Arch Toxicol (1989) 63: 13–17

Archives of
Toxicology

The in vitro effects of alkanes, alcohols, and ketones on rat lung cytochrome P450-dependent alkoxyphenoxazone dealkylase activities

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Abstract. The sensitivities of cytochrome P450 (EC 1.14.14.1)-dependent benzyloxy- and ethoxyphenoxazone dealkylase (BzOPh'ase and EtOPh'ase, respectively) activities towards a series of aliphatic hydrocarbons were measured in the microsomal fraction of lung obtained from β-naphthoflavone-treated rats. The unsubstituted hydrocarbons were straight-chain (*n*-hexane through *n*-undecane) and branch-chain (eight carbons). The substituted compounds were alcohols and ketones of hexane and octane. The data are expressed as I_{50} values, i.e. the hydrocarbon concentration required to cause 50% decrease in the rate of enzyme-catalyzed product (resorufin) formation. The unsubstituted aliphatic hydrocarbons exhibited I_{50} values towards BzOPh'ase from 0.76 μM (2,5-dimethylhexane) to 8.8 μM (*n*-hexane). The lung EtOPh'ase activity was insensitive towards the tested unsubstituted aliphatic hydrocarbons. When the alcohols and ketones of hexane and octane were tested against lung BzOPh'ase activity, I_{50} values ranged from 16 μM (1-octanol) to 4.8 mM (2,5-hexanedione). Lung EtOPh'ase activity exhibited some sensitivity towards the alcohols and ketones, and I_{50} values ranged from 0.52 mM (4-octanol) to 40.5 mM (2-hexanol). The data show rat lung BzOPh'ase and EtOPh'ase activites are differentially sensitive towards the selected unsubstituted aliphatic hydrocarbons and corresponding alcohols and ketones. The difference in sensitivities may reflect different requirements for an adventitious interaction between a hydrocarbon and enzyme active site.

Key words: Cytochrome P450 – Lung – Hydrocarbons – Alcohols – Ketones

Introduction

Aliphatic hydrocarbons are a continuing source of environmental and occupational contamination. Pollution by unsubstituted alkanes results from their use as additives and calibration standards in the petroleum and aircraft industries, and as intermediates and solvents in the manufacture of lubricants, degreasing agents, varnishes and printing inks. Aliphatic alcohols and ketones are used in the manufacture of lacquers and enamels, and are components of flavor ingredients, perfumes, and anti-foaming agents (Patty and Irish 1963; Merck Index 1976; Clayton and Clayton 1982; MacFarland et al. 1984).

Alkanes and their corresponding alcohols and ketones are physiologically active compounds that produce toxic responses including peripheral neuropathy, skin inflammation, pulmonary edema and respiratory disfunction (Patty and Irish 1963; Clayton and Clayton 1982). Co-carcinogenic and co-tumorogenic properties have been reported for higher chain alkanes (Sice 1966; Bingham and Falk 1969; Van Duuren and Goldschmidt 1976), and co-tumorogenic properties have been reported for the corresponding alcohols (Sice 1969). In addition to their primary toxic effects, some ketones are potentiators of exposure-related liver dysfunction (Plaa and Ayotte 1985).

At the membrane level, aliphatic hydrocarbons stimulate mitochondrial steroid synthesis (McNamara and Jefcoate 1988) and prevent Ca^{2+}-induced aggregation of phospholipid vesicles (Rabovsky et al. 1985). Metabolism of alipathic hydrocarbons is initiated in microsomal membranes by cytochrome P450-dependent monooxygenases (Ichihara et al. 1969; Frommer et al. 1972; Toftgard and Nilsen 1982; Toftgard et al. 1986). The metabolism of one alkane, hexane, has undergone detailed study because of the known adverse health effects associated with human exposure. The ultimate toxicant, 2,5-hexanedione, is the final metabolite in a complex pathway, initiated by the action of the membrane-bound cytochrome P450-dependent monooxygenases (Bus et al. 1985 and references therein).

Unsubstituted alkanes and their corresponding alcohols and ketones are therefore related not only through their chemistry and industrial use, but through their metabolism. In all cases, initial hydroxylation of the unsubstituted compound is under catalytic control of cytochrome P450-dependent activity, while subsequent steps may involve other enzymes (Bus et al. 1985). Because any effect on the initial cytochrome P450-dependent activity by a contaminant, its immediate product, or subsequent metabolite, may impact on the metabolism of additional pollutants [e.g. benzo[a]pyrene (Bingham et al. 1978)] it is important to understand how the various unsubstituted alkanes and their metabolites interact with the first enzyme on the pathway.

In a previous study, we investigated the interactions of alkanes and cytochrome P450-dependent enzymes by measuring the effect of alkanes on the catalytic activity of selected cytochrome P450-dependent activities (Rabovsky et

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al. 1986). Our results showed that straight-chain alkanes affected liver microsomal cytochrome P450-dependent activities towards two substrates and the affect was dependent on chain length and pre-exposure history of the animal. Our results also showed *n*-octane effectively inhibited ethoxycoumarin deethylase activity in liver microsomes from control rats, but only minimally in liver microsomes from β-naphthoflavone (BNF)-treated animals.

In the current study, we extended our observations to lung tissue, an important site of cytochrome P450-dependent monoxygenase activity (Baron and Kawabata 1983). Xenobiotics, including aliphatic hydrocarbons, can reach the lung by two routes, inhalation and systemic circulation. Uptake of alkanes by inhalation is proportional to carbon number and inversely proportional to vapor pressure (Dahl et al. 1988). Although the lung/air partition coefficients for the lower chain alkanes are less than those for other organs, they are greater than the blood/air coefficients, and in the cases of *n*-hexane and *n*-heptane, they are equal to or greater than 1.0. (Perbellini et al. 1985). Because the lung receives the total cardiac output, circulating alkanes and their metabolites also have access to lung tissue.

Although the low levels of cytochrome P450-dependent monoxygenases in lung tissue have previously rendered detailed studies difficult, application of the highly sensitive alkoxyphenoxazone dealkylase assay has greatly facilitated such an investigation. We have shown benzyloxyphenoxazone dealkylase (BzOPh'ase), a non-βNF-inducible, metyrapone-sensitive cytochrome P450-dependent activity, and ethoxyphenoxazone dealkylase (EtOPh'ase), a BNF-inducible, α-naphthoflavone-sensitive activity, were present at substantial levels in lung microsomes from BNF-treated rats (Rabovsky and Judy 1987). We were thus able to study two forms of cytochrome P450-dependent activities in one lung microsomal preparation and the sensitivity of each towards xenobiotics.

In the present communication, we describe and compare the effects of 21 aliphatic hydrocarbons including alcohols and ketones, on BzOPh'ase and EthOPh'ase activities in rat lung microsomes. In this way we have been able to study the specificity of hydrocarbon-cytochrome P450 interactions and further, to gain insight into potential changes in lung cytochrome P450-mediated metabolism that occur upon exposure to aliphatic hydrocarbons and their derivatives.

Methods and materials

Male Sprague-Dawley rats, 275–300 g, with free access to food and water were injected once with BNF (80 mg/kg, ip.) in corn oil, 48 h before sacrifice. At the time of sacrifice, animals were anesthetized with pentobarbital (65 mg/kg, ip.), lungs were removed and perfused with saline, and the microsomal fraction isolated by published procedures (Danner-Rabovsky and Groseclose 1982). Briefly, the perfused lungs were finely minced on a tissue chopper, homogenized in 0.05 M Tris, pH 7.4 + 0.15 M KCl, and the microsomal fraction separated from the homogenate by differential centrifugation. The published procedure was modified, such that the final microsomal pellet was suspended in 0.05 M Tris, pH 7.4 + 0.25 M sucrose + 20% (v/v) glycerol at a tissue concentration equivalent to 2 g lung tissue/ml and stored at −80° C until used.

O-Dealkylation was followed by a direct kinetic fluorometric procedure (Burke et al. 1977), that used benzyloxyphenoxazone (BzOPh) and ethoxyphenoxazone (EtOPh) as substrates. We have previously shown the former is a substrate for a non-inducible and perhaps a major constitutive cytochrome P450-dependent activity in rat lung microsomes, whereas the latter is a substrate for PAH-inducible lung P450 activity (Rabovsky and Judy 1987). Stock BzOPh and EtOPh substrate solutions, each at 0.25 mM were prepared in DMSO, and in the 2 ml reaction, the final substrate concentration was 2.5 μM ([DMSO] = 1%, v/v). The NADPH was provided by a generating system formed by incubating 5 mM $NADP^+$, 10 mM glucose-6-phosphate, 10 mM $MgCl_2$ and 2 units/ml glucose-6-phosphate dehydrogenase in 0.05 M Hepes pH 7.8 for 10 min at 37° C.

The 2 ml enzyme-catalyzed reaction was then carried out in the presence of 0.5 mM NADPH, 0.1 M NaCl, 0.05 M Hepes, pH 7.8 at 36°. Formation of the product, resorufin, was followed fluorometrically at 585 nm (excitation at 530 nm), and was quantified by use of an internal resorufin standard, whose concentration was based on $E = 40.0\ cm^{-1}\ mM^{-1}$ (Prough et al. 1978). During the interval in which the studies described in this paper were carried out, the specific activity (pmole resourfin per min per mg protein ± SEM, $n = 9$) was 687 ± 107 for BzOPh'ase and 308 ± 39 for EtOPh'ase.

Aliphatic hydrocarbon solutions were prepared in CH_3OH. A 20 μl aliquot of an appropriately diluted sample was added to the cuvette before the addition of substrate, and control reactions representing 100% activity contained 20 μl CH_3OH without test compound. The effect of the hydrocarbon on enzyme activity has been expressed as per cent activity remaining in the presence of test compound. Activation of BzOPh'ase or EtOPh'ase by the tested aliphatic hydrocarbons was not observed under the conditions of our experiments. The data were plotted as per cent inhibition versus logarithm alkane concentration. The concentration of hydrocarbon required for 50% inhibition of product release was determined and expressed as I_{50}. The values reported in Results are average values ± s.e.m. for at least three different microsomal preparations, and the data were analyzed by one-way analysis of variance.

BzOPh, EtOPh and resorufin were purchased from Molecular Probes (Eugene, OR). Alkanes were purchased from the following companies: *n*-hexane through-*n*-undecane, 2-methyl-, and 4-methylheptane, 2,5-dimethylhexane from Aldrich Chemical Co. (Milwaukee, WI); 1-, 2-, 3-, and 4-octanols, 2- and 3-octanones, 3-methylheptane, 2-methylhexane, 2-hexanol, and 2-hexanone from Fluka Chemical Corp. (Ronkonakoma, NY). According to manufacturers' labels, the purities of the alkanes were >97%, except for *n*-hexane which was 95%. They were used without further purification. Methanol was purchased from Mallinkrodt, Inc. (St. Louis, MO) and dimethylsulfoxide from Eastman-Kodak Co. (Rochester, NY). All other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Fluorometric analysis was carried out on a Farrand Mark 2 spectrofluorometer equipped with a water-jacketed cell holder and a Foci Model 300 recorder. The daily operation of the instrument was monitored with a pyrex standard (λ_{ex} = 315 nm and λ_{em} = 355 nm). The slits were set at 5 nm for excitation and 10 nm for emission.

Results

The straight chain alkanes, between 6 and 11 carbons, inhibited BzOPh'ase-mediated release of resorufin (Fig. 1). Analysis of the data showed that although no differences existed among the I_{50} values for *n*-heptane through *n*-undecane, the value for *n*-hexane was higher than the rest. The inhibition was directed at cytochrome P450-dependent monooxygenase activity. In a previous report (Rabovsky et al. 1986), we showed cytochrome P450 was not transformed into the inactive P420 form, and microsomal NADPH cytochrome c reductase activity was not affected by the compounds. EtOPh'ase activity was not affected by the straight chain alkanes up to the maximum concentrations allowed (1–10 mM).

To determine the effect of branching on the inhibitory potential of an unsubstituted alkane, branched heptane and hexane compounds were tested and the results compared to the effect of *n*-octane. In each case, the compound contained a total of eight carbons. The data in Fig. 2, obtained with BzOPh'ase activity, showed that the position of the methyl group affected the measured I_{50} value. Whereas 2-methylheptane was not different from *n*-octane in its inhibition of BzOPh'ase activity, the I_{50} values obtained for 3-methylheptane, 4-methylheptane and 2,5-dimethylhexane were reduced by about 80%. Inhibition of EtOPh'ase by the branched-chain aliphatic hydrocarbons was not observed.

Mono- and di-substituted hexanes were studied to determine the effect of different substituents and their positions on the inhibition of BzOPh'ase and EtOPh'ase activities (Table 1). Although the presence of a methyl group on carbon-2 of hexane had a negligible effect on the I_{50} value, the presence of the second methyl group (2,5-dimethylhexane) resulted in a significantly reduced I_{50} towards BzOPh. As with *n*-hexane, EtOPh'ase activity was not affected by the methylhexanes.

In contrast to the methylhexanes, the alcohols and ketones of *n*-hexane had a distinctly different effect on BzOPh'ase-mediated release of resorufin. The data in

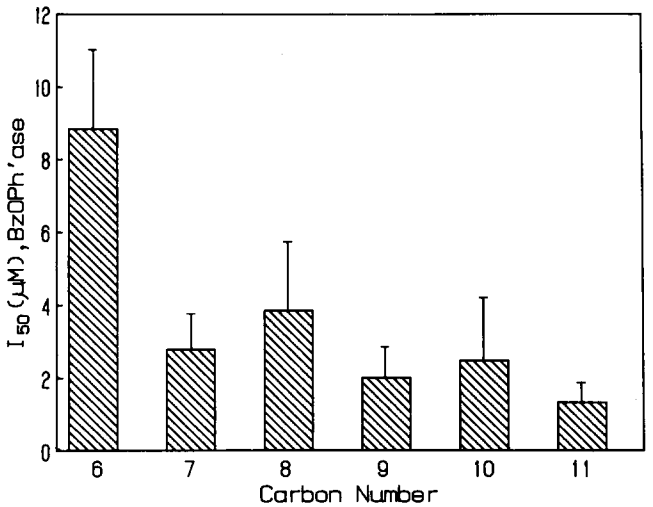

Fig. 1. Effect of straight-chain alkanes on BzOPh'ase-catalyzed release of resorufin in rat lung microsomes. Lung microsomes were obtained from rats pretreated with BNF for 48 h. EtOPh'ase activity in the same microsome preparations was not affected by the hydrocarbons up to concentrations permitted by their solubilities

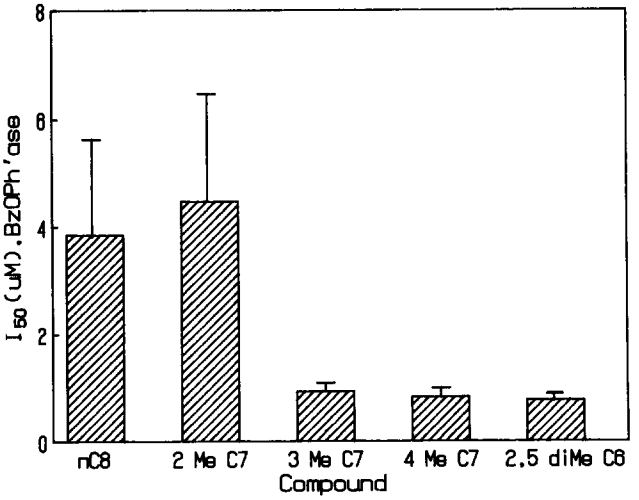

Fig. 2. Effect of straight- and branch-chain octanes on BzOPh'ase-catalyzed release of resorufin in rat lung microsomes. Lung microsomes were obtained from rats pretreated with BNF for 48 h. EtOPh'ase activity in the same microsome preparations was not affected by the hydrocarbons up to concentrations permitted by their solubilities

Table 1. Effect of hexane compounds on benzyloxy- and ethoxyphenoxazone dealkylase activities in lung microsomes from β-naphthoflavone-treated rats

Hexane derivative	I_{50} (μM ± sem)	
	BzOPh	EtOPh
n-Hexane	8.80±3.20	–[a]
2-Methylhexane	5.00±1.50	–
2,5-Dimethylhexane	0.76±0.14	–
2-Hexanol	418±161	9887±1919
2-Hexanone	556±184	40500±5352
2,5-Hexanediol	2145±103	–
2,5-Hexanedione	4826±1616	–

[a] 50% inhibition of product release was not observed at the highest concentration permitted by solubility

Table 2. Effect of octane substitution on benzyloxy- and ethoxyphenoxazone dealkylase activities in lung microsomes from β-naphthoflavone-treated rats

Octane derivative	I_{50} (μM ± sem)	
	BzOPh	EtOPh
n-Octane	3.8± 1.9	–[a]
1-Octanol	16 ± 2.7	863± 69
2-Octanol	90 ± 9.7	1153± 83.6
3-Octanol	47 ±18	552±164
4-Octanol	94 ± 9.0	516±155
2-Octanone	94 ± 5.6	919±376
3-Octanone	90 ± 9.7	1293± 80

[a] 50% inhibition of product release was not observed at the highest concentration permitted by solubility

Table 1 show a decreased sensitivity of lung microsomal BzOPh'ase towards the alcohol and carbonyl substituted hexanes, with the least reactivity being displayed toward the diol and dione derivatives. 2-Hexanol and 2-hexanone displayed some inhibitory potential towards the lung mi-

crosomal EtOPh'ase. When the diol and dione were tested, however, 50% inhibition was not observed at the highest concentrations (75 mM).

The effect of alcohol and carbonyl substitution was further investigated by measuring I_{50} values for a series of octanols and octanones and comparing them to the value obtained for *n*-octane (Table 2). Similar to the results obtained with hexane compounds, octanols and octanones displayed increased I_{50} values towards BzOPh'ase, compared to the unsubstituted *n*-octane. Similarly, EtOPh'ase activity was inhibited by the octanol and octanone compounds.

Discussion

The release of the product, resorufin, from BzOPh and EtOPh was inhibited or not affected by the aliphatic hydrocarbons, and lung microsomal BzOPh'ase activity was always more sensitive to the compounds than was EtOPh'ase activity. The differential sensitivities to the hydrocarbons may be compared to data obtained with *n*-octane and ethoxycoumarin deethylase in rat liver microsomes, wherein product release was affected to a lesser extent by *n*-octane when microsomes were obtained from BNF-treated rats than from control animals (Rabovsky et al. 1986). We had postulated that the activity in liver microsomes from BNF-treated rats represented a different cytochrome P450 form than did the same activity in control liver microsomes, and it was less reactive towards the straight-chain alkane.

Rat lung BzOPh'ase and EtOPh'ase also represent different cytochrome P450 forms, and the BzOPh'ase activity we measure in lung microsomes from BNF-treated rats has the characteristics of a non-BNF-inducible form (Rabovsky and Judy 1987). The lung cytochrome P450 study described herein shows BzOPh'ase activity is always more sensitive towards the tested aliphatic hydrocarbons than is the BNF-inducible EtOPh'ase activity. Hence, the data obtained with lung tissue reinforce our conclusion from the liver studies. Non-BNF-inducible cytochrome P450 activity is more sensitive to aliphatic hydrocarbons than is the BNF-inducible activity.

Regardless of the mechanism of inhibition of enzyme activity by the aliphatic hydrocarbons (direct inhibition or alternate substrate), the I_{50} data provide us with a way to assess the potential modulation of cytochrome P450-dependent metabolism in the presence of xenobiotics. The I_{50} data also allow us to determine an order of inhibitory potency of environmental/occupational pollutants towards specific forms of cytochrome P450. In our study, unsubstituted alkanes were more effective inhibitors of lung non-PAH-inducible BzOPh'ase than were those with hydroxyl or carbonyl groups. It is important to emphasize, however, that this inhibitory potential is specific for the form of cytochrome P450; BNF-inducible EtOPh'ase activity was intensitive towards the unsubstituted alkanes. Hence, an evaluation of the affect(s) of xenobiotics on pulmonary cytochrome P450-mediated metabolism must take into account the presence of multiple enzyme forms.

The observed inhibitory potencies may be related to membrane solubilities and/or enzyme active site properties. The order of inhibitory potency against BzOPh'ase (unsubstituted > alcohols, ketones) follows the solubility characteristics of aliphatic hydrocarbons in membrane lipids (Diamond and Katz 1974; Pope et al. 1984). However, the data of Krainer et al. (1988) show membrane proteins confer solubility characteristics on the native microsomal structure not observed in liposomal systems. Hence a definitive mechanism to explain our results will require additional studies to separate the contributions of all components.

A knowledge of the sensitivity of cytochrome P450-dependent enzyme activity towards metabolites of unsubstituted aliphatic hydrocarbons is important because of the potential for inhibition by reaction products such as that observed during benzo[a]pyrene metabolism (Keller et al. 1982). Our data suggest that for *n*-hexane and *n*-octane, the observed sensitivity of BzOPh'ase was due primarily to the parent compound, because the I_{50} values for the corresponding alcohols were substantially greater than for the unsubstituted alkanes. In a situation where the ketones may be present as a result of subsequent non-cytochrome P450-dependent metabolism (Bus 1985), little interference with the first step of alkane metabolism would be expected. In the case of cytochrome P450 form(s) responsible for EtOPh'ase activity, the potential for the inhibition of their enzyme activities by the alcohols or ketones would probably be minimal because of I_{50} values in the millimolar range.

In summary, we have shown that two pulmonary cytochrome P450-dependent activities are differentially sensitive towards aliphatic hydrocarbons and the corresponding alcohols and ketones. The difference in sensitivities appears to be associated with different cytochrome P450 forms (non PAH- and PAH-inducible forms) and may reflect differences in membrane solubilities and/or enzyme active site properties. Unsubstituted aliphatic hydrocarbons, which have low I_{50} values, have the potential to interfere with normal, non-PAH-inducible cytochrome P450-dependent metabolism. Because the inhibition of product release is less effective for the tested aliphatic alcohols and ketones, the presence of these compounds in lung tissue is unlikely to substantially alter cytochrome P450-dependent activity towards other substrates.

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Received June 29, 1988/Accepted October 3, 1988