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RAPID COMMUNICATIONS

NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD)-DEPENDENT OXIDATION OF NICOTINE-$\Delta^{1'(5')}$-IMINIUM ION TO COTININE BY RABBIT LIVER MICROSOMES

R. Scott Obach and Helen Van Vunakis*

Department of Biochemistry, Brandeis University, Waltham, MA 02254, U.S.A.

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Nicotine is metabolized to cotinine via the action of two sequential enzymatic reactions. Cytochrome P-450 oxidizes the 5' position to yield the intermediate nicotine-$\Delta^{1'(5')}$-iminium ion (1). Further oxidation of this compound to cotinine by cytosolic aldehyde oxidase has been described (2,3). The oxidation of tertiary amine xenobiotics to amides through an iminium ion intermediate is well documented as a general pathway of detoxication of this class of compounds (4). Since iminium ions are electrophiles with potentially deleterious effects, an understanding of their metabolism and disposition is important (5). During its metabolism, nicotine undergoes covalent binding to microsomal protein (6), while the iminium ion binds to sulfhydryl groups on macromolecules in the absence of enzymes (7).

In this report, we demonstrate the existence of a second pathway of cotinine formation from nicotine-$\Delta^{1'(5')}$-iminium ion (Fig. 1). This reaction is catalyzed by the microsomal fraction and requires NAD. The enzyme involved is distinguished from aldehyde oxidase by its subcellular location, oxidant specificity, and sensitivity to different inhibitors. This pathway uses water as the source of the oxygen in the production of cotinine.

MATERIALS AND METHODS

The sources of reagents were as follows: NAD, NADH disodium salt, NADP monosodium salt, methylene blue, disulfiram, coenzyme Q_0, nitroblue tetrazolium, and menadione (Sigma Chemical Co., St. Louis, MO); and 50% ^{18}O-water (Cambridge Isotope Laboratories, Woburn, MA). (S)-[*pyridyl*-^3H]-Nicotine-$\Delta^{1'(5')}$-iminium diperchlorate (7), cotinine (8), and nicotine-$\Delta^{1'(5')}$-iminium diperchlorate (9) were prepared as described. Subcellular fractions were obtained from the organs of New Zealand rabbits. Microsomal fractions were prepared as in Ref. 7 and washed three times with buffer (0.15 M KCl/0.05 M KH_2PO_4, pH 7.2) before use. Protein concentration was determined by the method of Lowry *et al.* (10).

Unless stated otherwise, a typical enzymatic assay mixture (consisting of 0.5 to 2.0 mg protein, 1.8 mM nicotine-$\Delta^{1'(5')}$-iminium diperchlorate, and 3.3 mM NAD in a total volume of 0.75 mL buffer) was shaken at 37° in covered tubes. The reactions were started upon addition of the microsomes and terminated by dilution into a 500-fold excess of cold buffer for the analysis of cotinine by radioimmunoassay (RIA) (11).

The oxygen-18 tracer study was performed in water enriched 20% in oxygen-18. After 1 hr, cotinine was extracted with 3 mL CH_2Cl_2. The solvent was removed under N_2, and the residue analyzed by GC/MS on a Hewlett-Packard SE-54 5% phenyl/1% vinyl column (0.3 mm x 50 m). The initial oven temperature was 150° and raised at a rate of 15°/min; the carrier gas (Helium) flow rate was 25 mL/min. The mass spectra were obtained by electron impact at 70 eV.

* To whom all correspondence should be addressed.

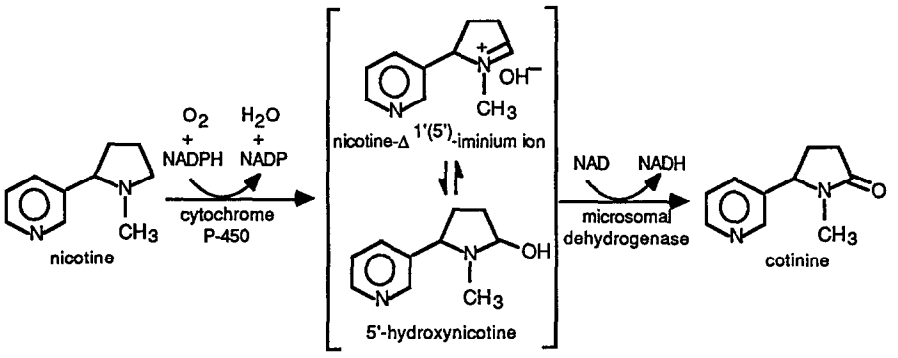

Fig. 1. Microsomal pathway of metabolism of nicotine to cotinine.

RESULTS AND DISCUSSION

When rabbit liver microsomes, nicotine-$\Delta^{1'(5')}$-iminium ion and NAD were incubated, cotinine was formed (Fig. 2). In addition to quantification of cotinine by a specific RIA, the identity of the product was confirmed by TLC utilizing [^{3}H]iminium ion as substrate, and by GC/MS (data to be discussed). The amount of NADH formed, as measured by absorbance at 340 nm, was equivalent to the amount of cotinine produced. Microsomes from other organs could catalyze this reaction, but not with the activity demonstrated by liver microsomes (Table 1). On a protein basis, kidney microsomes had approximately a third of the activity of liver; those from other organs were considerably less active.

Cytosolic aldehyde oxidase can use O_2, NAD, NADP, ferricyanide, alloxan, methylene blue, and nitroblue tetrazolium, in addition to many other compounds, as terminal electron acceptors (12). When tested with several different oxidants, the microsomal mediated oxidation of nicotine-$\Delta^{1'(5')}$-iminium ion was highly specific for NAD (Table 2). In contrast, a wide range of oxidants is sufficient for catalysis by the soluble fraction. Thus, the reaction mediated by microsomes is catalyzed by a distinct enzyme, and is not merely a membrane associated form of aldehyde oxidase. The microsomal enzyme can also use NADP, but much less efficiently than NAD.

Additional distinctions between the two enzyme systems were observed. Aldehyde oxidase is inhibited by arsenite ion and menadione (12). The inhibition by arsenite is believed to involve inactivation at the molybdenum cofactor center (12). The production of cotinine by microsomes was not affected by arsenite (Table 3), indicating that this activity does not reside in an isozyme of the molybdenum containing oxidase family. Menadione, a potent inhibitor of aldehyde oxidase, *enhanced* cotinine production in the microsome mediated reaction (Table 3). Menadione did not directly activate the enzyme or serve as an oxidant, but provided additional oxidizing capacity by cycling the NADH product back to NAD. Consistent with this explanation is the observation that cotinine was formed in incubation mixtures containing menadione and NADH (instead of NAD). Coenzyme Q_0, a quinone similar in structure to menadione, also enhanced cotinine production, whereas benzoquinone and vitamin K_2 did not (data not shown).

Oxygen-18 isotope tracer evidence conclusively showed that the lactam oxygen of cotinine is derived from water (Table 4). It remains to be determined whether the oxygen is inserted by an enzymatic or non-enzymatic mechanism. Theoretically, in aqueous solution, cyclic iminium ions can exist in carbinolamine and amino carbonyl tautomeric species or as the deprotonated enamine species (13). In the case of nicotine-$\Delta^{1'(5')}$-iminium ion, NMR spectroscopy indicates that only the iminium and carbinolamine forms exist, with the iminium form predominating at pH < 7.5 and the carbinolamine form predominating at pH > 8.9 (14). Because of this tautomerism, the microsomal enzyme could be an iminium ion, alcohol, or aldehyde dehydrogenase. Both aldehyde and alcohol dehydrogenases have

Table 1. NAD-dependent conversion of nicotine-$\Delta^{1'(5')}$-iminium ion to cotinine by rabbit microsomes isolated from various organs

Organ	% Activity (relative to liver)
Liver	100.0
Lung	5.8
Kidney	33.4
Heart	3.1
Bladder	9.7
Brain	4.5

Table 2. Metabolism of nicotine-$\Delta^{1'(5')}$-iminium ion to cotinine: Oxidant specificity of liver microsomal and soluble fractions*

Oxidant	% of Iminium ion converted to cotinine Microsomal	Soluble
O_2	0.9	9.0
NAD	28.5	26.2
NADP	4.1	33.3
$K_3Fe(CN)_6$	3.9	24.3
Methylene blue	1.2	25.1
Alloxan	1.3	13.8
Nitroblue tetrazolium	1.2	15.3

* Incubations were carried out for 1 hr under a N_2 atmosphere except in the case in which O_2 was tested as an oxidant. Oxidant concentrations were 1.8 mM.

Table 3. Metabolism of nicotine-$\Delta^{1'(5')}$-iminium ion to cotinine: Effect of aldehyde oxidase inhibitors on liver microsomal and soluble fractions*

Inhibitor	Cofactor	Conversion (% of control) Microsomal	Soluble
Control	NAD	100	100
$NaAsO_2$	NAD	96	60
Menadione	NAD	247	<3
Control	NADH	1	ND
Menadione	NADH	152	ND

* Arsenite and menadione concentrations were 1.8 mM. Menadione was added to the reaction mixture in dimethyl sulfoxide. DMSO itself had no effect in the amounts used. ND = not determined. Amount of cotinine formed in the control + NAD reaction = 57.3 µg (microsomal); 87.1µg (soluble).

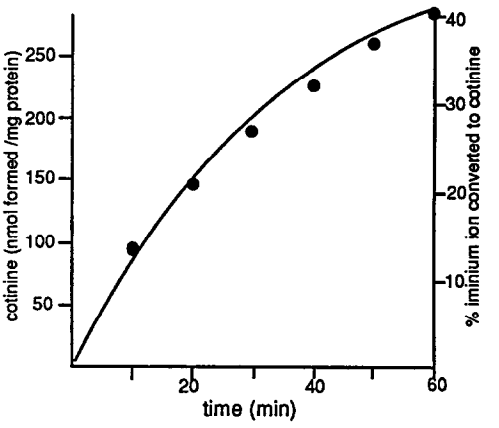

Fig. 2. Time course of conversion of nicotine-$\Delta^{1'(5')}$-iminium ion to cotinine catalyzed by rabbit liver microsomes.

Table 4. Mass spectrometry fragment abundances of cotinine formed from nicotine-$\Delta^{1'(5')}$-iminium ion by rabbit liver microsomes

Sample	*m/z*	Relative amount (%)
Synthetic (S)-cotinine (natural abundance)	98	99.7
	100	0.3
	176	99.4
	178	0.6
Synthetic (S)-cotinine (incubated in 20% $H_2{}^{18}O$)	98	99.5
	100	0.6
	176	99.0
	178	1.0
Enzymatic incubation in $H_2{}^{16}O$	98	99.6
	100	0.4
	176	99.2
	178	0.8
Enzymatic incubation in 20% $H_2{}^{18}O$	98	80.8
	100	19.2
	176	80.6
	178	19.4

m/z 176 = mass ion; *m/z* 98 = pyrrolidonyl fragment.

been detected in microsomal preparations (15,16,17). Initial studies using aldehyde dehydrogenase inhibitors {disulfiram, Mg^{2+}, diethyldithiocarbamic acid methanethiol mixed disulfide, chloral, and cyanamide with catalase activation (18,19,20)} or alcohol dehydrogenase inhibitors {imidazole, pyrazole, and 4-methylpyrazole (15)} showed little or no effect of these compounds on the microsomal conversion of nicotine-$\Delta^{1'(5')}$-iminium ion to cotinine. These studies do not eliminate the possibility that this reaction is catalyzed by an isozymic form of either enzyme that is insensitive to the inhibitors tested.

Several laboratories have shown that cytosolic aldehyde oxidase converts nicotine-$\Delta^{1'(5')}$-iminium ion to cotinine (2,3,21). We have presented evidence that this conversion can also be catalyzed by a microsomal dehydrogenase. Although the metabolism of nicotine has been studied extensively (22) this second pathway was probably missed because it specifically requires NAD or, to a lesser extent, NADP. The small amounts of cotinine detected when nicotine is incubated with liver microsomes and NADPH (6, *) may result from the action of this pathway utilizing, as the oxidant, the NADP

*Obach RS and Van Vunakis H, Radioimmunoassay of nicotine-$\Delta^{1'(5')}$-iminium ion, an intermediate formed during the metabolism of nicotine to cotinine. Manuscript submitted for publication.

produced during the cytochrome P-450 reaction. In *in vitro* incubations, rabbit liver soluble fraction has about twice the specific activity of the microsomal fraction. Based on the fact that there is 3.5-5 times as much protein in the soluble fraction, it can be estimated that the soluble fraction has approximately 7-10 times the capacity to oxidize nicotine-$\Delta^{1'(5')}$- iminium ion. However, this new pathway may play a major role in the detoxication of nicotine-$\Delta^{1'(5')}$-iminium ion (and perhaps other iminium ions) *in vivo* since it is located in the microsomal fraction where these electrophiles, capable of covalently binding to tissue macromolecules (6,7), are formed by cytochrome P-450. Underway are studies directed toward purifying, characterizing, and determining the substrate specificity of this dehydrogenase.

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