Role of CYP2A5 in the Clearance of Nicotine and Cotinine: Insights from Studies on a *Cyp2a5*-null Mouse Model^[S]

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ABSTRACT

CYP2A5, a mouse cytochrome P450 monooxygenase that shows high similarities to human CYP2A6 and CYP2A13 in protein sequence and substrate specificity, is expressed in multiple tissues, including the liver, kidney, lung, and nasal mucosa. Heterologously expressed CYP2A5 is active in the metabolism of both endogenous substrates, such as testosterone, and xenobiotic compounds, such as nicotine and cotinine. To determine the biological and pharmacological functions of CYP2A5 in vivo, we have generated a *Cyp2a5*-null mouse. Homozygous *Cyp2a5*-null mice are viable and fertile; they show no evidence of embryonic lethality or developmental deficits; and they have normal circulating levels of testosterone and progesterone. The *Cyp2a5*-null mouse and wild-type mouse were then used for determination of the roles of CYP2A5 in the metabolism of nicotine and its major circulating metabolite,

cotinine. The results indicated that the *Cyp2a5*-null mouse has lower hepatic nicotine 5'-hydroxylation activity in vitro, and slower systemic clearance of both nicotine and cotinine in vivo. For both compounds, a substantially longer plasma half-life and a greater area under the concentration-time curve were observed for the *Cyp2a5*-null mice, compared with wild-type mice. Further pharmacokinetics analysis confirmed that the brain levels of nicotine and cotinine are also influenced by the *Cyp2a5* deletion. These findings provide direct evidence that CYP2A5 is the major nicotine and cotinine oxidase in mouse liver. The *Cyp2a5*-null mouse will be valuable for in vivo studies on the role of CYP2A5 in drug metabolism and chemical toxicity, and for future production of CYP2A6- and CYP2A13-humanized mouse models.

The mouse *Cyp2a* gene subfamily encompasses four genes (*Cyp2a4*, *Cyp2a5*, *Cyp2a12*, and *Cyp2a22*), whereas the human CYP2A gene subfamily has three genes (CYP2A6, CYP2A7, and CYP2A13) (Wang et al., 2003). Human CYP2A6 and CYP2A13 are known to be functional, and they are most similar to mouse CYP2A5, with respect to tissue distribution and substrate specificity (Su and Ding, 2004). Both CYP2A6 and CYP2A13 are expressed in the olfactory mucosa and other tissues of the respiratory tract, whereas CYP2A6 is also

expressed in the liver; mouse CYP2A5 is expressed in many tissues, including tissues of the respiratory tract, liver, and kidney. CYP2A5 shares many substrates with CYP2A6 and/or CYP2A13, such as coumarin, nicotine, cotinine, testosterone, and the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and other nitrosamines (Su and Ding, 2004; Wong et al., 2005; Raunio et al., 2008a).

Nicotine is the pharmacologically active (and addictive) ingredient in cigarette products. Nicotine is also used as a therapeutic agent for smoking cessation, and it is being tested as a potential preventative agent for neurodegenerative diseases, both in animal models and in clinical trials (Ravina et al., 2003; Quik et al., 2007). Nicotine can be metabolized via several pathways in humans, including 5'-hydroxylation to yield cotinine, catalyzed mainly by CYP2A6 (Nakajima et al., 1996; Messina et al., 1997); *N*-demethylation (Murphy et al., 2005), also mediated by CYP2A6; *N*-

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ABBREVIATIONS: ES, embryonic stem; P450, cytochrome P450; UGT, UDP-glucuronosyltransferase; PCR, polymerase chain reaction; LC, liquid chromatography; HPLC, high-performance liquid chromatography; kb, kilobase pair; MS, mass spectrometer; AUC, area under the concentration-time curve; CL, clearance; F, bioavailability; cotinine-*d*₃, (±)-cotinine-methyl-*d*₃; G418, (2*R*,3*S*,4*R*,5*R*,6*S*)-5-amino-6-[(1*R*,2*S*,3*S*,4*R*,6*S*)-4,6-diamino-3-[(2*R*,3*R*,4*R*,5*R*)-3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl]oxy-2-hydroxycyclohexyl]oxy-2-(1-hydroxyethyl)oxane-3,4-diol; WT, wild type; psig, pound-force per square inch gauge.

oxidation (Cashman et al., 1992; Cashman, 2000), catalyzed by flavin-containing monooxygenase; and *N*-glucuronidation (Kuehl and Murphy, 2003; Kaivosari et al., 2007), catalyzed by various UDP-glucuronosyltransferases. In humans, ~75% of administered nicotine is converted to cotinine (Hukkanen et al., 2005), a γ -lactam metabolite formed via 5'-hydroxylation. Indeed, cotinine is used as a chemical marker for human exposure to cigarette smoking (de Leon et al., 2002). In addition, genetic polymorphisms in the human *CYP2A6* gene have been linked to interindividual differences in the rates of nicotine clearance (Benowitz et al., 2006).

Mouse *CYP2A5* is also believed to play a major role in the clearance of nicotine and cotinine, based on in vitro data and in vivo pharmacological evidence (Siu et al., 2006; Siu and Tyndale, 2007; Raunio et al., 2008b), although direct in vivo evidence, such as the disposition in a *Cyp2a5*-null mouse, has yet to be reported. However, a remarkable species difference exists between humans and mice in the rates of nicotine clearance, with the metabolism being much more rapid in mice than in humans. This notable species difference makes it difficult to extrapolate pharmacological and toxicological findings obtained in mouse experiments to human contexts. Furthermore, the rapid metabolism of nicotine in mice has hindered efforts to use this species for studies on the mechanisms of nicotine addiction, or for studies on the potential of nicotine as an agent for prevention of diseases such as Parkinson's disease (Quik et al., 2007). Biochemical studies have suggested that the species difference between mouse and humans in nicotine clearance is largely due to differences in the efficiencies of human *CYP2A6* and mouse *CYP2A5* in nicotine 5'-hydroxylation, with *CYP2A5* being much more efficient than *CYP2A6* is (Murphy et al., 2005). Indeed, other mouse hepatic P450 enzymes seem to be either similar to, or less efficient than, human *CYP2A6*, with respect to their enzymatic activities toward nicotine (Murphy et al., 2005; Siu and Tyndale, 2007). Thus, a *Cyp2a5*-null mouse will not only be valuable for determination of the specific roles of *CYP2A5* in drug metabolism and toxicity in a mouse model, but it will also represent a more "humanized" mouse model that is suitable for various studies concerning the pharmacology of nicotine and cotinine. A *Cyp2a5*-knockout mouse model has not been described previously.

In the present study, we have generated a *Cyp2a5*-null mouse through homologous recombination in embryonic stem (ES) cells derived from the C57BL/6 (B6) mouse strain. Homozygous *Cyp2a5*-null mice were characterized for viability and fertility, growth rates, potential compensatory expression in other enzymes that can influence nicotine clearance, and circulating levels of testosterone and progesterone, endogenous compounds that are among known *CYP2A5* substrates. The *Cyp2a5*-null mice were then compared with the WT B6 mice, for their abilities to clear nicotine and cotinine in vivo, and for the activities of their hepatic microsomes to metabolize nicotine in vitro. Pharmacokinetic analyses were then performed to determine the impact of the *Cyp2a5* deletion on levels of nicotine and cotinine in the brain (the pharmacological target organ), the liver (the major site of metabolic clearance), and the blood (the medium for biomonitoring).

Materials and Methods

Chemicals and Reagents. (–)-Cotinine, (±)-cotinine-methyl- d_3 (cotinine- d_3), (–)-nicotine hydrogen tartrate, (–)-nicotine, ammonium acetate, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). The sources of testosterone, progesterone, 16 α -hydroxyprogesterone, and all testosterone metabolite standards were the same as described previously (Ding and Coon 1988, 1994; Zhou et al., 2009). [3,4- ^{13}C]Progesterone was obtained from Cambridge Isotope Laboratories (Andover, MA). All solvents (acetonitrile, methanol, and water) were of high-performance liquid chromatography (HPLC) grade (Thermo Fisher Scientific, Houston, TX).

Targeting Vector Construction. The targeting vector (Fig. 1) was prepared in a pMC-lox-neo-lox vector (4.0 kb) (Millipore Corporation, Billerica, MA). It consists of a *tk*-neo-bpA cassette for the neomycin-resistance (neo) gene expression, using the thymidine kinase (*tk*) promoter; the neo was flanked by two loxP sites in the same orientation. A 1.6-kb PstI fragment encompassing exons 6 to 8 and a 5.6-kb BamHI-EcoRI fragment of the 3'-flanking region were obtained from the C57BL/6 (B6) mouse bacterial artificial chromosome clone RP23-165I8 (BACPAC Resources, Oakland, CA), which contains *Cyp2a5*, but not *Cyp2a4*. The two fragments were cloned into the pMC-lox-neo-lox vector, which was linearized with ClaI, before electroporation into ES cells.

Electroporation and Selection of ES Cells. The Bruce4 (C57BL/6J-derived) ES cells (Köntgen et al., 1993), provided by Dr. Colin Stewart (National Cancer Institute, Frederick, MD), were used for electroporation at the Transgenic and Knockout Core Facility of the Wadsworth Center. Recombinant ES cells were selected with the use of 250 μ g/ml G418. Positive ES cell clones were identified by PCR [using the primers 5'-ttagggcactgggtcacttc-3' (upstream of the 1.6-kb PstI fragment) and 5'-cgatctagaggtaccataacttct-3' (within the vector region), with an annealing temperature of 62°C], and were confirmed by Southern blot analysis with both internal (a 350-bp HindIII-PstI fragment upstream of the PvuII restriction site within the targeting vector) and external probes (a 1.1-kb BamHI-SacI fragment upstream of exon 6 of the *Cyp2a5* gene).

Blastocyst Injection and Animal Breeding. ES cells from positive clones were karyotyped, and the clone with the best karyotyping result was selected for expansion. ES cells were injected into the blastocysts from albino B6(Cg)-Tyrc-2J/J (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) female mice. Blastocysts were transferred into the uterus of a pseudopregnant B6CBAF1/J mouse for generation of offspring. Male chimera pups were identified by their black eyes and coat color. Adult chimeras were bred with C57BL/6J female mice to produce germline-transmission F1 mice that were heterozygous for the *Cyp2a5*-null allele. F2 homozygotes were used for breeding and characterization. B6 mice were used as WT controls in all experiments described. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center.

RNA-PCR. Tissues for RNA or microsome preparation were all collected between 9:00 and 10:00 AM local time. Total RNA was isolated with use of the RNeasy Mini kit (QIAGEN, Valencia, CA). All RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA) before reverse transcription. RNA-PCR analysis was performed as described previously (Zhang et al., 2005). Gene-specific PCR primers for amplification of exons 2 and 3 of *Cyp2a5* were the same as described elsewhere (Zhuo et al., 2004); the primers for amplification of exons 8 and 9 were 5'-gatgacaaggagcagtttaagaagaa-3' and 5'-gtgtaggttggtgggtatctgtg-3' (with an annealing temperature of 66°C). PCR products were validated by sequence analysis. Methods for real-time PCR analysis of *CYP2G1* and *CYP2A12* expression are described in the Supplemental Materials.

Pharmacokinetic Analysis for Nicotine and Cotinine. For studies on systemic clearance of nicotine and cotinine, mice were given a single injection (at 9:00–10:00 AM) of either nicotine tartrate (at 1.0 or 5.0 mg/kg i.p., free base) or cotinine (at 1.0 mg/kg i.p.) in

Fig. 1. Targeted disruption of the mouse *Cyp2a5* gene. Structures of the WT *Cyp2a5* allele and the *Cyp2a4/Cyp2a12* allele (A), the targeting vector (B), and the targeted allele (C) are depicted. Both external (probe E) and internal (probe I) probes were used for Southern blot analysis. The restriction site PvuII (P) was used to distinguish between *Cyp2a4/Cyp2a12* and *Cyp2a5* WT alleles. D, detection of the targeted *Cyp2a5* allele by Southern blot analysis of mouse tail genomic DNA. The 7.0-, 4.0-, and 2.0-kb bands (detected with probe E) represent the WT *Cyp2a5* allele (+), targeted *Cyp2a5* allele (-), and WT *Cyp2a4/Cyp2a12* alleles, respectively. E, absence of full-length or truncated CYP2A5 mRNA in tissues of the *Cyp2a5*-null mice. RNA-PCR was performed as described in *Materials and Methods*. E2_E3 and E8_E9 represent primer sets flanking exons 2 and 3 and sets flanking exons 8 and 9, respectively. OM, olfactory mucosa.

saline. Blood samples (~20 μ l each) were collected from the tail of individual mice, at various time points (5 min to 8 h) after the injection. The samples were centrifuged at 1000g, for 5 min, at 4°C, for preparation of plasma. For studies on tissue nicotine and cotinine levels, tissue samples and blood samples (from the heart) were obtained after animals were killed by CO₂ asphyxiation. Pharmacokinetic parameters were calculated by use of the noncompartmental method in the WinNonlin software (Pharsight, Mountain View, CA). Statistical significance of differences between two groups was examined with Student's *t* test. For total body clearance, the hybrid constant CL/F was used according to Statler et al., (2007), instead of CL (clearance), given that F (bioavailability) is not known.

For plasma samples, 0.1 ng of cotinine-d₃ was added as internal standard to 10 μ l of plasma, which had been diluted in 1.0 ml of 0.6 M phosphate buffer, pH 6.8, as described previously (Heavner et al., 2005). The resultant mixture was extracted with an Isolute Extraction Cartridge (C18; 1 ml/100 mg) (Biotage, Charlottesville, VA). The samples were eluted with 1 ml of methanol, dried under nitrogen, and then reconstituted with 50% (v/v) methanol in water for LC/MS analysis. The recovery of added nicotine and cotinine standards in blank plasma was >80%, at all concentrations tested. For tissue sample preparation, brain and liver (~400 mg each) were homogenized in 3 ml of saline (0.9% NaCl), followed by addition of 0.1 ng of cotinine-d₃. The mixtures were centrifuged, at 3000g, for 10 min, and the supernatant was then extracted with an Isolute Extraction Cartridge (C18; 3 ml/100 mg), as described above for plasma samples. The recovery of added standards in blank tissues was >75% at all concentrations tested.

The levels of nicotine and cotinine were determined by use of a LC/MS system composed of an Agilent 1200 Series HPLC and an ABI 4000 Q-Trap mass spectrometer (Applied Biosystems, Foster City,

CA), fitted with a 5- μ m Gemini C18 column (50 $\times$ 2.0 mm). The samples were eluted at a flow rate of 0.2 ml/min, with solvent A (10 mM ammonium acetate in water) and solvent B (100% acetonitrile); the column was equilibrated with 100% A for 1 min, and the solvent gradient consisted of linear increases from 0% B to 100% B between 1 and 8 min, followed by a wash at 100% B for 1 min. The MS was operated in the positive ion mode, using electrospray ionization. The parent/product ion pairs of *m/z* 163/130 (for nicotine), *m/z* 177/80 (for cotinine), and *m/z* 180/80 (for cotinine-d₃) were monitored in the Multiple Reaction Monitoring scan mode. The parameters for the chamber were as follows: curtain gas, 40 psig; heated nebulizer temperature, 350°C; ion spray voltage, 4000 V; nebulizer gas, 50 psig; turbo gas, 50 psig; declustering potential, 40 V; and entrance potential, 10 V. For both compounds, the detection limit was 0.5 pg/ μ l, or 5 pg on column.

Identification of Hepatic Microsomal Nicotine Metabolites by LC/MS. Assay mixtures contained 50 mM potassium phosphate buffer, pH 7.4, 10 μ M nicotine, 1.0 mg/ml liver microsomal protein, with or without 1 mM KCN, and 1.0 mM NADPH, in a final volume of 0.3 ml. Reactions were carried out at 37°C for 0 or 60 min, and were terminated by the addition of 0.3 ml of acetonitrile, followed by removal of denatured proteins through centrifugation. The supernatant was analyzed by use of an LC/UV/MS system consisting of a Waters 2690 separation module (Waters, Milford, MA), a Surveyor photodiode array detector (Thermo Fisher Scientific), and an LCQ Deca XP ion-trap mass spectrometer (Thermo Fisher Scientific). The chromatographic separation of metabolites was achieved on a 5- μ m Gemini C18 (150 $\times$ 2.0 mm) column (Phenomenex, Torrance, CA). The mobile phase consisted of solvent A (10 mM ammonium acetate in water) and solvent B (100% acetonitrile). The samples were eluted, at a flow rate of 0.5 ml/min, with 100% A for 20 min, followed

by linear increases from 0% B to 100% B between 21 and 25 min, and then 100% B for a further 10 min. The mass spectrometer was set for a data-dependent scan mode, and was operated in a positive ion mode with an atmospheric pressure chemical ionization source. The parameters for the chamber were: m/z range, 50 to 500; capillary temperature, 350°C; nitrogen sheath gas flow rate, 30 (arbitrary units); auxiliary gas, 10 (arbitrary units); spray voltage, 4 kV; capillary voltage, 10 V; and tube lens offset, 30 V.

In Vitro Metabolism of Testosterone and Nicotine. Microsomal preparation was carried out essentially as described previously (Ding and Coon, 1990); the postmicrosomal supernatant fraction was used as cytosol. Metabolism of testosterone was assayed essentially as described previously (Zhou et al., 2009). Reaction mixtures contained 5.0 or 10 μ M testosterone and 0.1 mg/ml liver microsomal protein, and the reaction was carried out for 10 min. Testosterone metabolites were identified by use of a LC/MS system consisting of an Agilent 1200 Series HPLC and an ABI 4000 Q-Trap mass spectrometer, essentially as described elsewhere (Zhou et al., 2009), with minor modifications, as detailed in the Supplemental Materials. For assay of nicotine C-5'-oxidation, the reaction mixture contained 1.0 or 10 μ M nicotine, 0.5 mg/ml liver microsomal protein, and 1.0 mg/ml cytosolic protein. The reactions were carried out under conditions that support constant rates of product formation, according to a protocol described previously (Siu et al., 2006). The reaction was stopped by the addition of 1 ml of methanol (containing 1 ng of cotinine- d_3), and the mixture was centrifuged, to precipitate protein. The supernatant was dried under nitrogen, and then reconstituted with 50% (v/v) methanol in water, for the measurement of cotinine by LC/MS analysis (as described above for the pharmacokinetics of plasma cotinine). Metabolite standard was added to reaction mixtures containing boiled microsomes, with recoveries being >85% at all concentrations tested.

Quantitative Analysis of Serum Testosterone and Progesterone by LC/MS. Two-month-old male and female mice, respectively, were used for testosterone and progesterone determination. For serum testosterone, samples were prepared and analyzed essentially as described previously (Zhou et al., 2009). For serum progesterone, the sample preparation method was modified from the protocol of Tai et al. (2006). The internal standard, [3,4- ^{13}C] progesterone, was added to serum samples at 1.0 ng/ml. Samples were extracted twice, each time with 10 volumes of hexane; the extracts were dried with nitrogen, and then reconstituted in 50% methanol for LC/MS analysis. To construct calibration curves, we added progesterone and stable isotope-labeled progesterone to charcoal-stripped bovine serum (Hyclone, Logan, UT). The recovery of added progesterone was >90%, at all concentrations tested. Details of LC/MS analysis for progesterone are included in the Supplemental Materials.

Results

Generation and Characterization of the *Cyp2a5*-Null Mouse. The structures of the WT *Cyp2a5* allele, the targeting construct, and the targeted *Cyp2a5*-null allele are shown in Fig. 1, A to C. A *Cyp2a5* bacterial artificial chromosome genomic clone, isolated from the B6 strain, was used for construction of the targeting vector. The strategy used for targeted disruption of the mouse *Cyp2a5* gene by homologous recombination in ES cells was to replace the last exon (exon 9), which encodes the active-site Cys residue, with a neomycin-resistance gene. ES cells from a homologous recombinant clone (clone 221) were used for subsequent injection into the blastocyst cavity of albino B6(Cg)-Tyrc-2J/J embryos from which a chimeric male was generated. When bred with WT B6 females, the chimera exhibited germline transmission. The absence of random integration of the targeting construct was confirmed by the use of both internal (probe I) and

external (probe E) probes for Southern blot analysis (data not shown). Homozygous *Cyp2a5*-null mice (*Cyp2a5*^{-/-}) were produced by cross-breeding between heterozygous littermates (*Cyp2a5*^{+/-}). Homozygotes, heterozygotes, and WT littermates were identified by the presence of the characteristic bands for the WT (7-kb) and the *Cyp2a5*-null (4-kb) alleles, on Southern blots (Fig. 1D). As a control, the integrity of the *Cyp2a4/12* genes, which are highly similar to *Cyp2a5* in genomic structure, is confirmed by the detection of the unique 2-kb genomic fragment in mice of all three genotypes.

The absence of *Cyp2a5* expression in various tissues of the *Cyp2a5*-null mice was confirmed by RNA-PCR, with use of two sets of gene-specific primers, which are complementary to sequences near either the 5' end (E2–E3) or the 3' end (E8–E9) of the CYP2A5 transcript. The results (Fig. 1E) indicated that neither the full-length CYP2A5 mRNA, nor a truncated CYP2A5 mRNA containing the first eight exons (which are intact), was present at detectable levels in any of the tissues examined of either male or female *Cyp2a5*-null mice.

Mice homozygous for the disrupted allele were indistinguishable from their WT littermates, or from WT B6 mice, in growth rate and reproductive ability. No deviation from Mendelian distribution of genotypes was observed in pups derived from F1 heterozygous breeding pairs (Supplemental Table 1), a result suggesting that CYP2A5 is not critical for embryonic development. Adult body and organ weights (liver, kidney, lung, and testis for males, and liver, kidney, and lung for females) were identical between *Cyp2a5*-null and WT B6 mice (Supplemental Table 2), indicating that CYP2A5 is not essential for normal growth. Homozygous breeding pairs were established to produce *Cyp2a5*-null mice for subsequent studies.

Given our previous finding of a neighboring effect of *Cyp2g1* gene disruption on the expression of the (downstream) *Cyp2a5* gene (Zhuo et al., 2004), we examined the potential effects of the disruption to *Cyp2a5*, on the expression of the neighboring *Cyp2g1*. We found that there was no significant difference between *Cyp2a5*-null and WT mice, in CYP2G1 mRNA levels in the olfactory mucosa, a site where CYP2G1 is uniquely expressed (Supplemental Fig. 1). Thus, the genomic disruption at *Cyp2a5* exon 9 did not lead to noticeable changes in the expression of the upstream *Cyp2g1* gene.

The expression of various *Cyp2b* genes, which are located further upstream of the *Cyp2g1* gene, also seemed to be unaffected by the neo insertion, as exemplified by the results from immunoblot analysis of CYP2B protein expression (Supplemental Fig. 2). It is noteworthy that immediately downstream of *Cyp2a5* is a cluster of *Cyp2a* genes and pseudogenes, including (in order of increasing distance from *Cyp2a5*) *Cyp2a23-ps*, *Cyp2a22*, *Cyp2a21-ps*, *Cyp2a12*, and *Cyp2a20-ps*, with *Cyp2a23-ps*, a pseudogene, located at a distance of >100 kb from *Cyp2a5* (Wang et al., 2003). The expression of *Cyp2a22*, a gene immediately downstream of *Cyp2a23-ps*, was not detected in the liver of either WT or *Cyp2a5*-null mice (data not shown); there has been no previous report of studies on *Cyp2a22* expression or function. Thus, the first functional gene downstream of *Cyp2a5* is *Cyp2a12* (Su and Ding, 2004). Hepatic expression of *Cyp2a12* was also not different between WT and *Cyp2a5*-null mice

(data not shown), thus further confirming the absence of any neighboring effects of the neo insertion.

To examine whether the loss of *Cyp2a5* expression in the liver led to compensatory increases in the expression of other biotransformation genes, we compared hepatic microsomal levels of several P450 proteins, namely, CYP2B, 2C, and 3A, as well as cytochrome P450 reductase, UGT1A, and UGT2B proteins, between *Cyp2a5*-null and WT mice. We found that the expression levels were similar between WT and *Cyp2a5*-null mice for each of these enzymes in liver microsomes (Supplemental Fig. 2). Furthermore, we examined the metabolism of testosterone, a common substrate for multiple P450 enzymes, in liver microsomal reactions (Table 1). We found that, whereas the rates of formation of 15 β -OH-testosterone, a testosterone metabolite known to be produced by CYP2A5, were lower (by ~50%), the rates of formation of two major testosterone metabolites, 16 α -OH-testosterone and 6 β -OH-testosterone, the preferred products formed by CYP2D9 (Wong et al., 1987) and CYP3A, respectively (Yanagimoto et al., 1992), were not changed in the *Cyp2a5*-null mice, compared with WT mice. In other studies not shown, we also observed lower rates of formation of 15 α -OH-testosterone (also a CYP2A5 product), but not 16 β -, 6 α -, or 11 α -OH-testosterone. Consistent with the lack of a substantial difference in the overall rate of hepatic microsomal testosterone metabolism in vitro, there was no significant difference in circulating testosterone levels between WT males and *Cyp2a5*-null males (Supplemental Fig. 3A). Likewise, there was also no significant difference in circulating progesterone levels between WT females and *Cyp2a5*-null females (Supplemental Fig. 3B). These in vivo results indicate that, although CYP2A5 is active toward testosterone and progesterone (Gu et al., 1999), it does not play a major role in the systemic clearance of testosterone or progesterone in the WT mice.

Role of CYP2A5 in the Metabolic Clearance of Nicotine and Cotinine. Systemic clearance of nicotine was compared between *Cyp2a5*-null and WT mice, with nicotine administered at either 1 mg/kg (Fig. 2A) or 5 mg/kg (Fig. 2B). As expected, clearance of nicotine in the WT mouse was very rapid, with an elimination half-life ($t_{1/2}$) value of ~13 min after an injection at a dose of 1 mg/kg i.p., and a $t_{1/2}$ value of ~70 min after an injection at the higher dose, 5 mg/kg i.p. (Table 2). Nicotine clearance was significantly slower (by >70% in CL/F values) in the *Cyp2a5*-null mice, with concomitant 3.2-fold and 6.1-fold increases in the values for maximal concentration (C_{max}) and AUC, respectively, at a dose of 1

mg/kg, and 3.4-fold and 2.7-fold increases in the values for C_{max} and AUC, respectively, at a dose of 5 mg/kg. Increases in the $t_{1/2}$ value were also observed: 2.8-fold and 1.4-fold, for nicotine doses of 1 mg/kg and 5 mg/kg, respectively. Consistent with the notion that CYP2A5 is the low- K_m enzyme in metabolizing nicotine, the impact of *Cyp2a5* deletion on nicotine clearance was more pronounced at the 1 mg/kg dose than at the 5 mg/kg dose, as indicated by the greater magnitude (low-dose group versus high-dose group) of differences between null and WT mice, in both AUC (6.1-fold versus 2.7-fold, respectively) and $t_{1/2}$ (2.8-fold versus 1.4-fold, respectively). A further examination of the AUC values obtained at the two nicotine doses indicated a greater difference in the WT mice (>8-fold, high-dose/low-dose) than in the *Cyp2a5*-null mice (<4-fold); this result is consistent with a more efficient clearance in the WT mice than in the *Cyp2a5*-null mice at the low dose.

The formation and clearance of cotinine, the main circulating metabolite of nicotine, were also monitored in nicotine-treated mice. The AUC and C_{max} values for cotinine were much higher (>24-fold and >6-fold, respectively) than the corresponding values for nicotine in WT mice treated at either nicotine dose (Table 2). In nicotine-treated *Cyp2a5*-null mice, the rates of formation of cotinine seemed to be substantially lower, as indicated by lower C_{max} values, and higher time at maximal concentration (T_{max}) values, compared with the values for the corresponding WT groups. However, rates of cotinine clearance also seemed to be lower, as reflected by significantly higher $t_{1/2}$ values.

The lower rate of cotinine clearance, as a result of the *Cyp2a5* deletion, was confirmed by additional studies in which circulating levels of cotinine were determined for cotinine-treated WT and *Cyp2a5*-null mice (Fig. 2C). As shown in Table 3, the rate of cotinine clearance was significantly lower (by 74% in CL/F value), whereas the AUC (by 3.7-fold) and $t_{1/2}$ (by 2- to 4-fold) values were higher, in the *Cyp2a5*-null mice than in the WT mice, at a cotinine dose of 1 mg/kg. It is noteworthy that, in cotinine-treated mice, the lower rates of cotinine clearance in the *Cyp2a5*-null mice were accompanied by only marginally higher (1.3-fold; $P > 0.05$) cotinine C_{max} values, compared with values for WT mice. This result can be explained by the much slower rate of first-pass metabolism of cotinine, compared with metabolism of nicotine, in the liver.

For determination of the role of CYP2A5 in the regulation of tissue levels of nicotine and cotinine, a pharmacokinetic study was also performed for liver, the main metabolic organ, and for brain, the target organ, with nicotine administered at 1 mg/kg. As shown in Fig. 2, D and E, the concentration-time curves for tissue nicotine and cotinine levels were similar between the two organs, and also similar to the curves determined for circulating nicotine and cotinine levels, respectively. Much higher levels of nicotine, and much lower levels of cotinine, were seen in both liver and brain of the *Cyp2a5*-null mice than in liver and brain of the WT mice, at multiple time points after dosing. A further examination of the pharmacokinetic parameters for tissue nicotine and cotinine (Table 4) confirmed that CYP2A5 has a major impact on the bioavailability of nicotine and cotinine in the brain and the liver. In the *Cyp2a5*-null mice, the tissue levels of nicotine were higher in both liver (2.3-fold higher AUC, 1.2-fold higher C_{max}) and brain (2.7-fold higher AUC, 2.0-fold higher C_{max}), whereas tissue levels of cotinine were lower in both organs (>75%

TABLE 1

In vitro metabolism of testosterone by liver microsomes of WT and *Cyp2a5*-null mice

Rates of formation of three major testosterone metabolites, 15 β -hydroxy-testosterone (15 β -OH-T), 16 α -OH-T, and 6 β -OH-T, were determined. Reaction mixtures contained 50 mM potassium phosphate buffer, pH 7.4, 5.0 or 10 μ M testosterone, 1.0 mM ascorbic acid, 0.1 mg/ml liver microsomal protein from 2-month-old male mice, and 1.0 mM NADPH. The values presented are means $\pm$ S.D. ($n = 3$).

Testosterone concentration	Genotype	Rates of Product Formation		
		15 β -OH-T	16 α -OH-T	6 β -OH-T
<i>pmol/min/mg protein</i>				
5 μ M	WT	4.6 $\pm$ 1.1	240 $\pm$ 50	100 $\pm$ 30
	<i>Cyp2a5-null</i>	2.3 $\pm$ 0.1 ^a	250 $\pm$ 30	100 $\pm$ 20
10 μ M	WT	6.1 $\pm$ 0.7	270 $\pm$ 30	130 $\pm$ 20
	<i>Cyp2a5-null</i>	2.9 $\pm$ 0.5 ^a	280 $\pm$ 50	140 $\pm$ 20

^a Significantly lower than the corresponding WT value ($P < 0.05$).

Fig. 2. Systemic and tissue levels of nicotine and cotinine at various time points after intraperitoneal administration of nicotine or cotinine. WT and *Cyp2a5*-null mice (2-month-old, male) were injected with nicotine or cotinine at the indicated dose, and levels of nicotine and/or cotinine were determined in the plasma, liver, and brain at various time points after the injection. Body weights were not different between the two mouse strains. A and B, plasma levels of nicotine and cotinine after an injection of nicotine at 1 mg/kg i.p. (A) or 5 mg/kg i.p. (B); C, Plasma levels of cotinine after an injection of cotinine at 1 mg/kg i.p. D and E, levels of nicotine and cotinine in the liver (D) and brain (E) after an injection of nicotine at 1 mg/kg i.p. Data represent means $\pm$ S.D. ($n = 4-5$, for plasma; $n = 3-4$, for tissue). **, $P < 0.01$, compared with levels in WT mice.

lower $C_{\max}$, and 20–30% lower AUC), compared with the levels for WT mice (Table 4).

Overall, the CYP2A5-associated pharmacokinetic changes for tissue nicotine and cotinine (Table 4) are similar to the changes seen in plasma (Table 2), and they are also comparable between brain and liver. This pattern is consistent with the notion that the impact of CYP2A5 is on systemic clearance, rather than on tissue distribution. In that context, the cotinine/nicotine abundance ratios were much higher for plasma than for liver and brain, in either WT or *Cyp2a5*-null

mice; however, the abundance ratios were uniformly decreased in the *Cyp2a5*-null mice (Supplemental Table 3). It is noteworthy that tissue nicotine and cotinine levels were found to differ somewhat between brains and livers: AUC and $C_{\max}$ values for both nicotine and cotinine were higher in the liver than in the brain, for either mouse strain (Table 4). This tissue difference can be explained, in part, by the fact that the liver is the portal-of-entry organ for intraperitoneally injected nicotine, and by the fact that the bulk of the cotinine was produced in the liver.

TABLE 2

Pharmacokinetic parameters for plasma nicotine and cotinine in mice treated with nicotine

Mice were given nicotine at either 1 or 5 mg/kg i.p. Values were derived from plots shown in Fig. 2, A and B. Results shown represent means $\pm$ S.D. ($n = 4-5$).

Nicotine Dose	Analyte	Mouse Strain	AUC _{0-240 min}	$t_{1/2}$	CL/F	T_{max}	C_{max}
mg/kg			ng·h/ml	min	ml/min	min	ng/ml
1	Nicotine	WT	12.7 $\pm$ 0.5	12.9 $\pm$ 3.2	31.9 $\pm$ 2.5	5.0 $\pm$ 0.0	43.2 $\pm$ 7.9
		<i>Cyp2a5</i> -null	77.3 $\pm$ 8.2*	35.5 $\pm$ 2.9*	5.72 $\pm$ 0.60*	5.0 $\pm$ 0.0	139 $\pm$ 38*
	Cotinine	WT	391 $\pm$ 50	38.2 $\pm$ 2.7	N/A	16.3 $\pm$ 10.3	267 $\pm$ 55
		<i>Cyp2a5</i> -null	253 $\pm$ 25*	119 $\pm$ 43*	N/A	52.5 $\pm$ 22.0*	89.2 $\pm$ 11.0*
5	Nicotine	WT	107 $\pm$ 21	70.5 $\pm$ 15.0	25.3 $\pm$ 4.1	5.0 $\pm$ 0.0	113 $\pm$ 36
		<i>Cyp2a5</i> -null	292 $\pm$ 61*	95.9 $\pm$ 14.0**	7.49 $\pm$ 2.50*	5.0 $\pm$ 0.0	385 $\pm$ 105**
	Cotinine	WT	2600 $\pm$ 340	55.0 $\pm$ 9.4	N/A	52.5 $\pm$ 15.0	1010 $\pm$ 80
		<i>Cyp2a5</i> -null	2140 $\pm$ 210	167 $\pm$ 47*	N/A	90.0 $\pm$ 35.0	449 $\pm$ 56*

N/A, not applicable

*, $P < 0.01$, compared with corresponding WT value**, $P < 0.05$, compared with corresponding WT value.

TABLE 3

Pharmacokinetic parameters for plasma cotinine in mice treated with cotinine

Mice were injected with cotinine at 1 mg/kg i.p. Values were derived from plots shown in Fig. 2C. Results shown represent means $\pm$ S.D. ($n = 4$).

Strain	AUC _{0-480 min}	$t_{1/2}$	CL/F	T_{max}	C_{max}
	ng·h/ml	min	ml/min	min	ng/ml
WT	847 $\pm$ 95	54.6 $\pm$ 3.1	0.43 $\pm$ 0.01	15.0 $\pm$ 0.0	627 $\pm$ 76
<i>Cyp2a5</i> -null	3140 $\pm$ 240*	131 $\pm$ 19*	0.11 $\pm$ 0.02*	22.5 $\pm$ 8.6	827 $\pm$ 136

*, $P < 0.01$, compared with corresponding WT value.

TABLE 4

Pharmacokinetic parameters for tissue nicotine and cotinine levels in mice treated with nicotine

Mice were given nicotine at 1 mg/kg i.p. Values were derived from plots shown in Fig. 2, D and E. Pharmacokinetic parameters were calculated by use of mean concentration values ($n = 3-4$) for each time point; each mouse was used for a single time point.

Tissue	Analyte	Strain	AUC _{0-240 min}	$t_{1/2}$	T_{max}	C_{max}
			ng·h/g	min	min	ng/g
Brain	Nicotine	WT	98.9	46.3	5.0	233
		<i>Cyp2a5</i> -null	263	44.8	5.0	477
	Cotinine	WT	219	37.8	15.0	218
		<i>Cyp2a5</i> -null	160	199	60.0	50.2
Liver	Nicotine	WT	157	32.4	5.0	658
		<i>Cyp2a5</i> -null	365	43.4	5.0	761
	Cotinine	WT	350	34.4	5.0	468
		<i>Cyp2a5</i> -null	275	149	30.0	109

It is noteworthy that, at a nicotine dose of 1 mg/kg, the tissue/plasma abundance ratios for nicotine were higher in the WT mice than in the *Cyp2a5*-null mice, whereas the ratios for cotinine were similar between the WT and *Cyp2a5*-null mice (Supplemental Table 4). The lower tissue/plasma abundance ratios for nicotine, but not for cotinine, in the *Cyp2a5*-null mice can probably be explained by a saturation of tissue binding for nicotine, but not for cotinine, because tissue levels of nicotine (but not the levels of cotinine) were much higher in the null mice than in the WT mice.

The lower rates of nicotine clearance in the *Cyp2a5*-null mice were probably due to the loss of CYP2A5-mediated cotinine formation in the liver, a notion that is supported by results from in vitro studies using hepatic microsomes from WT and *Cyp2a5*-null mice. Cotinine, formed through nicotine C-5'-oxidation (Fig. 3), is the major metabolite of nicotine in mouse hepatic microsomal reactions, as indicated by the relative abundance of all metabolites detected by LC/UV at 254 nm (data not shown). The conversion of the reactive intermediate to the stable cotinine was aided by the inclusion of aldehyde oxidase (contained in liver cytosol) (Beedham, 1987). As shown in Table 5, the rates of cotinine formation

were 85% and 73% lower for hepatic microsomes from the *Cyp2a5*-null mice than for hepatic microsomes from WT mice at nicotine concentrations of 1 and 10 μ M, respectively. It is noteworthy that the CYP2A5 contribution to microsomal nicotine metabolism in the WT mice was greater at the lower substrate concentration (1 μ M), a result further supporting the proposal that CYP2A5 is the low- K_m enzyme in metabolizing nicotine.

We detected two other nicotine metabolites in mouse hepatic microsomal reaction mixtures (Fig. 3). One, which was detected only when KCN was included in the reaction mixture, was identified as a cyanide adduct ($m/z = 188$), given the characteristic neutral loss of an HCN motif ($m/z = 27$), and the features of other fragment ions in the product ion spectrum of the metabolite (not shown). The formation of the cyanide adduct indicated the presence of an iminium ion intermediate, which we propose to be the dihydropyrolinium ion that is formed via the initial CYP2A5-mediated 5'-hydroxylation of nicotine (Fig. 3). The other metabolite ($m/z = 149$) had a mass fragmentation pattern consistent with that of nornicotine, which is formed from nicotine *N*-demethylation. Note that this metabolite does not represent a contaminant, because it was not detected in zero-minute incubations. The roles of CYP2A5 in the formation of these apparently less abundant metabolites were not determined.

Discussion

The in vivo role of CYP2A5 in nicotine and cotinine clearance has recently been studied by others, who made use of either CYP2A5 chemical inhibitors (Raunio et al., 2008b), or mouse strains (B6 and DBA/2) that have allelic differences in the structure and function of the CYP2A5 protein (Siu and Tyndale, 2007). Pharmacokinetic parameters for plasma nicotine and cotinine were determined both in the study by Siu and Tyndale (2007) and in the present study; therefore, it is tempting to compare the results from these two studies. However, although B6 WT mice were treated with nicotine

Fig. 3. Identification of nicotine metabolites produced in mouse liver microsomal reactions. The reaction mixtures contained 50 mM potassium phosphate buffer, pH 7.4, 10 μM nicotine, 1.0 mg/ml liver microsomal protein from 2-month-old male WT mice, and 1.0 mM NADPH, with or without 10 mM KCN. Metabolite analysis was performed with a LCQ Deca-XP LC/MS instrument, as described under *Materials and Methods*. Three metabolites (cotinine, normcotinine, and a cyanide adduct of nicotine) were identified; their structures and fragmentation patterns and the metabolic pathways that led to their formation are shown.

TABLE 5

Rates of in vitro metabolism of nicotine by liver microsomes from WT and *Cyp2a5*-null mice

Cotinine formation was determined as described under *Materials and Methods*. Reaction mixtures contained 50 mM potassium phosphate buffer, pH 7.4, 1.0 or 10 μM nicotine, 0.5 mg/ml liver microsomal protein, and 1.0 mg/ml liver cytosol protein (as a source of aldehyde oxidase) from 2-month-old male mice, and 1.0 mM NADPH. The values presented are means $\pm$ S.D. ($n = 3$).

Nicotine Concentration	Strain	Rates of Cotinine Formation	Ratio of Null/WT
		<i>pmol/min/mg</i>	
1.0 μM	WT	39.1 ± 1.2	0.15
	<i>Cyp2a5</i> -null	$5.7 \pm 0.8^*$	
10 μM	WT	221 ± 2	0.27
	<i>Cyp2a5</i> -null	$58.6 \pm 3.8^*$	

*, $P < 0.01$, compared with the corresponding WT value.

and cotinine (both at 1 mg/kg) in both studies, the experiments that correspond between the two studies in terms of compound and dose did not yield fully comparable results, given that the compounds were administered subcutaneously in the earlier study, but intraperitoneally in ours. An examination of the pharmacokinetic data from the two studies indicates a difference in clearance rate, e.g., the CL/F , AUC, and C_{max} values for nicotine were 5.2 ± 0.2 ml/min, 80.8 ± 3.2 ng·h/ml, and 160 ± 15 ng/ml, respectively, after subcutaneous injection (their study), and 31.9 ± 2.5 ml/min, 12.7 ± 0.5 ng·h/ml, and 43.2 ± 7.9 ng/ml, respectively, after intraperitoneal injection (our study). This difference in clearance rates can be explained by the essential role of hepatic P450 enzymes in nicotine clearance and the differing proportions of the injected dose that are subjected to hepatic first-pass metabolism. Although essentially all of the intraperitoneally injected dose of nicotine would be subjected to first-pass metabolism in the liver, before appearing in the systemic circulation, only a proportion of the subcutaneously injected dose of nicotine would be distributed to the liver in each pass.

The study design of Siu and Tyndale (2007) also differed from that of the present study, in that total (conjugated and unconjugated) nicotine and cotinine were determined there, but only unconjugated compounds were determined here. The extent of glucuronidation of nicotine or cotinine in mice is not known (Siu and Tyndale, 2007). In earlier work, UGT activities toward nicotine and cotinine had not been detected

in mouse liver microsomes in vitro (Ghosheh and Hawes 2002). Therefore, it is unlikely that the use or nonuse of a deconjugation step, between these two pharmacokinetics studies, would have affected the experimental outcome to any large extent.

It is noteworthy that, despite the difference in the route of injection (subcutaneous versus intraperitoneal) between the two studies, and the large differences in pharmacokinetic parameters seen for nicotine clearance, essentially identical pharmacokinetic parameters were seen for cotinine clearance (after comparable cotinine injections at 1 mg/kg to WT B6 mice) in the two studies. This apparent independence on the injection route for cotinine clearance is consistent with a rate of hepatic cotinine metabolism that is much lower than the rate of hepatic nicotine metabolism (as was shown by Siu and Tyndale, 2007). Although the high-affinity K_m values for B6 hepatic microsomal metabolism of nicotine and cotinine were similar (11.4 and 9.5 μM , respectively), the V_{max} values differed by >10-fold (0.50 and 0.04 nmol/min/mg). Thus, a much smaller proportion of the dose will be removed through first-pass clearance for intraperitoneally injected cotinine than for intraperitoneally injected nicotine; conversely, given the overall differences in clearance rate (slower for cotinine than for nicotine), the majority of subcutaneously injected cotinine will eventually have a chance to pass through the liver and be metabolized by hepatic CYP2A5.

The lower rate of nicotine clearance in the *Cyp2a5*-null mice was accompanied by a lower level of cotinine formation, compared with the values for WT mice. It is noteworthy that the impact of the *Cyp2a5* deletion on nicotine AUC values (2.7-fold to 6.1-fold increases, null versus WT) and the impact of the *Cyp2a5* deletion on cotinine C_{max} values (56–67% reductions), an impact that mainly reflects the lower rate of nicotine metabolism, were more robust than the impact on cotinine AUC values (18–35% reductions). The latter can be influenced by both rates of cotinine formation and rates of cotinine metabolism in nicotine-treated mice. These findings relating to the effects of the *Cyp2a5* gene deletion on pharmacokinetics of circulating nicotine and cotinine may aid in the interpretation of smoke-exposure-biomonitoring results for individuals with genetic polymorphisms in the ortholo-

gous *CYP2A6* gene. In that regard, the dependence of serum cotinine levels on rates of both formation and elimination has been found previously in human smokers (Pérez-Stable et al., 1998).

Human CYP2A6 is less efficient than CYP2A5 is in the metabolism of nicotine; heterologously expressed CYP2A6 was found to have a K_m value of 144 μ M and a V_{max}/K_m value of 0.01 in nicotine C-5'-oxidation, whereas heterologously expressed CYP2A5 had a K_m value of 7.7 μ M and a V_{max}/K_m value of 0.20 (Murphy et al., 2005). In liver microsomes from WT B6 mice, a high-affinity component (K_m , 11.4 μ M) and a low-affinity component (K_m , 306 μ M) were identified, with CYP2A5 being apparently responsible for the high-affinity site (Siu and Tyndale, 2007). Thus, in terms of the pharmacokinetics of nicotine clearance, the *Cyp2a5*-null mouse more closely resembles humans than the WT B6 mouse does. The residual activities toward nicotine in the *Cyp2a5*-null mice are probably contributed by other P450 enzymes that have either low affinity or low efficiency for this reaction, in particular, members of the mouse *Cyp2b* subfamily (Siu and Tyndale, 2007). In this context, human CYP2B6 and rat CYP2B1/2 have been found to be active in nicotine 5'-oxidation (Nakayama et al., 1993; Yamazaki et al., 1999; Schoedel et al., 2001). Efforts are underway to prepare a CYP2A6-humanized mouse model to enable direct study of CYP2A6-mediated nicotine metabolism in vivo.

It is important that we understand the impact of systemic metabolism on tissue levels of nicotine and cotinine in the brain, the pharmacological target organ for nicotine. Although cotinine does not have the same drug action as nicotine does, it does have the potential to modulate nicotine action through its binding to epibatidine-sensitive nicotinic receptors (Vainio and Tuominen, 2001). Our observation of much higher (assuming that 1 g of tissue is equivalent to 1 ml of plasma) levels of nicotine in brain tissue than in plasma most likely reflects the higher tissue binding of nicotine in brain, compared with plasma. In contrast, cotinine levels in the brain were actually slightly lower than levels in the plasma, of nicotine-treated mice. This finding may be explained by the slow rate of cotinine metabolism (relative to the rate of nicotine metabolism) in the liver; it may also reflect rate limitations for penetration of the blood-brain barrier by cotinine (Riah et al., 1998). Our findings concerning the effects of the *Cyp2a5* gene deletion on nicotine and cotinine levels, and on the cotinine/nicotine abundance ratios, in the brains and plasma of nicotine-treated mice should be taken into account when predictions are made on the basis of the measurable plasma levels, for levels of nicotine and cotinine in the brain in nicotine-exposed individuals.

The neuroprotective effects of nicotine against chemically induced neurotoxicity have been consistently demonstrated in experimental rat and monkey models, but conflicting results have been found in mice (Quik et al., 2007). Nicotine clearance is much faster in mice than in humans, monkeys, and rats; it is conceivable that we are not able to maintain adequate levels of nicotine in the mouse brain for a sufficiently long period to enable effective neuroprotection, in particular, for chronic effects involving nicotine-induced alterations in gene expression. Our finding that plasma nicotine $t_{1/2}$ values, as well as brain nicotine levels, are significantly higher in nicotine-treated *Cyp2a5*-null mice than in the similarly dosed WT mice suggests that the *Cyp2a5*-null

mouse model is valuable for efforts to establish whether effective neuroprotection by nicotine can be achieved in mouse models through suppression of systemic clearance.

In summary, several features of our *Cyp2a5*-null mouse make it a suitable model for applications in drug metabolism and toxicology research. These features include the B6 genetic background; the specific deletion of the single mouse *Cyp2a* gene (sparing *Cyp2a4* and *Cyp2a12*); the absence of a negative effect of the *Cyp2a5* disruption on the expression of neighboring *Cyp2a12*, *Cyp2g1*, and *Cyp2b* genes; the absence of compensatory changes in the expression of other biotransformation enzymes examined; and the lack of any concomitant reproductive or developmental defects. In a first application of the *Cyp2a5*-null model to in vivo drug metabolism studies, we have examined the role of CYP2A5 in systemic clearance of nicotine and cotinine. Our findings provided direct evidence that CYP2A5 plays an essential role in the in vivo metabolism of both nicotine and cotinine. Our pharmacokinetic analysis of plasma and tissue nicotine and cotinine levels in the *Cyp2a5*-null mice should have important implications for an understanding of the impact of genetic polymorphisms in the *CYP2A6* gene on pharmacokinetics of nicotine and cotinine in human brain and plasma. In addition, future studies on the efficacy of nicotine as a neuroprotective agent in the *Cyp2a5*-null mouse model are warranted.

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References

- Beedham C (1987) Molybdenum hydroxylases: biological distribution and substrate-inhibitor specificity. *Prog Med Chem* **24**:85–127.
- Benowitz NL, Swan GE, Jacob P III, Lessov-Schlaggar CN, and Tyndale RF (2006) CYP2A6 genotype and the metabolism and disposition kinetics of nicotine. *Clin Pharmacol Ther* **80**:457–467.
- Cashman JR (2000) Human flavin-containing monooxygenase: substrate specificity and role in drug metabolism. *Curr Drug Metab* **1**:181–191.
- Cashman JR, Park SB, Yang ZC, Wrighton SA, Jacob P III, and Benowitz NL (1992) Metabolism of nicotine by human liver microsomes: stereoselective formation of trans-nicotine N'-oxide. *Chem Res Toxicol* **5**:639–646.
- de Leon J, Diaz FJ, Rogers T, Browne D, Dinsmore L, Ghosheh OH, Dwoskin LP, and Crooks PA (2002) Total cotinine in plasma: a stable biomarker for exposure to tobacco smoke. *J Clin Psychopharmacol* **22**:496–501.
- Ding X and Coon MJ (1988) Purification and characterization of two unique forms of cytochrome P-450 from rabbit nasal microsomes. *Biochemistry* **27**:8330–8337.
- Ding X and Coon MJ (1990) Immunochemical characterization of multiple forms of cytochrome P-450 in rabbit nasal microsomes and evidence for tissue-specific expression of P-450s NMa and NMb. *Mol Pharmacol* **37**:489–496.
- Ding X and Coon MJ (1994) Steroid metabolism by rabbit olfactory-specific P450 2G1. *Arch Biochem Biophys* **315**:454–459.
- Ghosheh O and Hawes EM (2002) Microsomal N-glucuronidation of nicotine and cotinine: human hepatic interindividual, human intertissue, and interspecies hepatic variation. *Drug Metab Dispos* **30**:1478–1483.
- Gu J, Dudley C, Su T, Spink DC, Zhang QY, Moss RL, and Ding X (1999) Cytochrome P450 and steroid hydroxylase activity in mouse olfactory and vomeronasal mucosa. *Biochem Biophys Res Commun* **266**:262–267.
- Heavner DL, Richardson JD, Morgan WT, and Ogden MW (2005) Validation and application of a method for the determination of nicotine and five major metabolites in smokers' urine by solid-phase extraction and liquid chromatography-tandem mass spectrometry. *Biomed Chromatogr* **19**:312–328.
- Hukkanen J, Jacob P III, and Benowitz NL (2005) Metabolism and disposition kinetics of nicotine. *Pharmacol Rev* **57**:79–115.
- Kaivosari S, Toivonen P, Hesse LM, Koskinen M, Court MH, and Finel M (2007) Nicotine glucuronidation and the human UDP-glucuronosyltransferase UGT2B10. *Mol Pharmacol* **72**:761–768.

- Köntgen F, Süss G, Stewart C, Steinmetz M, and Bluethmann H (1993) Targeted disruption of the MHC class II Aa gene in C57BL/6 mice. *Int Immunol* **5**:957–964.
- Kuehl GE and Murphy SE (2003) N-glucuronidation of nicotine and cotinine by human liver microsomes and heterologously expressed UDP-glucuronosyltransferases. *Drug Metab Dispos* **31**:1361–1368.
- Messina ES, Tyndale RF, and Sellers EM (1997) A major role for CYP2A6 in nicotine C-oxidation by human liver microsomes. *J Pharmacol Exp Ther* **282**:1608–1614.
- Murphy SE, Raulinaitis V, and Brown KM (2005) Nicotine 5'-oxidation and methyl oxidation by P450 2A enzymes. *Drug Metab Dispos* **33**:1166–1173.
- Nakajima M, Yamamoto T, Nunoya K, Yokoi T, Nagashima K, Inoue K, Funae Y, Shimada N, Kamataki T, and Kuroiwa Y (1996) Role of human cytochrome P4502A6 in C-oxidation of nicotine. *Drug Metab Dispos* **24**:1212–1217.
- Nakayama H, Okuda H, Nakashima T, Imaoka S, and Funae Y (1993) Nicotine metabolism by rat hepatic cytochrome P450s. *Biochem Pharmacol* **45**:2554–2556.
- Pérez-Stable EJ, Herrera B, Jacob P III, and Benowitz NL (1998) Nicotine metabolism and intake in black and white smokers. *J Am Med Assoc* **280**:152–156.
- Quik M, O'Neill M, and Perez XA (2007) Nicotine neuroprotection against nigrostriatal damage: importance of the animal model. *Trends Pharmacol Sci* **28**:229–235.
- Raunio H, Hakkola J, and Pelkonen O (2008a) The CYP2A subfamily, in *Cytochrome P450 Role in Metabolism and Toxicity of Drugs and Other Xenobiotics* (Ioannides C ed) pp 151–171, Advancing the Chemical Sciences Publishing, Cambridge, UK.
- Raunio H, Pokela N, Puhakainen K, Rahnasto M, Mauriala T, Auriola S, and Juvonen RO (2008b) Nicotine metabolism and urinary elimination in mouse: in vitro and in vivo. *Xenobiotica* **38**:34–47.
- Ravina BM, Fagan SC, Hart RG, Hovinga CA, Murphy DD, Dawson TM, and Marler JR (2003) Neuroprotective agents for clinical trials in Parkinson's disease: a systematic assessment. *Neurology* **60**:1234–1240.
- Riah O, Courrière P, Dousset JC, Todeschi N, and Labat C (1998) Nicotine is more efficient than cotinine at passing the blood-brain barrier in rats. *Cell Mol Neurobiol* **18**:311–318.
- Schoedel KA, Sellers EM, and Tyndale RF (2001) Induction of CYP2B1/2 and nicotine metabolism by ethanol in rat liver but not rat brain. *Biochem Pharmacol* **62**:1025–1036.
- Siu EC and Tyndale RF (2007) Characterization and comparison of nicotine and cotinine metabolism in vitro and in vivo in DBA/2 and C57BL/6 mice. *Mol Pharmacol* **71**:826–834.
- Siu EC, Wildenauer DB, and Tyndale RF (2006) Nicotine self-administration in mice is associated with rates of nicotine inactivation by CYP2A5. *Psychopharmacology (Berl)* **184**:401–408.
- Statler PA, McPherson RJ, Bauer LA, Kellert BA, and Juul SE (2007) Pharmacokinetics of high-dose recombinant erythropoietin in plasma and brain of neonatal rats. *Pediatr Res* **61**:671–675.
- Su T and Ding X (2004) Regulation of the cytochrome P450 2A genes. *Toxicol Appl Pharmacol* **199**:285–294.
- Tai SS, Xu B, and Welch MJ (2006) Development and evaluation of a candidate reference measurement procedure for the determination of progesterone in human serum using isotope-dilution liquid chromatography/tandem mass spectrometry. *Anal Chem* **78**:6628–6633.
- Vainio PJ and Tuominen RK (2001) Cotinine binding to nicotinic acetylcholine receptors in bovine chromaffin cell and rat brain membranes. *Nicotine Tob Res* **3**:177–182.
- Wang H, Donley KM, Keeney DS, and Hoffman SM (2003) Organization and evolution of the Cyp2 gene cluster on mouse chromosome 7, and comparison with the syntenic human cluster. *Environ Health Perspect* **111**:1835–1842.
- Wong G, Kawajiri K, and Negishi M (1987) Gene family of male-specific testosterone 16 alpha-hydroxylase (C-P-450(16) alpha) in mouse liver: cDNA sequences, neonatal imprinting, and reversible regulation by androgen. *Biochemistry* **26**:8683–8690.
- Wong HL, Murphy SE, and Hecht SS (2005) Cytochrome P450 2A-catalyzed metabolic activation of structurally similar carcinogenic nitrosamines: N'-nitrosomorpholine, N-nitrosopiperidine, and N-nitrosopyrrolidine. *Chem Res Toxicol* **18**:61–69.
- Yamazaki H, Inoue K, Hashimoto M, and Shimada T (1999) Roles of CYP2A6 and CYP2B6 in nicotine C-oxidation by human liver microsomes. *Arch Toxicol* **73**:65–70.
- Yanagimoto T, Itoh S, Muller-Enoch D, and Kamataki T (1992) Mouse liver cytochrome P-450 (P-450IIIAM1): its cDNA cloning and inducibility by dexamethasone. *Biochim Biophys Acta* **1130**:329–332.
- Zhang X, Zhang QY, Liu D, Su T, Weng Y, Ling G, Chen Y, Gu J, Schilling B, and Ding X (2005) Expression of cytochrome p450 and other biotransformation genes in fetal and adult human nasal mucosa. *Drug Metab Dispos* **33**:1423–1428.
- Zhou X, Zhang X, Weng Y, Fang C, and Ding X (2009) High abundance of testosterone and salivary androgen-binding protein in the lateral nasal gland of male mice. *J Steroid Biochem Mol Biol* **117**:81–86.
- Zhuo X, Gu J, Behr MJ, Swiatek PJ, Cui H, Zhang QY, Xie Y, Collins DN, and Ding X (2004) Targeted disruption of the olfactory mucosa-specific *Cyp2g1* gene: impact on acetaminophen toxicity in the lateral nasal gland, and tissue-selective effects on Cyp2a5 expression. *J Pharmacol Exp Ther* **308**:719–728.

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