In Vivo and in Vitro Characterization of Chlorzoxazone Metabolism and Hepatic CYP2E1 Levels in African Green Monkeys: Induction by Chronic Nicotine Treatment

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ABSTRACT:

CYP2E1 metabolizes compounds, including clinical drugs, organic solvents, and tobacco-specific carcinogens. Chlorzoxazone (CZN) is a probe drug used to phenotype for CYP2E1 activity. Smokers have increased CZN clearance during smoking compared with nonsmoking periods; however, it is unclear which cigarette smoke component is causing the increased activity. The relationships between in vivo CZN disposition, in vitro CZN metabolism, and hepatic CYP2E1 have not been investigated in a within-animal design. In control-treated monkeys (Cercopithecus aethiops), the in vivo CZN area under the curve extrapolated to infinity (AUCinf) was 19.7 ± 4.5 μg × h/ml, τ1/2 was 0.57 ± 0.07 h, and terminal disposition rate constant calculated from last three to four points on the log-linear end of the concentration versus time curve was 1.2 ± 0.2 /h. In vitro, the apparent Vmax was 3.48 ± 0.02 pmol/min/μg microsomal protein, and the Km was 95.4 ± 1.8 μM.

Chronic nicotine treatment increased in vivo CZN disposition, as indicated by a 52% decrease in AUCinf (p < 0.01) and 52% decrease in T1/2 (p < 0.05) compared with control-treated monkeys. The log metabolic ratios at 0.5, 1, 2, and 4 h significantly negatively correlated with CZN AUCinf (p = 0.01–0.0001). Monkey hepatic CYP2E1 levels significantly correlated with both in vivo AUCinf (p = 0.03) and in vitro (p = 0.004) CZN metabolism. Together, the data indicated that nicotine induction of in vivo CZN disposition is related to the rates of in vitro CZN metabolism and hepatic microsomal CYP2E1 protein levels. Nicotine is one component in cigarette smoke that can increase in vivo CZN metabolism via induction of hepatic CYP2E1 levels. Thus, nicotine exposure may affect the metabolism of CYP2E1 substrates such as acetaminophen, ethanol, and benzene.

CYP2E1 is a drug-metabolizing enzyme that biotransforms many compounds, including clinical drugs such as acetaminophen and halothane (Caro and Cederbaum, 2004). CYP2E1 also metabolizes ethanol (Lieber, 1999) and can be induced by ethanol in rats (Howard et al., 2001), rabbits (Lieber, 1999), and humans (Oneta et al., 2002). CYP2E1 contributes to ethanol metabolism at high blood alcohol concentrations in humans (Salaspuro and Lieber, 1978) and rats (Matsumoto et al., 1996). Many low molecular weight compounds such as benzene and carbon tetrachloride are also CYP2E1 substrates (Caro and Cederbaum, 2004). CYP2E1 produces a high level of reactive oxygen species, without need of a ligand, that can result in cell damage from lipid peroxidation and DNA strand breaks (Caro and Cederbaum, 2004).

Chlorzoxazone (CZN), a clinically used muscle relaxant, is metabolized by CYP2E1 to 6-hydroxychlorzoxazone (6OHCZN), and this reaction has been established as a phenotypic measure of CYP2E1 activity in vivo in humans (Lucas et al., 1999) and in vitro in rats (Kobayashi et al., 2002) and cynomolgus monkeys (Amato et al., 1998). No studies linking in vivo CZN disposition, in vitro hepatic CZN metabolism, and hepatic CYP2E1 protein levels have been published in any species. The in vivo CZN disposition, in vitro CZN metabolism, and hepatic CYP2E1 protein levels have not yet been investigated in African green monkeys (vervets, Cercopithecus aethiops). This monkey is a useful model of human metabolism; it has been established as a model of CYP2A6 (Schoedel et al., 2003) and CYP2B6 (Lee et al., 2006) isozyme metabolism. Our model provides an opportunity to investigate the relationship between these three variables in a nonhuman primate. African green monkey CYP2E1 has not yet been sequenced, but it is expected to have similar amino acid homology to that of human CYP2E1. Other monkeys such as the cynomolgus monkey (Macaca fascicularis) and the rhesus monkey (Macaca mulatta) have 92 and 95% amino acid homology to human...
CYP2E1, respectively [cytogenetic accession number: P33266, GI: 461827 (Komori et al., 1992), rhesus accession number: AAT49269, GI: 4906635] (unpublished data).

Cigarette smoking can alter the activity of CYP2E1. In humans, periods of cigarette smoking increase the in vivo CZN clearance compared with nonsmoking periods (Benowitz et al., 2003). In mice, exposure to cigarette smoke increases CYP2E1 protein, mRNA, and activity in lung, kidney, and liver (Villard et al., 1994; Seree et al., 1996; Villard et al., 1998). CZN metabolism is not affected by carbon monoxide (Benowitz et al., 2003), which is produced by burning cigarettes, nor by cotinine, the primary metabolite of nicotine (Miciu et al., 2003). The increase in CYP2E1 is important because it may contribute to the harmful effects of smoking. CYP2E1 activates many procarcinogens found in cigarette smoke, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, N-nitrosodimethylamine, and N-nitrosodiethylamine (Kushida et al., 2000), and is associated with the development of some types of cancer (Caro and Cederbaum, 2004). Nicotine could be the inducing agent responsible for the increase in CZN clearance in smokers. Nicotine has been shown to increase CYP2E1 protein and in vitro activity in rats (Anandatheerthavarada et al., 1993; Bhagwat et al., 1998; Howard et al., 2001; Miciu et al., 2003). The effect of nicotine on CYP2E1 levels is of interest because current and former smokers may be exposed to nicotine via smoking or as a smoking cessation drug.

We hypothesize that there will be a significant relationship between in vivo CZN disposition, in vivo CZN metabolism, and hepatic CYP2E1 levels in monkeys. In addition, we hypothesize that chronic nicotine treatment will increase the rate of in vivo CZN disposition and the rate of in vitro CZN metabolism in hepatic microsomes, via an increase in hepatic CYP2E1 protein levels in monkeys.

### Materials and Methods

#### Materials
Nicotine bitartrate, CZN, and 2-benzoxazolinone were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). All the other chemical reagents were obtained from standard commercial sources. The protein assay dye reagent was purchased from Bio-Rad Laboratories (Hercules, CA). Prestained molecular weight protein markers were purchased from MBI Fermentas (Flamborough, ON, Canada). Hybond nitrocellulose membrane was purchased from Amersham Bioscience (Toronto, ON, Canada). Human CYP2A6, CYP2B6, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 expressed from cDNA, polyclonal anti-human CYP2E1 antibody for immunoblotting, and polyclonal anti-rat CYP2E1 antibody for immunohistochemistry were purchased from BD Gentest (Woburn, MA). Horseradish peroxidase-conjugated anti-goat secondary antibody was purchased from Chemicon International, Inc. (Temecula, CA). Avidin-biotin complex with peroxidase kit and 3,3′-diaminobenzidine were purchased from Vector Laboratories (Burlington, ON, Canada). Chemiluminescent substrate was purchased from Pierce Chemical Company (Rockford, IL). Autoradiographic film was purchased from Ulitdent (St. Laurent, PQ, Canada). Human liver was provided by Dr. Ted Inaba (University of Toronto, ON, Canada). Three freshly dissected livers from untreated, drug-free, male African green monkeys were donated by Aventis Pasteur Ltd. (formerly Connaught Laboratories, Ltd., Toronto, ON, Canada). Freshly dissected tissues were immediately frozen and transported to the laboratory.

#### Animals
The study consisted of 12 male adult African green monkeys (verevs, C. aethiops) housed at Caribbean Primates Ltd. (St. Kitts) and three untreated adult African green monkey livers. The 12 African green monkeys were drawn from a large, isolated, and nonendangered Caribbean population and housed outdoors in social groups (Ervin et al., 1990). Monkeys were given standard rations of Purina monkey chow supplemented with fresh fruit and vegetables. Drinking water was available ad libitum. The nicotine-treated monkeys (n = 6) were given nicotine bitartrate (milligram base in saline, pH 7.0) at 0.05 mg/kg (all the injections s.c., twice daily) for 2 days, then 0.15 mg/kg for 2 days, followed by 0.3 mg/kg for 18 days. The control-treated monkeys (n = 6) received sham nicotine injections (saline s.c., twice daily).

On day 14, all the monkeys received 7 mg/kg CZN intragastrically under ketamine anesthesia instead of nicotine or saline treatment, and a 6-ml blood sample was drawn at baseline, 10, 20, 30, 60, 120, 240, and 360 min after CZN administration. Blood samples were centrifuged, and the plasma removed and frozen for drug analyses. All the animals were sacrificed on day 22, 6 h after AM drug treatments under ketamine anesthesia. At the time of sacrifice, nicotine levels are estimated to be less than 5 ng/ml (Lee et al., 2006), and we have shown that nicotine does not interact with CYP2E1 (Howard et al., 2001), suggesting no interference in subsequent assays. Body weights of the monkeys did not decrease as a result of treatment. Organs 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 Behavioral Sciences Foundation and the University of Toronto Animal Care Committee. All the procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

#### In Vivo CZN and 6OH-CZN Plasma Assessments
Plasma CZN and 6OH-CZN levels were assayed based on the methods in Howard et al. (2001) with minor modifications. Briefly, plasma was centrifuged at 3000 g for 10 min, and 0.5 ml of the supernatant was removed for analysis. Internal standard of 2.0 mM 2-benzoxazolinone (25 µl) and β-glucuronidase (10 mg/ml, 250 µl) were added to 0.2 M acetate buffer (pH 5.0, 1.0 ml) and incubated overnight at 37°C. Following incubation, 10% perchloric acid (600 µl) and ethyl acetate/hexane (5 ml) were added, the sample was shaken for 30 min, centrifuged at 3500 g for 15 min, and the organic phase evaporated to dryness at 37°C. The sample was reconstituted into 200 µl of mobile phase consisting of 19% acetonitrile in 10 mM sodium acetate buffer (pH 4.5). CZN and 6OH-CZN were measured by high-performance liquid chromatography with UV detection at 287 nm. A Waters Spherisorb S5-ODS2 column (4.6 × 150 mm) (Waters, Bedford, MA) was used to separate CZN, 6OH-CZN, and 2-benzoxazolinone using a mobile phase performed with isocratic elution at a flow rate of 1 ml/min. The retention times for CZN, 6OH-CZN, and 2-benzoxazolinone were 19.6, 4.7, and 6.5 min, respectively.

#### Microsomal Membrane Preparation and Protein Assay
Monkeys or human liver tissue was homogenized in 100 mM Tris, 0.1 mM EDTA, 0.1 mM DDT, and 0.32 M sucrose (pH 7.4) for immunoblotting or in 1.15% w/v KCl for in vitro metabolism assessments and then centrifuged at 12,500 g for 30 min at 4°C. The supernatant was then centrifuged at 110,000 g for 90 min at 4°C, and the pellet was resuspended in 100 mM Tris, 0.1 mM EDTA, 0.1 mM DDT, 1.15% w/v KCl, and 20% v/v glycerol for immunoblotting or 1.15% w/v KCl for in vitro metabolism assays. Microsomes were aliquoted and stored at −80°C. The protein content of liver microsomes was assayed with the Bradford technique using a Bio-Rad Protein Assay kit.

#### In Vitro CZN and 6OH-CZN Assessments
CZN 6-hydroxylation was assayed according to the method of Leclercq et al. (1998) for rat liver microsomes with minor modifications for use with monkey tissues (Leclercq et al., 1998). African green monkey hepatic microsomes were mixed with 0.1 M phosphate buffer (pH 7.4), 250 µM CZN, 25 µM NADPH to a final volume of 500 µl and 5 mM NADPH to a final volume of 500 µl. Incubations were carried out with CZN in a shaking water bath at 37°C. Different protein concentrations and incubation times were tested to establish the linear incubation conditions for CZN metabolism. Final reactions included 0.4 mg of protein incubated for 20 min. Zinc sulfate (15% w/v, 0.2 ml) was added to stop the reaction, and 6.4 µg of the internal standard, 2-benzoxazolinone in Tris buffer, was added per reaction. Following centrifugation for 10 min at 12,700g, the supernatant was injected onto an Agilent Zorbax SB-C18 column (5 µm, 4.6 × 250 mm) (Agilent Technologies, Palo Alto, CA) with UV detection at 287 nm. Separation of peaks was performed using a mobile phase of 50 mM ammonium acetate (adjusted to pH to 4.0 with 1 M glacial acetic acid)/acetonitrile (65:35) at a flow rate of 0.7 ml/min. The retention times for CZN, 6OH-CZN, and 2-benzoxazolinone were 18.5, 7.5, and 10.1 min, respectively. Kinetic parameters (Vmax and Km) in untreated monkey liver microsomes were incubated with CZN at various concentrations.

#### Immunohistochemistry
Frozen monkey liver, fixed with 4% paraformaldehyde, was cut into 16-µm sections. Sections were washed twice for 5 min with phosphate-buffered saline (PBS) (10 mM sodium phosphate buffer, 0.9% sodium chloride, pH 7.4) and incubated for 1 h in a blocking solution (PBS with 1% w/v skim milk powder, 0.1% bovine serum albumin (BSA), 0.01% Triton X-100 and 2% normal horse serum). Tissue sections were then incu-
bated for 48 h at 4°C with polyclonal goat anti-rat CYP2E1 antibody diluted 1:1000 with 0.1% BSA, 0.01% Triton X-100, and 2% normal horse serum in PBS. Tissue sections were washed three times for 5 min each with 0.01% Triton X-100 in PBS and then reblocked for 30 min with 0.1% BSA, 2% normal horse serum, and 0.01% Triton X-100 in PBS. Sections were then incubated for 1 h with biotinylated anti-goat secondary antibody diluted 1:1500 with 0.1% BSA, 2% normal horse serum, and 0.01% Triton X-100 in PBS, followed by three washes of 5 min. Tissue sections were then quenched for 10 min with 0.3% hydrogen peroxide in PBS, followed by two washes of 5 min each with 0.01% Triton X-100 in PBS. The antigen-antibody complex was visualized using the avidin-biotin complex, followed by a reaction with 3,3'-diaminobenzidine and hydrogen peroxide. Tissue sections were then dehydrated and mounted onto silane-coated slides with Permount. Immunohistochemical control sections were incubated without primary antibody.

Immunoblotting. Monkey liver microsomes and cDNA expressed CYP2E1 were serially diluted to generate standard curves and establish the linear detection range for the assays. Liver microsomal proteins (3 μg) were separated with SDS-polyacrylamide gel electrophoresis using a 10% separating gel and a 4% stacking gel. Proteins were transferred overnight onto nitrocellulose membranes. To detect CYP2E1, membranes were incubated for 1 h in a blocking solution of 1% w/v skim milk powder, 0.1% BSA, 0.1% Triton X-100, and Tris-buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.4). Membranes were then incubated for 1 h with polyclonal anti-human CYP2E1 antibody diluted 1:3000 in 0.1% BSA, 0.1% Triton X-100, and TBS followed by three washes of 5 min each with TBS and 0.1% Triton X-100. Membranes were reblocked with the same initial blocking solution for 45 min. To detect CYP2E1, membranes were then incubated for 1 h with horseradish peroxidase-conjugated anti-goat secondary antibody diluted 1:2500 in 0.1% BSA, 0.1% Triton X-100, 0.1% Triton X-100, and TBS. CYP2E1 proteins were visualized on immunoblots using chemiluminescence, followed by exposure to autoradiographic film for 1 to 5 min. MCID Elite imaging software (Imaging Research, Inc., St. Catherines, ON, Canada) was used to analyze films. Baseline corrections for band intensities were made by subtracting the film background from the band intensity. Using a standard curve of cDNA-expressed CYP2E1, band intensities were extrapolated from the last quantifiable concentration plus additional area extrapolated to the last quantifiable concentration (AUCT.), terminal disposition rate expressed as mean S.D. (range). Parameters measured were area under the curve versus time curve (Kel), measurement of area under the curve up to the last quantifiable concentration plus additional area extrapolated to infinity calculated using Keq (AUCinf), terminal disposition rate constant calculated from last 3 to 4 points on the log-linear end of the concentration versus time curve (Keq), measurement of area under the curve up to the last quantifiable concentration plus additional area extrapolated to infinity calculated using Keq (AUCinf), terminal disposition rate constant calculated from last 3 to 4 points on the log-linear end of the concentration versus time curve (Kel).

Statistical Analysis. In vivo CZN and 6OHCZN pharmacokinetic parameters were calculated for each treatment group. Pharmacokinetic results are expressed as mean ± S.D. (range). Parameters measured were area under the curve to the last quantifiable concentration (AUCinf), terminal disposition rate constant calculated from last 3 to 4 points on the log-linear end of the concentration versus time curve (Kel), measurement of area under the curve up to the last quantifiable concentration plus additional area extrapolated to infinity calculated using Keq (AUCinf), Cmax, Tmax, and t1/2. All the pharmacokinetic statistical analyses were carried out using SAS software version 8.2 (SAS Institute, Cary, NC). Differences between groups were evaluated using a one-tailed Student’s t test and were deemed significant if p < 0.05. Before statistical analysis, plasma levels were corrected for any baseline values by subtraction of time 0 values from all the subsequent time points.

For in vitro CZN metabolism, the calibration curves were constructed by using peak area ratios (6OHCZN/internal standard), and least square linear regression analysis was used to determine slope, intercept, and correlation coefficients. The absolute recovery was calculated by comparing the peak area obtained with the standards in the Tris-HCl buffer, and relative recovery was obtained by calculating the ratio of the additional amount to the value calculated by the curve. The apparent Vmax and Km were calculated using nonlinear regression. The 6OHCZN formation activities between the treatment groups were evaluated using unpaired, one-tailed Student’s t tests. In vitro pharmacokinetics and the Hill coefficient were calculated using GraphPad Prism version 4.0 (GraphPad Software, Inc., San Diego, CA). CYP2E1 femtomole protein levels per microgram microsomal protein were extrapolated from expressed cDNA CYP2E1 and control-treated monkey microsomal protein dilution curves. CYP2E1 content of microsomes prepared from livers of control- and nicotine-treated monkeys were compared using unpaired one-tailed Student’s t tests. For in vitro kinetics and CYP2E1 protein measurements, results are expressed as mean ± S.D., which represents the average of the six animals per group from at least four different experiments. Groups were deemed significantly different if p < 0.05. Correlations between groups were calculated with Pearson correlation coefficients, and correlations were deemed significant if p ≤ 0.05.

Results

In Vivo CZN Disposition and In Vitro CZN Metabolism in Control-Treated Monkeys. Control-treated monkeys (n = 6) had a CZN AUCinf of 19.7 ± 4.5 μg × h/mL, a Cmax of 9.2 ± 2.1 μg/mL, a T1/2 of 0.57 ± 0.07 h, and a Kel of 1.2 ± 0.2/h. The parameters for 6OHCZN included an AUCinf of 6.6 ± 0.7 μg × h/mL, a T1/2 of 0.68 ± 0.26 h, and a Kel of 1.2 ± 0.4/h. Concentration-time curves are presented in Fig. 1, A and B, and other pharmacokinetic values for control-treated monkeys are listed in Table 1.

The in vitro pharmacokinetic parameters of CZN metabolism were determined in hepatic microsomes prepared from three untreated African green monkey livers. The absolute and relative recoveries of both CZN and 6OHCZN were superior when centrifugation was used compared with liquid-liquid extraction with ethyl acetate and filtration. The average absolute and relative recoveries of 6OHCZN were 90.4 and 99.4%, respectively. The linear detection range for 6OHCZN formation was 0.5 to 16 μg/mL, and intraday and interday S.D. were less than 1%. During assay optimization, we established that the formation of 6OHCZN was linear up to microsomal protein concen-
trations of 800 μg/ml (Fig. 2A) and up to an incubation time of 40 min (Fig. 2B). Incubations lacking NADPH, or using heat denatured monkey microsomes, produced no detectable 6OHCZN (data not shown).

The formation of 6OHCZN displayed monophasic Michaelis-Menten kinetics (Fig. 3). The apparent V\textsubscript{max} and K\textsubscript{m} for the formation of 6OHCZN in the three untreated monkeys were 3.48 ± 0.02 pmol/min/μg microsomal protein and 95.4 ± 1.8 μM, respectively (Fig. 3). The average V\textsubscript{max}/K\textsubscript{m} is 34.2 ± 2.3 μl/min/μg microsomal protein. The Hill coefficient was 0.99, suggesting the involvement of one catalytic site on one enzyme, although a second enzyme with identical catalytic characteristics cannot be ruled out.

### Kinetic parameters for CZN and 6OHCZN in control- and nicotine-treated monkeys, n = 6 per treatment group, expressed as mean ± S.D. (range)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Nicotine</th>
<th>Control</th>
<th>Nicotine</th>
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</thead>
<tbody>
<tr>
<td>AUC\textsubscript{tr} (μg × h/ml)</td>
<td>19.7 ± 4.5 (14.4–27.1)</td>
<td>9.5 ± 3.2* (5.9–14.3)</td>
<td>6.6 ± 0.7 (6.0–7.6)</td>
<td>4.7 ± 1.2** (3.3–6.1)</td>
</tr>
<tr>
<td>AUC\textsubscript{inf} (μg × h/ml)</td>
<td>19.7 ± 4.5 (14.4–27.2)</td>
<td>9.5 ± 3.2* (5.9–14.3)</td>
<td>6.6 ± 0.7 (6.0–7.6)</td>
<td>4.8 ± 1.2** (3.3–6.2)</td>
</tr>
<tr>
<td>C\textsubscript{tr} (μg/ml)</td>
<td>9.2 ± 2.1 (7.0–11.8)</td>
<td>7.8 ± 2.8 (5.9–11.1)</td>
<td>2.7 ± 0.5 (2.0–3.2)</td>
<td>2.8 ± 1.4 (1.6–5.6)</td>
</tr>
<tr>
<td>T\textsubscript{1/2} (h)</td>
<td>0.75 ± 0.27 (0.50–1.00)</td>
<td>0.36 ± 0.07** (0.33–0.50)</td>
<td>1.08 ± 0.49 (0.50–2.00)</td>
<td>0.61 ± 0.31 (0.33–1.00)</td>
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<tr>
<td>V\textsubscript{max} (pmol/min/μg protein)</td>
<td>0.57 ± 0.07 (0.59–0.66)</td>
<td>0.60 ± 0.08 (0.47–0.70)</td>
<td>0.68 ± 0.26 (0.38–1.03)</td>
<td>0.76 ± 0.23 (0.32–0.93)</td>
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<tr>
<td>K\textsubscript{m} (h)</td>
<td>1.22 ± 0.17 (1.06–1.55)</td>
<td>1.18 ± 0.16 (0.99–1.49)</td>
<td>1.16 ± 0.45 (0.68–1.81)</td>
<td>1.05 ± 0.54 (0.74–2.13)</td>
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**Significantly different from the relative control, *p ≤ 0.001, **p ≤ 0.01.

#### Hepatic CYP2E1 Expression and Protein Levels in Control-Treated Monkeys

Nicotine treatment increased in vivo CZN disposition, which was related to induction of in vitro CYP2E1 metabolism and hepatic CYP2E1 protein levels. Nicotine treatment increased in vivo CZN disposition in monkeys. Nicotine-treated monkeys had a 52%...
lower CZN AUC<sub>inf</sub> (p < 0.01) and a 52% decrease in T<sub>max</sub> compared with control-treated monkeys (p < 0.05) (Fig. 1A). Other CZN pharmacokinetic parameters were not significantly different between treatments (Table 1).

Monkey CZN AUC<sub>inf</sub> values correlated significantly with the log plasma metabolic ratio (6OHCZN/CZN) calculated at 0.5, 1, 2, and 4 h (Table 2; Fig. 1C), indicating a strong relationship between CZN AUC<sub>inf</sub> and the metabolic ratio. The in vivo CZN disposition and hepatic CYP2E1 protein levels in control- and nicotine-treated monkeys were significantly correlated (r = −0.62, p = 0.03) (Fig. 6A), with higher CYP2E1 protein levels associated with lower CZN AUC<sub>inf</sub>. Nicotine treatment resulted in a 1.35-fold increase in CYP2E1 levels per microgram microsomal protein compared with control treatment (62 ± 21 versus 46 ± 11 pmol/µg microsomal protein, p = 0.07), although this was not significant (Fig. 6B). There was large interanimal variation in CYP2E1 levels within the treatment groups shown in a representative immunoblot (Fig. 5D).

The log plasma metabolic ratio at 1 h approached a significant correlation with hepatic CYP2E1 levels (p = 0.07) and 6OHCZN AUC<sub>inf</sub> (r = 0.53, p = 0.17). The 6OHCZN clearance increased in nicotine-treated monkeys, indicated by a 29% decrease in 6OHCZN AUC<sub>inf</sub> (p < 0.01) (Fig. 1B; Table 1). One nicotine-treated and one control-treated monkey were outliers (greater than 2 S.D. away from the mean of their group). When their values were omitted (n = 10), significant correlations existed between both the log plasma metabolic ratio and hepatic CYP2E1 protein content (r = 0.88, p < 0.0001; r = 0.58, p = 0.05, respectively). This data indicated a significant relationship existed between the log plasma metabolic ratio, hepatic CYP2E1 protein levels, and in vivo CZN and 6OHCZN disposition in most monkeys.

The in vitro CZN velocity at 950 µM CZN (approximate V<sub>max</sub>) and hepatic CYP2E1 protein levels in control- and nicotine-treated monkeys were significantly correlated (r = 0.74, p = 0.004) (Fig. 7A), with higher CYP2E1 protein levels associated with higher rates of CZN oxidation in vitro. Nicotine treatment resulted in a 1.27-fold increase in velocity at 950 µM CZN (approximate V<sub>max</sub>) compared with control treatment (2.13 ± 0.83 versus 1.67 ± 0.04 pmol/min/µg microsomal protein, p = 0.14), but this did not reach significance.

<table>
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<tr>
<th>Correlation Variables</th>
<th>r</th>
<th>p Value</th>
</tr>
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<tr>
<td>CZN AUC&lt;sub&gt;inf&lt;/sub&gt; vs. log (6OHCZN/CZN) plasma at 0.5 h</td>
<td>−0.78</td>
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<td>1 h</td>
<td>−0.91</td>
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<td>2 h</td>
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<td>4 h</td>
<td>−0.70</td>
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</tr>
<tr>
<td>6 h</td>
<td>0.18</td>
<td>0.58</td>
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Fig. 5. CYP2E1 protein detection in immunoblots of monkey liver microsomes. A, standard curve for CYP2E1 immunoblotting in monkey liver microsomes in one control-treated monkey. CYP2E1 detection was linear up to 12 µg of protein. B, monkey CYP2E1 comigrates with both human microsomal CYP2E1 and cDNA-expressed human CYP2E1 protein. Human liver (HL, lane 1) loaded at 10 µg; expressed human CYP2E1 loaded at 0.1, 0.2, 0.3, 0.4, and 0.5 pmol (lane 2–6); monkey CYP2E1 at 1, 3, 5, 7.5, 10, and 12.5 µg microsomal protein (lane 7–12). Migration of CYP2E1 is increased with increasing amount of protein loaded. C, anti-human CYP2E1 antibody did not cross-react with other human P450 isozymes. Each P450 is loaded in pairs of lanes at 0.3 and 3.0 pmol. CYP2E1 is indicated with an arrow. D, representative immunoblot of CYP2E1 in control- and nicotine-treated monkeys (six per group). Monkey CYP2E1 is indicated with an arrow.

Fig. 6. Relationship between in vivo CZN AUC<sub>inf</sub> and hepatic CYP2E1 protein in control- and nicotine-treated monkeys (six per group). A, there was a significant negative correlation between in vivo CZN AUC<sub>inf</sub> and hepatic CYP2E1 protein, indicating a relationship between lower in vivo CZN AUC<sub>inf</sub> and higher CYP2E1 protein levels. B, nicotine treatment increased CYP2E1 protein levels by 1.35-fold (nonsignificant, p = 0.07).
Control-treated African green monkeys had similar CZN disposition compared with humans. Studies that administered 500-mg CZN doses (approximately 7 mg/kg for an average 70-kg person) to healthy human volunteers reported AUC_\text{inf} from 25 to 34 µg × h/ml and a C_{\text{max}} of 11 µg/ml (Girre et al., 1994; Dreisbach et al., 1995). Control-treated monkeys given a 7-mg/kg CZN dose had an average AUC_\text{inf} of 19.7 ± 4.5 µg × h/ml and an average C_{\text{max}} of 9.2 ± 2.1 µg/ml. Monkey CZN t_{1/2} (0.57 ± 0.07 h) was faster than humans (1.45 ± 0.44 h) (Dreisbach et al., 1995), and monkey CZN and 6OHCZN T_{\text{max}} values were 2-fold smaller than values reported for humans (Frye et al., 1998). The African green monkeys studied here have a 1.5-fold higher turnover rate for CYP2E1-mediated CZN metabolism compared with humans (Court et al., 1997), which can contribute to the faster half-life and T_{\text{max}}. In humans, the metabolic ratio (6OHCZN/CZN) is optimal for detection of CZN metabolism at 2 through 4 h post-CZN (Frye et al., 1998). In African green monkeys, we observed significant correlations between the log metabolic ratio calculated at 0.5, 1, 2, and 4 h with CZN AUC_\text{inf} (p = 0.01–0.0001) (Table 2), further indicating that these monkeys are a good model of human CZN metabolism.

The Hill coefficient of 0.99 for in vitro CZN metabolism suggested that only one catalytic site was involved in CZN metabolism, providing further support for the specificity of CYP2E1-mediated CZN metabolism in these monkeys. In vitro CZN metabolism is mediated by CYP2E1 in humans (Lucas et al., 1999), rats (Kobayashi et al., 2002), and cynomolgus monkeys (Amato et al., 1998), consistent with the single-site kinetics observed in these African green monkeys and further supporting the role of CYP2E1 as the sole enzyme importantly involved in CZN metabolism.

The characteristics of in vitro CZN metabolism in African green monkeys are similar to humans. In humans, the K_m ranges from 77 to 149 µM (Court et al., 1997; Lejus et al., 2002), and in African green monkeys, the K_m was 95.4 µM. In humans, the V_{\text{max}} ranges from 1.43 to 3.19 pmol/min/µg (Court et al., 1997; Lejus et al., 2002; Muzeeb et al., 2005); the V_{\text{max}} in African green monkeys was 3.5 pmol/min/µg, and in cynomolgus monkeys it ranges from 0.52 to 3.38 pmol/min/µg (Court et al., 1997; Tanaka et al., 2000). The amount of CYP2E1 protein detected in microsomes from African green monkeys is similar to the levels of CYP2E1 in human microsomes, assuming equal detection of the two proteins by the antibody, consistent with the similar V_{\text{max}} values for CZN metabolism between African green monkeys and humans (Court et al., 1997; Lejus et al., 2002; Muzeeb et al., 2005). Monkey CYP2E1 is mainly expressed around the central veins in liver tissue (Fig. 4), similar to the expression in humans (Cohen et al., 1997).

There were significant correlations between in vitro CYP2E1 protein content with in vivo CZN AUC_\text{inf} (p = 0.03) (Fig. 6) and between in vitro CYP2E1 protein content with velocity at 950 µM CZN (approximate V_{\text{max}}) (p = 0.004) (Fig. 7), indicating a consistent relationship between in vivo CZN AUC_\text{inf} in vitro CZN velocity, and CYP2E1 protein levels among the monkeys and strongly suggesting that the increase in in vivo CZN disposition is mediated by an increase in hepatic CYP2E1 protein, which is also reflected by the increased in vitro CZN metabolism.

Nicotine-treated monkeys had significantly increased in vivo CZN disposition. The amount of nicotine a 70-kg smoker receives per day is estimated to range from 0.2 to 1.1 mg/kg (Benowitz and Jacob, 1984). The total daily dose of nicotine administered to the monkeys (0.6 mg/kg/day) is similar to the average amount received by a smoker. Although the frequency of administration differs from smoking, this dosing regimen has previously been shown to alter other...
P450 to a similar extent to that seen in smokers (Schoedel et al., 2003).

In humans, periods of smoking increase in vivo CZN clearance compared with nonsmoking periods (Benowitz et al., 2003). Previous studies have shown that carbon monoxide does not increase CZN metabolism (Benowitz et al., 2003). During cigarette smoking, the CZN clearance increases by 24% (p < 0.05), whereas the half-life does not change, suggesting that cigarette smoking increases CZN first-pass metabolism (Benowitz et al., 2003). Chronic nicotine treatment in monkeys resulted in similar kinetic changes to those seen in humans with cigarette smoking, with a 52% decrease in CZN AUC

\[ V_{\text{max}} \]

and no change in half-life (Table 1). These data suggested that nicotine increased the first-pass metabolism and decreased the oral bioavailability of CZN, as seen in human smoking, where CZN clearance increased without a change in half-life. The decrease in CZN \( T_{\text{max}} \) and nonsignificant decrease in \( C_{\text{ss}} \) are consistent with a larger first-pass metabolism.

Differences in hepatic enzyme levels have been previously shown to alter these pharmacokinetic aspects of p.o. administered drugs. For example, subjects with higher hepatic CYP2D6 metabolism showed a decrease in debrisoquine AUC

\[ \text{in vitro} \]

but no change in half-life compared with subjects with lower CYP2D6 metabolism (Sloan et al., 1983; Dalen et al., 1999). This indicates that nicotine induced CYP2D6 levels in the nicotine-treated monkeys were similar to each other (1.27-fold compared with 1.35-fold, respectively) and to the increase in CZN clearance seen in smokers (1.24-fold) (Benowitz et al., 2003). There was no difference in velocity at 950 \( \mu \text{M CZN} \) (approximate \( V_{\text{max}} \)) and CYP2E1 protein levels in the nicotine-treated monkeys were similar to each other (Table 1). These effects of nicotine may be importantly increasing CYP2E1 metabolism of drugs and toxins such as alcohol, acetaminophen, and nitrosamines.

In conclusion, we assessed in vivo CZN disposition, in vitro CZN metabolism, and hepatic CYP2E1 protein levels within 12 monkeys. Monkeys are good models of human CYP2E1 activity and protein because the in vivo CZN disposition, in vitro CZN metabolism, and CYP2E1 expression are highly related to each other and similar to that seen in humans. Chronic nicotine treatment increased in vivo CZN clearance by increasing first-pass metabolism, likely via increased hepatic CYP2E1, which is reflected in the increased in vitro metabolism. These effects of nicotine may be importantly increasing CYP2E1 metabolism of drugs and toxins such as alcohol, acetaminophen, and nitrosamines.

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Hashimoto T, Yoneda M, Shimada T, Kurosawa M, and Terano A (2004) Intraportal nicotine administration without altering systemic metabolism. The increases in velocity at 950 \( \mu \text{M CZN} \) (approximate \( V_{\text{max}} \)) and CYP2E1 protein levels in the nicotine-treated monkeys were similar to each other (1.27-fold compared with 1.35-fold, respectively) and to the increase in CZN clearance seen in smokers (1.24-fold) (Benowitz et al., 2003). There was no difference in velocity at 95 \( \mu \text{M CZN} \) (approximate \( K_{\text{m}} \)) between control- and nicotine-treated monkeys, suggesting that nicotine treatment does not alter the affinity of the enzyme for CZN, but rather acts via an increase in hepatic CYP2E1 levels. The removal of nicotine during smoking cessation may reverse the induction of CYP2E1 protein and activity such that CYP2E1-metabolized drugs, such as acetaminophen, may require dose adjustments because of the decreased level of hepatic CYP2E1 (Frishman et al., 2006).

It is possible that the decrease in CZN AUC

\[ \text{in vitro} \]

may be caused by nicotine’s effect on hepatic blood flow, but this is unlikely. In rats, the decreased hepatic blood flow after intraperitoneal nicotine administration returns to baseline after 15 min of ceasing treatment (Hashimoto et al., 2004). The monkeys were administered CZN more than 12 h after the last nicotine treatment; therefore, any effect on hepatic blood flow should have ceased.

In this study, we did not investigate the mechanism of CYP2E1 induction by nicotine in monkeys. Our laboratory has shown that in rats, nicotine does not increase hepatic CYP2E1 by increasing transcription (Howard et al., 2001), by protein stabilization, or by regulation via cotinine, the primary metabolite of nicotine (Micu et al., 2003). Kocarek et al. (2000) have shown that translational efficiency of CYP2E1 mRNA is increased if the 5'-UTR region of the CYP2E1 mRNA is deleted. It is possible that chronic nicotine treatment results in a direct or indirect interaction with the 5'-UTR region of CYP2E1 mRNA to increase transcription.

Nicotine-treated monkeys also had a decrease in 60HCZN AUC

\[ \text{in vitro} \]

Table 1), suggesting an increase in 60HCZN disposition. 60HCZN is glucuronidated and excreted quickly compared with its rate of formation (Dreisbach et al., 1995). Cigarette smoking can increase glucuronidation in humans (Benowitz and Jacob, 2000), suggesting that nicotine may increase 60HCZN glucuronidation, leading to the observed increase in 60HCZN disposition.

Nicotine induction of CYP2E1 is of interest because it could result in metabolic cross-tolerance via increased metabolism of alcohol by CYP2E1. In humans, smokers have faster alcohol metabolism than nonsmokers (Kopun and Propping, 1977; Morabia et al., 1999), and smoking increases alcohol consumption (Barrett et al., 2006). In rats, nicotine increases ethanol consumption and can result in reinstatement of alcohol-seeking behavior (Le et al., 2003). This suggests that nicotine, for example via cigarette smoking, can increase CYP2E1 levels, which may result in increased alcohol metabolism, possibly leading to increased consumption of alcohol.


