2′-Hydroxylation of nicotine by cytochrome P450 2A6 and human liver microsomes: Formation of a lung carcinogen precursor

Stephen S. Hecht*, J. Bradley Hochalter, Peter W. Villalta, and Sharon E. Murphy

University of Minnesota Cancer Center, Minneapolis, MN 55455

Edited by Allan H. Conney, Rutgers, The State University of New Jersey, Piscataway, NJ, and approved September 1, 2000 (received for review May 8, 2000)

Smokers or people undergoing nicotine replacement therapy excrete approximately 10% of the nicotine dose as 4-oxo-4-(3-pyridyl)butanoic acid (keto acid) and 4-hydroxy-4-(3-pyridyl)butanoic acid (hydroxy acid). Previously, these acids were thought to arise by secondary metabolism of the major nicotine metabolite cotinine, but our data did not support this mechanism. Therefore, we hypothesized that nicotine is metabolized by 2′-hydroxylation, which would ultimately yield keto acid and hydroxy acid as urinary metabolites. This pathway had not been established previously in mammalian systems and is potentially significant because the product of nicotine 2′-hydroxylation, 4-(methylamino)-1-(3-pyridyl)-1-butanone (aminoketone), can be converted to the potent tobacco-specific lung carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Incubation of nicotine with cytochrome P450 2A6 and cofactors did indeed produce aminoketone, which was identified as its *N*-benzoyl derivative by GC-MS. The rate was 11% of that of cotinine production. Incubation of human liver microsomes with nicotine gave keto acid by using aminoketone as an intermediate; keto acid was not formed from cotinine. In 10 human liver samples, rates of formation of keto acid were 5.7% of those of cotinine and production of these metabolites correlated. These results provide definitive evidence for mammalian 2′-hydroxylation of nicotine and elucidate a pathway by which endogenous formation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone could occur in humans.

As an addictive agent that prevents smokers from quitting, nicotine is arguably responsible for more adverse health consequences than any other single compound. Smoking causes an estimated 430,000 deaths per year in the U.S., including 30% of all cancer deaths (1). Lung cancer alone will kill over 150,000 people in the U.S. in 2000, and cigarette smoking is directly responsible for 87% of lung cancer mortality (1). An understanding of nicotine metabolism provides a critical framework for deciphering the mechanisms by which tobacco products cause disease.

An overview of nicotine metabolism is presented in Fig. 1 (2). Nicotine is hydroxylated at the 5′ position yielding an unstable intermediate, 5′-hydroxynicotine (**5**), which exists in equilibrium with $\Delta^{1'(5')}$ iminium ion **6**. 5′-Hydroxylation of nicotine is catalyzed mainly by cytochrome P450 2A6 (P450 2A6) in humans, with some contribution of P450s 2B6 and 2D6 (2–5). 5′-Hydroxynicotine is oxidized by aldehyde oxidase to cotinine (**8**; ref. 2). Cotinine, in turn, is metabolized further to cotinine-Gluc (**9**), *trans*-3′-hydroxycotinine (**12**), and *trans*-3′-hydroxycotinine-Gluc (**13**; ref. 2). Cotinine and its metabolites account for 70–80% of nicotine metabolism in humans (6–9). Nicotine also is metabolized to nicotine-Gluc (**2**) and several other minor metabolites not shown in Fig. 1 (2, 6–9).

Recently, we developed analytical methods to quantify 4-oxo 4-(3-pyridyl)butanoic acid (keto acid; **11**) and 4-hydroxy-4-(3-pyridyl)butanoic acid (hydroxy acid; **14**) in the urine of smokers and people using the nicotine patch (10). We found that the sum of keto acid and hydroxy acid accounted for approximately 10–15% of urinary nicotine metabolites (9, 10). In the 1960s, McKennis and coworkers (11, 12) proposed that keto acid and hydroxy acid were formed by further metabolism of cotinine. However, recently we analyzed the urine of nonsmokers who had been dosed with cotinine and found that keto acid and hydroxy acid accounted for less than 0.5% of the dose (S.E.M., unpublished data). Therefore, we hypothesize that the actual source of keto acid and hydroxy acid is 2′-hydroxylation of nicotine. As shown in Fig. 1, 2′-hydroxylation of nicotine would give 2′-hydroxynicotine (**4**), which spontaneously yields $\Delta^{1'(2')}$ iminium ion **3** and 4-(methylamino)-1-(3-pyridyl)-1-butanone (aminoketone; **7**), also known as pseudooxynicotine (13, 14). Aminoketone (**7**) could ultimately be converted metabolically to keto acid by keto aldehyde (**10**). Although this pathway of nicotine metabolism apparently occurs in bacterial systems (15–18), there is scant and questionable evidence for its existence in mammalian systems (2, 19–21). Aminoketone (**7**) is the direct precursor to the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK; Fig. 2), which can be formed from aminoketone by simple nitrosation (22). In fact, chemical synthesis of NNK is carried out by nitrosation of aminoketone (23). Therefore, metabolic production of aminoketone from nicotine potentially could provide a direct link between nicotine and lung cancer. Consequently, we carried out experiments to test our hypothesis that aminoketone is a mammalian metabolite of nicotine.

Materials and Methods

Chemicals and Reagents. (*S*)-(−)-Nicotine, (*S*)-(−)-cotinine, and benzoyl chloride were obtained from Aldrich Chem (Milwaukee, WI). The purity of (*S*)-(−)-nicotine was determined by using HPLC system A with UV detection at 254 nm; there were no detectable impurities. Keto acid, hydroxy acid, [2,2,3,3,4-D_5]hydroxy acid, and aminoketone (**7**) dihydrochloride were synthesized (10, 12, 23). 4-(Methyl-*N*-benzoylamino)-1-(3-pyridyl)-1-butanone (*N*-benzoylaminoketone; **15**) was prepared as described (13). A mixture of 40.9 mg of (163 μmol) aminoketone **7** dihydrochloride, 2 ml of 2 M NaOH, and 200 μl of benzoyl chloride was stirred vigorously overnight. The reaction mixture was extracted with $CHCl_3$, and the extracts were dried and concentrated. The residue was purified by using HPLC system B to give **15**: ^{1}H-NMR ($CDCl_3$) δ9.09 (0.6H, bs, *pyr-2H*, *E* isomer), 8.95 (0.4H, bs, *pyr-2H*, *Z* isomer), 8.68 (bs, 1H, *pyr-6H*), 8.23 (0.6H, m, *pyr-4H*, *E* isomer), 8.11 (0.4H, m, *pyr-4H*, *Z* isomer), 7.41–7.26 (m, 6H, *pyr-5H* + *ArH*), 3.58 (m, 1.2H, *4-H*, *E* isomer), 3.31 (m, 0.8H, *4-H*, *Z* isomer), 3.15 (s, 1.8H, CH_3, *E* isomer), 3.07 (m, 1.2H, *2H*, *E* isomer), 2.94 (s, 1.2H, CH_3, *Z* isomer), 2.79 (m, 0.8H, *2H*, *Z* isomer), 2.08 (m, 1.2H, *3H*, *E* isomer),

This paper was submitted directly (Track II) to the PNAS office.

Abbreviation: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

*To whom reprint requests should be addressed at: University of Minnesota Cancer Center, Box 806 Mayo, 420 Delaware Street SE, Minneapolis, MN 55455. E-mail: hecht002@tc.umn.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.220207697. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.220207697

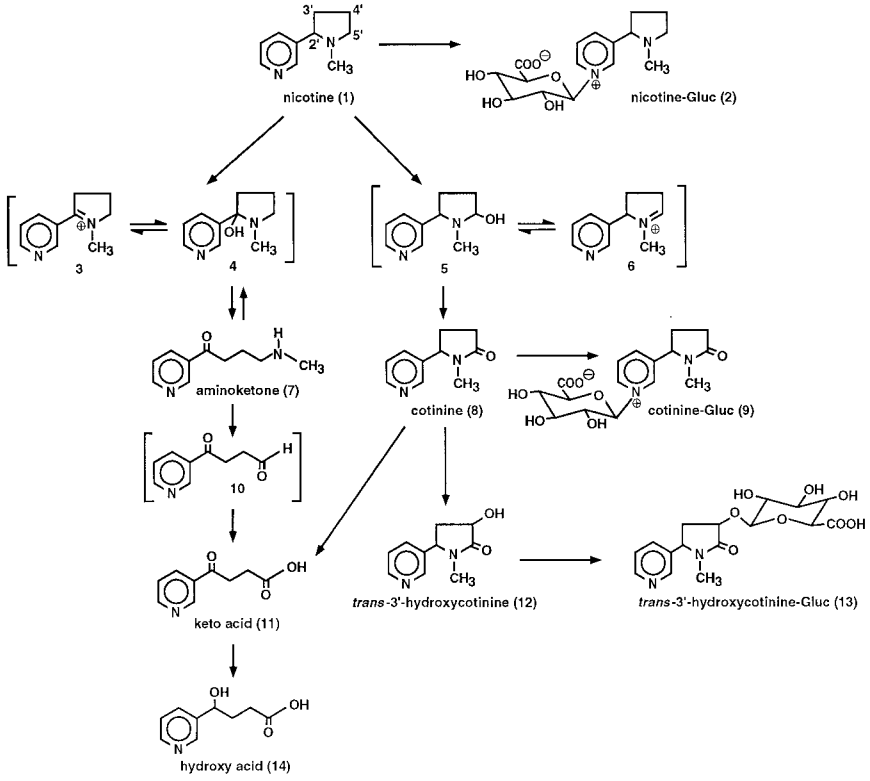

Fig. 1. Pathways of mammalian nicotine metabolism initiated by 5′-hydroxylation and 2′-hydroxylation. Other pathways not shown include *N*-oxidation, *N*-demethylation, and *N*-methylation. See ref. 2 for details.

1.94 (m, 0.8H, *3H*, *Z* isomer); chemical ionization-MS (*m*/*z*, relative intensity) 283 (M + 1, 100); electron impact-MS, 147 (32), 105 (100), 77 (77).

Trifluoroacetic acid, sequanal grade, was obtained from Pierce. NADPH-P450 oxidoreductase was obtained from Panvera (Madison, WI). P450 2A6 was expressed from human *CYP*2A6 cDNA by using a *baculovirus* expression system. *Baculovirus*-infected insect cells were used to prepare the microsomes as described (24). All other biochemical reagents were obtained from Sigma. Human liver microsomes were prepared as described from 10 liver samples generously provided by F. P. Guengerich (Vanderbilt University, Nashville, TN) and obtained from organ donors through the Tennessee Donor Services (Nashville, TN) and the Veterans Administration Medical Center (Little Rock, AR). Human liver cytosol was prepared from liver samples kindly provided by R. P. Remmel (University of Minnesota, Minneapolis) and obtained through the Anatomic Gift Foundation.

Nicotine Metabolism by P450 2A6 or Human Liver Microsomes. For each analysis, the incubation mixture contained nicotine (30.9 nmol, 5 μg, 61.7 μM), 5 mM $MgCl_2$, an NADPH generating system (0.4 mM NADP, 10 mM glucose-6-phosphate, 0.4 unit/ml glucose-6-phosphate dehydrogenase), 50 μl of human liver cytosol (4.87 mg protein per ml) when required, and either P450 2A6 (0.044 mg) or human liver microsomes (0.5–1.2 mg) in 0.5 ml of 50 mM potassium phosphate buffer, pH 7.4. In incubations with P450 2A6, 0.7 nmol/ml concentration NADPH/P450 oxidoreductase was included.

The samples were incubated at 37°C with shaking. The reactions were terminated by addition of either 75 mg of Na_2CO_3 in 0.25 ml of H_2O or, if keto acid and hydroxy acid were to be determined, 37.5 mg of Na_2CO_3 in 1.5 ml of H_2O containing 0.47 μg of [2,2,3,3,4-D_5]hydroxy acid as an internal standard. Nicotine, cotinine, and aminoketone were extracted into 2 ml of $CHCl_3$, which was separated from the aqueous layer by using an empty 15-ml Bond Elute column (Varian) fitted with a 0.45 μm × 25 mm Acrodisc CR PTFE syringe filter (Gelman). The aqueous layer was stored at −80°C until analysis for hydroxy acid and keto acid. The $CHCl_3$ phase was then extracted with 0.4 ml of H_2O that had been adjusted to pH 1.56 with trifluoroacetic acid. Aminoketone **7** is stable in acid, but is unstable at neutral or basic pH. The aqueous extracts were stored at −80°C until analysis.

Analysis of Metabolites. HPLC was carried out with a Waters Associates system (Millipore) equipped with a Hitachi D-250 Chromato-Integrator, a Shimadzu SPD-10Avp detector, and a Waters 717 plus autosampler.

The following HPLC systems were used: (A) a 0.46 × 25 cm Luna octadecyl silane (ODS) 5 μm C-18 column (Phenomenex, Torrance, CA) eluted with H_2O adjusted to pH 1.56 with trifluoroacetic acid, at a flow rate of 0.7 ml/min with UV detection at 254 nm; (B) a 0.46 × 25 cm Ultrasphere ODS 5 μm C-18 column (Beckman Coulter) eluted with a linear gradient from 25 to 53% CH_3OH in H_2O over 20 min at a flow rate of 0.7 ml/min with UV detection at 254 nm; (C) the same column as in B eluted with a linear gradient from 20 to 60% CH_3OH in H_2O over 30 min at a flow rate of 1 ml/min with UV detection at 254

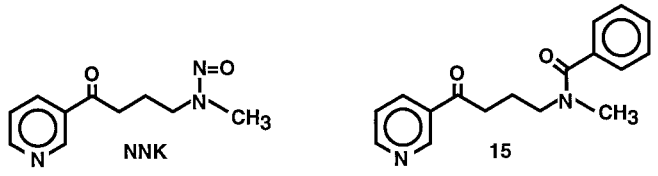

Fig. 2. Structures of NNK and *N*-benzoylaminoketone (**15**).

nm; (D) one column, or two columns connected in series, as in B, with a column temperature of 30°C, and eluted isocratically with 50% CH_3OH in H_2O at a flow rate of 0.7 ml/min with detection by tandem MS; (E) as in A, with a column temperature of 30°C and eluted with H_2O adjusted to pH 1.8 with trifluoroacetic acid.

Nicotine and cotinine (pH 1.56 samples) were quantified by using HPLC system A. Aminoketone (pH 1.56 samples) was quantified by using two methods as follows.

Method 1. The samples were concentrated to dryness on a Speedvac, redissolved in 100 μl of H_2O/trifluoroacetic acid (pH 1.56), and injected onto HPLC system A. The aminoketone region (20.5 to 25 min) was collected and then concentrated to dryness in a 250-μl insert in a 1-ml vial. To each insert 50 μl of H_2O was added, which contained 14.4 μmol of both Na_2CO_3 and $NaHCO_3$, and 0.5 μl of benzoyl chloride. The samples were vortexed, centrifuged, and then sonicated for 1 h; this procedure was repeated twice. They were then diluted with 50 μl of H_2O and injected onto HPLC system B. The eluant containing *N*-benzoylaminoketone (**15**) (26 to 30 min) was collected and concentrated to dryness in a 250-μl insert in a 1-ml vial, then taken up in 20 μl of toluene. Gas chromatography-chemical ionization MS with selected ion monitoring was performed on a Finnigan TSQ 7000 instrument (Finnigan-MAT, San Jose, CA) interfaced with a CTC A200SE autosampler (Leap Technologies, Carrboro, NC) and a HP5890 series II GC (Hewlett–Packard) fitted with a DB-17MS (50% dimethyl/50% diphenylpolysiloxane) capillary column (0.25 × 30 m, 0.15 μm film thickness; J&W Scientific, Folsom, CA). A sample (2 μl) was injected in the splitless mode at an injector temperature of 300°C, with the carrier gas flow (He) set at 2.5 ml/min. The GC oven was programmed as follows: 60°C for 2 min, increase to 300°C at 20°C/min, maintain at 300°C for 7 min. The transfer line to the MS was maintained at 300°C. The MS apparatus was operated in the positive-ion chemical ionization mode, acquiring ion m/z 283, corresponding to M + H of **15**. MS settings were as follows: source temperature, 150°C; manifold temperature, 70°C; electron energy, 150 ev (1 eV = 1.602×10^{-19} J); filament current, 700 μA. Total scan time was 0.25 s, scan width was 0.5 atomic mass unit, and the chemical ionization (methane) gas pressure was 3300 mT. The data were acquired in profile mode with Finnigan's ICIS data acquisition and processing software. GC-MS full scan of **15** was performed as described above with the MS apparatus operated in the positive-ion electron impact mode, acquiring ions from 50 to 400 atomic mass units, with the following modifications: 4-μl sample injection, electron energy 70 ev, and filament current 400 μA.

Method 2. Aliquots (50 μl) were quantified by liquid chromatography electrospray ionization MS in the positive-ion mode by using HPLC system E. MS settings were as follows: capillary temperature, 200°C; electrospray ionization, 2 kV and 53 μA; scan time, 6 s; scan width, 0.3 atomic mass unit; and sheath and auxiliary gas on. Data were acquired in profile mode and smoothed seven times by using the Gaussian algorithm provided with Finnigan's ICIS data acquisition and processing software.

Keto acid and hydroxy acid were quantified essentially as described (10). To each sample to be analyzed for hydroxy acid 0.25 ml of H_2O containing 37.5 mg of Na_2CO_3 was added, and to each sample to be analyzed for keto acid 0.25 ml of 0.1 M NaOH containing 6.46 mg of $NaBH_4$ was added. The samples were allowed to stand overnight at room temperature. Each sample was acidified with 1 ml of 2 M HCl, concentrated to dryness on a Speedvac, dissolved in 0.4 ml of freshly prepared 3% (vol/vol) concentrated H_2SO_4 in CH_3OH, and allowed to stand overnight at room temperature to allow formation of hydroxy acid methyl ester. The reaction mixture was neutralized with 1 ml of 16.7% (wt/vol) $KHCO_3$, and the methyl ester was extracted into 2 ml of $CHCl_3$. The $CHCl_3$ layer was concentrated to dryness in a 4-ml vial, and the residue was immediately treated with 200 μl of benzene containing 12 μl of trimethylamine and 8 μl of (*S*)-(−)-α-methylbenzyl isocyanate. The vial was sealed with a Teflon-coated cap and heated at 75°C for 24 h. Excess reagents were removed by concentration on a Speedvac. The residue was dissolved in 0.22 ml of 60% aqueous CH_3CN and further purified by HPLC system C. Material eluting from 30 to 40 min was collected. After concentration, the residue was dissolved in 150 μl of CH_3OH/H_2O (50/50). Aliquots (50 μl) were quantified by liquid chromatography-atmospheric pressure-chemical ionization-tandem MS with HPLC system D as described (10).

Results

Human P450 2A6 and cofactors were incubated with nicotine for 70 min at 37°C. At the end of the incubation, products were extracted into trifluoroacetic acid and analyzed by *Method 1*. The HPLC retention time corresponding to aminoketone (**7**) was collected and derivatized with benzoyl chloride, producing *N*-benzoylaminoketone (**15,** Fig. 2). This material was analyzed by gas chromatography-chemical ionization-MS with selected ion monitoring for m/z 283, which is M + 1 of **15**. The peak illustrated in Fig. 3*A* has the correct retention time for **15**. The MS of **15** was obtained under electron impact conditions. As shown in Fig. 3 *B* and *C*, this spectrum was identical to that of a standard. These results demonstrate conclusively that aminoketone (**7**) is produced on incubation of nicotine with human P450 2A6. We did not detect **7** in incubations that contained nicotine but lacked P450 2A6.

Rates of metabolism of nicotine by P450 2A6 to aminoketone (**7**) and cotinine were compared. In these experiments, aminoketone was analyzed by *Method 2*, by using liquid chromatography-electrospray ionization-MS, whereas cotinine was quantified by HPLC with UV detection. Cytosol, as a source of aldehyde oxidase, was added to incubations in which cotinine was to be quantified. Formation of these metabolites was linear for at least 40 min. The rates of formation of aminoketone and cotinine were 0.18 and 1.64 nmol/mg of protein per min, respectively. Thus, the rate of formation of aminoketone was 11% of that of cotinine.

In the next series of experiments, we incubated nicotine with human liver microsomes and cofactors. Aminoketone was not detected by liquid chromatography-electrospray ionization-MS, but we did obtain preliminary evidence for the presence of keto acid (Fig. 1, **11**). These results suggested that aminoketone was being converted to keto acid in human liver microsomes. Therefore, we carried out the experiments summarized in Table 1. Each sample was analyzed for keto acid, hydroxy acid, and cotinine. We detected keto acid in incubation mixtures containing human liver microsomes, nicotine, and NADPH, whether or not we included cytosol (Experiments 1 and 2), but keto acid was not detected when NADPH was omitted (Experiment 3). We did not detect keto acid in similar incubations with cotinine as substrate (Experiments 4–6). However, we did detect keto acid in incubation mixtures containing human liver microsomes and aminoketone, independent of the presence of cytosol or NADPH (Experiments 7–9). These results demonstrate that aminoketone is formed from nicotine in human liver microsomes, then converted to keto acid. Aminoketone was not formed from cotinine. Production of hydroxy acid from keto acid was inefficient in these *in vitro* systems.

Metabolism of nicotine to keto acid by human liver microsomes was linear for at least 40 min. Rates of keto acid formation from nicotine were 20.8 and 17.1 pmol/mg of protein per min, respectively, in the presence and absence of cytosol, in incubations with one human liver microsomal sample. Cotinine was formed at a rate of 362 pmol/mg of protein per min. In 10 human liver microsomal samples, the rate of formation of keto acid was 13.0 ± 7.9 (SD) pmol/mg of protein per min (range 0.1–26.9), whereas the corresponding values for cotinine were 230 ± 146

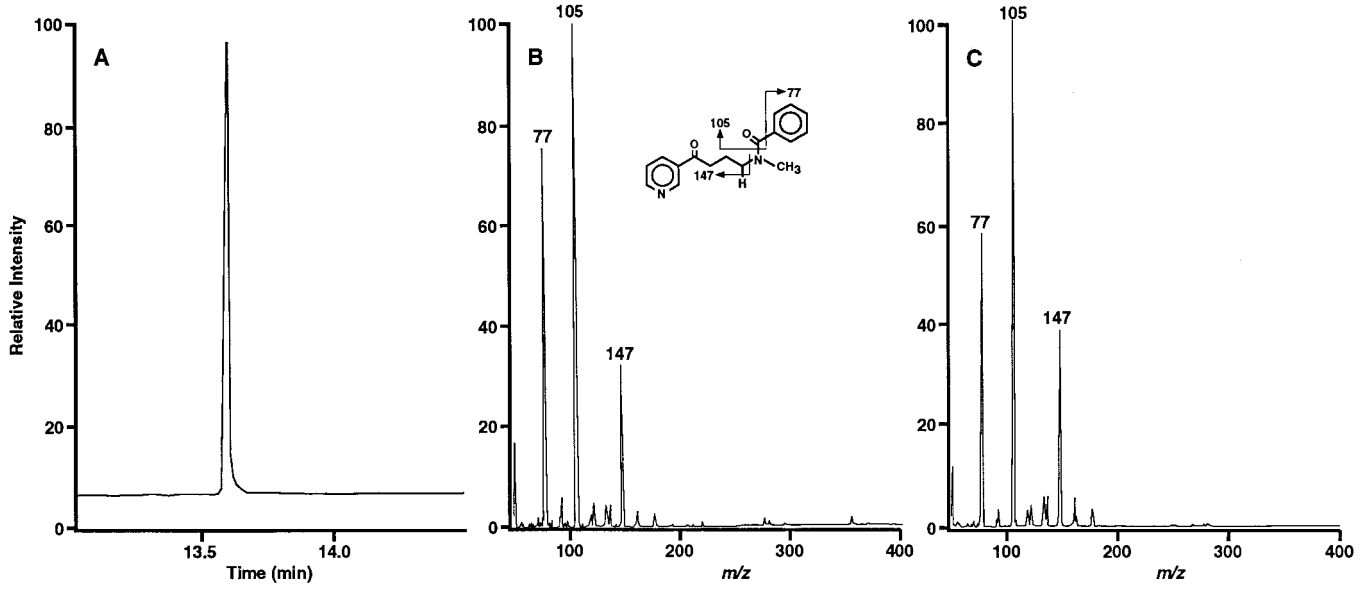

Fig. 3. (*A*) Gas chromatography-chemical ionization MS with selected ion monitoring analysis at m/z 283 for *N*-benzoylaminoketone (**15**). Nicotine, P450 2A6, and cofactors were incubated for 70 min and analyzed by *Method 1*, as described in *Materials and Methods*. The peak has the correct retention time for **15**. (*B*) Electron impact-MS of standard **15**. (*C*) **15** isolated from metabolism of nicotine by P450 2A6.

pmol/mg of protein per min (range 0–532). These rates correlated ($r = 0.92$, $P = 0.0002$).

Discussion

The results presented here clearly demonstrate that 2′-hydroxylation is a mammalian metabolic pathway of nicotine. We observed substantial amounts of aminoketone (**7**) in incubations of nicotine with human P450 2A6; the rate of formation of **7** was about 11% of that of cotinine, formed by 5′-hydroxylation. In human liver microsomes, aminoketone was formed from nicotine, then further metabolized to keto acid (**11**), presumably by keto aldehyde (**10**). The rate of formation of keto acid was 5.7% of that of cotinine in these incubations. Consistent with these observations, we have previously reported that keto acid and hydroxy acid account for approximately 10–15% of urinary nicotine metabolites in smokers and ex-smokers using the nicotine patch, whereas metabolites formed by 5′-hydroxylation of nicotine comprise about 80% of urinary nicotine metabolites (9, 10). McKennis and coworkers (11, 25) demonstrated that keto acid and hydroxy acid were metabolites of cotinine in dogs and rats, accounting for less than 2% of the dose. We found that urinary keto acid and hydroxy acid comprised less than 0.5% of the cotinine dose in humans (S.E.M., unpublished data). Collectively, these results demonstrate that although keto acid and hydroxy acid are metabolites of cotinine, their main source in humans is 2′-hydroxylation of nicotine, as illustrated in Fig. 1. Depending on the system investigated, metabolic 2′-hydroxylation of nicotine was 5.7–13% as great as the well established 5′-hydroxylation pathway was (Table 2).

Bacterial metabolism of nicotine apparently produces aminoketone (**7**), according to studies in the older literature (15–18). Although the identification of aminoketone in those investigations was probably correct, the techniques used at that time do not conform to modern standards of metabolite characterization. There is controversy in the literature regarding mammalian 2′-hydroxylation of nicotine. Neurath *et al.* (19) reported detection of a cyanide adduct of $\Delta^{1'(2')}$ iminium ion **3** in smokers' urine, but the veracity of this report has been challenged (20).

Table 1. Metabolism of nicotine, cotinine, and aminoketone by human liver microsomes

	Reaction mixtures containing human liver microsomes and					Products (nmol)		
Experiment	Nicotine	Cotinine	Aminoketone	Cytosol	NADPH	Keto acid	Hydroxy acid	Cotinine
1	+	−	−	+	+	0.51	ND	8.3
2	+	−	−	−	+	0.36	ND	ND
3	+	−	−	−	−	ND	ND	ND
4	−	+	−	+	+	ND	ND	13
5	−	+	−	−	+	ND	ND	13
6	−	+	−	−	−	ND	ND	14
7	−	−	+	+	+	12	0.44	ND
8	−	−	+	−	+	6.0	ND	ND
9	−	−	+	−	−	6.5	ND	ND
10	−	−	−	+	+	ND	ND	ND
11	−	−	−	−	+	ND	ND	ND
12	−	−	−	−	−	ND	ND	ND

Incubation mixtures contained nicotine (61.7 μM), cotinine (31.8 μM), or aminoketone (21.5 μM), human liver microsomes, and cofactors in a total volume of 0.5 ml, as described in *Materials and Methods*. Reactions were carried out at 37°C for 30 min; keto acid, hydroxy acid, and cotinine were quantified as described in *Materials and Methods*. ND, not detected.

Table 2. Metabolism of nicotine by 2′- and 5′-hydroxylation

System	2′-Hydroxylation as percent of 5′-hydroxylation
P450 2A6	11*†
Human liver microsomes	5.7†‡
Smokers′ urine	13§

*Based on relative rates of formation of aminoketone and cotinine.
†Data from a single nicotine concentration (61.7 μM). Values may differ at lower nicotine concentrations. Rates are estimates because cotinine is a secondary product.
‡Based on relative rates of formation of keto acid and cotinine.
§Percent keto acid plus hydroxy acid (of nicotine metabolites in smokers′ urine) divided by products of 5′-hydroxylation (from ref. 9).

Based on our data, it seems unlikely that substantial amounts of **3** could reach the urine unchanged. Gorrod and Schepers (2) reported detection of $\Delta^{1'(2')}$ iminium ion **3** in tissue preparations during nicotine metabolism, but this work has not been published.

The equilibrium among 2′-hydroxynicotine (**4**), $\Delta^{1'(2')}$ iminium ion **3**, aminoketone (**7**), and 2′,3′-dehydronicotine, which would result from dehydration of **4**, has been studied by NMR (13,14). These studies demonstrate that, at neutral pH, the equilibrium mixture is comprised of approximately 50% each of $\Delta^{1'(2')}$ iminium ion **3** and aminoketone (**7**), with no **4** or 2′,3′-dehydronicotine being detected. At acidic and basic pH, aminoketone predominates. Thus, metabolic 2′-hydroxylation of nicotine will presumably yield a mixture of $\Delta^{1'(2')}$ iminium ion **3** and aminoketone (**7**). As **7** is removed by further metabolism to keto acid, this equilibrium may shift producing more **7**, or **3** may simultaneously be trapped by cellular nucleophiles other than H_2O.

Our results are potentially significant because aminoketone is the direct precursor to the tobacco-specific lung carcinogen NNK, which is believed to play a significant role as a cause of lung cancer in smokers (26, 27). Aminoketone is easily nitrosated, with an intrinsic rate constant similar to that of other secondary amines such as pyrrolidine (22, 28). Because the pH maximum for nitrosation of secondary amines, such as **7**, is typically 3–4, this reaction would occur most readily in the stomach (29). However, nitrosation also occurs at neutral pH under a variety of conditions (29). Iminium ions such as **3**, which is in equilibrium with **7**, are nitrosated at neutral pH (30). Nitric oxide and peroxynitrite react with secondary amines—via N_2O_3, N_2O_4 and other intermediates—to produce nitrosamines at neutral pH (31–33). Nitric oxide and peroxynitrite are formed endogenously under conditions of chronic inflammation or infection, leading to endogenous nitrosamine formation (33–36), and substantial amounts of nitrogen oxides are present in cigarette smoke, resulting in endogenous nitrosamine formation in smokers (29, 34, 35, 37). Although we did not find evidence for endogenous production of NNK in exsmokers using nicotine replacement theory, that conclusion was based on analysis of urinary NNK metabolites (38). Local formation of NNK in certain tissues would not be detectable by analysis of urinary metabolites. We also did not detect increased levels of NNK metabolites in the urine of rats treated with nicotine and nitrite; however, rats lack a hepatic enzyme related to P450 2A6 and therefore may not produce significant amounts of **7** from nicotine (39, 40). These considerations lead to the realistic possibility that NNK could be formed endogenously in people who use tobacco products, resulting in exposure to this carcinogen above and beyond the amounts already present in these products. It is also possible that NNK could be formed endogenously during nicotine replacement therapy, particularly under conditions of long-term therapy.

In summary, this study provides definitive evidence for mammalian 2′-hydroxylation of nicotine. Although nicotine metabolism has been studied for 50 years, virtually all previous work has focused on 5′-hydroxylation, *N*-oxidation, and conjugation reactions. The pathway described here, although quantitatively less important than 5′-hydroxylation, could have important toxicologic consequences.

We thank Pramod Upadhyaya for providing aminoketone (**7**). This study was supported by Grant CA-81301 from the National Cancer Institute.

1. American Cancer Society (2000) *American Cancer Society* (Atlanta, GA), pp. 28–31.
2. Gorrod, J. W. & Schepers, G. (1999) in *Analytical Determination of Nicotine and Related Compounds and Their Metabolites*, eds. Gorrod, J. W. & Jacob, P., III (Elsevier, Amsterdam), pp. 45–67.
3. Nakajima, M., Yamamoto, T., Nunoya, K.-I., Yokoi, T., Nagashima, K., Inoue, K., Funae, Y., Shimada, N., Kamataki, T. & Kuroiwa, Y. (1996) *Drug Metab. Dispos.* **24,** 1212–1217.
4. Yamazaki, H., Inoue, K., Hashimoto, M. & Shimada, T. (1999) *Arch. Toxicol.* **73,** 65–70.
5. Messina, E. S., Tyndale, R. F. & Sellers, E. M. (1997) *J. Pharmacol. Exp. Ther.* **282,** 1608–1614.
6. Benowitz, N. L., Jacob, P., Fong, I. & Gupta, S. (1994) *J. Pharmacol. Exp. Ther.* **268,** 296–303.
7. Byrd, G. D., Chang, K. M., Greene, J. M. & deBethizy, J. D. (1992) *Drug Metab. Dispos.* **20,** 192–197.
8. Neurath, G. B. (1994) *Clin. Invest.* **72,** 190–195.
9. Hecht, S. S., Carmella, S. G. & Murphy, S. E. (1999) *Cancer Epidemiol. Biomarkers Prev.* **8,** 907–913.
10. Hecht, S. S., Hatsukami, D. K., Bonilla, L. E. & Hochalter, J. B. (1999) *Chem. Res. Toxicol.* **12,** 172–179.
11. McKennis, H., Jr. (1965) in *Tobacco Alkaloids and Related Compounds*, ed. von Euler, U. S. (Macmillan, New York), pp. 53–74.
12. McKennis, H., Schwartz, S. L., Turnbull, L. B., Tamaki, E. & Bowman, E. R. (1964) *J. Biol. Chem.* **239,** 3981–3989.
13. Brandäge, S., Lindblom, L., Pilotti, Å. & Rodriguez, B. (1983) *Acta Chem. Scand.* B **37,** 617–622.
14. Zoltewicz, J. A., Bloom, L. B. & Kem, W. R. (1989) *J. Org. Chem.* **54,** 4463–4468.
15. Wada, E. & Yamasaki, K. (1954) *J. Am. Chem. Soc.* **76,** 155–157.
16. Wada, E. (1957) *Arch. Biochem. Biophys.* **72,** 145–162.
17. Hylin, J. W. (1959) *Arch. Biochem. Biophys.* **83,** 528–537.
18. Gherna, R. L., Richardson, S. H. & Rittenberg, S. C. (1965) *J. Biol. Chem.* **240,** 3669–3674.
19. Neurath, G. B., Dünger, M. & Orth, D. (1992) *Med. Sci. Res.* **20,** 853–858.
20. Byrd, G. D., Robinson, J. H., Caldwell, W. S. & deBethizy, J. D. (1994) *Recent Adv. Tob. Sci.* **20,** 5–31.
21. Nguyen, T.-L., Gruenke, L. D. & Castagnoli, N. (1979) *J. Med. Chem.* **22,** 259–263.
22. Caldwell, W. S., Greene, J. M., Dobson, G. P. & deBethizy, J. D. (1993) *Ann. N.Y. Acad. Sci.* **686,** 213–228.
23. Hecht, S. S., Chen, C. B., Dong, M., Ornaf, R. M., Hoffmann, D. & Tso, T. C. (1977) *Beitr. Tabakforsch.* **9,** 1–6.
24. Patten, C. J., Smith, T. J., Murphy, S. E., Wang, M.-H., Lee, J., Tynes, R. E., Koch, P. & Yang, C. S. (1996) *Arch. Biochem. Biophys.* **333,** 127–138.
25. Schwartz, S. L. & McKennis, H., Jr. (1963) *J. Biol. Chem.* **238,** 1807–1812.
26. Hecht, S. S. (1998) *Chem. Res. Toxicol.* **11,** 559–603.
27. Hecht, S. S. (1999) *J. Natl. Cancer Inst.* **91,** 1194–1210.
28. Mirvish, S. S. (1975) *Toxicol. Appl. Pharmacol.* **31,** 325–351.
29. Mirvish, S. S. (1995) *Cancer Lett.* **93,** 17–48.
30. Keefer, L. K. & Roller, P. P. (1973) *Science* **181,** 1245–1247.
31. Masuda, M., Mower, H. F., Pignatelli, B., Celan, I., Friesen, M. D., Nishino, H. & Ohshima, H. (2000) *Chem. Res. Toxicol.* **13,** 301–308.
32. Kirsch, M., Korth, H.-G., Sustmann, R. & deGroot, H. (2000) *Chem. Res. Toxicol.* **13,** 451–461.
33. Leaf, C. D., Wishnok, J. S. & Tannenbaum, S. R. (1989) *Cancer Surv.* **8,** 323–334.
34. Bartsch, H., Ohshima, H., Pignatelli, B. & Calmels, S. (1989) *Cancer Surv.* **8,** 335–362.
35. Bartsch, H. & Spiegelhalder, B. (1996) *Eur. J. Cancer Prev.* **5,** 11–18.
36. Liu, R. H., Baldwin, B., Tennant, B. C. & Hotchkiss, J. H. (1991) *Cancer Res.* **51,** 3925–3929.
37. International Agency for Research on Cancer (1986) *IARC Monogr. Eval. Carcinog. Risk Chem. Hum.* **38,** 95.
38. Hecht, S. S., Carmella, S. G., Chen, M., Koch, J. F. D., Miller, A. T., Murphy, S. E., Jensen, J. A., Zimmerman, C. L. & Hatsukami, D. K. (1999) *Cancer Res.* **59,** 590–596.
39. Carmella, S., Borukhova, A., Desai, D. & Hecht, S. S. (1997) *Carcinogenesis* **18,** 101–106.
40. Honkakoski, P. & Negishi, M. (1997) *Drug Metab. Rev.* **29,** 977–996.