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Role of Cytochrome P-450 in the Bioactivation of Nicotine

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Abstract □ Nicotine (100 μM) was incubated with microsomes (1 mg/m*l*) prepared from New Zealand White rabbits. On the basis of microsomal weight, the rate of nicotine oxidation was slightly slower in lung compared to liver. However, when the rates of nicotine oxidation were calculated on the basis of cytochrome P-450 concentration, the specific activity of the metabolic oxidation catalyzed by lung was approximately 4 times greater than liver (6.4 *vs* 1.65 nmoles nicotine oxidized. nmole cytochrome $P\text{-}450^{-1}$ min^{-1}). These studies employed several methods of altering activities of specific isozymes present in pulmonary microsomes, including the use of the isozyme 2 and 6 specific inhibitor α-methylbenzyl ABT, metabolite inhibitors, norbenzphetamine and N-hydroxyamphetamine, TCDD induction and Arochlor 1260 pretreatment. These results support the conclusion that nicotine metabolism by rabbit lung microsomes is mediated primarily by cytochrome P-450 isozyme 2.

Keywords □ Nicotine oxidation, cytochrome P-450, suicide inhibitor, metabolite inhibitor, α-methylbenzyl ABT, norbenzphetamine, *n*-hydroxyamphetamine, TCDD, Arochlor 1260.

Nicotine, a major component of tobacco, is an alkaloid which is suspected to contribute to some of the irreversible tissue lesions and other long term toxic effects observed in human who are exposed chronically to tobacco products. One mechanism which has been shown to contribute to the chronic toxicity of various xenobiotics, including known carcinogens such as benzo(a)pyrene, involves the oxidative metabolism of such compounds to reactive electrophilic species which are capable of interacting irreversibly with tissue macromolecules. Although the metabolic fate of nocotine has been examined extensively[1], the possibility that it may be biotransformed to chemically reactive intermediates capable of forming covalent bonds to tissue macromolecules has not been investigated. Recently, through studies with rabbit liver and lung microsomal preparations, we have shown that nicotine is metabolized in a time and NADPH dependent manner to reactive intermediates which covalently bind to microsomal protein[2]. Covalent binding to liver microsomal biomacromolecules was inhibited by SKF-525A, cytochrome C and *n*-octylamine, indicating the involvement of the cytochrome P-450 in the bioactivation of this tobacco alkaloid.

Although it is well accepted that liver contributes to most of the oxidative metabolism of xenobiotics, the lung may contribute significantly to the metabolism and bioactivation of certain compounds. A prime example of this is the lung toxicant 4-ipomeanol, a sweet potato mold. Oxidative metabolism of this compounds has been shown to result in the formation of reactive species which covalently bind to protein and which cause extensive pulmonary damage in animals possessing good pulmonary cytochrome P-450 monooxygenase activity. In this regard, the metabolism of nicotine by lung tissue is obvious interest since this organ represents the site of initial absorption and is the primary target organ for tobacco induced toxicities. Lung metabolism of nicotine has previously been demonstrated in perfusion studies[3]. Our interest in identifying metabolic pathways involved in the bioactivation of nicotine in this target organ encouraged the present study

on the biodisposition of this alkaloid in rabbit lung microsomal systems. So in our efforts to characterize the metabolic disposition of nicotine, studies were undertaken to difine the interactions between this compound and cytochrome P-450, the effects of inducing agents on the *in vitro* oxidative metabolism by lung microsomes and through the utilization of biochemical probes isozyme inhibitors-the specific pulmonary cytochrome P-450 isozymes.

EXPERIMENTAL METHODS

Chemicals

Sodium dithionite, KCl, HEPES, D-glucose-6-phosphate, $NADP^+$, and TRIS were obtained from Sigma Chemical Co. Acetonitrile, methanol, methylene chloride were HPLC grade solvents obtained from Fisher Chemical Co., *n*-propylamine and triethylamine were obtained from Aldrich Chemical Co.. Glucose-6-phosphate dehydrogenase was purchased from Calbiochem-Behring Co. Sucrose and sodium phenobarbital were obtained from Mallinckrodt, Inc. α-methylbenzylaminobenzotriazole, TCDD, and Aroclor 1260 were the generous gifts of Dr. James M. Mathews (National Institute of Environmental Health Science, NIH). Norbenzphetamine and N-hydroxyamphetamine were kindly provided by Dr. Michael Franklin (Dept. Biochemical Pharmacology and Toxicology, University of Utah). Nicotine-$\Delta^{1',5'}$-iminium ion bisperchlorate[4], the diastereomeric nicotine N-oxides[5], cotinine[6], and nornicotine[7] were prepared as described previously.

Sources of tissue

The liver and lungs of New Zealand White male rabbits (2.5-3 kg) were used for the preparation of microsomes. For studies using phenobarbital treated animals, rabbits received 0.1% (w/v) sodium phenobarbital, pH adjusted to 7.0 with 0.1 N HCl, administered in drinking water for 6 days before sacrifice. A 1 mg/m*l* solution of TCDD (tetrachlorodibenzodioxin) was prepared by dissolving 2.3 mg in 2.3 m*l* corn oil. The stock solution was diluted to a final concentration of 100 μg/m*l*. Injected lower left caudal portion of the abdomen of three New Zealand White rabbits with 0.75 m*l* of the 100 μg/m*l* solution *via* a 20 gauge needle. A neat solution of Aroclor 1260 was injected as described for TCDD such that a concentration of 200 mg/kg was delivered. Animals were sacrificed 72 hours later.

Preparation of rabbit liver microsomal fractions

After carbon dioxide asphyxiation, the liver was perfused *in situ via* the portal vein with 250 m*l* of ice cold 0.25 M sucrose buffered at pH 7.4 and with 0.05 M Tris/0.05 M NaOH. The liver was minced with scissors and then, homogenized in a Potter-Elvehjem apparatus in 3 volumes (w/v) of the same solution. The homogenate was centrifuged at 10,000 g for 20 min and the resulting supernatant fraction was centrifuged at 100,000 g for 75 min. The pellet was resuspended in 5 m*l* of 0.15 M KCl buffered at pH 7.4 with 0.02 M KH_2PO_4 and this mixture was centrifuged a second time at 100,000 g. The resulting pellet was homogenized in this buffer at a concentration of approximately 50 m*l*/g of microsomal protein per m*l* and stored under nitrogen at −70℃ for up to 1 month. Protein concentration was determined by the method of Lowry *et al.*[8].

Preparation of rabbit lung microsomes

Immediately after the animal was sacrificed by carbon dioxide asphyxiation, the lungs were perfused *via* the pulmonary artery with 10-15 m*l* of ice cold 0.15 M KCl/0.02 M KH_2PO_4 buffer containing 100 μ/m*l* heparin (Elkin-Sinn, Cherry Hill, NJ). The isolated perfused lungs were coarsely minced in a solution consisting of 0.02 M Tris, 0.15 M KCl, 0.2 mM EDTA, and 0.5 mM dithiotreitol and the resulting mince was homogenized in a Waring Blendor with two 10 sec bursts. The contents were transferred to a Potter Elvehjem homogenizer and homogenized with six passes of a Teflon pestle. The resulting homogenate was centrifuged at 18,000 g for 20 min. The postmitochondrial supernatant fraction was centrifuged for an additional 60 min. at 100,000 g. The microsomal pellet was resuspended in 5 m*l* of 0.02 M Tris and 0.15 M KCl, the pH was adjusted to 7.4 with 1 N NaOH, and the resulting suspension was centrifuged at 100,000 g for an additional 60 min. The pellet was resuspended in this buffer, homogenized, and stored under nitrogen at a concentration of approximately 15-25 mg/m*l* at −70℃ for up to 1 month.

Determination of cytochrome P-450 concentration

The concentration of cytochrome P-450 was determined using an Aminco DW-2 UV/visible spec-

trophotometer by measuring UV absorbance difference between the dithionite-reduced carbon monoxide treated sample and an unreduced carbon monoxide treated reference sample[9].

Metabolism studies

Microsomes isolated from untreated New Zealand White rabbits were studied for their nicotine oxidase activity. Nicotine (100 μM) was subjected to metabolic incubation mixtures consisting of either liver (0.5 mg/m*l*) or lung microsomes (1 mg/m*l*) and the appropriate cofactors such as EGTA (1 mM) in 0.1 M HEPES buffer, pH 7.6 and an NADPH regenerating system (0.5 mM $NADP^+$, 8 mM glucose-6-phosphate, 1 unit/m*l* glucose-6-phosphate dehydrogenase, and 4 mM $MgCl_2$). Since preincubation of the incubation mixture was limited to 5 minutes, glutathione was not included. Incubations were conducted over a period of 60 min at 37℃ and samples were taken at timed intervals. Incubations were terminated by the addition of an equal volume of ice cold K_2CO_3 followed by the internal standard. Nicotine and its metabolites were extracted into CH_2Cl_2. The methylene chloride extract was separated by normal phase HPLC and quantitation of nmoles of nicotine metabolized was accomplished by determining the peak height ratio of nicotine to the internal standard.

HPLC analysis normal phase

To examine the overall lung and liver microsomal metabolism of nicotine, a normal phase silica HPLC system was utilized. This assay which detects nicotine and its base extracts nicotine-$\Delta^{1',5'}$-iminium ion and continine was used to estimate the overall metabolism of nicotine. Lung microsomal incubation (0.4 m*l*) was terminated by the addition of 0.4 m*l* of 1 M K_2CO_3 followed by the addition of the internal standard N-methyl-N-(3-pyridyl)methylpropionamide and extracted with 1 m*l* CH_2Cl_2. This mixture was extracted by vortexing vigorously for 1 min followed by centrifugation at 1000×'s g for 2-3 min. A volume of 50 μ*l* of the resulting methylene chloride extract was injected. The HPLC system consisted of a Beckman 110A solvent delivery system and a Hitachi 100-10 spectrophotometer/flow cell combination. The precolumn consisted of a direct connect precolumn assembly and a precolumn cartrige 5 μ adsorbosphere silica, Alltech. A Chemco 75 mm×4.6 mm 3 μ silica column (Dychrom, Sunnyvale CA) was utilized for the analytical seperation of nicotine and its metabolites, The mobile phase consisted of 0.3% *n*-propylamine in acetonitrile. UV absorbance was monitored at 260 nm, the λ_{max} for nicotine.

Isozyme studies: Isozyme specificities toward in the oxidation on nicotine

α-methylbenzyl ABT studies

Incubations with α-methylbenzyl ABT utilized concentrations of 1 μM, 2.5 μM and 1 mM. α-methyl ABT was dissolved in methanol to a stock concentration of 10 mM. Dilutions of the stock solution was made and approprite volumes of the methanolic solutions were transferred such that 0.4, 1 and 400 nmoles of α-methylbenzyl ABT to sample tubes. Prior to adding the other components of the incubation mixture methanol was evaporated with a gentle stream of nitrogen. Components of this mixture included EGTA (1.0 mM), $NADP^+$ (0.5 mM) and a glucose 6-phosphate based regenerating system (glucose 6-phosphate 8.0 mM, glucose 6-phosphate dehydrogenase 1 unit/m*l*, and magnesium chloride 4.0 mM, lung microsomes from untreated male NZW rabbits (1 mg/m*l*) and glutathione (1 mM) in a final incubation volume of 0.4 m*l*. Incubation mixtures were preincubated in the presence of the inhibitor for 30 min at 37℃ prior to the addition of the substrate nicotine (100 μM). Samples were worked up as described previously each condition was performed N>3.

Metabolite inhibitor (MI) studies

The effect of MI complex formation on the oxidation of nicotine was tested by preincubating untreated New Zealand White rabbits (4 mg/m*l*) with the following combinations of inhibitor: 1) 100 μM norbenzphetamine only 2) 667 μm N-hydroxyamphetamine only 3) 100 μM norbenzphetamine plus 667 μm N-hydroxyamphetamine. The concentrations used above were derived from studies which established maximal rates of MI complex formation[10]. For maximal inhibition, these compounds were preincubated with NADPH (0.8 mM) and lung microsomes for 30 min at room temperature. Following the preincubation, microsomes treated with the respective conbination of inhibitor was transferred to sample tubes which included the components/co-

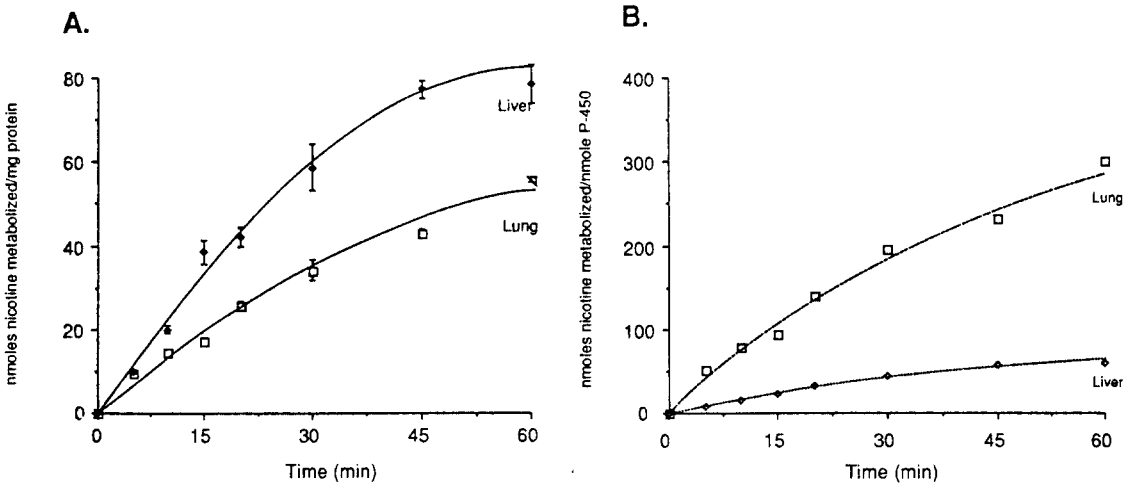

Fig. 1. Rates of metabolism of nicotine in rabbit lung and liver microsomal preparations plotted per milligram of microsomal protein (panel A) and per nanomole of cytochrome P-450 (panel B).

factors necessary for cytochrome P-450 monooxygenase activity. These incubations, containing 1 mg/ml of the MI complexed lung microsomes, were incubated with nicotine (100 μM) for an additional 20 min at 37℃.

RESULTS AND DISCUSSION

Comparison between lung and liver nicrosomal oxidation of nicotine

Studies on the biodisposition of nicotine by liver and lung microsomes were investigated in order to provided a better understanding of its fate in tissues. Nicotine (100 μM) was incubated with microsomes (1 mg/ml) prepared from NZW rabbits. The time course of oxidation was observed over a 60 min period. (Fig. 1) Liver microsomal metabolism of 100 μM nicotine appeared linear over the first 15-20 minutes and proceeded with a rate of approximately 2.11 nmoles nicotine oxidized·mg^{-1}·min^{-1}. Lung microsomal metabolism of 100 μM nicotine was similarly linear over the first 20 min and proceeded at a rate of 1.28 nmoles nicotine oxidized·mg^{-1}·min^{-1}. On the basis of microsomal weight, the rate of nicotine oxidation was slightly slower in lung compared to liver. However, estimation of cytochrome P-450 content in lung and liver revealed a large difference in the absolute concentration of this enzyme. Cytochrome P-450 levels in liver microsomes 1.28± 0.14 nmoles/mg protein compared to 0.02± 0.20 nmoles/mg protein in lung. Therefore, when the rates of nicotine oxidation were calculated on the basis of cytochrome P-450 concentration, the specific activity of lung was approximately 4 times greater than liver (6.4 *vs.* 1.65 nmoles nicotine oxidized·nmole cytochrome P-450^{-1}·min^{-1}).

Isozyme studies: Isozyme specificities toward the oxidation of nicotine

The differences in the specific activities of the microsomal preparations isolated from these two tissues is consistent with the paticipation of lung isozymes 2 and/or 5 in the oxidation of this compound, since these two forms represent most of the cytochrome P-450 (estimated to account for up to 85%) activity present in lung. The results of the lung microsomal metabolism of nicotine encouraged us to pursue studies which could elucidate the isozyme selectivities toward the oxidation of nicotine. These studies employed several methods of altering activities of specific isozymes present in pulmonary microsomes, including the use of the isozyme 2 and 6 specific inhibitor α-methylbenzyl ABT, metabolic inhibitor (MI) complex formation, TCDD induction and Arochlor 1260 pretreatment. Although the highest microsomal monooxigenase activities are normally found in the liver, the lung also contains an active P-450 system. The rabbit pulmonary monooxigenase system is composed of two major and one minor P-450 isozyme. The major isozymes are forms 2 and 5, which are present in approximately equal amounts, are indistinguishable from those P-

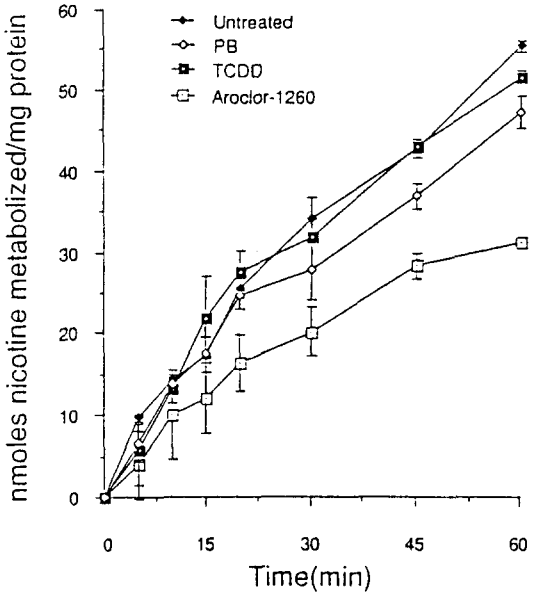

Fig. 2. Effect of various pretreatments on lung microsomal metabolism of nicotine.

450 isozymes induced in rabbit liver by treatment with phenobarbital and account for up to 80% of total pulmonary P-450[11,12]. In our experiments, treatment of the animals with phenobarbital had increased metabolism of nicotine in the liver but little effect on the overall lung microsomal oxidation. So we thought that cytochrome P-450 isozyme is not induced in rabbit lung by treatment with phenobarbital. The minor form, isozyme 6, is induced in liver and lung of rabbit by treatment with 2,3,7,8 tetrachlorodibenzo-*p*-dioxin and it occurs in amount of 1 to 30% of total P-450 in pulmonary microsomes prepared from untreated rabbits[13]. Treatment of the animals with TCDD had little effect on the overall lung microsomal oxidation and the metabolite profile of nicotine consistent with their known uninducing effects on isozyme 2 (Fig. 2). Pretreatment of animals with Aroclor 1260, a mixture of polychlorinated biphenyls, which has been shown to depress isozyme 2 but induce isozyme 6 was shown to decrease benzphetamine N-demethylation by >70% and the overall metabolism of nicotine by 35%. Pretreatment with TCDD or Aroclor resulted increase in isozyme 6 catalyzed ethoxyresolrufin *O*-deethylation[13]. The failure of either TCDD or Aroclor 1260 to induce nicotine metabolism suggests that isozyme 6 probably does not contribute significantly to the

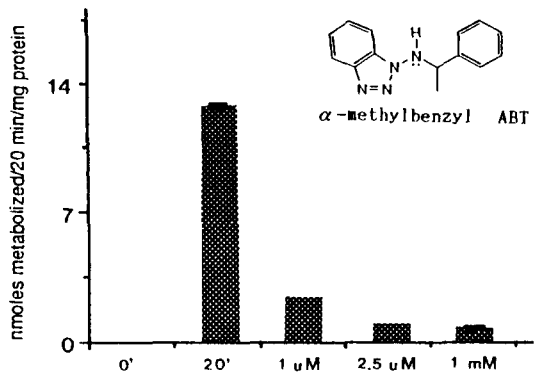

Fig. 3. Effect of α-methyl ABT on the metabolism of nicotine by UINZW rabbit lung microsomes.

overall oxidation of nicotine by lung microsomes.

Studies utilized α-methylbenzyl ABT, the suicide inactivator described previously[14]. Lung microsomes isolated from untreated New Zealand White rabbits were preincubated with 1 μM, 2.5 μM and 1 mM α-methylbenzyl ABT, concentrations which have been reported to inhibit 84.93 and 98% of the isozyme 2 specific *n*-demethylation of benzphetamine and 41,70 and 100% of the isozyme 6 catalyzed *O*-deethylation of ethoxyresolrufin. Similar incubations with nicotine resulted in 82.7, 93.7 and 95.0% inhibition of its overall metabolism at the concentrations of α-methylbenzyl ABT described above (Fig. 3). Inhibition of benzphetamine N-demethylation corresponded to the values reported by Mathwes *et al.*. The results of this inhibition study would appear to indicate that nicotine is metabolized predominantly by pulmonary isozyme 2 and possibly 6.

To further evaluate the effect of various pulmonary isozymes on the metabolism of this compound, the metabolite intermediate (MI) complex forming agents norbenzphetamine and N-hydroxyamphetamine were utilized. These agents, characterized by their ability to inhibit cytochrome P-450 monooxygenase activity following metabolic oxidation, display a 455 nm chromophore and have been shown to be useful as probes to quantitate cytochrome P-450 isozymes 2 and 5 in lung microsomes[15]. These inhibitors form stable noncovalent interactions with the heme of cytochrome P-450 in a process which requires NADPH and O_2. There is an evidence that the complex is a result of a ligand interaction between an unstable nitroso intermediate and the reduced ferrous cytochrome P-450[16]. In the case of

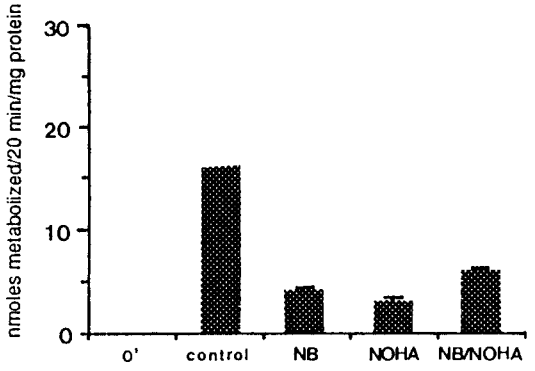

Fig. 4. Effect of MI inhibitors on the oxidation of nicotine by UINZW rabbit lung microsomes.
NB: Norbenzphetamine, NOHA: N-hydroxyamphetamine

norbenzphetamine, oxidative metabolism leads to the formation of an intermediate which binds tightly only to pulmonary isozyme 2. In this respect, the inhibitory properties of norbenzphetamine and α-methylbenzyl ABT are similar. N-hydroxyamphetamine, on the other hand, is a less specific inhibitor of both isozymes 2 and 5. The differential inhibition exhibited by these 2 MI complex substrates can provide information on the role of isozyme 5 in the oxidation of nicotine. Preincubation of lung microsomes with norbenzphetamine resulted in a marked decrease in nicotine metabolism (Fig. 4). N-hydroxyamphetamine did not appear to inhibit the oxidation of nicotine any better than norbenzphetamine. These results support the conclusion derived from the α-methylbenzyl ABT inhibition studies that nicotine metabolism by rabbit lung microsomes is mediated primarily by cytochrome P-450 isozyme 2.

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