Evidence of Quinone Metabolites of Naphthalene Covalently Bound to Sulfur Nucleophiles of Proteins of Murine Clara Cells after Exposure to Naphthalene

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Naphthalene-induced Clara cell toxicity in the mouse is associated with the covalent binding of electrophilic metabolites to cellular proteins. Epoxide and quinone metabolites of naphthalene are proposed to be the reactive metabolites responsible for covalent binding to proteins. To identify the nature of reactive metabolites bound to proteins (cysteine residues), we alkaline-permethylated proteins obtained from mouse Clara cells incubated with 0.5 mM naphthalene *in vitro*. Alkaline permethylation of protein adducts produced (methylthio)naphthalene derivatives detected by GC−MS. 3,4-Dimethoxy(methylthio)naphthalene was observed to be a predominant (methylthio)naphthalene derivative formed in the alkaline-permethylated protein sample obtained from Clara cells after exposure to naphthalene. This indicates that 1,2-naphthoquinone is a major metabolite covalently bound to cysteine residues of the cellular proteins. We have developed an immunoblotting approach to detect 1,2-naphthoquinone covalently bound to cysteine residues of proteins [Zheng, J., and Hammock, B. D. (1996) *Chem. Res. Toxicol.* **9**, 904−909]. To identify 1,2-naphthoquinone covalently bound to sulfur nucleophiles of proteins, homogenates obtained from naphthalene-exposed Clara cells were separated by SDS−PAGE followed by Western blotting and immunostaining with the antibodies. Two protein bands with 24 and 25 kDa were detected by the antibodies, further supporting the view that 1,2-naphthoquinone is a reactive metabolite of naphthalene which binds to Clara cell proteins *in vitro*.

Introduction

Intraperitoneal administration of naphthalene produces selective necrosis of pulmonary nonciliated bronchiolar cells in mice. Previous studies have shown cytochrome P450-mediated metabolism of naphthalene results in dose-dependent bronchiolar necrosis in mice and in the formation of reactive metabolites which deplete glutathione and bind covalently to cellular proteins (*1*−*4*). Depletion of glutathione by diethyl maleate enhances the lung injury induced by naphthalene as well as the covalent binding of reactive metabolites of naphthalene to pulmonary proteins (*4, 5*). There is a good correlation between the covalent binding of reactive metabolites in the lung and the extent and severity of naphthalene-induced bronchiolar injury in mice.

Naphthalene epoxide and naphthoquinones have been proposed to be the reactive metabolites of naphthalene responsible for the covalent modification of pulmonary proteins. However, little direct evidence has been reported supporting the structure of the naphthalene metabolite-modified proteins. Epoxides and quinones are known as electrophilic agents reactive toward nucleophiles. Nucleophilic sulfhydryl groups on cysteine side chains are reactive targets for epoxide and quinone metabolites of naphthalene. As shown in Scheme 1, the nucleophilic sulfhydryl groups of cysteine residues of proteins react with both naphthalene oxide (epoxide) by S_N2 and S_N1 reactions and quinone metabolites of naphthalene (1,4- and 1,2-naphthoquinone) by 1,4-addition (Michael reaction), respectively.

To understand the mechanism of naphthalene-induced cytotoxicity, it is important to identify the reactive metabolites responsible for covalent binding of cellular proteins. Here, we report evidence of 1,2-naphthoquinone as an important reactive metabolite of naphthalene which binds covalently to cysteine residues of proteins in mouse lung Clara cells incubated with naphthalene.

Experimental Procedures[1]

Chemicals and Instruments. Chemical reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Mouse albumin, *N*-acetylcysteine, and immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). HPLC analysis and purification of synthetic chemicals were performed using a Varian 9010 solvent delivery system equipped with a Varian 9050 UV−vis detector. A reverse phase C_{18} chromatography column (250 × 4.6 mm) purchased from Vydac (Hesperia, CA) was used for HPLC analysis and purification of synthetic compounds. ^{1}H-NMR spectra were obtained from a QE-300 spectrometer (General Electric; 300 MHz). Synthetic chemicals and alkaline-permethylated samples were analyzed on a Hewlett-Packard GC−MS system [HP 5890 Series II gas chromatograph with a 30 m DB-5 capillary column (J & W scientific, Folsom, CA), HP 5971A mass-selective detector, and HP 59940A MS

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[1] Abbreviations: ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline Tween; SIM, selected ion monitoring.

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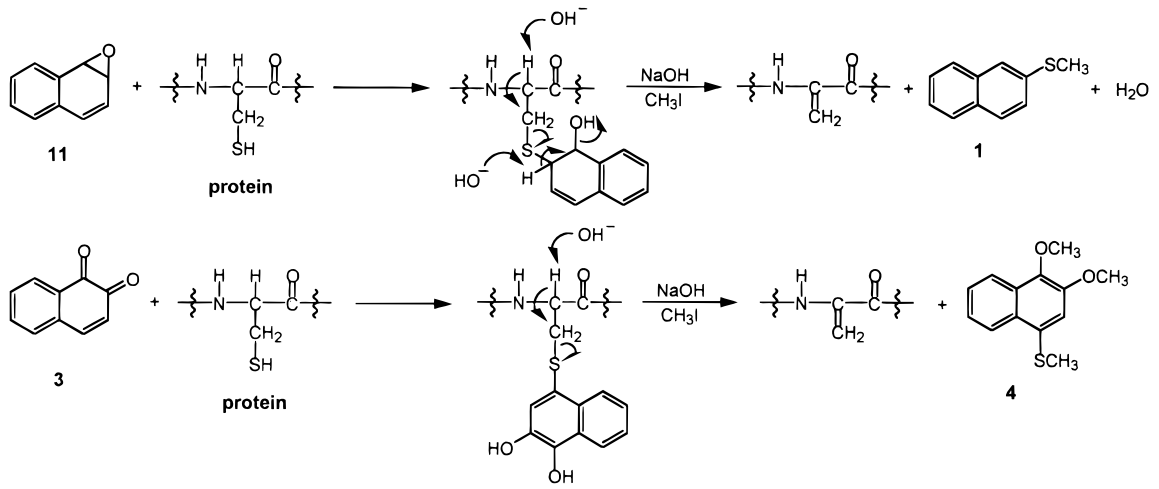
Scheme 1



ChemStation (HP-UX series) controller] using 70 eV electron ionization. A temperature program from 100 to 250 °C at 10 °C/min was used. Immunoassays were performed with polystyrene 96-well microtiter plates purchased from Nunc (Maxisorb, Roskilde, Denmark). The absorbances were read with a V_{max} microplate reader (Molecular Devices, Menlo Park, CA). Curve fitting was performed using SigmaPlot.

Syntheses. 2-(Methylthio)naphthalene (1). The (methylthio)naphthalene **1** (Scheme 2) was synthesized by methylation of 2-thionaphthol (*6*). 2-Thionaphthol (500 mg) was placed in a round bottom flask followed by addition of 10 mL of ethanol, 0.5 mL of methyl iodide, and 200 mg of sodium carbonate. The mixture was refluxed under nitrogen for 2.0 h. Following the reaction, the crude product was filtered, and compound **1** was purified using flash chromatography on silica gel eluted with hexane/chloroform. An 87% yield was obtained. ^{1}H-NMR ($CDCl_3$): δ 2.58 (s, 3H, SCH_3), 7.4−7.5 (m, 3H, H aromatic), 7.59 (s, 1H, H aromatic), 7.7−7.8 (m, 3H, H aromatic). EIMS: 174 (M^+), 159, 141, 128, 115.

2-(Ethylthio)naphthalene (2). Synthesis of compound **2** in 79% yield was conducted using the similar procedure as for the preparation of compound **1** except ethyl iodide was used as an alkylating agent. The yield of ethylation was 79%. ^{1}H-NMR ($CDCl_3$): δ 1.36 (t, J = 7.4 Hz, 3H, SCH_2CH_3), 3.05 (q, J = 7.4 Hz, 2H, SCH_2CH_3), 7.7−7.8 (m, 4H, H aromatic), 7.59 (s, 1H, H aromatic), 7.7−7.8 (m, 3H, H aromatic). EIMS: 188 (M^+), 173, 160, 128, 115.

1,2-Naphthoquinone (3). 1,2-Naphthoquinone was prepared by the method of Fieser (*7*). Briefly, 1-aminonaphthol (1.5 g) was dissolved in 100 mL of 0.5 N HCl, and the insoluble solid was removed by filtration. To this solution was added 15 mL of 10% (w/v) $FeCl_3$ dissolved in 3 N HCl dropwise over 10 min, with vigorous stirring at room temperature. The mixture was stirred at room temperature for 30 min, and the resulting brown crystals were recrystallized from hexane and ethyl acetate, giving bright orange crystals in 72% yield.

3,4-Dimethoxy(methylthio)naphthalene (4). *N*-Acetylcysteine (500 mg, 3 mmol) was dissolved in 5.0 mL of water which had been purged with nitrogen gas. 1,2-Naphthoquinone (310 mg, 2 mmol) was dissolved in 5.0 mL of nitrogen-purged ethanol and added dropwise to the cysteine solution under nitrogen. After 30 min of stirring at room temperature, the mixture was transferred to a 50 mL glass culture tube containing 10 mL of 4 M NaOH and 2 mL of CH_3I. The resulting mixture was heated at 80 °C for 4 h in a tube sealed with a Teflon-lined cap. The crude products were extracted with $CHCl_3$, $CHCl_3$ was removed by rotary evaporation, and compound **4** was purified by flash chromatography on silica gel eluted with hexane/chloroform. Total yield was 17%. ^{1}H-NMR ($CDCl_3$): δ 2.00 (s, 3H, SCH_3), 3.98 (s, 3H, OCH_3), 4.01 (s, 3H, OCH_3), 7.33 (s, 1H, H aromatic), 7.4−7.6 (m, 2H, H aromatic), 8.18 (dd, J = 8.4, 33.9 Hz, 2H, H aromatic). EIMS: 234 (M^+), 219, 191, 176, 129, 115.

1,4-Dimethoxy-2-(methylthio)naphthalene (5). Compound **5** was synthesized using a synethetic procedure similar to that for the synthesis of compound **4** except that 1,4-naphthoquinone rather than 1,2-naphthoquinone was used as starting material. Total yield was 22%. ^{1}H-NMR ($CDCl_3$): δ 2.56 (s, 3H, SCH_3), 3.98 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 6.71 (s, 1H, H aromatic), 7.48 (m, 2H, H aromatic), 8.18 (dd, J = 8.4, 33.9 Hz, 2H, H aromatic). EIMS: 234 (M^+), 219, 187, 174, 159, 129, 115.

***N*-Acetyl-*S*-[2-(1,2-dihydro-1-hydroxynaphthyl)]cysteine (6).** Compound **6** was synthesized by the reaction of naphthalene oxide with *N*-acetylcysteine as described by Marco and co-workers (*8*).

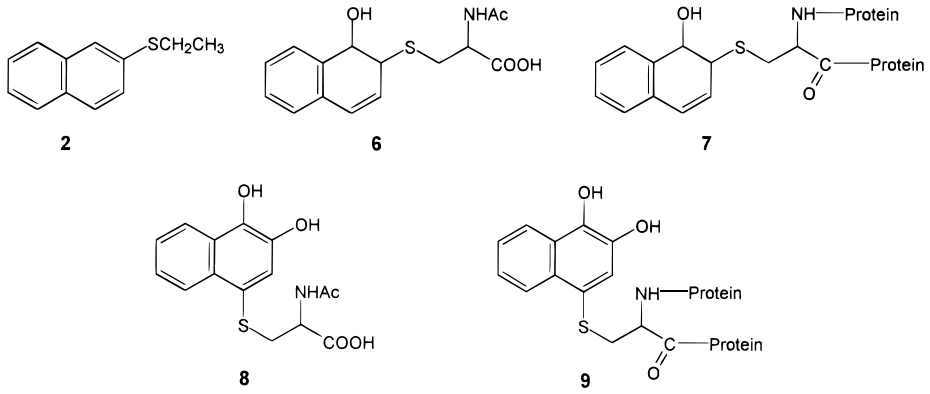
***N*-Acetyl-*S*-[4-(1,2-dihydroxynaphthyl)]cysteine (8).** Compound **8** was prepared by conjugating 1,2-naphthoquinone (**3**) with *N*-acetylcysteine as described previously (*9*).

Isolation of Clara Cells. Mouse Clara cells were isolated by previously published techniques (*10*, *11*). Briefly, animals were euthanized by pentobarbital overdose, the trachea was cannulated, and the lungs were inflated and thoroughly perfused via the pulmonary artery to remove blood. Alveolar macrophages were removed by lavage (eight washes), and lungs were filled with elastase (4.3 U/mL). Following digestion, cells were allowed to adhere to plates coated with IgG. Nonadherent cells were removed, and viability was checked by exclusion of erythrocin B. This isolation method yields approximately 2.5−3 × 10^6 cells/animal with a viability of >80%. Electron microscopic evaluation of the final isolates showed that this preparation contained an average of 72% Clara cells, 8% ciliated cells, and 11% alveolar type II cells (*11*). Contamination by red blood cells averaged 1−3%.

***In Vitro* Studies.** Freshly isolated Clara cells were incubated at a final concentration of 10^6 cells/mL in F-12/DME medium. Septum-stopped vials were flushed with an atmosphere of 95% O_2 and 5% CO_2, and naphthalene dissolved in ethanol was added in 5.0 μL/mL incubation to allow final concentration of 0.5 mM. Incubations were conducted at 37 °C for 1.0 h. Control incubation contained Clara cells in the absence of naphthalene. Following the incubation, the resulting Clara cells were lysed in 1% SDS. Unbound metabolites were removed by extraction with ethyl acetate (4 × 4 mL), and samples were dialyzed extensively against 0.1% SDS (six changes). The dialyzed samples were subjected to alkaline permethylation.

Alkaline Permethylation of Clara Cell Proteins. Alkaline permethylations of protein samples were conducted as described previously by Slaughter *et al.* (*12*, *13*). Briefly, the dialyzed protein samples (4.0 mL) were placed in culture tubes and mixed with 4.0 mL of 8 M NaOH followed by addition of 1.0 mL of CH_3I (the protein samples, NaOH solution, and CH_3I had been prepurged with nitrogen). The tubes were purged with nitrogen, sealed with Teflon-lined caps, and heated at 80 °C for

Chart 1



4 h. Following the reaction, the mixture was cooled to room temperature, spiked with (ethylthio)naphthalene **2** (Chart 1) as an internal standard, and extracted with pentane. The pentane extracts were dehydrated by anhydrous Na_2SO_4, concentrated by distillation, and analyzed by GC−MS.

GC−MS Analysis. The pentane extracts obtained from alkaline-permethylated protein samples were analyzed in the selected ion monitoring (SIM) mode to gain increased sensitivity and specificity. Molecular ions at *m*/*z* 174, 188, and 234 were selected to monitor (methylthio)naphthalene derivatives such as compound **1**, (ethylthio)naphthalene **2**, and dimethoxy(methylthio)naphthalenes **4** and **5**, respectively. Structural identification of (methylthio)naphthalene derivatives formed in the alkaline permethylation of the protein samples was conducted by mass matching and comparing the relative retention times of peaks observed in the reconstructed ion chromatograph with those of the synthetic authentic standards to the internal standard. In addition, following the first round analysis, the remaining sample was consecutively spiked with synthetic authentic standards. The resulting spiked sample was reanalyzed by GC−MS in the same SIM mode for further structural confirmation.

Competitive Enzyme-Linked Immunosorbent Assay (ELISA). Mouse albumin-1,2-naphthoquinone conjugate was synthesized for use as a coating antigen, as described by Zheng and Hammock (*9*). To each well of 96-well microtiter plates was added 100 μL of the coating antigen at 2.0 μg/mL (200 ng/well) in PBS buffer (0.2 M sodium phosphate, pH 7.4), and plates were sealed and incubated at 4 °C overnight. Serial dilutions of the analytes were prepared in PBST buffer (PBS containing 0.05% Tween-20, pH 7.4) and 1% ethanol. The resulting analyte solution was mixed (1:1, v:v) with primary antisera (1:1000 dilution) in 2% nonfat milk dissolved in PBST buffer followed by incubation at 4 °C overnight. The following day the plates were washed five times with PBST buffer and blocked by addition of 1.0% nonfat milk in PBST buffer (120 μL/well) and incubation for 1 h at room temperature. The plates were washed as described above. The preincubated antisera with analytes such as compounds **6** and **8** at various concentrations were added to the plates (100 μL/well) and incubated at room temperature for 2 h. After the same process of washing, 100 μL of a 1/12000 diluted goat anti-rabbit IgG-horseradish peroxidase (HRP) solution was added to each well. The mixture was incubated for an additional 1 h, and after another wash step, 100 μL of a substrate solution containing 0.3 mM tetramethylbenzidine and 0.1 mM H_2O_2 in 0.1 M acetate buffer (pH = 5.5) was added and incubated at room temperature for 30 min followed by addition of 50 μL of a 4 M sulfuric acid solution to quench colorimetric development. The absorbance was read at dual wavelength (450 nm minus 650 nm).

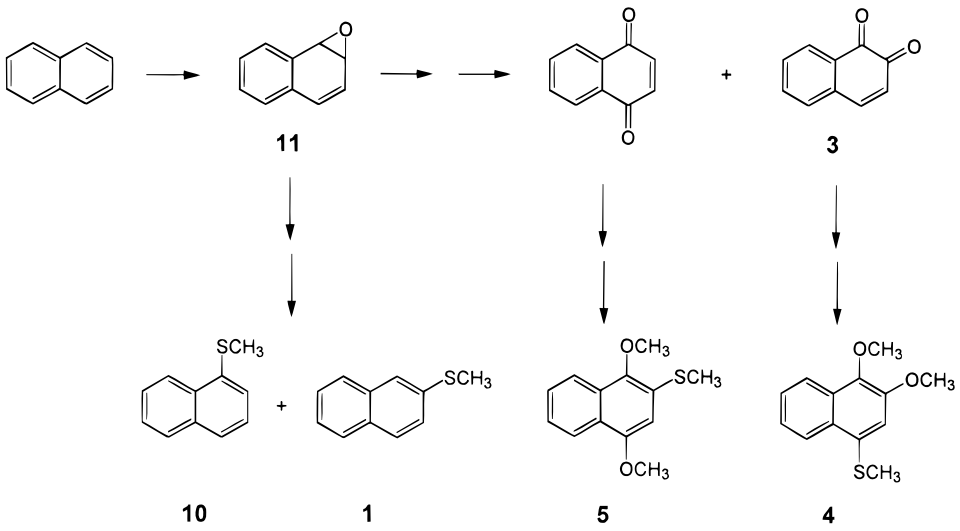
Electrophoresis and Immunoblotting. Clara cell proteins were resolved by SDS−polyacrylamide gel electrophoresis (Mini-Protean II, Bio-Rad) according to Laemmli (*14*) using 3.3% stacking and 12% resolving gels. Proteins were transferred to polyvinylidene difluoride microporous membranes (Immobilon-P transfer membranes, Millipore) by an electroblotter (LKB Novablot electrophoretic transfer kit). After 3.0 h of transferring (43 mA) blots were blocked by shaking overnight in 5% nonfat dry milk in PBST buffer. The blotted polyvinylidene difluoride membranes were incubated for 1.0 h with a 1/2000 dilution of primary rabbit antiserum in PBST buffer with 3% nonfat milk. The immunoblots were incubated for 1.0 h with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (1/4000 in PBST buffer; Sigma). The blotted protein bands were detected by chemiluminescence using ECL Western blotting kits (Amersham International plc, England).

Results and Discussion

The mechanism of naphthalene-induced cytotoxicity is attributed to reactive metabolites of naphthalene covalently bound to cellular proteins which play important roles in physiological and biochemical processes required for cell survival. Both epoxide and quinone metabolites of naphthalene are suggested to be the reactive metabolites which are involved in the modification of cellular proteins and associated with naphthalene-induced cell injury. However, structural evidence for the generation of the reactive metabolites responsible for the formation of protein adducts is currently lacking.

Hanzlik and co-workers developed an alkaline permethylation process which allows the detection of epoxide- and quinone-derived bromobenzene metabolites covalently bound to sulfur nucleophiles of proteins (*12*, *13*). We extended this methodology to the detection of reactive metabolites of naphthalene covalently modifying sulfur nucleophiles of cellular proteins. Scheme 1 illustrates the process of alkaline permethylation of protein adducts presumably formed by conjugation of epoxide or quinone metabolites of naphthalene to cysteine residues of cellular proteins. In principle, the bond linking sulfur and β-carbon is cleaved through "retro-Michael reaction" induced by base (4 M NaOH). The resulting thiol, as well as the phenol groups of the quinone-cysteine adducts, is methylated by iodomethane *in situ*. A process of dehydration (aromatization) occurs during the alkaline permethylation of the epoxide-cysteine adducts. The alkaline permethylation of the protein adducts generates (methylthio)naphthalene derivatives which are dependent on the origins of the reactive metabolites of naphthalene modifying cysteine residues of proteins (Scheme 2). These (methylthio)naphthalene derivatives are chemically stable and can be readily detected by GC−MS. Structural identification of epoxide or/and quinone metabolites of naphthalene responsible for the covalent binding to sulfur nucleophiles of proteins can be obtained by determination of the structures of the (methylthio)-naphthalene derivatives. Alkaline permethylation of

Scheme 2



naphthalene epoxide-cysteine protein adducts would generate (methylthio)naphthalenes **1** and **10** (Scheme 2), whereas alkaline permethylation of quinone-cysteine protein adducts should produce dimethoxy(methylthio)-naphthalenes **4** and **5** resulting from 1,4- and 1,2-naphthoquinones responsible for the covalent binding to sulfur nucleophiles of cysteine residues of proteins (Scheme 2).

Mercapturic acids **6** and **8**, used as models of alkaline permethylation, were synthesized as described previously (*8*, *9*) and alkaline-permethylated as described in the experimental section. As expected, these two mercapturic acids were converted to compounds **1** and **4**, and their yields from the corresponding mercapturic acids were 65% and 47%, respectively.

Previous studies showed naphthalene produced highly selective necrosis of Clara cells in murine lung (*15*, *16*). Incubation of Clara cells with [^{3}H]naphthalene produced time-dependent covalent binding of radioactive materials to cellular proteins (*17*). To identify the reactive metabolites of naphthalene responsible for the modification of cellular proteins, we incubated freshly isolated mouse Clara cells with naphthalene (0.5 mM) at 37 °C for 1 h, and the resulting protein adducts were alkaline-permethylated as described above. Control incubations containing Clara cells were treated identically except for the absence of naphthalene. After alkaline permethylation, the resulting protein samples were extracted with pentane, and the pentane extracts were analyzed by GC−MS. Ions at *m*/*z* 174, 188, and 234 representing the molecular weight of compounds **1**, **2**, and **4**−**5,** respectively, were monitored using SIM mode. As shown in Figure 1C, a large peak (peak 3) with a retention time at 14.2 min, which was absent in the alkaline-permethylated control group (Figure 1B), consistent with the retention time of authentic compound **4** (Figure 1A), was observed in the alkaline-permethylated protein sample obtained from naphthalene-exposed Clara cells. As expected, a large peak at the same retention time was observed in a reconstructed ion chromatogram for *m*/*z* 234 (not shown) which is the molecular weight of compound **4**. In addition, an appreciable peak (peak 1) with retention time at 10.4 min was observed in the alkaline-permethylated protein sample obtained from naphthalene-exposed Clara cells (Figure 1C). The retention time of peak 1 was the same as that of synthetic authentic compound **1** (Figure 1A). The reconstructed

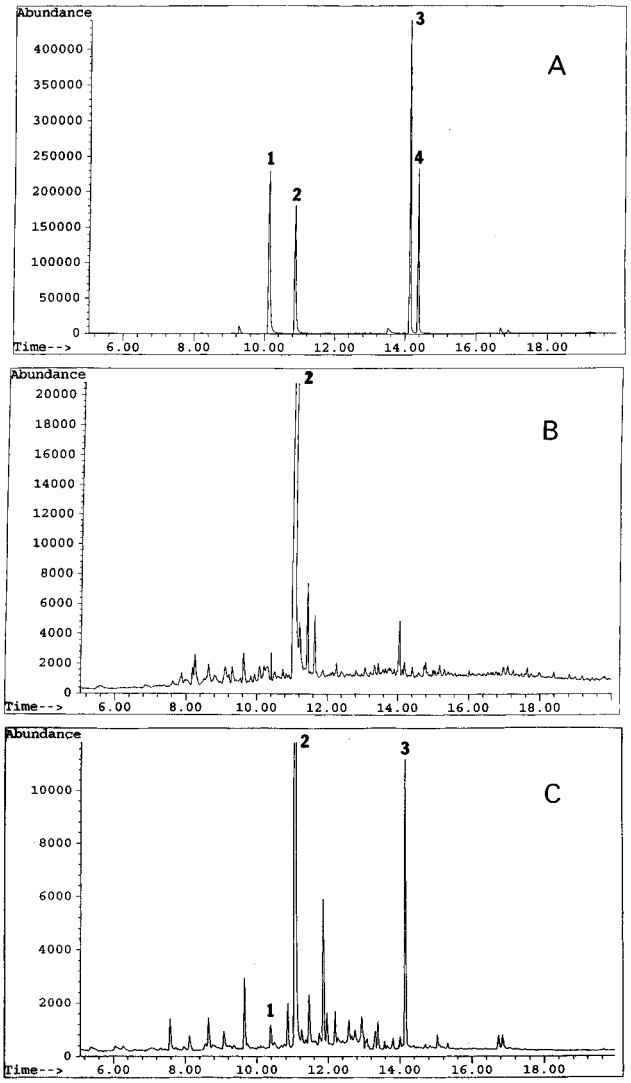


Figure 1. GC−MS profiles of authentic standards (A) and alkaline-permethylated protein samples obtained from mouse Clara cells after exposure to vehicle (B) or 0.5 mM naphthalene (C).

ion chromatograph at *m*/*z* 174 (not shown) showed a major peak with retention time at 10.4 min consistent with the retention time of compound **1**. Each peak of interest observed in the first round of GC−MS analysis was confirmed by spiking of authentic compounds **1** and

4 separately and reanalyzing by GC−MS. By peak mapping of the GC−MS profiles before and after spiking and assistance of a reconstructed ion chromatograph, peaks 1 and 3 were assigned to be compounds **1** and **4** which are apparently derived from naphthalene epoxide and 1,2-naphthoquinone covalently modifying the cysteine residues of Clara cell proteins. Interestingly, compound **5** was not observed in the alkaline-permethylated protein sample obtained from Clara cells after exposure to naphthalene, indicating absence of the 1,4-naphthoquinone proposed as a quinone metabolite of naphthalene covalently bound to cysteine residues of cellular proteins.

Compounds **1** and **4** generated in the alkaline permethylation of the protein sample obtained from Clara cells after exposure to naphthalene were quantitated by integration of peak area followed by standardization in consideration of mass response factors and alkaline permethylation yields. The area of peak 2 (compound **2**) was used as quantitative internal standard. The ratio of compound **4** vs **1** was found to be 32:1 in this experiment. In addition, the attribution of 1,2-naphthoquinone metabolite to the covalent binding toward sulfur nucleophiles may be underestimated, since some protein adduct **9** may be further oxidized to the quinone form which may not be released upon alkaline permethylation. The quantitative data indicate 1,2-naphthoquinone as a predominant reactive metabolite covalently bound to sulfur nucleophiles of proteins.

Immunoblotting has been successfully applied to identify protein adducts by the detection of the moiety of reactive metabolites as ligands covalently bound to proteins (*18*, *19*). Antibodies used for immunoblotting can be raised by immunization of animals with rationally designed immunogens which have the same or similar structures of reactive metabolites covalently bound to proteins. We successfully prepared polyclonal antibodies against 1,2-naphthoquinone-derived protein adducts by immunizing rabbits with keyhole limpet hemocyanin 1,2-naphthoquinone adducts (*9*). The specificity of the antibodies was determined by competitive ELISA using *N*-acetyl-*S*-[4-(1,2-dihydroxynaphthyl)]cysteine (**8**) as a competitor. Compound **8** is a 1,2-naphthoquinone-derived *N*-acetylcysteine adduct whose structure is similar to compound **9**, an adduct of 1,2-naphthoquinone on a cysteine residue of proteins. Competitive ELISAs were conducted to examine the specificity of antibodies. As demonstrated in Figure 2, a sigmoid curve was obtained from the competitive ELISA by plotting optical density measured at 450 nm minus 650 nm against the logarithm of the various concentrations of competitor **8**. The resulting curve was fitted by a four-parameter logistic equation. An IC_{50} of 2.82 nM was observed with slope = 0.98 and $r = 0.987$ (r: regression coefficient).

Naphthalene epoxide appears to be a reactive metabolite of naphthalene bound to cysteine residues of proteins of Clara cells which have been exposed to naphthalene. In this study, covalent binding of naphthalene epoxide metabolite bound to sulfur nucleophiles of proteins was observed in naphthalene-exposed Clara cells although binding of the epoxide was minor relative to that of 1,2-naphthoquinone. It is important to determine the cross-reactivity of the quinone selective antibodies toward the naphthalene epoxide cysteine adduct. The cross-reactivity was tested by a parallel competitive ELISA using *N*-acetyl-*S*-[2-(1,2-dihydro-1-hydroxynaphthyl)]cysteine (**6**) as a competitor. As shown in Chart 1, compound **6** is

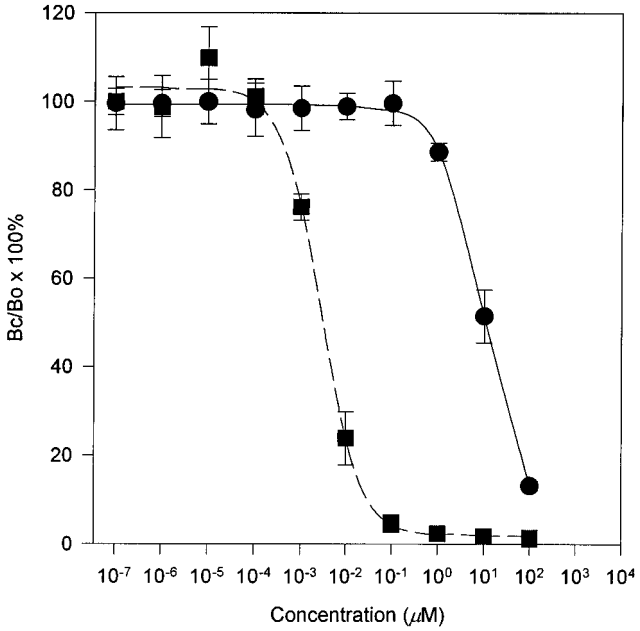


Figure 2. Analysis of *N*-acetyl-*S*-[4-(1,2-dihydroxynaphthyl)]-cysteine (■) and *N*-acetyl-*S*-[2-(1,2-dihydro-1-hydroxynaphthyl)]-cysteine (●) in a competitive ELISA using an antiserum raised against 1,2-naphthoquinone adduct on keyhole limpet hemocyanin in a microtiter plate coated with 1,2-naphthoquinone adduct on mouse serum albumin. Error bars represent standard deviation of separate runs on different days. The values are mean ± SD for triplicate assays.

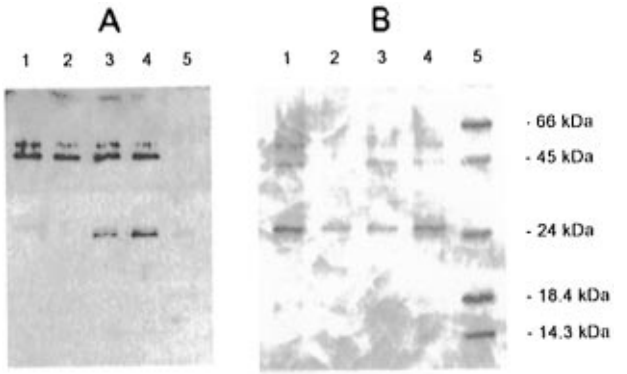
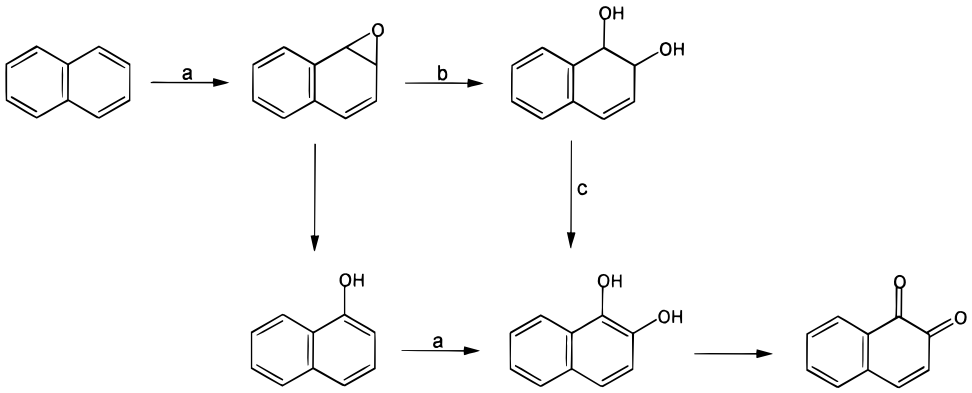


Figure 3. Immunochemical detection (A) and Coomassie blue staining (B) of proteins obtained from mouse Clara cells after exposure to vehicle (lane 1) and 0.5 mM naphthalene (lanes 2−4); 40, 5, 10, and 20 μg of protein were loaded in lanes 1, 2, 3, and 4, respectively. Lane 5 contains molecular weight standards.

the structural mimic of the naphthalene epoxide adduct of cysteine residues of proteins (**7**). We found very weak cross-reactivity (0.01%, $IC_{50} = 10.2$ μM) of the antibodies toward *N*-acetyl-*S*-[2-(1,2-dihydro-1-hydroxynaphthyl)]-cysteine (**6**) (Figure 2).

Data from the competitive ELISA showed very high specificity of the antibodies toward 1,2-naphthoquinone cysteine adducts. We took advantage of the antibody recognition as a probe to identify 1,2-quinone metabolites of naphthalene covalently bound to sulfur nucleophiles of cellular proteins of Clara cells after exposure to naphthalene. Homogenates containing 40 μg of proteins obtained from Clara cells which had been exposed to vehicle as the control group were loaded onto a 12% SDS−polyacrylamide gel. In addition, homogenates containing 20, 10, or 5 μg of protein obtained from Clara cells exposed to 0.5 mM naphthalene were loaded onto the gel. Proteins were resolved by electrophoresis,

Scheme 3[a]



[a] (a) Cytochrome P450; (b) epoxide hydrolase; (c) dihydrodiol dehydrogenase.

transferred to Immobilon-P transfer membranes, and incubated with the primary antibodies we raised and then secondary antibodies (goat anti-rabbit IgG antibody HRP). The resulting membranes were developed using a chemiluminescence ECL kit. Two chemiluminescent bands at 24 and 25 kDa, which were absent in the control group lane (Figure 3A) loaded with 40 μg of protein, were clearly observed in the lanes loaded with 20 μg and even 10 μg protein samples (lanes 4 and 3, respectively, Figure 3A) obtained from Clara cells after exposure to naphthalene. These two chemiluminescent bands were not observed in the lane loaded with 5 μg of protein (Figure 3A, lane 2) from the naphthalene-exposed Clara cells. Perhaps, the amount of protein loaded was below the limit of detection by the antibodies. We also found that the intensity of the 24 and 25 kDa protein bands detected by the antibodies (Figure 3A, lane 4) was disproportionately high relative to staining by Coomassie blue (Figure 3B, lane 4). This further indicates that the immunostaining of 24 and 25 kDa protein bands results from the modification of protein cysteine residues by the 1,2-naphthoquinone metabolite rather than antibody cross-reactivity toward the proteins. This also implies that the covalent binding by the 1,2-naphthoquinone metabolite is not proportional to intracellular protein abundance.

As shown in Figure 3A, a chemiluminescent band at 52 kDa was observed in protein samples of both control and the naphthalene-exposed Clara cells. This probably resulted from cross-reactivity of the polyclonal antiserum to this particular cellular protein. However, this immuno-cross-reactivity does not affect the identification of the quinone metabolite covalently bound to the 24 and 25 kDa proteins which were not observed in controls.

The results of the alkaline permethylation/GC−MS, along with the immunoblotting, demonstrated strong evidence for the formation of the 1,2-naphthoquinone metabolite which modifies cysteine residues of proteins of Clara cells after exposure to naphthalene. However, both techniques unlikely detected other nucleophiles such as nitrogen in addition to sulfur of proteins. We propose the metabolic pathway of the formation of the 1,2-quinone metabolite of naphthalene as shown in Scheme 3. Naphthalene metabolism is initiated by the formation of naphthalene epoxide mediated by cytochrome P450. The resulting epoxide metabolite is spontaneously and enzymatically hydrolyzed by epoxide hydrolase to dihydrodiol and further oxidized to 1,2-dihydroxynaphthalene (1,2-naphthohydroquinone) by dihydrodiol dehydrogenase. Alternatively, naphthalene oxide can chemically rearrange to 1-naphthol which can then be hydroxylated by cytochrome P450 to 1,2-dihydroxynaphthalenes (naphthohydroquinones). The resulting 1,2-hydroquinone metabolites of naphthalene are chemically (by reactive oxygen species) and enzymatically (by dioxygenases) oxidized to 1,2-naphthoquinone which possesses high chemical reactivity toward nucleophiles of intracellular proteins forming protein adducts.

In conclusion, we have identified 1,2-naphthoquinone as a major reactive metabolite of naphthalene which covalently modifies cysteine residues of proteins of mouse Clara cells after exposure to naphthalene. By the use of immunostaining, we observed that two cellular proteins with weights of 24 and 25 kDa were attacked by the 1,2-quinone metabolite of naphthalene through the modification of protein cysteine residues. The role of protein modification by the reactive metabolite in naphthalene-induced cytotoxicity is under investigation.

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References

(1) Reid, W. D., Ilett, K. F., Glick, K. F., and Krishna. (1973) Metabolism and binding of aromatic hydrocarbons in the lung: Relationship to experimental bronchiolar necrosis. *Am. Rev. Respir. Dis.* **107**, 539−551.

(2) Mahvi, D., Bank, H., and Harley, R. (1977) Morphology of a naphthalene-induced bronchiolar lesion. *Am. J. Pathol.* **86**, 559−571.

(3) Tong, S. S., Lowe, M. C., Trush, M. A., Minmaugh, E. G., Ginsburg, E., Hirokata, Y., and Gram, T. A. (1982) Bronchiolar epithelial damage and impairment of pulmonary microsomal monooxygnase activity in mice by naphthalene. *Exp. Mol. Pathol.* **37**, 358−369.

(4) Warren, D. L., Brown, D. L., Jr., and Buckpitt, A. R. (1982) Evidence for cytochrome P450 mediated metabolism in the bronchiolar damage by naphthalene. *Chem.-Biol. Interact.* **40**, 287−303.

(5) Buckpitt, A. R., and Warren, D. L. (1983) Evidence for hepatic formation, export and covalent binding of reactive naphthalene metabolites in extrahepatic tissues *in vivo*. *J. Pharmacol. Exp. Ther.* **225**, 8−16.

(6) Zheng, J., and Hanzlik, R. P. (1992) Bromomonohydroxyphenyl mercapturic acids. A new class of mercapturic acids from bromobenzene treated rats. *Drug Metab. Dispos.* **20**, 688−694.

(7) Fieser, L. F. (1943) 1,2-Naphthoquinone. *Org. Synth.* **2**, 430−433.

(8) Marco, M., Nasiri, M., Kurth, M. J., and Hammock, B. D. (1993) Enzyme-linked immunosorbent assay for the specific detection of the mercapturic acid metabolites of naphthalene. *Chem. Res. Toxicol.* **6**, 284−293.

(9) Zheng, J., and Hammock, B. D. (1996) Development of polyclonal antibodies for detection of protein modification by 1,2-naphthoquinone. *Chem. Res. Toxicol.* **9**, 904−909.
(10) Walker, S., Hale, S., Malkinson, A., and Manson, R. (1989) Properties of isolated nonciliated bronchiolar cells from mouse lung. *Exp. Lung Res.* **15**, 553−573.
(11) Chichester, C., Philpot, R., Weir, A., Buckpitt, A. R., and Plopper, C. G. (1991) Characterization of the cytochrome P450 monooxygenase system in nonciliated bronchiolar epithelial (Clara) cells isolated from the mouse lung. *Am. J. Respir. Cell Mol. Biol.* **4**, 179−186.
(12) Slaughter, D. E., and Hanzlik, R. P. (1991) Identification of epoxide- and quinone-derived bromobenzene adducts to protein sulfur nucleophiles. *Chem. Res. Toxicol.* **4**, 349−359.
(13) Slaughter, D. E., Zheng, J., Harriman, S., and Hanzlik, R. P. (1993) Identification of covalent adducts to protein sulfur nucleophiles by alkaline permethylation. *Anal. Biochem.* **208**, 288−295.
(14) Laemmli, U. K. (1970) Cleavage of Structural proteins during the assembling of the head of bacteriophage T4. *Nature* **227**, 680−685.
(15) Plopper, C. G., Suverkropp, C., Morin, D., Nishio, S., and Buckpitt, A. R. (1992) Relationship of cytochrome P-450 activity to Clara cell cytotoxicity. I. Histopathologic comparison of the respiratory tract of mice, rats, and hamsters after parenteral administration of naphthalene. *J. Pharmacol. Exp. Ther.* **261**, 353−363.
(16) Plopper, C. G., Macklin, J., Nishio, S. J., Hyde, D. M., and Buckpitt, A. R. (1992) Relationship of cytochrome P-450 activity to Clara cell cytotoxicity. III. Morphometric comparison of changes in the epithelial populations of terminal bronchioles and lobar bronchi in mice, hamsters, and rats after parenteral administration of naphthalene. *Lab. Invest.* **67**, 553−565.
(17) Cho, M., Chichester, C., Morin, D., Plopper, C. G., and Buckpitt, A. R. (1994) Covalent interactions of reactive naphthalene metabolites with proteins. *J. Pharmacol. Exp. Ther.* **269**, 881−889.
(18) Hinson, J. A., Pumford, N. R., and Roberts, D. W. (1995) Mechanisms of acetaminophen toxicity: immunochemical detection of drug-protein adducts. *Drug Metab. Rev.* **29**, 73−92.
(19) Martin, J. L., Meinwald, J., Radford, P., Liu, Z., Graf, M. L., and Pohl, L. R. (1995) Stereoselective metabolism of halothane enantiomers to trifluoroacetylated liver proteins. *Drug Metab. Rev.* **27**, 179−189.

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