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Metabolism-Dependent Covalent Binding of S(−)-^{3}H-Nicotine to Lung Microsomes in Vitro

Bong Hee Kim[1] and Mark K. Shigenaga[2]

College of Pharmacy, Chung Nam National University, Taejeon 305-764, Korea and [2]Department of Pharmacology and Division of Toxicology, University of California, San Francisco, CA 94143, USA

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Incubation of S(−)-^{3}H-nicotine with rabbit lung microsomes in the presence of dioxygen and NADPH results in the formation of metabolites that bind covalently to microsomal macromolecules. The addition of cytochrome P-450 monooxygenase inhibitors, α-methylbenzyl aminobenzotriazole and aroclor 1260, inhibited both (S)-nicotine metabolism and covalent binding. The relative rates of oxidation of nicotine $\Delta^{1',5'}$ iminium ion to cotinine indicates that lung 100,000×g supernatant catalyzed this oxidation approximately 18 times slower than that of liver system based on mg of protein, and increased covalent interactions. Since the activity of lung iminium oxidase appears much lower than the liver, it is tempting to speculate that localized concentrations of nicotine $\Delta^{1',5'}$ iminium ion in the lung will survive for a longer period of time. These results support that cytochrome P-450 catalyzed oxidation of nicotine leads to the formation of reactive and electrophilic intermediates capable of chemical interactions with biomacromolecules.

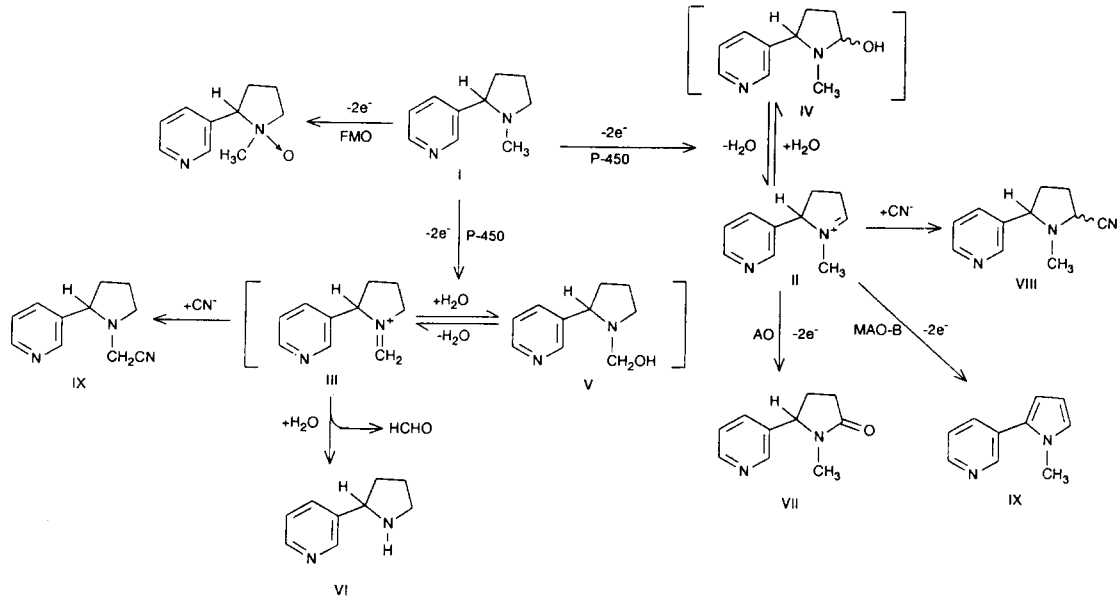
Key words: S(−)-^{3}H-Nicotine oxidation, Cotinine, Nicotine $\Delta^{1',5'}$ iminium ion, Cytochrome P-450 monooxygenase inhibitors, α-Methylbenzylaminobenzotriazole, Aroclor 1260

INTRODUCTION

(S)-Nicotine (in Scheme I), the principal alkaloid presents in tobacco, is suspected of contributing to some of the irreversible tissue lesions and other toxic effects observed in humans and animals who are exposed chronically to tobacco products (Sudan and Sterboul, 1981; Bock, 1980). Although the metabolic fate of (S)-nicotine had been examined extensively (Gorrod and Jenner, 1975), the possibility that it may be biotransformed to chemically reactive intermediates capable of forming covalent bonds to tissue macromolcules has not been investigated. The present paper reports the results of our efforts to characterize such bioactivation pathways.

Previous studies have shown that (S)-nicotine undergoes cytochrome P-450 dependent oxidative metabolism principally at the C-5′ position of the pyrrolidine ring and, to a minor extent, at the N-methyl position (Hucker *et al.*, 1960). These two electron α-carbon oxidations generate the electrophilic iminium species, II and III, which will be in equilibrium with the corresponding α-carbinol amines, IV and V, respectively (Scheme 1). The spontaneous breakdown of V yields nornicotine (VI). The (S)-nicotine $\Delta^{1',5'}$ iminium species undergoes further oxidation in vivo in a reaction catalyzed by a cytosolic oxidase (Brandange and Lindblom, 1979) to yield the lactam (S)-cotinine (VII), the principal urinary metabolite of (S)-nicotine in mammals (Gorrod and Jenner, 1975). Recently, through studies with rabbit lver 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 (Kim and Trevor, 1991). Covalent binding to liver microsomal biomacromolecules was inhibited by SKF-525 A, cytochrome C and N-octylamine, indicating the involvement of the cytochrome P-450 in the bioactivation of this tobacco alkaloid (Shigenaga *et al.*, 1988). Although it is well accepted that liver contributes to the 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 compound 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 monooxyge-

Correspondene to: Bong Hee Kim, College of Pharmacy, Chung Nam National University, Taejeon 305-764, Korea



Scheme 1. Major pathways for the metabolic oxidation of (S)-nocotine.

nase activity. In this regard, the metabolism of nicotine by lung tissue is of obvious interest since this organ represents in site of initial absorption and is the primary target organ for tobacco induced toxicities. The demonstration that (S)-nicotine $\Delta^{1',5'}$ iminium species, a potentially reactive nicotine metabolite, exists other than transiently, has encouraged us to examine the covalent binding of (S)-nicotine under conditions favorable for cytochrome P-450 monooxygenase activity. In the present study we have employed HPLC analytical methods for direct quantitation of the (S)-nicotine $\Delta^{1',5'}$ iminium species and have shown it to be a major metabolite formed from (S)-nicotine by microsomes prepared from rabbit lung tissue.

MATERIALS AND METHODS

Chemicals

N-octylamine, sodium dithionite, KCl, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), D-glucose-6-phosphate, $NADP^+$, and TRIS were obtained from the Sigma Chemical Co. (St. Louis, MO). Acetonitrile, methanol, methylene chloride were HPLC grade which were purchased from the Fisher Scientific Co. (Springfield, NJ). The HPLC internal standard, N-methyl-N-(3-pyridyl) methylpropan amide was a gift from Dr. Peyton Jacob III (San Francisco General Hospital, CA). n-Propylamine and triethylamine were products from Aldrich Chemical Co. (Milwaukee, WI). α-Methylbenzylaminobenzotriazole (α-MB), and Aroclor 1260 were the generously donated from Dr. James M. Mathews (National Institute of Environmental Health Science, NIH, Bethesda, MA). Norbenzphetamine and N-hydroxyamphetamine were kindly donated by Dr. Michael Franklin (Dept. Biochemical Pharmacology and Toxicology, University of Utah). Nicotine-$\Delta^{1',5'}$-iminium ion bisperchlorate (Peterson *et al.*, 1987), and cotinine (Bowman and McKennis, 1963) were prepared as described previously. (S)-^{3}H nicotine was prepared (Shigenaga *et al.*, 1987) by carrier-free catalytic fritiolysis of (S)-5-bromonicotine at the National Tritiation Facility (University of California, Berkeley, CA). and the final product was greater than 99% pure by HPLC using both UV and radiochemical detectors and had a specific activity of 32 Ci/mmol. Flow Scint II scintillation fluid was obtained from Radiomatic Instruments and Chemical Co. Inc. (Tampa, FL).

Sources of tissue

A neat solution of Aroclor 1260 was injected lower left caudal portion of the abdomen of three male New Zealand White rabbits (2.5-3.0 Kg) with a concentration of 200 mg/kg via a 20 gauge needle. Animals were sacrified 72 hours later and the liver and lung of the rabbits were used for the preparation of microsomes.

Preparation of rabbit lung microsomes

Immediately after the animal was sacrified by carbon dioxide asphyxiation, the lungs were perfused via the pulmonary artery with 10-15 ml of ice cold 0.15 M KCl/0.02 M KH_2PO_4 buffer containing 100 U/ml heparin (Elkin-Sinn, Cherry Hill, NJ). The isolated perfused lungs were coarsely minced in a solution consisting of 0.02 M of Tris, 0.15 M of KCl, 0.2 mM of EDTA, and 0.5 mM of dithiotreitol and homogenized in a Waring Ble-

ndor 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 ml of 0.02 M of Tris and 0.15 M of KCl, and the pH was adjusted to 7.4 with 1 N NaOH, and finally the resulting suspension was centrifuged at 100,000×g for an additional 60 min. The pellet was resuspended in the buffer, homogenized, and stored under nitrogen at a concentration of approximately 15-25 mg/ml at −70℃ for up to 1 month. Protein concentration was determined by the method of Lowry et al.(1951).

Determination of cytochrome P-450 concentrations

The concentrations of cytochrome P-450 were determined using an Aminco DW-2 UV/visible spectrophotometer by measuring UV absorbance difference between the dithionite-reduced carbon monoxide treated sample and an unreduced carbon monoxide treated reference sample (Estabrook *et al.*, 1972).

Metabolism studies

Incubation mixtures (final volume of 1.0 ml) consisted of 1.0 mg of microsomal protein, 1 mM of (S)-nicotine $\Delta^{1',5'}$ iminium ion, 100 μm of (s)-^{3}H-nicotine (50 mCi), EGTA (1 mM) in 0.1 M of HEPES buffer, pH 7.6 and an NADPH regenerating system (0.5 mM of $NADP^+$, 8 mM of glucose-6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase, and 4 mM of $MgCl_2$). Incubations were conducted over a period of 60 min at 37℃ under air and samples were taken at designed time 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 with CH_2Cl_2. Sample workup followed the procedure described below for the construction of standard curves.

HPLC analysis and standard curves

Standard curves for (S)-nicotine, (S)-nicotine-$\Delta^{1',5'}$-iminium perchlorate, and (S)-cotinine were constructed as follows: A mixture of the above compounds (20-80 nmol) in 0.1 ml of 0.01 N HCl was added to suspension of 2.0 mg of microsomal protein in 0.9 ml of 0.1 M HEPES, pH 7.6 buffer. To these mixtures was added N-metyl-N-(3-pyridyl)methyl-propionamide (23 nmol) in 40 μl of HEPES buffer as internal standard followed by 1 ml of 1 M K_2CO_3. HPLC grade methylene chloride (2 ml) was added and the resulting mixtures was vortexed for 30 sec. Centrifugation at 1000 g for 2-3 min provided a clear organic layer, which was subjected to HPLC analysis as described below. Plots of peak height ratios of analyte to internal standard against analyte concentrations gave straight lines, which were used in estimating analyte concentrations in sample incubation mixtures. Recoveries were estimated to be greater than 95%. The HPLC assay used a Beckman 110A solvent delivery system and a Hitachi 100-10 spectrophotometer/flowcellcombination.Thepre-column (4.6-mm×5-cm) was packed with Lichrosorb Si 60, 30-μm particle size (Merck, Darmstadt, FRG) and the analytical column (4.6-mm×25-cm) with 10-μm Lichrosorb. The mobile phase consisted of acetonitrile plus 1% of n-propylamine (v/v) and the flow rate was 2.0 ml/min. UV absorption was monitored at 260 nm, the λ_{max} for (S)-nicotine. Samples were run in triplicate.

Quantitation of covalent binding

This was assayed by a minor modification of a filtration method reported by Bulger *et al.* (1983). Post-incubation precipitates in acetonitrile were filter-trapped on GF/B glass microfiber filters (Whatman, Clifton, NJ) using a 10-well vacuum filtration apparatus (Bio-Rad, Richmond, CA). Filters were presaturated with 2.0 ml of 100 mM nicotine to reduce nonspecific binding of ^{3}H-nicotine. The precipitates were washed with ethanol (10 ml), hexane (20 ml), methanol : ether (3 : 1, 30 ml) and methanol:ether (1 : 3, 20 ml). No radioactivity was detected in the final solvent wash. The filters were transferred to scintillation vials containing 5 ml of Aquasol scintillation cocktail and radioactivity was determined using a Packard Prias PLD Tri-Carb instrument. The results obtained by this method were almost identical to those obtained by comparative studies using more laborious acid/solvent extraction methods (Bulger *et al.*, 1983).

RESULTS AND DISCUSSION

Metablism-dependent covalent binding of (S)-[5-^{3}H] nicotine to the lung microsomes *in vitro*

The incubation of (S)-[5-^{3}H]nicotine with control rabbit lung microsomal preparations resulted in the NADPH-dependent covalent binding of radioactive metabolites derived from (S)-nicotine to microsomal macromolecules (Fig. 1). As expected, incubation of lung microsomes isolated from rabbits with the cytochrome P-450 monooxygenase inhibitors, α-mb and Aroclor 1260 decreased both the rate of covalent binding. 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 covalent binding with rabbit lung macromolecules by >65%. α-MB, rabbit lung microsomal cytochrome P-450 isozyme 2 and 6 suicide

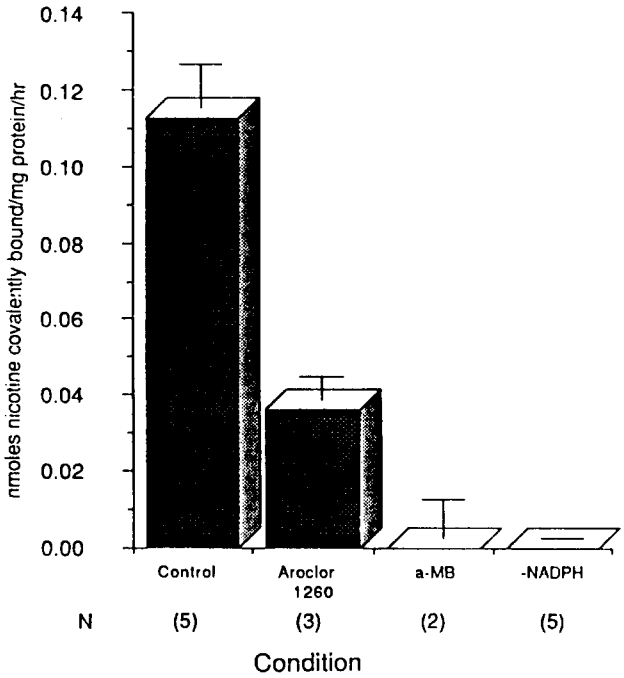


Fig. 1. Effect of inhibitors of rabbit lung microsomal cytochrome P-450 isozyme-2 on the covalent of 5-(S)-^{3}H-Nicotine.

substrate, inhibited covalent binding almost completely. This suggests that isozyme 6 probably does not contribute significantly to the overall oxidation of nicotine by lung microsomes. Therefore, we have demonstrated the requirement for lung cytochrome P-450 isozyme 2 in the bioativation of nicotine to reactive species which covalently bind to biomacromolecules.

Biodisposition of nicotine $\Delta^{1',5'}$ iminium ion in the lung

Since the lung represents the most important target tissue for the expression of tobacco related deseases, assessing the potential for alkylation of reactive intermediates of nicotine to pulmonary biomacromolecules is an important issue. In this regard, characterization of the biodisposition of nicotine $\Delta^{1',5'}$ iminium ion to cotinine by the lung soluble enzymes is important since levels of this activity could be related inversely to the local concentration of the electrophilic and reactive iminium species. The biodisposition of nicotine and its primary 2 electron oxidized metabolite, nicotine $\Delta^{1',5'}$ iminium ion is a much more complex situation in the lung than the liver because of the diversity of cells present in the pulmonary system. For example, there have been approximately 40 cell types identified in the lung compared to 3 in the liver. Of these 40 cell types, the Clara and the alveolar type II cells are the only known to possess cytochrome P-450 activity. It is likely that most of the pulmonary oxidative metabolism of nicotine is carried out by

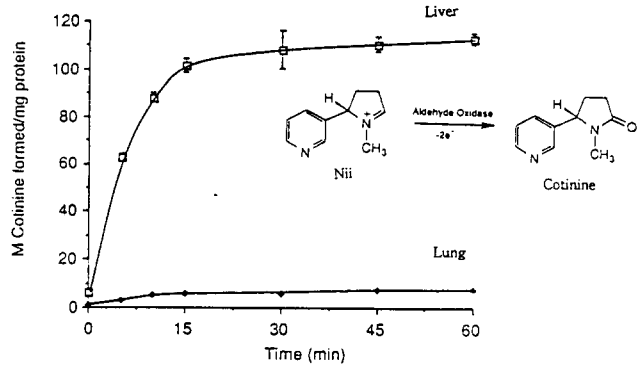


Fig. 2. Rates of oxidation of nicotine $\Delta^{1',5'}$ iminium ion (Nii) to cotinine by 100,000×g supernatant of lung and liver.

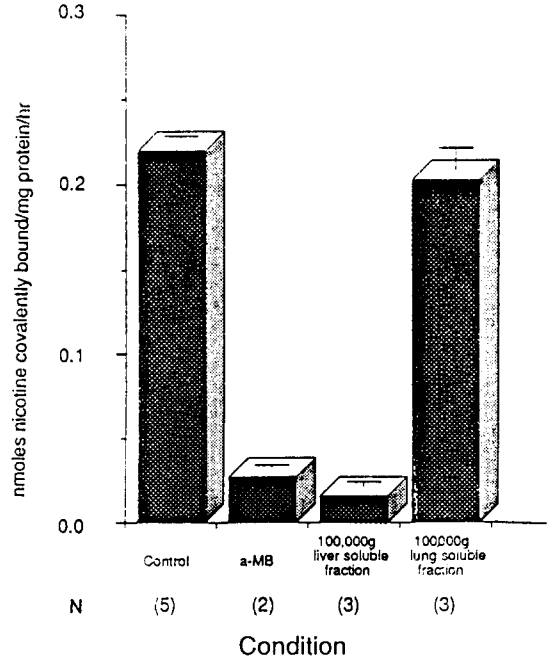


Fig. 3. Effect of various treatments on the covalent binding of 5-(S)-^{3}H-Nicotine to lung microsomal protein.

these cells. Characterization of the aldehyde oxidase catalyzed oxidation of nicotine $\Delta^{1',5'}$ iminium ion to cotinine by the pulmonary tissue has not been documented.

To evaluate nicotine $\Delta^{1',5'}$ iminium ion oxidase activity in lung 100,000×g supernatant, formation of cotinine was measured. Control activity was assessed by liver 100,000×g supernatant catalyzed oxidation of nicotine $\Delta^{1',5'}$ iminium ion to cotinine (Fig. 2). The iminium ion was rapidly oxidized over the first 10-15 minutes (Kcat=12.6 nmoles nicotine iminium ion oxidized mg protein^{-1}min^{-1}). When nicotine $\Delta^{1',5'}$ iminium ion was subjected to metabolic incubations containing the lung 100,000×g supernatant, small but measurable quantities of cotinine were detected (Kcat=0.7 nmoles nicotine iminium ion oxidized mg protein^{-1}min^{-1}).

Similar to the observations with liver, lung 100,000×g supernatant catalyzed oxidation of nicotine $\Delta^{1',5'}$ iminium ion was linear over the first 10 min with activity abruptly leveling off between 15 to 60 min. By 1 hour approximately only 1% of the iminium ion was converted to cotinine. The relative oxidation rates of nicotine $\Delta^{1',5'}$ iminium ion to cotinine described above indicates that lung 100,000×'s g suernatant catalyzes this oxidation approximately 18 times slower than the liver system per mg of protein. Since the activity of lung iminium oxidase appears much lower than the liver, it is tempting to speculate that localized concentrations of nicotine $\Delta^{1',5'}$ iminium ion in lung will survive for a longer period of time. Possible consequences of this lower activity may be expressed as increased covalent binding.

Results of these incubation encouraged us to pursue studies on the covalent binding of nicotine to liver and lung 100,000×g soluble fraction microsomal protein (Fig. 3). As expected, the results show that the covalent binding of nicotine to the lung 100,000×g supernatant more increased compared to that of the liver.

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