

Characterization and Comparison of Nicotine and Cotinine Metabolism in Vitro and in Vivo in DBA/2 and C57BL/6 Mice

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ABSTRACT

DBA/2 and C57BL/6 are two commonly used mouse strains that differ in response to nicotine. Previous studies have shown that the nicotine-metabolizing enzyme CYP2A5 differs in coumarin metabolism between these two strains, suggesting differences in nicotine metabolism. Nicotine was metabolized to cotinine in vitro by two enzymatic sites. The high-affinity sites exhibited similar parameters (K_m , 10.7 ± 4.8 versus 11.4 ± 3.6 μ M; V_{max} , 0.58 ± 0.18 versus 0.50 ± 0.07 nmol/min/mg for DBA/2 and C57BL/6, respectively). In vivo, the elimination half-lives of nicotine (1 mg/kg, s.c.) were also similar between DBA/2 and C57BL/6 mice (8.6 ± 0.4 versus 9.2 ± 1.6 min, respectively); however, cotinine levels were much higher in DBA/2 mice. The production and identity of the putative cotinine metabolite 3'-hydroxycotinine in mice was confirmed by

liquid chromatography/mass spectrometry/mass spectrometry. The in vivo half-life of cotinine (1 mg/kg, s.c.) was significantly longer in the DBA/2 mice compared with the C57BL/6 mice (50.2 ± 4.7 versus 37.5 ± 9.6 min, respectively, $p < 0.05$). The in vitro metabolism of cotinine to 3'-hydroxycotinine was also less efficient in DBA/2 than C57BL/6 mice (K_m , 51.0 ± 15.6 versus 9.5 ± 2.1 μ M, $p < 0.05$; V_{max} , 0.10 ± 0.01 versus 0.04 ± 0.01 nmol/min/mg, $p < 0.05$, respectively). Inhibitory antibody studies demonstrated that the metabolism of both nicotine and cotinine was mediated by CYP2A5. Genetic differences in *Cyp2a5* potentially contributed to similar nicotine but different cotinine metabolism, which may confound the interpretation of nicotine pharmacological studies and studies using cotinine as a biomarker.

Nicotine is the primary component of cigarettes that is responsible for the addictive properties of smoking, which include feelings of pleasure and reward (Henningfield and Keenan, 1993). Rodents, particularly mice, have been widely used for studying the pharmacological effects of nicotine (Aschhoff et al., 1999; Stolerman et al., 1999). Two of the most commonly used mouse strains for studying nicotine behavioral effects are DBA/2 and C57BL/6. A large number of studies have examined various aspects of nicotine-mediated behaviors such as discrimination, self-administration, tolerance, and withdrawal, and the majority of these studies have

found some differences in nicotine effects between these strains (Aschhoff et al., 1999; Stolerman et al., 1999).

The amino acid sequence of CYP2A5 is 84% identical with the human CYP2A6, the main enzyme responsible for the metabolic inactivation of nicotine (Nakajima et al., 1996b; Messina et al., 1997). The mouse *Cyp2a5* gene is genetically polymorphic (Lindberg et al., 1992). Specifically, the DBA/2 mice express the amino acid Val¹¹⁷ in hepatic CYP2A5 and metabolized coumarin, a selective probe substrate for mouse CYP2A5 and human CYP2A6, much more efficiently than C57BL/6 mice, which express the amino acid Ala¹¹⁷ (Lindberg et al., 1992). Likewise, mutagenesis of CYP2A6, substituting the valine with alanine at the same position, also significantly reduced its catalytic efficiency for coumarin (He et al., 2004).

Genetic variation in human CYP2A6 can alter nicotine metabolism, resulting in altered smoking behaviors (Schoedel et al., 2004; Malaiyandi et al., 2006). For instance, individuals who are homozygous for the *CYP2A6* deletion variant (*CYP2A6**4) produce minimal cotinine (Yamanaka et al., 2004). These individuals smoke fewer cigarettes and are less likely to be dependent on tobacco (Schoedel et al., 2004;

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ABBREVIATIONS: HPLC, high-performance liquid chromatography; MS, mass spectrometry; LC, liquid chromatography; EMS, enhanced mass scan; AUC, area under the concentration; C_{max} , maximum concentration; T_{max} , time at maximum concentration; CL, clearance; F, bioavailability; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

Malaiyandi et al., 2006). Likewise, in male mice, we have shown previously that lower nicotine self-administration behaviors were associated with lower CYP2A5 protein levels and rates of nicotine metabolism (Siu et al., 2006). Furthermore, inhibition of CYP2A5-mediated nicotine metabolism significantly enhanced the pharmacological (i.e., antinociceptive) effects of nicotine in mice (Damaj et al., 2007). These data together suggest that, as in humans, nicotine metabolism can significantly affect nicotine-mediated behaviors in mice. Therefore, the main objective of the study was to characterize nicotine and cotinine metabolism (both in vitro and in vivo) in both the DBA/2 and C57BL/6 mouse strains. Such differences may account for the variations observed in the pharmacological effects of nicotine in these mice.

Materials and Methods

Animals. Adult male C57BL/6 and DBA/2 mice (22–24 g) were obtained from Charles River Laboratories Inc. (Saint-Constant, PQ, Canada). Animals were housed in groups of three to four on a 12-h light cycle and had free access to food and water. We restricted the study to male mice because we have found previously large variation in CYP2A levels and nicotine metabolism among female mice (Siu et al., 2006), which may be due to hormonal influence (mouse estrous cycle is approximately 3–7 days) because estrogen-only oral contraceptives increased nicotine metabolism in human females (Benowitz et al., 2006). In addition, a second CYP2A enzyme, CYP2A4, is present in female mice that may metabolize nicotine (Murphy et al., 2005), complicating the interpretation of our CYP2A5 studies.

Reagents. (–)-Nicotine hydrogen tartrate and (–)-cotinine were purchased from Sigma-Aldrich (St. Louis, MO). Both nicotine and cotinine were dissolved in physiological saline (0.9% sodium chloride) for use in in vivo studies. *Trans*-3'-hydroxycotinine was custom-made by Toronto Research Chemicals Inc. (Toronto, ON, Canada). The internal standard 5-methylcotinine was a generous gift from Dr. Peyton Jacob III at the University of California, San Francisco. All doses are expressed as the free base of the drug. Inhibitory antibodies against human CYP2A6, CYP2B6, and CYP2D6 were purchased from BD Biosciences (Mississauga, ON, Canada).

Membrane Preparations. Microsomal membranes were prepared from mouse livers for in vitro nicotine metabolism assays as described previously (Messina et al., 1997; Siu et al., 2006) and stored at –80°C in 1.15% KCl. The cytosolic fractions were acquired during membrane preparation and were used as a source of aldehyde oxidase. All livers were collected and frozen before 3 PM to avoid the circadian effect on CYP2A5 expression.

Nicotine C-Oxidation Assay. Before determining the in vitro kinetic parameters (K_m and V_{max}) for nicotine metabolism in C57BL/6 and DBA/2 mice, assay conditions were optimized as described previously (Siu et al., 2006). Linear formation of cotinine from nicotine was obtained under assay conditions of 0.5 mg/ml protein concentration with an incubation time of 15 min. Incubation mixtures contained 1 mM NADPH and 1 mg/ml mouse liver cytosol in 50 mM Tris-HCl buffer, pH 7.4, and were performed at 37°C in a final volume of 0.5 ml. The reaction was stopped with a final concentration of 4% v/v Na_2CO_3 . After incubation, 5-methylcotinine (70 μg) was added as the internal standard, and the samples were prepared and analyzed for nicotine and metabolites by HPLC system I as described previously (Siu et al., 2006). The limits of quantification were 5 ng/ml for nicotine, 12.5 ng/ml for cotinine, and 10 ng/ml for 3'-hydroxycotinine.

Cotinine Hydroxylation Assay. Before determining the in vitro kinetic parameters (K_m and V_{max}) of cotinine metabolism in C57BL/6 and DBA/2 mice, assay conditions were optimized. Linear formation of 3'-hydroxycotinine from cotinine was obtained under assay conditions of 1 mg/ml protein with an incubation time of 20 min. The incubation mixture was the same as above with the exception that

aldehyde oxidase was not added, because cotinine metabolism to 3'-hydroxycotinine does not require this cytosolic enzyme. Samples were then analyzed by HPLC system I.

In Vivo Nicotine and Cotinine Treatments and Plasma Nicotine, Cotinine, and 3'-Hydroxycotinine Measurements. To determine the in vivo kinetic parameters of nicotine and cotinine in C57BL/6 and DBA/2 mice, animals were injected with nicotine (1 mg/kg, s.c.) or cotinine (1 mg/kg, s.c.). Blood samples were drawn by cardiac puncture at baseline from untreated animals and from treated animals at various times after the injections. Immediately after collection, plasmas were prepared by centrifugation at 3000g for 10 min and frozen at –20°C until analysis. Sample collection took place before 3 PM. Total nicotine, cotinine, and 3'-hydroxycotinine levels (free and glucuronides) were measured after deconjugation by β -glucuronidase at a final concentration of 5 mg/ml in 0.2 M acetate buffer, pH 5.0, at 37°C overnight. Samples were then analyzed by HPLC system I.

LC/MS/MS Analysis of Cotinine Metabolite. An alternative HPLC system (system II) suitable for separation of eluate for mass spectrometry was used for the characterization of the cotinine metabolite. This system was similar to that described previously with minor modifications (Murphy et al., 1999). In brief, using the same column as HPLC system I, cotinine and its metabolites were eluted with a linear gradient from 100% A' (10 mM ammonium acetate buffer, pH 6.5) to 70% A' and 30% acetonitrile over the course of 30 min at a flow rate of 1 ml/min.

Mass spectrometry analysis was performed at the Proteomic and Mass Spectrometry Centre at the University of Toronto (Toronto, ON, Canada). Data were acquired with the Q TRAP LC/MS/MS System (Applied Biosystems/MDS Sciex, Toronto, ON, Canada). The sample was injected into the sample loop and delivered to the mass spectrometer by 65% acetonitrile and 0.1% formic acid in water at 20 $\mu\text{l}/\text{min}$. Liquid chromatography conditions were as described above (system II), except a flow rate of 0.8 ml/min was used. The liquid was introduced to the mass spectrometer directly after 40:1 splitting. Electrospray ionization was performed in enhanced mass scan (EMS) mode with positive ionization. Nitrogen was used as curtain gas (25 psi), nebulizer gas (25 psi), and heater gas (0 psi). The spray needle voltage was set at 5.5 kV, and collision-induced dissociation gas was set at high. The decluster potential was 20 V, collision energy was 30 eV, and entrance potential was 10 V. Enhanced product ion was performed at a collision energy of 30 eV, all other parameters were the same as described for EMS.

Antibody Inhibition of Nicotine and Cotinine Metabolism. We have demonstrated previously that the anti-CYP2A6 antibody was able to cross-react with mouse CYP2A5 (Siu et al., 2006). Microsomes were preincubated with antihuman selective P450 antibodies (anti-CYP2A6, anti-CYP2B6, and anti-CYP2D6), at concentrations of 0, 2, 40, and 80 μl antibodies per milligram of microsomal protein, for 15 min on ice according to manufacturer's instruction. Substrate concentrations used represented the high-affinity K_m value concentrations for nicotine and cotinine metabolism, specifically 11 μM for nicotine and 51 μM for cotinine for DBA/2 mice microsomes and 11 μM for nicotine and 9.5 μM for cotinine for C57BL/6 mice microsomes.

In Vitro Kinetic and Pharmacokinetic Parameters Analyses. The Michaelis-Menten kinetic parameters K_m and V_{max} from in vitro metabolism studies were calculated using GraphPad Prism (GraphPad Software Inc., San Diego, CA) and were verified by the Eadie-Hofstee method. The equation used to determine K_m and V_{max} for one and two enzymatic sites were $v = V_{max}[S]/(K_m + [S])$ and $v = [V_{max1}[S]/(K_{m1} + [S])] + [V_{max2}[S]/(K_{m2} + [S])]$, respectively, where [S] denotes substrate concentration.

The in vivo pharmacokinetic parameters were determined using noncompartmental analysis: AUC_{0-480} , peak plasma concentration (C_{max}), and maximum plasma concentration (T_{max}). AUC_{0-480} was calculated using the trapezoidal rule. Elimination half-life ($t_{1/2}$) was estimated by the terminal slope. Because the bioavailabilities (F) of

nicotine and cotinine were unknown after subcutaneous injection in mice, CL (clearance) was determined as a hybrid parameter CL/F and was calculated as $dose/AUC_{0-480}$. The average weights of the animals of the strains were similar (24.8 ± 1.7 versus 25.5 ± 1.1 g for DBA/2 and C57BL/6, respectively, $n = 50$ for each strain); therefore, the dose of $25 \mu\text{g}$ (1 mg/kg) was used for the calculation of CL/F for nicotine.

Statistical Analyses. Statistical analyses of in vitro kinetic parameters were tested by Mann-Whitney U test. Assessment of in vivo nicotine, cotinine, and 3'-hydroxycotinine plasma levels for the entire time course was not possible from individual animals due to limited blood volume; therefore, each time point represented data from multiple mice. Due to this experimental design, pharmacokinetic parameters (e.g., half-life) were estimated by resampling methods using the PKRandTest software (H. L. Kaplan, Toronto, ON, Canada) (Damaj et al., 2007).

Results

In Vitro Nicotine C-Oxidation in DBA/2 and C57BL/6 Mice. We first assessed the in vitro kinetic parameters of nicotine C-oxidation in hepatic microsomes prepared from DBA/2 and C57BL/6 mice. Nicotine metabolism to cotinine, demonstrated with Michaelis-Menten kinetics (Fig. 1A) and Eadie-Hofstee plotting (Fig. 1B), revealed two enzymatic sites in both strains. The high-affinity sites for hepatic microsomes from both DBA/2 and C57BL/6 showed similar K_m , V_{\max} , and V_{\max}/K_m values for nicotine (Table 1). In contrast, the low-affinity enzymatic sites exhibited differing and much

lower nicotine metabolic activities with a slight nonsignificantly higher K_m for C57BL/6 and V_{\max} for DBA/2. The V_{\max}/K_m value was significantly higher for the DBA/2 microsomes (Table 1).

Characterization of In Vivo Nicotine Metabolism in DBA/2 and C57BL/6 Mice. Because the rate of drug metabolism in vitro does not necessarily reflect drug clearance in vivo (e.g., presence of nonhepatic elimination processes), we determined whether the in vivo clearance of nicotine was similar between the two mouse strains. Adult male mice from both strains were treated with 1 mg/kg subcutaneous nicotine, a dose used previously in nicotine behavioral studies (Zarrindast et al., 2003). In both mouse strains, nicotine concentrations peaked at 10 min with DBA/2 mice having a significantly greater maximum concentration compared with C57BL/6 mice (Fig. 2A and Table 2). The overall AUC_{0-480} value of nicotine was also modestly higher for DBA/2 than for C57BL/6 mice. Both strains had similar elimination half-lives for nicotine, but the clearance of nicotine was slower in the DBA/2 mice compared with C57BL/6 mice.

When examining the disposition kinetics of cotinine formed from injected nicotine, we observed that the appearance of the cotinine metabolite was rapid and similar between the two mouse strains and achieved peak concentrations at approximately 15 min (Fig. 2B). In contrast, compared with C57BL/6 mice, DBA/2 mice showed a significantly larger AUC_{0-480} and longer elimination half-life (Table 2).

LC/MS/MS Characterization of the Putative Cotinine Metabolite 3'-Hydroxycotinine. In humans, cotinine is metabolized exclusively to *trans*-3'-hydroxycotinine by CYP2A6 (Nakajima et al., 1996a; Dempsey et al., 2004; Yamanaka et al., 2004). To our knowledge, no prior studies have examined or confirmed the production of *trans*-3'-hydroxycotinine from cotinine in mice; therefore, our immediate goal was to determine whether mice metabolize cotinine to 3'-hydroxycotinine. In a preliminary in vitro study, we identified a cotinine metabolite that displayed the same retention time as the *trans*-3'-hydroxycotinine standard (Fig. 3A). To confirm the identity of the putative *trans*-3'-hydroxycotinine compound, a second HPLC system compatible with MS/MS analysis was used. Both the *trans*-3'-hydroxycotinine standard and the cotinine metabolite eluted with the same retention time (10.4 min) using the new HPLC system (Fig. 3B).

In the LC/MS/MS analyses, EMS indicated that the *trans*-3'-hydroxycotinine standard had a retention time of 11.8 min with an m/z of 193 (Fig. 3C), and when the metabolite of in vitro cotinine metabolism was monitored at m/z 193, a major peak was present at 11.1 min (Fig. 3D). Fragmentation of the

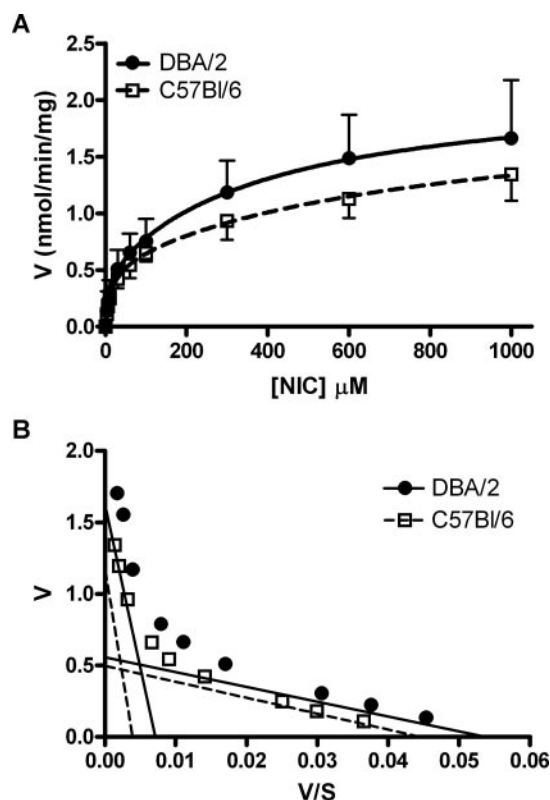


Fig. 1. In vitro metabolism of nicotine to cotinine in DBA/2 and C57BL/6 mice. In vitro kinetic parameters of nicotine metabolism were investigated using hepatic microsomes. A, rate of formation of cotinine from nicotine (NIC) (mean \pm S.D. of four to five mice). B, Eadie-Hofstee plots of the kinetic data for DBA and C57BL/6 mice. See Table 1 for values of kinetics parameters. V, velocity; S, substrate.

TABLE 1

In vitro nicotine metabolism (C-oxidation) parameters

	DBA/2 ($n = 5$ mice)	C57BL/6 ($n = 4$ mice)
	mean \pm S.D.	
High affinity		
K_m (μM)	10.7 ± 4.8	11.4 ± 3.6
V_{\max} (nmol/min/mg)	0.58 ± 0.18	0.50 ± 0.07
V_{\max}/K_m	0.05 ± 0.03	0.04 ± 0.02
Low affinity		
K_m (μM)	234 ± 77	306 ± 126
V_{\max} (nmol/min/mg)	1.60 ± 0.63	1.18 ± 0.36
V_{\max}/K_m	$0.007 \pm 0.001^*$	0.004 ± 0.001

* $p < 0.05$ compared with C57BL/6 mice.

trans-3'-hydroxycotinine standard ion (*m/z* 193) gave two fragments of *m/z* 80 and *m/z* 134 (Fig. 3E). Fragmentation of the cotinine metabolite at *m/z* 193 also gave two major ions of *m/z* 80 and *m/z* 134 (Fig. 3F). The peak area ratios of *m/z* 80/134 for the *trans*-3'-hydroxycotinine and the cotinine metabolite were 3.44 and 3.41, respectively. The *m/z* 80 and the *m/z* 134 fragments corresponded to (C₅H₅N)H⁺ and pyridyl-C₃H₄O⁺, respectively (Murphy et al., 1999).

Characterization of In Vivo Cotinine Metabolism in DBA/2 and C57BL/6 Mice. Having confirmed that 3'-hydroxycotinine is produced from cotinine in mice, we proceeded with in vivo injections of cotinine (1 mg/kg, s.c.). Plasma cotinine concentrations were maximal between 5 and 15 min and were similar for both DBA/2 and C57BL/6 mice (Fig. 4A and Table 3). Similar to cotinine derived from nicotine injection, after cotinine injection, the cotinine AUC_{0–180} was much higher in the DBA/2 mice compared with the

C57BL/6 mice. The clearance of cotinine was slower in DBA/2 mice, which resulted in longer elimination half-life of cotinine compared with C57BL/6 mice.

The plasma levels of 3'-hydroxycotinine formed from cotinine injections were also monitored. The plasma AUC_{0–180} of 3'-hydroxycotinine was higher in the DBA/2 mice compared with the C57BL/6 mice (Fig. 4B and Table 3).

In Vitro Cotinine Metabolism in DBA/2 and C57BL/6 Mice. To determine whether cotinine was metabolized to 3'-hydroxycotinine differently between the two mouse strains, accounting for the differences in cotinine plasma concentrations seen in vivo, we performed in vitro cotinine metabolism studies. We found that cotinine metabolism to 3'-hydroxycotinine was characterized by Michaelis-Menten kinetics (Fig. 5A), mediated by a single enzymatic site in both strains (Fig. 5B). The DBA/2 mice had a significantly higher *K_m* value compared with the C57BL/6 mice (Table 4), whereas the *V_{max}* for cotinine was much greater for DBA/2 than for C57BL/6 mice. This resulted in an overall lower catalytic efficiency (*V_{max}*/*K_m*) for DBA/2 compared with C57BL/6.

Inhibition of In Vitro Nicotine and Cotinine Metabolism. The mouse CYP2A5 has been identified as the enzyme responsible for the high-affinity metabolism of nicotine using cDNA-expressed CYP2A5 (Murphy et al., 2005). To extend these studies characterizing the enzyme involved, we tested the effect of inhibitory antibodies on in vitro nicotine metabolism. Anti-CYP2A6 inhibitory antibodies dose-dependently inhibited the formation of cotinine from nicotine in DBA/2 microsomes with maximal inhibition of 70% at 40 μl of antibody/mg of microsomal protein (Fig. 6A, filled symbols). Similar results were seen in hepatic microsomes from C57BL/6 mice, tested at 80 μl of antibody/mg of protein (Fig. 6A, open symbols). Inhibitory antibodies against CYP2B6 and CYP2D6, enzymes postulated to be involved in the remaining small percentage of metabolism of nicotine in humans (Nakajima et al., 1996b; Messina et al., 1997; Yamazaki et al., 1999) did not inhibit nicotine metabolism in either mouse strain (Fig. 6A).

In humans, CYP2A6 is exclusively responsible for the metabolism of cotinine to *trans*-3'-hydroxycotinine (Nakajima et al., 1996a; Dempsey et al., 2004). To determine whether mouse CYP2A5 was also responsible for this metabolic pathway, we performed inhibition studies on cotinine metabolism. Anti-CYP2A6 inhibitory antibodies dose-dependently inhibited the formation of 3'-hydroxycotinine from cotinine in DBA/2 mouse liver microsomes (Fig. 6B, filled symbols), with a maximal inhibition of 90% at 40 μl of antibody/mg of microsomal protein. Anti-CYP2A6 inhibitory antibodies also

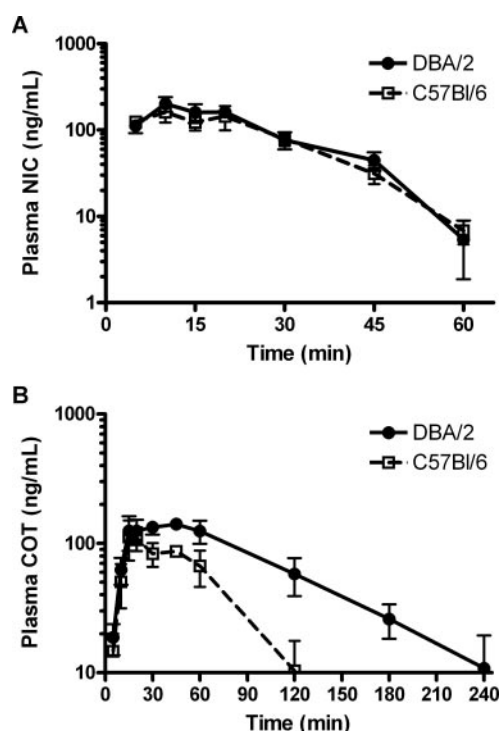


Fig. 2. Plasma nicotine and cotinine concentrations after nicotine treatment. Adult male DBA/2 and C57BL/6 mice were injected with subcutaneous nicotine (1 mg/kg). A, the AUC_{0–480} of nicotine (NIC) was 15% higher, whereas the elimination half-life was similar in the DBA/2 mice compared with C57BL/6 mice. B, the AUC_{0–480} of cotinine (COT) in the DBA/2 mice was twice that of the C57BL/6 mice. Each time point represents mean (± S.D.) of five to seven animals for each strain. Values below the limit of quantification are not shown.

TABLE 2

Pharmacokinetic parameters of plasma nicotine and cotinine in mice treated with nicotine (1 mg/kg, s.c.)

Results are derived using data from five to seven animals at each time point.

	Nicotine		Cotinine	
	DBA/2	C57BL/6	DBA/2	C57BL/6
AUC _{0–480} (ng · h/ml)	92.9 ± 2.9*	80.8 ± 3.2	245 ± 10*	120 ± 7
<i>t</i> _{1/2} (min)	8.6 ± 0.4	9.2 ± 1.6	51.0 ± 4.1*	23.7 ± 2.0
CL/F (ml/min)	4.5 ± 0.1*	5.2 ± 0.2	N.D.	N.D.
<i>T</i> _{max} (min)	10.0 ± 2.2	10.0 ± 4.7	45.0 ± 13.3*	20.0 ± 13.3
<i>C</i> _{max} (ng/min)	201 ± 15*	160 ± 15	141 ± 7*	112 ± 12

N.D., not determined.

* *p* < 0.05 compared with C57BL/6 mice.

inhibited cotinine metabolism (no detectable metabolite peak) in C57BL/6 hepatic microsomes (Fig. 6B, open symbols). Inhibitory antibodies against CYP2B6 and CYP2D6 had no effect on cotinine metabolism (Fig. 6B).

Discussion

In the present study, we examined both in vitro metabolism and in vivo pharmacokinetics of nicotine and cotinine in two commonly used inbred mouse strains, DBA/2 and C57BL/6. Mice have been used extensively for the study of nicotine behaviors; however, the effects of nicotine vary widely between strains (Aschhoff et al., 1999; Stolerman et al., 1999). One potential contributing factor may be differences in nicotine pharmacokinetics, which can significantly alter its pharmacology. CYP2A5 metabolizes nicotine (Murphy et al., 2005), and previous studies found that DBA/2 and C57BL/6 mice differed in CYP2A5 structure and function (Lindberg et al., 1992). Therefore, the primary goal of this

study was to determine whether nicotine and cotinine metabolism differed between these two strains, which may be contributed by the *Cyp2a5* polymorphism between these mice (Lindberg et al., 1992).

The in vitro metabolism of nicotine to cotinine was mediated by a high- and a low-affinity enzyme site in both mouse strains. The K_m values for the high-affinity sites reported here are consistent with those seen using cDNA-expressed CYP2A5 ($7.7 \pm 0.8 \mu\text{M}$; 129/J mouse strain) (Murphy et al., 2005) but have modestly higher affinity relative to hepatic microsomes from ICR mice ($18.6 \pm 5.9 \mu\text{M}$) (Damaj et al., 2007). The identity of the high-affinity site was confirmed, because CYP2A5 inhibitory antibodies inhibited up to 70% of nicotine metabolism at K_m for nicotine in both strains. The low-affinity sites in our mice could potentially belong to the 2B family. In humans, cDNA-expressed CYP2B6 metabolizes nicotine but with much lower affinity and activity compared with CYP2A6 (Yamazaki et al., 1999). In monkeys, CYP2B6agm is a minor enzyme compared with CYP2A6agm

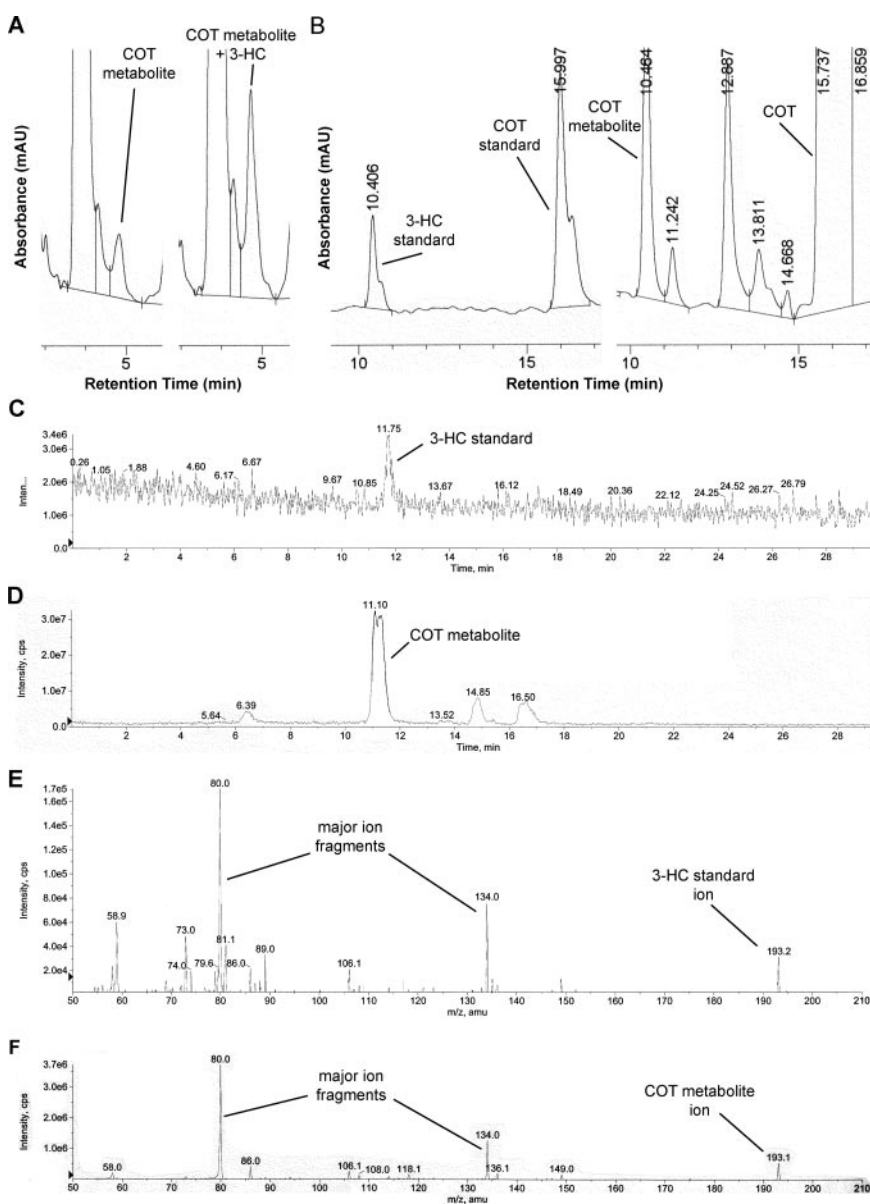


Fig. 3. Characterization of 3'-hydroxycotinine in mice by HPLC and LC/MS/MS. A, cotinine and its metabolite (COT metabolite) from a pilot in vitro cotinine metabolism study using mouse liver microsomes (DBA/2) were separated with HPLC system I in the absence (left) or presence (right) of the *trans*-3'-hydroxycotinine standard (3-HC). B, cotinine and *trans*-3'-hydroxycotinine standards (left) and products from a pilot in vitro cotinine metabolism study (right) were separated with HPLC system II. C, enhanced mass scan at m/z 193 showed that the *trans*-3'-hydroxycotinine standard had a retention time of 11.8 min. D, liquid chromatography of products from in vitro cotinine metabolism monitored at m/z 193 identified a putative 3'-hydroxycotinine compound with a retention time of 11.1 min. E, fragmentation of the m/z 193 ion (from C) at 30 eV produced m/z 80 and m/z 134 ions. F, fragmentation of the m/z 193 ion (from D) at 11.1 min at 30 eV produced m/z 80 and m/z 134 ions.

for the metabolism of nicotine to cotinine (Schoedel et al., 2003). In contrast, rat CYP2B1/2 is the primary enzyme responsible for this process (Nakayama et al., 1993). In our experiments, however, no indication of inhibition of nicotine metabolism was seen at the highest CYP2B antibody concentration tested. Considering that at the highest plasma nicotine concentrations observed (~ 200 ng/ml ≈ 1.2 μ M) after nicotine injection, the estimated contribution of the low-affinity enzymes to nicotine metabolism was only $\leq 10\%$, so the identity of this enzyme was not pursued.

To determine whether nicotine was metabolized similarly between DBA/2 and C57BL/6 mice in vivo, we administered nicotine subcutaneously because this route kinetically mimics, somewhat, the route of nicotine intake from smoking in that it bypasses first-pass metabolism. The in vivo kinetics of

nicotine in these two strains differed, although not dramatically. The higher nicotine C_{\max} value in DBA/2 mice may be due to a smaller volume of distribution of nicotine: male DBA/2 mice have, on average, 34 to 40% more body fat and 10% lower lean mass compared with C57BL/6 mice (Mouse Phenome Database, The Jackson Laboratory, Bar Harbor, ME), which could result in higher levels of nicotine in the plasma (and other highly perfused organs such as liver, kidneys, and the lung) (Urakawa et al., 1994). Despite similar nicotine clearance, however, we found that cotinine was removed more slowly in DBA/2 mice, as demonstrated by the 2-fold longer elimination half-life and higher cotinine AUC_{0-480} .

In humans, the main metabolites of cotinine recovered in urine are *trans*-3'-hydroxycotinine and its glucuronide, which account for 40 to 60% of the total administered dose of nicotine (Hukkanen et al., 2005). Initially, we demonstrated that mice produced 3'-hydroxycotinine from cotinine with LC/MS/MS. We then confirmed that the metabolism of cotinine to 3'-hydroxycotinine was mediated by CYP2A5—up to 90% of cotinine metabolism to 3'-hydroxycotinine was inhibited by anti-CYP2A6 inhibitory antibodies. This is consistent with the metabolism of cotinine to 3'-hydroxycotinine being mediated exclusively by human CYP2A6 in vitro and in vivo (Nakajima et al., 1996a; Dempsey et al., 2004). After cotinine injections, DBA/2 mice showed slower clearance of cotinine compared with C57BL/6 mice, which was consistent with the pharmacokinetics of cotinine formed from nicotine injections. It is likely that DBA/2 mice have a slower hepatic (intrinsic) clearance of cotinine to 3'-hydroxycotinine compared with C57BL/6; this is supported by our in vitro findings that cotinine was metabolized to 3'-hydroxycotinine significantly more slowly in the DBA/2 compared with C57BL/6 mice. Even at the maximum plasma cotinine concentrations observed (~ 760 ng/ml ≈ 4.3 μ M), our in vitro data indicated that the DBA/2 mice metabolized cotinine slower than the C57BL/6 mice ($v = 0.008$ versus 0.0012 nmol/min/mg, respectively).

When examining the plasma concentrations of 3'-hydroxycotinine formed from cotinine, we found that DBA/2 mice had a larger AUC_{0-180} value compared with C57BL/6 mice. The higher level of 3'-hydroxycotinine in DBA/2 mice was probably due to reduced elimination of 3'-hydroxycotinine. This could occur through slower rates of conjugation to *O*-glucuronide and/or slower renal excretion of 3'-hydroxycotinine and its glucuronidated metabolite. In mice, *N*-glucuronides of nicotine and its proximal metabolites have not been detected or identified (although *O*-glucuronides were not measured) (Ghosheh and Hawes, 2002), whereas in humans $\sim 80\%$ of

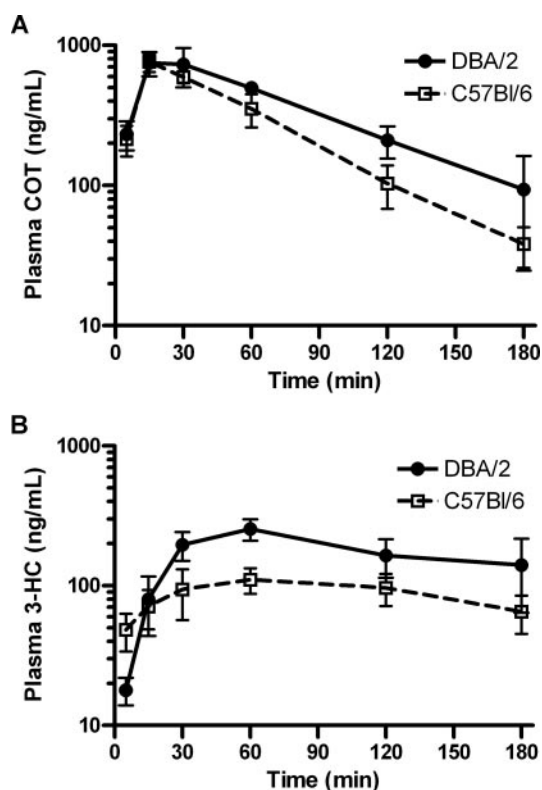


Fig. 4. Plasma cotinine and 3'-hydroxycotinine concentrations after cotinine injections. Adult male DBA/2 and C57BL/6 mice were injected with subcutaneous cotinine (1 mg/kg). A, the AUC_{0-180} of cotinine (COT) was 37% higher, and its elimination half-life was 34% longer in DBA/2 mice compared with C57BL/6 mice. B, the AUC_{0-180} of 3'-hydroxycotinine (3-HC) was 94% higher in the DBA/2 mice compared with C57BL/6 mice. Each time point represents mean \pm S.D. of three to eight animals for each strain.

TABLE 3

Pharmacokinetic parameters of plasma cotinine and 3'-hydroxycotinine in mice treated with cotinine (1 mg/kg, s.c.)

Results are derived using data from three to eight animals at each time point.

	Cotinine		3'-Hydroxycotinine	
	DBA/2	C57BL/6	DBA/2	C57BL/6
AUC_{0-180} (ng \cdot h/ml)	1087 \pm 33*	796 \pm 33	518 \pm 30*	268 \pm 19
$t_{1/2}$ (min)	50.2 \pm 4.7*	37.5 \pm 9.6	N.D.	N.D.
CL/F (ml/min)	0.38 \pm 0.01*	0.52 \pm 0.02	N.D.	N.D.
T_{\max} (min)	15.0 \pm 7.5	15.0 \pm 0.0	60.0 \pm 0.0	60.0 \pm 23.6
C_{\max} (ng/ml)	748 \pm 35	759 \pm 39	255 \pm 23*	110 \pm 10

N.D., not determined.

* $P < 0.05$ compared with C57BL/6.

trans-3'-hydroxycotinine is excreted unchanged (Hukkanen et al., 2005). Thus, we believe the higher level of 3'-hydroxycotinine was most probably due to slower renal excretion in the DBA/2 mice.

This study showed that the *in vitro* and *in vivo* metabolism of nicotine was similar between DBA/2 and C57BL/6 mice. In contrast to nicotine, DBA/2 mice metabolized cotinine to 3'-hydroxycotinine with lower efficiency compared with C57BL/6 mice both *in vitro* and *in vivo*. These data indicate that genetic differences in the structure of CYP2A5 between the two strains can potentially alter the rate of metabolism depending on the specific substrate. The DBA/2 CYP2A5, which has valine at position 117, is more efficient at metabolizing coumarin compared with C57BL/6, which has an alanine in this position (Lindberg et al., 1992; van Iersel et al., 1994). In contrast, this genetic variant does not seem to alter the metabolism of nicotine, but rather, it reduces the catalytic activity for cotinine. Mouse CYP2A5 oxidizes nicotine to $\Delta^{5(1')}$ -iminium ion followed by conversion to cotinine by aldehyde oxidase (Murphy et al., 2005); however, no functional polymorphisms for the mouse aldehyde oxidase genes have

been reported (Mouse Genome Informatics, The Jackson Laboratory), and our *in vivo* data were consistent with our *in vitro* data suggesting that the variation in aldehyde oxidases were not contributing substantively to our observations. Substrate-selective metabolisms by genetic variants of human CYP2A6 have been observed. For example, the CYP2A6*7 variant has reduced nicotine metabolic activity, but the coumarin metabolism was minimally affected (Ariyoshi et al., 2001). In addition, different levels of cotinine after similar nicotine intake have been observed in smokers (Benowitz et al., 1999), and this may be partly related to genetic variations in CYP2A6 that have minor impacts on the metabolism of nicotine relative to the impact on cotinine metabolism. Finally, these observations warrant further studies on the metabolic activation of CYP2A5/6 substrates such as NNK, a tobacco-specific nitrosamine known to cause lung cancer (Miyazaki et al., 2005), and the consequence of these genetic variants on NNK activation. Future studies will focus on the expression of variants V117A (found in CYP2A5 [Lindberg et al., 1992] and CYP2A13 [National Center for Biotechnology Information]) and F118L (found in CYP2A6 [National Center

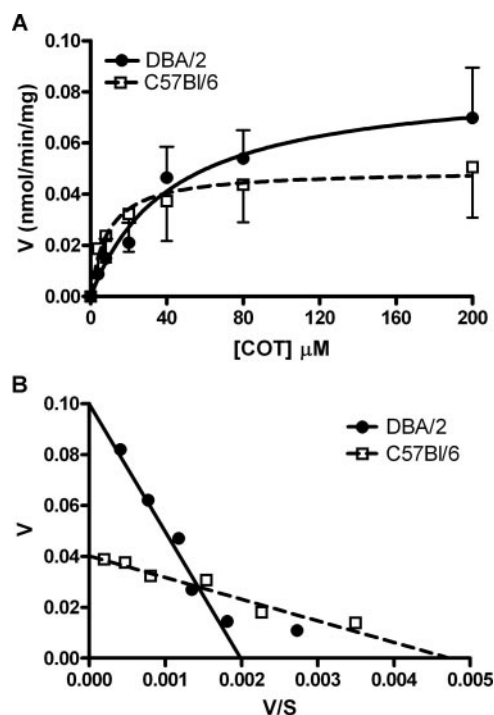


Fig. 5. *In vitro* metabolism of cotinine to 3'-hydroxycotinine in DBA/2 and C57BL/6 mice. *In vitro* kinetic parameters of cotinine metabolism were investigated using hepatic microsomes. A, rate of formation of 3'-hydroxycotinine from cotinine (mean \pm S.D.; $n = 4$ samples per strain). DBA/2 and C57BL/6 mice. Regression lines and intercepts for both DBA/2 and C57BL/6 were calculated from K_m and V_{max} values derived from Michaelis-Menten fitting, and no data points were censored. See Table 4 for values of kinetic parameters. V, velocity; S, substrate.

TABLE 4

In vitro cotinine hydroxylation parameters

	DBA/2 ($n = 4$)	C57BL/6 ($n = 4$)
	mean \pm S.D.	
K_m (μ M)	51.0 \pm 15.6*	9.5 \pm 2.1
V_{max} (nmol/min/mg)	0.10 \pm 0.01*	0.04 \pm 0.01
V_{max}/K_m	0.002 \pm 0.000*	0.005 \pm 0.002

* $p < 0.05$ compared with C57BL/6 mice.

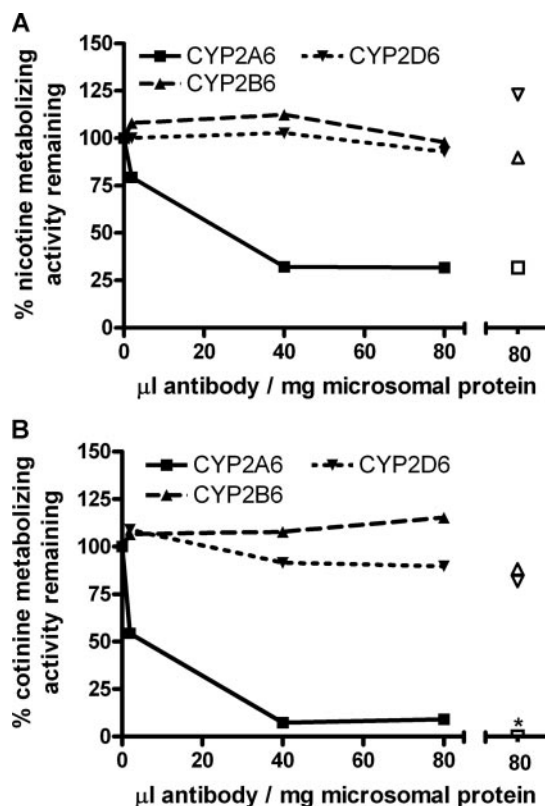


Fig. 6. Antibody inhibits *in vitro* nicotine and cotinine metabolism in DBA/2 and C57BL/6 mice. A, At K_m (11 μ M) nicotine, anti-CYP2A6 inhibitory antibodies inhibited cotinine formation maximally by 70% in hepatic microsomes in DBA/2 mice (filled symbols). At K_m (11 μ M) nicotine, anti-CYP2A6 inhibitory antibodies (80 μ l of antibodies/mg of protein) inhibited cotinine formation by 70% in C57BL/6 hepatic microsomes (open symbols). B, at K_m (51 μ M) cotinine, anti-CYP2A6 inhibitory antibodies inhibited 3'-hydroxycotinine formation by >90% in hepatic microsomes DBA/2 mice (filled symbols). At K_m (9.5 μ M) cotinine, anti-CYP2A6 inhibitory antibodies inhibited 3'-hydroxycotinine formation in C57BL/6 hepatic microsomes (open symbols). *, activity of cotinine metabolism was lower than the limit of quantification. Anti-CYP2B6 and anti-CYP2D6 antibodies had no effect on nicotine or cotinine metabolism. Values are expressed as a percentage of activity in the absence of antibodies and were determined from a pooled sample of two animals.

for Biotechnology Information]) in all three enzymes and their impact on multiple substrates, including NNK and Tegafor.

The finding that cotinine is differentially metabolized relative to nicotine, between strains, has implications with respect to interpreting nicotine pharmacological studies. Cotinine can pass through the blood-brain barrier (Lockman et al., 2005). In rats, cotinine can bind to epibatidine-sensitive nicotinic receptors in frontal cortex and hippocampus tissues, although with lower affinity than nicotine (Vainio and Tuominen, 2001). Furthermore, administration of cotinine in rat striatal tissues evoked dopamine overflow in a dose-dependent manner (Dwoskin et al., 1999). Thus, differing levels of cotinine may result in altered pharmacological effects, despite similar nicotine levels between the two mouse strains and the effects may be erroneously attributed to nicotine pharmacology. On the other hand, the effects of cotinine in humans are less clear (Crooks and Dwoskin, 1997; Buccafusco and Terry, 2003). Cotinine alone did not show pharmacological effects, but it did interfere with the ability of nicotine patch to reduce withdrawal symptoms (Hatsukami et al., 1998b). Cotinine also increased plasma nicotine levels in smokers, possibly through increased smoking to compensate for the interference of nicotine action by cotinine (Hatsukami et al., 1998a). In other studies, cotinine seemed to have some effects in reducing withdrawal symptoms (Benowitz et al., 1983; Keenan et al., 1994).

Consideration should also be taken when using cotinine as biomarker for environmental tobacco smoke exposure (Benowitz, 1999) and cigarette intake (de Leon et al., 2002) in humans or nicotine consumption in mice (Sparks and Pauly, 1999), because cotinine can be metabolized at different rates by human CYP2A6 and mouse CYP2A5. Differing levels of cotinine could be erroneously interpreted as different levels of exposure rather than differing rates of removal.

In conclusion, we have characterized nicotine and cotinine metabolism in two mouse strains that differed in CYP2A5 enzyme structure (Lindberg et al., 1992). Although coumarin metabolism differed (Lindberg et al., 1992), we observed no substantial difference in nicotine metabolism. In contrast, CYP2A5-mediated cotinine metabolism to 3'-hydroxycotinine was different between the mouse strains, which may confound interpretation of pharmacological and biomarker studies.

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