Differential Effects of Nicotine Treatment and Ethanol Self-Administration on CYP2A6, CYP2B6 and Nicotine Pharmacokinetics in African Green Monkeys

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ABSTRACT
In primates, nicotine is metabolically inactivated in the liver by CYP2A6 and possibly CYP2B6. Changes in the levels of these two enzymes may affect nicotine pharmacokinetics and influence smoking behaviors. This study investigated the independent and combined effects of ethanol self-administration and nicotine treatment (0.5 mg/kg b.i.d. s.c.) on hepatic CYP2A6 and CYP2B6 levels (mRNA, protein, and enzymatic activity), in vitro nicotine metabolism, and in vivo nicotine pharmacokinetics in monkeys. CYP2A6 mRNA and protein levels and in vitro coumarin (selective CYP2A6 substrate) metabolism were decreased by nicotine treatment but unaffected by ethanol. CYP2B6 protein levels and in vitro bupropion (selective CYP2B6 substrate) metabolism were increased by ethanol but unaffected by nicotine treatment; CYP2B6 mRNA levels were unaltered by either treatment. Combined ethanol and nicotine exposure decreased CYP2A6 mRNA and protein levels, as well as in vitro coumarin and nicotine metabolism, and increased CYP2B6 protein levels and in vitro bupropion metabolism, with no change in CYP2B6 mRNA levels. Chronic nicotine resulted in higher nicotine plasma levels achieved after nicotine administration, consistent with decreased CYP2A6. Ethanol alone, or combined with nicotine, resulted in lower nicotine plasma levels by a mechanism independent of the change in these enzymes. Thus, nicotine can decrease hepatic CYP2A6, reducing the metabolism of its substrates, including nicotine, whereas ethanol can increase hepatic CYP2B6, increasing the metabolism of CYP2B6 substrates. In vivo nicotine pharmacokinetics are differentially affected by ethanol and nicotine, but when both drugs are used in combination the effect more closely resembles ethanol alone.

Introduction
Nicotine is responsible for the reinforcing effects of cigarette smoking, and dependent smokers will adjust their smoking behavior (cigarettes/day, inhalation volume, puff frequency) to maintain desired plasma nicotine levels (Scherer, 1999). Therefore, factors that influence nicotine pharmacokinetics (PK), either genetic or environmental (Swan et al., 2009), can have an impact on smoking behaviors and ultimately the risk for certain smoking-related diseases (Liu et al., 2011; Strasser et al., 2011; Wassenaar et al., 2011). Identifying factors that modify nicotine pharmacokinetics is an important step toward understanding smoking behaviors.

In humans, nicotine is eliminated mainly via metabolic inactivation to cotinine in the liver (Benowitz and Jacob, 1994). This reaction is mediated primarily by CYP2A6, with some contribution from CYP2B6 (Messina et al., 1997; Yamanaka et al., 2005). Cotinine is further metabolized to trans-3-hydroxycotinine via a reaction mediated exclusively by CYP2A6 (Nakajima et al., 1996; Yamazaki et al., 1999; Yamanaka et al., 2005). Cigarette smoking decreases nicotine metabolism, which is in contrast to the well known effect of cigarette smoking on accelerating the metabolism of many other drugs (Zevin and Benowitz, 1999). This paradox is presumably caused by the down-regulation of hepatic CYP2A6 by nicotine, because chronic nicotine treatment decreased hepatic CYP2A6 mRNA, protein, and activity levels...
in monkeys (Schoedel et al., 2003). CYP2B6 levels and activity are unaffected by smoking, and nicotine treatment does not affect hepatic CYP2B6 mRNA, protein, or activity levels in monkeys (Schoedel et al., 2003; Hesse et al., 2004). Elevated levels of CYP2A6 and CYP2B6 protein have been reported in livers from alcoholics (Niemelä et al., 2000; Hesse et al., 2004). Chronic ethanol administration induces protein levels of hepatic CYP2A5 (orthologue to human CYP2A6) in mice (Lu et al., 2011) and hepatic CYP2B1/2 (orthologue to human CYP2B6) in rats (Howard et al., 2001), suggesting that ethanol exposure may be responsible for the higher levels of CYP2A6 and CYP2B6 in alcoholics; however, the induction of these hepatic enzymes by ethanol has not yet been demonstrated in primates.

Despite the large proportion of smokers who regularly consume alcohol, there is limited information about the combined effects of smoking and alcohol consumption on CYP2A6, CYP2B6, and nicotine pharmacokinetics. There have been, however, many studies showing that alcohol consumption can modify smoking behaviors (Shiffman and Balabanis, 1995). For example, smokers consume a greater number of cigarettes and inhale more deeply while drinking alcohol (Mintz et al., 1985; Witkiewitz et al., 2012). The association between smoking and alcohol consumption may be caused in part by ethanol-induced changes in nicotine pharmacokinetics, possibly involving the induction of CYP2A6 and/or CYP2B6.

The African green monkey (AGM) is an ideal animal model for studying hepatic CYP2A6 and CYP2B6 regulation, nicotine metabolism, and nicotine pharmacokinetics. The expression, substrate specificity, and regulation of AGM CYP2A6 (also referred to as CYP2A26) and CYP2B6 are similar to their respective human orthologues (Schoedel et al., 2003; Uno et al., 2011). Like humans, AGMs eliminate nicotine mainly via metabolic inactivation to cotinine in the liver, with CYP2A6 having a major role in mediating this reaction and CYP2B6 having a minor contribution (Schoedel et al., 2003). The apparent $k_m$ value (mean ± S.D.) for in vitro nicotine metabolism in AGM hepatic microsomes is 29.1 ± 8.6 μM ($n = 6$) (Schoedel et al., 2003), which is comparable with the apparent $k_m$ value of 64 ± 32.7 μM ($n = 31$) in human hepatic microsomes (Messina et al., 1997). Consistent with these similarities, the plasma half-life for nicotine ranges from 1 to 4 h in both AGMs and humans (Hukkanen et al., 2005; Lee et al., 2006). AGMs are also an important model for complex human behaviors and are routinely used in alcohol research because they will voluntarily self-administer alcohol at levels comparable with human consumption (Ervin et al., 1990).

In this study we characterized the effects of chronic nicotine treatment and ethanol self-administration, alone and in combination, on hepatic CYP2A6 and CYP2B6 levels, in vitro nicotine metabolism, and in vivo nicotine pharmacokinetics in AGMs. First, we showed that nicotine treatment can decrease hepatic CYP2A6 levels and ethanol self-administration can increase hepatic CYP2B6 levels. Second, we demonstrated that the metabolism of nicotine to cotinine in monkey liver microsomes is mediated primarily by CYP2A6 and reduced by nicotine treatment but unaffected by ethanol exposure. Finally, we showed that exposure to ethanol and nicotine, either alone or in combination, can modify in vivo nicotine pharmacokinetics.

### Materials and Methods

#### Animals

Adult male African green monkeys (vervets; Chlorocebus sabaues) were provided by and housed at the Behavioral Sciences Foundation (St. Kitts). The study timeline is shown in Fig. 1 and has been described previously in detail (Ferguson et al., 2011). In brief, the first 14 days of the study consisted of an ethanol preference screening phase, where monkeys were allowed to self-administer 10% (v/v) ethanol in 0.5% (w/v) sucrose solution for 4 h/day. Forty monkeys that voluntarily consumed more than 1 g of ethanol/kg per day were randomized into four groups based on daily ethanol consumption ($n = 10$/group): a no-drug control group, an ethanol-only group, a nicotine-only group, and an ethanol + nicotine group. The following 14 days (days 15–28) consisted of a washout period, during which monkeys were not exposed to any ethanol or nicotine. From days 29 to 42, monkeys in the ethanol-only and ethanol + nicotine groups were allowed to self-administer 10% ethanol in 0.5% sucrose solution for 4 h/day, whereas the other groups consumed 0.5% sucrose solution on the same schedule. From days 43 to 63, in addition to ethanol (or sucrose), monkeys in the nicotine-only and ethanol + nicotine groups were given subcutaneous injections of nicotine bitartrate (milligram base in saline, pH 7.0, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) twice daily at a dose of 0.05 mg/kg on day 43,

![Fig. 1. Overview of the study timeline. Monkeys were assessed for ethanol preference and then randomized into four study groups ($n = 10$/group) based on daily ethanol consumption. The groups consisted of a no-drug control group, an ethanol-only group, a nicotine-only group and an ethanol + nicotine group. In vivo nicotine PK testing was performed before drug administration on day 22 (during the washout period) and again after drug administration on day 50. Nicotine treatment and alcohol access were suspended on day 50 to allow for pharmacokinetic testing. Monkeys were sacrificed on day 63.](image-url)
0.1 mg/kg on day 44, 0.25 mg/kg on day 45, and 0.5 mg/kg on subsequent days. The first injection was given 30 min before the ethanol (or sucrose) access period, and the second injection was given 10 h later. Monkeys in the ethanol-only and control groups were given saline injections (as a vehicle control for nicotine bitartrate) on the same schedule. On day 50, nicotine treatment and ethanol access were suspended to conduct pharmacokinetic testing. Throughout the study, monkeys were fed standard rations of Purina (St. Louis, MO) monkey chow supplemented with fresh fruit and vegetables twice a day, and drinking water was available ad libitum. Body weights at the start of the study (average = 5.9 ± 0.5 kg) were not significantly different from body weights at sacrifice (average = 5.7 ± 0.5 kg) (paired t-test; p > 0.05). There were no significant differences in body weights among groups at the start of the study [one-way analysis of variance (ANOVA); F3,36 = 0.3784; p > 0.05] or at sacrifice (one-way ANOVA; F1,36 = 1.860; p > 0.05). Animals were sacrificed by exsanguination via the femoral artery under ketamine anesthesia, and livers were immediately dissected and flash-frozen in liquid nitrogen and stored at −80°C until further use. The experimental protocol was reviewed and approved by the Institutional Review Board of the Behavioral Sciences Foundation and the University of Toronto Animal Care Committee. All procedures were conducted in accordance with the guidelines of Declaration of Helsinki and the Canadian Council on Animal Care.

Microsomal Membrane Preparation. Monkey liver microsomal protein was homogenized in 100 mM Tris, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.32 M sucrose, pH 7.4, for immunoblotting or in 1.15% (w/v) KCl for in vitro metabolism assays, and then centrifuged at 12,500 g for 30 min at 4°C. The supernatant was then centrifuged at 110,500 g for 90 min at 4°C, and the pellet was resuspended in 100 mM Tris, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1.15% (w/v) KC1, and 20% (v/v) glycerol for immunoblotting or 1.15% (w/v) KCl for in vitro metabolism assays. The protein content of liver microsomes was assayed with the Bradford (1976) technique using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Microsomes were stored at −80°C until further use.

Immunoblotting. Monkey liver microsomal protein was serially diluted to generate a standard curve and establish the linear detection range for the immunoblotting assays (Supplemental Fig. 1). Liver microsomal proteins (10 μg for the CYP2A6 assay and 5 μg for the CYP2B6 assay) were separated by SDS-polyacrylamide gel electrophoresis (4% stacking and 8% separating gels). A portion of each gel was stained with Coomassie blue R-250 to ensure equal loading of protein among lanes, and the remaining gel was transferred overnight onto nitrocellulose membranes (Pall Corporation, East Hills, NY). To detect CYP2A6 and CYP2B6 protein, the membranes were first blocked with 1% skim milk in 50 mM Tris-buffered saline (TBS) containing 0.1% (w/v) bovine serum albumin and 0.1% (v/v) Triton X-100 for 1 h. Membranes were then incubated with either polyclonal anti-human CYP2A6 antibody (Fitzgerald Industries, Acton, MA) or anti-human CYP2B6 antibody (Fitzgerald Industries) diluted 1:2000 for 2 h, followed by three 5-min washes with TBS containing 0.1% (v/v) Triton X-100. The membranes were then blocked again with the initial blocking solution for 1 h and incubated with peroxidase-conjugated rabbit anti-sheep antibody (Millipore Corporation, Billerica, MA) diluted 1:5000 for 1 h, followed by three 5-min washes with TBS containing 0.1% (v/v) Triton X-100. Proteins were visualized by using chemiluminescence (Thermo Fisher Scientific, Mississauga, ON, Canada) followed by exposure to autoradiographic film (Ultident, St. Laurent, QC, Canada).

In Vitro Nicotine C-Oxidation Assay. Nicotine C-oxidation (NCO) was assayed as described previously (Schoedel et al., 2003). For the NCO chemical inhibition assays, pooled samples containing equal amounts of microsomal protein from all monkeys in a study group were used. The reaction mixtures were preincubated for 15 min with chemical inhibitors. The inhibitor concentrations were approximately equal to Kᵢ and 10 times higher than Kᵢ for the target cytochromes P450 in human liver microsomes: pilocarpine (CYP2A6, 4 and 40 μM; Sigma-Aldrich Canada Ltd) (Zhang et al., 2001), C8-xanthate (CYP2B6, 1 and 10 μM; Toronto Research Chemicals, Toronto, ON, Canada) (Bourrie et al., 1996), and quinidine (CYP2D6, 0.4 and 4 μM; Sigma-Aldrich Canada Ltd) (Bourrie et al., 1996). Chemically inhibited NCO was compared with reactions performed in the presence of appropriate vehicle controls but not chemical inhibitors. Samples were then analyzed for nicotine and cotinine concentrations.

In Vivo Nicotine and Cotinine Plasma Assays. To determine in vivo kinetic parameters for nicotine and cotinine, on days 22 and 50 monkeys were anesthetized with ketamine and injected with 0.1 mg/kg nicotine subcutaneously (milligram base in saline, pH 7.0; Sigma-Aldrich Canada Ltd). Blood samples (6 ml) were drawn at baseline and 10, 20, 30, 60, 120, 240, and 360 min after the injection; because of the need for continuous anesthesia, later sample collection times were not feasible. The blood samples were centrifuged, and the plasma was removed and frozen at −20°C for subsequent drug analyses. Total nicotine and cotinine was measured after deconjugation by an overnight incubation with β-glucuronidase (15 mg/ml, 250 μl per 0.5 ml of plasma) at 37°C.

Nicotine and Cotinine Measurements. The method for nicotine and cotinine measurement was adapted from a previous study (Siu et al., 2006). In brief, samples (either plasma or reaction mixtures from the in vitro NCO assays) were extracted with dichloromethane, and the organic phase was dried under nitrogen. Samples were reconstituted with 105 μl of 0.01 M HCl and 90 μl of each sample was analyzed by HPLC with UV detection (260 nm). Separation of nicotine and cotinine was achieved by using a ZORBAX Bonus-RP column (5 μm, 150 × 4.6 mm; Agilent Technologies, Mississauga, ON, Canada) and a mobile phase consisting of acetonitrile/potassium phosphate buffer (10:90 v/v, pH 5.07) containing 3.3 mM heptane sulfonic acid and 0.5% triethylamine. The separation was performed with a flow rate of 0.9 ml/min. Nicotine and cotinine sample concentrations were determined from standard curves. The quantitation limits were 5 ng/ml for nicotine and 12.5 ng/ml for cotinine. Cotinine concentrations were adjusted to account for baseline plasma cotinine present in monkeys from the nicotine-only and the ethanol + nicotine groups by using the following equation: 

\[ C_{\text{adjusted}} = C_t - (C_0 \times e^{-kt}) \]

where \( C_{\text{adjusted}} \) is baseline adjusted cotinine concentration, \( C_t \) is the actual cotinine plasma concentration measured at time \( t \), and \( C_0 \) is the concentration of cotinine immediately before nicotine dosing, \( k \) is the elimination rate constant, and \( t \) is the postdosing interval at which \( C_t \) was sampled.

In Vitro Coumarin Hydroxylation Assay and 7-OH-Coumarin Measurement. The methods used for the in vitro coumarin hydroxylation assays and the measurement of coumarin and 7-OH-coumarin were adapted from previous studies (Bogan and O'Kennedy, 1996; Li et al., 1997). Monkey hepatic microsomal protein (0.25 mg/ml) was mixed with 50 mM Tris buffer, pH 7.6, and 1.0 mM NADPH. After a 2-min preincubation, reactions were initiated by the addition of coumarin (30 μM, Sigma-Aldrich Canada Ltd) making the final volume 200 μl. Incubations were for 5 min at 37°C, and 50 μl of acetonitrile was added to stop the reaction. Trichloroacetic acid (10 μl, 20% v/v) and the internal standard 4-hydroxycoumarin (20 μl, 1 mg/ml) were added to the reaction mixture, which was then vortexed and spun at 16,500g for 10 min. A 100-μl aliquot of each sample was analyzed by HPLC with UV detection (315 nm). Separation of 7-OH-coumarin was achieved by using a ZORBAX SB C18 column (5 μm, 250 × 4.6 mm; Agilent Technologies) and a mobile phase consisting of acetonitrile, water, and acetic acid (25:75:0.1, v/v/v). The separation was performed with a flow rate of 1 ml/min. The concentration of 7-OH-coumarin was determined from a standard curve. The quantitation limit for 7-OH-coumarin was 25 ng/ml.

In Vitro Bupropion Hydroxylation Assay and Hydroxybupropion Measurement. The methods used for the in vitro bupropion hydroxylation assays and the measurement of hydroxybupropion were adapted from previous studies (Hesse et al., 2000; Loboz et al., 2005). Monkey hepatic microsomal protein (0.25 mg/ml) was
mixed with 50 mM Tris buffer, pH 7.6, and 1.0 mM NADPH. After preincubation for 2 min, the reactions were initiated by the addition of bupropion (300 μM; Sigma-Aldrich Canada Ltd.), making the final volume 250 μl. Incubations were for 20 min at 37°C, and sodium carbonate (100 μl, 20% v/v) was added to stop the reaction. Timolol (25 μl, 100 μg/ml) was added as an internal standard, and the mixture was vortexed and spun at 16,500 g for 20 min. A 100-μl aliquot of each sample was analyzed by HPLC with UV detection (210 nm). Separation of hydroxybupropion was achieved by using a ZORBAX Bonus-RP column (250 × 4.6 mm, 5 μM, Agilent Technologies) and a mobile phase consisting of methanol and 0.05 M phosphate buffer (pH 5.8, 45:55 v/v). The separation was performed with a flow rate of 1 ml/min. The concentration of hydroxybupropion was determined from a standard curve. The quantitation limit for hydroxybupropion was 10 ng/ml.

**Isolation, cDNA Synthesis, and mRNA Quantification.** Liver tissue (50–100 mg) was homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA), and total RNA was isolated according to the TRIzol reagent protocol. RNA concentrations were determined spectrophotometrically, and total RNA integrity was confirmed by electrophoresis on a 1.2% agarose gel (Onbio, Inc., Richmond Hill, ON, Canada) stained with ethidium bromide. Total mRNA from one animal from the nicotine-only group was not included in our analysis because of poor RNA integrity. cDNA was synthesized by using 1 μg of total RNA, random hexamers (Invitrogen), RiboLock RNAase inhibitor (Fermentas, Burlington, ON, Canada), and Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to protocols provided by Invitrogen. Primers for real-time PCR amplification of CYP2A6, CYP2B6, and β-actin were as follows: CYP2A6 forward primer (CYP2A6ex1), 5'-CTGGCAAGCAGAGGA-CAGCAA-3'; CYP2A6 reverse primer (CYP2A6ex2), 5'-GGCTC- CCTG AGCGGATCATA-3'; CYP2B6 forward primer (CYP2B6ex2), 5'-ATTGGGGAGGCTCTGTTCA-3'; CYP2B6 reverse primer (CYP2B6ex3), 5'-ATCGACGACATGGATCTCC-3'; β-actin forward primer (ACTBex3), 5'-CAG AGC AGA AGA GGC ATC CT-3'; and β-actin reverse primer (ACTBex4), 5'-GGT CTC AAA CAT GAT CTG GGT C-3'. The sequences for AGM CYP2A6 and CYP2B6 are not known; primer specificity was based on the nicotine-only group and CYP2A6 and CYP2B6 sequences from human and rhesus macaque (Macaca mulatta). Amplification and fluorescent detection were performed by using the Applied Biosystems Viia7 Real-Time PCR system (Invitrogen). The real-time PCR amplification mixture (20 μl) contained 1 μl of synthesized cDNA, 10 μl of 2× Fast SYBR-Green Mix (Invitrogen), and 0.3 μM concentrations of each primer. Cycling conditions consisted of an initial activation of AmpliTaq Fast DNA polymerase followed by 40 cycles of denaturing (95°C for 15 s) and annealing/extension (58°C for 20 s). Dilutions of monkey and human cDNA were used to determine the range of log-linear detection. CYP2A6 and CYP2B6 mRNA levels were normalized to β-actin mRNA by using the comparative Ct method for relative quantification as described by the manufacturer (Real-Time PCR Chemistry Guide; Invitrogen).

**Data Analysis.** In vivo pharmacokinetics were derived by using noncompartmental analysis. Elimination half-life was estimated by the terminal slope of the concentration versus time curve. The area under the concentration curve from 0 to 6 h (AUC_0–6h) was calculated by using the trapezoidal rule and the area under the concentration curve from 0 to infinity (AUC_0–∞) was calculated by using the equation: AUC_0–∞ = AUC_0–6h + C_0/k, where C_0 is the concentration at 6 h after nicotine dosing and k is the elimination rate constant. Apparent volume of distribution was estimated by using the equation: [nicotine dose/AUC_0–∞] × 1/k. The effect of nicotine and ethanol on hepatic CYP2A6 and CYP2B6 protein, in vitro activity, and mRNA were assessed by using a two-way ANOVA (nicotine treatment × ethanol self-administration), followed by a one-way ANOVA and least significant difference post hoc test. Paired Student’s t tests were used to assess differences in in vivo nicotine and cotinine pharmacokinetic parameters measured before and after drug administration. Statistical significance was set at p < 0.05.

**Results**

**Monkeys Voluntarily Self-Administered Alcohol.** The amount of alcohol consumed by the monkeys in this study has been described previously (Ferguson et al., 2011). In brief, average daily alcohol consumption during the 4-h alcohol access session was 38.12 ± 7.8 ml/kg (3.0 g ethanol/kg). There was no significant difference in mean ethanol consumption between the ethanol-only and the ethanol + nicotine groups, indicating no effect of nicotine treatment on voluntary alcohol consumption (Ferguson et al., 2011).

**Nicotine and Ethanol Alter Hepatic Levels of CYP2A6 and CYP2B6, Respectively.** There was a significant main effect of nicotine treatment on hepatic CYP2A6 protein (F_1,36 = 23.73; p < 0.001; Fig. 2A), in vitro activity (F_1,36 = 27.18; p < 0.001; Fig. 2C), and mRNA levels (F_1,35 = 13.2; p < 0.001; Fig. 2E). Compared with the control group, monkeys in the nicotine-only and nicotine + ethanol groups had a 40% (p < 0.01) and 47% (p < 0.05) decrease in hepatic CYP2A6 protein, respectively (Fig. 2A). CYP2A6 protein levels in the two nicotine-treated groups were also significantly lower compared with the ethanol-only group (Fig. 2A). Hepatic CYP2A6 in vitro activity, measured by the rate of coumarin metabolism to 7-OH-coumarin in monkey liver microsomes, showed a pattern of reduction similar to the CYP2A6 protein levels, with a 55% (p < 0.05) and 35% (p < 0.05) decrease in activity in the nicotine-only and ethanol + nicotine groups, respectively (Fig. 2C). Heparic CYP2A6 mRNA was reduced by 46% (p < 0.01) in the nicotine-only group and 37% (p < 0.05) in the nicotine + ethanol group compared with the control group (Fig. 2E). Ethanol self-administration did not significantly affect hepatic CYP2A6 protein (F_1,36 = 0.02; p = 0.9; Fig. 2A), activity (F_1,36 = 0.16; p = 0.76; Fig. 2C), or mRNA (F_1,35 = 0.16; p = 0.17; Fig. 2E) levels. There was no interaction effect between ethanol and nicotine on hepatic CYP2A6 protein (F_1,36 = 0.39; p = 0.89; Fig. 2A) or mRNA (F_1,35 = 1.93; p = 0.17; Fig. 2E) levels. However, a significant drug interaction on CYP2A6 in vitro activity was observed (F_1,36 = 13.02; p < 0.01; Fig. 2C). This may be a spurious finding, because post hoc testing revealed no significant difference in vitro CYP2A6 activity between the nicotine-only group and the ethanol + nicotine group.

There was also significant main effect of ethanol self-administration on hepatic CYP2B6 protein (F_1,36 = 18.81; p < 0.001; Fig. 2B) and in vitro (F_1,36 = 12.16; p = 0.001; Fig. 2D) activity. Compared with the control group, monkeys in the ethanol-only and the ethanol + nicotine groups had a 1.96-fold (p < 0.01) and 1.73-fold (p < 0.05) increase in hepatic CYP2B6 protein, respectively. CYP2B6 protein levels in the ethanol-consuming groups were also significantly higher compared with the nicotine-only group (Fig. 2B). Hepatic CYP2B2 activity, measured by the rate of bupropion metabolism to hydroxybupropion in monkey liver microsomes, showed a pattern of increase similar to the CYP2B6 protein levels, with a 1.26-fold (p > 0.05) and 1.51-fold (p < 0.05) increase in activity in the ethanol-only and ethanol + nicotine groups, respectively. Ethanol self-administration did not significantly affect CYP2B6 mRNA levels in the liver (F_1,35 = 0.13, p = 0.7; Fig. 2F). As a positive control for transcriptional induction of CYP2B6, we used hepatic mRNA from AGMs treated with phenobarbital, a known inducer of CYP2B6 mRNA and protein (Lee et al., 2006). A 6.5-fold increase in mRNA...
Nicotine Treatment but Unaffected by Ethanol Self-Administration. The rate of NCO in monkey liver microsomes was assessed at two nicotine concentrations: 30 \( \mu \text{M} \) (\( \sim K_m \) for NCO) and 300 \( \mu \text{M} \) (\( \sim V_{\text{max}} \) for NCO) (Schoedel et al., 2003). At the 30 \( \mu \text{M} \) concentration, there was a significant main effect of nicotine treatment on the rate of in vitro NCO (\( F_{1,36} = 14.08; p < 0.001 \)), with a 47\% (\( p < 0.05 \)) decrease in the nicotine-only group and a 32\% (\( p > 0.05 \)) decrease in the nicotine + ethanol group compared with the control group (Fig. 3A). Likewise, at the 300 \( \mu \text{M} \) substrate concentration, there was also a significant effect of nicotine on the rate of in vitro NCO (\( F_{1,36} = 11.12; p < 0.001 \)), with a 42\% (\( p < 0.05 \)) decrease in the nicotine-only group and a 20\% (\( p > 0.05 \)) decrease in the nicotine + ethanol group compared with the ethanol-only group.
Nicotine C-Oxidation Is Mediated Primarily by CYP2A6 in Monkey Liver. Pilocarpine, a selective chemical inhibitor of CYP2A6, strongly inhibited NCO in monkey liver microsomes from all four study groups to a similar extent. At 30 μM nicotine (≈ K_m for NCO), a concentration of pilocarpine equivalent to K_i decreased NCO by 68 to 74%, and a concentration 10 times higher than K_i decreased NCO by approximately 94% (Fig. 4). Results were similar at 300 μM nicotine (≈ V_max for NCO) with 36 to 44% inhibition at a concentration of pilocarpine equivalent to K_i and 75 to 79% NCO inhibition at 10 times K_i (Supplemental Fig. 2). The selective CYP2B6 inhibitor C8-xanthate used at a concentration equivalent to K_i had little effect on NCO at 30 or 300 μM nicotine in monkey liver microsomes from all four groups. At 10 times K_i C8-xanthate decreased NCO by 35 to 38% at 30 μM nicotine (Fig. 4) and 18 to 27% at 300 μM nicotine (Supplemental Fig. 2). Quinidine, a selective CYP2D6 inhibitor, was used as a negative control and did not inhibit NCO at any concentration of nicotine or inhibitor (Fig. 4; Supplemental Fig. 2).

There was a significant positive correlation between hepatic CYP2A6 protein levels and in vitro NCO at nicotine concentrations at both 30 μM (r = 0.64, p < 0.001; Supplemental Fig. 3A) and 300 μM (r = 0.52, p < 0.001; Supplemental Fig. 3C). Hepatic CYP2B6 protein levels did not correlate with NCO at either 30 μM (R = -0.20, p = 0.13; Supplemental Fig. 3B) or 300 μM (R = -0.09, p = 0.3; Supplemental Fig. 3D) nicotine concentrations.

Ethanol and Nicotine Modify In Vivo Nicotine Pharmacokinetics. Like humans, monkeys have substantial interindividual variation in nicotine pharmacokinetics (Fig. 5, control day 22), hence a within-animal design was used to assess changes in in vivo nicotine disposition. After chronic nicotine treatment, monkeys in the nicotine-only group had a 1.39-fold increase in nicotine half-life (p < 0.01), a 1.50-fold increase in nicotine AUC_0–6h (p < 0.05), and a 1.66 increase in nicotine AUC_∞ (p < 0.05) (Table 1; Figs. 5 and 6). After chronic ethanol self-administration, monkeys in the ethanol-only group had a 72% reduction in the maximal concentration of nicotine (C_max) (p < 0.05), a 52% reduction in nicotine AUC_0–6h (p < 0.01), and a 1.96-fold increase in nicotine AUC_∞ (p < 0.001) (Figs. 5 and 6). There was also a significant positive correlation between hepatic CYP2B6 protein levels and nicotine AUC_0–6h and AUC_∞ (r = 0.82, p < 0.001; Fig. 3B) nicotine concentrations at both 30 μM nicotine (r = 0.99, p = 0.001; Fig. 3B) nicotine concentrations at both 30 μM nicotine (r = 0.99, p = 0.001; Fig. 3B) nicotine concentrations at both 30 μM nicotine (r = 0.99, p = 0.001; Fig. 3B) nicotine concentrations at both 30 μM nicotine (r = 0.99, p = 0.001; Fig. 3B) nicotine concentrations at both 30 μM nicotine (r = 0.99, p = 0.001; Fig. 3B) nicotine concentrations at both 30 μM nicotine (r = 0.99, p = 0.001; Fig. 3B).
in nicotine AUC\textsubscript{0–6h} (p < 0.05), and a 50% reduction in nicotine AUC\textsubscript{0–\infty} (p < 0.05), (Table 1; Figs. 5 and 6). Combined ethanol self-administration and nicotine treatment increased nicotine half-life by 1.26-fold and decreased the nicotine C\textsubscript{max} by 76% (p < 0.05), AUC\textsubscript{0–6h} by 62% (p < 0.01), and AUC\textsubscript{0–\infty} by 54% (p < 0.01) (Table 1; Figs. 5 and 6). Therefore, combined ethanol self-administration and nicotine treatment increased nicotine half-life, an effect that presumably is mediated by nicotine treatment, and decreased nicotine AUC and C\textsubscript{max}, an effect that seems to be mediated by ethanol. No significant changes in nicotine kinetic parameters were observed in the control group between pharmacokinetic challenge days (Table 1; Figs. 5 and 6).

After chronic nicotine treatment, monkeys in the nicotine-only group had a 2.48-fold increase in cotinine AUC (p < 0.01) and a 2.52-fold increase in cotinine C\textsubscript{max} (p < 0.01) (Table 2; Fig. 7). Likewise, combined ethanol self-administration and nicotine treatment increased cotinine AUC by 1.89-fold (p < 0.001) and increased cotinine C\textsubscript{max} by 1.82-fold (p < 0.01) (Table 2; Fig. 7). No significant changes in cotinine kinetic parameters were observed in the control or ethanol-only groups between pharmacokinetic challenge days (Table 2; Fig. 7). Therefore, the effect of combined ethanol and nicotine treatment on cotinine pharmacokinetics seems to be an effect mediated by nicotine treatment, not by ethanol.

**Discussion**

Nicotine treatment and/or ethanol exposure did not significantly change the relative proportion of NCO mediated by hepatic CYP2A6 and CYP2B6. For example, monkeys exposed to nicotine and ethanol in combination had approximately a 50% reduction in hepatic CYP2A6 protein and 2-fold increase in hepatic CYP2B6 protein. However, NCO in these animals was still mediated primarily by CYP2A6 with very little contribution from CYP2B6. These results argue against the concept that CYP2B6 plays a greater role in nicotine metabolism among individuals with reduced CYP2A6 activity (Ring et al., 2007). CYP2A6 protein was significantly correlated with NCO activity, whereas variability in CYP2B6 was not associated with changes in NCO. Similar results have also been shown in human liver microsomes (Al Koudsi and Tyndale, 2010; Al Koudsi et al., 2010), suggesting that
CYP2A6, but not CYP2B6, hepatic activity is an important factor influencing nicotine metabolism.

Nicotine treatment increased the plasma half-life of nicotine by approximately 1.3-fold in monkeys, suggesting that nicotine is the agent in cigarette smoke responsible for the quantitatively similar decrease in nicotine clearance (12–27%) during smoking compared with nonsmoking (Benowitz and Jacob, 1993, 2000). The total daily dose of nicotine administered to the monkeys (1.0 mg/kg/day) was similar to the average amount of nicotine acquired from smoking (0.2–1.1 mg/kg) (Benowitz and Jacob, 1984). This dose produced mean nicotine plasma levels of approximately 25 ng/ml for over 16 h/day in AGMs, which is within the range of nicotine plasma levels achieved by a smoker during waking hours (10–50 ng/ml) (Benowitz and Jacob, 1990). Our nicotine treatment paradigm was shown to down-regulate CYP2A6, leading to slower nicotine metabolism and decreased nicotine clearance in vivo. In contrast, the administration of a 42-mg transdermal nicotine patch twice a day for 10 days did not affect nicotine clearance in humans (Huukkanen et al., 2010). In that human study, the measurement of hepatic CYP2A6 mRNA and protein was not feasible, and the impact of nicotine on in vivo CYP2A6 activity was not assessed. Our study, however, was specifically designed to concurrently investigate the impact of nicotine treatment on CYP2A6 levels, nicotine metabolism, and in vivo nicotine disposition.

Nicotine treatment also had a significant impact on cotinine pharmacokinetics, resulting in an increased plasma cotinine AUC after nicotine administration. Cotinine is metabolized to its major metabolite trans-3-OH-cotinine in a reaction mediated exclusively by CYP2A6 (Nakajima et al., 1996). Although nicotine and cotinine both are metabolized by hepatic CYP2A6, cotinine has a much lower hepatic extraction ratio; thus, changes in the level of hepatic CYP2A6 will have a greater impact on cotinine clearance compared with nicotine clearance (Huukkanen et al., 2005). Therefore, it was expected that the down-regulation of hepatic CYP2A6 would decrease the rate of cotinine metabolism to a greater extent than the rate of cotinine formation, resulting in an overall increase in cotinine levels.

The daily amount of alcohol self-administered by the monkeys was comparable with moderate human alcohol consumption (Ferguson et al., 2011). At this level of intake, ethanol did not affect hepatic CYP2A6 protein levels or activity. Our findings are consistent with a human study that showed no association between the level of alcohol intake and in vivo CYP2A6 activity in a population of moderate alcohol consumers (Mwenifumbo et al., 2007). It has been proposed that hepatic CYP2A6 may be induced in response to ethanol-mediated oxidative stress and/or liver damage (Lu et al., 2011). Treatment with 100 mM ethanol induced CYP2A6 in a human monocytic cell line, and this induction was blocked by pretreatment with the antioxidant vitamin C, suggesting a role for oxidative stress in the regulation of CYP2A6 (Jin et al., 2012). This mechanism may contribute to the elevated levels of CYP2A6 found in the livers of alcoholics compared with nonalcoholics (Niemela¨ et al., 2000). However, moderate alcohol consumption, which was modeled in our study, did not induce CYP2A6 mRNA, protein levels, or activity.

Ethanol induced hepatic CYP2B6 protein and activity in AGMs, suggesting that ethanol exposure is responsible, at least in part, for the higher levels of CYP2B6 in livers from alcoholics compared with nonalcoholics (Hesse et al., 2004). Hepatic CYP2B6 mRNA was not significantly altered by ethanol, indicating a nontranscriptional mechanism of induction at this dose and duration of ethanol exposure. In rats, both transcriptional and nontranscriptional mechanisms for the induction of CYP2B6 by ethanol have been reported previously (Namjji et al., 1994; Schoedel et al., 2001).

The induction of CYP2B6 by ethanol did not significantly alter in vitro NOC; however, chronic ethanol self-administration substantially decreased nicotine Cmax and AUC by more than 50%. Postethanol in vivo nicotine pharmacokinetic testing was performed 24 h after the last ethanol self-administration session, at which point blood ethanol levels would be negligible, based on an average ethanol elimination rate of 40 to 62 mg%/h in AGMs (Grant and Bennett, 2003). Therefore, the changes in nicotine pharmacokinetic parameters observed in the ethanol-exposed monkeys were not likely to be caused by the acute effects of ethanol. This is consistent with human studies showing no effect of short-term ethanol exposure on nicotine pharmacokinetics (Benowitz et al., 1986). We speculate that chronic ethanol exposure may affect the distribution kinetics of nicotine. Monkeys that self-adminis-

**Fig. 7.** The cotinine concentration versus time curves after a 0.1 mg/kg s.c. nicotine injection for monkeys pretreatment (day 22) and post-treatment (day 50). Each point represents the mean cotinine concentration for 10 monkeys.
tered ethanol had a noticeable, but nonsignificant, increase in nicotine plasma levels compared with those who do not (Withkiewitz et al., 2012). Nicotine and ethanol seem to have opposing effects on nicotine pharmacokinetics, but when nicotine and ethanol are presented in combination, the effect of ethanol on nicotine plasma levels dominates. Thus, it is important that researchers and clinicians are aware that smokers who regularly consume alcohol may have lower plasma nicotine levels compared with smokers who have the same nicotine intake but rarely consume alcohol. This may also have implications for the efficacy of nicotine replacement therapies.

Both CYP2A6 and CYP2B6 metabolize a variety of compounds other than nicotine; these include clinical drugs, toxins, procarcinogens, drugs of abuse, and endogenous molecules (Honkakoski and Negishi, 1997; Mo et al., 2009). The down-regulation of CYP2A6 by nicotine and the induction of CYP2B6 by ethanol in humans could potentially alter the efficacy of clinical drugs, the susceptibility to chemical toxicity and carcinogenesis, and vulnerability to drug abuse.

In conclusion, our findings demonstrate that nicotine treatment can decrease hepatic CYP2A6 levels, ethanol self-administration can induce hepatic CYP2B6 levels, and combined nicotine and ethanol exposure can alter levels of both enzymes, respectively. In addition, our results suggest that chronic exposure to nicotine and ethanol, either alone or in combination, can modify in vivo nicotine pharmacokinetics in humans.

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Authorship Contributions
Participated in research design: Ferguson, Miksys, Palmour, and Tyndale.

Conducted experiments: Ferguson, Miksys, and Palmour.

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Erdos E, Albericio F, and Tyndale RF (2010) Nicotine down-regulated its own CYP2A6-mediated metabolism and decreased in vivo nicotine clearance, a paradoxical effect. Chronic ethanol consumption substantially decreased the nicotine plasma levels achieved after nicotine administration, an effect that cannot be attributed to altered CYP2A6, CYP2B6, or nicotine metabolism. The lower nicotine plasma levels achieved after ethanol exposure may contribute to the greater number of cigarettes/day smoked by individuals who regularly consume alcohol compared with those who do not (Withkiewitz et al., 2012). Nicotine and ethanol seem to have opposing effects on nicotine pharmacokinetics, but when nicotine and ethanol are presented in combination the effect of ethanol on nicotine plasma levels dominates. Thus, it is important that researchers and clinicians are aware that smokers who regularly consume alcohol may have lower plasma nicotine levels compared with smokers who have the same nicotine intake but rarely consume alcohol. This may also have implications for the efficacy of nicotine replacement therapies.

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