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Independent and combined effects of ethanol self-administration and nicotine treatment on

hepatic CYP2E1 in African Green monkeys

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Running Title: Ethanol and Nicotine Induce Hepatic CYP2E1

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ABSTRACT

Cytochrome P450 2E1 (CYP2E1) 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 non-human primate model. Monkeys self-administered ethanol and nicotine was administered as subcutaneous injections (0.5 mg/kg bid), alone and in combination compared to control animals (4 groups, N=10/group). Chlorzoxazone 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 mmol/L) and was unaffected by nicotine treatment. Ethanol self-administration and nicotine treatment, alone and in combination, significantly increased *in vivo* CZN disposition compared to 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 (CYP2E1) 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 pro-carcinogens 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).

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* RsaI 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 S., 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

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of hepatic CYP2E1 protein compared 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) is 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 (Ernstgard et al., 2004; Lee et al., 2006b). As in other species, CZN-6-hydroylation 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 involve 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 to either drug alone, due 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 if the effects of ethanol were dependent on the level of alcohol intake.

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METHODS

Materials. Chlorzoxazone (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). Hybond nitrocellulose membrane was purchased from Amersham Biosciences (Toronto, ON, Canada). Human cDNA expressed CYP2E1, CYP2A6, CYP2A1, CYP2A2, CYP2D6, CYP3A4 and CYP2B6 were purchased from BD Biosciences (Franklin Lakes, NJ). Polyclonal anti-rat CYP2E1 antibody was purchased from Fitzgerald Industries (Acton, MA). Horseradish peroxidase-conjugated anti-sheep secondary antibody was purchased from Millipore. (Billerica, MA). Chemiluminescent substrate was purchased from Pierce Chemical Company (Rockford, IL). Autoradiographic film was purchased from Ultident (St. Laurent, PQ, Canada).

Animals. Adult male African green monkeys (vervets, *C. aethiops*) were housed outdoors in social groups at Caribbean Primates Ltd (St Kitts). They were acquired from a large, isolated, and non-endangered 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 Figure 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 hours per day. Forty monkeys that voluntarily

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consumed more than 1g ethanol/kg/day were selected and randomized into four groups based on

daily ethanol consumption (n=10 per group). The following 14 days (day 15-28) consisted of a

washout period, where monkeys had no exposure to ethanol or nicotine. During the second

phase of the study, from day 29-42, 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 hours a day while the other groups (1 and 3) consumed 0.5% sucrose solution on the same

schedule. During the third phase of the study, from day 43-63, in addition to alcohol (or sucrose),

monkeys in the nicotine-only (group 3) and ethanol + nicotine (group 4) were given s.c 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 prior to the alcohol (or sucrose) access period. The second

injection was given 10 hours later. Monkeys in 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 was suspended in order to conduct

pharmacokinetic testing. Monkeys received 7 mg/kg CZN intragastrically under ketamine

anesthesia, and blood samples were drawn at t-10 min (10 minutes before CZN administration),

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 removed and frozen for subsequent

drug analyses. A commercial enzymatic assay kit (Sigma Diagnostics, St Louis, MO) was used

to determine plasma blood levels of ethanol. Body weights at the start of the study (average = 5.9

kg $\pm$ 0.5) were not significantly different from body weights at sacrifice (average = 5.7 kg $\pm$ 0.5)

(paired t-test, p>0.5). There was no significant differences in body weights between groups at the

start of the study (one way ANOVA, F (3, 36) = 0.3784, p>0.05) or at sacrifice (one way

ANOVA, F (3, 36) = 1.860, p>0.05). Animals were sacrificed by exsanguination via the femoral

artery under ketamine anesthesia and livers were immediately dissected and flash-frozen in

liquid nitrogen and stored at -80°C until further use. Upon visual inspection of the livers, there

were no apparent signs of injury or cirrhosis. 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 Plasma Assessments. Plasma CZN levels were assayed based on the methods in

Howard et al. (2001). Briefly, plasma was centrifuged at 3000*g* for 10 min, after which 100 µl

of the supernatant was removed and incubated with β-glucuronidase (10 mg/ml, 50 µl) overnight

at 37°C. Following incubation, internal standard of 2-benzoxazolinone (2.0 mM, 25 µl), 10%

perchloric acid (120 µl) and ethyl acetate/hexane (3 ml) were added, the sample was shaken for

30 min, centrifuged at 3500*g* for 15 min, and the organic phase was evaporated to dryness at

37°C. The sample was reconstituted into 110µl of mobile phase consisting of 50 mM ammonium

acetate (adjusted to pH to 4.0 with 1 M glacial acetic acid)/acetonitrile (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 x 250 mm) (Agilent Technologies, Palo Alto, 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 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 12,500g 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 DDT, 1.15% w/v KCl, and 20% v/v glycerol for immunoblotting or 1.15% w/v KCl for in vitro metabolism assays. The protein content of liver microsomes was assayed with the Bradford technique using a Bio-Rad Protein Assay kit. Microsomes were stored at –80°C.

In Vitro CZN and 6OHCZN Assessments. CZN 6-hydroxylation was assayed according to the protocol established by Leclercq et al., in which the protein concentration and incubation times were optimized for linear formation of 6OHCZN (Leclercq et al., 1998). 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 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 x 250 mm) (Agilent Technologies, Palo Alto, CA) with UV detection at 287 nm. The mobile phase consisted of 50 mM ammonium acetate (adjusted to pH to 4.0 with 1 M glacial acetic acid)/acetonitrile (65:35) with a flow rate of 0.7 ml/min. The retention times for CZN, 6OHCZN, and 2-benzoxazolinone were 18.5, 7.5, and 10.1 min, respectively. The absolute and relative recoveries of 6OHCZN 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 ug) were separated by SDS-polyacrylamide gel electrophoresis (4% stacking and 8% separating gels), and then transferred overnight onto 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, 0.1% v/v Triton X-100 for 1 hr. 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 hr and then incubated with peroxidase-conjugated rabbit anti-sheep antibody diluted 1:5000 for 1 hr, 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 subtracting background.

Isolation, cDNA synthesis and mRNA quantification. Liver tissue (50-100 mg) was homogenized in TRIzol reagent (Invitrogen) and total RNA was isolated according to the TRIzol reagent protocol. RNA concentrations were determined spectrophotometrically and total RNA integrity was confirmed by electrophoresis on a 1.2% agarose gel (OnBio, ON, Canada) stained

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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) 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 CTT CCC ATC ATC GGG AAC-3', CYP2E1 reverse primer (CYP2E1ex1): 5'-GCG CTT TCA CCG CCT TGT A-3', β-actin forward primer (ACTBFex3): 5'-CAG AGC AAG AGA GGC ATC CT- 3', β-actin reverse primer (ACTBRex4/): 5'-GGT CTC AAA CAT GAT CTG GGT 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 ABI ViiA7 Real-Time PCR system (Life Technologies). Real-time PCR amplification mixture (20 μl) contained 1 μl of synthesized cDNA, 10 μl of 2× Fast SYBR-Green Mix (Life Technologies), and 0.3 μM of each primer. Cycling conditions consisted of an initial activation of AmpliTaq Fast DNA polymerase followed by 40 cycles of denaturing (95°C for 15 s) and annealing/extension (58°C for 20s). Dilutions of monkey and human cDNA were used to determine the range of log linear detection. CYP2E1 mRNA levels were obtained by normalized to β-actin and using the comparative CT method for relative quantification as described by the manufacturer (Real-Time PCR Chemistry Guide, Life Technologies)

Statistics. Differences in alcohol (or sucrose) consumption and BEL 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

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version 8.2 (SAS Institute, Cary, NC). One-tailed paired Student's t-tests were used to assess

differences in *in vivo* CZN pharmacokinetic parameters measured pre and post-drug

administration. One tailed unpaired Student's t-test was used to compare the change in CZN

AUC_{0-6hr} among groups. One-way ANOVA followed by post-hoc tests (Kruskal Wallis 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.

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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 four hour sessions in phases II and III ranged from 23.6-54.6 ml/kg (1.9-4.4g ethanol/kg), resulting in average consumption of 38.12 ± 7.8 ml/kg (3.0g of 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 phase II and II (Fig. 2B and Table 1).

Blood ethanol levels (BEL) were measured on two occasions, once during phase II (day 38) and again during phase III (day 59). A paired-test reveals no significant difference in BEL measured during phase II and phase III 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

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A within animals design was use to assess changes in *in vivo* CZN metabolism due to animal variation in CZN pharmacokinetics. A comparison of *in vivo* CZN metabolism pre- and post-drug administration indicated that the nicotine-only group had a 34% decrease in CZN area under the curve (AUC) (p=0.002) and a 42% reduction in the maximum plasma CZN concentration (C_{max}) (p<0.001) compared to 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), C_{max} by 35% (p=0.003) and the time to maximum CZN concentration (T_{max}) by 35% (p=0.04). Kinetic parameters were not significantly different between groups pre-drug administration (day 22) and control monkeys (group 1) did not show significant changes in CZN pharmacokinetic parameters assessed on day 22 compared to day 50 (Supplemental Fig. 1).

There was a significant correlation between post-ethanol CZN AUC_{0-6h} and mean daily consumption of 10 % ethanol (r = 0.42, p = 0.03) (Fig. 4A). The high ethanol consumers had a post-ethanol CZN AUC_{0-6h} of 34 h.µg/ml, which was significantly lower than the CZN AUC_{0-6h} of 64 h.µg/ml seen in the low consumers (p<0.05) (Fig. 4B). Only in the high consumer group was there a significant change in CZN AUC post 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

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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 2-20 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 co-migrated with cDNA-expressed human CYP2E1 (Fig. 5B).

Compared to 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. 6A and 6B). Comparing mean CYP2E1 levels across all the groups, there is a significant linear trend; ethanol or nicotine exposure alone increased CYP2E1 levels compared to no drug exposure, and CYP2E1 levels were further increased with combined exposure to both drugs ($p_{trend}=0.0002$). Ethanol and nicotine exposure, either alone or in combination, did not significantly alter CYP2E1 mRNA levels in the liver ($F(3, 33)=2.423$, $p=0.083$).

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 control monkeys, while low consumers had a 65% increase. Comparing mean CYP2E1 levels among monkeys in the control, low ethanol consumer and high ethanol consumer groups, there was a significant linear trend, indicating increasing CYP2E1 protein levels with higher levels of ethanol consumption ($p_{trend} = 0.0004$) (Fig. 6C). CYP2E1 mRNA levels in the liver were not significantly different among the high consumer, low consumers or control animals ($F(2, 27)=2.349$, $p=0.1147$).

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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 6OHCZN was measured *in vitro* using monkey liver microsomes. There was a significant positive correlation between hepatic CYP2E1 levels and *in vitro* 6OHCZN formation velocity at 950 μM (approximate Vmax) (r=0.45 p=0.002) (Fig. 7A). Compared to control monkeys, ethanol self-administration alone and nicotine treatment alone resulted in an 11% increase in 6OHCZN 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. Comparing mean CZN metabolism velocity across all the groups, there is a significant linear trend, with monkeys in the ethanol and nicotine combined group having the highest rate of CZN metabolism (p_{trend}=0.003).

The impact of ethanol consumption level on *in vitro* CZN metabolism was investigated. High ethanol consumers had a 19% increased in velocity of CZN metabolism compared control monkeys, whereas the low consumers had a 13% increase. 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_{trend}=0.003) (Fig. 7C).

DISCUSSION

Humans and AGM have similar hepatic CYP2E1 levels, *in vivo* CZN disposition and *in vitro* CZN metabolism (Hayashi et al., 1991; Lee et al., 2006b), and while the AGM CYP2E1 has not been sequenced, both the cynomolgus monkey (Macaca fascicularis) and the rhesus monkey (Macaca mulatta), which are closely related to AGM, have a greater than 90% amino acid homology to human CYP2E1. Thus, AGM 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 AGM voluntarily self-administering between 0.8-3.5 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 BEL ranging from 5-20 mmol/L (Eckardt et al., 1998). In our study, monkeys achieved an average BEL of 26 ± 2.55 mmol/L comparable to moderate consumption in humans; using BEL to compare ethanol consumption takes into consideration the two-fold greater rate of ethanol elimination in AGM compared to 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 to non-alcoholics, suggesting that elevated CYP2E1 levels can affect ethanol metabolism (Vidal et al., 1990). Based on this premise, induction of

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CYP2E1 in the ethanol-consuming monkeys may have contributed to metabolic tolerance, allowing monkeys to consume more ethanol without a corresponding rise in BEL.

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 while others have shown that nicotine treatment has no effect or even decreases ethanol consumption (Blomqvist et al., 1996; Le et al., 2000; Olausson et al., 2001; Sharpe and Samson, 2002). Differences in rats 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 per week, and only smoking on two days per week) consumed more alcohol when smoking nicotine-containing cigarettes compared to de-nicotinized cigarettes (Barrett et al., 2006). A similar study in regular smokers (smoking more than 10 cigarettes per day) showed that smokers deprived of nicotine had a greater urge to consumed alcohol compared to non-deprived 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 where 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 compared to the low consumers, however they demonstrated nearly a 50% greater reduction in post-ethanol CZN AUC *in vivo*. These results are interesting from a public health perspective, as our results

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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 10-50 ng/ml during the day (Benowitz NL, 1990). The total daily nicotine dose administered to the monkeys was at the high end of this range (1.0 mg/kg/day) to compensate for the slightly faster nicotine metabolism in AGM (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 what is observed in human smokers (Lee et al., 2006a, Benowitz NL, 1990). 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 following 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 nicotine's effect on blood flow. Monkeys were administered CZN more than 12 hr 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, similarly, 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., 2009). This discrepancy may be due to differences in the dose and

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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 toward monkeys in the ethanol and nicotine combined group having the highest CYP2E1 protein levels and fastest rates of *in vitro* CZN metabolism compared to 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 post-drug 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 four weeks resulted in a gradual increase in CYP2E1 activity measured by CZN clearance. CZN clearance was significantly faster on day 28 compared to day 21 (Oneta et al., 2002). In rats, 7 days of nicotine treatment is 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). Previously 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 in this study (7 vs. 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 non-transcriptional mechanism. Consistent with our findings, ethanol-treated rats with BEL similar to our monkeys showed induction of hepatic CYP2E1 without a corresponding increase in CYP2E1 mRNA levels (Ronis et al., 1993).

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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 involve an increase in translational efficiency (Wu et al., 1997; Micu et al., 2003). Thus ethanol and nicotine may increase CYP2E1 in monkey liver using similar post transcriptional mechanisms as 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). Since a large proportion of the population is exposed to ethanol, nicotine or often both, these findings have important implications for public health, health risk assessment and disease prevention.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Miksys, Palmour, Tyndale

Conducted experiments: Ferguson, Miksys, Palmour

Performed data analysis: Ferguson

Wrote or contributed to the writing of the manuscript: Ferguson, Tyndale

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Overview of the study timeline. Monkeys were randomized into four study groups (n=10 per group); Study groups consisted of a no-drug control group (group 1), an ethanol-only group (group 2), a nicotine-only group (group 3) and an ethanol + nicotine group (group 4). Groups were named based on drug exposure during the last phase of the study (phase III). Blood ethanol levels (BEL) were measured near the end of phase II and phase III. *In vivo* CZN metabolism was assessed during the washout period prior to phase II, and at the end of phase III. On day 50, nicotine treatment and alcohol access was suspended in order to conduct pharmacokinetic testing.

Figure 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 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 while 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 in the nicotine-only (group 3) and ethanol + nicotine (group 4) groups were given nicotine injections while monkeys in the other groups (1 and 2) were give 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 phase II and III. B) Unfilled symbols represent monkeys in ethanol-only

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group (group 2), filled symbol represent monkeys in the ethanol + nicotine group (group 4) and horizontal lines indicated group means. Significantly different from low consumers, *p<0.05.

Figure 3. Ethanol self-administration and nicotine treatment reduced chlorzoxazone AUC. A) Mean CZN plasma concentration over time curves for treatment groups, n=10 per group. B) Lines represent the change in plasma CZN AUC_{0-6hr} for individual monkeys pre-drug administration (day 22) and post-drug administration (day 50). Diamonds with error bars represent mean $AUC_{0-6hr} \pm$ SE for the group pre- and post drug administration; for the ethanol-only group, the filled diamonds indicate the mean including the monkey with a noticeably high CZN AUC_{0-6hr} both pre- and post-ethanol exposure (indicated by the dashed line) and the unfilled diamonds indicate the mean excluding this monkey. Significantly different from same group of monkeys pre-drug administration, *p<0.05.

Figure 4. Correlation between ethanol consumption and chlorzoxazone AUC. A) There was a significant negative correlation between mean consumption of 10% ethanol (ml/kg/day) during phase II and II and CZN AUC_{0-6hr} post-drug administration (day 50). With the exclusion of the monkey with noticeably high post-ethanol CZN AUC (circled), the correlation remains significant (r=0.43, p=0.04). B) High ethanol consumers (see fig 2B) had a significantly reduced CZN AUC_{0-6hr} compared low consumers (p<0.05), n=10 per group. C) Lines represent the change in plasma CZN AUC_{0-6hr} for high or low ethanol consumers pre-drug administration (day 22) and post-drug administration (day 50). Diamonds with error bars represent mean $AUC_{0-6hr} \pm$ SE for the group pre- and post drug administration with (filled) or without (unfilled) the inclusion of a monkey with a noticeably high CZN AUC_{0-6hr} both pre- and post-ethanol exposure

(indicated by the dashed line). Significantly different from same group of monkeys pre-drug administration, *p<0.05.

Figure 5. Detection of CYP2E1 monkey hepatic protein was linear and specific. A) A dilution curve of monkey hepatic microsomal protein showed linear detection of CYP2E1 from 2-20 μg. B) The anti-CYP2E1 antibody used for the CYP2E1 immunoblotting did not cross react with cDNA-expressed human CYP2A6, CYP2A1, CYP1A2, CYP2D6, CYP3A4, CYP2B6. Monkey hepatic CYP2E1 co-migrated with cDNA-expressed human CYP2E1.

Figure 6. Ethanol self-administration and nicotine treatment, alone and in combination, induced hepatic CYP2E1 protein levels. A) Representative immunoblot for hepatic CYP2E1 protein from monkeys in the four study groups (showing n = 4 of 10 per group). Coomassie blue staining was used to confirm equal protein loading among lanes. B) Mean percent increase in CYP2E1 protein relative to control group. A significant linear trend was observed, with animals in the ethanol + nicotine group (group 4) having the highest levels of CYP2E1. Significantly different from control group, *p<0.05, **p<0.001. n=10 per group. C) Mean percent increase in hepatic CYP2E1 protein in high and low ethanol consumers (see Fig. 2B) relative to control group. A significant linear trend was observed, demonstrating increased CYP2E1 levels with higher levels of alcohol consumption. Significantly different from control group,*p<0.05, **p<0.001. n=10 for each group.

Figure 7. Ethanol self-administration and nicotine treatment increased *in vitro* chlorzoxazone metabolism. A) Hepatic CYP2E1 protein levels were positively correlated with 6OHCZN

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formation velocity at 950 μM CZN (approximate v_{max}). B) Mean percent increases in the

6OHCZN 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 per group. C) Mean percent increases

in velocity of CZN metabolism in high and low ethanol consumers (see 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 for each group.

Table I. Consumption of 10% ethanol increase over time with no increase in blood

ethanol levels (values expressed as mean ± SE, n=10/group)

	Phase II		Phase III	
	Consumption (ml/kg/day)	BEL (mmol/L)	Consumption (ml/kg/day)	BEL (mmol/L)
Group 3: EtOH-only	35.4 ± 1.2	24.6 ± 3.9	42.4 ± 1.9*	26.3 ± 9.3
Group 4: EtOH + Nicotine	37.8 ± 2.2	23.8 ± 2.1	40.7 ± 3.1*	29.7 ± 4.7
Low consumers	32.9 ± 1.6	22.9 ± 3.8	35.5 ± 1.6*	24.7 ± 4.0
High consumers	37.3 ± 1.6[†]	25.5 ± 2.2	45.1 ± 1.9* [‡]	31.7 ± 4.0

Consumption and BEL were not significantly different between groups during either phase

*p<0.05, significantly different from phase II

[†]p<0.05, [‡]p<0.001, significantly different from low consumer

Table II. Kinetic parameters (mean ± SE, n=10) form CZN pre-drug administration (day 22) and

post drug administration (day 50)

CZN	Pre-EtOH	Post-EtOH	Pre-nicotine	Post-nicotine	Pre-EtOH + nicotine	Post-EtOH + nicotine
AUC (hr.ug/ml)	54.8 ± 7.9	56.6 ± 13.6	44.8 ± 6.2	29.5 ± 5.6*	56.8 ± 4.6	41.6 ± 3.2*
Cmax (ug/ml)	17.5 ± 2.7	27.7 ± 5.7	20.2 ± 3.6	11.7 ± 2.5*	25.3 ± 3.0	16.4 ± 2.0*
tmax (hr)	1.3 ± 0.2	0.9 ± 0.2	1.1 ± 0.2	1.3 ± 0.2	1.1 ± 0.2	0.7 ± 0.1*
t1/2 (hr)	1.3 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.8 ± 0.3

Kinetic parameters were not significantly different between groups pre-drug administration

*p<0.05, significantly different from pre-drug administration within the same treatment group
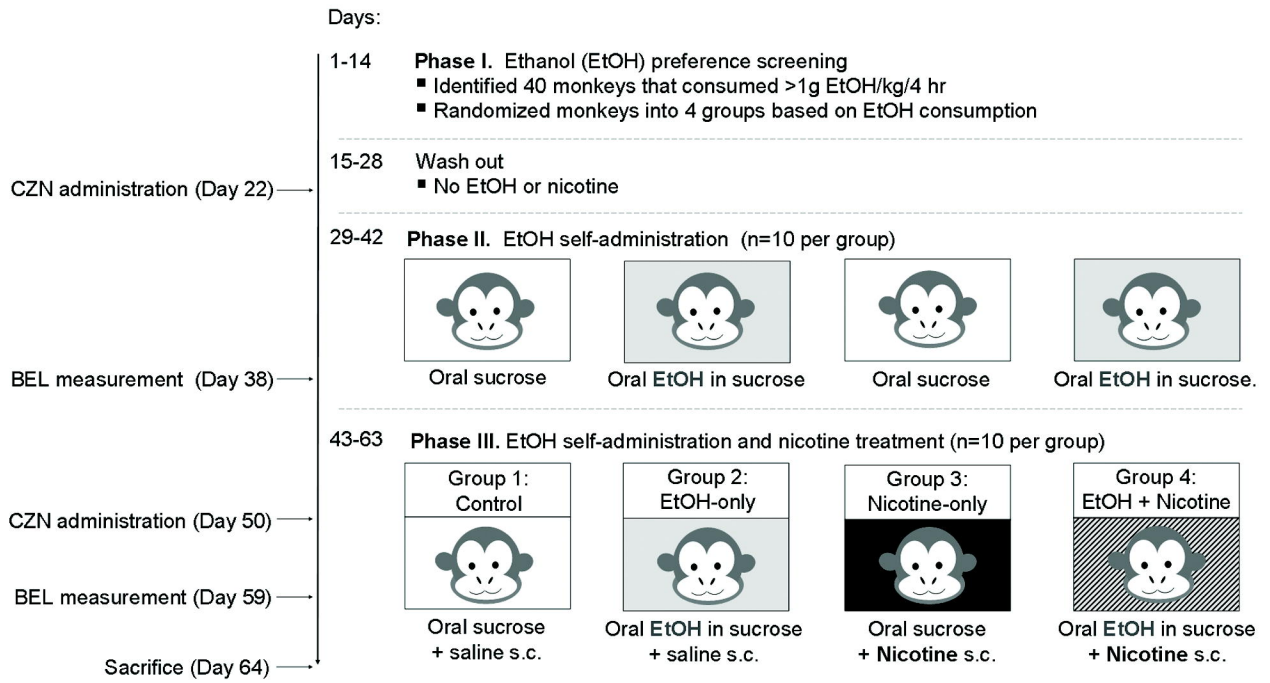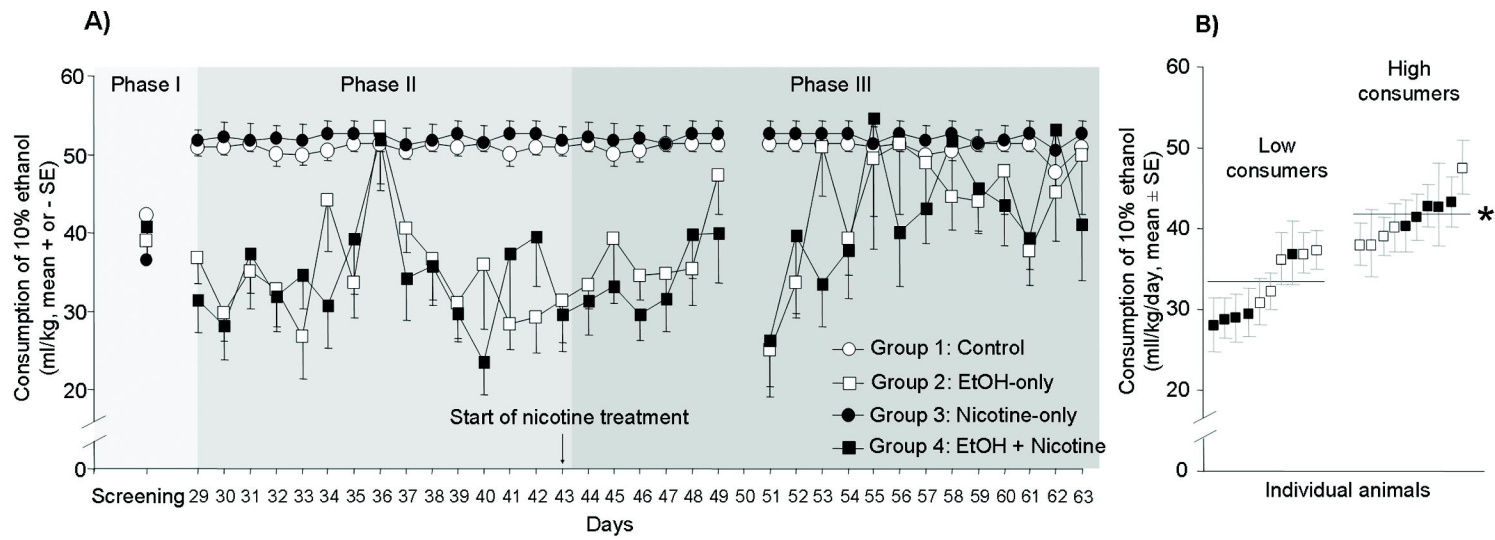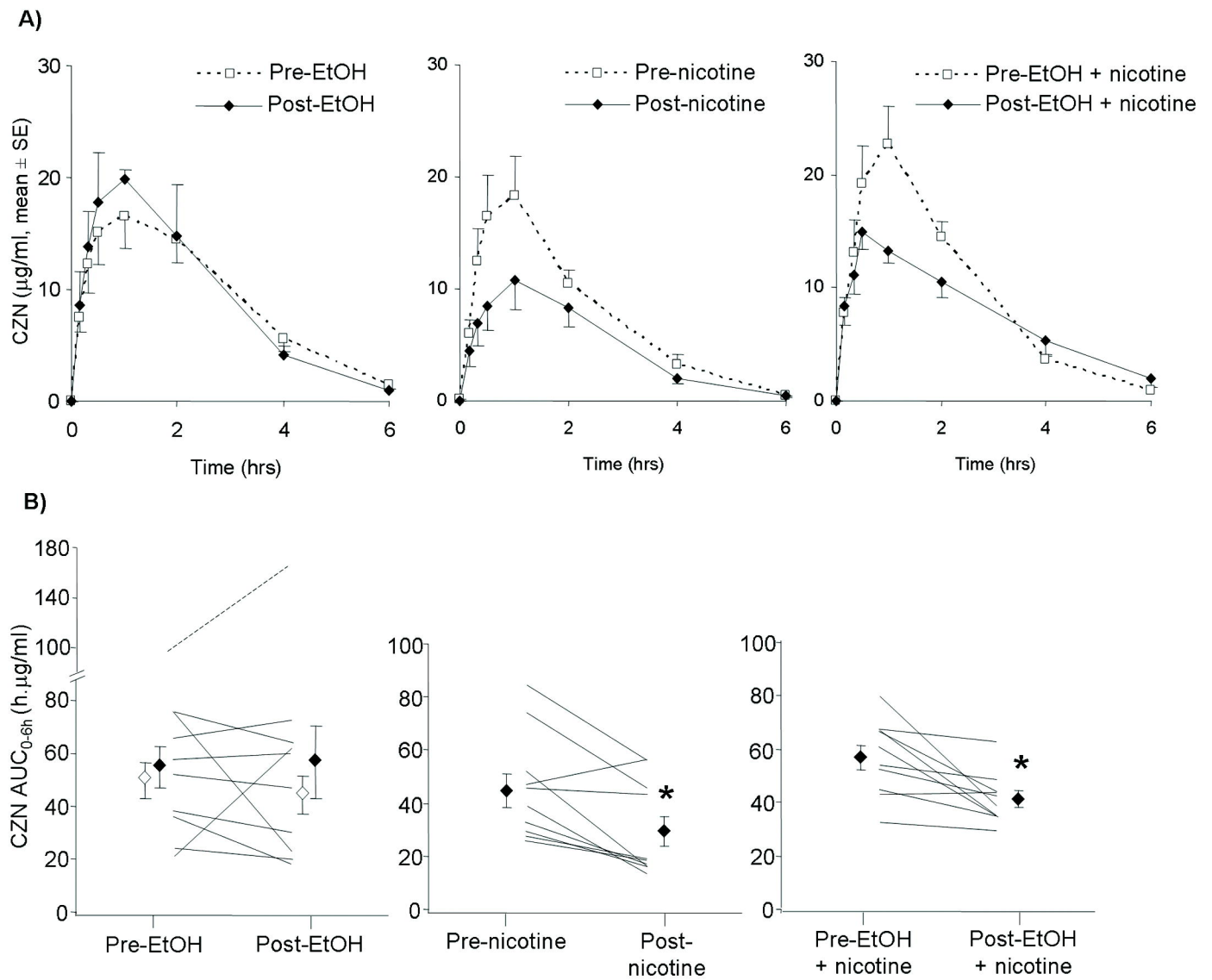
Figure 1

Figure 2

Figure 3

A)

B)

Figure 4

Figure 5

A)

B)

Figure 6

A)

120 kDa →

55 kDa →

| Group1: Control | Group 2: EtOH-only | Group 3 Nicotine-only | Group 4: EtOH + Nicotine |

B)

☐ Group 1: Control
▨ Group 2: EtOH-only
■ Group 3: Nicotine-only
▨ Group 4: EtOH+Nicotine

p_{trend} = 0.0002

CYP2E1 protein
(% of control group, mean + SE)

C)

☐ Group 1: Control
▨ Low consumers
▨ High consumers

p_{trend} = 0.0004

CYP2E1 protein
(% of control group, mean + SE)

Figure 7