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RABBIT NASAL CYTOCHROME P-450 NMa HAS HIGH ACTIVITY AS A NICOTINE OXIDASE

David E. Williams*+, Xinxin Ding# and Minor J. Coon#

*Department of Food Science & Technology and Toxicology Program, Oregon State University, Corvallis, OR 97331

#Department of Biological Chemistry, Medical School, The University of Michigan, Ann Arbor, MI 48109

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SUMMARY: Rabbit nasal olfactory and respiratory microsomes demonstrate high activity toward [^{3}H]-(S)-nicotine, with specific activities of 22.2 and 6.5 nmol/min/mg protein, respectively. The major metabolite produced is (S)-nicotine $\Delta^{1',5'}$-iminium ion, with lesser amounts of nornicotine and the N'-oxide. Reconstitution of the rabbit nasal microsomal system with cytochromes P-450 NMa and NMb indicated that only P-450 NMa has significant activity toward nicotine, and the metabolite profile and turnover are similar to that observed with nasal microsomes. The low K_m (35 μM) and high V_{max} (28 min^{-1}) suggest that a significant portion of inhaled nicotine is metabolized by nasal tissues in the rabbit.

(S)-Nicotine is the major pharmacologically active component of inhaled tobacco and is primarily responsible for tobacco addiction. The metabolism of nicotine (reviewed in ref. 1) in mammals is catalyzed predominantly by two microsomal monooxygenase systems, the cytochrome P-450-dependent mixed-function oxidase system and the flavin-containing monooxygenase. Two major metabolites of nicotine are derived from oxidation at either the 5'-carbon to yield initially the $\Delta^{1',5'}$-iminium ion, which is further metabolized by cytosolic aldehyde oxidase to cotinine, or at the pyrrolidine nitrogen to give the N'-oxide. The formation of the $\Delta^{1',5'}$-iminium ion is catalyzed by cytochromes P-450, and numerous studies have indicated that the P-450s belonging to the IIB sub-family have high activity in this reaction (2-5). N'-oxidation of (S)- and (R)-nicotine is catalyzed efficiently by liver flavin-containing monooxygenase (6). In a general sense, P-450-dependent C5'-oxidation can be regarded as bioactivation, as most covalent binding observed following exposure to nicotine occurs through the iminium ion (7). Tertiary amine N-

+To whom correspondence should be addressed.

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oxides, however, such as nicotine N'-oxide, are highly hydrophilic, easily excreted metabolites generally thought to represent detoxication (8).

Most studies performed on nicotine metabolism have focused on liver. However, as nasal and other respiratory tissues are the initial tissues exposed, it is of importance to determine the capacity of these tissues for metabolism of this alkaloid. A number of studies have recently demonstrated that the levels and activity of cytochromes P-450 are relatively high in nasal tissues of a number of mammals (9,10) with specific contents ranging from 8-41% of that found in liver (10). The activity of nasal microsomes toward a number of xenobiotics, when expressed on a per nmol P-450 basis are actually significantly higher than activities seen in liver microsomes. These examples include known or suspected nasal carcinogens such as hexamethylphosphoramide and N-nitrosodiethylamine (11).

The relative roles of cytochromes P-450 and flavin-containing monooxygenase in the metabolism of (S)-nicotine in rabbit lung has recently been examined (12). The major metabolite produced by rabbit lung microsomes is the iminium ion, and P-450 form 2 (IIB4) accounts for essentially all of the P-450-dependent formation of this metabolite. Rabbit lung flavin-containing monooxygenase, which has previously been demonstrated to exhibit substrate specificities which distinguish it from the liver enzyme (13-15), has very low activity toward N'-oxidation of (S)-nicotine. The high C5'-oxidation by P-450 2, the low N'-oxidation by flavin-containing monooxygenase, and the relatively low levels of aldehyde oxidase in lung may all be contributing factors to the relatively high levels of covalent binding to macromolecules observed upon incubation of [^{3}H]-(S)-nicotine with rabbit lung microsomes (7).

Recently, two novel forms of P-450 (P-450s NMa and NMb) have been purified from rabbit nasal microsomes (11). Rabbit P-450 NMa exhibits high activity toward a number of xenobiotics, including hexamethylphosphoramide, phenacetin, N-nitrosodiethylamine and ethanol, whereas P-450 NMb displays high activity toward an endogenous substrate, testosterone. In this study, we have found that rabbit P-450 NMa very efficiently catalyzes the oxidation of (S)-nicotine, producing predominantly the $\Delta^{1',5'}$-iminium ion. The metabolic profile of P-450 NMa in the reconstituted system and the

high turnover number (28 min^{-1}) are consistent with the postulate that the high nicotine oxidase activity observed with rabbit nasal microsomes is due to P-450 NMa.

EXPERIMENTAL PROCEDURES

Materials: (S)-Nicotine, (S)-[5'-^{3}H]-nicotine, (S)-nicotine $\Delta^{1',5'}$-iminium ion bisperchlorate, nicotine N'-oxide and nornicotine were generously provided by Dr. Neal Castagnoli, Jr., (Virginia Polytechnic Inst. and State University, Blacksburg, VA). NADPH, dilauroylphosphatidylcholine (DLPC), DL-isocitrate and isocitrate dehydrogenase (EC 1.1.1.42, Type IV from porcine heart) were purchased from Sigma Chem. Co., (St. Louis, MO). Triethylamine (gold label) was obtained from Aldrich Chem. Co., (Milwaukee, WI).

Tissues and enzymes: Frozen nasal tissue from male adult New Zealand White (NZW) rabbits was obtained from Pel-Freez Biol., (Rogers, AR) and microsomes were prepared as described previously (16). The nasal maxillary turbinals were used for the preparation of respiratory microsomes, while the ethmoturbinals and the septa were combined for the preparation of olfactory microsomes. The purification and partial characterization of rabbit nasal cytochromes P-450 NMa and NMb have been published (11). Rabbit lung P-450 2 was purified from lung microsomes of pregnant NZW rabbits (Pel-Freez) using the procedure of Guengerich (17). NADPH-cytochrome P-450 reductase was purified from rabbit liver microsomes using the procedure of Yasukochi and Masters (18).

Nicotine oxidation: The microsomal incubations were performed in microcentrifuge tubes in a total volume of 0.1 ml. The assay system was composed of 0.05 mg of microsomal protein in 0.05 M Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA with an NADPH-regenerating system (0.02 M isocitrate, 0.025 U isocitrate dehydrogenase and 1 mM NADPH). (S)-[5-^{3}H]-Nicotine was present at a concentration of 0.5 mM (64 µCi/µmol, added from a 10 mM stock in 20% ethanol, 0.1 N HCl). All of the reaction components, excluding the microsomes, were preincubated at 37°C for 2 min prior to initiation of the reaction by the addition of the microsomes. The reaction was carried out at 37°C with shaking for 0-40 min and terminated by the addition of 0.1 ml of methanol. The tubes were incubated on ice for 30 min and then the precipitated proteins were pelleted by centrifugation at 16,000 rpm in a microcentrifuge (Beckman Microfuge E) at 4°C for 10 min. An aliquot of the supernatant layer was injected directly onto the HPLC column (Beckman Ultrasphere ODS, 5 m, 4.6 x 250 mm) and [^{3}H]-nicotine and metabolites were resolved by an isocratic system of 20% acetonitrile:methanol (7:3) and 80% 0.03 M sodium phosphate, pH 7.0, containing 0.1% triethylamine, at a flow rate of 1 ml/min (Shimadzu Model LC-6A pumps and SCL-6A system controller). Quantitation was performed with an on-line radioisotope detector (Beckman Model 171) equipped with a liquid flow cell; the scintillation fluid (RPI 3a70B) flow rate was 2 ml/min. Peak identification was by co-elution (uv detection at 260 nm with a Shimadzu Model SPD-6AV) with the following standards: (S)-nicotine N'-oxide (3.2 min), nornicotine (3.7 min), cotinine (5.7 min), (S)-nicotine $\Delta^{1',5'}$ iminium ion (7.2 min) and (S)-nicotine (10.5 min). The results were plotted as A_{260} and ^{3}H on two separate channels and the radioactivity peaks integrated utilizing a ChromatoGraphics software program from Beckman.

Rabbit lung cytochrome P-450 nicotine oxidase was assayed by reconstituting 0.01-0.05 nmol of P-450 with a 2.5-fold molar excess of NADPH-cytochrome P-450 reductase and 2 μg of DLPC (added from a 0.1% stock in water, sonicated just prior to use) at room temperature for 10 min. Following the preincubation, the remaining assay components were added, excluding NADPH, and the system was preincubated for 2 min at 37°C prior to initiation of the reaction with NADPH. (S)-[5-^{3}H]-Nicotine was added at levels of 0.01-0.5 mM. The reaction time was varied (10-40 min) in the kinetic studies, with the (S)-nicotine concentration adjusted to maintain initial velocity rates as much as possible. The subsequent termination of the reaction and metabolite analysis was as described above.

RESULTS AND DISCUSSION

Incubation of rabbit olfactory and respiratory nasal microsomes, at a concentration of 0.5 mg of microsomal protein per ml, with 0.5 mM (S)-[^{3}H]-nicotine resulted in a roughly linear rate of nicotine oxidase activity between 2-40 min (Fig. 1). Essentially all of the ^{3}H incorporated into metabolites could be accounted for as (S)-nicotine-N'oxide, nornicotine and the iminium ion. Rabbit nasal microsomes resemble rabbit lung microsomes in producing primarily the iminium ion, with lesser amounts of nornicotine and the N'-oxide (Table I). The sum of total metabolites derived from (S)-nicotine was 22.2 and 6.46 nmol/min/mg protein for rabbit nasal olfactory and respiratory microsomes, respectively. Although, on a per mg protein basis, the olfactory microsomes display almost 4-fold higher

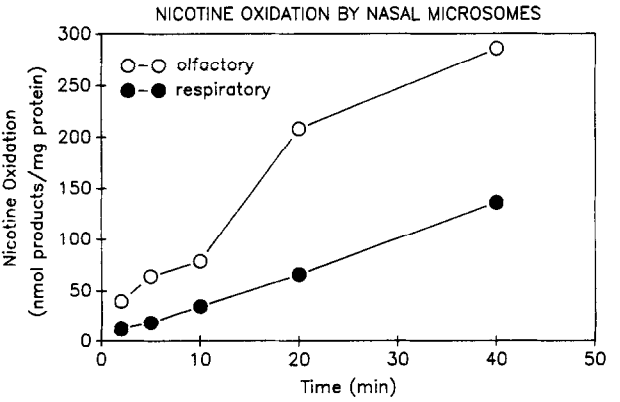

Figure 1. The metabolism of (S)-[5-^{3}H]-nicotine by rabbit nasal microsomes as a function of time. The total nicotine oxidase (N'-oxide, nornicotine and iminium ion) activity of rabbit nasal olfactory (open circles) and respiratory (closed circles) microsomes was determined by incubating 0.05 mg of microsomal protein with 0.5 mM (S)-[5-^{3}H]-nicotine at 37°C with shaking for 2, 5, 10, 20 or 40 min. The remaining assay components were as described in Materials and Methods. Data points represent the mean of duplicates.

Table I

Metabolism of (S)-Nicotine by Microsomes From Rabbit Nasal and Lung Tissue

Microsomes	Nicotine Oxidase Activity (nmol products/min/mg protein)[a]			
	N'-oxide	Nornicotine	Iminium Ion	Total
Nasal Olfactory	6.67	5.17	10.4	22.2 (35.8)[b]
Nasal Respiratory	0.84	1.57	4.05	6.46 (28.1)
Lung	3.01	0.95	4.67	8.63 (18.8)

[a]The values given represent the average of duplicate determinations.
[b]Values in parentheses are calculated as nmol/min/nmol total P-450.

activity, if the results are expressed as turnover numbers (nmol of nicotine oxidized/min/nmol total microsomal P-450), the activities are roughly equal (35.8 and 28.1 min^{-1} for olfactory and respiratory, respectively). The microsomes from both regions of the nasal tissue displayed activities (on a per nmol P-450 basis) about 2-fold higher than rabbit lung microsomes (Table I). The lung microsomes were obtained from pregnant NZW rabbits and the nasal microsomes were from adult male NZW rabbits (both from Pel-Freez Biol.), and, therefore, a direct comparison of activities between tissues may not be appropriate.

Previous studies, using immunoblotting techniques to determine the isozyme composition of rabbit tissues had documented that nasal microsomes contain P-450s 2, 3a, 4 and 5 (19,20). Williams et al. (12) and McCoy et al. (21) have shown that, of these four isozymes, P-450 2 displays the highest activity toward nicotine. The activities of reconstituted rabbit nasal P450s NMa and NMb toward (S)-nicotine were compared to rabbit lung P-450 2 (Table II). (S)-Nicotine oxidation was catalyzed most efficiently by P-450 NMa, which displayed 10-fold higher activity than P-450 NMb. These results would be consistent with the finding of Ding and Coon (11) that P-450 NMa displays higher activity than P-450 NMb toward xenobiotics such as hexamethylphosphoramide, phenacetin, N-nitrosodiethylamine and ethanol, whereas NMb has a higher turnover with the endogenous substrate testosterone. The metabolite profile of purified P-450 NMa in the reconstituted system resembles that of the nasal microsomes in that the iminium ion is the major product, but there are also significant amounts of nornicotine and N'-oxide produced. Rabbit nasal P-450 NMa oxidized (S)-nicotine at least 4 times as rapidly as P-450 2 (Table II). The turnover number given for P-450 NMa (9.01

Table II

Nicotine metabolism by rabbit P-450s in the reconstituted system

Rabbit P-450	Nicotine Oxidase Activity (nmol/min/nmol P-450)[a]			
	N'-oxide	Nornicotine	Iminium Ion	Total
Nasal P-450 NMa	1.11	2.15	5.75	9.01
Nasal P-450 NMb	0.62	ND[b]	0.31	0.93
Lung P-450 2	0.42	0.74	0.91	2.07

[a]Rabbit P-450s (0.05 nmol) were reconstituted with a 2.5 molar excess of NADPH-cytochrome P-450 reductase and 2 μg of DLPC as described in Materials and Methods. The reaction was allowed to proceed at 37°C for 40 min.
[b]Not detectable.

min^{-1}) was not a true V_{max}, as under the conditions used in Table I (0.05 nmol, 40 min incubation with 0.5 mM (S)-nicotine), 70-80% of the parent compound had been metabolized. (See Fig. 2.)

In order to get a more accurate estimate of the V_{max} and K_m of P-450 NMa-dependent oxidation of (S)-nicotine, the activity of the reconstituted enzyme system with concentrations of (S)-nicotine from 0.01-0.5 mM was determined under conditions which ensured initial

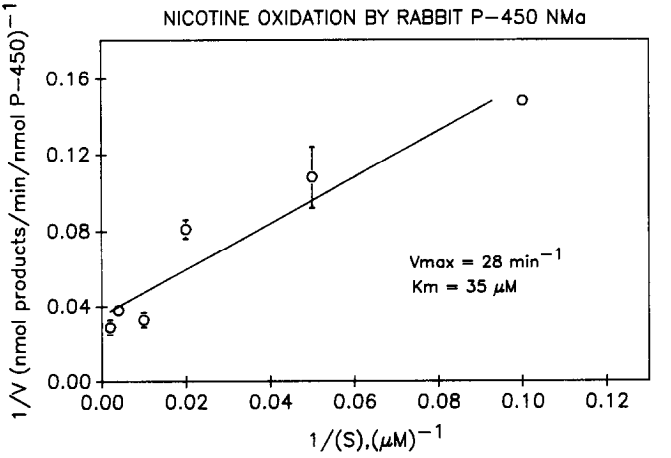

Figure 2. Kinetics of (S)-[5-^{3}H]-nicotine oxidation by nasal P-450 NMa in the reconstituted system. P-450 NMa (0.01 nmol) was reconstituted with rabbit liver NADPH-cytochrome P-450 reductase (0.025 nmol) and 2 μg DLPC as described in Materials and Methods. The metabolism of (S)-[5-^{3}H]-nicotine was determined at substrate concentrations of 0.01, 0.02, 0.05, 0.1, 0.25 and 0.5 mM. The incubation times were 10, 20 and 40 min for the lowest, middle and highest substrate concentrations, respectively (in order to ensure initital velocities). The values shown are the mean (± S.E.) of triplicates or the mean of duplicates.

rates (see Materials and Methods). Linear regression analysis of the double-reciprocal plot obtained yielded a V_{max} of 28 min^{-1} (total metabolites) and a K_m of 35 μM. This turnover number with reconstituted P-450 NMa compares well with the turnover exhibited by nasal microsomes (Table I), suggesting that P-450 NMa catalyzes most of the nicotine oxidation in these microsomes. For comparison, previous work (12) had determined that rabbit P-450 2 had a V_{max} of 1.5 min^{-1} (iminium ion only) and a K_m of 70 μM. Therefore, the rabbit P-450 which had previously been demonstrated to have the highest activity toward nicotine had a maximum velocity about 10-fold less than rabbit nasal P-450 NMa and a K_m about 2-fold higher. In a study by McCoy et al. (21), rabbit P-450 2 exhibited a turnover of 35 min^{-1} (C- plus N-oxidation) and the turnover with other rabbit P-450s (3a, 3b, 3c, 4 and 6) ranged from 2.4-38.0 min^{-1}. These incubations were performed with 30 mM nicotine, and the differences between our results and those of McCoy et al. are similar to those observed in previously published turnover numbers of various rabbit P-450 isozymes with N-nitrosodimethylamine (22). At a concentration of 0.1 mM N-nitrosodimethylamine, only rabbit P-450 form 3a displayed significant N-demethylation activity, whereas all the P-450s displayed significant activities at a substrate concentration of 100 mM.

In summary, we have demonstrated that rabbit nasal microsomes readily metabolize (S)-nicotine to (S)-nicotine $\Delta^{1',5'}$-iminium ion, nornicotine and (S)-nicotine N'-oxide. Most of the nicotine oxidation in rabbit nasal microsomes appears to be due to the activity of a novel form of P-450 recently purified by Ding and Coon (11) and termed P-450 NMa. P-450 NMa also exhibits relatively high activity toward hexamethylphosphoramide, phenacetin, N-nitrosodiethylamine and ethanol. The formation of relatively large amounts of nicotine $\Delta^{1',5'}$-iminium ion, an electrophilic metabolite responsible for much of the nicotine-derived covalent binding to macromolecules in nasal tissue, is consistent with previous studies documenting covalent adducts in nasal tissue from tobacco smoke (23) and a possible epidemiological correlation between smoking and cancers of the nasal cavity (24).

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