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NON-METABOLIC COVALENT BINDING OF NICOTINE-$\Delta^{1'(5')}$-IMINIUM ION TO LIVER MICROSOMES AND SULFHYDRYL-CONTAINING POLYAMINO ACIDS

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In users of tobacco products, nicotine is metabolized by several distinct enzyme systems to yield more than a dozen products (1). Cotinine, an abundant metabolite, is formed by two sequential enzymatic reactions: the 5'-position of nicotine is oxidized by the cytochrome P-450 system to nicotine-$\Delta^{1'(5')}$-iminium ion (2), which is further metabolized by cytosolic aldehyde oxidase to form cotinine (3) (Fig. 1). During its metabolism, nicotine can also undergo cytochrome P-450 dependent covalent binding in the range of 0.2 to1.3 nmol of nicotine derived product per mg microsomal protein (4 and †). However, while the iminium ion has been proposed as a likely participant in this binding (4), direct evidence for the identities of the reactive metabolite(s) and protein groups involved in adduct formation is lacking. Cytochrome P-450 dependent binding of tertiary amines to protein nucleophiles occurs with 1-benzylpyrrolidine (5) and phencyclidine (6), and their respective iminium ions have been implicated as the species responsible for the binding. Toxicity, carcinogenicity, and teratogenicity of iminium ions have been proposed but have yet to be determined (7).

Here we show that, in the absence of enzymes, nicotine-$\Delta^{1'(5')}$-iminium ion can covalently bind to the sulfhydryl groups of polycysteine, but not to polyamino acids having other reactive groups (i.e. hydroxyl, amino, carboxyl, imidazole). Also presented is evidence indicating that the nicotine-$\Delta^{1'(5')}$-iminium ion and nicotine-$\Delta^{4'(5')}$-enamine are interconvertible. The tautomeric form that prevails is dependent upon whether aqueous or anhydrous conditions are used in the isolation procedures.

MATERIALS AND METHODS

The sources of reagents were as follows: (S)-nicotine (Eastman Kodak Co., Rochester, NY); glutathione, poly-S-carbobenzoxycysteine, bovine serum albumin, and methylene blue (Sigma Chemical Co., St. Louis, MO); sodium borohydride and 5,5'-dithiobis-(2-nitrobenzoic acid) (Aldrich Chemical Co., Milwaukee, WI). (S)-[*pyridyl*-^{3}H]-Nicotine (1.02 Ci/mmol) was prepared by catalytic exchange by New England Nuclear, Boston, MA. Polycysteine (8), (S)-cotinine (9), (S)-5'-cyanonicotine and (S)-nicotine-$\Delta^{1'(5')}$-iminium diperchlorate (10) were prepared as described. The buffer used in all experiments was 0.15 M potassium chloride/0.05 M potassium phosphate, pH 7.2.

For the synthesis of (S)-[^{3}H]-nicotine-$\Delta^{1'(5')}$-iminium salt (10,11), 6.0 mg of [^{3}H]-nicotine (4.3 mCi) was added to a reaction mixture containing 0.7 mg methylene blue, 4.3 mg sodium pyruvate, and 3.0 mg KCN dissolved in 1.0 ml of methanol. The reaction tube was placed in a glass water bath maintained at room temperature, positioned 6 cm away from a 250-watt incandescent lamp, and oxygen was bubbled through for 12 hr. After evaporation of the solvent, the 5'-cyanonicotine was purified by silica gel TLC (R_f=0.5-0.7) using as a solvent system methylene chloride/acetonitrile/n-propylamine (400:100:1) and eluted from the plate with methanol to yield 4.1 mg product (60%). This material, combined with 300 mg unlabeled 5'-cyanonicotine, was reacted with perchloric acid as described (10) to obtain 329 mg [^{3}H]-nicotine-$\Delta^{1'(5')}$-iminium diperchlorate (57%) (m.p.= 233-240^o [dec.]).

* To whom all correspondence should be addressed.
† Unpublished data.

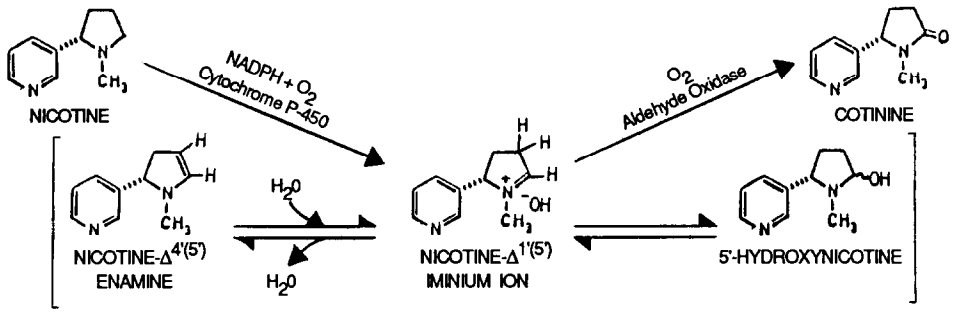

Fig. 1. Pathway of metabolism of nicotine to cotinine.

Livers were obtained from New Zealand rabbits killed by asphyxiation with CO_2. Microsomes were prepared from livers of freshly killed animals or from tissue that had been frozen at -70°. The livers were homogenized in buffer, centrifuged for 30 min (15,000 *g*) at 4° to remove coarse debris, then centrifuged for 1 hr at 100,000 *g* to pellet the microsomes. The supernatant fluid from this high speed centrifugation was kept frozen at -20° and used as the source of the aldehyde oxidase. The pellet was resuspended in buffer using a teflon homogenizer, centrifuged two additional times at 100,000 *g* as described, suspended again in buffer, and stored at -70°. The protein concentration was determined by the method of Lowry *et al.* (12).

RESULTS AND DISCUSSION

To determine the extent of non-metabolic covalent binding of iminium ion to microsomes, the reactants were incubated as described in Table 1. NADPH, a requirement of the enzymatic dependent binding (4 and *), was omitted. Approximately 3.4 nmol of iminium ion bound per mg of microsomal protein, 3-10 times more than when [^{3}H]-nicotine was incorporated enzymatically. Various nucleophiles were tested for their abilities to inhibit iminium ion binding (Table 1). Cyanide ion and glutathione react with nicotine-$\Delta^{1'(5')}$-iminium ion to form 5'-cyanonicotine and 5'-S-glutathionylnicotine respectively (13), but we found the glutathione adduct to be labile to isolation (*). Cyanide ion, glutathione, and β-mercaptoethanol diminished iminium ion binding to microsomal protein (Table 1); n-propylamine was ineffective. Cyanide adduct(s) were identified in the reaction mixture containing KCN, demonstrating that loss of the iminium functional group resulted in decreased binding to protein. Both glutathione and cyanide ion diminished cytochrome P-450 dependent binding of nicotine to microsomal protein (4 and *). However, the effect of the cyanide ion could be attributed to inhibition of metabolism rather than to reaction with the nicotine iminium ion.

In the presence of aldehyde oxidase, nicotine-$\Delta^{1'(5')}$-iminium ion is oxidized to cotinine, which would be expected to reduce the amount of reactive species available for covalent binding. The inclusion of aldehyde oxidase (0.5 ml 100,000 *g* supernatant fluid) in the reaction mixture diminished iminium ion binding to the protein by 80%. Approximately 25% of the iminium ion was metabolized to cotinine as determined by silica gel TLC (ethyl acetate/methanol/acetic acid [17:2:1]) and by radioimmunoassay (14). In the absence of aldehyde oxidase, cotinine formation was undetectable. After blockage of the sulfhydryl groups of the microsomes by prior treatment with 1.0 mM 5,5'-dithiobis-(2-nitrobenzoic acid), binding of [^{3}H]-iminium ion was reduced 75% compared to the untreated controls, implicating sulfhydryl groups in nucleophilic attack on the iminium ion. During a time course study of iminium ion binding to microsomal protein, maximal binding was observed at 30 min. The binding slowly

* Unpublished data.

Table 1. Non-metabolic covalent binding of nicotine-$\Delta^{1'(5')}$-iminium ion to microsomes in the presence of various nucleophiles*

Nucleophile	Binding (nmol/mg protein)
Control	3.3
+ 1.0 mM KCN	0.3
+10.0 mM Glutathione	1.9
+10.0 mM β-Mercaptoethanol	2.4
+10.0 mM n-Propylamine	3.6

* [^{3}H]-Iminium salt (1.0 mg) and 13.0 mg microsomal protein in 2 ml buffer were incubated at 37° for 1 hr in the presence and absence of nucleophiles. The protein was precipitated by adding 2.5 ml ethanol. After centrifugation, the pellet was washed with 2.5-ml portions of ethanol seven additional times and then dissolved in 1 M NaOH for radioactivity measurements. The results shown represent the average of at least two values differing by no greater than 10% except for the KCN values which differed by 20%.

Table 2. Binding of nicotine-$\Delta^{1'(5')}$-iminium ion to polyamino acids*

Polyamino acid	Binding (nmol/mg protein)
Polycysteine	115
Poly-S-carbobenzoxycysteine	1
Polyhistidine	2
Polyglutamic acid	2
Polylysine	1
Polylysyltyrosine (1:9)	2

* [^{3}H]-Iminium salt (1.0 mg) was added to 3.0 mg of various polyamino acids suspended in 2 ml buffer, and incubated for 1 hr at 37°. To provide bulk to the precipitate, 10 mg bovine serum albumin (0.5 ml of 20 mg/ml) was added followed immediately by 5 ml ethanol. The precipitate, collected and washed with 2.5-ml portions of ethanol seven additional times, was then dissolved in 1 M NaOH for radioactivity measurements. Results shown represent the average of two values that differed by no greater than 6%.

decreased to about 80% of its maximal value and leveled off over the next 3.5 hr. Addition of an equal amount of unlabeled iminium ion after 1 hr did not accelerate the release of radioactivity beyond that of the control reaction. The reaction between the iminium ion and protein was essentially irreversible within the time frame studied.

When polyamino acids containing nucleophilic groups commonly found in proteins (sulfhydryl, amino, carboxyl, imidazole, and phenolic) were allowed to react with the iminium ion, only polycysteine bound the labeled compound (115 nmol/mg polycysteine) (Table 2). Polyhistidine, polylysine, polyglutamic acid, polylysyltyrosine (1:9), and poly-S-carbobenzoxycysteine (which contained no free sulfhydryl groups) bound iminium ion to a negligible extent (1-2 nmol/mg polyamino acid). To measure the extent of reaction between the iminium ion and free sulfhydryl groups, 1.0 mg polycysteine and 1.0 mg of the radiolabeled iminium ion were incubated for 1 hr at 37°. Estimation of free sulfhydryl groups using 5,5'-dithiobis-(2-nitrobenzoic acid) indicated that only 16% of the total residues in the polycysteine were reduced. (The remainder may have been oxidized, e.g. formed disulfide bonds.) Under these conditions, one out of ten free sulfhydryl groups in polycysteine reacted with the iminium ion.

Whether iminium ion-protein adducts exist in users of tobacco products remains to be determined. The concentration of the iminium ion in a particular cell type would be dependent upon the enzyme systems involved in its formation and catabolism as well as the pharmacodynamic factors that govern its distribution and excretion. The reaction catalyzed by the cytochrome P-450 system (Fig. 1) may not be the only metabolic route by which the iminium ion can be formed *in vivo*. Recently, nicotine-$\Delta^{4'(5')}$-enamine has been identified in incubation mixtures containing nicotine, arachidonic acid, and a source of prostaglandin H synthase, i.e. microsomes from lung or seminal vesicles (15). (Covalent binding of the metabolic product to microsomes was observed during this reaction.) The enamine was also isolated from urine samples obtained from a smoker and rabbits receiving nicotine (15). Mattammal *et al.* (15) characterized the enamine as a new, distinct metabolite that may serve as a marker for prostaglandin H synthase dependent nicotine metabolism. Our results indicate that the enamine represents a form of the iminium ion that was isolated because of the anhydrous conditions used in their procedures. The enamine, iminium ion, carbinolamine, and amino aldehyde are equivalent forms of the same compound and the maintenance of a particular structure is determined by the properties of the compound and its

environment (16) (Fig. 1). NMR evidence (17) indicates that the nicotine iminium form is prevalent in neutral aqueous solution while the enamine form is undetectable. The enamine, chemically synthesized in the manner outlined (15) (and with structure confirmed by infrared spectroscopy and mass spectrometry), demonstrates properties identical to the iminium ion in neutral aqueous solution. It undergoes two reactions characteristic of the iminium ion: it reacts with cyanide ion to yield 5'-cyanonicotine and with sodium borohydride to yield nicotine. It is also metabolized to cotinine by aldehyde oxidase at the same rate as the iminium ion. When we subjected the chemically synthesized iminium ion to the anhydrous procedures that were used to isolate the prostaglandin H synthase product (15), the enamine tautomer was formed. This ability to interconvert the enamine and iminium ion by altering environmental conditions (i.e. anhydrous and aqueous) would argue against the claim that the enamine is a new metabolite of nicotine metabolism (15). Under physiological conditions, the iminium ion would be the expected tautomeric form of the reaction product. Further study is required to determine the extent to which the individual enzymatic reactions contribute to the formation of nicotine-$\Delta^{1'(5')}$-iminium ion and to its covalent binding to macromolecules *in vivo*.

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