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COMPARISON OF THE ARACHIDONIC ACID AND NADPH-DEPENDENT MICROSOMAL METABOLISM OF NAPHTHALENE AND 2-METHYLNAPHTHALENE AND THE EFFECT OF INDOMETHACIN ON THE BRONCHIOLAR NECROSIS*

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Abstract—Naphthalene and 2-methylnaphthalene cause a highly organo- and species-selective lesion of the pulmonary bronchiolar epithelium in mice. Naphthalene- but not 2-methylnaphthalene-induced pulmonary bronchiolar injury is blocked by prior administration of the cytochrome P-450 monooxygenase inhibitor piperonyl butoxide, thus suggesting that metabolism by enzymes other than the P-450 monooxygenases may be important in 2-methylnaphthalene-induced lung injury. Since many of the polycyclic aromatic hydrocarbons are metabolized by the prostaglandin endoperoxide synthetase system and because detectable xenobiotic metabolizing activity has been associated with the prostaglandin synthetases in the Clara cell, the studies reported here were done to compare NADPH- versus arachidonate-dependent metabolism of naphthalene and 2-methylnaphthalene *in vitro* and to determine whether indomethacin, a potent inhibitor of prostaglandin biosynthesis, was capable of blocking the *in vivo* toxicity of these two aromatic hydrocarbons. The NADPH-dependent metabolism of naphthalene and 2-methylnaphthalene to covalently bound metabolites in lung or liver microsomal incubations occurred at easily measurable rates. Renal microsomal NADPH-dependent metabolism of either substrate was not detected. The formation of covalently bound naphthalene or 2-methylnaphthalene metabolites was dependent upon NADPH and was inhibited by the addition of reduced glutathione, piperonyl butoxide, and SKF 525A. Covalent binding of radioactivity from [^{14}C]2-methylnaphthalene also was strongly inhibited by incubation in a nitrogen atmosphere or at 2°. The arachidonic acid-dependent formation of reactive metabolites from naphthalene or 2-methylnaphthalene was undetectable in microsomal incubations from lung, liver or kidney. Indomethacin, 1 hr before and 6 hr after the administration of 300 mg/kg naphthalene or 2-methylnaphthalene, failed to block the pulmonary bronchiolar injury induced by these aromatic hydrocarbons. These studies suggest that the major enzymes involved in the metabolic activation of naphthalene or 2-methylnaphthalene *in vitro* are the cytochrome P-450 monooxygenases and that cooxidative metabolism by the prostaglandin synthetases appears to play little role in the formation of reactive metabolites *in vitro*.

There is now ample experimental evidence demonstrating the importance of the prostaglandin H synthetase system in the cooxidation of a wide variety of xenobiotic substrates *in vitro* [1–3]. In many cases the prostaglandin synthetases have been shown to complement the cytochrome P-450 monooxygenases in the metabolic formation of ultimate carcinogenic metabolites (such as the dihydrodiol epoxides derived from polycyclic aromatic hydrocarbons like benzo[*a*]pyrene [4]). Moreover, recent studies have demonstrated arachidonic acid-dependent formation of tetrols and covalently bound derivatives from benzo[*a*]pyrene in microsomes prepared from tissues that have low cytochrome P-450 monooxygenase activity such as rat, guinea pig and human lung microsomes [5] and in highly enriched fractions of Clara cells [6], a pulmonary cell type that is exquisitely sensitive to the cytotoxic and carcinogenic effects of a number of chemicals [7]. Thus, while the ability of the prostaglandin synthetase system to cooxidize chemicals *in vitro* has been documented thoroughly, the relevance of this enzyme system in the metabolism of chemicals *in vivo* and in the toxic and carcinogenic effects mediated by metabolic activation of inert compounds remains to be established.

The widespread industrial use of naphthalene and 2-methylnaphthalene combined with the human

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exposures that occur from such diverse sources as drinking water and cigarette smoke [8–10] underscores the possible importance of the finding that both compounds result in dose-dependent injury to the pulmonary bronchiolar epithelium in the mouse after intraperitoneal administration [11–14]. Although the target tissue, cell and species for both naphthalene and 2-methylnaphthalene are identical (Clara cell of the mouse) [11, 12, 14], data on the metabolic fate and its relationship to the pulmonary lesion by these two compounds differ markedly. Naphthalene is rapidly metabolized to reactive metabolites that become covalently bound to tissue macromolecules in the lung, liver and kidney. There is a glutathione threshold for covalent binding, and the dose at which this threshold occurs corresponds to the dose at which prominent lung lesions are first observed. The covalent binding, depletion of glutathione and the bronchiolar lesion are inhibited by prior treatment with piperonyl butoxide and are enhanced considerably by the glutathione depletor diethyl maleate. However, data indicating that the levels of covalently bound metabolite in lung, liver and kidney *in vivo* did not correlate with the tissue selectivity of naphthalene-induced toxicity raised the possibility that reactive metabolites were not involved in the series of events leading to lung injury [13]. In contrast to these data with naphthalene, 2-methylnaphthalene-induced bronchiolar injury was not affected by prior treatment with either piperonyl butoxide or with diethyl maleate [15]. Moreover, an interrelationship between covalent binding, glutathione depletion and the pulmonary lesion was not apparent with 2-methylnaphthalene, thus suggesting that the mechanisms for the lung toxicity by these compounds might differ.

A further anomalous finding in the studies with naphthalene/2-methylnaphthalene was that the NADPH-dependent metabolism of naphthalene or 2-methylnaphthalene by renal microsomal enzymes is slow or nondetectable, yet the levels of covalently bound metabolites in the kidney *in vivo* are as high if not higher than tissues that are capable of the rapid conversion of these compounds to covalently bound metabolites [15–17]. These data combined with the studies showing that 2-methylnaphthalene-induced bronchiolar injury or the covalent binding of 2-methylnaphthalene metabolites was not affected substantially by prior treatment with piperonyl butoxide suggested the possibility of an alternate enzymatic pathway in their activation. Since the prostaglandin synthetases have been shown to catalyze the metabolism of aromatic hydrocarbons and dihydrodiols derived therefrom and because detectable activities of this enzyme have been demonstrated in Clara cells of the rat, it seemed possible that this enzyme system might play a role in mediating the formation of covalently bound and/or lung toxic metabolites of naphthalene and 2-methylnaphthalene. Therefore, the aim of the studies presented here was to compare arachidonic acid- versus NADPH-dependent metabolism of naphthalene and 2-methylnaphthalene to covalently bound metabolites and to determine whether the cyclooxygenase inhibitor, indomethacin, could modulate the pulmonary bronchiolar necrosis of either of these hydrocarbons.

MATERIALS AND METHODS

Radiochemicals. [^{14}C]Naphthalene (5 mCi/mmole) was purchased from Amersham/Searle, Arlington Heights, IL, and was shown to be >99.5% radiochemically pure by high pressure liquid chromatography on a C_{18} column eluted with 70% methanol/water. 2-Methylnaphthalene (8-^{14}C, >99% radiochemical purity [16]) was purchased from California Bionuclear, Sun Valley, CA, at a specific activity of 5 mCi/mmole.

Chemicals. Components of the NADPH-generating system (NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase), arachidonic acid and indomethacin were purchased from the Sigma Chemical Co., St. Louis, MO. Reduced glutathione was from Calbiochem, La Jolla, CA. Piperonyl butoxide was purchased from the Chemical Dynamics Corp., South Plainfield, NJ, and SKF 525A was a gift of the Smith Kline, Beckman Co., Philadelphia, PA. All other chemicals were reagent grade or better.

Animals and drug treatments. Male, Swiss–Webster mice (20–25 g) were purchased from the Charles River Breeding Laboratories, Wilmington, MA, and were maintained over hardwood bedding in a HEPA/activated carbon laminar flow cage rack for at least 5 days after receipt from the supplier. Mice were allowed free access to food and water. Phenobarbital was added to the drinking water at 0.1% (w/v) for 5 days followed by 1 day of fresh water to induce the cytochrome P-450 monooxygenases as noted in the legends to the tables. Indomethacin was dissolved in a small volume of 0.1 M NaOH, the solution was buffered to pH 8.0, and sufficient NaCl was added to bring the solution to 0.9% (w/v). Indomethacin was administered at 4 mg/kg 1 hr before and 5 hr after naphthalene or 2-methylnaphthalene. This dose had been shown previously to result in significant inhibition of the prostaglandin synthetases in the mouse [18] without significant inhibition of the cytochrome P-450 monooxygenases [19]. Naphthalene and 2-methylnaphthalene were dissolved in corn oil and administered at a dose of 300 mg/kg. All compounds were administered intraperitoneally in 5 ml solution/kg body wt.

Preparation of microsomes and incubations. Pulmonary, hepatic and renal microsomes were prepared by ultracentrifugation by techniques described in detail previously [17]. The final microsomal pellet was resuspended in 0.1 M phosphate buffer, pH 7.4. All incubations were prepared on ice, substrate was added last, and the vessels were tightly capped and transferred to a shaking water bath at 37°. Specific details of the incubations are given in the table legends. Reactions were stopped by immersing the incubation vessels in an ice bath and adding 2 vol. of ice-cold methanol.

Covalent binding assay. Radioactivity covalently bound to microsomal protein was assessed after exhaustive solvent extraction as described previously [13].

Histology. Animals were killed by an overdose of pentobarbital, the chest cavity was opened, and Karnovsky's fixative [20] was infused intratracheally. Lungs were removed, placed in fixative and later

Table 1. Effects of various incubation conditions on hepatic microsome-catalyzed covalent binding of reactive metabolites from 2-methylnaphthalene and naphthalene

Incubation conditions*	Covalent binding† (nmoles/min/mg protein) 2-Methylnaphthalene	% Control	Naphthalene‡	% Control
None	0.005 ± 0.000		0.001 ± 0.000	
NADPH	0.185 ± 0.002	100	0.682 ± 0.017	100
NADPH + GSH	0.045 ± 0.005	24	0.524 ± 0.023	77
NADPH + piperonyl butoxide	0.113 ± 0.012	61	0.186 (N = 2)	27
NADPH + SKF 525A	0.114 ± 0.005	62	0.209 ± 0.007	31
NADPH + N_2 atmosphere	0.056 ± 0.005	30		
NADPH + inc. at 2°	0.090 ± 0.009	49		

* All incubations contained phenobarbital-induced microsomes (4 mg protein for the naphthalene incubations and 5 mg protein for the 2-methylnaphthalene incubations) and substrate (0.5 mM, 1242 dpm/nmole naphthalene, 1090 dpm/nmole 2-methylnaphthalene). NADPH-generating system, and SKF 525A (0.5 mM, dissolved in 0.1 M, pH 7.4, phosphate buffer), piperonyl butoxide (0.5 mM, added in 10 μl methanol) or glutathione (1 or 5 mM final concentration in the naphthalene and 2-methylnaphthalene incubations, respectively) were added as indicated. Incubations were conducted for 15 (naphthalene) or 10 (2-methylnaphthalene) min at 37°.

† Covalent binding values are the mean ± S.E. for three incubations.

‡ Data on naphthalene are taken from ref. 21 for comparison.

embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. The severity of bronchiolar injury was graded in a blind fashion according to the rating scheme presented in the legend to Table 3.

RESULTS

To determine whether the generation of reactive metabolites from 2-methylnaphthalene could be modulated *in vitro* by inhibitors of the cytochrome P-450 monooxygenase system, liver microsomes from phenobarbital-induced mice were incubated with [^{14}C]2-methylnaphthalene under various conditions. The data in Table 1 indicate that metabolism of 2-methylnaphthalene to covalently bound derivatives was dependent upon the presence of NADPH and was inhibited by the addition of piperonyl butoxide, SKF 525A or by incubation in an N_2 atmosphere. Inclusion of reduced glutathione or incubation at 2° resulted in markedly decreased levels of covalent binding. The rates of NADPH-dependent formation of covalently bound metabolites from naphthalene were 3- to 4-fold higher than with 2-methylnaphthalene, a result which is consistent with the higher levels of covalently bound metabolites from naphthalene versus 2-methylnaphthalene observed *in vitro* [13, 17]. The data in Table 1 indicate that the inhibition of reactive metabolite formation from naphthalene is considerably more pronounced than that observed in similar studies with 2-methylnaphthalene.

To examine the potential involvement of arachidonic acid-dependent prostaglandin synthetase in the metabolism of naphthalene and 2-methylnaphthalene to covalently bound derivatives *in vitro*, lung, liver and kidney microsomes were incubated in the presence of either NADPH-generating system or arachidonic acid. Consistent with previous studies on the covalent binding of reactive naphthalene metabolites *in vitro* [17], the data in Table 2 indicate that the rate of NADPH-dependent formation of covalently bound products by pulmonary microsomal enzymes was slightly higher than with hepatic microsomal enzymes. Similar results were obtained with 2-methylnaphthalene except that the overall rate of reactive metabolite formation was substantially slower. NADPH-dependent metabolism of either substrate was undetectable in renal microsomal incubations. Use of arachidonic acid as a cofactor in the incubations at concentrations similar to those used to study prostaglandin synthetase-dependent xenobiotic cooxidation [6] failed to catalyze the formation of covalently bound metabolites in any of the tissues studied. 1-Naphthol and naphthalene dihydrodiol were easily quantitated by high performance liquid chromatography (HPLC) in lung and liver microsomal incubations containing NADPH but were undetectable in incubations containing arachidonic acid as a cofactor (data not shown).

Although arachidonic acid supported metabolism of either naphthalene or 2-methylnaphthalene to reactive metabolites could not be demonstrated *in vitro*, the possibility that prostaglandin synthetase-dependent metabolism of one of the oxygenated metabolites derived from the cytochrome P-450-dependent biotransformation of naphthalene or 2-methylnaphthalene was the rate-limiting step in the formation of cytotoxic metabolites could not be excluded. Thus, groups of mice were treated with indomethacin (4 mg/kg, i.p., 1 hr before and 6 hr after a lung toxic dose of either naphthalene or 2-methylnaphthalene) to determine whether inhibition of prostaglandin biosynthesis would alter the pulmonary injury induced by these aromatic hydrocarbons. (This dose of indomethacin has been shown previously not to cause inhibition of hepatic monooxygenase activity [19] and has been shown to result in significant inhibition of prostaglandin biosynthesis [18].) Lung injury was not detected in any of the animals treated with saline and corn oil or indomethacin and corn oil. Light microscopic examination of lungs of mice treated with saline and naphthalene or saline plus 2-methylnaphthalene

Table 2. NADPH versus arachidonic acid dependent metabolism of naphthalene or 2-methylnaphthalene to covalently bound metabolites

Microsomes	Substrate	Cofactor	Covalent binding (nmoles/mg protein/min)
Lung	Naphthalene	NADPH	1.240
		Arachidonic acid	0.011
		None	0.021
Liver	Naphthalene	NADPH	0.926
		Arachidonic acid	0.088
		None	0.023
Kidney	Naphthalene	NADPH	0.005
		Arachidonic acid	0.005
		None	0.005
Lung	2-Methylnaphthalene	NADPH	0.260
		Arachidonic acid	0.022
		None	0.023
Liver	2-Methylnaphthalene	NADPH	0.248
		Arachidonic acid	0.033
		None	0.027
Kidney	2-Methylnaphthalene	NADPH	0.028
		Arachidonic acid	0.025
		None	0.023

In a total volume of 2 ml, incubations contained: 2 mg microsomal protein, 0.1 mM substrate (6,117 dpm/nmole naphthalene; 23,560 dpm/nmole 2-methylnaphthalene added in 5 μl methanol), NADPH-generating system, or arachidonic acid (100 μM, prepared immediately before use and added in 5 μl methanol). Incubations were for 6 min at 37°.

showed the typical bronchiolar injury associated with administration of these compounds to mice. This included moderate to severe vacuolation and exfoliation of cells from the epithelial airways. Treatment with indomethacin failed to protect against the bronchiolar necrosis caused by either hydrocarbon (Table 3).

DISCUSSION

The pulmonary Clara cell has been shown to be an important locus of cytochrome P-450 monooxygenase activity in the lung, and the metabolic activation of compounds like 4-ipomeanol and CCl_4 by enzymes localized in this pulmonary cell has been closely related to the cytotoxicity of these compounds in the lung [7, 22, 23]. Although both naphthalene and 2-methylnaphthalene result in a Clara cell lesion in the mouse and are both metabolized to reactive metabolites that bind covalently to macromolecules in the lung, the involvement of the cytochrome P-450 monooxygenases in either the formation of covalently bound 2-methylnaphthalene metabolites or the pulmonary injury induced by this compound cannot be demonstrated. In contrast, the prior administration of piperonyl butoxide blocks both the covalent binding and the pulmonary injury by naphthalene. However, while there is a good correlation between the levels of covalently bound naphthalene metabolites in the lung and the severity of the lung lesion, the precise role of reactive metabolites in naphthalene-induced lung injury is uncertain. Unlike 4-ipomeanol where the tissue selectivity for the necrosis was reflected in the overall levels of covalent

Table 3. Effect of pretreatment with indomethacin on the pulmonary bronchiolar necrosis by naphthalene or 2-methylnaphthalene

Treatment protocol			Severity of bronchiolar necrosis			
T = 0 hr	T = 1 hr	T = 6 hr	None	Mild	Moderate	Severe
Saline	Corn oil	Saline	5/5			
Indo.	Corn oil	Indo.	5/5			
Saline	Naphthalene	Saline			1/5	4/5
Indo.	Naphthalene	Indo.				5/5
Saline	2-Methylnaphthalene	Saline			3/5	2/5
Indo.	2-Methylnaphthalene	Indo.			4/5	1/5

Groups of five mice each were treated with saline, corn oil, indomethacin (4 mg/kg), naphthalene or 2-methylnaphthalene (300 mg/kg) according to the treatment protocol. Mice were killed by pentobarbital overdose 24 hr after hydrocarbon administration (T = 25 hr). Lung injury was graded in a blind manner as: mild, swelling of bronchiolar epithelial cells with disruption of the normal cuboidal appearance of the bronchiolar lining cells but no exfoliation of cells; moderate, some cellular vacuolation and some exfoliation of cells particularly in the terminal airways; severe, extensive sloughing of airway epithelial cells in both terminal and larger airways with exfoliation of entire sections of the epithelial layer.

binding in the target tissue [7], the levels of covalently bound naphthalene or 2-methylnaphthalene metabolites are higher in nontarget than in target tissues. There are several possibilities to explain these apparent discrepancies which are still consistent with the view that biotransformation of these hydrocarbons within the Clara cell plays an important role in the bronchiolar lesion. For example, naphthalene is metabolized to a number of different reactive metabolites all of which can potentially bind covalently to macromolecules in the lung only some of which are toxicologically active [24, 25, *]. In addition, the finding that reactive metabolites of naphthalene are sufficiently stable to circulate [17] and that naphthalene oxide is capable of leaving intact hepatocytes [26] suggests that measurement of *in vivo* covalent binding levels may not necessarily reflect the rate of formation of reactive metabolites within that tissue. Because many of the differences in the data on naphthalene versus 2-methylnaphthalene and on covalent binding *in vivo* versus *in vitro* in various tissues could be explained if an enzyme system such as the prostaglandin synthetases were active in the formation of reactive and/or cytotoxic metabolites from these aromatic hydrocarbons, we have examined the possibility that this system is contributing in whole or part to the pathology and/or metabolism of naphthalene and 2-methylnaphthalene.

The apparent inability of piperonyl butoxide or SKF 525A to block 2-methylnaphthalene-induced bronchiolar necrosis or result in a substantial decrease in the levels of covalent binding of reactive 2-methylnaphthalene metabolites *in vivo* simply may be due to the fact that these compounds are not potent inhibitors of the particular P-450 isozyme(s) responsible for producing the cytotoxic and/or reactive metabolite(s) from 2-methylnaphthalene. The data in Table 1 indicate that both P-450 inhibitors result in only moderate decreases in the rate of formation of covalently bound 2-methylnaphthalene metabolites while under the same conditions both inhibitors produce a substantial decrease in the covalent binding of reactive naphthalene metabolites. Naphthalene and 2-methylnaphthalene are appreciably volatile at room temperature and thus are likely to be excreted unchanged by the lung. Therefore, pretreatment with the cytochrome P-450 monooxygenase inhibitors is likely to decrease the hepatic clearance of naphthalene/2-methylnaphthalene and result in increased quantities of unchanged hydrocarbon available for pulmonary excretion. In the case of 2-methylnaphthalene, increases in the amount of parent compound reaching the target organ may outweigh any inhibitory effects of piperonyl butoxide or SKF 525A on the P-450 system with a resultant lack of effect on the toxicity and formation of covalently bound metabolites. Similarly, while piperonyl butoxide blocked naphthalene-induced pulmonary bronchiolar necrosis, prior administration of SKF 525A did not. Comparison of the levels of pulmonary covalent binding in animals pretreated with either of these drug metabolism inhibitors indicated that the levels of binding of reactive naphthalene metabolites in piperonyl butoxide pretreated animals were approximately half those in SKF 525A treated animals [13]. Again the increased amounts of naphthalene reaching the lungs in SKF 525A pretreated animals compared to vehicle pretreated animals may be sufficient to overcome the inhibition in pulmonary metabolism provided by SKF 525A.

The fact that the formation of reactive 2-methylnaphthalene metabolites is dependent upon the presence of NADPH-generating system and that the rate of covalent binding is decreased by incubation under conditions which inhibit the P-450 system combined with the inability to demonstrate any prostaglandin synthetase dependent metabolism of the compound *in vitro* suggests that *in vivo* covalent binding of reactive metabolites from this compound is likely to be a P-450-dependent process. The question of the role that this process plays in 2-methylnaphthalene-induced pulmonary injury cannot be answered with the data currently available.

Despite the finding that arachidonic acid was incapable of supporting the metabolism of either naphthalene or 2-methylnaphthalene to covalently bound or oxygenated metabolites *in vitro* and that indomethacin did not alter the extent or severity of the pulmonary lesion induced by these compounds *in vivo*, a role for the prostaglandin synthetase system in the cooxidation of these compounds cannot be ruled out entirely. As mentioned previously, dihydrodiols and phenols produced by initial P-450-dependent metabolism of the parent hydrocarbons may be good substrates for the cyclooxygenase system and thus may play an important, but perhaps not rate-limiting, role in the formation of the ultimate cytotoxic metabolites. Moreover, even though indomethacin at the doses used in the present study has been reported to cause significant inhibition of prostaglandin biosynthesis, there is no way to be certain that the synthetase enzyme in the target cells is sufficiently inhibited to result in a noticeable decrease in the pulmonary toxicity of naphthalene or 2-methylnaphthalene. Furthermore, indomethacin may cause alterations in the disposition or in the detoxication pathways for naphthalene and 2-methylnaphthalene and, as a result, there may be no overall shift in the quantity of toxicologically active agent present in the target tissue/cells.

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