent diethylmaleate (1 g/kg i.p. dose; 1.0 M solution in corn oil) 1 hour prior to starting exposure; a dose and time combination that would produce maximal GSH depletion in the lung and liver (unpublished results) before the naphthalene vapor exposure was started. An equivalent volume of corn oil was given to animals not treated with DEM (NA only animals), as a control for the DEM/corn oil treatment. Animals were euthanized with an overdose of pentobarbital sodium.

HPLC analysis of GSH. A previously described method (Lakritz et al., 1997) was employed for the quantitation of GSH in tissues. Briefly, acidic tissue homogenates were subjected to reverse-phase HPLC coupled with electrochemical detection to directly measure reduced GSH. The response was linear from 1.6 to 5200 nmol of GSH (R² = 0.995). GSH levels were measured in liver, lung, proximal (near the trachea) and distal (near the alveoli) intrapulmonary airways, and in nasal ethmoid turbinates. Intrapulmonary airways were isolated by microdissection procedures described in detail by Plopper et al. (1991) with the modification that the lungs were perfused free of blood with ice-cold 1 mM EDTA/saline while still in the chest. To isolate nasal tissue, the head was split along the nasal septum with Teflon (PTFE)-coated razor blades, and the ethmoid turbinates were physically removed under a dissecting microscope with forceps. At least 4 animals were evaluated at each time point for each treatment. GSH measurements were normalized to protein levels (Lowry et al., 1951).

High-resolution histopathology. A minimum of 3 mice were compared for each treatment at each time point. Lungs were inflated in situ via a tracheal cannula with 4% formaldehyde, buffered to pH 7 with phosphate at 30 cm water pressure. Nasal tissue was fixed by forcing fixative through the nose with a syringe via a cannula inserted into the nasopharynx followed by immersion in the fixative for at least 3 days. Blocks of fixed lung tissue (3–5 mm thick) were sampled in orientations both parallel and perpendicular to the main airway path. Careful sampling was necessary because inhaled toxicants do not produce uniform injury patterns, because of a combination of airflow dynamics (e.g., laminar vs turbulent) and the complex three-dimensional branching pattern of the airways, which causes localized differences in dose (Overtan et al., 1989; Plopper et al., 1998). The lateral side of the first airway generation of the left lobe and the terminal bronchioles were selected to represent the proximal and distal airways, respectively. These two regions were examined separately for injury (and for GSH levels) because studies have shown differential susceptibility of these regions to NA; and this may be due to phenotypic differences in the Clara cell populations in proximal compared to distal regions (Plopper et al., 1992; West et al., 2001). Careful sampling of nasal tissue was also required, and four sagittal sections were prepared along the length of the nose for embedding. Prior to sampling, the fixed nasal tissues were de-calciﬁed by immersion in 13% formic acid for 4 days (Eggett and Germain, 1979), followed by extensive rinsing with water. Areas of ethmoid turbinate with high amounts of olfactory epithelium (relative to respiratory epithelium) were chosen for imaging. All tissues were embedded in glycol methacrylate resin. Two-micron thick sections were stained with methylene blue/azure II, and fields were recorded at 256× magnification on an Olympus BX41 microscope (Olympus International, Melville, NY) equipped with a Q-imaging cooled CCD-camera (Burnaby, B.C., Canada).

Naphthalene metabolism. Mice were either treated with diethylmaleate (1 g/kg; 1.0 M corn oil solution) or an equivalent volume of corn oil. One hour later, animals were euthanized and intrapulmonary airways were isolated by microdissection. Following isolation, airways were allowed to recover in Waymouth’s media for 30 min in a shacking water bath at 37°C.2 ml Teflon (PTFE) microcentrifuge tubes (Upchurch Scientific, Oak Harbor, WA). At the end of the 30-min incubation a small portion of each sample was removed to measure tissue GSH levels as described above. To the remaining tissues, each in
500 μl of media, was added 1 μl of 20 mM [14C]-naphthalene in methanol (50 mCi/mmole), to reach a final naphthalene concentration of 0.2 mM. [Note: 0.2 mM is well above the Km of CYP2F2 (3μM), the predominant cytochrome P450 monooxygenase isozyme responsible for naphthalene metabolism, effectively making these incubations saturated with naphthalene.] After 60 min at 37°C, 1 ml of acetone was added to quench the reactions before storing the incubation mixtures overnight at –20°C. Tissues were separated from media by centrifugation at 14,000 × g for 15 min. Unmetabolized naphthalene is volatile, whereas the metabolites are not, so the resulting tissue pellets were dried under reduced pressure. Once dry, they were washed exhaustively with acetone, dried a second time, and dissolved in 1 N NaOH for analysis by liquid scintillation counting. The incubation media was also dried under reduced pressure before dissolving it in 50/50 methanol/water for analysis of water-soluble NA metabolites by HPLC. Aliquots were taken for liquid scintillation counting, and an aliquot of the dissolved tissue fraction was used to determine the protein content (Lowry et al., 1951) using BSA as a standard.

**HPLC analysis of metabolites.** Individual naphthalene metabolites from the incubation media were separated by reverse phase HPLC as described (Lakritz et al., 1996) with the minor modification that the mobile phase consisted of buffer A (0.6% triethylamine-phosphate, pH 3.1) developed against pure acetonitrile. The gradient was generated as follows: starting with 98% buffer A and 2% acetonitrile, moving to 11% acetonitrile by 60 min, 85% acetonitrile by 90 min, and 100% acetonitrile from 95 to 110 min, and ending at 120 min. Metabolite standards were prepared (see below) containing diastereomeric mixtures of the glutathionyl-NA, cysteiny1-glycyl-NA, cysteinyl-NA, and N-acetyl-cysteine-NA conjugates of naphthalene epoxide (e.g., 1,2-dihydro-1-hydroxy-2-cysteinyl-naphthalene), as well as naphthalene and naphthalene dihydriodiol. Samples were spiked with the mixture of standards before HPLC analysis, and the elution order of the metabolites was determined by monitoring the absorbance at 260 nm. Eluate fractions were collected every minute, and the radioactivity was determined by liquid scintillation counting. Radioprofiles were matched with UV absorbance traces from HPLC so that the amounts of the individual metabolites could be calculated; they are presented as nmoles of metabolite formed per mg protein per 60 min.

Standards were prepared by reacting racemic NA-epoxide [0.2 mmol; prepared as described by (Yagi and Jerina, 1975)] with 0.1 mmoles of the appropriate thiol compound (e.g., GSH, cysteine, etc.) in 5 ml of 0.1 M sodium phosphate buffer at pH 8.5 under an argon atmosphere. Unreacted epoxide was extracted into diethyl ether, and the aqueous phase was loaded onto a styrene divinylbenzene solid phase extraction column pre-equilibrated with 2% acetic acid. The column was washed with 10 volumes of 2% acetic acid, and the thiol derivatives were eluted with 75% acetonitrile before lyophilization. The purity and structure of the individual conjugates was checked by HPLC (described above) and electrospray mass-spectrometry after loop injection in 0.1% formic acid/50% acetonitrile in negative ion mode (VG Quattro BQ, Fisons Instrument, Atrincham, England). An equimolar mixture was prepared in methanol from the individual conjugates, NA and NA diol, and stored at –80°C until used.

**Statistical analysis.** All data are reported as the mean ± 1 standard deviation. Statistical differences were determined by one-way analysis of variance (ANOVA) and the Bonferroni-Dunn post-hoc testing method for pairwise comparisons at p < 0.05 significance level.

**RESULTS**

**Glutathione Levels after NA Inhalation**

Naphthalene vapor at 15 ppm for 4 h caused a rapid and substantial decrease in GSH levels throughout the respiratory tract but did not significantly alter hepatic GSH levels (Fig. 1). In contrast, DEM pretreatment led to severe depletion of GSH in pulmonary, nasal, and hepatic tissues. Depletion was more marked in the airways than it was in homogenates of the whole lung, in terms of percent of control GSH loss. Exposure to 15 ppm NA caused such a drastic decrease in airway GSH (90% loss) that DEM did not exacerbate the depletion. In mice
treated with an equivalent dose of DEM but not exposed to NA vapor, GSH levels dropped to 11% and 22% of control values in lung and liver, respectively, 1 hour after administration of DEM, and returned to near control levels within 6 h (data not shown). GSH loss in the ethmoid turbinates was not significant at any time point after NA exposure, except in animals pretreated with DEM. Hepatic GSH losses in animals exposed to NA alone, were only statistically significant at the 8 hour time point. However, statistically non-significant decreases in GSH levels were also observed at the 2 h (29% loss; \( p < 0.06 \)) and 4 h (22% loss; \( p < 0.07 \)) time points. Four hours after the end of exposure, GSH levels had recovered to near control values in all tissues, except for the liver in animals exposed to both NA and DEM. At 1.5 ppm NA, moderate GSH losses were observed in the respiratory tract and liver of mice after 2 h of exposure (Fig. 2), but were only statistically significant for the whole lung homogenates. In contrast, DEM pretreatment lead to substantial additional GSH loss in all tissues exposed to 1.5 ppm NA except distal airways.

Response of Respiratory Epithelium to Inhalation of NA

Severe Clara cell injury occurred in response to inhalation of 15 ppm NA in both proximal and distal intrapulmonary airways (Fig. 3–4). Signs of cellular injury (swelling and vacuolization) first became apparent 4–8 h after the start of the 4 h exposure (Fig. 3B and 4B) and progressed rapidly. Twenty-four hours after the exposure, large patches of airway epithelium lacked Clara cells and were covered with squamated ciliated cells (Fig. 3D and 4D). Necrotic Clara cells were exfoliated into the airway lumen. No changes were apparent in the olfactory epithelium at the end of the 15 ppm exposure (Fig. 5B) when compared with filtered air controls (Fig. 5A). However, 24 h after 15 ppm NA inhalation, the olfactory epithelium was nearly devoid of cells (Fig. 5D). In both the airways and nasal epithelium, the injury pattern was focal in nature, with portions of the epithelium having swollen but intact cells adjacent to areas devoid of any cells (not shown).

Diethymaleate pretreatment accelerated the injury caused by 15 ppm NA throughout the respiratory tract. In mice pretreated with DEM, a substantial number of Clara cells were missing from the proximal airways (Fig. 3C) at the completion of the 4 h exposure. In the distal airways of these same mice, the Clara cells had abundant cytoplasmic vacuoles and condensed nuclei (Fig. 4C). The extent and severity of injury in DEM pretreated mice was significantly different from animals exposed to NA alone at this time point (Fig. 3B and 4B). By 24 h after the exposure to NA and DEM, nearly all Clara cells had been lost throughout the airway tract. In the olfactory tissue of mice treated with both DEM and 15 ppm NA, injury was evident within 2 h of the start of exposure (not shown) and prominent by the end of the 4 h exposure (Fig. 5C). Twenty-four hours after termination of the exposure, the nasal olfactory epithelium looked similar in animals exposed to 15 ppm NA, with or without DEM pretreatment (Fig. 5D–E), with nearly all epithelial cells being lost. In mice that were only treated with an equivalent dose of DEM (1 g/kg), no Clara cell necrosis was observed (data not shown).

In contrast with the alterations in airway epithelium after exposure to 15 ppm NA, the lower dose of 1.5 ppm resulted in no observable effects on Clara cells at either airway level (Fig. 6A and 6C). However, in animals exposed to both DEM
FIG. 3. Histopathologic comparison of proximal conducting airways from mice exposed to 15 ppm NA for 4 h. Airway epithelium in control mice exposed to filtered air (3A) was of even thickness and contained a mixture of ciliated (arrowheads) and Clara cells (arrows). Clara cells in mice exposed to NA alone for 4 h (3B) showed early signs of injury (e.g., swelling and vacuolization, see asterisks), while animals treated with DEM and NA showed more advanced stages of NA induced injury with areas of epithelium devoid of Clara cells (3C). Maximal injury was apparent 24 h after the end of exposure, with necrotic Clara cells having been exfoliated into the airway lumen of mice exposed to NA alone (3D) or to DEM and NA (3E). Ciliated cells spread out to cover the areas vacated by necrotic Clara cells. Bar = 50 microns.

FIG. 4. Histopathologic comparison of distal conducting airways from mice exposed to 15 ppm NA for 4 h. Airway epithelium in control mice exposed to filtered air (4A) was of even thickness and contained a mixture of ciliated (arrowheads) and Clara cells (arrows). Clara cells in mice exposed to NA alone for 4 h (4B) were vacuolated (asterisks) and the airways were generally thicker, while in animals treated with DEM and NA airway thickness was decreased and cells appeared denser, even though some Clara cells were vacuolated (4C). Twenty-four h after the end of exposure, necrotic Clara cells had been exfoliated into the airway lumen of mice exposed to NA alone (4D), or to DEM and NA (4E). Ciliated cells spread out to cover the areas vacated by necrotic Clara cells. Bar = 50 microns.
FIG. 5. Histopathologic comparison of olfactory epithelium from the ethmoid turbinate region of the nasal tracts of mice exposed to 15 ppm NA for 4 h. The olfactory epithelium (OE) is a densely packed pseudo-stratified layer of tissue consisting of sustentacular, basal, and sensory cells (i.e. neurons); it sits atop the cartilaginous lamina propria (LP) layer, which contains abundant blood vessels. The olfactory epithelium was of even thickness in filtered air control animals (5A) and in mice exposed to NA alone for 4 h (5B). At the end of the exposure period for mice exposed to NA and DEM, the epithelium was greatly disrupted, with many cells of the olfactory epithelium missing (5C; arrow). Twenty-four hours after the exposures, nearly all epithelial cells had been lost in mice treated with NA alone (5D), or NA and DEM (5E), and the apical surface of the lamina propria (LP) was exposed (arrowheads). Bar = 50 microns.

FIG. 6. Mice were exposed to 1.5 ppm NA (+/− DEM) for 2 h and their lungs and nasal passages were fixed for high-resolution histopathology 24 h after the end of the exposure. Proximal intrapulmonary airways of mice treated with NA and DEM showed clear signs of cellular injury such as swelling or vacuolization (6B; asterisks), while those treated with NA alone were unchanged (6A; see 3D for comparison); arrows identify Clara cells. Likewise, with 15 ppm NA distal airways of mice treated with NA alone showed no signs of cellular injury (6C; see 4D for comparison), while those treated with NA and DEM (6D) contained swollen and vacuolated (asterisks) Clara cells. Olfactory epithelium from the ethmoid turbinate region showed mild disruption (‡) in mice exposed to NA alone (6E), and complete loss of cells in mice exposed to NA and DEM (6F), leaving the lamina propria exposed (arrowhead). Bars = 50 microns.
and 1.5 ppm NA, Clara cell necrosis was apparent in the
proximal airways (Fig. 6B) and initial signs of NA-induced
injury (i.e., swelling) were evident in the distal airways
(Fig. 6D). In the olfactory epithelium, 1.5 ppm NA caused
some cell loss (Fig. 6E), and DEM pretreatment greatly
increased the severity of the injury, with large portions of the
ethmoid turbinates being devoid of epithelium (Fig. 6F).

**Metabolism and Adduct Formation in Airways**

Glutathione levels were much lower in the airway explants
isolated from animals pretreated with DEM (Fig. 7A). Following
incubation with [14C]-NA, the levels of protein adducts formed
within airways nearly doubled in airway explants from mice
treated with DEM (Fig. 7B). At the same time, the amount of
water-soluble NA metabolites (thiol conjugates and diol) sig-
nificantly decreased (Fig. 7C). The decrease of metabolites in the
media (4.29 nmol/mg protein/60 min) was greater than the
increase in protein binding (0.32 nmol/mg protein), resulting
in a net inhibition of NA metabolism. To further investigate
how DEM treatment was impacting airway metabolism of
NA, the individual metabolites were resolved by reverse-
phase HPLC (Fig. 8) and quantitated (Table 1). Because of
the decreased availability of GSH, we had anticipated a decrease
in GSH conjugates of NA in DEM treated animals, and an
accompanying increase in NA diol. However, decreases in
both NA diol and GSH conjugates of NA were observed. Over-
all, these results suggest a general inhibition of NA metabolism
in Clara cells brought on by DEM treatment. About 30% of the
total radioactivity eluted between 73–88 min in a series of small
peaks that could not be resolved fully (see Fig. 8C and Table 1).
However, we did find that a number of known NA metabolites
produced in our lab or available commercially) elute in this
region of our system, including naphthalene-glucuronides,
N-acetyl-glutathionyl-hydroxy-dihydronaphthalene, naphthol,
naphthol-sulfate, 1,2- and 1,4-naphthoquinone, and 1,4-
napthoquinone-sulfate. Likewise, many of these metabolites,
as well as a series of trihydroxy-naphthalene species, have been
identified in the urine of rodents exposed to NA (Pakenham et al.,
2002; Stillwell et al., 1982). As the individual metabolites could
not be resolved, the total radioactivity eluting in this region was
summed together and is labeled as “unidentified” in Table 1.
Only 0.1% of the total radioactivity in the metabolite fraction
(Fig. 7) was attributed to unmetabolized [14C]-NA, not removed
during the washing and vacuum drying steps described in the
methods section.

**DISCUSSION**

This study employed inhalation of NA vapor to test the
hypothesis that NA acts directly on respiratory tissues to
cause the loss of GSH. By following GSH loss and injury pro-
gression during the first 24 h after inhalation of NA, we were able

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**FIG. 7.** One hour after treatment of mice with DEM or corn oil (carrier)
conducting airways were isolated by microdissection to evaluate the in vitro
metabolism of NA. GSH levels were measured by HPLC, coupled with
electrochemical detection, in homogenates of small samples removed from
each airway incubation before the addition of NA (7A). At the end of the
1 hour incubation, airway proteins were precipitated, washed repeatedly, and
metabolites of [14C]-NA covalently bound to protein were quantitated by
scintillation counting (7B). The total amount of water-soluble NA metabolites
in the incubation media was determined by liquid scintillation counting (7C),
as described in methods. The values for the DEM treated animals are also
listed as a percentage of the values obtained for the corresponding corn oil
treated animals (inset on graph bars). Data are the mean ± S.D.; n = 10
airways. *Significantly different from corn oil treated control animals,
\( p < 0.05 \).
HPLC method for resolving and quantitating NA metabolites

A

B

C

FIG. 8. Sample chromatograms and radioprofiles from HPLC separation of NA metabolites. UV (260 nm) absorbance profile of standards (8A) overlaid with percent of acetonitrile in the mobile phase. An actual sample spiked with standards (8B) shows baseline resolution of individual NA-conjugate stereoisomers. Inset frame (8B) shows 4 glutathionyl-naphthalene stereoisomers: 1 = (1S,2R)-1-hydroxy-2-glutathionyl-naphthalene, 2 = (1R,2S)-1-hydroxy-2-glutathionyl-naphthalene, 3 = (1S,2R)-1-gutathionyl-2-hydroxy-naphthalene, 4 = (1R,2S)-1-gutathionyl-2-hydroxy-naphthalene. Phenol red, a component of the cell culture media the cells were incubated in, was identified as a peak eluting at 76 min. [14C]-NA metabolites were quantitated by scintillation counting in fractions collected in 1-minute intervals, and the values were plotted to generate a radioprofile (8C). Peak elution times from UV profile were aligned with the radioprofile to assign measured radioactivity to specific metabolites. Several unidentified peaks were noted in the radioprofile, totaling 30% of the measured radioactivity; they corresponded to very small peaks in the UV absorbance profiles. Cys-NA = cysteinylnaphthalene; Cys-gly-NA = cysteinyl-glycynaphthalene; GS-NA = glutathionyl-naphthalene; Diol = 1,2-dihydroxy-1,2-dihydronaphthalene; NAC-NA = N-acetyl-cysteinyl-naphthalene; NA = naphthalene.

to show that GSH loss occurs within the pulmonary airways independent of liver function, and hence strengthens the argument that GSH loss is requisite for NA cytotoxicity. The respiratory tract experiences the highest oxygen tension in the body (21%) and turns over GSH pools rapidly (DeLeve and Kaplowitz, 1991). GSH levels drop 60% after passage through the lung, indicating significant uptake (Martensson et al., 1989). The liver is the primary source of circulating vascular GSH (Meister, 1981), and intraperitoneal doses of NA cause significant hepatic GSH loss (Warren et al., 1982), potentially limiting the ability of the liver to supply GSH to other organs. Therefore the respiratory tract GSH depletion occurring in response to intraperitoneal NA
doses could reflect decreased hepatic supply. If this were true, then it would not be clear how critical GSH loss at the site of injury was to NA toxicity. By demonstrating inhaled NA can deplete respiratory tract GSH without substantial hepatic GSH losses, we have shown that GSH depletion and cellular injury are manifest at the same site. Further, we have demonstrated that GSH depletion via diethylmaleate (DEM) promotes NA adduct formation in isolated airways (i.e., independent of circulating metabolites of hepatic origin) and increases the toxicity of inhaled naphthalene in vivo. These observations provide support for the hypothesis that reactive metabolites of NA are formed by in situ metabolism and are responsible for GSH depletion and Clara cell toxicity.

Previous studies have shown that inhaled NA is toxic to the respiratory tract (National Toxicology Program, 2000; West et al., 2001), but this is the first study correlating GSH loss with inhaled NA respiratory toxicity. Toxicity was only apparent in tissues severely depleted of GSH (1.5 ppm NA + DEM or 15 ppm NA ± DEM) suggesting that substantial GSH loss must occur for injury to take place. The intrapulmonary airways lost the greatest percentage of GSH (Fig. 1B-C) and were the most extensively injured (Fig. 3–4), whereas the liver maintained more GSH and is not susceptible to injury (O’Brien et al., 1985; Plopper et al., 1992; Shopp et al., 1984). It should be noted that in mice exposed to NA alone, hepatic GSH never dropped below 57.6 nmol/mg protein, substantially higher than lowest levels measured in the lung and airways, 9.7 and 1.0 nmol/mg protein, respectively (Fig. 1). Therefore, even though the liver did experience a decrease in GSH levels, the absolute concentration of GSH within the liver may have always been high enough to protect the cells from injury. Additionally, it should be noted that when DEM is given by itself, hepatic and pulmonary GSH levels can drop by 50–90% within 1 h, recovering to steady-state values within 2–6 h (Deneke et al., 1985; Gerard-Monnier et al., 1992). Van Winkle et al. have found that soon after Clara cells are exposed to NA the endoplasmic reticulum and mitochondria dilate, increasing cell volume about 50% (Van Winkle et al., 1995; Van Winkle et al., 1999). Epithelial swelling was evident at the end of the 15 ppm NA 4 hour exposure (Fig. 3B and 4B). However, at the same time point the Clara cells were smaller and denser than normal in animals pretreated with DEM (Fig. 3C and 4C), resembling the later stages of NA-induced Clara cell death. This suggests that DEM treatment is accelerating the injury process by removing GSH needed for NA detoxification.

The olfactory epithelium was more sensitive to NA inhalation than the intrapulmonary airways, with injury developing from exposure to 1.5 ppm for 2 h. GSH depletion with DEM greatly enhanced NA injury to the olfactory epithelium (Fig. 5 and 6E–F), however, it was not clear if exposure to NA alone caused GSH losses in the nasal tissues. Because we were unable to clear the nasal capillaries of blood during tissue collection, our GSH measurements might be skewed by the high content of GSH found in red blood cells (2.4 mM; (Anderson et al., 1985)). Chronic inhalation of NA vapors has been shown to induce olfactory neuroblastomas in rats in a concentration dependent manner (National Toxicology Program, 2000), prompting the listing of NA as a probable human carcinogen (National Toxicology Program, 2003). The current OSHA limits for human exposure to NA are 10 ppm TWA for 8 h with a standard threshold of 15 ppm (OSHA, 2003). Given that 1.5 ppm NA is much lower than the current exposure limit and that it produced significant injury (Fig. 6E), our results may raise concerns over the adequacy of current limits, although it has not been established how good of a model mice are for NA respiratory toxicity in humans.

The data in this study reinforce the concept that GSH loss sensitizes cells to injury from bioactivated toxicants such as NA by allowing more adducts to form. In DEM treated animals the levels of protein adducts nearly doubled (0.33 to 0.65 nmol/mg protein) but was countered by a large decrease in water-soluble metabolites (decreasing from 8.88 to 4.59 nmol/mg protein/60 min). The result was a net decrease in total NA metabolism (43%). By quantifying individual NA metabolites, we were able to determine that an overall decrease in metabolism was occurring, not simply a shift in the types of metabolites formed. However,

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<th>Individual Metabolites of Naphthalene (NA)</th>
<th>Corn oil control (N = 10)</th>
<th>DEM (N = 10)</th>
<th>DEM as % Control</th>
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<tbody>
<tr>
<td>Cysteinyl-naphthalene</td>
<td>0.65 ± 0.33</td>
<td>0.39 ± 0.19</td>
<td>60</td>
</tr>
<tr>
<td>Cysteinyl-glycine-naphthalene</td>
<td>0.26 ± 0.20</td>
<td>0.17 ± 0.06</td>
<td>65</td>
</tr>
<tr>
<td>Glutathionyl-naphthalene</td>
<td>1.70 ± 0.87</td>
<td>0.89 ± 0.51*</td>
<td>52</td>
</tr>
<tr>
<td>N-acetyl-cysteinyl-naphthalene</td>
<td>0.11 ± 0.06</td>
<td>0.06 ± 0.04*</td>
<td>55</td>
</tr>
<tr>
<td>All thiol-based conjugates</td>
<td>2.72 ± 1.27</td>
<td>1.50 ± 0.81*</td>
<td>55</td>
</tr>
<tr>
<td>Naphthalene dihydrodiol</td>
<td>3.63 ± 1.89</td>
<td>1.18 ± 1.01*</td>
<td>32</td>
</tr>
<tr>
<td>Cysteinyl-glycine-naphthalene</td>
<td>3.33 ± 0.99</td>
<td>4.50 ± 2.66</td>
<td>135</td>
</tr>
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<td>1.18 ± 1.01*</td>
<td>32</td>
</tr>
<tr>
<td>Unidentified</td>
<td>3.33 ± 0.99</td>
<td>4.50 ± 2.66</td>
<td>135</td>
</tr>
<tr>
<td>Total</td>
<td>9.68 ± 4.15</td>
<td>7.18 ± 4.47*</td>
<td>74</td>
</tr>
</tbody>
</table>

Note: Data expressed as mean ± standard deviation. Names abbreviated for space (e.g., cysteinyl-naphthalene represents a diastereomeric mixture of the R and S isomers of 1-cysteinyl-2-hydroxy-1,2-dihydronaphthalene and 2-cysteinyl-1-hydroxy-1,2-dihydronaphthalene).

*Significantly different from control, p < 0.05.
the total decrease in metabolism was smaller (26%) when determined by summing the individual metabolites resolved by HPLC when compared with analyzing all of the metabolites before separation (43%; compare Fig. 7 with Table 1). A decrease NA-GSH conjugates might be anticipated given the decreased availability of GSH in tissues isolated from DEM treated animals, but it was surprising that NA diol, a product of epoxide hydrolase, was also decreased by 45%. There was a slight increase in a group of unidentified metabolites (Table 1), which may represent particularly toxic intermediates (e.g., naphthoquinones). Regardless of the nature of these metabolites, the number of NA adducts formed nearly doubled in airsly depleted of GSH with DEM and may be responsible for the accelerated injury seen in DEM treated animals.

A few reports have questioned the use of DEM as a GSH depleting agent on the basis that it might produce changes in cellular and toxicant metabolism not solely related to GSH depletion (Costa and Murphy, 1986; Krack et al., 1980; Reiter and Wendel, 1982). However, it was also noted that preparations of DEM can be contaminated up to 20% diethylfumarate (Plummer et al., 1981), which can inhibit enzymes of the citric acid cycle. It is unclear how DEM inhibits NA metabolism but it does not likely involve contaminants, as we used the highest purity DEM available (98.4% by gas chromatography). Severe GSH loss has the potential to effect toxicant metabolism by affects DEM available (98.4% by gas chromatography). Severe GSH loss has the potential to effect toxicant metabolism by

ACKNOWLEDGMENTS

Advice on assessing respiratory tract injury patterns provided by Drs. Laura S. Van Winkle, Kent. E. Pinkerton, and Dallas M. Hyde, was greatly appreciated. Proofreading assistance by Dr. Suzette Smiley-Jewell was very helpful. Financial support was provided by the National Institute of Environmental Health Sciences (R01 ES0431, R01 ES06700, R01 ES04699, and T32 ES0759). UC Davis is a National Institute of Environmental Health Sciences Center (P30 ES05707), and support for core facilities used in this work is gratefully acknowledged.

REFERENCES


apparently, the reservoir of GSH in the liver is not available to balance loss in the respiratory tract within a time frame sufficient to modulate or prevent injury. GSH depletion by DEM enhances the inhaled NA respiratory tract toxicity and increases covalent binding of reactive NA metabolites to protein.

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Airways are the best current method for conducting in vitro incubations.

This study represents an important step in defining the relationship between the status of the intracellular GSH pool and P450-mediated cytotoxicity in Clara cells and nasal olfactory epithelium. GSH loss in the respiratory tract is due to the direct effect of the bioactivated cytotoxicant NA on target tissues and is not dependent on the hepatic supply of GSH or its precursors.


