Low Doses of Nicotine and Ethanol Induce CYP2E1 and Chlorozoxazone Metabolism in Rat Liver

LISA A. HOWARD, ALINA L. MICU, EDWARD M. SELLERS, and RACHEL F. TYNDALE

Departments of Pharmacology (L.A.H., A.L.M., E.M.S., R.F.T.) and Psychiatry (E.M.S.), Centre for Addiction and Mental Health (E.M.S., R.F.T.), University of Toronto, Toronto, Ontario, Canada

Received April 3, 2001; accepted July 26, 2001 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

The use of ethanol and nicotine is strongly linked; 80 to 95% of heavy alcohol users are also smokers. In humans, cigarette smoking significantly enhances CYP2E1 activity, as measured by increased metabolism of chlorzoxazone in vivo. CYP2E1 metabolizes ethanol and can generate toxic intermediates. CYP2E1 also bioactivates tobacco smoke and other procarcinogens and several hepatotoxins. We hypothesized that, like ethanol, nicotine increases CYP2E1 activity. Rats were treated once daily with saline, ethanol (0.3, 1.0, and 3.0 g/kg p.o.), or nicotine bitartrate (0.1, 0.3, and 1.0 mg base/kg s.c.) for 7 days. After ethanol or nicotine administration, immunostaining for CYP2E1 was increased in the centrilobular regions of rat liver. Western blot analyses revealed that hepatic CYP2E1 levels were increased by ethanol (1.6–2.4-fold) and nicotine (1.3–1.7-fold). In vitro chlorzoxazone 6-hydroxylation analyses demonstrated elevated $V_{\text{max}}$ values (compared with saline-treated animals) by using hepatic microsomes from high-dose ethanol (2.27 ± 0.12 versus 1.18 ± 0.23 nmol/mg/min, p < 0.001) or nicotine-treated rats (2.35 ± 0.04 versus 1.32 ± 0.55 nmol/mg/min, p < 0.005), with no change in affinity. The magnitude of enhanced chlorzoxazone metabolism by microsomes from drug-treated animals is consistent with the observed increase in CYP2E1 protein by immunoblot. These data suggest that nicotine may increase CYP2E1-induced toxicity and contribute to cross-tolerance in smokers and people treated with nicotine (e.g., smokers, patients with Alzheimer’s disease, ulcerative colitis, neuropsychiatric motor disorders).

Cytochromes P450 (CYP) are mixed function oxidases that are predominantly expressed in the liver and biotransform drugs, endogenous compounds, dietary constituents, and environmental toxins (Lieber, 1997; Woodroof and Novak, 1998; Zhukov and Ingelman-Sundberg, 1999). Although alcohol dehydrogenase (ADH) is the major contributor to the metabolism of ethanol (Crabb, 1995), the CYP2E1 enzyme has been proposed to account for approximately 20% of ethanol metabolism at physiologically relevant blood alcohol concentrations (Lieber, 1994; Matsumoto et al., 1996). Ethanol consumption leads to increased CYP2E1 mRNA transcript levels in alcohol abusers (Lieber, 1999), but ethanol can also increase CYP2E1 levels by stabilizing the enzyme and preventing its degradation (Zhukov and Ingelman-Sundberg, 1999). ADH is not induced by ethanol (Lieber, 1994), suggesting that the induction of CYP2E1 by chronic ethanol is responsible for the 2-fold increase in ethanol metabolism observed in heavy drinkers (Lieber, 1994, 1999).

CYP2E1 activity is also induced by endogenous substances (e.g., acetone) as well as by pathophysiological states such as diabetes, obesity, and hypertriglyceridemia (Lieber, 1997, 1999). The underlying mechanisms involve transcriptional, post-transcriptional, and post-translational regulation (Lieber, 1997, 1999). CYP2E1 has been proposed to contribute to gluconeogenesis because it metabolizes acetone to an intermediate of this energy-producing pathway (Lieber, 1997). This physiological role may contribute to its high functional conservation across species; human CYP2E1 exhibits catalytic properties that are similar to its rat ortholog and they appear to share many regulatory features (Lieber, 1999).

There is mounting evidence that CYP2E1 is a key factor in the pathogenesis of alcoholic liver disease (ALD), a risk factor for hepatocellular carcinoma (Jarvelainen et al., 2000). Due to its high NADPH oxidase activity (Zhukov and Ingelman-
Sundberg, 1999), CYP2E1 generates ethanol- and oxygen-derived free radicals that can initiate lipid peroxidation, oxidative stress, and Kupffer cell activation, thereby propagating cellular injury and DNA strand breaks (Jarvelainen et al., 2000). Furthermore, CYP2E1 induction correlates with lipid peroxidation and pathological severity during chronic ethanol exposure (Jarvelainen et al., 2000).

This enzyme also bioactivates cytotoxins, including carbon tetrachloride, many organic solvents, and acetaminophen, and its activity strongly correlates with degree of tissue injury induced by these toxins (Lieber, 1997; Woodcroft and Novak, 1998). The enhanced toxicity associated with the 5- to 10-fold induction of CYP2E1 during chronic ethanol intake may explain the increased vulnerability of alcohol abusers to therapeutically and industrially used xenobiotics, such as acetaminophen and bromobenzene (Lieber, 1999).

Epidemiological findings have established an interactive influence of ethanol drinking and tobacco smoking on cancer development; the synergistic risk for certain cancers is about 50% higher than the sum of the risks from cigarette smoking or ethanol drinking alone. CYP2E1 bioactivates tobacco smoke and other procarcinogens (Lieber, 1997); the enzyme is implicated in benzene-induced hematological malignancies in humans (Rothman et al., 1997) and nitrosodimethylamine-induced tumorigenesis in animals (Lieber, 1997). Tobacco smoke has been shown to induce CYP2E1 activity in animal models and in humans (Villard et al., 1998; Benowitz et al., 1999). Therefore, the induction of CYP2E1 by both tobacco smoke and ethanol may contribute to their synergistic increase in risk of liver disease development.

A higher percentage of alcoholics smoke (80–95%) compared with nonalcoholics (25–30%) (Batel et al., 1995), and twin studies indicate that, for equal ethanol consumption, heavy smokers had higher ethanol elimination rates compared with nonsmokers (Kopun and Propping, 1977). The incidence of alcoholism is 10 times more likely in smokers than nonsmokers (Batel et al., 1995). Among nonalcoholics, smokers report drinking alcohol at levels that are about twice that of nonsmokers (Batel et al., 1995). Consistent with the idea that smoking increases ethanol metabolism leading to increased consumption, long-term nicotine exposure was shown to enhance ethanol consumption in rats (Blomqvist et al., 1996). Ethanol effects on central nicotinic receptors or other central or peripheral receptor systems have been postulated to contribute to this interaction between ethanol and nicotine (Blomqvist et al., 1996; Ericson et al., 2000). However, cigarette smoke has been shown to increase CYP2E1 activity (Villard et al., 1998; Benowitz et al., 1999). Therefore, we postulated that in addition to pharmacodynamic alterations, the higher ethanol consumption among smokers may be partly due to tobacco smoke constituent(s) (i.e., nicotine) increasing the metabolism of ethanol (metabolic tolerance), requiring dependent individuals to drink more ethanol for the same effect. The purpose of this study is to examine whether of behaviorally and pharmacologically relevant doses of ethanol and nicotine increase CYP2E1 protein and activity in rat liver.

**Experimental Procedures**

**Materials.** Protease inhibitor cocktail tablets and chemiluminescence blotting substrate were purchased from Roche Diagnostics (Laval, QC, Canada). Recombinant viral-expressed rat CYP2E1 in lymphoblastoid cells; baculoviral-expressed rat CYP1A1, CYP2B1, CYP3A4, CYP2C11, and CYP2A2 in insect cells; and expressed rat CYP2A1 Supersomes were purchased from GENTEST (Woburn, MA) and pyridine-induced rat liver microsomes were purchased from Oxford Biomedical Research, Inc. (Oxford, MI). Protran nitrocellulose membranes were purchased from Schleicher & Schuell (Keene, NH). Biotinylated anti-rabbit IgG secondary antibody, ABC Elite kit, and DAB/hydrogen peroxide kit were purchased from Vector Laboratories (Burlington, ON, Canada), and Neutravidin-conjugated goat anti-rabbit IgG secondary antibody, ABC Elite kit, and DAB/hydrogen peroxide kit were purchased from Vector Laboratories (Burlington, ON, Canada). Ethanol, nicotine bitartrate, chlorozoxazon (CZN), and 2-benzoxazolinolone were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

**Animals.** Four groups (n = 4/group) of adult male Wistar rats (250–300 g; Charles River, St-Constant, QC, Canada) were injected subcutaneously, once per day, for 7 days with either 0, 0.1, 0.3, or 1.0 mg of nicotine base per kilogram of body weight, in the form of nicotine bitartrate in sterile saline (pH adjusted to 7.4). Saline and ethanol (in saline), at doses of 0.3, 1.0, and 3.0 g/kg of body weight (n = 4/group), were administered by gavage to food-deprived (2–4 h, to facilitate constant absorption) male Wistar rats once daily for 7 days. Animals were sacrificed by decapitation 4 h after the last drug injection and livers were removed, frozen immediately in liquid nitrogen, and stored at −80°C until processed for protein, activity, and mRNA studies. All experimental procedures described in this study were carried out in accordance with the guidelines for the care and use of laboratory animals and approved by the Animal Care Committee of the University of Toronto.

In this study, behaviorally relevant doses of chronically administered ethanol were chosen to model light social drinking to heavy binge ethanol drinking. Most studies of the effects of ethanol on CYP2E1 have used 3- to 4-week administration of the Lieber-DeCarli liquid ethanol diet, which leads to relatively high ethanol dosing (approximately 12–13 g/kg/day of ethanol) (Eliasson et al., 1988). Based on the extensive ethanol pharmacokinetic data in the literature (Xie et al., 1994; Buczek et al., 1997), we estimate that the doses of ethanol used in our study of 0.3, 1.0, and 3.0 g/kg will produce peak ethanol levels of 41, 86, and 180 mg/dl, respectively, similar to plasma concentrations achieved in humans after 1, 4, and 10 standard drinks, respectively (Wilkinson et al., 1977; Sadler et al., 1996). These doses of ethanol are also associated with changes in neurophysiology (i.e., γ-aminobutyric acid and serotonergic receptor alterations) and behavior (i.e., self-administration, reinforcement, and motor impairment) (Le and Israel, 1994; Buczek et al., 1997).

In this study, plasma nicotine levels were measured in rats 0.5, 2, and 4 h after subcutaneous injection of 0.0 and 1.0 mg/kg nicotine. The level of nicotine rapidly peaked by 1 h and declined by 4 h postinjection in the nicotine-treated animals, whereas no nicotine was detected in vehicle-treated animals (data not shown). From our studies and previous investigations (Pratt et al., 1983; London et al., 1990), subcutaneous nicotine administration at doses of 0.1, 0.3, and 1.0 mg/kg nicotine lead to peak plasma nicotine levels (after 30–60 min) of 32, 106, and 202 ng/ml, respectively. Smokers acquire plasma nicotine levels of approximately 10 ng/ml/cigarette, suggesting that our chosen doses are similar to total doses acquired from 3, 10, and 20 cigarettes (Le Houezec et al., 1993). In addition, subcutaneous nicotine injections at 1.0- to 2.0-mg/kg doses have been associated with central nicotinic receptor adaptation, a pharmacodynamic change observed in brain regions of smokers that is hypothesized to be one pathway by which nicotine exerts its behavioral effects such as tolerance (Rowell and Li, 1997; Perry et al., 1999). In addition, chronic injections of 0.8 mg/kg nicotine increase ethanol self-administration by male Wistar rats (Blomqvist et al., 1996).
Membrane Preparation. Portions of livers were homogenized manually in 100 mM Tris, pH 7.4, with 0.1 M EDTA, 0.32 M sucrose, 0.1 M dithiothreitol, and protease inhibitor cocktail (1 tablet/50 ml of homogenizing buffer) on ice. Homogenates were centrifuged twice at 3000g for 3 min, and the membrane fractions were prepared by 110,000g centrifugation (Sorvall RC2-B Combi Plus Ultraspread centrifuge; Sorvall, New York, CT) of the supernatant fraction. The resulting membrane pellets were resuspended in 100 mM Tris, pH 7.4, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1.15% w/v KCl, 20% v/v glycerol, and stored at −80°C. Microsomes from pyridine-induced rat liver, as well as from rat CYP2E1-expressed lymphoblastoid cells, were used as positive controls. For kinetic studies, liver microsomes were prepared in cold 1.15% KCl by centrifugation at 9,000g followed by 100,000g.

Immunoblotting. Untreated rat liver membranes were serially diluted and used to construct standard curves to establish conditions of linearity. Membrane proteins (2.5 μg of hepatic membranes and 0.18 μl of expressed CYP2E1 were used as a standard) were separated by SDS-polyacrylamide gel electrophoresis (4% stacking and 8% separating gels), transferred onto nitrocellulose membrane, and probed with a rabbit polyclonal anti-rat CYP2E1 antibody at a 1:4000 dilution for 1.5 h; this antibody has been shown to be selective for CYP2E1 at concentrations used in this study (Hansson et al., 1990). Membranes were incubated with a secondary bioinylated goat anti-rabbit IgG (1:6000 dilution) for 1 h, followed by Neutravidin-conjugated horseradish peroxidase (1:40,000 dilution) for 20 min before detection by chemiluminescence and analyzed with an imaging system (Imaging Research Inc., St. Catherines, ON, Canada). Control blots were processed without primary antibody.

Immunohistochemistry. Liver samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, cryoprotected in 20% sucrose in phosphate buffer, and rapidly frozen in isopentane cooled on dry ice. Sections (16 μm thick) were cut on a freezing microtome, collected in well plates in PBS (10 mM sodium phosphate buffer, 0.9% sodium chloride) (pH 7.4). Sections were washed and blocked with PBS containing 4% Triton, 5% normal goat serum, 2% bovine serum albumin, and 1% skimmed milk for 2 h at room temperature. Sections were incubated at 4°C for 4 days in rabbit anti-rat CYP2E1 antibody (used in immunoblotting assays) diluted 1:1000 in PBS containing 2.5% Triton, 1% normal goat serum, and 1% bovine serum albumin. Sections were washed with PBS, incubated for 1 h at room temperature with biotinylated goat anti-rabbit IgG diluted 1:2000, and rewashed in PBS. Endogenous peroxidase activity in the sections was quenched with 0.3% hydrogen peroxide in PBS for 5 min. The antigen-antibody complex was visualized using the avidin-biotin complex technique followed by reaction with 3,3′-diaminobenzidine. Sections were dehydrated and mounted using xylene in Permount; control sections were incubated without primary antibody.

Chlorzoxazone Metabolism and Chemical Inhibition Assays. CZN 6-hydroxylation was assayed according to the method of Leclercq et al. (1998) for rat liver microsomes with the following modifications. For kinetic parameter studies, rat hepatic microsomes (0.1 mg) were mixed with 25 μl of 10 mM β-NADPH, 25 μl of 50 mM MgCl₂ in Tris-HCl buffer, pH 7.4, to a final volume of 250 μl. Incubations were carried out at 37°C. Initial studies indicated that this reaction was linear up to 1.6 mg of microsomal protein and to an incubation time of 40 min; final assays used incubations of 0.1 mg of microsomal protein for 20 min. CZN stock solutions were prepared in methanol and all other stock solutions were made in Tris-HCl. CZN solutions were dried under N₂ and reconstituted in Tris-HCl prior to incubations. The reaction was stopped by the addition 2 ml of ethyl acetate and 100 μl of 400 mM 2-benzoxazolinone (internal standard) and subsequently extracted. The organic phase was evaporated to dryness and reconstituted into 150 μl of mobile phase consisting of 19% acetonitrile in 10 mM sodium acetate buffer, pH 4.5. Formation of 6-hydroxy-CZN (6-OH-CZN) was measured by high-performance liquid chromatography with UV detection at 285 nm. A Waters Spherisorb S5 ODS2 column (4.6 × 150 mm; Waters, Bedford, MA) was used to separate CZN, 6-OH-CZN, and internal standard by using a mobile phase. The separation was performed with isocratic elution at a flow rate of 1 ml/min. The retention times for CZN, 6-OH-CZN, and 2-benzoxazolinone were 19.6, 4.7, and 6.5 min, respectively. CZN metabolic assays (5–400 μM) were performed to obtain kinetic parameters (Kₘ and Vₘₐₓ) in rat liver microsomes. Chemical inhibition studies were performed using 150 μM CZN, 0.1 mg of hepatic microsomal protein, or 11.5 pmol of expressed CYP2E1, incubated for 20 min at 37°C with either 10 or 100 μM nicotine bitartrate, or 100 or 500 μM aniline. Because aniline was dissolved in methanol and low methanol concentrations strongly inhibit CYP2E1 activity, solutions for aniline experiments were dried under N₂ and reconstituted prior to incubation. Because diethylthiocarbamate is a mechanism-based inhibitor of CYP2E1, microsomes were preincubated for 20 min with 31.5 or 315 μM DDC prior to the addition of CZN. Substrate and inhibitor concentrations were chosen based on our current experimental data and published data (Jayyosi et al., 1995; Bourrie et al., 1996; Eagling et al., 1998). Negative controls consisted of incubations without NADPH or with heat-denatured microsomal protein.

RNA Slot Blot Analysis. Total RNA from rat livers was isolated using StrataPrep Total RNA kit and its quality assessed by electrophoresis in 1.2% agarose gel. Yeast tRNA was added to all samples (9.5 μg) and cDNA standards (10 μg) to reduce background. Total liver RNA (2.5 μg), serially diluted full-length CYP2E1 cDNA (0.63–20 pg, to ensure linearity of the assay and as a positive control), cDNAs for CYP2B6, CYP2A6, CYP2D6, and CYP3A4 (1.0 and 10.0 pg each, as negative controls), were applied directly to nylon membranes under vacuum and denaturing conditions by using a Bio-Dot microfiltration apparatus according to manufacturer’s instructions. Membranes were prehybridized for at least 60 min at 43°C in buffer [50% formamide, 120 mM Na₂HPO₄ (pH 7.2), 7% SDS, and 250 mM NaCl] and then hybridized for 16 h with either 1.4 × 10⁷ cpm/ml [α-32P]dCTP random-primed full-length human CYP2E1 cDNA or with two end-labeled rat oligonucleotide hybridization probes (each 2.0 × 10⁵ cpm/ml [γ-32P]dATP) in the same buffer. Specifically, the rat oligonucleotides that are the reverse complement to make sequences to 5′-TTATTCGACAGCATTTTTCC-3′ and 5′-GAAGTTTCTAATGACAAACT-3′ that correspond to positions 721 to 740 and 613 to 633 of CYP2E1 mRNA, respectively, were used as probes; these sequences were specific for CYP2E1 mRNA. In addition, blots were probed with a 1.3 × 10⁷ cpm/ml [α-32P]dCTP randomly labeled 517-base pair PCR product of β-actin (a loading control), which was made using the forward primer 5′-CACCACACGCTGAGGGGAAACTGGTCGATGA-3′, the reverse primer 5′-ATTTCGGGTACGATGGAGGCGCGACT-3′, and rat β-actin cDNA template followed by gel extraction of the PCR product. A serial dilution of the β-actin PCR product (0.008–1000 pg) was also probed to verify the linearity of our detection system. Blots were washed at room temperature sequentially in 2× then 0.5× saline sodium citrate with 0.1% SDS for 15 min each and exposed to Kodak OMAT-XR film for 1 to 8 days at −80°C. All films were analyzed using a digital imaging system (Imaging Research Inc.).

Determination of Plasma Nicotine Levels. Plasma concentrations of nicotine in frozen rat plasma samples were determined by a well-established gas-liquid chromatography technique with 5-methylnicotine as the internal standard (Pacifici et al., 1993). The limit of detection of the assay was 0.5 ng/ml and there was a linear relationship between detected chromatographic peak and nicotine concentration (data not shown).

Analysis. The kinetic parameters (Kₘ and Vₘₐₓ) for CZN metabolism by liver microsomes from rats treated with saline, nicotine, or ethanol were analyzed using Enzfit® computer software, version 1.05 (Elsevier Biosoft Inc., Cambridge, UK). Treatment groups were considered to be significantly different from control if p < 0.05, using unpaired student t-tests.
Results

CYP2E1 Is Induced by Low Doses of Ethanol and Nicotine. An immunoblotting assay was developed to measure hepatic CYP2E1. Detection of serially diluted CYP2E1 indicated that the immunoblotting signal was linear up to 10 μg of protein from untreated liver membranes (Fig. 1A); no band was detected in the absence of primary antibody (Fig. 1A). The specificity of the rabbit antibody for rat CYP2E1 has been demonstrated (Hansson et al., 1990); we have confirmed this finding because no cross-reactivity was observed with expressed rat CYP2C11, 2B1, 3A2, 1A1, 2A1, or 2A2 isozymes under immunoblotting conditions used in this study (Fig. 1B). The immunoreactive band from expressed rat CYP2E1 microsomes comigrated with liver microsomes from rats treated with the CYP2E1 inducer pyridine in vivo and untreated liver membranes (Fig. 1B). A 7-day ethanol treatment dose dependently induced CYP2E1 protein in rat hepatic membranes (Fig. 2, A and B). Ethanol doses of 0.3, 1.0, and 3.0 g/kg dose dependently induced CYP2E1 by 1.6-, 1.9-, and 2.4-fold, respectively, compared with saline-treated animals. In addition, 0.1-, 0.3-, and 1.0-mg/kg doses of nicotine for 7 days significantly increased CYP2E1 in rat liver by 1.4-, 1.8-, and 1.5-fold compared with saline controls. The apparent saturation of the level of CYP2E1 induction at the low-to-middle nicotine doses has been observed with other CYP2E1 inducers (Woodcroft and Novak, 1998).

Region-Specific CYP2E1 Induction by Behaviorally Relevant Doses of Ethanol and Nicotine. Immunohistochemical analyses clearly demonstrate that a short 7-day treatment with a 3.0-g/kg dose of ethanol markedly increased CYP2E1 in rat liver relative to saline-treated animals. In addition, 0.1-, 0.3-, and 1.0-mg/kg doses of nicotine for 7 days significantly increased CYP2E1 in rat liver by 1.4-, 1.8-, and 1.5-fold compared with saline controls. The apparent saturation of the level of CYP2E1 induction at the low-to-middle nicotine doses has been observed with other CYP2E1 inducers (Woodcroft and Novak, 1998).

In Vitro Kinetics of Chlorzoxazone Hydroxylation in Rat Liver. We determined protein concentrations and incubation times that resulted in the linear formation of 6-OH-CZN with less than 15% CZN disappearance. Microsomes incubated without NADPH or heat denatured rat microsomes did not produce detectable product (data not shown).
Ten substrate concentrations ranging from 5 to 400 μM and Eadie-Hofstee analyses were used to determine the kinetic parameters ($K_m$ and $V_{max}$) of CZN hydroxylation in rat liver microsomes. Kinetic analyses of CZN hydroxylation by hepatic microsomes from saline and ethanol (3 g/kg)-treated animals yielded similar $K_m$ values of 150 ± 21 and 221 ± 65 μM, respectively (mean ± S.D., $p = 0.100$) (Table 1). Liver microsomes from saline and nicotine-treated (1.0 mg/kg) rats produced similar $K_m$ affinity constants of 109 ± 22 and 131 ± 18 μM ($p = 0.250$) (Table 1).

Liver microsomes from ethanol-treated animals exhibited a significantly 1.9-fold higher $V_{max}$ (2.27 ± 0.12 nmol/mg/min) compared with saline-treated animals (1.18 ± 0.23 nmol/mg/min) ($p < 0.001$) (Table 1). Furthermore, animals treated with 1.0 mg/kg nicotine had a significantly increased $V_{max}$ (2.35 ± 0.04 nmol/mg/min), which was 1.7-fold higher than for saline-treated animals (1.32 ± 0.55 nmol/mg/min) ($p < 0.005$) (Table 1).

### Inhibition CYP2E1 Hydroxylation of Chlorzoxazone.

At our estimated $K_m$ value of 150 μM CZN, expressed rat CYP2E1 catalyzed 6-hydroxylation of CZN with a velocity of 51.7 (pmol/pmol CYP2E1/min), which was completely abolished by 100 μM aniline, a CYP2E1 competitive inhibitor, and was significantly inhibited by 31.5 μM DDC, a mechanism-based inhibitor (Fig. 4B; $p < 0.01$). Inhibitors were tested at concentrations equal to reported IC50 values (Bourrie et al., 1996; Eagling et al., 1998). Nicotine, at 100 μM, did not inhibit expressed CYP2E1-mediated CZN metabolism.

DDC concentrations of 31.5 (IC50) and 315 μM (10× IC50), significantly inhibited CZN 6-hydroxylation by microsomes from ethanol control animals by 61 and 90% ($p < 0.001$ and $p < 0.001$, respectively). Aniline, at 100 (IC50) and 500 μM (5× IC50), significantly inhibited 6-hydroxylation of CZN by hepatic microsomes from ethanol control animals by 33 and 94% ($p < 0.05$ and $p < 0.001$, respectively) and from 3.0 g/kg ethanol-treated animals by 44 and 79% ($p < 0.05$ and $p < 0.001$, respectively). Aniline, at 100 (IC50) and 500 μM (5× IC50), significantly inhibited 6-hydroxylation of CZN by nicotine control animals by 33 and 94% ($p < 0.05$ and $p < 0.001$, respectively) and from 3.0 g/kg ethanol-dosed animals by 37 and 88% ($p < 0.05$ and $p < 0.001$) (Fig. 5A). The percentage of inhibition by DDC at 31.5 and 315 μM was 78 and 88% in nicotine control and 63 and 85% in nicotine-treated rat microsomes, respectively (all $p < 0.001$). Concentrations of 100 and 500 μM aniline inhibited

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$K_m$ (μM)</th>
<th>$p$ Value</th>
<th>$V_{max}$ (nmol/mg/min)</th>
<th>$p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>150 ± 21</td>
<td>0.100</td>
<td>1.18 ± 0.23</td>
<td>0.001</td>
</tr>
<tr>
<td>Ethanol (3.0 g/kg)</td>
<td>221 ± 65</td>
<td>0.250</td>
<td>2.27 ± 0.12</td>
<td>0.005</td>
</tr>
<tr>
<td>Saline</td>
<td>109 ± 22</td>
<td>0.250</td>
<td>1.32 ± 0.55</td>
<td>0.005</td>
</tr>
<tr>
<td>Nicotine (1.0 mg/kg)</td>
<td>131 ± 17</td>
<td>0.250</td>
<td>2.35 ± 0.04</td>
<td>0.005</td>
</tr>
</tbody>
</table>

### Fig. 3.

Behaviorally relevant doses of ethanol and nicotine increased CYP2E1 immunoreactivity in the centrilobular region of rat liver. Immunohistochemical analysis demonstrates that a 7-day treatment with 3.0 g/kg ethanol (B), relative to its saline control (A), and 1.0 mg/kg nicotine (D), relative to its saline control (C), increased hepatic CYP2E1 immunostaining in the region surrounding the central vein (v) of the liver lobule. E, no staining was observed in sections processed without primary antibody. Scale bar, 500 μm.
6-hydroxylation of CZN by 58 and 89% for nicotine control (both \( p < 0.05 \)) and 47 and by 92% for 1.0 mg/kg nicotine-treated animals (\( p < 0.05 \) and \( p < 0.001 \)) (Fig. 5B). The presence of nicotine at 10 and 100 \( \mu \)M did not significantly inhibit CZN 6-hydroxylation in any of the treatment groups (Fig. 5, A and B).

**Effects of Ethanol and Nicotine on CYP2E1 mRNA Levels.** Total RNA was extracted from rat livers and slot blot analysis was used to compare CYP2E1 mRNA between different treatment groups. A serial dilution of CYP2E1 cDNA was used to determine the linear range of the assay, and all samples fell within this range (Fig. 6A). No signal was detected from 1 and 10 pg of cDNAs of CYP2B6, CYP2A6, CYP2D6, and CYP3A4, indicating the specificity of the CYP2E1 probes (Fig. 6B). No significant difference in CYP2E1 mRNA levels was observed between livers from ethanol and vehicle-treated (2.24 \( \pm \) 0.16 and 2.15 \( \pm \) 0.22) \( p = 0.56 \) or nicotine-treated animals compared with their saline controls (2.28 \( \pm \) 0.30 and 2.19 \( \pm \) 0.14) \( p = 0.59 \)) (Fig. 6, C and D); similar results were observed with blots hybridized with rat CYP2E1 oligonucleotide probes (data not shown). The \( \beta \)-actin levels between livers from ethanol and vehicle-dosed animals (0.78 \( \pm \) 0.11 and 1.01 \( \pm \) 0.26) \( p = 0.18 \) or between livers from nicotine and vehicle-dosed animals (1.21 \( \pm \) 0.14 and 1.32 \( \pm \) 0.30) \( p = 0.55 \) were also not significantly different; likewise, no significant differences were observed in the levels of CYP2E1/\( \beta \)-actin between ethanol and nicotine and their respective control groups.

**Discussion**

In this study, behaviorally relevant doses of ethanol and nicotine were chosen (see Experimental Procedures) to model the effects of ethanol drinking and tobacco consumption on the levels and activity of hepatic CYP2E1. We showed that low-level ethanol treatments caused a dose-dependent increase in CYP2E1 protein. We also found that short-term exposure to nicotine at low doses induces CYP2E1. Our kinetic data are the first to show an increase in hepatic CZN metabolism after low-level ethanol or nicotine treatment specifically related to induction of CYP2E1 protein. Because the enzyme’s affinity for CZN was not altered, the increased \( V_{\text{max}} \) is consistent with an increased amount of active CYP2E1 enzyme after both ethanol and nicotine treatments (Table 1), and the increase in \( V_{\text{max}} \) is similar in magnitude to the observed CYP2E1 protein elevation detected by immunoblotting (Fig. 2; Table 1).

These findings further elucidate, and supported by the results from previous studies, that suggest cigarette smoke...
Cigarette smoking also significantly enhanced the metabolism of CZN in humans (Benowitz et al., 1999), reflecting an induction of hepatic CYP2E1 that is specific to the centrilobular region, which may exacerbate the hepatotoxicity of the CYP2E1 substrates ethanol, acetaminophen, carbon tetra-chloride, and N-nitrosodimethylamine in this hepatic region (Tsutsumi et al., 1989). Our immunohistochemical analyses also suggest that CYP2E1 induction, as estimated by liver homogenates used in immunoblotting and kinetic studies, may underestimate the levels of CYP2E1 attained centrilobularly in rat liver.

High-dose ethanol is a well established inducer of CYP2E1 in animal models and in human alcohol abusers (Lieber, 1999). Chronic high-dose ethanol treatment (12–13 g/kg/day) for 20 to 30 days increases CYP2E1 protein 6- to 9-fold via ligand stabilization (Eliasson et al., 1988). When a blood alcohol concentration of 300 mg/dl is attained, there is a 5- to 6-fold elevation in mRNA, which is accompanied by 12- to 13-fold induction of CYP2E1 protein in rats (Ronis et al., 1993). Our findings demonstrate that considerably lower doses of ethanol for 7 days dose dependently induce CYP2E1 protein and CYP2E1-mediated 6-OH-CZN activity in rat.

Intermittent and chronic doses of nicotine (similar to those used in this study) have been shown to increase ethanol consumption (Blomqvist et al., 1996). Some studies have suggested that central nicotinic receptors are responsible for this behavioral interaction (Blomqvist et al., 1996), whereas other findings dispute this proposal (Dyr et al., 1999) or implicate other receptor systems as the underlying mediators of this interplay (Ericson et al., 2000). Interestingly, nicotine was observed to decrease hepatic ADH activity (Bhagwat et al., 1998). We have shown that nicotine increased hepatic CYP2E1 and may account for one component of the cross-tolerance observed between ethanol and nicotine (Ericson et al., 2000). Of interest, we have also found that CYP2B1/2, the nicotine-metabolizing CYPs in rats, were induced by ethanol in the livers of the same animals used in this study and that the magnitude of induction of CYP2B1/2 was similar to that observed for CYP2E1 (2.0- to 3.0-, and 2.7-fold for 0.3-, 1.0-, and 3.0-g/kg doses, respectively) (Schoedel et al., 2001). These findings provide strong evidence that the induction of
ethanol and nicotine metabolic pathways may synergistically contribute to the development of cross-tolerance.

One mechanism by which nicotine could increase CYP2E1 is by transcriptional regulation. However, we found that there were no differences in mRNA levels between livers from saline- or nicotine-treated animals, similar to the results following ethanol treatments (Fig. 6). An alternative mechanism is protein stabilization, a process whereby many CYP2E1 substrates, including ethanol and low-molecular weight ligands, increase CYP2E1 levels by slowing its high NADPH oxidase activity and its generation of reactive oxygen species (Zhukov and Ingelman-Sundberg, 1999). These reactive oxygen metabolites are thought to oxidize and modify the enzyme, labeling CYP2E1 for auto-degradation by the proteosomal complex (Zhukov and Ingelman-Sundberg, 1999). Therefore, we assessed if nicotine induction involved an interaction of nicotine with CYP2E1 (i.e., nicotine did not inhibit CYP2E1-hydroxylation of CZN) suggesting it does not interact with the catalytic site. However nicotine may still act as a heme ligand (as does imidazole, a structurally similar compound) to stabilize the enzyme (Eliasson et al., 1988; Lieber, 1999; Zhukov and Ingelman-Sundberg, 1999). Alternatively, nicotine also bears structural similarity to pyridine, which translationally activates CYP2E1 (Lieber, 1999), and may be the mechanism underlying nicotine’s induction of the enzyme. Current experiments are underway to assess 1) whether and how nicotine acts to stabilize CYP2E1, and 2) whether nicotine can increase CYP2E1 levels at lower doses than tested here. These mechanistic studies, in combination with the latter dose-response experiments that will identify lower nonsaturating doses of nicotine that “induce” the enzyme, will enable us to optimize the design of combination experiments of nicotine and ethanol to test whether their inductive effects are additive or synergistic.

The generation of hydroxethyl radicals, reactive oxygen species, and acetaldehyde by CYP2E1 in heavy drinkers is implicated in the pathogenesis of ALD (Lieber, 1999). In addition, alcohols without clinical symptoms of liver disease exhibited lower CZN metabolism compared with alcoholic patients with ALD, further implicating CYP2E1 in the development of ALD (Albano et al., 1999). CYP2E1 has also been linked to the development of nonalcoholic steatohepatitis via its generation of oxidative stress (Leclercq et al., 1999). Therefore, nicotine-induced CYP2E1 may not only synergistically increase ethanol-mediated liver damage, but also increase the susceptibility to a number of nonalcoholic-associated liver diseases.

In vivo cigarette smoke exposure induced CYP2E1 in mice, which was associated with a concomitant increase in cigarette smoke-induced DNA strand breaks, implicating CYP2E1 in this tobacco-related toxicity. Of the tobacco constituents implicated in toxicity, chronic treatment with 0.6 mg/kg nicotine was shown to increase liperoxides, hydroperoxides, conjugated dienes, and free fatty acids in liver, lungs, and heart compared with control rats (Ashakumary and Vijayammal, 1996). Furthermore, ethanol and nicotine produced an additive enhancement of lipid peroxidation and depletion of antioxidants (Ashakumary and Vijayammal, 1996). Our data suggest that nicotine-induced CYP2E1 may contribute to nicotine-induced oxidative stress and free radical generation.

In summary, we showed that like chronic ethanol, low, behaviorally relevant doses of nicotine induced CYP2E1 protein and activity in the centrilobular region of rat liver. In addition to smokers, millions of nonsmokers may be exposed therapeutically to nicotine during smoking cessation therapy and, potentially, during treatment for ulcerative colitis (Guslandi, 1999), Alzheimer’s disease (White and Levin, 1999), and Parkinson’s disease and other neuropsychiatric movement disorders (Erdmann, 1996; Kelton et al., 2000). Chronic nicotine may contribute to the observed cross-tolerance between ethanol and nicotine by increasing the clearance of ethanol. Considering the magnitude of induction and CYP2E1’s role in oxidative stress generation and procarcinogenic bioactivation, the enhanced levels of CYP2E1 by nicotine exposure may also contribute to susceptibility to alcoholic and nonalcoholic liver diseases. It remains to be determined whether nicotine and ethanol synergistically induce CYP2E1; whether nicotine alone, or with ethanol, induces CYP2E1 in humans; and whether this induction of CYP2E1 is associated with any advantageous, or detrimental, effects.

Acknowledgments

We thank Sharon Miksys, Wenjiang Zhang, Ewa Hoffmann, and Helma Nolte for technical assistance; Magnus Ingelman-Sundberg (Department of Physiological Chemistry, Karolinska Institute, Stockholm, Sweden) for providing the rabbit anti-rat CYP2E1 polyclonal antibody; Frank J. Gonzalez (National Cancer Institute, Bethesda, MD) for providing CYP cDNAs; and Neal Benowitz (Division of Clinical Pharmacology and Therapeutics at University of California, San Francisco, San Francisco, CA) for providing 5-methylnicotine.

References


Perry DC, Davila-Garcia MI, Stockmeier CA, and Kellar KJ (1999) Increased nico-


Schoedel KA, Sellers EM, and Tyndale RF (2001) Induction of CYP2B1/2 and nico-
tine metabolism by ethanol in rat liver but not rat brain. Biochem Pharmacol, in press.


Address correspondence to: Rachel F. Tyndale, Department of Pharmacol-
ogy, 1 King’s College Circle, University of Toronto, Toronto, ON, Canada M5S 1A8. E-mail: rtyndale@utoronto.ca