Independent and Combined Effects of Ethanol Self-Administration and Nicotine Treatment on Hepatic CYP2E1 in African Green Monkeys

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ABSTRACT:
Cytochrome P450 2E1 metabolizes ethanol and also bioactivates many toxins and procarcinogens. Elevated levels of hepatic CYP2E1 are associated with an increased susceptibility to chemical toxicity and carcinogenesis. This study investigated the induction of hepatic CYP2E1 by ethanol and nicotine, alone and in combination, in a nonhuman primate model. Monkeys that self-administered ethanol and that received subcutaneous injections of nicotine (0.5 mg/kg b.i.d.), alone and in combination, were compared with control animals (four groups, n = 10/group). Chlorzoxazone (CZN) was used as a probe drug to phenotype in vivo CYP2E1 activity before and after chronic ethanol and/or nicotine exposure. CYP2E1 protein levels and in vitro chlorzoxazone metabolism were assessed in liver microsomes. Average daily ethanol consumption was ~3.0 g/kg (blood ethanol levels ~24 mM) and was unaffected by nicotine treatment. Ethanol self-administration and nicotine treatment, alone and in combination, significantly increased in vivo CZN disposition compared with that in control animals. The effect of ethanol was only observed at higher levels of intake. Ethanol and nicotine increased CYP2E1 protein levels and in vitro CZN metabolism, with combined exposure to both drugs resulting in the greatest increase. The effect of ethanol was also dependent on level of intake. Chronic exposure to ethanol and nicotine induced hepatic CYP2E1 activity and protein levels, particularly when both drugs were used in combination and when ethanol intake was high. These results have important implications for public health, given the association between elevated CYP2E1 and disease, and the large proportion of individuals who are exposed to ethanol and nicotine, often in combination.

Introduction
Cytochrome P450 2E1 is a drug-metabolizing enzyme that is responsible for the biotransformation of numerous low-molecular-weight compounds, including ethanol, several commonly used industrial solvents, environmental pollutants, and various clinical drugs (Lieber, 1997). Many of these substrates are procarcinogens or cytotoxins that are bioactivated by CYP2E1. CYP2E1 is also known to generate high levels of reactive oxygen species that can cause cell damage via lipid peroxidation and DNA strand breaks (Caro and Cederbaum, 2004). This study was supported by the Centre for Addiction and Mental Health; Canadian Institute of Health Research [MOP97751]; Canadian Foundation for Innovation [20289 and 16014]; Ontario Ministry of Research and Innovation; Canada Research Chair in Pharmacogenetics (to R.F.T.); Canadian Liver Foundation and Scholarship Program for Interdisciplinary Capacity Enhancement. R.F.T. has shares in Nicogen Research Inc. Funds were not received from Nicogen for these studies, nor was the manuscript reviewed by individuals associated with Nicogen.

Elevated levels of CYP2E1 are associated with increased susceptibility to chemical toxicity and carcinogenesis. Several polymorphisms have been identified in the human CYP2E1 gene. Individuals with the CYP2E1 Rsal c2 allele, associated with increased CYP2E1 transcriptional activity (Hayashi et al., 1991), are more susceptible to toxicity from industrial chemicals bioactivated by CYP2E1 such as vinyl chloride (Wang et al., 2010) and n-hexane (Zhang et al., 2006). This and other CYP2E1 genetic variants have been associated with increased risk for hepatocellular (Munaka et al., 2003), colorectal (Morita et al., 2009), and esophageal cancer (Liu et al., 2007).

Hepatic CYP2E1 can be induced by a variety of compounds, many of which are substrates. Ethanol is an inducer of hepatic CYP2E1 protein and activity in humans and monkeys (Lieber, 1997; Ivester et al., 2007). Smoking increases CYP2E1 activity in humans (Benowitz et al., 2003), and chronic nicotine treatment increases hepatic CYP2E1 protein levels and activity in monkeys (Lee et al., 2006b).

Approximately 90% of smokers also consume alcohol (Shiffman and Balbanis 1995), yet little is known about the combined effects of ethanol and nicotine on hepatic CYP2E1 levels. It was previously shown that rats exposed to both ethanol and nicotine had significantly greater levels of hepatic CYP2E1 protein compared with rats exposed to either drug alone (Yue et al., 2009). This enhancement of CYP2E1 protein was partially attributed to a nicotine-stimulated increase in
ethanol consumption. There was no indication as to how the observed increases in CYP2E1 protein levels would affect in vitro and in vivo CYP2E1 activity. Understanding the impact of ethanol and nicotine on CYP2E1-mediated metabolism is crucial, given the large proportion of the population that is exposed to both drugs and the potential for elevated CYP2E1 to cause toxicity and disease.

The African green monkey (AGM) has been established as an excellent animal model of human CYP2E1 expression and activity (Lee et al., 2006b). Chlorzoxazone (CZN), a clinically used muscle relaxant that is metabolized by CYP2E1 to 6-hydroxychlorzoxazone (6OHCZN), is a validated probe drug for the measurement of CYP2E1 activity in both humans and monkeys (Ernstgård et al., 2004; Lee et al., 2006b). As in other species, CZN 6-hydroxylation in monkey liver microsomes can be inhibited by anti-CYP2E1 antibodies and selective chemical inhibitors of CYP2E1, providing evidence that CYP2E1 is the primary enzyme involved in the 6-hydroxylation of CZN (Amato et al., 1998). AGMs are also useful in modeling human alcohol consumption. These monkeys will voluntarily self-administer alcohol at levels comparable to human consumption and are therefore routinely used in alcohol research (Palmour et al., 1997).

We investigated the independent and combined effects of ethanol self-administration and nicotine treatment on in vivo CZN disposition, hepatic CYP2E1 protein levels, and in vitro CZN metabolism. We hypothesized that ethanol and nicotine would independently induce CYP2E1 levels, resulting in increased CZN clearance in vivo and in vitro CZN metabolism. In addition, combined ethanol and nicotine exposure was hypothesized to result in greater induction of CYP2E1 protein and in vitro CZN activity compared with those of either drug alone, owing to both the direct effects of the inducers and a nicotine-mediated increase in alcohol consumption. The induction of CYP2E1 by ethanol is dose-dependent in humans (Millonig et al., 2011); therefore, we also investigated whether the effects of ethanol were dependent on the level of alcohol intake.

Materials and Methods

Materials. CZN and 2-benzoxazolinone were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). All other chemicals were obtained from standard commercial sources. Protein estimation was performed with dye reagent purchased from Bio-Rad Laboratories (Hercules, CA). Prestained molecular weight protein markers were purchased from MBI Fermentas (Flamborough, ON, Canada). Hydantoin nitrocellulose membrane was purchased from Pall Corporation (Pensacola, FL). Human cDNA-expressed CYP2E1, CYP2A6, CYP2A1, CYP2A2, CYP2D6, CYP3A4, and CYP2B6 were purchased from BD Biosciences (San Diego, CA). Polyclonal anti-rat CYP2E1 antibody was purchased from Fitzgerald Industries (Acton, MA). Horseradish peroxidase-conjugated anti-sheep secondary antibody was purchased from Millipore Corporation (Billerica, MA). Chemiluminescent substrate was purchased from Thermo Fisher Scientific (Mississauga, ON, Canada). Autoradiographic film was purchased from Ulitdent (St. Laurent, PQ, Canada).

Animals. Adult male African green monkeys (verrets, Chlorocebus aethiops) were housed outdoors in social groups at Caribbean Primates Ltd. (St. Kitts). They were acquired from a large, isolated, and nonendangered Caribbean population (Palmour et al., 1997). Monkeys were given standard rations of Purina monkey Chow supplemented with fresh fruit and vegetables twice a day and were allowed to feed ad libitum. Drinking water was also available ad libitum.

Drug Treatment. The study timeline is shown in Fig. 1. The first 14 days of the study consisted of an ethanol preference screening phase, where monkeys were given access to 10% v/v alcohol 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 selected and randomized into four groups based on daily ethanol consumption (n = 10/group). The following 14 days (days 15–28) consisted of a washout period, during which monkeys had no exposure to ethanol or nicotine. During the second phase of the study, from days 29 to 42, monkeys in the ethanol-only (group 2) and ethanol + nicotine (group 4) groups were allowed to self-administer 10% alcohol in 0.5% sucrose solution for 4 h/day, whereas the other groups (1 and 3) consumed 0.5% sucrose solution on the same schedule. During the third phase of the study, from days 43 to 63, in addition to alcohol (or sucrose), monkeys in the nicotine-only (group 3) and ethanol + nicotine (group 4) were given subcutaneous injections of nicotine bitartrate (milligram base in saline, pH 7.0) twice daily at a dose of 0.05 mg/kg on day 43, 0.1 mg/kg on day 44, 0.25 mg/kg on day 45, and 0.5 mg/kg for subsequent days. The first injection was given 30 min before the alcohol (or sucrose) access period. The second injection was given 10 h later. Monkeys in the ethanol-only (group 2) and control (group 1) groups were given saline injections (as a vehicle control for nicotine bitartrate) on the same schedule. On day 50, nicotine treatment and alcohol access were suspended to conduct pharmacokinetic testing. Monkeys received 7 mg/kg CZN intragastrically under ketamine anesthesia, and blood samples were drawn at t = 10 min (10 min before CZN administration) and at 10, 20, 30, 60, 120, 240, and 360 min after CZN administration. Blood samples (2 ml) were drawn immediately after the alcohol access period on days 38 and 59 to determine blood ethanol levels. All blood samples were centrifuged, and the plasma was removed and frozen on dry ice.

Drug treatment was suspended near the end of phase II and phase III. Blood ethanol levels (BELs) were measured near the end of phase II and phase III. In vivo CZN metabolism was assessed during the washout period before phase II and at the end of phase III. Blood ethanol levels (BELs) were measured at the end of the study (one-way ANOVA, F(1, 9) = 0.3784, p > 0.05). There were no significant differences in body weights among groups at the start of the study (one-way ANOVA, F(3, 36) = 5.7, p < 0.05).
CYP2D6, CYP3A4, and CYP2B6 were used as positive or negative controls. Immunoblotting assays. To determine cross-reactivity of the primary antibody, a standard curve and to establish the linear detection range for the 6-OH-CZN were 90.4 and 99.4%, respectively.

Mobile phase consisted of 50 mM ammonium acetate (adjusted to pH to 4.0 with perchloric acid (120 M)). The sample was shaken for 30 min and centrifuged at 3500g for 15 min, and the organic phase was evaporated to dryness at 37°C. The sample was reconstituted into 110 µl of mobile phase consisting of 50 mM ammonium acetate (adjusted to pH 4.0 with 1 M glacial acetic acid)-acetone (65:35). CZN was measured by high-performance liquid chromatography with UV detection at 287 nm. An Agilent Zorbax SB-C18 column (5 µm, 4.6 × 250 mm; Agilent Technologies, Santa Clara, CA) was used to separate CZN and 2-benzoxazolinone using a flow rate of 0.7 ml/min. The retention times for CZN and 2-benzoxazolinone were 18.5 and 10.1 min, respectively.

Microsomal Membrane Preparation. Monkey liver tissue 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 assessments and then centrifuged at 12,500g for 30 min at 4°C. The supernatant was then centrifuged at 110,000g 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 KCl, and 20% v/v glycerol for immunoblotting or 1.15% w/v KCl for in vitro metabolism assessments. The protein content of liver microsomes was assayed with the Bradford (1976) technique using a Bio-Rad Protein Assay kit. Microsomes were stored at −80°C.

In Vitro CZN and 6OH-CZN Assessments. CZN 6-hydroxylation was assayed according to the protocol established by Leclercq et al. (1998), in which the protein concentration and incubation times were optimized for linear formation of 6OH-CZN. Monkey hepatic microsomal protein (0.4 mg) was mixed with 0.1 M Tris buffer at pH 7.6, 10 mM magnesium chloride, 5 mM NADPH, and 950 µM CZN to a final volume of 500 µl. The reaction mixture was incubated for 20 min at 37°C. 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 after incubation 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) with UV detection at 287 nm. The mobile phase consisted of 50 mM ammonium acetate (adjusted to pH 4.0 with 1 M glacial acetic acid)-acetone (65:35) with 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. The absolute and relative recoveries of 6OH-CZN were 90.4 and 99.4%, respectively.

Immunoblotting. Monkey liver microsomal protein was serially diluted to generate a standard curve and to establish the linear detection range for the immunoblotting assays. To determine cross-reactivity of the primary antibodies, cDNA-expressed human CYP2E1, CYP2A6, CYP1A1, CYP2A2, CYP2D6, CYP3A4, and CYP2B6 were used as positive or negative controls.

Liver microsomal proteins (4 µg) were separated by SDS-polyacrylamide gel electrophoresis (4% stacking and 8% separating gels) and then were transferred overnight into nitrocellulose membranes. Gels were stained with Coomassie Blue R-250 to ensure equal loading of protein among lanes. To detect hepatic CYP2E1, 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 anti-CYP2E1 antibody diluted 1:1000 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-rabbit antibody 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 using chemiluminescence followed by exposure to autoradiography film. Immunoblots were analyzed using MCID Elite software (InterFocus Imaging Ltd., Linton, UK), and the relative density of each band was expressed as arbitrary density units after background was subtracted.

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 (Orbiso, Inc., Richmond Hill, ON, Canada) stained with ethidium bromide and inspection of the 28S and 18S ribosomal bands. cDNA was synthesized using 1 µg of total RNA, random hexamers (Invitrogen), Ribolock RNase inhibitor (Fermentas, Burlington, ON, Canada) and MMLV Reverse Transcriptase (Invitrogen) according to protocols provided by the manufacturers. Primers for real-time PCR amplification of CYP2E1 and β-actin were as follows: CYP2E1 forward primer (CYP2E1ex1), 5′-CCG CCT CCC ATC ATC GGC AAC-3′; CYP2E1 reverse primer (CYP2E1ex1), 5′-GGG CTC TCA CCC CAG TTT ATC-3′; β-actin forward primer (ACTBex3), 5′-CAG AGC AAG AGA AGA GTC CT-3′; and β-actin reverse primer (ACTBex4), 5′-GTT CTC AAA CAT GAT CTG GTT C-3′. The sequence of CYP2E1 in African green monkey is not known; primer specificity was based on human CYP2E1 and rhesus macaque (Macaca mulatta) CYP2E1. Amplification and fluorescence detection were performed 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 1 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. CYP2E1 mRNA levels were obtained by normalization to β-actin and use of the comparative CT method for relative quantification as described by the manufacturer (Real-Time PCR Chemistry Guide; Invitrogen).

Statistics. Differences in alcohol (or sucrose) consumption and blood ethanol levels (BELs) were assessed by one-tailed Student’s t test, unpaired tests for between-group comparisons, and paired tests for within-group comparisons. All pharmacokinetic statistical analyses were performed using SAS software (version 8.2; SAS Institute, Cary, NC). One-tailed paired Student’s t tests were used to assess differences in vivo CZN pharmacokinetic parameters measured before and after drug administration. A one-tailed unpaired Student’s t test was used to compare the change in CZN AUC0–t,h among groups. One-way ANOVA followed by post hoc tests (Kruskal-Wallis test and test for linear trend) were used to determine differences in CYP2E1 protein levels, mRNA levels, and in vitro CZN metabolism between groups. Correlations were calculated with Pearson correlation coefficients.

Results

Monkeys Voluntarily Self-Administered Ethanol. Monkeys consistently self-administered high levels of 10% ethanol throughout the study (Fig. 2A). Mean daily ethanol consumption during the 4-hour ethanol access sessions in phases II and III ranged from 23.6 to 54.6 mg/kg (1.9–4.4 g ethanol/kg), resulting in average consumption of 38.12 ± 7.8 ml/kg (3.0 g ethanol/kg). A gradual increase in mean daily ethanol consumption was observed as monkeys progressed from phase II to phase III of the study. This increase was statistically significant for both the ethanol-only (p < 0.001) and the ethanol + nicotine groups (p = 0.04) (Table 1). There was no significant difference in mean ethanol consumption between the ethanol-only and the ethanol + nicotine groups (phase III; Table 1), indicating no effect of nicotine treatment on voluntary alcohol consumption. Sucrose consumption remained constant throughout the study.

Considerable individual variation in alcohol intake was observed. Monkeys were divided into high and low ethanol consumers by performing a median split based on mean daily consumption of ethanol during phases II and III (Fig. 2B; Table 1). BELs were measured on two occasions, once during phase II (day 38) and again during phase III (day 59). A paired t test revealed no significant difference in BELs measured during phase II and phase III.
Fig. 2. Monkeys voluntarily self-administered ethanol. A, average daily consumption by monkeys self-administering alcohol in sucrose (squares) or sucrose alone (circles). Phase I is the ethanol (EtOH) preference screening period (consumption levels at screening represent average consumption during this phase). During phase II monkeys in the ethanol-only (group 2) and ethanol + nicotine (group 4) groups self-administered ethanol in sucrose solution, whereas the other groups (1 and 3) consumed sucrose solution only on the same schedule. During phase III in addition to alcohol (or sucrose), monkeys in the nicotine-only (group 3) and ethanol + nicotine (group 4) groups were given nicotine injections, whereas monkeys in the other groups (1 and 2) were given saline injections on the same schedule. B, a median split was used to categorize monkeys into low and high ethanol consumers based on mean daily consumption of 10% ethanol during phases II and III. B, Open symbols represent monkeys in the ethanol-only group (group 2), filled symbols represent monkeys in the ethanol + nicotine group (group 4), and horizontal lines indicated group means. Significantly different from low ethanol consumers: *p < 0.05.

for either the ethanol-only and ethanol + nicotine groups or for the high and low consumer groups, despite increases in ethanol consumption (Table 1).

In Vivo CZN Disposition Is Influenced by Nicotine Treatment and the Level of Daily Ethanol Intake. A within-animals design was used to assess changes in in vivo CZN metabolism due to animal variation in CZN pharmacokinetics. A comparison of in vivo CZN metabolism before and after drug administration indicated that the nicotine-only group had a 34% decrease in CZN AUC (p = 0.002) and a 42% reduction in the maximum plasma CZN concentration (Cmax) (p = 0.001) compared with values before treatment (Table 2). Mean values for all the assessed CZN pharmacokinetic parameters were not significantly altered in the ethanol-only group; however, several monkeys showed a substantially decreased CZN AUC after ethanol self-administration (Fig. 3). In the ethanol + nicotine group, there was a significant reduction in CZN AUC by 27% (p = 0.02), Cmax by 35% (p = 0.003), and the time to maximum CZN concentration (Tmax) by 35% (p = 0.04). Kinetic parameters were not significantly different among groups before drug administration (day 22), and control monkeys (group 1) did not show significant changes in CZN pharmacokinetic parameters assessed on day 22 compared with those on day 50 (Supplemental Fig. 1).

T A B L E 1
Consumption of 10% ethanol increase over time with no increase in blood ethanol levels

<table>
<thead>
<tr>
<th>Phase II</th>
<th>Phase III</th>
<th>Consumption</th>
<th>BEL</th>
<th>Consumption</th>
<th>BEL</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>ml/kg per day</td>
<td>mmol/l</td>
<td>ml/kg per day</td>
<td>mM/l</td>
</tr>
<tr>
<td>Group 3: EtOH-only</td>
<td></td>
<td>35.4 ± 1.2</td>
<td>24.6 ± 3.9</td>
<td>42.4 ± 1.9*</td>
<td>26.3 ± 9.3</td>
</tr>
<tr>
<td>Group 4: EtOH + nicotine</td>
<td></td>
<td>37.8 ± 2.2</td>
<td>23.8 ± 2.1</td>
<td>40.7 ± 3.1*</td>
<td>29.7 ± 4.7</td>
</tr>
<tr>
<td>Low consumers</td>
<td></td>
<td>32.9 ± 1.6</td>
<td>22.9 ± 3.8</td>
<td>35.5 ± 1.6*</td>
<td>24.7 ± 4.0</td>
</tr>
<tr>
<td>High consumers</td>
<td></td>
<td>37.3 ± 1.6†</td>
<td>25.5 ± 2.2</td>
<td>45.1 ± 1.9*</td>
<td>31.7 ± 4.0</td>
</tr>
</tbody>
</table>

EtOH, ethanol.
* p < 0.05, significantly different from phase II.
† p < 0.05 and ‡ p < 0.001, significantly different from low consumer.

There was a significant correlation between post-ethanol CZN AUC0–6 h and mean daily consumption of 10% ethanol (r = 0.42, p = 0.03) (Fig. 4A). The high ethanol consumers had a post-ethanol CZN AUC0–6 h of 34 h · µg/ml, which was significantly lower than the CZN AUC0–6 h of 64 h · µg/ml seen in the low ethanol consumers (p < 0.05) (Fig. 4B). Only in the high consumer group was there a significant change in CZN AUC after ethanol consumption (p < 0.05) (Fig. 4C). Taken together, these results show a reduction in CZN AUC by ethanol that is dependent on the level of intake.

Hepatic CYP2E1 Protein Levels Are Induced by Ethanol and Nicotine, Particularly When Both Drugs Are Present in Combination and at Higher Ethanol Intakes. An immunoblotting assay was established to measure CYP2E1 protein levels in monkey liver. Detection of CYP2E1 in serially diluted liver microsomal protein from a control monkey was shown to be linear from 2 to 20 µg of protein (Fig. 5A). All subsequent immunoblots were loaded with 5 µg of microsomal protein. The CYP2E1 antibody did not cross-react with other cDNA-expressed human cytochromes P450 and monkey hepatic CYP2E1 comigrated with cDNA-expressed human CYP2E1 (Fig. 5B).

Compared with control monkeys, ethanol self-administration alone resulted in a 56% increase in CYP2E1 levels (p < 0.05), nicotine treatment alone resulted in a 55% increase (p < 0.05), and combined ethanol self-administration and nicotine treatment resulted in a 106% increase (p < 0.001), suggesting an additive effect (Fig. 6, A and B). In comparing mean CYP2E1 levels across all the groups, there was a significant linear trend: ethanol or nicotine exposure alone increased CYP2E1 levels compared with no drug exposure, and CYP2E1 levels were further increased with combined exposure to both drugs (pdrug = 0.0002). Ethanol and nicotine exposure, either alone or in combination, did not significantly alter CYP2E1 mRNA levels in the liver (F1,33 = 2.423, p = 0.083).

The effect of ethanol consumption level on CYP2E1 protein levels was also examined. High ethanol consumers had a 97% increase in CYP2E1 levels relative to those of control monkeys, whereas low ethanol consumers had a 65% increase. In comparing mean CYP2E1 levels among monkeys in the control, low ethanol consumer, and high ethanol consumer groups, there was a significant linear trend, indi-
cating increasing CYP2E1 protein levels with higher levels of ethanol consumption ($p_{\text{trend}} = 0.0004$) (Fig. 6C). CYP2E1 mRNA levels in the liver were not significantly different among the high ethanol consumer, low ethanol consumer, or control animals [$F(2, 27) = 2.349$, $p = 0.1147$].

In Vitro CZN Metabolism Is Induced by Ethanol and Nicotine, Particularly When Both Drugs Are Present in Combination and at Higher Ethanol Intakes. The rate of CZN metabolism to 6OH-CZN was measured in vitro using monkey liver microsomes. There was a significant positive correlation between hepatic CYP2E1 levels and in vitro 6OH-CZN formation velocity at 950 μM (approximate $V_{\text{max}}^r$: $r = 0.45$, $p = 0.002$) (Fig. 7A). Compared with control monkeys, ethanol self-administration alone and nicotine treatment alone resulted in an 11% increase in 6OH-CZN formation velocity, although these increases were not significant after post hoc testing ($p > 0.05$) (Fig. 7B). Combined ethanol self-administration and nicotine treatment resulted in a 21% increase ($p < 0.05$), suggesting an additive effect. In comparing mean CZN metabolism velocity across all the groups, there was a significant linear trend, with monkeys in the ethanol and nicotine combined group having the highest rate of CZN metabolism ($p_{\text{trend}} = 0.003$). The impact of ethanol consumption level on in vitro CZN metabolism was investigated. High ethanol consumers had a 19% increase in velocity of CZN metabolism compared with control monkeys, whereas the low ethanol consumers had a 13% increase. In comparing mean CZN metabolism velocity among monkeys in the control, low ethanol consumer, and high ethanol consumer groups, there was a significant linear trend, demonstrating that the rate of CZN metabolism increases with higher levels of ethanol consumption ($p_{\text{trend}} = 0.003$) (Fig. 7C).

### Discussion

Humans and AGMs have similar hepatic CYP2E1 levels, in vivo CZN disposition, and in vitro CZN metabolism (Hayashi et al., 1991; Lee et al., 2006b), and although the AGM CYP2E1 has not been sequenced, CYP2E1s of both the cynomolgus monkey (Macaca fascicularis) and the rhesus monkey (M. mulatta), which are closely related to the AGM, have a greater than 90% amino acid homology to human CYP2E1. Thus, AGMs offer a valuable model for predicting the impact of ethanol and nicotine on CYP2E1 induction and metabolism in humans.

Monkeys consistently self-administered high levels of ethanol throughout the study. Previous alcohol research classified AGMs

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### Table 2

<table>
<thead>
<tr>
<th>CZN Parameter</th>
<th>EtOH</th>
<th>Nicotine</th>
<th>EtOH + Nicotine</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>AUC (hr × μg/ml)</td>
<td>54.8 ± 7.9</td>
<td>56.6 ± 13.6</td>
<td>44.8 ± 6.2</td>
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<tr>
<td>$C_{\text{max}}$ (μg/ml)</td>
<td>17.5 ± 2.7</td>
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<tr>
<td>$t_{\text{max}}$ (h)</td>
<td>1.3 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>0.9 ± 0.1</td>
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</table>

EtOH, ethanol.

* $p < 0.05$, significantly different from before drug administration within the same treatment group.
voluntarily self-administering between 0.8 and 3.5 g/kg ethanol/day as moderate consumers (Palmour et al., 1997). In our study, average daily ethanol intake was approximately 3.0 g/kg, identifying these monkeys as moderate consumers. Moderate alcohol consumption in humans has been described as intakes resulting in BELs ranging from 5 to 20 mM (Eckardt et al., 1998). In our study, monkeys achieved an average BEL of 26.25 mM, comparable to moderate consumption in humans; use of BELs to compare ethanol consumption takes into consideration the 2-fold greater rate of ethanol elimination in AGMs compared with humans (Ervin et al., 1990).

Ethanol consumption steadily increased throughout the study, whereas BEL remained unchanged over time, suggesting increased rates of ethanol elimination in the monkeys. In humans, rats, and other mammals, ethanol metabolism is primarily mediated by alcohol dehydrogenase and to a lesser extent by CYP2E1 (Matsumoto et al., 1996; Lands, 1998). Human alcoholics have higher levels of CYP2E1, no change in alcohol dehydrogenase, and an increased capacity to eliminate ethanol compared with nonalcoholic humans, suggesting that elevated CYP2E1 levels can affect ethanol metabolism (Vidal et al., 1990). On the basis of this premise, induction of CYP2E1 in the ethanol-consuming monkeys may have contributed to metabolic tolerance, allowing monkeys to consume more ethanol without a corresponding rise in BELs.

Nicotine treatment did not affect voluntary consumption of 10% ethanol in AGM. Some studies in rats have shown an increase in ethanol consumption with chronic or repeated nicotine treatment, whereas others have shown that nicotine treatment has no effect or even decreases ethanol consumption (Blomqvist et al., 1996; Lê et al., 2000; Olausson et al., 2001; Sharpe and Samson, 2002). Differences in rat strains, methods used to initiate ethanol consumption, and duration of the ethanol access period may contribute to the inconsistent results. A limited number of human studies have investigated the effect of nicotine on alcohol consumption. Occasional smokers (smoking an average of 10 cigarettes/week and only smoking on 2 days/week) consumed more alcohol when they smoked nicotine-containing cigarettes compared with denicotinized cigarettes (Barrett et al., 2006). A similar study in regular smokers (smoking more than 10 cigarettes/day) showed that smokers deprived of nicotine had a greater urge to consume alcohol compared with nondeprived smokers (Cooney et al., 2003). Therefore, the effect of nicotine on ethanol consumption in either rats or humans is complex and requires further investigation. Our study is the first to look at the effect of nicotine on voluntary ethanol self-administration in monkeys in which we observed no significant increase or decrease in ethanol consumption from the nicotine administration under the conditions tested.

Here we show that variation in ethanol intake, within the range of moderate drinking, can lead to very different CYP2E1 levels and activity as measured by CZN metabolism. High ethanol-consuming monkeys were only drinking 25% more ethanol per day than the low ethanol consumers; however, they demonstrated nearly a 50% greater reduction in postethanol CZN AUC in vivo. These results are interesting from a public health perspective, because our results suggest...
that among moderate consumers, those with higher daily ethanol intakes may be at elevated risk for CYP2E1-associated diseases.

The average smoker has a total daily nicotine intake of 0.2 to 1.1 mg/kg (Benowitz and Jacob, 1984), resulting in plasma levels of 10 to 50 ng/ml during the day (Benowitz et al., 1990). The total daily nicotine dose administered to the monkeys was at the high end of this range (1.0 mg/kg per day) to compensate for the slightly faster nicotine metabolism in AGMs (Lee et al., 2006a). Although the pattern of nicotine intake in this study differs from smoking, the levels and duration of nicotine in the plasma were estimated to be comparable to those observed in human smokers (Benowitz et al., 1990; Lee et al., 2006a). Nicotine increased CYP2E1 protein, CYP2E1 in vitro activity, and in vivo CZN clearance in the monkeys, consistent with the results of a previous study that assessed the effect of chronic nicotine treatment on CYP2E1 and CZN disposition in African green monkeys (Lee et al., 2006b). The increase in CZN clearance after chronic nicotine exposure, without any change in CZN half-life, suggests that nicotine increases CZN first-pass metabolism. It is unlikely that the change in CZN clearance could be attributed to the effect of nicotine on blood flow. Monkeys were administered CZN more than 12 h after the last nicotine injection, at which point any effect on hepatic blood flow would have subsided (Hashimoto et al., 2004). In humans, smoking increases CZN clearance by 25% (Benowitz et al., 2003). Nicotine-treated monkeys, likewise, showed a 34% decrease in CZN AUC, supporting a role for nicotine as the CYP2E1-inducing agent in cigarette smoke. Our results are not consistent with the conclusions from a recent study showing that administration of a 42-mg transdermal nicotine patch twice a day for 10 days did not affect CZN clearance in humans (Hukkanen et al., 2010). This discrepancy may be due to differences in the dose and duration of nicotine achieved in the plasma with a transdermal nicotine patch versus smoking, which our study was designed to model.

There was a trend for monkeys in the ethanol and nicotine combined group having the highest CYP2E1 protein levels and fastest rates of in vitro CZN metabolism compared with monkeys in either the ethanol-only or nicotine-only groups. This trend was not present in the in vivo CZN pharmacokinetic parameters, which may be due to the timing of the CZN pharmacokinetic testing. The postdrug CZN challenge was performed on day 50 and protein levels and in vitro activity were assessed in tissue from monkeys sacrificed on day 64. Ongoing induction of CYP2E1 by ethanol and nicotine may have occurred between days 50 and 64 of the study, resulting in a lack of correlation between in vivo and ex vivo assessment of CYP2E1 activity. In humans, moderate consumption of alcohol (40 g/day) over a period of 4 weeks resulted in a gradual increase in CYP2E1 activity measured by CZN clearance. CZN clearance was significantly faster on day 28 than on to day 21 (Oneta et al., 2002). In rats, 7 days of nicotine treatment are sufficient to induce CYP2E1 levels in the liver; however, whether there is further induction of CYP2E1 beyond 7 days is not known (Joshi and Tyndale, 2006). In a previous study, a correlation between in vivo CZN clearance and CYP2E1 protein levels was shown in nicotine-treated monkeys; however, the duration between the in vivo and ex vivo testing was substantially shorter compared with that in this study (7 versus 14 days) (Lee et al., 2006b).

Hepatic CYP2E1 mRNA levels were not significantly altered by nicotine or ethanol exposure, suggesting that, at these doses and durations of ethanol and nicotine exposure, induction of CYP2E1 protein occurs via a nontranscriptional mechanism. Consistent with our findings, ethanol-treated rats with BELs similar to those of our monkeys showed induction of hepatic CYP2E1 without a corresponding increase in CYP2E1 mRNA levels (Ronis et al., 1993). Ethanol induces CYP2E1 protein levels in rats by protecting the enzyme from degradation (Roberts et al., 1995). Nicotine does not induce rat hepatic CYP2E1 via transcription or by protein stabilization and may
involves an increase in translational efficiency (Wu et al., 1997; Micu et al., 2003). Thus, ethanol and nicotine may increase CYP2E1 in monkey liver using post-transcriptional mechanisms similar to those observed for rat liver CYP2E1.

In conclusion, ethanol and nicotine increased hepatic CYP2E1 protein and in vitro CZN metabolism, leading to increased CZN clearance in vivo. The effect of ethanol was dependent on the level of daily ethanol intake and combined exposure to ethanol and nicotine resulted in the highest levels of hepatic CYP2E1 protein and activity. Nicotine treatment did not affect ethanol consumption. The induction of hepatic CYP2E1 by nicotine and ethanol may mediate some of the negative health effects of smoking and alcohol consumption via increased metabolic bioactivation of many commonly used industrial chemicals, environmental pollutants, and drugs into toxic metabolites (Lieber, 1997). Because a large proportion of the population is exposed to ethanol, nicotine, or both, these findings have important implications for public health, health risk assessment, and disease prevention.

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References


Lee AM, Micysk S, Palmour R, and Tyndale RF (2006a) CYP2B6 is expressed in African Green monkey liver.

Lee AM, Miksys S, Palmour R, and Tyndale RF (2006a) CYP2B6 is expressed in African Green monkey liver.

Lee AM, Miksys S, Sellers EM, Koop DR, and Tyndale RF (2003) Rat hepatic CYP2E1 is inducible and is involved in the production of carcinogenic DNA-lesions.


FIG. 7. Ethanol (EtOH) self-administration and nicotine treatment increased in vitro chlorzoxazone metabolism. A, hepatic CYP2E1 protein levels were positively correlated with 60OHZN formation velocity at 950 μM CZN (approximate Vmax). B, mean percentage increases in the 60OHZN formation velocity relative to the control group. A significant linear trend was observed, with animals in the ethanol + nicotine group (group 4) having the highest velocity. Significantly different from control group: * p < 0.05, n = 10/group. C, mean percentage increases in velocity of CZN metabolism in high and low ethanol consumers (Fig. 2B) relative to the control group. A significant linear trend was observed, demonstrating increased CZN velocity with higher levels of alcohol consumption. Significantly different from control group: * p < 0.05, n = 10/group.

Authorship Contributions

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