

# Down-Regulation of Hepatic Nicotine Metabolism and a CYP2A6-Like Enzyme in African Green Monkeys after Long-Term Nicotine Administration

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## ABSTRACT

Nicotine metabolism is decreased in smokers compared with nonsmokers, but the mechanism(s) responsible for the slower metabolism are unknown. Nicotine is inactivated to cotinine by CYP2A6 in human liver [nicotine C-oxidation (NCO)]. CYP2B6 also metabolizes nicotine to cotinine but with lower affinity than CYP2A6. To evaluate the effects of long-term nicotine treatment on hepatic levels of CYP2A6 and CYP2B6, and nicotine metabolism, an African green monkey (AGM) model was developed. As in humans, approximately 80 to 90% of in vitro hepatic NCO is mediated by a CYP2A6-like protein (CYP2A6agm) in this species, as determined by inhibition studies. Male AGM ( $n = 6$  per group) were treated for 3 weeks with nicotine (s.c., 0.3 mg/kg, b.i.d.), phenobarbital (oral, 20 mg/kg, as a positive control for P450 induction), and/or saline (s.c., b.i.d.). Immunoblotting demonstrated a 59% decrease ( $p < 0.05$ )

in hepatic CYP2A6agm protein in nicotine-treated animals. A CYP2B6-like protein (CYP2B6agm) was modestly and insignificantly decreased (14%,  $p = 0.11$ ). In vitro NCO was decreased by 41% in the nicotine-treated group ( $p < 0.05$ ), mediated by a decrease in CYP2A6agm, as demonstrated using inhibitory antibodies. CYP2A6agm mRNA (33%,  $P \leq 0.05$ ) and CYP2B6agm (35%,  $p < 0.01$ ) mRNA were also significantly decreased in the nicotine-treated group. Phenobarbital-treated animals demonstrated an increase in CYP2B6agm (650%,  $p < 0.001$ ), but not CYP2A6agm (20%,  $p = 0.49$ ). NCO was increased in the phenobarbital-treated group (55%,  $p < 0.05$ ) by an increase in CYP2B6agm-mediated NCO. Consistent with the slower nicotine metabolism observed in smokers, nicotine may decrease its own metabolism in primates by decreasing the expression of the primary nicotine-metabolizing enzyme CYP2A6.

In vitro, CYP2A6 has been found to be the principle hepatic enzyme responsible for nicotine's inactivation to cotinine in humans (80–90%) (Nakajima et al., 1996; Messina et al., 1997). In vivo, approximately 70 to 80% of nicotine is metabolized to cotinine (Benowitz et al., 1994); persons with genetically inactive CYP2A6 produce little or no cotinine (Benowitz et al., 2001). Together, these findings indicate that CYP2A6 is the primary enzyme, in vitro and in vivo, that inactivates nicotine to cotinine. CYP2B6 may also metabolize nicotine to cotinine, but it has lower affinity and variable expression in human liver (Yamazaki et al., 1999).

Nicotine is responsible for the reinforcing effects of cigarette smoking (Benowitz, 1988). Because dependent smokers regulate their tobacco consumption to maintain desirable plasma nicotine concentrations (McMorrow and Foxx, 1983), variations in rates of nicotine metabolism could influence individual smoking behaviors (Tyndale and Sellers, 2001). Our group (unpublished data) and others have found that nicotine metabolism is decreased in smokers versus nonsmokers (Benowitz and Jacob, 1993, 2000). Smokers also had slower nicotine clearance after an overnight abstinence period compared with a 7-day abstinence period (Lee et al., 1987). These studies administered deuterium-labeled (*S*)-nicotine (*d*2-nicotine) to distinguish it from nicotine inhaled from cigarette smoke and environmental sources. Other studies found increased nicotine clearance and/or decreased

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**ABBREVIATIONS:** AGM, African green monkey; P450, cytochrome P450; NCO, nicotine C-oxidation; kb, kilobase pair(s).

nicotine terminal half-life in smokers versus nonsmokers (Kyerematen et al., 1982, 1990). These studies administered very low doses of racemic nicotine (2.4–2.7  $\mu\text{g/kg}$ ). It is possible that at the lower doses, nicotine might not be present at concentrations observed in smokers (Benowitz and Jacob, 1993), making small changes in clearance difficult to detect. Using a within-subject design, Benowitz and Jacob (2000) found a significant decrease in nicotine clearance during the cigarette-smoking phase, compared with the placebo and carbon monoxide phases of the study (Benowitz and Jacob, 2000). Given the relatively large interindividual differences in nicotine kinetics (Benowitz et al., 1997), this type of study design has increased power to detect changes in nicotine metabolism during smoking and nonsmoking conditions.

The factor(s) responsible for reduced nicotine metabolism during smoking have not been identified. One possibility is that constituents of tobacco smoke inhibit nicotine metabolism. There are many compounds in tobacco smoke that could alter drug-metabolism; many of these factors, however, may not be present in concentrations high enough to alter nicotine metabolism. Two compounds with higher concentrations, carbon monoxide (from cigarette smoke) and cotinine (the primary metabolite of tobacco-derived nicotine), do not inhibit nicotine metabolism in vivo (Zevin et al., 1997; Benowitz and Jacob, 2000). A second possibility is that compound(s) in cigarette smoke, such as nicotine, down-regulate CYP2A6, resulting in slower nicotine metabolism. Nicotine has been shown to increase hepatic and respiratory CYP1A1/2 (Iba et al., 1999), hepatic CYP2E1 (Howard et al., 2001), and brain CYP2B1 (Miksys et al., 2000). The effects of nicotine on hepatic CYP2A6 expression have not yet been studied.

Rodent CYP2A enzymes differ from their human counterparts in terms of substrate selectivity and regulation (Sharer et al., 1995). Rat hepatic CYP2A enzymes do not appreciably metabolize nicotine; the conversion of nicotine to cotinine is one of several nicotine metabolic pathways and is probably mediated primarily by CYP2B1/2 (Kyerematen et al., 1988; Nakayama et al., 1993). Therefore, rats may not be an ideal model for human nicotine metabolism and regulation of CYP2A6.

Nonhuman primates may provide a better model for human CYP2A6-mediated metabolism of substrates such as nicotine. Coumarin 7-hydroxylation is a specific probe for human CYP2A6 activity (Pelkonen et al., 2000). In vitro, this pathway is similar between humans and African green monkeys (AGM) ( $K_m$  of 2.1  $\mu\text{M}$  and  $V_{\text{max}}$  of 0.79 nmol/mg of protein/min in humans compared with  $K_m$  of 2.7  $\mu\text{M}$  and  $V_{\text{max}}$  of 0.52 nmol/mg of protein/min in AGM) (Li et al., 1997). This CYP2A6-mediated pathway is also similar between cynomolgus monkeys and humans in terms of proportions and rates (Pearce et al., 1992). In vivo, the terminal half-life of nicotine in macaque monkeys is 1.6 h compared with 1.4 h in humans (Seaton et al., 1991).

Although a CYP2A enzyme has not been cloned from a nonhuman primate species, other nonhuman primate P450s demonstrate 88 to 94% nucleotide and/or amino acid sequence identity with their human counterparts (CYP3A, CYP2D, CYP1A in marmoset, and CYP2B in rhesus monkey) (Igarashi et al., 1997; Ohmori et al., 1998). Given the similarities between human and nonhuman primate CYP2A metabolic activity and the high degree of sequence identity generally found among primate P450s, nonhuman primate

species may be more suitable for the study of nicotine metabolism and of CYP2A6 activity and regulation.

Given the observations that nicotine metabolism is reduced in smokers and that nicotine is known to alter P450 expression, we hypothesized that long-term nicotine use itself could down-regulate its own metabolism. The purpose of this study was to test the effects of long-term nicotine treatment on nicotine metabolism and the expression of a CYP2A6-like (CYP2A6agm) and a CYP2B6-like enzyme (CYP2B6agm). We developed an African green monkey model for this purpose.

## Materials and Methods

**Materials.** Chemiluminescence Blotting Substrate was purchased from Roche Diagnostics (Laval, PQ, Canada). Recombinant baculovirus-expressed human CYP2B6 and CYP2A6 Supersomes, human selective Western blotting antibodies to CYP2A6 and CYP2B6, and human selective inhibitory antibodies to CYP2A6, CYP2B6, CYP2E1, CYP3A4, CYP2C, and CYP1A1/2 were purchased from Gentest Corporation (Woburn, MA). Protran nitrocellulose membranes were purchased from Schleicher and Schuell Inc. (Keene, NH). Biotinylated anti-mouse IgG secondary antibody was purchased from Vector Laboratories Inc. (Burlington, ON, Canada) and Neutravidin-conjugated horseradish peroxidase was purchased from Pierce Chemical Company (Rockford, IL). Protein assay kit, prestained molecular markers, and Zeta-Probe nylon membrane were purchased from Bio-Rad Laboratories (Hercules, CA). Strataprep Total RNA Mini-prep kit was purchased from Stratagene (La Jolla, CA). Potassium octylxanthate (C8 xanthate) was custom synthesized by Toronto Research Chemicals (Toronto, ON, Canada). 5-Methylnicotine was generously provided by Peyton Jacob III (University of California, San Francisco, CA). Nicotine bitartrate, 8-methoxypsoralen, and other chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Human CYP2A6, CYP2B6, CYP2D6, CYP2E1, and CYP3A4 plasmid cDNAs were kindly provided by Frank Gonzalez (National Institutes of Health, Bethesda, MD). Human liver (K19) was generously provided by Ted Inaba (University of Toronto, Toronto, ON, Canada). Untreated AGM liver (AG44) was obtained as described previously (Li et al., 1997).

**Animals.** Subjects were 18 young male African green monkeys (*Chlorocebus aethiops*) housed at Behavioural Sciences Foundation, St. Kitts; each treatment group comprised 6 animals. Long-term treatment with nicotine bitartrate (mg base in saline, pH 7.4) was given at 0.05 mg/kg (s.c., b.i.d.) for 2 days, 0.15 mg/kg (s.c., b.i.d.) for 2 days followed by 0.3 mg/kg (s.c., b.i.d.) for 18 days. Phenobarbital at 20 mg/kg was given once a day in the morning in sweetened water, as a positive control for CYP2B and 2A regulation. The saline and phenobarbital groups received sham nicotine injections (saline, s.c., b.i.d.). The saline and nicotine groups also received sham phenobarbital drinks (50 ml of sweetened water) once a day. Animals were fed throughout with normal rations of Purina monkey chow, supplemented with fresh fruit and produce, and fresh drinking water was available ad libitum. Body weight did not decrease as a consequence of the experimental regimen. All animals were sacrificed on day 22 under ketamine anesthesia, 6 h after the morning drug treatment. Organs were immediately dissected, flash frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until further use. The experimental protocol was reviewed and approved by the Institutional Review Board of Behavioural Sciences Foundation, as well as the University of Toronto Animal Care Committee. All procedures were conducted according to the guidelines of the Canadian Council on Animal Care.

**Membrane Preparations.** Microsomes were prepared from AGM livers for the in vitro nicotine metabolism assay as described previously for human nicotine metabolism (Messina et al., 1997), aliquoted into small volumes, and stored at  $-80^\circ\text{C}$  in 1.15% KCl. The

cytosolic fractions from rat livers were used as a source of aldehyde oxidase. For immunoblotting, membranes were prepared in the same way but stored in 100 mM Tris, pH 7.4, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1.15% (w/v) KCl, and 20% (v/v) glycerol. Protein concentrations were determined according to manufacturer's instructions (Bio-Rad).

**Immunoblotting.** To determine the linear range of detection for the assay, AGM liver microsomes were diluted serially and used to construct standard curves (1.25 to 10  $\mu$ g for CYP2B6agm and 3.25 to 50  $\mu$ g for CYP2A6agm). Standard curves of Sf-9 cDNA-expressed human CYP2A6 and CYP2B6 were also generated. Membrane proteins from livers (3  $\mu$ g for CYP2B6agm, 15  $\mu$ g for CYP2A6agm) were separated by SDS-polyacrylamide gel electrophoresis (4% stacking and 8% separating gels), and transferred overnight onto nitrocellulose membranes. Sf-9 cDNA-expressed human CYP2A6 and CYP2B6 were used as standards. For detection of CYP2A6, nitrocellulose membranes were preincubated for 1.5 h in a blocking solution containing 1% skim milk powder (w/v), and 0.1% bovine serum albumin (w/v) in Tris-buffered saline-Triton X-100 [50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% (v/v) Triton X-100]. Membranes were probed with a monoclonal antibody to human CYP2A6 (1:2000 dilution), a biotinylated anti-mouse secondary antibody (1:3000 dilution), followed by incubation with a tertiary Neutravidin-conjugated horseradish peroxidase (1:80,000 dilution). For detection of CYP2B6, nitrocellulose membranes were preincubated in 0.5% skim milk, and 0.1% bovine serum albumin in TBST. Membranes were then incubated with rabbit polyclonal human-selective CYP2B6 primary antibody (1:750), a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:500), followed by detection using enhanced chemiluminescence. Controls included immunoblots that were incubated without primary antibody. Nitrocellulose membranes were exposed to Kodak X-OMAT-AR film (Eastman Kodak, Rochester, NY) for 0.5 to 2 min. Immunoblots were analyzed using an imaging system (Imaging Research Inc., St. Catharines, ON, Canada).

**Nicotine C-Oxidation Assay.** NCO activity was measured according to the method of Messina et al. (1997) with minor modifications. Briefly, incubation mixtures contained 1 mM NADPH and 20  $\mu$ l of rat liver cytosol (a source of aldehyde oxidase) in Tris-HCl buffer, pH 7.4. Incubations were carried out at 37°C. The reaction was stopped, 5-methylnicotine (50  $\mu$ l of 2  $\mu$ g/ml) was added as the internal standard, samples were extracted with 3 ml of dichloromethane, and the organic phase was dried under nitrogen. Samples were reconstituted with 200  $\mu$ l of 0.01 M HCl and 50  $\mu$ l of each sample was subjected to HPLC analysis with a UV detector (set at 260 nm). Separation of nicotine and cotinine was achieved using a Supercosil LC-8DB column (5 mm, 150  $\times$  4.6 mm; Supelco, Bellefonte, PA) and a mobile phase consisting of acetonitrile/potassium phosphate buffer [10:90 (v/v), pH 4.6] containing 1 mM octanesulfonic acid and 0.5% triethylamine. The separation was performed with isocratic elution at a flow rate of 1 ml/min. Cotinine concentrations were determined by extrapolation from a standard dilution (0.0875 to 5.6 nmol of cotinine).

Protein and time course studies were performed at 30 and 300  $\mu$ M nicotine to establish assay linearity. From these studies, a protein concentration of 50  $\mu$ g and an incubation time of 20 min were selected for all subsequent assays, because cotinine formation at these points was within the linear portions of the protein concentration-time curves for both substrate concentrations. There was no cotinine formation when NADPH was excluded from the reaction mixture or when microsomal protein was heat-denatured. The amount of rat aldehyde oxidase was not rate-limiting for the conversion of the nicotine iminium ion to cotinine. There was some (44%) cotinine formation in the absence of rat cytosol, indicating residual aldehyde oxidase in liver microsomes, and/or spontaneous formation of cotinine from the enzymatically formed nicotine iminium ion.

**Characterization of NCO in AGM.** The following antibody and chemical inhibition studies were performed using liver microsomes from an untreated male AGM (AG44) whose coumarin (Li et al.,

1997) and nicotine kinetics had been previously assessed. Subsequent metabolic studies were performed accordingly at 30 and 300  $\mu$ M nicotine, approximately equal to  $K_m$  and to  $V_{max}$  ( $10 \times K_m$ ) for NCO.

Anti-human selective P450 antibodies were preincubated with the reaction mixtures according to manufacturer's instruction as follows; 30 min at room temperature for anti-CYP2C and anti-CYP1A1/2, or 15 min on ice for anti-CYP2A6, anti-CYP2B6, anti-CYP2E1, anti-CYP2D6, and anti-CYP3A4. Antibody concentrations were as follows; 2.5, 5, 10, and 20  $\mu$ l for anti-CYP2C, 10 and 20  $\mu$ l for anti-CYP1A1/2, 1 and 5  $\mu$ l for anti-CYP2E1, anti-CYP2D6 and anti-CYP3A4, and 0.5, 1, 2.5 and 5  $\mu$ l for anti-CYP2A6 and anti-CYP2B6 according to manufacturer's instructions. Incubations with preimmune serum (5 and 20  $\mu$ l) were also included, and all results were compared with NCO without preincubation with antibodies.

Various P450-selective chemical inhibitors were dissolved in methanol, and evaporated before addition of other reaction components (coumarin, methoxsalen, quinidine, budipine, ketoconazole, sulfaphenazole,  $\alpha$ -naphthoflavone, and aniline), or dissolved in water (pilocarpine, C8 xanthate, and DDC). Reaction mixtures were preincubated for 15 min with chemical inhibitors; concentrations were approximately equal to  $K_i$  and 10-fold higher than the  $K_i$  for the target P450 (indicated below in brackets). The concentrations that were used are as follows: coumarin (CYP2A6, 2.5 and 25  $\mu$ M) (Li et al., 1997), methoxsalen (CYP2A6, 0.5 and 5  $\mu$ M) (Zhang et al., 2001), pilocarpine (CYP2A6, 4 and 40  $\mu$ M) (Bourrie et al., 1996), C8 xanthate (CYP2B6, 1 and 10  $\mu$ M) (Yanev et al., 1999), quinidine (CYP2D6, 0.4 and 4  $\mu$ M) (Bourrie et al., 1996), budipine (CYP2D6, 0.5 and 5  $\mu$ M) (Ramamoorthy et al., 2001), ketoconazole (CYP3A4, 0.015 and 0.15  $\mu$ M) (Bourrie et al., 1996), sulfaphenazole (CYP2C9, 0.3 and 3  $\mu$ M) (Bourrie et al., 1996),  $\alpha$ -naphthoflavone (CYP1A1/2, 0.01 and 0.1  $\mu$ M) (Bourrie et al., 1996), aniline (CYP2E1, 100  $\mu$ M and 1 mM) (Bourrie et al., 1996), and DDC (CYP2E1, 110  $\mu$ M and 1.1 mM) (Eagling et al., 1998). Chemically inhibited NCO was compared with reactions performed in the absence of chemical inhibitors.

**Hepatic NCO in Saline-, Nicotine-, and Phenobarbital-Treated AGM.** Reaction mixtures containing liver microsomes from saline- ( $n = 6$ ), nicotine- ( $n = 6$ ), or phenobarbital-treated ( $n = 6$ ) AGM were preincubated for 15 min on ice with human selective anti-CYP2A6 or anti-CYP2B6 antibodies or buffer before the addition of 30  $\mu$ M nicotine; cotinine formation was assessed as described above.

**RNA Northern Blot Analysis.** Total RNA was isolated using guanidinium thiocyanate according to manufacturer's instructions (Strataprep Total RNA mini-prep kit). Total RNA (5  $\mu$ g for CYP2B6 and 10  $\mu$ g for CYP2A6) was loaded into formaldehyde gels (1.2% agarose), electrophoresed, and transferred overnight by capillary action onto nylon membranes (Zeta-Probe). RNA was fixed by UV cross-linking, followed by baking for 1 h in an 80°C oven. Membranes were prehybridized for 1 h at 43°C in prehybridization buffer (50% formamide, 120 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.2, 7% SDS, and 250 mM NaCl). Membranes were then hybridized overnight at 43°C with human CYP2B6 (1.88 kb) or CYP2A6 (1.78 kb) [ $\alpha$ - $^{32}\text{P}$ ]dCTP-labeled cDNA probes, standardized to a total activity of  $1 \times 10^7$  cpm. Membranes were washed and exposed to X-OMAT-AR (Kodak) film for 2 to 12 days. Membranes were re-probed with [ $\alpha$ - $^{32}\text{P}$ ]dCTP-labeled human  $\beta$ -actin cDNA (500-base-pair fragment). This probe was obtained by polymerase chain reaction amplification from human liver genomic DNA using primers that have been described previously (Howard et al., 2001). Selectivity of CYP2A6 and CYP2B6 cDNA probes was tested using human cRNAs for various P450s (CYP2A6, CYP2B6, CYP2D6, CYP3A4, CYP2E1) transcribed in vitro from BlueScript BIIISK- expression plasmids containing P450 cDNAs using T7 or T3 RNA polymerases according to manufacturer's instructions (Promega in vitro transcription kit). Band sizes were determined using RNA markers (Sigma).

**Statistics.** Treatment groups were compared with saline using unpaired, two-sided Student's  $t$  tests. Treatment groups were con-



sidered to be significantly different from the saline treatment group if  $p \leq 0.05$ .

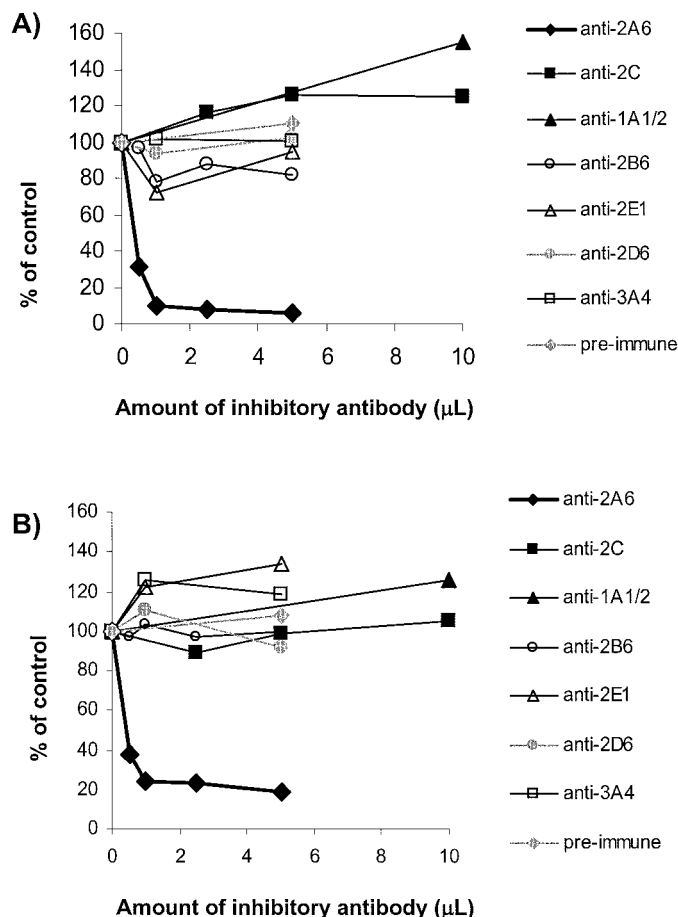
## Results

**NCO Is Mediated Primarily by CYP2A6agm in AGM Liver.** The  $K_m$  and  $V_{max}$  for NCO in liver microsomes from AG44 were  $24.1 \mu\text{M}$  and  $203.3 \text{ nmol/mg/h}$ , respectively.  $K_m$  values for this animal were similar to values obtained in liver microsomes from other AGM ( $29.1 \pm 8.6 \mu\text{M}$ ,  $n = 6$ ) (unpublished data). Selective anti-human CYP2A6 inhibitory antibodies strongly inhibited liver microsomal NCO from an untreated male AGM (AG44) (approximately 90%,  $30 \mu\text{M}$  nicotine, Fig. 1A). There was also a slight effect of anti-CYP2B6 (approximately 10–20% inhibition, across all antibody concentrations, Fig. 1A) at this substrate concentration. Although anti-CYP2E1 seems to have an effect at the lower concentration of antibody (Fig. 1A), this has returned to baseline at the higher antibody concentration. Anti-CYP2D6, anti-CYP3A4, anti-CYP2C, anti-CYP1A1/2, and preimmune serum did not inhibit NCO. Results were similar at  $300 \mu\text{M}$  nicotine ( $\approx V_{max}$ , Fig. 1B) with approximately 80% inhibition

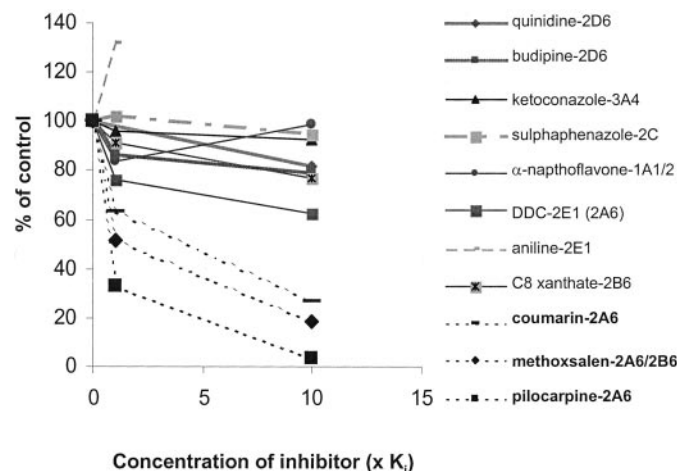
by anti-CYP2A6; no apparent effect of other anti-P450 inhibitory antibodies was observed. A primary role of CYP2A6agm in NCO was confirmed using various P450-selective chemical inhibitors (Fig. 2). At  $30 \mu\text{M}$  nicotine ( $\approx K_m$ ), inhibitors, at concentrations equivalent to the  $K_i$  of these compounds for human CYP2A6 (Bourrie et al., 1996; Li et al., 1997; Zhang et al., 2001), decreased NCO by approximately 50% (2.5  $\mu\text{M}$  coumarin, 37%; 0.5  $\mu\text{M}$  methoxsalen, 49%; and 4  $\mu\text{M}$  pilocarpine, 67%). At concentrations 10-fold higher than  $K_i$ , coumarin, methoxsalen, and pilocarpine inhibited NCO by 73, 81, and 97%, respectively. DDC also inhibited NCO; however, at these concentrations (110  $\mu\text{M}$  and 1.1 mM) DDC is not selective for human CYP2E1 but also inhibits human CYP2A6 and CYP2B6 (Chang et al., 1994). Aniline, another CYP2E1 inhibitor, had no inhibitory effect on cotinine formation (Fig. 2). Other chemical P450 inhibitors did not inhibit NCO even at concentrations approximately 10 times  $K_i$ ; quinidine (0.4  $\mu\text{M}$ ) and budipine (0.5  $\mu\text{M}$ ) for CYP2D6, ketoconazole (0.015 and 0.15  $\mu\text{M}$ ) for CYP3A4, sulfaphenazole (0.3 and 3  $\mu\text{M}$ ) for CYP2C9,  $\alpha$ -naphthoflavone (0.01 and 0.1  $\mu\text{M}$ ) for CYP1A1/2, and C8 xanthate (1 and 10  $\mu\text{M}$ ) for CYP2B6 (Fig. 2).

The above results were obtained using the liver microsomes from an untreated AGM (AG44). At  $30 \mu\text{M}$  nicotine, anti-CYP2A6 antibodies significantly inhibited liver microsomal NCO in the saline-treated group of AGM ( $n = 6$ ) by 82% ( $P = 0.001$ ). There was also a small nonsignificant inhibitory effect of anti-CYP2B6 on NCO in these liver microsomes (28% inhibition,  $P = 0.12$ ).

**Long-Term Nicotine Treatment Decreases CYP2A6agm in AGM Liver.** An immunoblotting assay was developed using a monoclonal antibody directed against human CYP2A6. A single band was detected in AGM liver microsomes that migrated slightly more slowly than the single band seen with human liver microsomes and cDNA-expressed CYP2A6 (Fig. 3A). Standard curves of human cDNA-expressed CYP2A6 were generated to estimate the quantity of CYP2A6agm. Assuming equivalent detection, approximate quantities were 0.013 pmol of CYP2A6/ $\mu\text{g}$  of protein and  $0.039 \pm 0.015$  pmol of CYP2A6agm/ $\mu\text{g}$  of protein for human



**Fig. 1.** Anti-CYP2A6 inhibits NCO in AGM liver microsomes. A, at  $30 \mu\text{M}$  nicotine, selective anti-human CYP2A6 antibodies inhibit NCO by 90% in liver microsomes from an untreated male AGM (AG44). Minor inhibition occurred with anti-CYP2B6 antibodies. B, at  $300 \mu\text{M}$  nicotine, approximately 80% of NCO is inhibited by anti-CYP2A6, whereas anti-CYP2B6 has no effect. All values are expressed as a percentage of NCO performed in the absence of antibodies. Anti-CYP2D6, anti-CYP2E1, anti-CYP1A1/2, anti-CYP3A4, and anti-CYP2C had no effect at either substrate concentration.



**Fig. 2.** Human CYP2A6 chemical inhibitors significantly inhibit NCO in AGM liver microsomes. At  $30 \mu\text{M}$  nicotine, CYP2A6 inhibitors [coumarin (2.5 and  $25 \mu\text{M}$ ), pilocarpine (4 and  $40 \mu\text{M}$ ), and methoxsalen (0.5 and  $5 \mu\text{M}$ )] inhibit cotinine formation by untreated male AGM (AG44) liver microsomes. Values are expressed as percentage change from NCO performed in the absence of chemical inhibitors.

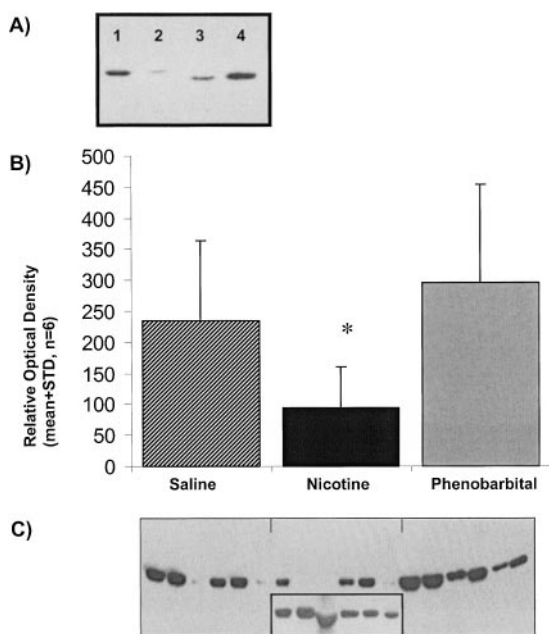
( $n = 1$ ; K20) and AGM ( $n = 7$ ; six saline-treated animals and one untreated animal) liver microsomes, respectively. This assay was used to investigate changes in the expression of CYP2A6agm in the livers of AGM ( $n = 6$ ) who underwent long-term treatment with nicotine. Compared with the saline-treated group, CYP2A6agm protein expression was significantly decreased in the long-term nicotine treatment group (59%;  $p = 0.04$ ; Fig. 3, B, and C). Long-term phenobarbital treatment did not significantly increase CYP2A6agm expression compared with saline (20%;  $p = 0.49$ ; Fig. 3, B and C). Because CYP2A6agm protein in some of the animals was at or below the limits of detection of the assay when 15  $\mu\text{g}$  of hepatic protein was loaded (Fig. 3C), experiments were conducted to ensure that measurements of CYP2A6agm protein were accurate. Samples were diluted or concentrated to achieve an equal density measurement (within the linear range of the assay) across the samples. For example, samples from nicotine-treated animals with very low CYP2A6agm (using 15  $\mu\text{g}$  of protein per lane) were quantifiable when more protein was loaded (Fig. 3C, inset). The relative optical densities were then corrected for the factor by which the samples were diluted or concentrated.

CYP2B6agm expression was examined using an anti-human CYP2B6 antibody-based immunoblotting assay. CYP2B6agm also migrated slightly more slowly than human liver and expressed CYP2B6 (Fig. 4A). Assuming equal detection, approximate quantities (derived from standard curves of expressed human CYP2B6) were 0.021 pmol of CYP2B6/ $\mu\text{g}$  of protein and  $0.083 \pm 0.016$  pmol CYP2B6agm/ $\mu\text{g}$  of protein for human ( $n = 1$ ; K20) and AGM ( $n = 7$ ; six saline-treated animals and one untreated

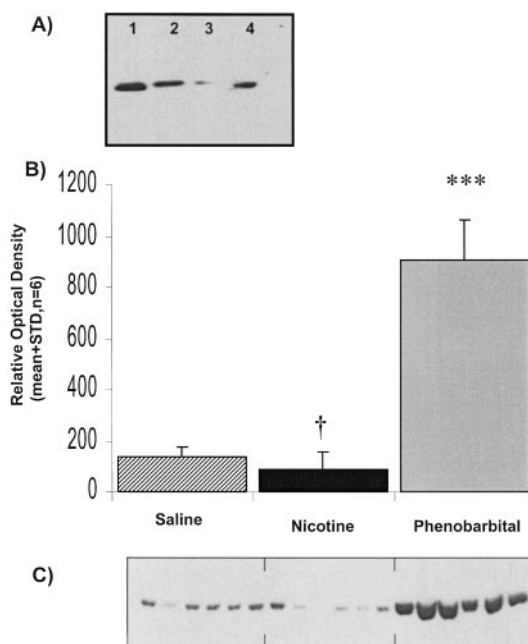
animal) liver microsomes, respectively. Hepatic CYP2B6agm expression was slightly but insignificantly decreased (approximately 14%) in the long-term nicotine treatment group ( $P = 0.11$ ; Fig. 4, B and C). As expected, CYP2B6agm protein was significantly increased in the phenobarbital-treated animals (650%;  $P < 0.001$ ; Fig. 4, B and C).

**Long-Term Nicotine Treatment Decreases NCO in AGM Liver.** NCO was measured in liver microsomes from AGM ( $n = 6$ ) treated long-term with saline, nicotine, and phenobarbital. Total NCO was decreased by 41% in the nicotine treatment group compared with the saline treatment group ( $18.2 \pm 5.0$  pmol of cotinine formed/mg of protein/min in the saline-treated group compared with  $10.73 \pm 4.4$  pmol of cotinine formed/mg of protein/min in the nicotine-treated group, Figs. 5 and 6,  $P = 0.02$ ).

We used human selective inhibitory antibodies to determine whether this decrease was related to the observed decreases in CYP2A6agm or CYP2B6agm protein expression. As illustrated in Fig. 5, the portion of NCO mediated by CYP2A6agm was decreased by 39% in the nicotine group compared with the saline group ( $14.9 \pm 4.2$  pmol of cotinine formed/mg of protein/min for the saline group compared with  $9.1 \pm 2.6$  pmol of cotinine formed/mg of protein/min for the nicotine group,  $P = 0.02$ ). There was no significant change in the portion of NCO not mediated by CYP2A6agm ( $3.3 \pm 2.6$  pmol of cotinine formed/mg of protein/min for the saline group compared with  $2.1 \pm 2.5$  pmol of cotinine formed/mg of protein/min for the nicotine group,  $P = 0.42$ , Fig. 5). The CYP2A6agm-mediated NCO, as determined by inhibitory antibodies, was not different between the saline and phenobarbital groups ( $14.9 \pm 4.2$  compared with  $15.3 \pm 6.9$  pmol of



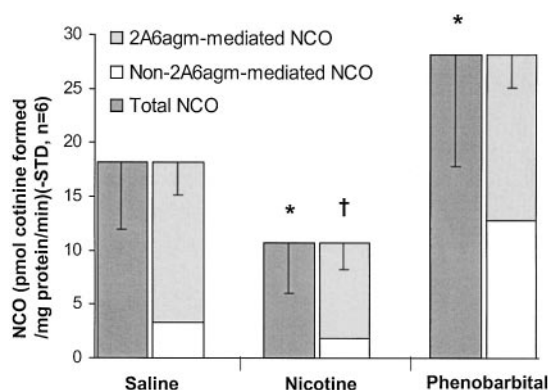
**Fig. 3.** Long-term, in vivo nicotine treatment significantly decreases CYP2A6agm protein expression in AGM liver. A, lane 1 contains AGM liver microsomes (15  $\mu\text{g}$ ), lane 2 contains AGM liver microsomes (7.5  $\mu\text{g}$ ), lane 3 contains human liver microsomes (15  $\mu\text{g}$ ), and lane 4 contains expressed human CYP2A6 (0.6 pmol). B, histogram demonstrating that hepatic CYP2A6agm protein is decreased by 59% in nicotine-treated AGM (five experiments). Values are expressed as mean  $\pm$  S.D. ( $n = 6$  animals per group). C, representative immunoblot from each animal. Inset showing measurable levels of protein in animals with low levels of CYP2A6agm (when more protein was loaded). \*,  $p \leq 0.05$ ; significantly different from saline.



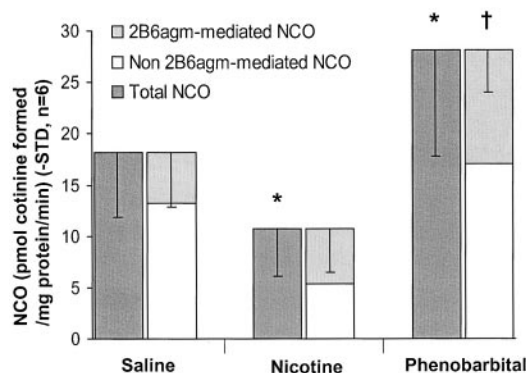
**Fig. 4.** Long-term, in vivo phenobarbital treatment significantly increases CYP2B6agm protein in AGM liver. A, lane 1 contains AGM liver (3  $\mu\text{g}$ ), lane 2 contains AGM liver (1.5  $\mu\text{g}$ ), lane 3 contains human liver (K20) (3  $\mu\text{g}$ ), and lane 4 contains expressed human CYP2B6 (0.025 pmol). B, histogram (five experiments) showing induction of CYP2B6agm by phenobarbital and lack of effect by nicotine. Values represent mean  $\pm$  S.D. ( $n = 6$  animals per group). C, representative immunoblot of each animal. \*\*\*,  $p < 0.001$ ; significantly different from saline; †,  $p = 0.11$

cotinine formed/mg of protein/min,  $P = 0.90$ , Fig. 5). However, there was a significant increase in the portion of NCO, which was not mediated by CYP2A6agm in the phenobarbital group ( $3.3 \pm 2.6$  pmol of cotinine formed/mg of protein/min for the saline group compared with  $12.8 \pm 2.9$  pmol of cotinine formed/mg of protein/min for the phenobarbital group,  $P = 0.001$ , Fig. 5).

Total NCO was increased by 55% in phenobarbital-treated animals ( $18.2 \pm 5.0$  pmol of cotinine formed/mg of protein/min in the saline group compared with  $28.1 \pm 6.6$  in the phenobarbital group,  $P = 0.01$ , Figs. 5 and 6). Inhibitory



**Fig. 5.** Long-term nicotine treatment reduces total NCO and CYP2A6agm-mediated NCO in AGM liver. Solid dark gray bars indicate mean total NCO [ $\pm$ S.D. (STD)] in liver microsomes from saline-, nicotine-, and phenobarbital-treated AGM ( $n = 6$  per group) showing decreased total NCO in nicotine-treated AGM and increased total NCO in phenobarbital-treated AGM. Stacked bars indicate the mean portion of NCO mediated by CYP2A6agm ( $\pm$ S.D., light gray portion) and the mean remaining NCO that was not mediated by CYP2A6agm (white portion, NCO remaining after incubation with anti-CYP2A6 antibodies). In liver microsomes from saline-, nicotine-, and phenobarbital-treated AGM ( $n = 6$  per group), the CYP2A6agm-mediated NCO is decreased in the nicotine group but unchanged in the phenobarbital group. \*,  $p \leq 0.05$ , total NCO significantly different from saline group; †,  $p \leq 0.05$ , CYP2A6agm-mediated NCO significantly different from saline group.



**Fig. 6.** Long-term phenobarbital treatment increases total NCO and CYP2B6-mediated NCO in AGM liver. Solid dark gray bars indicate mean total NCO [ $\pm$ S.D. (STD)] in liver microsomes from saline-, nicotine-, and phenobarbital-treated AGM ( $n = 6$  per group) showing decreased total NCO in nicotine-treated AGM and increased total NCO in phenobarbital-treated AGM (as in Fig. 5). Stacked bars indicate the mean portion of NCO mediated by CYP2B6agm ( $\pm$ S.D., light gray portion) and the mean remaining NCO that was not mediated by CYP2B6agm (white portion, NCO remaining after incubation with anti-CYP2B6 antibodies). In liver microsomes from saline-, nicotine-, and phenobarbital-treated AGM ( $n = 6$  per group), the CYP2B6agm-mediated NCO is increased in the phenobarbital group but does not differ significantly between the saline-treated and nicotine-treated groups. \*,  $p \leq 0.05$ , total NCO significantly different from saline group; †,  $p \leq 0.05$ , CYP2B6agm-mediated NCO significantly different from saline group.

antibodies were again used to determine whether this increase was mediated by CYP2B6agm, consistent with the increase observed in CYP2B6agm protein. Compared with the saline-treated group, CYP2B6agm-mediated NCO was increased by 221% in the phenobarbital-treated animals ( $5.5 \pm 3.9$  versus  $11.1 \pm 4.0$  pmol of cotinine formed/mg of protein/min,  $P = 0.001$ , Fig. 6). In contrast, whereas the NCO mediated by CYP2B6 was similar in the saline and nicotine groups ( $5.5 \pm 3.9$  pmol of cotinine formed/mg of protein/min in the saline group compared with  $7.4 \pm 2.2$  pmol of cotinine formed/mg of protein/min in the nicotine group,  $P = 0.32$ , Fig. 6), the portion of NCO that was not mediated by CYP2B6agm was significantly decreased in the nicotine group ( $13.2 \pm 5.3$  pmol of cotinine formed/mg of protein/min in the saline group compared with  $5.3 \pm 4.3$  pmol of cotinine formed/mg of protein/min in the nicotine group,  $P = 0.02$ , Fig. 6). The portion of NCO that was not mediated by CYP2B6agm was insignificantly increased when the saline and phenobarbital-treated groups were compared ( $13.2 \pm 5.3$  pmol of cotinine formed/mg of protein/min in the saline group compared with  $17.0 \pm 4.0$  pmol of cotinine formed/mg of protein/min in the phenobarbital group,  $P = 0.20$ , Fig. 6).

**RNA Analysis.** We used human CYP2A6 and CYP2B6 cDNA probes to measure CYP2A6agm and CYP2B6agm in the AGM treatment groups. We tested these probes for cross-reactivity with human cRNAs for CYP2E1, CYP3A4, and CYP2D6, as well as CYP2B6 and CYP2A6. The CYP2A6 cDNA probe was strongly selective for the human CYP2A6 cRNA. Using the CYP2A6agm assay (Fig. 7A, inset), we detected an mRNA band that comigrated with a band from human liver. The size of the band detected with the CYP2A6 probe was 3.2 kb. Another mRNA band was detected at approximately 1 kb in both the AGM and human livers. The CYP2B6 cDNA probe was strongly selective for the CYP2B6cRNA. The CYP2B6 probe detected mRNA bands at 2.5 kb and 500 base pairs in both human and AGM livers (Fig. 7B, inset). However, we encountered difficulties in extracting sufficient quantities of quality RNA from the AGM liver samples; some samples yielded insufficient amounts of RNA. For this reason, two or three animals in the saline group and one animal in the nicotine group were not measured, and the experiments were completed only once for each P450. Keeping these limitations in mind, we did find a significant decrease in the 3.2-kb CYP2A6agm mRNA band (33%,  $P = 0.05$ , Fig. 7A) in the nicotine-treated group compared with the saline-treated group, with no significant change in the phenobarbital-treated group ( $P = 0.23$ ). The 2.5-kb CYP2B6agm mRNA band was also significantly decreased in the nicotine-treated group (35%,  $P = 0.002$ , Fig. 7B) and significantly increased in the phenobarbital-treated group (170%,  $P < 0.001$ , Fig. 7B).

## Discussion

Using AGM as a model, we found that long-term, in vivo nicotine treatment significantly decreases in vitro nicotine metabolism by approximately 40% and the expression of a CYP2A6-like protein in AGM liver by approximately 60%. Because nicotine metabolism is dose-independent at the levels of nicotine self-administered by smokers (Benowitz and Jacob, 1993), changes in CYP2A6 protein would be expected to alter nicotine metabolism. Experiments using anti-



CYP2A6 inhibitory antibodies strongly suggest that the decrease in nicotine metabolism after long-term nicotine administration is mediated by the decrease observed in the CYP2A6agm-mediated portion of the total NCO (40%), similar to the reduction observed in CYP2A6agm protein (60%). This is quantitatively similar to the decreases in nicotine clearance observed during smoking compared with nonsmoking (27–34%; Lee et al., 1987; Benowitz and Jacob, 1993). Our observation that CYP2A6agm protein and nicotine metabolism is reduced in AGM that underwent long-term treatment with nicotine supports the concept that nicotine metabolism is reduced in smokers via down-regulation of CYP2A6. In addition, this finding argues against the concept that peripheral

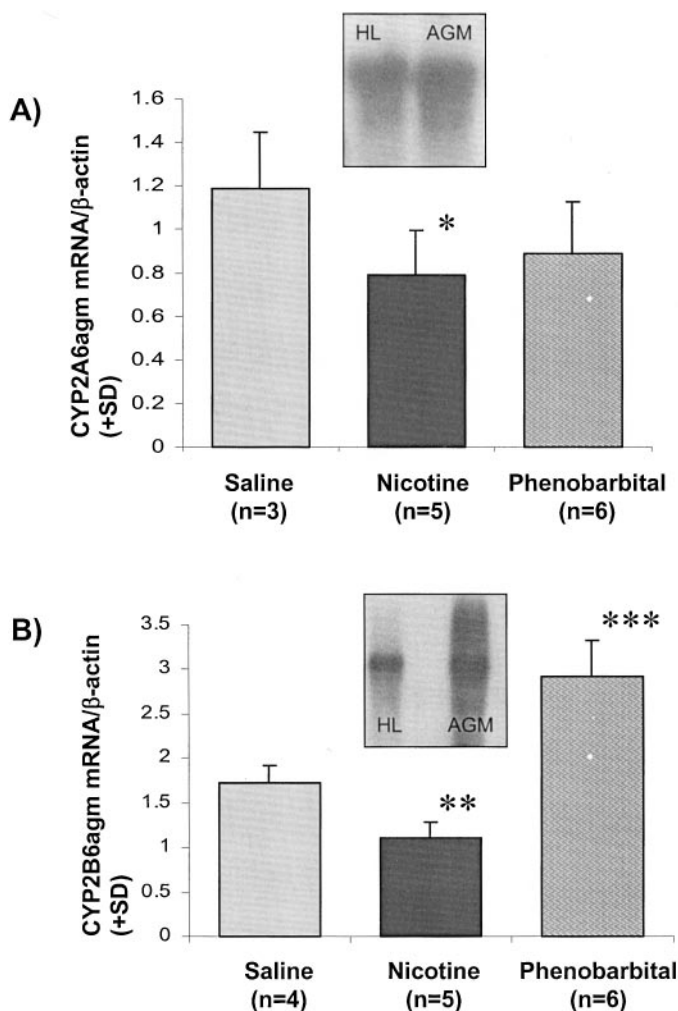
metabolic tolerance contributes to smoking initiation and/or escalation.

An early study reported a decrease in nicotine metabolism in mice after 3 days of nicotine pretreatment (Stalhandske and Slanina, 1970). The study described here is the first report of nicotine-mediated down-regulation of CYP2A expression and nicotine metabolism in nonhuman primates. Thus, it seems that nicotine can decrease CYP2A in nonhuman primates consistent with the decrease in NCO observed after nicotine treatment in mice or smoking in humans. Nicotine metabolism to cotinine was also decreased in rats after long-term exposure to cigarette smoke compared with rats after single-dose exposure to cigarette smoke (Rotenberg and Adir, 1983). In rats, NCO is mediated primarily by CYP2B1/2 (Nakayama et al., 1993). Although CYP2B6agm expression was modestly decreased in AGM that underwent long-term treatment with nicotine (14%, not significant), the amount of NCO mediated by CYP2B6agm was not decreased. Long-term treatment with nicotine also had no significant effect on rat hepatic CYP2B1 expression (Miksys et al., 2000); therefore, it is possible that in rats, some other component of cigarette smoke mediates the decrease in NCO.

NCO was significantly increased in the phenobarbital-treated group. We found that CYP2B6agm was increased by phenobarbital treatment as expected, and although there have been reports that primate CYP2A enzymes are induced by phenobarbital (Ohmori et al., 1993), we did not see a significant induction of CYP2A6agm by phenobarbital with these doses. In our study, the induction in nicotine metabolism by long-term treatment with phenobarbital was caused by an increase in CYP2B6-mediated NCO, as determined with inhibitory antibodies. These results suggest that NCO may be affected by the phenobarbital-mediated induction of CYP2B6, which may account for some of the interindividual variation observed in nicotine metabolism. In the nicotine-treated AGM with decreased CYP2A6agm-mediated NCO, the proportion of NCO mediated by CYP2B6agm seems to be increased (i.e., from approximately 20% of mediated by CYP2B6agm in the saline group to approximately 50% in the nicotine group, Fig. 6). This suggests that CYP2B6 may become more important for NCO in persons with reduced CYP2A6 activity.

Cigarette smoking induces the metabolism of a number of drugs that are substrates of CYP1A (Miller, 1990). Recently, nicotine itself (in addition to the polycyclic aromatic hydrocarbons found in cigarette smoke) has been postulated to mediate CYP1A1 induction (Iba et al., 1999). Low doses of nicotine can also induce rat hepatic CYP2E1 expression (Howard et al., 2001), and rat brain CYP2B1 expression (Miksys et al., 2000). These data together suggest that nicotine can regulate multiple P450s in liver and other organs.

Changes in CYP2A6 expression caused by long-term exposure to nicotine may alter elimination or activation of substrates metabolized by this enzyme, including drugs and procarcinogens (Tiano et al., 1994). Smokers may have decreased elimination rates of CYP2A6 substrates; for example, coumarin metabolism (a CYP2A6 probe substrate) is decreased in smokers (Poland et al., 2000). Activation of prodrugs may also be altered by changes in CYP2A6 expression. It has been shown that the level of CYP2A6 expression is correlated to activation of the chemotherapeutic prodrug tegafur (Murayama et al., 2001). Thus, smoking may alter



**Fig. 7.** CYP2A6agm and CYP2B6agm mRNA is decreased in nicotine-treated AGM liver. A, histogram showing a decrease in CYP2A6agm mRNA in the nicotine-treated group ( $n = 5$ ,  $0.79 \pm 0.20$ ) compared with the saline-treated group ( $n = 3$ ,  $1.18 \pm 0.26$ ) and no significant change in the phenobarbital-treated group ( $n = 6$ ,  $0.89 \pm 0.23$ ). Inset, comigration of human (HL) and AGM liver mRNA bands detected by a human CYP2A6 cDNA probe. B, histogram showing a decrease in CYP2B6agm mRNA in the nicotine-treated group ( $n = 4$ ,  $1.72 \pm 0.21$ ) compared with the saline-treated group ( $n = 5$ ,  $1.11 \pm 0.17$ ), and an increase in CYP2B6agm in the phenobarbital-treated group ( $n = 6$ ,  $2.92 \pm 0.40$ ). Inset, comigration of human (HL) and AGM (phenobarbital-treated) liver mRNA bands detected by a human CYP2B6 cDNA probe. \*,  $p \leq 0.05$ ; \*\*,  $p < 0.01$ ; and \*\*\*,  $p < 0.001$ ; significantly different from saline-treated group. Values are given as means of the relative optical density measurements from each animal divided by the relative optical density measurements of the  $\beta$ -actin mRNA in each animal.

the clinical efficacy of this and other drugs by decreasing CYP2A6-mediated metabolism.

In addition to nicotine from cigarette smoke, nicotine is also currently administered to patients in the form of nicotine replacement therapies as an aid for smoking cessation. Nicotine replacement therapies are now available in multiple forms, including nicotine gum, inhalers, nasal spray, and transdermal patches (Karnath, 2002). Nicotine has also demonstrated efficacy in the treatment of ulcerative colitis and has been postulated for the treatment of Alzheimer's disease and Parkinson's disease (Baron, 1996). Therefore, long-term nicotine use may decrease CYP2A6-mediated drug metabolism and carcinogen activation in these populations as well as in smokers.

In humans, nicotine is metabolized to the major metabolite cotinine by CYP2A6 (Messina et al., 1997). We characterized AGM nicotine metabolism to evaluate this species' potential as a model for human nicotine metabolism. AGM have three to four times more CYP2A6 and CYP2B6 protein per microgram of liver microsomes than the control human liver (K20). The expression of both enzymes is known to vary widely between individual subjects. In the current study, we have measured CYP2A6 and CYP2B6 in only one human liver; however, the mean content of CYP2A6 in K20 (0.012 pmol) was similar to that of a group of 30 human livers [ $0.012 \pm 0.013$  pmol of CYP2A6 protein, calculated from (Li et al., 1997), where K20 CYP2A6 protein was also measured by immunoblotting]. Consistent with the higher levels of CYP2A6 protein, AGM have an approximately 2-fold higher  $V_{\max}$  for NCO, and a 2-fold lower  $K_m$ . Using inhibitory antibodies and chemical inhibitors, we determined that 80 to 90% of NCO in AGM is mediated by a CYP2A6-like enzyme in AGM liver, which is the same as what has been found in humans (Messina et al., 1997).

Nonhuman primate CYP2A is also similar to the human homolog in other substrate profiles and in its regulation (Li et al., 1997), in contrast to rat CYP2A enzymes, which do not metabolize nicotine (Nakayama et al., 1993). Therefore, AGM may be a more suitable model for human nicotine metabolism and CYP2A than rodents.

Using Northern analysis with human CYP2A6 and CYP2B6 probes, we found a significant decrease in CYP2A6 and CYP2B6 mRNA bands in the nicotine-treated group and a significant increase in the CYP2B6 mRNA band in the phenobarbital-treated group. These results suggest that one mechanism for the decreased levels of CYP2A6 protein and activity may involve regulation of mRNA levels. However, because of the low RNA extraction yields and inferior quality of RNA from some animals, studies determining the changes in RNA will need to be verified. Further work is needed to clarify the mechanism of CYP2A6 down-regulation by nicotine (i.e., decreased transcription, and/or increased protein or mRNA degradation).

A general limitation of this study is the use of human-selective antibodies and probes to study AGM P450s. Whereas the similarity between AGM and human P450 enzymes is expected to be fairly high (based on 88 to 94% amino acid or nucleotide sequence similarity in other primate species), CYP2A or CYP2B have not yet been cloned, sequenced, or purified from AGM. It is possible that the human-selective probes are not cross-reacting with their intended targets, but

rather with unknown P450 (not a CYP2A or CYP2B enzyme) or other protein and/or RNA found in this species. However, we have used a variety of approaches to measure CYP2A6 and CYP2B6 (i.e., immunoblotting, NCO assays with and without inhibition antibodies, and Northern analysis) with consistent results, which increases the strength of our interpretation.

The twice-daily 0.3 mg/kg dose of nicotine (total 0.6 mg/kg per day) is similar to the average daily intake of nicotine by smokers; 0.53 mg/kg of nicotine per day (0.14 to 1.1 mg/kg; 10–79 mg of nicotine/day for a 70-kg man) (Benowitz et al., 1989). However, smokers achieve these total doses over the course of the day through small increments (approximately 1 mg of nicotine per cigarette), with nicotine plasma levels accumulating over 6 to 8 h (Benowitz et al., 1989), whereas our animals received a larger dose of nicotine twice per day. Given the approximately 1- to 2-h half-life of nicotine [in humans and nonhuman primates (Seaton et al., 1991)], plasma nicotine would be expected to be fully eliminated 12 h after administration and therefore would not accumulate to steady-state plasma levels in these animals. Thus, it is possible that a longer lasting metabolite of nicotine, rather than nicotine itself, is mediating the down-regulation. For example, whereas a single dose of cotinine did not decrease nicotine metabolism (Zevin et al., 1997), it is possible that long-term use of cotinine might produce an effect on CYP2A6 expression.

In conclusion, we developed a nonhuman primate AGM model for nicotine metabolism. CYP2A6 accounts for 80 to 90% of NCO, and long-term nicotine exposure decreases CYP2A6 protein and nicotine metabolism in AGM liver. These results strongly suggest that the decreased nicotine clearance observed in smokers is caused by the ability of nicotine to down-regulate hepatic CYP2A6.

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