Alkylnaphthalene. XI. Pulmonary Toxicity of Naphthalene, 2-Methylnaphthalene, and Isopropylnaphthalenes in Mice

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Pulmonary toxicity of naphthalene (NAP), 2-methylnaphthalene (2-MN), 2-isopropylnaphthalene (2-IPN) and 2,6-diisopropylnaphthalene (2,6-DIPN) was studied in mice. Twenty four h after the intraperitoneal (i.p.) administration of NAP (200 mg/kg (1.6 mmol)) or 2-MN (400 mg/kg (2.8 mmol)), pulmonary damage was detected. Prior treatment with diethyl maleate resulted in enhancement of NAP and 2-MN-induced bronchiolar damage. In contrast to the effects of NAP and 2-MN, injections of 2-IPN (3000 mg (17.6 mmol)/kg) and 2,6-DIPN (3000 mg (14.2 mmol)/kg) did not cause detectable pulmonary damage. Injections of NAP and 2-MN caused considerable depletion of pulmonary reduced glutathione (GSH), while injections of 2-IPN and 2,6-DIPN caused only a slight depletion. There were general decreases in the binding of the compounds to lung slices with increasing number of carbons of the alkyl substituent. Pretreatment with a cytochrome P-450 inducer (β-naphthoflavone) increased the binding of NAP, 2-MN, and 2-IPN to lung slices. Treatments with NAP, 2-MN, 2-IPN and 2,6-DIPN did not affect the lipid peroxidation or phospholipid contents in the lung.

These results suggest that the difference in pulmonary toxicity among NAP, 2-MN, 2-IPN, and 2,6-DIPN may be dependent on the ability of these compounds to irreversibly bind to lung tissue.

Keywords naphthalene; 2-methylnaphthalene; isopropylnaphthalene; pulmonary toxicity; Clara cell; reduced glutathione; binding to lung slice; lipid peroxidation; phospholipid

Isopropylnaphthalenes have recently been used as solvents for duplicating papers and as heat transfer media as one of the substitutes for polychlorinated biphenyls.[1,2] In view of the increase in the use of isopropylnaphthalenes, it is desirable to study their toxicity and biological fate. Recently, we reported the absorption, tissue distribution, and excretion of 2-isopropylnaphthalene (2-IPN) in rats after single and repeated oral administration of the substance,[3] and the identification of urinary and biliary metabolites of 2-IPN in the animals.[4,5] We also reported the identification of metabolites of 2,6-diisopropylnaphthalene (2,6-DIPN) in rats.[6,7]

Naphthalene (NAP) and 2-methylnaphthalene (2-MN) produce pulmonary damage in mice, affecting primarily the nonciliated bronchiolar lining (Clara) cells and secondarily, the bronchiolar ciliated cells.[8,9] Whether similar damage can be produced by isopropylnaphthalenes has not, to our knowledge, been reported. We therefore investigated the pulmonary toxicity of 2-IPN and 2,6-DIPN to compare with that of NAP and 2-MN in mice.

Experimental

Materials Pure 2,6-DIPN (mp 69.5 °C) was a gift from Kureha Chemical Co. (Tokyo). 2-IPN was purchased from Tokyo Kasei Chemical Co. (Tokyo) and purified by column chromatography. NAP, 2-MN, thiobarbituric acid, piperonyl butoxide (PIP), osmium tetraoxide, disodium ethylenediaminetetraacetate (EDTA) and organic solvents were purchased from Wako Pure Chemical Ind. (Osaka). β-Naphthoflavone (BNF) was purchased from Nacalai Tesque. Inc. (Tokyo). Tissue culture reagents (Dulbecco's minimal essential medium, Ham's F-12k amino acid mixture,[10] and fetal bovine serum (FBS)) were purchased from Flow Lab. Inc. (Irvine, Scotland). All other chemicals were of reagent grade.

Animals Male ddY mice, weighing 18—23 g, were purchased from Inoue Breeding Lab. (Kumamoto). The animals were housed on hard wood bedding and allowed free access to food and water. They were not used sooner than 3 d after receipt from the supplier.

Microscopy For the histomorphological experiments involving scanning electron microscopy, mice were dosed intraperitoneally (i.p.) with varying amounts of NAP, 2-MN, 2-IPN or 2,6-DIPN in olive oil. Diethyl maleate (DEM) (600 μl/kg) was given 1 h prior to the administration of these compounds, and the mice were sacrificed with pentobarbital (0.15 ml of 50 mg/ml solution) at 24 h after the administration. The thorax was opened, the trachea cannulated, and the lungs perfused with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) until the lungs were fully expanded. On complete lung expansion, the trachea was ligated and the lungs excised and placed in a 20-ml vial with 2% glutaraldehyde. After 24 h, the lungs were sectioned with a razor blade into multiple cross sections varying in size from 5 × 3 to 9 × 3 mm, washed with 0.1 M phosphate buffer (pH 7.2) followed by postfixation with osmium tetraoxide and dehydration in an ascending alcohol series. The tissues were dried with a Hitachi critical point dryer. After drying, the specimens were mounted on stubs, coated with gold–palladium and examined with a Hitachi S-510 scanning electron microscope at magnifications of 2000× and 5000× at an accelerating voltage of 15 kV.

Assay for Reduced Glutathione (GSH) in Plasma and Lung Mice were injected i.p. with NAP (1, 2 or 3 mmol/kg), 2-MN (1, 2 or 3 mmol/kg), 2-IPN (1, 2, 3, 5.9 (1000 mg) or 17.6 (3000 mg) mmol/kg) or 2,6-DIPN (1, 2, 3, 4.7 (1000 mg) or 14.2 (3000 mg) mmol/kg). Animals were then sacrificed by an overdose of pentobarbital sodium (0.15 ml of 50 mg/ml solution) at 6 and 12 h after the administration. The chest was opened, and the blood samples were removed from the heart with syringes containing 0.1 ml of 0.025 M EDTA, 0.1 mg of heparin and 0.9 mg of NaCl to prevent clotting and oxidation. Next, the lung was perfused by injecting ice-cold 0.1 M phosphate buffer (pH 8.0, containing 0.05% EDTA) into the ventricle of the heart after severing the descending aorta. The lung was removed, blotted dry, weighed, and used immediately. The lung, usually 100 to 250 mg, was homogenized on ice using a potter Elvehjem homogenizer. The solution used for homogenization consisted of 5.0 ml of 0.1 M phosphate buffer (pH 8.0, containing 0.05% EDTA) and 1.0 ml of 25% H_3PO_4. The homogenate was centrifuged at 4 °C at 105000 *g* for 30 min to obtain the supernatant for the assay of GSH. Determination of GSH was performed by a modification of the method of Hissin and Hilf.[11] Briefly, to 0.3 ml of the supernatant, 5.0 ml of the phosphate buffer (pH 8.0, containing 0.05% EDTA), and 0.3 ml of *ortho*-phthalaldehyde methanol solution (1 mg/ml) were added. After mixing and incubation at room temperature for 15 min, the solution was transferred to a quartz cuvette. Fluorescence at 420 nm was determined with the activation at 350 nm. The blood samples were immediately centrifuged. The plasma was rapidly deproteinized by mixing it vigorously with one-half volume of 25% H_3PO_4 and centrifuging the mixture. The deproteinized samples were analyzed within 10 min after the samples were withdrawn.

Preparation and Incubation of Lung Slices To induce cytochrome P-450 enzymes, mice were pretreated i.p. with BNF (80 mg/kg) for 3 d. To inhibit cytochrome P-450 enzyme, they were pretreated i.p. with PIP (1600 mg/kg) in olive oil prior to the experiment. Control mice received olive oil alone.

Lungs of the mice were inflated *via* a tracheal cannula with a solution containing 2% agar in a phosphate-buffered nutrient solution maintained at 43°C to keep the agar fluid. The nutrient solution consisted of Dulbecco's phosphate buffered saline, F12K amino acid mixture and 1% FBS. After filling, the lungs were transferred to ice-cold nutrient solution to allow the agar to harden. Uniform slices of mouse lung were prepared by the method of Rasmussen.[12] Substrate solution (liposome) was prepared by the method of Batzri and Korn.[13] One half ml of ethanol solution containing 35.4 mg of egg lecithin and 5 μmol of substrate (NAP, 2-MN, 2-IPN or 2,6-DIPN) was rapidly injected through a Hamilton syringe into 7.5 ml of purified water, and the mixture was dialyzed for 1 h against one liter of purified water. The concentration of the substrate solution was adjusted to one-half μmol/ml. Lung slices were equilibrated in gassed (O_2/CO_2 : 95/5) nutrient solution with 1% FBS at 37 °C for 10—20 min before use. After equilibration, slices (100—200 mg) were transferred to freshly gassed nutrient solution with 1% FBS to which was added 0.2 ml of the substrate (100 nmol). After incubation at 37 °C for 0.5, 1.0 and 2.0 h, the slices were homogenized with 2 ml of ice-cold methanol. The homogenate was centrifuged at 3000 rpm for 10 min. The supernatant was subjected to high pressure liquid chromatography (HPLC) to determine the concentration of the compound (A nmol/mg protein). The concentration in the medium was determined by HPLC directly (B nmol/mg protein), and A–B (nmol/mg protein) was defined as the concentration of the compound bound to slices irreversibly.

HPLC HPLC was carried out with a Hitachi model 665 system on column packed with RiChrosorb RP-18 (25 × 0.26 cm). The solvent system was CH_3CN–H_2O (1 : 2, v/v) containing 0.002 M tetra-*n*-butylammonium hydroxide. The flow rate was 1.5 ml/min. A ultraviolet (UV) deterctor was used to measure absorbance at 230 nm.

Phospholipid (PL) Determination Mice were dosed i.p. with NAP (753 mg (5.9 mmol)/kg), 2-MN (835 mg (5.9 mmol)/kg), 2-IPN (1,000 mg (5.9 mmol)/kg) or 2,6-DIPN (1,248 mg (5.9 mmol)/kg), then were sacrificed by an overdose of pentobarbital sodium at 6 and 12 h after the administration. The lung was removed by the method described above using 1.15% KCl as refluxing solution. The minced tissues were homogenized with 9 volumes of ice-cold 1.15% KCl. The total PL of lung homogenate was extracted with chloroform–methanol (2 : 1, v/v) according to the procedure of Folch *et al.*[14] The extract was evaporated to dryness (total PL), and the lipid residue obtained was separated by thin-layer chromatography on silica gel G plate, using chloroform–methanol–H_2O (65 : 24 : 4, v/v) as the developing solvent. The lipids were located by staining with iodine vapor. The area of silica corresponding to phosphatidylcholine (PC) was scraped off into a glass tube. PC was extracted from the silica with methanol and the methanol extract was evaporated to dryness (PC fraction). The total PL and PC were determined as phosphorus by the method of Bartlett.[15]

Determination of Thiobarbituric Acid (TBA) Value Mice were dosed i.p. with NAP (200 (1.6 mmol) or 400 (3.1 mmol) mg/kg), 2-MN (200 (1.4 mmol) or 400 (2.8 mmol) mg/kg), 2-IPN (1000 (5.9 mmol) or 3000 (17.6 mmol) mg/kg) or 2,6-DIPN (1000 (4.7 mmol) or 3000 (14.2 mmol) mg/kg), then were sacrificed by an overdose of pentobarbital sodium at 12 h after the administration. The lung was removed by the method described above using 0.01 M phosphate buffer (pH 7.4) containing 1.15% KCl and 0.025% EDTA as refluxing solution. The minced tissues were homogenized with 19 volumes of ice-cold 0.01 M phosphate buffer (pH 7.4) containing 1.15% KCl and 0.025% EDTA. TBA values were determined by the method of Uchiyama and Mihara.[16] The assay mixture contained 0.75 ml of homogenate, 3 ml of 1% phosphoric acid, and 1.0 ml of 0.6% TBA in 50% acetate solution. The mixture was heated in a boiling water bath for 45 min and cooled in water. Then, the reaction products by TBA were extracted with 2.0 ml of *n*-butanol. The butanol phase was separated by centrifugation and the absorbance of *n*-butanol solution at 532 nm was measured using a Hitachi 100-60 spectrophotometer. 1,1,3,3-Tetraethoxypropane was used as the standard of malondialdehyde.

Protein Determination The determination of protein was carried out by the method of Lowry *et al.*[17] with bovine serum albumin as standard.

Results

Pulmonary Toxicity and Microscopy Examination by scanning electron micrography of the lungs of mice treated with 200 mg (1.6 mmol)/kg of NAP or 400 mg (2.8 mmol)/kg of 2-MN indicated abnormalities in the Clara cells (Fig. 1 b,c) compared to controls (Fig. 1a). At either doses of 200—600 mg (1.6—4.7 mmol)/kg of NAP or 400—600 mg (2.8—4.2 mmol)/kg of 2-MN, there was a dose-dependent increase in lung damage, mainly involving the bronchiolar region. At the higher dose of NAP (600 mg (4.7 mmol)/kg) or 2-MN (600 mg (4.2 mmol)/kg), a number of exfoliated Clara cells were observed in bronchiolar lumen. No pulmonary damage was detected in mice at the dose of NAP (100 mg (0.78 mmol)/kg) or 2-MN (100 or 200 mg (0.70 or 1.41 mmol)/kg). These results confirm and extend earlier reports on bronchiolar damage by NAP and 2-MN.[8,9,18] In contrast to the effects of NAP and 2-MN, no pulmonary damage was observed in mice injected i.p. 24 h earlier with either 2-IPN (100—3000 mg (0.59—17.6 mmol)/kg) or

Table I. Dose–Response Pulmonary GSH Depletion at 6 h after i.p. Administration of the Test Compounds

Compound	GSH (mg/g wet tissue)[a]		
	Dose (mmol/kg body weight)		
	1	2	3
NAP	0.223 ± 0.002[b]	0.144 ± 0.047[b]	0.070 ± 0.020[b]
2-MN	0.217 ± 0.022[b]	0.207 ± 0.001[b]	0.096 ± 0.030[b]
2-IPN	0.220 ± 0.014[b]	0.227 ± 0.022[b]	0.157 ± 0.010[b]
2,6-DIPN	0.238 ± 0.030	0.237 ± 0.017	0.174 ± 0.013[b]
Control		0.269 ± 0.014	
DEM (600 μl/kg body)		0.170 ± 0.011[b]	

a) The values represent means ± S.D. for 3 to 5 animals. *b*) Significantly different from control ($p < 0.05$).

Table II. Dose–Response Pulmonary GSH Depletion at 12 h after i.p. Administration of the Test Compounds

Compound	GSH (mg/g wet tissue)[a]		
	Dose (mmol/kg body weight)		
	1	2	3
NAP	0.229 ± 0.038	0.224 ± 0.053	0.126 ± 0.012[b]
2-MN	0.173 ± 0.008[b]	0.181 ± 0.042[b]	0.132 ± 0.003[b]
2-IPN	0.179 ± 0.020[b]	0.199 ± 0.049[b]	0.137 ± 0.046[b]
2,6-DIPN	0.224 ± 0.017	0.212 ± 0.009[b]	0.209 ± 0.020[b]
Control		0.254 ± 0.007	
DEM (600 μl/kg body)		0.187 ± 0.013[b]	

a) The values represent means ± S.D. for 3 to 5 animals. *b*) Significantly different from control ($p < 0.05$).

Table III. Dose–Response Pulmonary GSH Depletion at 6 and 12 h after i.p. Administration of 2-IPN and 2,6-DIPN

Compound	Dose (mg/kg body) (mmol/kg body)	Time (h)	GSH[a] (mg/g wet tissue)
2-IPN	1000 (5.9)	6	0.148 ± 0.022[b]
	1000 (5.9)	12	0.116 ± 0.011[b]
	3000 (17.6)	6	0.141 ± 0.030[b]
	3000 (17.6)	12	0.135 ± 0.021[b]
2,6-DIPN	1000 (4.7)	6	0.184 ± 0.033[b]
	1000 (4.7)	12	0.168 ± 0.017[b]
	3000 (14.2)	6	0.179 ± 0.024[b]
	3000 (14.2)	12	0.166 ± 0.005[b]
Control			0.254 ± 0.007

a) The values represent means ± S.D. for 3 to 5 animals. *b*) Significantly different from control ($p < 0.05$).

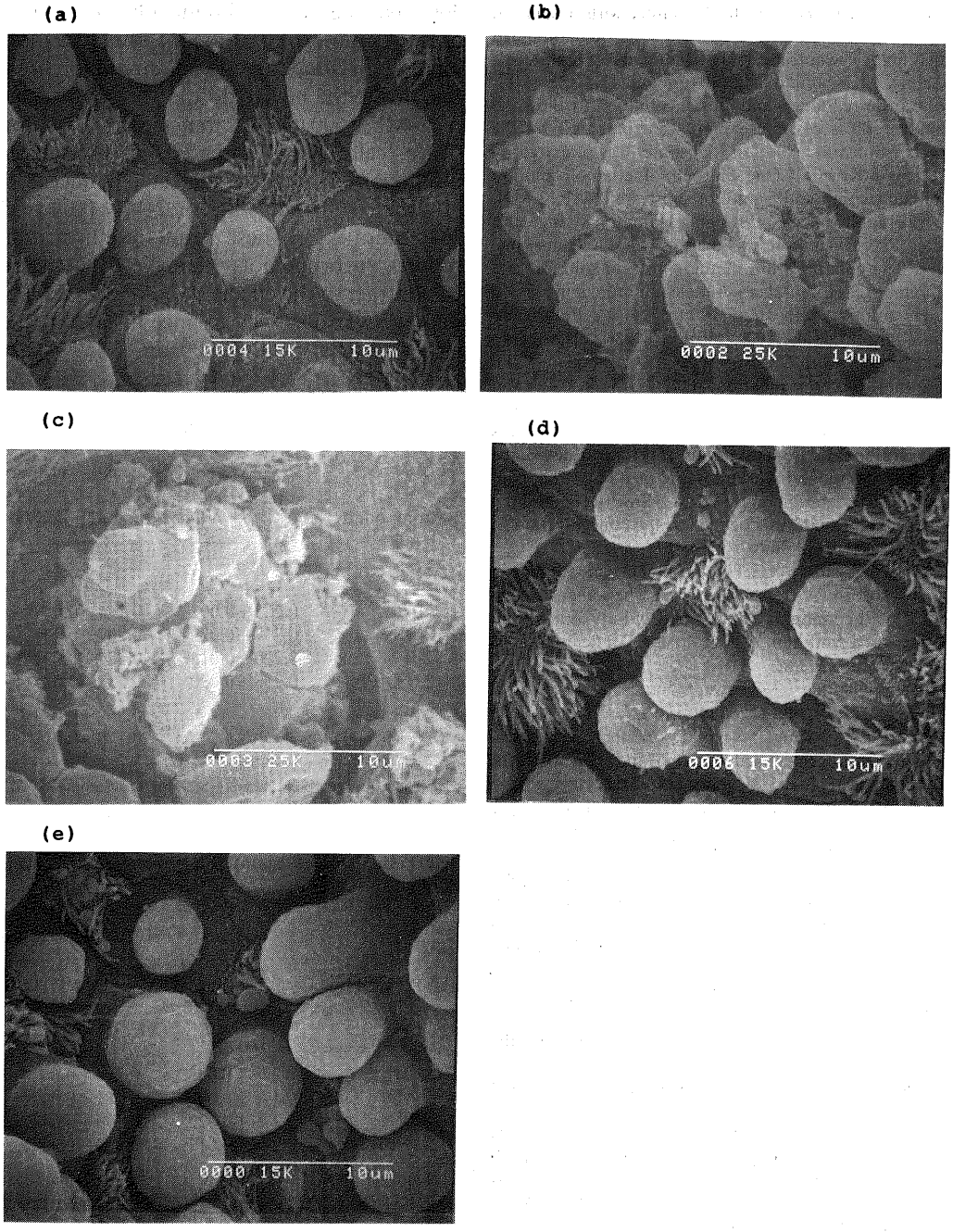

Fig. 1. Scanning Electron Micrographs of Clara Cells of Mice Following i.p. Injection of Olive Oil, NAP, 2-MN, 2-IPN, or 2,6-DIPN

(a) control (olive oil, 0.2 ml), (b) NAP (200 mg (1.6 mmol)/kg), (c) 2-MN (400 mg (2.8 mmol)/kg), (d) 2-IPN (3000 mg (17.6 mmol)/kg), (e) 2,6-DIPN (3000 mg (14.2 mmol)/kg).

2,6-DIPN (100—3000 mg (0.47—14.2 mmol)/kg) (Fig. 1 d,e). Prior treatment with DEM resulted in a substantial enhancement of NAP and 2-MN induced bronchiolar damage (Fig. 2). Extensive sloughing and exfoliation of bronchiolar epithelial cells occurred in the lungs of all mice injected with 100 mg (0.78 mmol)/kg of NAP or 200 mg (1.4 mmol)/kg or 2-MN after treatment with DEM, whereas such a phenomenon was not observed in mice treated with 100 mg (0.78 mmol)/kg of NAP or 200 mg (1.4 mmol)/kg of 2-MN alone. No pulmonary damage was detected in mice injected with 3000 mg (17.6 mmol)/kg of 2-IPN or 3000 mg (14.2 mmol)/kg of 2,6-DIPN after DEM treatment.

Dose Response for GSH Depletion Increasing doses of NAP and 2-MN from 1 to 3 mmol/kg resulted in a decrease in pulmonary GSH levels. As the dose was increased from 1 mmol to 3000 mg (17.6 or 14.2 mmol)/kg of 2-IPN or 2,6-DIPN, there was a moderate decrease in GSH levels (Tables I, II and III). GSH levels in the lung at the doses of 3 mmol/kg of NAP and 3 mmol/kg of 2-MN were less than 50% of control levels at 6 h after the administration. Treatments with NAP, 2-MN, 2-IPN and 2,6-DIPN in mice did not affect the plasma GSH levels at 6 h after the administrations (Table IV).

Binding of NAP, 2-MN, 2-IPN and 2,6-DIPN to Lung Slices Binding of either NAP, 2-MN, 2-IPN or 2,6-DIPN to lung slices was time dependent, reaching a plateau in about 1 h (Fig. 3). The binding of the compounds to slices decreased with increasing number of carbon of alkyl

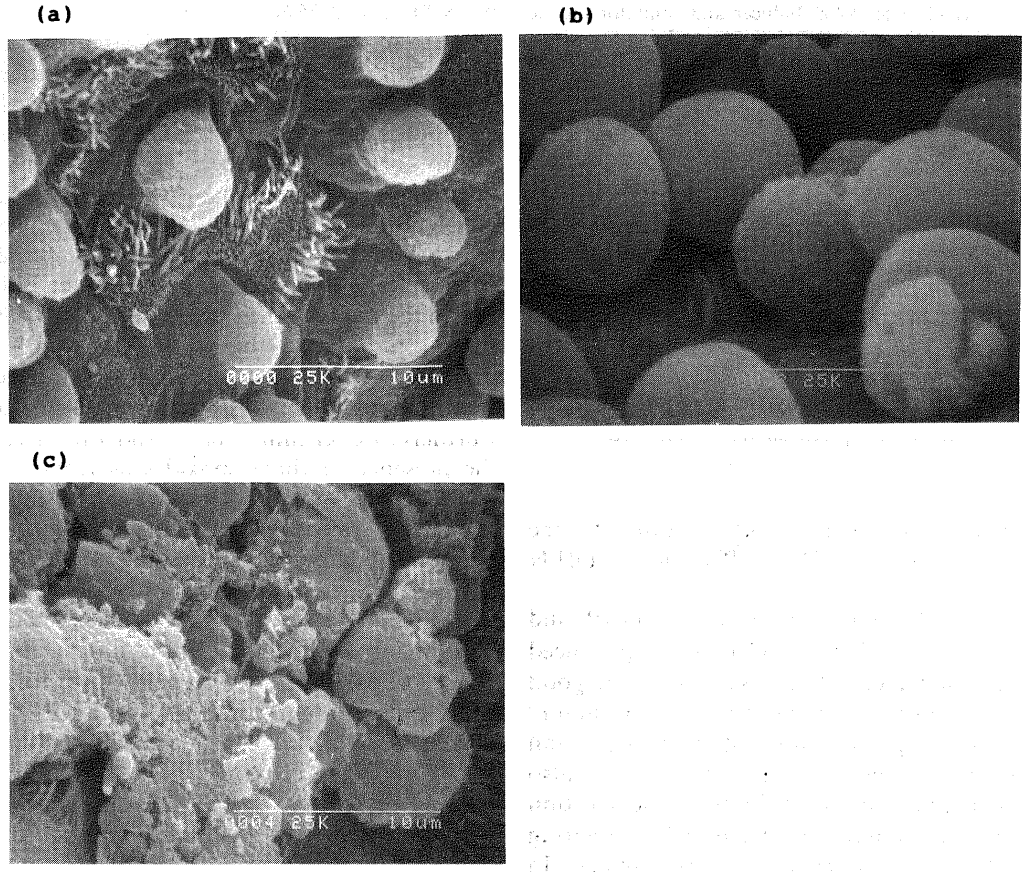

Fig. 2. Scanning Electron Micrographs of Clara Cells of Mice

DEM (600 μl/kg) was i.p. injected 1 h prior to i.p. injection of olive oil, NAP, or 2-MN. (a) DEM + olive oil, (b) DEM + NAP (100 mg (0.78 mmol)/kg), (c) DEM + 2-MN (200 mg (1.4 mmol)/kg).

TABLE IV. Plasma GSH Levels at 6 h after i.p. Administration of the Test Compounds

Treatment	Dose (mg/kg)	GSH (μg/ml plasma)[a]
Control		13.6±2.7
NAP	3 mM	13.8±4.3
2-MN	3 mM	13.7±3.3
2-IPN	3 mM	14.2±5.6
	5.9 mM (1000 mg)	13.6±5.6
	17.6 mM (3000 mg)	13.9±1.4
2,6-DIPN	3 mM	14.7±5.2
	4.7 mM (1000 mg)	14.1±4.9
	14.2 mM (3000 mg)	14.1±3.1
DEM	600 μl	11.2±3.9

a) The values represent means±S.D. for 6 animals.

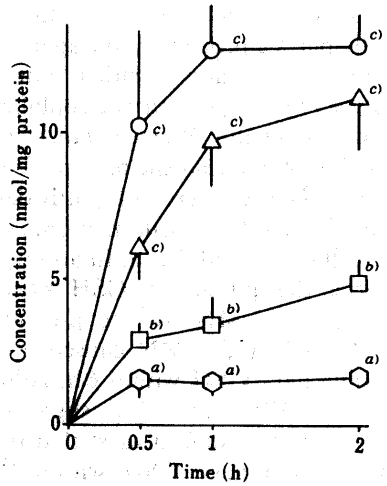

Fig. 3. The Binding of NAP, 2-MN, 2-IPN, or 2,6-DIPN to Mouse Lung Slices

Concentration of NAP (○), 2-MN (△), 2-IPN (□) or 2,6-DIPN (○) bound to lung slices (nmol/mg protein). The values represent means±S.D. for 4 separate incubations. *a*) Significantly different from NAP, 2-MN and 2-IPN, $p<0.05$, *b*) Significantly different from NAP, 2-MN and 2,6-DIPN, $p<0.05$, *c*) Significantly different from 2-IPN and 2,6-DIPN, $p<0.05$.

substituent.

Whether reactive metabolites of the compounds, which were produced by cytochrome P-450 enzymes, participate in the binding of the compounds to lung slices was examined (Table V). Metabolic induction using BNF, an inducer of cytochrome P-450 enzymes, increased the binding of NAP, 2-MN and 2-IPN to lung slices, although this increase was only significant in the binding of NAP. In contrast, pretreatment with PIP, an inhibitor of cytochrome P-450 enzymes, markedly decreased the binding of NAP, 2-MN and 2-IPN. Pretreatments with BNF and PIP did not affect the binding of 2,6-DIPN to lung slices.

Effect on Pulmonary PL The levels of PL and PC in the lung of mice at 6 or 12 h after the administration of NAP, 2-MN, 2-IPN or 2,6-DIPN were determined. Treatments with these compounds did not affect the levels of PL and PC in the lung.

Effect on Pulmonary Peroxidation The TBA values in the lung of mice treated with NAP, 2-MN, 2-IPN or

TABLE V. Effects of Cytochrome P-450 Inducer and Inhibitor on the Binding of NAP, 2-MN, 2-IPN, or 2,6-DIPN to Mouse Lung Slices

Compound	Concentration (nmol/mg protein)[a]		
	Control	BNF	PIP
NAP	15.1±1.6	21.3±4.4[b]	10.9±0.3[b]
2-MN	8.4±1.5	10.7±1.4	5.2±1.3[b]
2-IPN	4.6±1.1	5.2±0.4	1.4±0.4[b]
2,6-DIPN	1.9±1.1	1.8±0.4	0.8±0.2

a) The values represent means±S.D. for 4 animals. *b*) Significantly different from control ($p<0.05$).

2,6-DIPN were determined. No change in lipid peroxidation in lungs exposed to these compounds was observed.

Discussion

The present study was carried out to evaluate the pulmonary toxicity of NAP, 2-MN, 2-IPN and 2,6-DIPN in mice.

I.p. injections of 200 mg (1.6 mmol)/kg of NAP and 400 mg (2.8 mmol)/kg of 2-MN into mice produced pulmonary cellular damage. These results were in good agreement with the reports of Nahvi *et al.*[8] and Griffin *et al.*[9] Lung damage was increased substantially by depletion of GSH after DEM treatment (Fig. 2b, c); this result also concurred with data of Griffin *et al.*[9] However, the injection of 2-IPN (3000 mg (17.6 mmol)/kg) or 2,6-DIPN (3000 mg (14.2 mmol)/kg) did not produce pulmonary damage. In spite of pretreatment with DEM, no pulmonary damage was produced by 2-IPN or 2,6-DIPN injection. These results suggest that the pulmonary toxicity depends markedly on the number of carbon of the substituent and the damage tends to decrease with an increase in this number.

GSH generally plays a protective role against the toxicity of many xenobiotics by reacting, either spontaneously or in enzyme catalyzed reactions, with electrophilic metabolites to form nontoxic conjugates. Warren *et al.*[18] reported that reactive metabolites of naphthalene bind preferentially with GSH, but, when GSH levels were sufficiently depleted, these metabolites bind covalently to tissue macromolecules, which might result in cell injury and death. In addition, Griffin *et al.*[19] also reported that pulmonary GSH is decreased after the administration of 2-MN. In the present study, administrations of NAP and 2-MN caused a considerable depletion of pulmonary GSH within 6 h. Administrations of 2-IPN and 2,6-DIPN caused a slight depletion of pulmonary GSH. These results suggest that the considerable depletion of pulmonary GSH within 6 h after the administration reflects pulmonary damage. The depletion of GSH markedly reflected the pulmonary bronchiolar damage and the binding of the compounds to lung slices.

It has been reported that many drugs cause pulmonary lipid peroxidation and phospholipidosis, as an example of lung toxicity resulting from their accumulation and persistence.[20,21] In the present study, we attempted to confirm the relationship between pulmonary toxicity and pulmonary lipid peroxidation or PL. Treatments with NAP, 2-MN, 2-IPN and 2,6-DIPN did not change the pulmonary lipid peroxidation and PL concentration in the lung. Thus, it is suggested that the lipid peroxidation and phospholipidosis are not involved in the pulmonary damage by NAP and 2-MN.

Warren *et al.*[18] reported that cytochrome P-450 dependent metabolic activation of NAP plays a role in the bronchiolar damage; that is, NAP is metabolized to reactive metabolite, such as an epoxide, which covalently binds to macromolecules. Further, it has been reported that phenobarbital pretreatment produces a striking increase in covalent binding of NAP to macromolecules in the lung.[22] Horning *et al.*[23] and Jerina *et al.*[24] reported the presence of epoxide intermediate as reactive metabolites of NAP in rat. In the present study, the binding of NAP to mouse lung slices was increased by pretreatment with BNF and was decreased by pretreatment with PIP, indicating that NAP was metabolized to epoxide to bind to lung slices. Furthermore, Griffin *et al.*[19] and Breger *et al.*[25] reported the presence of three epoxides as reactive metabolites of 2-MN in mice and rats. Griiffin *et al.*[19] suggested that these epoxides covalently bind to macromolecules and produce pulmonary damage. The binding of 2-IPN and 2,6-DIPN to lung slices was not significantly affected by pretreatment with BNF. These results suggest that 2-IPN and 2,6-DIPN bind mainly hydrophobically to lung slices.

We propose a possible explanation for the damage to Clara cells produced by NAP and 2-MN. Due to the high degree of cellular localization of pulmonary cytochrome P-450 in Clara cells,[26-28] the formation and covalent binding of reactive metabolites of NAP and 2-MN may occur in these cells, resulting in the cell damage. Further studies will be needed to determine the mechanism of the pulmonary damage induced by reactive metabolites of NAP and 2-MN.

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