Rat Hepatic CYP2E1 is Induced by Very Low Nicotine Doses: An investigation of Induction, Time Course, Dose Response and Mechanism

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## Abstract

CYP2E1 is an ethanol- and drug-metabolizing enzyme that can also activate procarcinogens and hepatotoxicants and generate reactive oxygen species; it has been implicated in the pathogenesis of liver diseases and cancer. Cigarette smoke increases CYP2E1 activity in rodents and in humans and we have shown that nicotine (0.1-1.0 mg/kg s.c. x 7 days) increases CYP2E1 protein and activity in the rat liver. In the current studies we have shown that the induction peaks at 4 hours post nicotine (1 mg/kg s.c. X 7 days) treatment and recovers within 24 hrs. No induction was observed following a single injection and 18 days of treatment did not increase the levels beyond that found at 7 days. We found that CYP2E1 is induced by very low doses of chronic (x 7 days) nicotine with an $ED_{50}$ of 0.01 mg/kg s.c.; 0.01 mg/kg in a rat model results in peak cotinine levels (nicotine metabolite) similar to those found in people exposed to environmental tobacco smoke (passive smokers; 2-7 ng/ml). Previously we have shown no change in CYP2E1 mRNA, and our current mechanistic studies indicate that nicotine does not regulate CYP2E1 expression by protein stabilization. We postulated that a nicotine metabolite could be causing the induction but found that cotinine (1 mg/kg x 7 days) did not increase CYP2E1. Our findings indicate that nicotine increases CYP2E1 at very low doses and may enhance CYP2E1-related toxicity in smokers, passive smokers and people treated with nicotine (e.g. smokers, patients with Alzheimer's disease, ulcerative colitis or Parkinson's disease).

CYP2E1 is a member of the cytochrome P450 superfamily of heme-containing enzymes involved in the biotransformation of a variety of endogenous and exogenous compounds. CYP2E1 is of interest due to its potentially important role in the toxicity of a variety of chemicals and in the propagation of hepatic diseases.    A unique characteristic of CYP2E1 is its high NADPH oxidase activity (Gorsky et al., 1984) which results in increased production of reactive oxygen species including $O_2^-$ (superoxide radical) and $H_2O_2$ relative to other CYP isoforms (Gorsky et al., 1984). These reactive  intermediates can initiate lipid peroxidation, oxidative stress and Kupffer cell activation, thereby propagating cellular injury and DNA strand breaks (Jarvelainen et al., 2000).

CYP2E1 activates many xenobiotics to hepatotoxic or carcinogenic products including drugs such as acetaminophen (Raucy et al., 1989), industrial solvents such as carbon tetrachloride ($CCl_4$) (Manno et al., 1996) and nitrosamines such as N-nitrosodimethylamine (Lin et al., 1998). These hepatotoxicants and carcinogens are known to cause selective injury predominantly in the perivenular area, providing further evidence for the involvement of CYP2E1 as the presence and induction of CYP2E1 occur predominantly in this zone of the liver lobule (Tsutsumi et al., 1989). Furthermore, CYP2E1 activity strongly correlates with degree of tissue injury induced by these toxicants (Lieber, 1997).

CYP2E1 activity is induced by a variety of compounds many of which are also substrates of this enzyme. Ethanol is the best characterized CYP2E1 substrate and inducer. It is estimated that CYP2E1 is responsible for

approximately 20% of ethanol metabolism at pharmacologically relevant blood alcohol concentrations (Matsumoto et al., 1996). Moreover, CYP2E1 is increased 4- to 10-fold in liver biopsies of recently alcohol drinking patients (Tsutsumi et al., 1989) and CYP2E1 is thought to contribute to the increased ethanol metabolism and subsequent metabolic tolerance that develops in alcoholics (Lieber, 1999). Induction of CYP2E1 by agents such as ethanol can lead to a more pronounced formation of toxic and reactive metabolites which could lead to a higher risk of organ damage and carcinogenicity in people exposed to substrate chemicals and solvents. This may explain the increased vulnerability to hepatic toxicity of alcohol abusers upon exposure to therapeutically and industrially used xenobiotics such as acetaminophen and $CCl_4$ (Seeff et al., 1986).

In addition CYP2E1 activity can be induced by endogenous substrates and by pathophysiological states that result in the accumulation of ketones, such as fasting, diabetes and obesity (Lieber, 1997; Lieber, 1999). This physiological role may explain CYP2E1's high functional and regulatory conservation across species and within the human population (Lieber, 1997).

The molecular regulation of CYP2E1 induction is complex involving transcriptional, post-transcriptional and post-translational mechanisms (Lieber, 1997). The mechanism involved in CYP2E1 induction may depend on the dose, duration, and/or route of inducer treatment, as well on the specific structurally diverse inducing agent. For example, CYP2E1 induction by ethanol seems to occur by a two-step mechanism: post-translational mechanisms at low ethanol levels and transcriptional mechanisms at high ethanol levels (Badger et al., 1993;

Ronis et al., 1993).

Cigarette smoke induces CYP2E1 activity in rodents and in humans (Villard et al., 1998; Benowitz, 1999b). By inducing the activity and expression of CYP2E1, cigarette smoke may enhance the production of carcinogenic and toxic metabolites, further increasing the risk for cancer and organ damage in smokers. These findings could perhaps explain the fact that alcohol abuse and smoking interact synergistically as etiological factors for cancers (Garro and Lieber, 1990). Furthermore, since CYP2E1 substrates are commonly found in the industrial workplace (Raucy et al., 1993; Wang et al., 1996), smokers in these environments may be more sensitive to chemical injury than non-smokers.

Our laboratory has demonstrated that nicotine (0.1-1.0 mg/kg s.c. x 7 days), which is the most abundant chemical in cigarette smoke, increases CYP2E1 protein and activity in the rat liver (Howard et al., 2001). Since the observed level of CYP2E1 induction (1.5-1.8 fold) by nicotine was saturated at the doses tested in this initial study, we hypothesized that lower nicotine doses can induce hepatic CYP2E1. Such lower doses may be important in terms of passive smokers or people on nicotine replacement therapy (NRT) who may also be at risk of liver damage associated with increased CYP2E1 enzyme. The purpose of the current study was to further characterize this effect in terms of the dose-response relationship, time-course for induction, recovery time-course of induction, mechanisms of regulation, and potential involvement of the nicotine metabolite cotinine in CYP2E1 induction.

## Methods

### *Materials*

Nicotine bitartrate and cotinine were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Recombinant viral-expressed rat CYP2E1 in lymphoblastoid cells was obtained from GENTEST (Woburn, MA). The protein assay dye reagent concentrate was purchased from BioRad Laboratories (Hercules, CA). Pre-stained molecular markers were purchased from MBI Fermentas. Rabbit anti-rat CYP2E1 polyclonal antibody was generously provided by Magnus Ingelman-Sundberg (Department of Physiological Chemistry, Karolinska Institute, Stockholm, Sweden). Horseradish peroxidase-conjugated goat anti-rabbit IgG and SuperSignal West Pico Chemiluminescence Substrate were purchased from Pierce (Rockford, IL).  Hybond ECL Nitrocellulose Membrane was purchased from Amersham Pharmacia Biotech (Toronto, ON, Canada). All other chemical reagents were obtained from standard commercial sources.  GM0637 cells were obtained from NIGMS Human Genetic Mutant Cell Repository (Camden, NJ), and CHO-K1 cells were obtained from ATCC (Rockville, MD).

### *Animals*

Adult male Wistar rats (250-300g; Charles River, St-Constant, PQ, Canada), were used throughout these experiments. Upon arrival in the Animal Care Facility the animals were housed two per cage and allowed to adapt to the novel environment for one week. The animals were kept in a controlled environment with a 12 hour artificial light-dark cycle (light on at 6:00 a.m.). The

animals received rat chow and water ad libitum throughout the study period. All procedures described in the present study were conducted in accordance with the guidelines for the care and use of laboratory animals and were approved by the Animal Care Committee of the University of Toronto.

### Drug Treatment

The rats received subcutaneous (s.c.) injections of either nicotine bitartrate or cotinine dissolved in sterile saline. Solutions of nicotine containing 1 mg/ml (6.16 $\mu$mole/ml) nicotine base were prepared fresh daily by adding 28.51 mg of nicotine salt to 10 ml of sterile saline (0.9 % NaCl). This stock solution was titrated to pH 7.4 with 1N NaOH, and then was serially diluted to achieve desired nicotine concentrations. All nicotine doses cited are calculated as the base and were given per kg of body weight. Cotinine solution, at a concentration of 1.086 mg/ml (6.16 $\mu$mole/ml) cotinine, was also prepared daily by adding 4.5 mg cotinine to 4.14 ml of saline.  Storage bottles were kept wrapped in aluminum foil due to the light sensitivity of both nicotine and cotinine.  Control animals were treated with vehicle (saline) using an identical administration protocol. Rats were sacrificed by decapitation 4 hours after the last drug treatment unless specified otherwise. Livers were rapidly removed, frozen immediately in liquid nitrogen and stored at -80°C until used for microsomal preparation.

### Recovery Time-Course Study

In a time-course study, rats were treated with 0 or 1 mg/kg nicotine (n=3/group) for 7 consecutive days and were sacrificed at 0.5, 2, 4, 8, 12, 18 and

24 hours after the last drug treatment. Saline controls were included for each time point in order to eliminate any possible contribution of a diurnal cycle effect on CYP2E1 levels (Bruckner et al., 2002).

### Induction Time-Course Study

Rats (n=4/group) received a single, acute injection of 0 or 1.0 mg/kg nicotine s.c. and were sacrificed 4 hours later; these findings were contrasted to animals treated for 5, 7 and 18 days.

### Nicotine Dose-Response Study

Dose-response studies included 9 groups of rats (n=3/group), injected for 7 consecutive days with 0, 0.001, 0.003, 0.005, 0.01, 0.02, 0.03, 0.1 or 1.0 mg/kg nicotine s.c.. We have previously demonstrated that treatment of rats with 0.1 and 1.0 mg/kg nicotine results in a statistically significant increase in CYP2E1 protein level in rat liver (Howard et al., 2001); these doses were thus included as positive controls in the present study. The nicotine doses employed in this study are of behavioral and pharmacological relevance. Subcutaneous nicotine administration of 1.6 and 1.2 mg/kg have been associated with central nicotinic receptor adaptation, a pharmacodynamic change observed in brain regions of smokers and hypothesized to be one pathway by which nicotine exerts its behavioral effects such as tolerance (Rowell and Li, 1997; Perry et al., 1999). This range also includes doses that rats will self-administer (0.03 and 0.06 mg/kg per infusion or 0.3 and 0.6 mg/kg in 2 hrs) (Shoaib and Stolerman, 1999) and doses at which nicotine exerts its discriminative stimulus in rats ($ED_{50} = 0.14$

mg/kg s.c.) (Pratt et al., 1983). In addition chronic injections of 0.8 to 1.6 mg/kg nicotine increase ethanol self-administration by male Wistar rats (Blomqvist et al., 1996).

### Cotinine Study

Rats (n=3/group) were injected with cotinine at 1.086 mg/kg s.c. for 7 consecutive days and were sacrificed at 0.5, 4 and 8 hours after the last treatment.

### Microsomal Preparation

Liver microsomes were prepared as described previously (Miksys et al., 2000), aliquoted into small volumes and stored at -80°C until use.

### Western Blotting

Immunoblotting was performed as previously reported (Howard et al., 2001), with minor modifications. In brief, proteins (1.5 µg/liver sample) were separated by SDS-polyacrylamide gel electrophoresis (4% stacking and 10% separating gels), transferred overnight onto nitrocellulose membrane, and probed with a polyclonal rabbit anti-rat CYP2E1 antibody (1:4000 dilution for 1h). Microsomes from rat CYP2E1-expressing-lymphoblastoid cells were used as positive control. A mini standard curve consisting of 0, 1.0, 1.5 and 2.0 µg of hepatic microsomal protein was included in all experiments to allow the analysis of only those blots with samples that fell within the linear region of detection of the standard curve. Membranes were incubated with a peroxidase conjugated

secondary antibody (dilution 1:25000 for 1h) followed by detection by chemiluminescence. Membranes were exposed to autoradiography film (Ultident, St. Laurent, P.Q., Canada) for 0.25 to 2 minutes. Digital images of immunoblots were analyzed using MCID Elite software (Imaging Research Inc., St. Catherines, ON, Canada). The relative density of each band was corrected for the density of the film background and expressed as arbitrary density units.

### *Stability of CYP2E1*

Established methods for assessing CYP2E1 protein stabilization were performed according to published protocols (Barmada et al., 1995; Huan and Koop, 1999). In brief, Chinese hamster ovary cells (CHO-K1) that constitutively express rabbit CYP2E1 protein (Barmada et al., 1995) and human fibroblast cells (GM0637) that express rat CYP2E1 (Lin et al., 1998) were treated with various concentrations of nicotine (5 to 0.00005 $\mu$M). CYP2E1 catalytic activity was assessed by the 6-hydroxylation of chlorzoxazone. After overnight treatment with or without nicotine, cells were incubated with 200 $\mu$M chlorzoxazone for 6 h. Media was then extracted and 6-OH-chlorzoxazone was determined by HPLC (Barmada et al., 1995). Cells were allowed to lyse passively, insoluble debris was removed by centrifugation and the supernatants were frozen until use for immunoblot analysis. Western blotting was performed to measure the levels of immunodetectable CYP2E1 protein. Methylpyrazole can stabilize CYP2E1 in this *in vitro* model (Huan and Koop, 1999) and was used here as a positive control.

### *Determination of Plasma Nicotine and Cotinine*

Trunk blood (6-8 ml) was collected at the time of sacrifice. Plasma was

prepared by centrifugation at 3000g for 10 min and was stored at –20°C. Nicotine

and cotinine plasma concentrations were measured by standard HPLC

techniques using 5-methylnicotine as the internal standard (Xu et al., 2002). The

limit of detection of the assay was 1.5 ng/ml and there was a linear relationship

between detected chromatographic peak and nicotine and cotinine

concentrations.

### Statistical Analyses

Results are expressed as mean ± standard deviation (SD), which

represents the average values obtained from 3 different animals per treatment

group, from at least 3 separate experiments. In all experiments, statistical

significance of the difference between control and treated groups was

determined using unpaired Student's t-test. For the dose-response study, one-

way analysis of variance (ANOVA) was used for comparisons of multiple group

means. Statistically significant differences were determined at the 5% level

(p<0.05).

## Results

An immunoblotting assay was established to measure hepatic CYP2E1. Detection of serially diluted CYP2E1 indicated that the immunoblotting signal was linear up to 3.0 µg of microsomal protein from saline treated animals; no signal was detected in the absence of primary antibody (results not shown). The specificity of the rabbit antibody raised against rat CYP2E1 has been previously demonstrated (Hansson et al., 1990) and we have reconfirmed this finding (Howard et al., 2001).

### CYP2E1 Recovery Time-Course.

The return to basal levels of CYP2E1 following induction, after 7 days of treatment with 1.0 mg/kg nicotine, was assessed as a function of time over 24 hours.  Western blot analyses revealed significant increases in CYP2E1 levels of 1.2- ($p<0.05$), 1.3- ($p<0.05$)-, 1.6- ($p<0.01$), 1.4- ($p<0.05$) and 1.3-fold ($p<0.01$) at 0.5, 2, 4, 8 and 12 hours post-treatment compared to their respective control groups (Fig. 1A). The higher levels of CYP2E1 in nicotine treated rats returned to the levels found in saline treated animals by 18 hours post-treatment. In this study, the concentration of plasma nicotine and its metabolite, cotinine, were determined as a function of sacrifice time after the last drug treatment. The plasma level of nicotine was highest at 30 minutes (first time tested) and declined by 4 hours post-injection (Fig. 1B). The level of cotinine peaked at 4 hours and then slowly declined to near control levels by 24 hours post-treatment (Fig. 1B). The maximal increases in CYP2E1 protein were observed at 4 hours post-treatment, a time when nicotine levels were essentially zero, whereas cotinine

levels were at their peak. Neither nicotine nor cotinine were detected in vehicle-treated animals.

### Nicotine Induction Time-Course Study

Time-course studies revealed that CYP2E1 induction by nicotine returned to basal levels by 24 hours. This finding prompted us to examine whether the necessary changes leading to induction require a chronic (7 days) treatment or whether these changes occur following an acute (single) treatment. Western blot analyses (Fig. 2B) were carried out to determine the effect of a single, acute dose of nicotine on the expression of hepatic CYP2E1. Treatment of rats with 1.0 mg/kg nicotine for one day failed to alter CYP2E1 protein levels (p=0.36) (Fig. 2A). However in rats treated chronically with the same dose for 5, 7 and 18 consecutive days we observed a 1.4- (p=0.3), 1.7- (p<0.01) and 1.5- fold (p<0.05) increase in CYP2E1 levels.  Plasma nicotine and cotinine levels were measured in this study at the time of sacrifice (4 hours post treatment).  We observed no statistically significant (p=0.18 and p=0.06) accumulation of nicotine (12, 14, 20 and 15 ng/ml) or cotinine (229, 250, 272 and 215 ng/ml) in rats treated for 1, 5, 7 or 18 days respectively.

### Dose-Dependent Induction of CYP2E1 by Nicotine.

Previous studies conducted in our laboratory have demonstrated that treatment of rats with nicotine at 0.1, 0.3 and 1.0 mg/kg for 7 consecutive days resulted in increased CYP2E1 protein (1.4, 1.8 and 1.5-fold respectively) (Howard et al., 2001). Moreover we observed increased CYP2E1 activity

(increased $V_{max}$, no change in $K_m$) in the same animals, of similar magnitude to the observed increase in CYP2E1 protein, as assessed by chlorzoxazone metabolism (Howard et al., 2001). We confirmed that the enhancement of chlorzoxazone hydroxylation was mediated by CYP2E1 by chemical inhibitory studies with specific CYP2E1 inhibitors. The apparent saturation of CYP2E1 protein induction prompted us to examine whether nicotine can increase CYP2E1 at doses lower than 0.1 mg/kg. In the present study, western blot analyses were carried out to investigate the dose-response relationship of nicotine on hepatic CYP2E1 protein levels. Chronic (7 day) treatment of rats with nicotine at the doses of 0, 0.001, 0.003, 0.005, 0.01, 0.02, 0.03, 0.1 and 1.0 mg/kg/day, resulted in 1.02-, 1.05-, 1.17-, 1.40- ($p < 0.005$), 1.50- ($p < 0.05$), 1.57- ($p < 0.01$), 1.55- ($p < 0.001$) and 1.62-fold ($p < 0.01$) increases in CYP2E1 protein levels compared to saline controls (Fig. 3). The $ED_{50}$ value for the observed increase in CYP2E1 protein level was approximately 0.01 mg/kg. Results for treatment with 0.1 and 1.0 mg/kg nicotine were consistent with our previous findings (Howard et al., 2001).

Plasma nicotine and cotinine levels were also measured in this study. Due to the low doses used in this study, and due to the sacrifice time (4 hours post treatment), plasma nicotine levels were undetectable in rats treated with 0.001 to 0.03 mg/kg s.c. nicotine (Table 1). Even at the highest doses tested (0.1 and 1.0 mg/kg) the plasma nicotine levels were very low (1.6 and 19.7 ng/ml respectively). Cotinine, which has a much longer elimination half-life compared to nicotine, was detectable for doses ranging between 0.005 to 1.0 mg/kg (4.0 to

272.0 ng/ml respectively) (Table 1). Nicotine and cotinine levels measured in plasma were reflective of the administered nicotine dose which demonstrates that our treatment was effective.

### Nicotine does not induce CYP2E1 by protein stabilization.

The mechanism by which the majority of CYP2E1 inducers, including ethanol and low-molecular weight ligands, increase CYP2E1 is by protein stabilization, particularly at low doses. Previous studies indicated that substrates or ligands for CYP2E1 can stabilize the protein and inhibit its degradation resulting in an increase in the steady state level of the enzyme.  In the present study there was no change in chlorzoxazone (CYP2E1 substrate) metabolism, or in the levels of immunodetectable CYP2E1 protein in nicotine treated cells suggesting that the addition of nicotine failed to inhibit the degradation of CYP2E1 during the overnight incubation in cells expressing either rabbit or rat CYP2E1 (data not shown). Addition of the CYP2E1 ligand 4-methylpyrazole decreased the degradation of CYP2E1 as previously reported (Huan and Koop, 1999), resulting in a 2.5-fold increase in immunodectable CYP2E1 protein. These results suggest that nicotine does not inhibit the degradation of CYP2E1, and that the induction of CYP2E1 by nicotine does not occur via stabilization of the enzyme.

### Effects of Cotinine on CYP2E1 Protein Levels.

Our mechanistic studies suggest that nicotine does not increase CYP2E1

protein by two common induction mechanisms, specifically transcriptional regulation (Howard et al., 2001)or protein stabilization. Furthermore, in the time-course studies we observed similar patterns of CYP2E1 induction and plasma cotinine levels over time; nicotine plasma levels were undetectable at 4 hours post-treatment when we observed maximal CYP2E1 induction. These findings suggested the possibility that the major nicotine metabolite, cotinine, may be responsible for the observed CYP2E1 induction by nicotine. Treatment of rats with 1.086 mg/kg s.c. cotinine (dosing for equal molarity to 1.0 mg/kg nicotine) for 7 days, resulted in plasma cotinine levels of 837, 489 and 295 ng/ml at 0.5, 4 and 8 hours post-treatment (Fig. 4B). Despite the high plasma cotinine levels achieved with this treatment we observed no significant increase in CYP2E1 protein levels (Fig. 4 A and B). At 4 and 8 hours post treatment plasma cotinine levels (Fig. 4B) were similar to those found at 4 hours post nicotine (1.0 mg/kg) treatment (Fig. 1B) when maximal CYP2E1 induction was observed in nicotine treated rats (Fig. 1A). However, CYP2E1 levels were not elevated in cotinine treated rats at 4 or at 8 hours after the last injection (Fig. 4A). These results indicate that cotinine does not induce CYP2E1.

## Discussion

Previous studies conducted in our laboratory demonstrated that relatively low doses of nicotine induce CYP2E1 protein and activity in the rat liver (Howard et al., 2001). The objective of the current study was to further characterize this effect in terms of the dose-response relationship, the on and off time-course of induction, and to investigate mechanisms for CYP2E1 regulation by nicotine.

Based on our findings, the induction of hepatic CYP2E1 by nicotine requires multiple doses (Fig. 2). After 7 days of treatment the induction was rapid, as evidenced by the significant elevation in protein levels within 0.5 hours of nicotine treatment (Fig 1A). This induction is also best characterized as short-lasting, because the elevated levels of CYP2E1 in nicotine treated rats returned to near control levels by 18-24 hours after the last nicotine injection (Fig. 1A). We have also established that CYP2E1 levels peak at 4 hours after nicotine treatment which suggests that the highest risk in terms of CYP2E1 associated toxicity may be at approximately 4 hours following nicotine exposure at which time the production of CYP2E1-mediated toxic metabolites is expected be at its peak.

CYP2E1 inducers, including ethanol (Petersen et al., 1982), acetone (Forkert et al., 1994) and pyridine (Kim and Novak, 1990), are known to exert their stimulatory effects on CYP2E1 in both an acute and a chronic setting. In contrast, treatment of rats with a single dose of nicotine failed to alter hepatic CYP2E1 protein levels (Fig. 2). This finding implies that induction requires more than one exposure to nicotine suggesting a required priming effect and is

consistent with the idea that the induction of CYP2E1 by nicotine is not via protein stabilization. These data also suggest that a single exposure to nicotine may be insignificant in enhancing CYP2E1-associated hepatotoxicity, at least at the dose and paradigm tested here.

We have also shown that CYP2E1 induction is very sensitive to chronic nicotine with an estimated $ED_{50}$ of 0.01 mg/kg s.c.. Plasma nicotine levels in rats treated with 0.01 mg/kg s.c. were undetectable at 4 hours post-treatment, whereas cotinine levels were approximately 7.7 ng/ml. Blood or plasma concentrations of nicotine in smokers generally range from 10 to 50 ng/ml (Benowitz, 1999a). Plasma cotinine levels lower that 15-17.5 ng/ml are considered suggestive of exposure to second hand-smoke or environmental tobacco smoke (ETS). From our studies and previous investigations (Pratt et al., 1983), subcutaneous nicotine administration to rats leads to peak nicotine plasma levels after 30-60 minutes. While for the dose-response studies we have measured nicotine and cotinine plasma levels at 4 hours after the last treatment (Table 1), plasma nicotine and cotinine levels were measured at 30 minutes following a 7 day treatment with 1.0 mg/kg s.c. nicotine in our recovery-time course studies (Fig. 3B) where we found plasma nicotine of approximately 210 ng/ml and cotinine of 120 ng/ml. Based on the information available we can deduce that at the $ED_{50}$ (0.01 mg/kg s.c.) for effect on CYP2E1, peak plasma nicotine in rats at 30 minutes would be approximately 2.1 ng/ml and peak cotinine plasma levels at 4 hours would be 2.4 - 7.7 ng/ml. Of note, plasma cotinine in non-smoking adults living with a smoking versus a non-smoking

partner have been estimated at 2 ng/ml and 0.31 ng/ml respectively (Jarvis et al., 2001). Similar plasma cotinine levels have also been observed in young children living with at least one smoking parent (2.9 ng/ml) versus children with non-smoking parents (0.26 ng/ml) (Tang et al., 1999). Based on the similarities of plasma nicotine and cotinine levels, we can postulate that nicotine doses of 0.01 mg/kg s.c. ($ED_{50}$), at which we observed induction of CYP2E1, would be comparable to levels of ETS exposure in humans.

Therefore, in addition to smokers and people on nicotine replacement therapies, individuals exposed to ETS may also have increased CYP2E1 levels and increased risk of CYP2E1-related toxicity and cancer development via amplified activation of tobacco-smoke and other procarcinogens. Nevertheless, while smokers have higher levels of CYP2E1 in liver (Benowitz, 1999b) and brain (Howard et al., 2003) the induction by nicotine needs to be explicitly tested in humans, and the consequences of this induction with clinical and toxicological outcomes in passive and active smokers investigated.

We have previously demonstrated that nicotine does not regulate CYP2E1 expression by transcriptional mechanisms (Howard et al., 2001); no change in mRNA levels was observed following CYP2E1 induction by nicotine. An alternative mechanism of CYP2E1 induction is protein stabilization. Many CYP2E1 inducers, including ethanol (at low doses), acetone and pyrazole, interact with CYP2E1's active site and increase CYP2E1 levels by slowing its high NADPH oxidase activity and the subsequent generation of reactive oxygen species (Zhukov and Ingelman-Sundberg, 1999). These reactive metabolites are

thought to oxidize and modify the enzyme labeling CYP2E1 for auto-degradation by cellular proteolytic systems (Zhukov and Ingelman-Sundberg, 1999). Therefore, the binding of such substrates/inducers prolongs CYP2E1 half-life by inhibiting the fast-phase component associated with this enzyme's normal degradation. Since nicotine does not appear to regulate CYP2E1 by transcriptional mechanisms, we have conducted studies to examine whether nicotine can induce CYP2E1 by protein stabilization (Barmada et al., 1995; Huan and Koop, 1999). Using CHO cells that constitutively express rabbit CYP2E1 and human fibroblast cells that express rat CYP2E1 no change in chlorzoxazone metabolism and no elevation in CYP2E1 protein levels following nicotine exposure was detected. These findings suggest that nicotine does not inhibit the degradation of CYP2E1 by interacting with its active site, and that in vivo, the induction of CYP2E1 by nicotine is unlikely to occur via stabilization of the enzyme by nicotine. However, the molecular triggers that target CYP2E1 for degradation are still unclear and require further investigation. Thus, it is possible that *in vivo*, nicotine may slow-down CYP2E1 degradation by altering some transduction pathways or targeting events that are not present, or are different, in the CHO and in the human fibroblast cell lines. This would however distinguish the induction mechanism of nicotine from that of ethanol which has been shown to stabilize CYP2E1 in this experimental model (McGehee et al., 1994). In addition, we have demonstrated that nicotine does not inhibit CYP2E1-hydroxylation of chlorzoxazone in rat liver microsomes (Howard et al., 2001). We interpret this as evidence that nicotine does not interact with CYP2E1's catalytic

site, and does not induce CYP2E1 by protein stabilization directly.

Alternatively, nicotine could indirectly cause CYP2E1 protein stabilization in vivo, via its conversion to metabolites such as cotinine, which would not occur in the in vitro system in which we tested the stabilization of CYP2E1.  Nicotine is readily metabolized to cotinine in humans primarily by hepatic CYP2A6, while in rats hepatic CYP2B1 is the main catalyst in the conversion of nicotine to cotinine (Nakayama et al., 1993).  Interestingly, in our recovery time-course study we observed that plasma cotinine levels and CYP2E1 protein induction displayed similar patterns over 24 hours following nicotine treatment. Moreover, nicotine plasma levels were undetectable at 4 hours post-treatment when we detected maximal CYP2E1 induction. Based on these finding we hypothesized that cotinine could be responsible for the observed CYP2E1 induction in nicotine treated rats. However, cotinine treatment did not result in a significant increase in hepatic CYP2E1 levels (Fig. 4A); the role of intermediate metabolites  such as the nicotine-$\Delta^{1'(5')}$iminium ion remains to be investigated. While in rats the metabolism of nicotine to cotinine is less extensive, and other nicotine metabolites are more predominant than in humans, the induction of this enzyme by smoking or nicotine has been observed in humans (Benowitz, 1999b) and in non-human primates (Lee, Miksys and Tyndale unpublished observations) suggesting that a similar mechanism or compound is responsible.  In addition, the acute nicotine dose is unable to induce CYP2E1 which suggests that the mechanism does not involve protein stabilization by any of the rat metabolites. Other CYP2E1 inducers, such as pyridine, increase CYP2E1 protein by

enhancing CYP2E1 mRNA translational efficiency (Kim et al., 1990; Lieber, 1999). Since nicotine bears structural similarity to pyridine, translational activation of CYP2E1 may be the mechanism underlying nicotine's induction of this enzyme. Further investigations are necessary to elucidate the mechanism involved in CYP2E1 induction by nicotine.

In conclusion we have demonstrated that very low doses of nicotine, that are comparable to ETS exposure in humans, can induce hepatic CYP2E1 in the rat liver. Induction of CYP2E1 results in increased oxidative stress, activation of tobacco smoke, other procarcinogens and hepatototoxicants, which may increase the risk for organ damage and cancer development. Therefore, our data suggest that nicotine may increase CYP2E1 related toxicity in smokers, passive-smokers, and people treated with nicotine (e.g. smokers, patient's with Alzheimer's disease, ulcerative colitis and neuropsychiatric motor disorders).

**Acknowledgements**

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**Footnotes**

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*Nicotine and Tobacco.*

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**Fig. 1.**   Recovery time-course of hepatic CYP2E1 induction by 1.0 mg/kg s.c nicotine for 7 days.  (A) Fold-induction in CYP2E1 levels relative to saline (Sal) treated animals over 24 hours post-treatment.  Each point represents the mean + SD of at least three different experiments (n=3 rats).  Significant difference from saline is indicated by * (p<0.05).  (B) Corresponding  plasma nicotine (Nic) and cotinine (Cot) levels over 24 hours post-treatment.

**Fig. 2.**  Acute vs Chronic effects of nicotine on CYP2E1 levels.  (A) No significant difference was observed in rats treated with a single dose of 1.0 mg/kg nicotine when compared with their respective saline controls, whereas chronic treatment for 7 and 18 days results in a significant increase in CYP2E1 levels.  Significance from saline is indicated by *(p<0.05) and ** (p<0.01).   (B) Representative immunoblot of 1 day (acute) vs 7 day (chronic) nicotine treatment and their representative saline controls; 3 animals per treatment group.
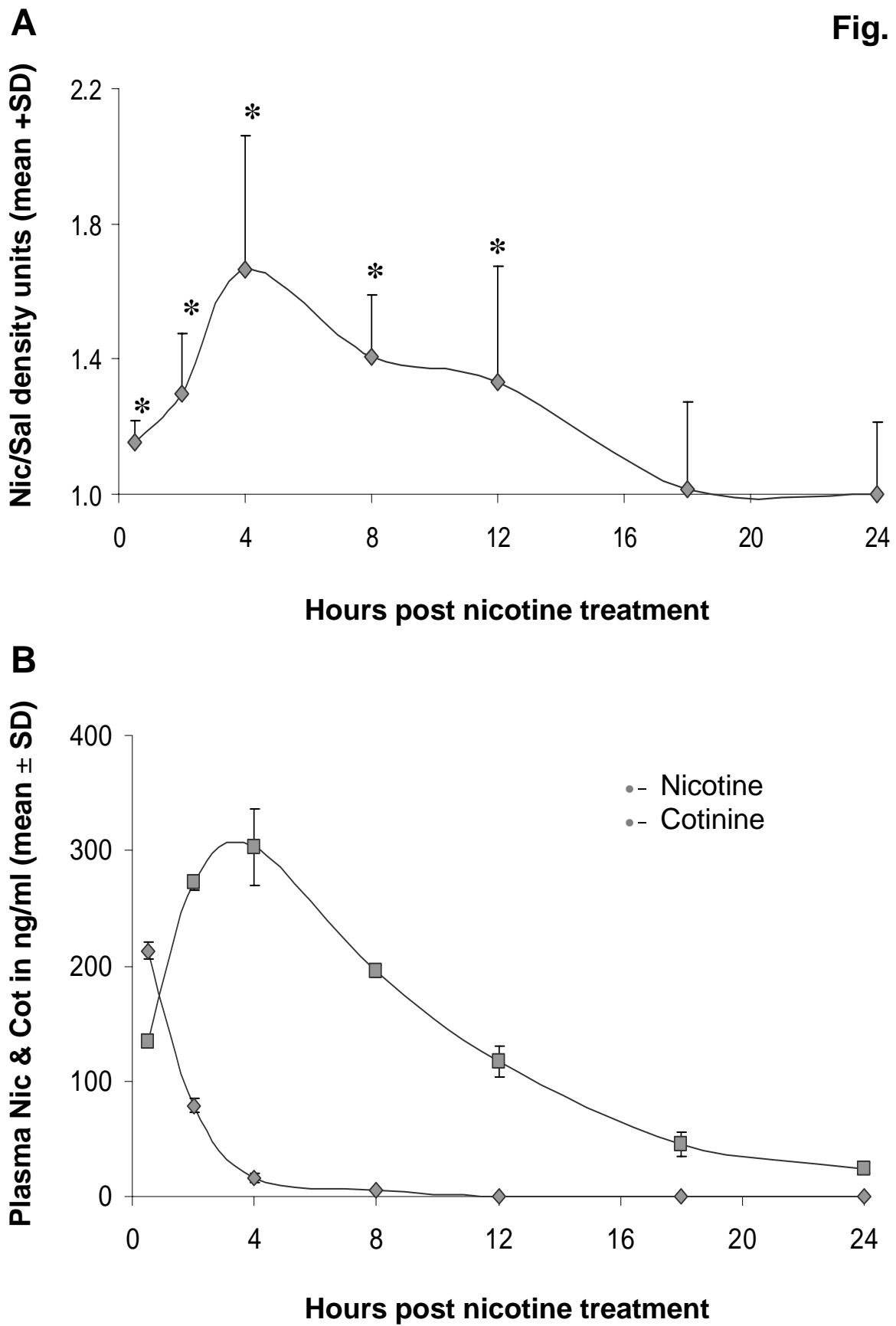
**Fig. 3.**   Dose-dependent induction of hepatic CYP2E1 by nicotine. (A) Dose-response curve of CYP2E1 induction as a function of nicotine (Nic) relative to saline (Sal) treated animals.   Each point represents mean Nic/Sal of three animals/group + SD.  Significant difference from saline is indicated by * (p<0.05). (B) Representative immunoblot of rats treated with 0, 0.01, 0.1 and 1.0 mg/kg Nic; three animals per treatment group.

**Fig. 4.** Effects of cotinine (Cot) treatment on CYP2E1 protein level. (A) No significant increase was observed in CYP2E1 levels at 0.5, 4 and 8 hours post cotinine treatment relative to saline (Sal) treated animals. (B) Plasma cotinine levels over time. Each point represents the mean ± SD of 3 animals/group.
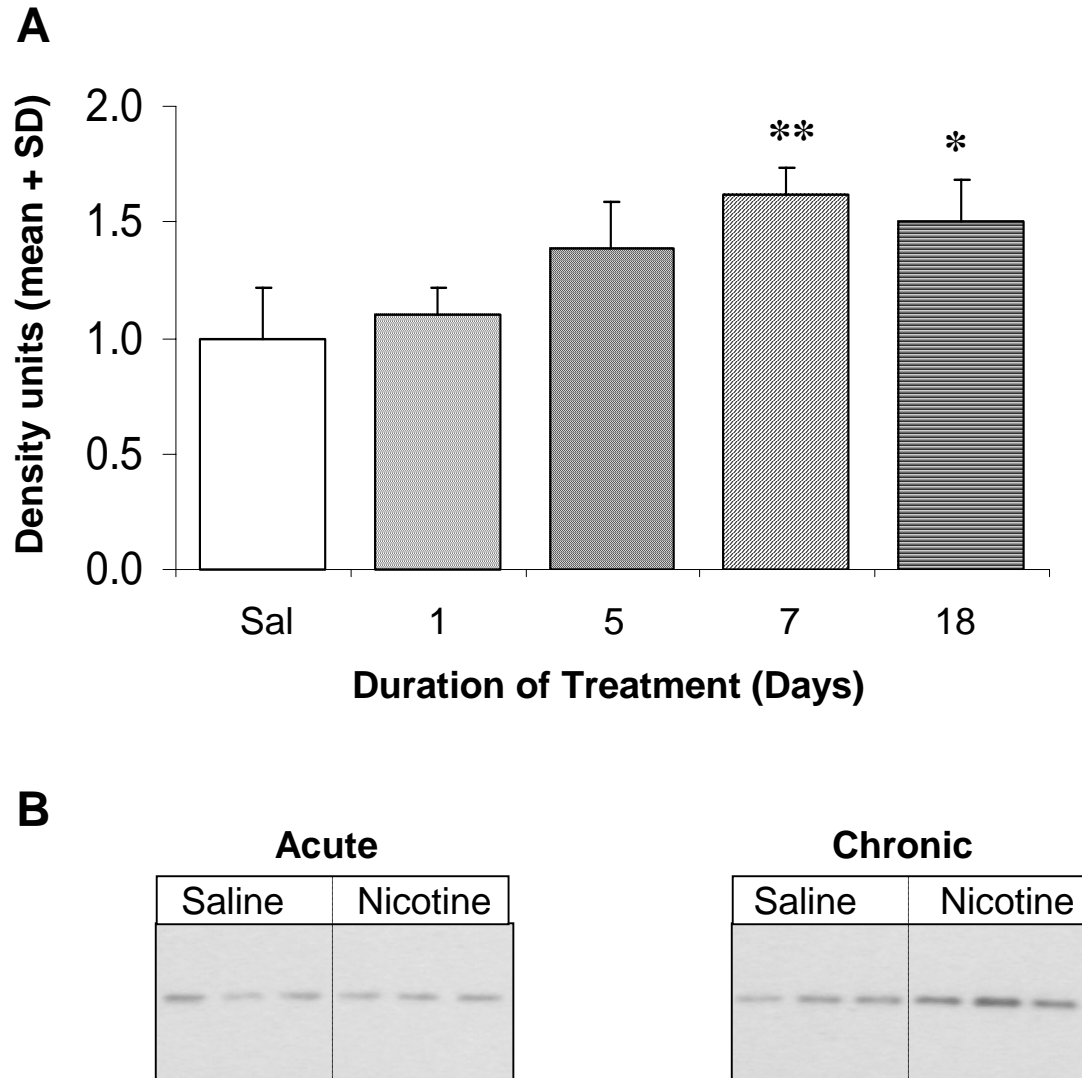
**Table 1.** Plasma nicotine and cotinine levels following s.c. injections of 0.001 to 1.0 mg/kg nicotine.  Rats were sacrificed at 4 hours post last nicotine treatment.  Data are means ± SD of 3 animals in each treatment group.

| Nicotine Dose | Plasma levels (ng/ml) | |
|---|---|---|
| mg/kg s.c | Nicotine | Cotinine |
| 0.001 | <1.5 | <1.5 |
| 0.003 | <1.5 | <1.5 |
| 0.005 | <1.5 | 4.0 ± 1.0 |
| 0.01 | <1.5 | 7.7 ± 1.53 |
| 0.02 | <1.5 | 10.0 ± 1.0 |
| 0.03 | <1.5 | 10.0 ± 2.65 |
| 0.1 | 1.6 ± 0.6 | 24.3 ± 2.08 |
| 1.0 | 19.7 ± 1.3 | 272.0 ± 28.2 |

**A**

**Fig. 1**



**B**
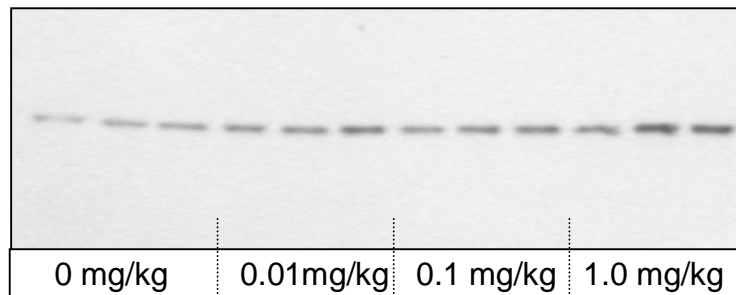
**Fig. 2**

**A**



**B**

**Fig. 3**

**A**



**B**

**Fig. 4**

**A**

**B**