# AN INVESTIGATION OF THE INDUCTION OF HEPATIC CYP2E1 BY LOW DOSES OF NICOTINE IN THE RAT

by

Alina L. Micu

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# Abstract

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Alina L. Micu, M.Sc. 2003, Department of Pharmacology, University of Toronto

CYP2E1 is an ethanol and drug-metabolizing enzyme that can also bioactivate hepatotoxins and procarcinogens 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 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 treatment and requires multiple exposures to nicotine. We have found that CYP2E1 is induced by very low doses of nicotine with an $ED_{50}$ of 0.01 mg/kg s.c.. Our mechanistic studies indicate that nicotine does not regulate CYP2E1 expression by transcriptional mechanisms or by protein stabilization. Cotinine, the main nicotine metabolite is not involved in CYP2E1 induction in our rat model. Our findings indicate that nicotine may increase CYP2E1-induced toxicity in smokers, passive smokers and people treated with nicotine.

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# 1. Introduction

## 1.1 Cytochromes P450

Cytochromes P450 (CYPs) are a superfamily of heme-containing enzymes involved in the biotransformation of a wide variety of exogenous and endogenous compounds. This enzymatic system is characterized by its broad and overlapping substrate specificity, and its enzyme multiplicity (multiple molecular forms of CYPs are present in any given specie); features which enable the handling of a vast and diverse range of substrates (Parkinson, 2001).

CYP enzymes have been detected in many living species from bacteria, to plants, to humans (Nelson *et al.*, 1996). In mammals, CYPs are most abundantly expressed in the liver endoplasmic reticulum (microsomes), but they have also been identified in nearly every tissue (Nelson and Strobel, 1988; Parkinson, 2001).

The primary catalytic function of CYPs is monoxygenation, which involves the incorporation of one oxygen atom into a substrate while the other is reduced to water with reducing equivalents usually derived from NADPH (Anzenbacher and Anzenbacherova, 2001; Riddick, 1998). This reaction generally results in a more water soluble, hydrophillic compound, which can either be eliminated from the body or it can be further modified by phase II enzymes (conjugation reactions) (Parkinson, 2001; Riddick, 1998). Although CYP enzymes generally convert xenobiotics to less toxic, more water-soluble products, the reactions can involve the formation of reactive oxygen species (ROS), and the activation of protoxins and procarcinogens increasing the risk of organ damage and carcinogenicity (Anzenbacher and Anzenbacherova, 2001; Rendic and Di Carlo, 1997).

The superfamily of CYP enzymes has been classified into different families and subfamilies according to the degree of homology of amino acid sequences in their protein

structures (Nelson *et al.*, 1996). CYPs that have at least 40% homology in their amino acid sequence are classified as members of the same family, which is designated by a number (e.g. CYP1 family). Enzymes within a subfamily share >55% sequence homology and are designated by capital letters (e.g. subfamily CYP1A). Individual enzymes within a subfamily are designated by a number (e.g. CYP1A1, CYP1A2).

The level and activity of CYP enzymes varies considerably among individuals due to environmental and/or genetic factors (Meyer and Zanger, 1997; Shimada *et al.*, 1994). Increased CYP levels and activity are commonly observed in response to certain substances or physiological states known as inducers. CYP inducers include therapeutic (e.g. barbiturates, anticonvulsants) and recreational drugs (e.g. alcohol, caffeine), environmental contaminants (e.g. carbon tetrachloride), dietary constituents (e.g. cruciferous vegetables), and disease status (e.g. diabetes, viral and bacterial infections) (Parkinson, 2001; Riddick, 1998). Various molecular mechanisms of CYP induction have been characterized. These include transcriptional mechanisms (e.g. increased DNA transcription), post-transcriptional mechanisms (e.g. mRNA stabilization, translational efficiency) and post-translational mechanisms (e.g. protein stabilization) (Riddick, 1998).

CYP induction results in accelerated rates of xenobiotic biotransformation which may have pharmacological and toxicological implications (Parkinson, 2001). Clinically important consequences of cytochrome P450 induction include pharmacokinetic tolerance (larger doses of drug must be administered to achieve desired drug concentrations), enhanced production of ROS, and greater activation of procarcinogens and protoxins. For these reasons, induction of CYPs and their role in drug tolerance and disease continues to be an exciting and extensively studied research field.

## 1.2 Cytochrome P450 2E1

### 1.2.1 Human and Rat CYP2E1

In humans and in rats, the CYP2E subfamily is represented by only one member, the CYP2E1 isozyme (Morel *et al.*, 1999). The rat CYP2E1 gene has been isolated and localized to chromosome 7 (Umeno *et al.*, 1988b) and the human gene to chromosome 10 (Umeno *et al.*, 1988a). Immunoinhibition, immunoquantification and structural studies have demonstrated that the structure and activity of rat and human hepatic CYP2E1 are remarkably similar (Wrighton *et al.*, 1986). Additional studies have confirmed that human CYP2E1 and its rat orthologue have substantially overlapping substrate specificity as well as similar regulatory features (Lechevrel and Wild, 1997; Takahashi *et al.*, 1993; Wrighton and Stevens, 1992). Thus, the rat appears to be an excellent animal model for studying human CYP2E1 expression, function and regulation by various substrates and inducers.

### 1.2.2 Substrates of CYP2E1

CYP2E1 metabolizes a wide variety of chemically and structurally diverse agents (over 70 different substrates), many of which are also inducers of this enzyme, including both endogenous and exogenous compounds. Endogenous compounds that have been shown to be metabolized by CYP2E1 include acetone and long-chain fatty acids such as arachidonic acid and linoleic acid (French *et al.*, 1997; Johansson *et al.*, 1988). Among exogenous substrates, ethanol is the most widely used and thus most extensively studied CYP2E1 substrate. It is estimated that CYP2E1 is responsible for approximateley 20% of ethanol metabolism at pharmacologically relevant blood alcohol concentrations (Lieber, 1994; Matsumoto *et al.*, 1996). Moreover,

CYP2E1 is increased 4-fold in liver biopsies of recently drinking patients (Tsutsumi *et al.*, 1989) with a corrresponding rise in mRNA (Takahashi *et al.*, 1993), and CYP2E1 is thought to contribute to the metabolic tolerance to ethanol that develops in alcoholic drinkers (Lieber, 1994; 1999).

In addition to ethanol metabolism, CYP2E1 is known to take part in the biotransformation of a large number of low-molecular weight compounds, many of which are industrial solvents of toxicological and carcinogenic significance. These include carbon tetrachloride, chloroform, vinylidine chloride and benzene (Kim *et al.*, 1997; Raucy *et al.*, 1993).

Among drugs, CYP2E1 is known to be involved in the metabolism of several halogenated anaesthetics [eg. halothane (Kharasch *et al.*, 1995), sevoflurane (Kharasch *et al.*, 1995; Kharasch and Thummel, 1993), enflurane (Garton *et al.*, 1995), isoflurane (Kharasch and Thummel, 1993)], analgesics such as acetominophen (Raucy *et al.*, 1989), the muscle relaxant chlorzoxazone (Vesell *et al.*, 1995), and the antiepilectic drug trimethadione (Tanaka and Funae, 1996).

CYP2E1 activity can be easily monitored by enzymatic assays using sensitive and specific substrates. Among these, chlorzoxazone (Carriere *et al.*, 1993) and 4-nitrophenol (PNP) (Koop, 1986) are widely used. Hydroxylation of chlorzoxazone to 6-hydroxychlorzoxazone and of PNP to 4-nitrocatechol are common probes used to determine the metabolic potential of CYP2E1 in vitro as well as in vivo (Carriere *et al.*, 1993; Koop, 1986). Chlorzoxazone has been used as an indicator of exposure to organic solvents (Ernstgard *et al.*, 1999; Jayyosi *et al.*, 1995; Nedelcheva, 1996), and to monitor liver function before and after transplantation (Burckart *et al.*, 1998), as well as to assess severity of alcoholic liver disease (Dilger *et al.*, 1997; Dupont *et al.*, 1998).

### 1.2.3 Tissue Distribution

CYP2E1 is predominantly expressed in the endoplasmic reticulum of liver hepatocytes as demonstrated in various species including rats and humans (Eliasson *et al.*, 1992; Riley *et al.*, 1993). Kupffer cells also express significant levels of CYP2E1 but the overall content in rat liver is approximately 10 times lower in Kupffer cells than in hepatocytes of the same animals (Koivisto *et al.*, 1996). Within the liver, CYP2E1 is not homogeneously distributed. Both constitutively expressed, and the increased CYP2E1 resulting from induction by various agents, is most abundant in the centrilobular region of the liver (Song, 1996). More specifically, CYP2E1 expression is most abundant in the 3-4 layers of hepatocytes surrounding the central vein. This region-specific hepatic expression is of particular interest, because ethanol, acetominophen and other CYP2E1-substrates/inducers cause cellular damage mainly in the centrilobular region of the liver (Lindros, 1997).

CYP2E1 has also been detected in many extrahepatic tissues at concentrations which are lower than in the liver (Lieber, 1999). For example, constitutively expressed and/or induced CYP2E1 has been detected in the rat pancreas (Kessova *et al.*, 1998), kidney (Ronis *et al.*, 1998), brain (Hansson *et al.*, 1990), lung (Powley and Carlson, 1999), cheek mucosa (Shimizu *et al.*, 1990), tongue (Shimizu *et al.*, 1990), esophagus (Shimizu *et al.*, 1990), as well as in human liver (Kim *et al.*, 1997), lung (Raunio *et al.*, 1999), brain (Upadhya *et al.*, 2000), and breast tissue (Iscan *et al.*, 2001).

### 1.2.4 Physiological Function

CYP2E1's broad substrate specificity and its high level of expression in the liver, which filters all circulating blood and traps xenobiotics entering the body through the gastrointestinal tract, suggest a role for CYP2E1 as a defense mechanism against the penetration of toxic xenobiotics (Lieber, 1999). CYP2E1 appears to play at least two physiological roles: one of detoxification and the other of nutritional support (Chen *et al.*, 1994; Lieber, 1997).

In terms of detoxification, one of CYP2E1's most significant roles is its adaptive response to high blood ethanol levels with a corresponding acceleration of ethanol metabolism (Pikkarainen and Lieber, 1980; Salaspuro and Lieber, 1978). CYP2E1's inducibility by ethanol may reduce the risk of reaching excessive/toxic levels of ethanol in the circulation, and has been suggested in experimental animals (Lieber and DeCarli, 1972), as well as in humans (Salaspuro and Lieber, 1978). Although alcohol dehydrogenase (ADH) is the main enzyme responsible for ethanol metabolism (Crabb, 1995), CYP2E1 has been proposed to account for approximateley 20% of ethanol metabolism at pharmacologically relevant blood alcohol concentrations (Lieber, 1994; Matsumoto *et al.*, 1996). Furthermore, 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).

In terms of a nutritional role, CYP2E1 has been proposed to contribute to ketone utilization and fatty acid metabolism in physiological states such as starvation (Koop and Tierney, 1990), obesity (Salazar *et al.*, 1988) and diabetes (Enriquez *et al.*, 1999). For example, a ketone such as acetone, which is both a substrate and an inducer of CYP2E1 (Koop, 1992; Patten *et al.*, 1986), is thought to be a significant gluconeogenic precursor in fasting humans accounting for approximately 10% of the gluconeogenic demands of humans who have fasted for

21 days (Reichard *et al.*, 1979). These physiological roles may explain CYP2E1's high functional and regulatory conservation across species and within the human population (Lieber, 1997).

### 1.2.5 CYP2E1 Induction

CYP2E1 can be induced by a variety of compounds, many of which are also substrates. The number of compounds that induce the activity of CYP2E1 is very extensive and includes ethanol (Perrot *et al.*, 1989; Takahashi *et al.*, 1993), pyrazole (Dicker and Cederbaum, 1991), pyridine (Kaul and Novak, 1987), isoniazid (Ryan *et al.*, 1985) and acetone (Patten *et al.*, 1986). In addition CYP2E1 activity is also induced by pathophysiological states that result in the accumulation of acetone or ketones, such as fasting, diabetes and obesity (Lieber, 1997; 1999). In addition to a more rapid hepatic elimination of xenobiotics, induction of drug metabolism leads to a more pronounced and faster formation of metabolites. In terms of CYP2E1, which is best known for its ability to bioactivate many hepatotoxins and procarcinogens, induction can lead to a more pronounced formation of toxic and/or reactive metabolites; this could lead to a higher risk of organ damage and carcinogenicity in people exposed to these various chemicals and solvents (see section 1.2.7 for more details on CYP2E1 related toxicity).

### 1.2.6 Regulation of CYP2E1 Expression

#### 1.2.6.1 CYP2E1 Degradation

The rat hepatic CYP2E1 gene is transcriptionally activated one day after birth, and this activation is accompanied by a demethylation of cytosine residues located within the 5'-flanking region of the gene (Ueno and Gonzalez, 1990). CYP2E1 is one of the most unstable of all liver microsomal cytochrome P450 forms (Barmada *et al.*, 1995; Roberts *et al.*, 1995; Song *et al.*, 1989). In the absence of a ligand, the CYP2E1 enzyme is degraded with a half-life of 6-7 hours, followed by a slower secondary half-life of about 37 hours (Roberts *et al.*, 1995). Several mechanisms are thought to be involved in CYP2E1 degradation, including cAMP-dependent phosphorylation of CYP2E1, followed by degradation by serine proteases present in the endoplasmic reticulum (Eliasson *et al.*, 1992; Zhukov *et al.*, 1993) or by ubiquitin-dependent or independent proteosome systems (Roberts, 1997; Yang and Cederbaum, 1996). However, the molecular triggers that predispose CYP2E1 to rapid degradation by the various proteolytic systems remain unclear. One possibility is that the CYP2E1 molecule possesses some unique and sensitive structural features making it a better substrate for cellular proteases than more stable proteins such as CYP2B1 (Eliasson *et al.*, 1992). An alternative possibility is that degradation is initiated during CYP2E1's catalytic cycling. A unique feature of CYP2E1 that distinguishes it from other CYP isoforms is its especially high NADPH-oxidase activity in the substrate-free form (Ekstrom and Ingelman-Sundberg, 1989; Guengerich and Johnson, 1997). This results in an elevated production of reactive oxygen species, which are known to cause oxidative modifications to proteins resulting in increased proteolytic susceptibility (Goasduff and Cederbaum, 1999). Two recent studies have investigated the potential role of CYP2E1 catalytic cycling in initiating its rapid degradation. Treatment of Fao rat hepatoma cells with diphenylene

iodonium (DPI), a suicide inhibitor of flavin oxidoreductase, resulted in NADPH-cytochrome P450 reductase inhibition and a concomitant decrease in $H_2O_2$ formation. In addition, by blocking the supply of reducing equivalents to CYP2E1, its half-life increased from 4 to 26 hours in this in vitro system (Zhukov and Ingelman-Sundberg, 1999). Goasduff and Cederbaum (1999) have also found decreased rates of CYP2E1 degradation in a HepG2 cell line expressing human CYP2E1, in the presence of DPI or of antioxidants. These findings suggest that NADPH-dependent production of ROS by CYP2E1 may result in oxidative modifications of CYP2E1, followed by rapid degradation.

1.2.6.2 Mechanisms of CYP2E1 Induction

As previously discussed CYP2E1 is induced by a variety of endogenous and exogenous compounds (see section 1.2.5). Mechanisms of induction have been studied extensively in rats and other laboratory animals, however, the regulation of CYP2E1 expression is complex involving transcriptional, post-transcriptional and post-translational mechanisms (Lieber, 1997).

Investigations have determined that many low-molecular weight chemicals such as ethanol, acetone, imidazole, 4-methylpyrazole, pyrazole and pyridine increase CYP2E1 protein in the absence of any significant alterations in CYP2E1 mRNA levels (Johansson *et al.*, 1988; Khani *et al.*, 1987; Porter *et al.*, 1989; Song *et al.*, 1989). Thus, a post-transcriptional mechanism, namely protein stabilization is currently recognized as the most common mechanism leading to CYP2E1 induction (Parkinson, 2001; Riddick, 1998). In this model of induction, CYP2E1 substrates/inducers are thought to prolong the in vivo half-life of CYP2E1 by stabilizing the protein against the fast-phase component associated with this enzyme's normal degradation (Huan and Koop, 1999; Song *et al.*, 1989). Substrates such as ethanol are thought

to increase CYP2E1 levels by interacting with the catalytic site resulting in decreased NADPH activity and generation of ROS (Zhukov and Ingelman-Sundberg, 1999). Alternatively, haem ligands such as imidazole, may stabilize the enzyme by providing steric protection against conformational changes that lead to degradation (Schmalix *et al.*, 1995).

Other studies suggest that increased mRNA levels and/or increased efficiency of mRNA translation, resulting in enhanced de novo enzyme synthesis, are also mechanisms of CYP2E1 induction. Studies in hamsters, have demonstrated that treatment with ethanol and pyrazole results in increased CYP2E1 protein and mRNA levels (Kubota *et al.*, 1988). Kim and Novak (1990) observed increased rates of [$^{14}$C] leucine incorporation into CYP2E1 protein after treatment of rats with pyridine in the absence of any change in CYP2E1 mRNA levels. These findings suggests that at least in rats, pyridine can induce CYP2E1 by enhancing CYP2E1 mRNA translational efficiency (Kim and Novak, 1990). Elevations in CYP2E1 protein and mRNA levels have also been observed in rats treated with ethanol (Diehl *et al.*, 1991; Ingelman-Sundberg *et al.*, 1988), as well as in currently ethanol/alcohol drinking patients (Takahashi *et al.*, 1993). Moreover, the latter study found significant correlation between cellular levels of CYP2E1 protein and mRNA in humans, providing support for the hypothesis that mRNA stabilization and/or transcriptional activation are the mechanisms involved in ethanol-mediated CYP2E1 induction in humans (Takahashi *et al.*, 1993).

To complicate this matter even further, the mechanism involved in CYP2E1 induction may also depend on the dose of the inducer. 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). Furthermore, the mechanism of induction varies even among closely related inducers. For

example, although fasting and diabetes both increase the levels of CYP2E1 mRNA by approximately 10-fold, the increase with diabetes results from mRNA stabilization, whereas the increase with fasting results from increased gene transcription (Johansson *et al.*, 1990; Peng and Coon, 1998). Therefore, the duration and/or mode of inducer treatment, and/or the distinct mechanisms of induction by the various pharmacologically and structurally diverse inducing agents, may contribute to the observed variety of mechanisms of CYP2E1 induction.

### 1.2.7 CYP2E1 Related Toxicity and Disease

Since its discovery, CYP2E1 has received considerable attention due to its potentially important role in the toxicity of a variety of chemicals, and its potential involvement in the propagation of hepatic diseases.

#### 1.2.7.1 ROS-dependent Toxicity

A unique characteristic of CYP2E1 is its especially high NADPH oxidase activity (Gorsky *et al.*, 1984; Ingelman-Sundberg and Johansson, 1984), which results in the production of ROS such as $O_2^-$ (superoxide radical) and $H_2O_2$ in high amounts relative to other CYP isoforms (Ekstrom and Ingelman-Sundberg, 1989; Gorsky *et al.*, 1984). These reactive oxygen intermediates can initiate lipid peroxidation, oxidative stress, and Kupffer cell activation, thereby propagating cellular injury and DNA strand breaks (Jarvelainen *et al.*, 2000). Moreover, CYP2E1 induction correlates with lipid peroxidation and pathological severity during chronic ethanol exposure (Jarvelainen *et al.*, 2000); this effect is blocked by inhibitors of CYP2E1 (Jaeschke *et al.*, 2002).

1.2.7.2 Hepatotoxin and Carcinogen Associated Toxicity

CYP2E1 also has the capacity to activate many xenobiotics to hepatotoxic or carcinogenic products. These include the commonly used analgesic acetominophen (Raucy *et al.*, 1989), industrial solvents such as carbon tetrachloride (Manno et al., 1996), bromobenzene (Hetu *et al.*, 1983), and vinylidene chloride (Siegers *et al.*, 1983), anaesthetics such as enflurane (Tsutsumi *et al.*, 1990) and halothane (Takagi *et al.*, 1983), as well as nitrosamines such as N-nitrosodimethylamine (NDMA) (Lin and Hollenberg, 2001). CYP2E1 knock-out mice have proven to be a useful tool to investigate the involvement of CYP2E1 in the development of hepatotoxicity by various protoxins which are substrates for CYP2E1. For example, CYP2E1 null-mice experienced significantly less hepatotoxicity than wild-type mice upon exposure to $CCl_4$ (Wong *et al.*, 1998) or to acetominophen (Zaher *et al.*, 1998).

These hepatotoxins and/or carcinogens are known to cause selective injury predominantly in the perivenular area, which provides further evidence for the involvement of CYP2E1 since it is well established that 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 toxins (Lieber, 1997; Woodcroft and Novak, 1998). Thus induction of CYP2E1 by agents such as ethanol could result in greater formation of potentially active metabolites of these toxins. This may explain the increased vulnerability to hepatic toxicity of alcohol abusers upon exposure to therapeutically and industrially used xenobiotics such as acetominophen (Seeff *et al.*, 1986) and $CCl_4$ (Manno *et al.*, 1996). Hundreds of cases of acetominophen hepatotoxicity associated with regular intake of alcohol have been reported (Zimmerman and Maddrey, 1995). Also, the potentiation of occupational $CCl_4$ toxicity by ethanol has been observed in fire-fighters, as illustrated in the following example. During the

extinction of fire seven workers were exposed to $CCl_4$. However only two workers with a high ethanol intake and presumably high levels of CYP2E1, developed severe hepatotoxicity (Manno *et al.*, 1996).

1.2.7.3 CYP2E1 Associated Diseases

There is increasing evidence that CYP2E1 is a key factor in the pathogenesis of alcoholic liver disease , a risk factor for hepatocellular carcinoma (Jarvelainen *et al.*, 2000) . Moreover, CYP2E1 induction is thought to contribute to the hepatopathology of non-alcoholic steatohepatitis (Weltman *et al.*, 1998), and to be implicated in benzene-induced hematological malignancies in humans (Rothman *et al.*, 1997) and nitrosodimethylamine-induced tumorigenesis in animals (Lieber, 1997).

### 1.2.8 Tobacco Smoke and CYP2E1 Interactions

Tobacco smoking is still a worldwide, major health concern. It has been estimated that approximately one third of the global population 15 years of age and older smokes, and that smoking related diseases claim approximately 3,000,000 deaths per year (World Health Organization, 1998). In Canada alone, it is estimated that approximately 30% of overall cancer cases, 80% of lung cancer cases and over 40,000 deaths per year are associated with smoking cigarettes (Canadian Cancer Statistics, 2000). Although it is well established that smoking cigarettes is a major risk factor in the development and progression of various types of cancers, as well as cardiovascular and respiratory diseases, people world-wide continue to smoke.

Among its various biological effects, cigarette/tobacco smoke has been associated with the induction of several drug-metabolizing enzymes including CYPs (Kawamoto *et al.*, 1993;

Villard *et al.*, 1994). It is well established that many tobacco procarcinogens are activated by CYP enzymes to active carcinogens. Thus, induction of these enzymes by cigarette smoke may be important for development of cancer.

Several studies demonstrated that tobacco smoke can induce CYP2E1 activity in animals and in humans (Benowitz, 1999b; Villard *et al.*, 1998). Villard et al. (1998) has shown that in mice exposed to tobacco smoke increased CYP2E1 activity, as measured by chlorzoxazone 6-hydroxylation, was correlated with hepatic CYP2E1 protein levels. In addition, this isozyme appeared to be the most tobacco smoke-inducible CYP among the different isoforms investigated in this study (Villard *et al.*, 1994).

The inductive effect on CYP2E1 could be due to the presence in cigarette smoke of components such as pyridine, acetone and benzene, which are potent inducers of CYP2E1 (Koop and Tierney, 1990). As previously mentioned CYP2E1 is implicated in the bioactivation of procarcinogens including nitrosamines and numerous low molecular weight compounds such as benzene, styrene, vinylchloride and urethane, which are all present in tobacco smoke (Gonzalez and Gelboin, 1994; Guengerich *et al.*, 1991). Therefore, by inducing the activity and expression of CYP2E1, cigarette smoke would 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 (ethanol is an inducer of CYP2E1) and smoking interact synergistically as etiological factors for cancers (Garro and Lieber, 1990). Furthermore, since CYP2E1 substrates are commonly found in the industrial workforce (Raucy *et al.*, 1993; Wang *et al.*, 1996), smokers in these environments may be more sensitive to chemical injury than non-smokers.

Tobacco smoke has also been associated with DNA damage in several human and other

mammalian cell types exposed in vitro to cigarette smoke, by a mechanism that involves reactive oxygen species (Spencer *et al.*, 1995). These reactive species are commonly produced by CYPs, especially by CYP2E1. One study has demonstrated that cigarette smoke exposure in vivo significantly induces DNA single-strand breaks (SSB) in the liver (Villard *et al.*, 1998). Furthermore, this study demonstrated that increased DNA strand breaks was associated with CYP2E1 induction, since treatment of mice with the CYP2E1 inhibitor propylene glycol resulted in decreased DNA strand breakage.

Together these data suggest that constituents of cigarette smoke can induce CYP2E1, and that the increased levels may result in some of the damage associated with smoking.

### 1.2.9 Nicotine and CYP2E1 Interactions

Nicotine is the major constituent of tobacco smoke that is responsible for producing and maintaining tobacco dependence (Henningfield *et al.*, 1985). In addition to its role in tobacco addiction, nicotine can affect a variety of cellular processes ranging from induction of gene expression, to secretion of hormones and modulation of enzymatic activities (Benowitz, 1988; Dani and Heinemann, 1996; Maritz and Burger, 1992). Recent studies have demonstrated that in vivo treatment with nicotine results in increased oxydative stress in rat liver and lung microsomes (Bhagwat *et al.*, 1998). More interestingly, p-nitrophenol hydroxylase activity, a known marker for CYP2E1 was also induced by nicotine treatment in these rats (Bhagwat *et al.*, 1998). Therefore, the observed increase in oxidative stress associated with nicotine treatment in this particular study, may in fact be due to CYP2E1 induction by nicotine leading to the subsequent production of reactive oxygen species. However, no further experiments were conducted to validate the possible involvement of CYP2E1 in the observed oxidative stress or

increased activity associated with nicotine treatment.

These studies prompted us to examine whether nicotine, which is quantitatively the most abundant chemical in cigarette smoke and which structurally resembles other CYP2E1 inducers such as pyridine, can in fact increase CYP2E1 protein and activity in the rat liver. Recent studies conducted in our laboratory have demonstrated for the first time that treatment of rats with relatively low doses of nicotine (0.1, 0.3 and 1.0 mg/kg s.c.) for 7 consecutive days leads to increased hepatic CYP2E1 protein levels (Fig. 1.1) (Howard *et al.*, 2001). From our studies and previous investigations (London *et al.*, 1990; Pratt *et al.*, 1983), 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 doses of 0.1, 0.3 and 1.0 mg/kg in rats are similar to total nicotine acquired from 3, 10 and 20 cigarettes (Le Houezec *et al.*, 1993). Moreover, our kinetic studies (Fig. 1.2) have demonstrated increased CYP2E1 activity in the same animals, as assessed by chlorzoxazone metabolism. Furthermore, the increase in activity is of similar magnitude to the observed increase in CYP2E1 protein levels. We confirmed that the enhancement of chlorzoxazone hydroxylation was mediated by CYP2E1 by chemical inhibitory studies with aniline, a competitive CYP2E1 inhibitor, and diethyldithriocarbamate (DDC), a mechanism-based CYP2E1 inhibitor (Fig. 1.3). From the observed inhibition of chlorzoxazone hydroxylation by these fairly selective CYP2E1 inhibitors, we estimated that CYP2E1 contributes to 80% or more of the chlorzoxazone metabolism in microsomes from saline and drug-treated animals. Minor contributions from CYP3A and CYP1A1 that can metabolize chlorzoxazone in rats may also occur (Jayyosi *et al.*, 1995).

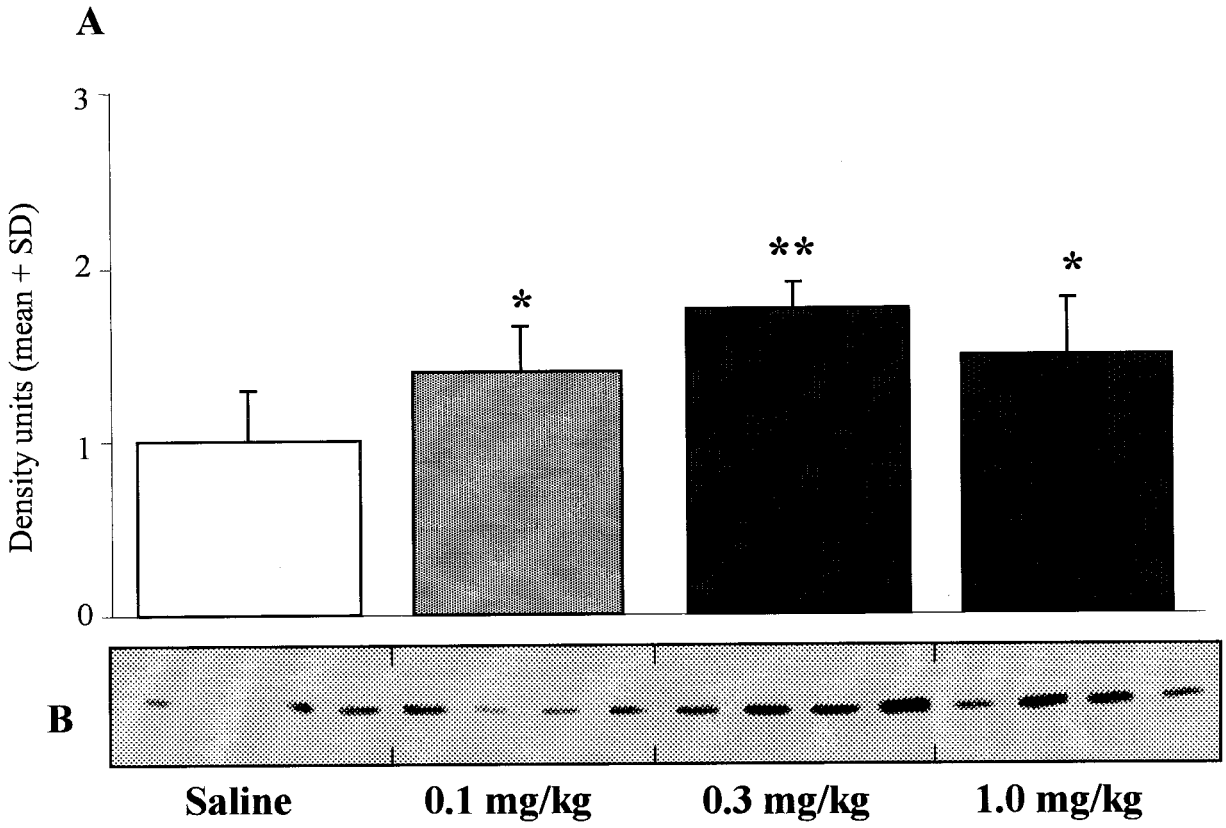


**Fig. 1.1** Chronic nicotine in vivo induces CYP2E1 in the rat liver. (A) Nicotine induces hepatic CYP2E1 in the rat relative to saline treated animals; mean of four animals /group (+ SD). Significant differences are indicated by * $p < 0.05$ and ** $p < 0.001$.
(B) Representative immunoblot (Based on findings from Howard *et al.*, 2001).

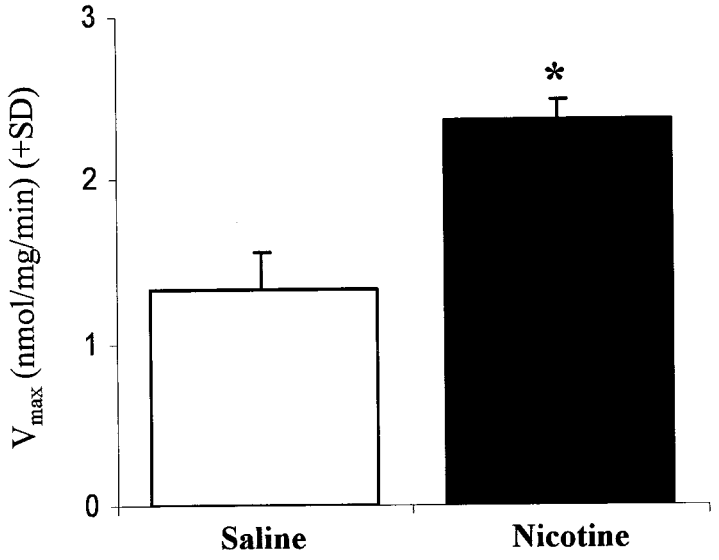


**Fig. 1.2** Kinetics of chlorzoxazone 6-hydroxylation. Rats treated with 1.0 mg/kg nicotine s.c. for 7 days had a significantly increased $V_{max}$ which was 1.7-fold higher than for saline-treated animals. Significance from saline is indicated by * ($p < 0.005$) (Based on findings from Howard *et al.*, 2001).

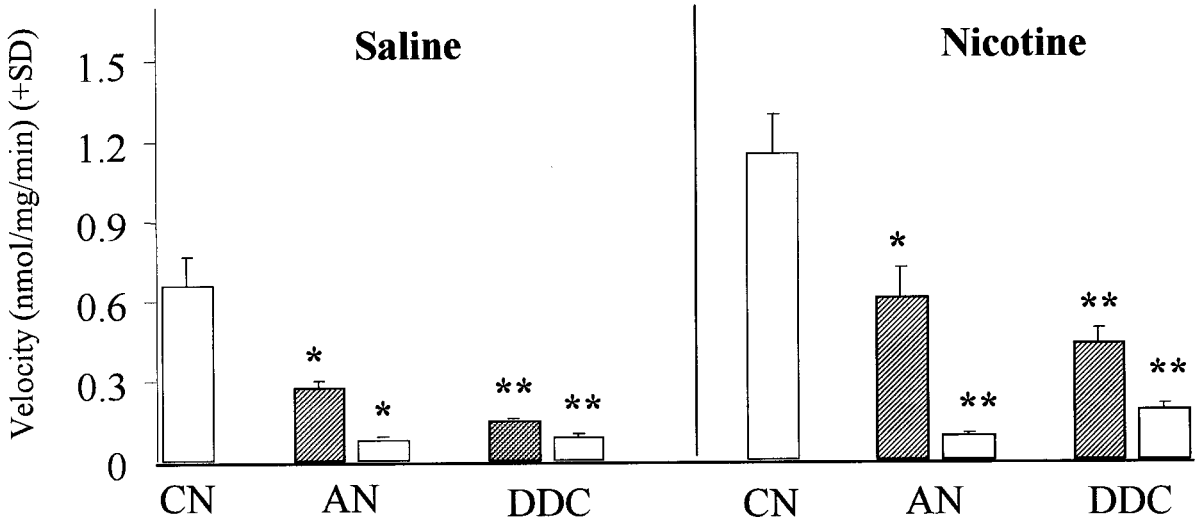


**Fig. 1.3** Effects of CYP2E1 inhibitors on 6-hydroxylation of chlorzoxazone (CN). Liver microsomes from rats treated with saline or nicotine, were incubated with 150 μM CN alone or with 100 (hatched) and 500 μM (solid) analine (AN), or 31.5 (hatched) and 315 μM (solid) diethyldithriocarbamate (DDC). Each column represents mean velocities calculated for four individual animals for each treatment group (+ SD). Significant inactivation is indicated by * $p < 0.05$ and ** $p < 0.001$ (Based on findings from Howard et al., 2001).

Immunohistochemical analyses (Fig. 1.4) have demonstrated that CYP2E1 induction by nicotine is specific to the centrilobular region of the liver. Therefore, induction of CYP2E1 by nicotine may exacerbate the hepatotoxicity of the CYP2E1 substrates ethanol, acetominophen, carbon tetracholoride, and N-nitrosodimethylamine in this hepatic region (Tsutsumi *et al.*, 1989). Our immunohistochemical analyses also suggest that the magnitude of induction, as estimated by whole lobular micorosomal homogenates used in immunoblotting and kinetic studies, may underestimate the levels of CYP2E1 attained in the centrilobular region of liver. From these findings we can postulate that nicotine may contribute to the CYP2E1 induction observed in rodents (Villard *et al.*, 1998) and humans (Benowitz, 1999b) exposed to cigarette smoke. In addition, nicotine may contribute to CYP2E1 related toxicity and organ damage in smokers.

The observed level of CYP2E1 induction by nicotine was saturated at the doses tested in this initial study, suggesting that lower nicotine doses should be examined. Such lower doses may be important in terms of passive smokers or people on nicotine replacement therapy (NRT) which may also be at risk of liver damage associated with increased CYP2E1 enzyme.

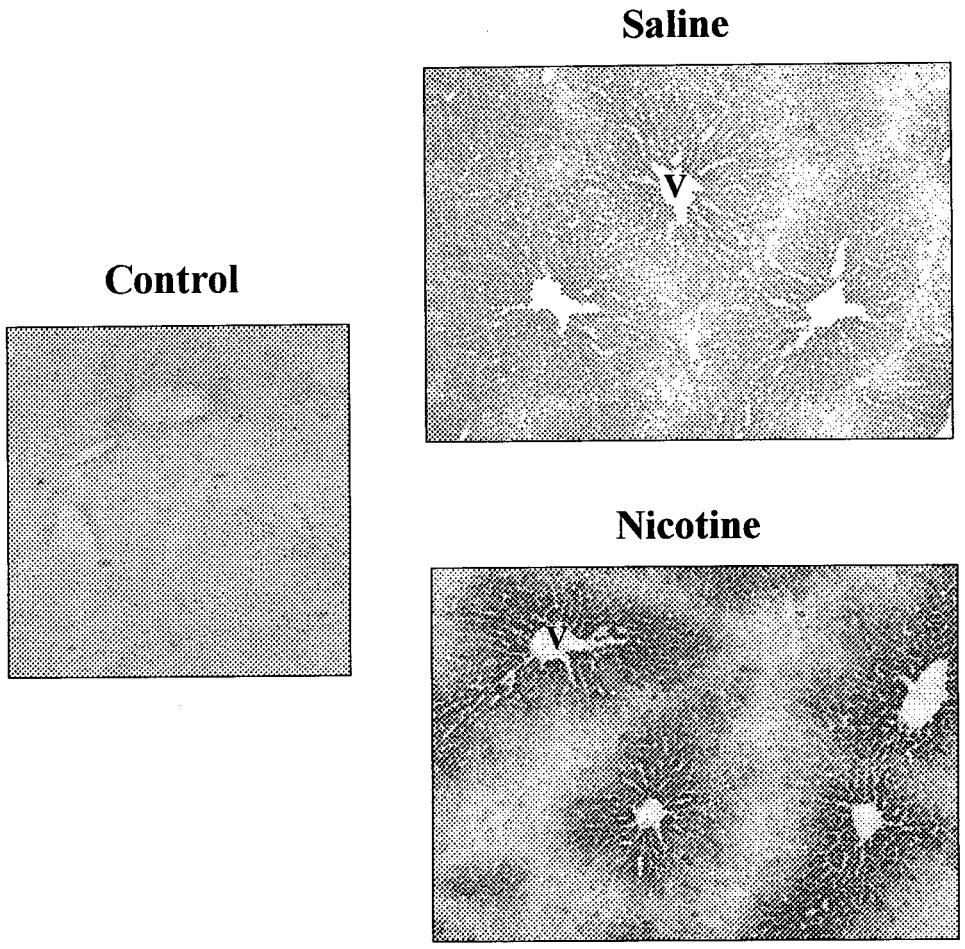


**Fig. 1.4** Behaviourally relevant doses of nicotine increased CYP2E1 immunoreactivity in the centrilobular region of the rat liver. Immunhistochemical analyses demonstrated that a 7 day treatment with 1.0 mg/kg s.c. nicotine relative to its saline control, increased hepatic CYP2E1 immunostaining in the region surrounding the central vein (V) of the liver lobule. No staining was observed in control sections processed without primary antibody (Based on findings from Howard *et al.*, 2001).

### 1.2.10 Nicotine/Cotinine Pharmacokinetics

Nicotine is a tertiary amine consisting of a pyridine and a pyrrolidine ring (Benowitz, 1996). It is readily absorbed from cigarette smoke and is rapidly distributed to body tissues. Nicotine is rapidly and extensively metabolized in the liver to cotinine (70-80%) and to nicotine-N'-oxide (4%) (Benowitz and Jacob, 1994). In humans, hepatic CYP2A6 is the primary enzyme responsible for nicotine metabolism (Messina *et al.*, 1997). In rats hepatic CYP2B1 is the primary enzyme responsible for nicotine metabolism to cotinine (Nakayama *et al.*, 1993). The rat CYP2B1 orthologue in humans is CYP2B6, however, this enzyme is found in low levels in the human liver and makes only a small contribution to human hepatic nicotine metabolism (Messina *et al.*, 1997). Conversely, in rats the CYP2A enzymes do not metabolize nicotine (Nakayama *et al.*, 1993). Thus nicotine is metabolized to cotinine by hepatic CYP2A6 in humans and hepatic CYP2B1 in rats. In humans, nicotine half-life is about 1-2 hours (Benowitz, 1988), while in rats it is about 20 minutes (Sastry *et al.*, 1995).

Cotinine does not have any known, significant pharmacological effects and is unable to substitute for nicotine in smoking behavior (Benowitz *et al.*, 1983). Cotinine is extensively metabolized in humans with only 13-17 % excreted unchanged in the urine (Benowitz *et al.*, 1983). Several metabolites of cotinine have been reported in humans that include trans-3'-hydroxycotinine (Benowitz *et al.*, 1982), 5'-hydroxycotinine (Murphy *et al.*, 1999), cotinine N-oxide (Shulgin *et al.*, 1987) and the cotinine methonium ion (Murphy *et al.*, 1999). In rats a number of cotinine metabolites have been identified including cotinine-N-oxide, 5'-hydroxycotinine and 5'-hydroxycotinine-N-oxide (Schepers *et al.*, 1999). The clearance of cotinine is much slower when compared to its precursor nicotine, with an elimination half-life that ranges between 16-20 hours in humans (Benowitz and Jacob, 1994) and about 6-7 hours in

rats (Adir *et al.*, 1976; Miller *et al.*, 1977). As a result of its long half-life, cotinine levels remain more constant throughout the day when compared to nicotine in passive and active smokers (Benowitz and Jacob, 1994). For these reasons, cotinine plasma concentration is commonly used as an index of smoking or environmental tobacco smoke exposure in non-smokers (Benowitz, 1983; Hofer *et al.*, 1991).

For the purposes of the subsequent studies we have treated rats with a range of nicotine doses and measured nicotine and cotinine plasma levels. The use of plasma levels allows a comparison to human nicotine levels (from smokers or those exposed therapeutically to nicotine, or to ETS) independent of the different isozymes (CYP2A6 vs CYP2B1) which metabolize nicotine in these two species.

### 1.2.11. Nicotine Exposure in Non-smokers

In addition to smokers, millions of nonsmokers may be exposed therapeutically to nicotine during smoking cessation therapy. Nicotine replacement therapy products (NRTs) are now available over-the counter (OTC). As a result, the sales of NRTs have more than doubled since its switch to OTC in 1996, which reflects increased use and thus increased exposure to nicotine (Pierce and Gilpin, 2002; Shiffman *et al.*, 1997; Thorndike *et al.*, 2002). In addition to its therapeutic use in smoking cessation, nicotine is also currently being investigated as a treatment for ulcerative colitis (Guslandi, 1999), Alzheimer's disease (White and Levin, 1999), Parkinson's disease (Kelton *et al.*, 2000) and other neuropsychiatric movement disorders (Erdmann, 1996). Moreover, billions of non-smokers worldwide may be chronically exposed to nicotine via environmental tobacco smoke (ETS) especially in home and workplace environments. In the U.S. alone it has been estimated that 43% of children 2 months to 11 years

lived in a home with at least one smoker, and 37% of adult non-tobacco users lived in a home with a smoker or reported ETS exposure at work (Pirkle *et al.*, 1996). Considering the high prevalence of nicotine exposure in the human population, it is of great importance to investigate possible adverse effects associated with nicotine use/exposure.

## 1.3 Rationale, Objectives and Hypotheses

Previous studies conducted in our laboratory have demonstrated that relatively low doses of nicotine (0.1, 0.3 and 1.0 mg/kg s.c. for 7 days) induce CYP2E1 protein and activity in the rat liver (Howard et al., 2001). However, the observed induction of CYP2E1 was saturated at the nicotine doses tested in this initial study, suggesting that lower doses of nicotine may also increase CYP2E1. These rats were sacrificed at 4 hours following the last nicotine injection however it has not been established whether this is the time of maximal CYP2E1 induction, nor have we investigated how long the induction of CYP2E1 lasts. We have established that chronic (7 day) nicotine treatment in rats results in hepatic CYP2E1 induction however, the effect of acute nicotine treatment on hepatic CYP2E1 has not been investigated. Moreover, the mechanism involved in the induction of CYP2E1 by nicotine and the potential contribution of nicotine metabolites to CYP2E1 induction remain unknown. Therefore, the overall objective of the studies presented in this manuscript is to further characterize the induction of CYP2E1 by nicotine in the rat liver. The specific objectives of these studies and the corresponding hypotheses are as follows:

### 1.3.1 Objectives

1. To assess the induction of CYP2E1 by nicotine over 24 hours following the last drug treatment in order to determine how long this effect persists and when it peaks.
2. To investigate the effect of acute (single dose) nicotine exposure on hepatic CYP2E1 levels.
3. To identify lower doses of nicotine that can induce CYP2E1 and to determine the $ED_{50}$ for this effect.
4. To investigate potential mechanisms of CYP2E1 regulation by nicotine.
5. To investigate whether nicotine metabolites may be involved in CYP2E1 induction.

### 1.3.2 Hypotheses

1. Hepatic CYP2E1 induction peaks at 4 hours following nicotine treatment.
2. Acute exposure to nicotine results in increased hepatic CYP2E1 levels.
3. Nicotine doses lower than 0.1 mg/kg s.c. can induce hepatic CYP2E1.
4. Nicotine treatment will result in increased CYP2E1 protein and mRNA levels.
5. Cotinine, the main nicotine metabolite, can induce CYP2E1.

# 2. Materials and Methods

## 2.1 Chemicals and Laboratory Reagents

Nicotine bitartrate and cotinine were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Recombinant viral-expressed rat CYP2E1 in lymphoblastoid cells, baculoviral-expressed rat CYP1A1, CYP2B1, CYP3A4, CYP2C11, and CYP2A2 in insect cells (BTI-TN-5B1-4), and expressed rat CYP2A1 Supersomes were obtained from GENTEST (Woburn, MA). The protein assay dye reagent concentrate, the Zeta-Probe nylon membrane and the Bio-Dot microfiltration apparatus were 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 secondary-antibody 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). Full-length cDNAs for CYP2E1, CYP2B6, CYP2A6, CYP2D6 and CYP3A4 were generously provided by Frank.J. Gonzalez of the National Institute of Health. A Strata-prep Total RNA Mini-prep kit was purchased from Stratagene (San-Diego, CA). Yeast tRNA was purchased from Gibco BRL (Gaithersburg, MD). Milipore Ultrafree-DA Centrifugal Filter for DNA extraction was purchased from Millipore Corporation (Bedford, MA). All other chemical reagents were obtained from standard commercial sources.

## 2.2 Animals

Adult male Wistar rats weighing 250-300 g, obtained from Charles River (St-Constant, QC, 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. and off at 6:00 p.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.

## 2.3 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 μm/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 carefully titrated to pH 7.4 with 1N NaOH, and then was serially diluted to achieve desired nicotine concentrations. All references to nicotine doses are based on the free-base form of the drug and refer to mg of nicotine base per kg of body weight. Cotinine solution, at a concentration of 1.086 mg/ml (6.16 μm/ml) cotinine, was also prepared fresh daily by adding 4.5 mg cotinine to 4.14 ml of saline. Due to the light sensitive property of cotinine, both the cotinine storage bottle as well as the cotinine solution vial prepared for rat treatments were kept wrapped in aluminum foil at all times. 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 or total RNA extraction.

### 2.3.1 Recovery Time-Course Study

In a time-related 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 CYP2E1 diurnal cycle, since rats were sacrificed at different times throughout the day; CYP2E1 has been reported to exhibit diurnal rhythms (Bruckner *et al.*, 2002).

### 2.3.2 Acute 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 were contrasted to animals treated for 7 days.

### 2.3.3 Nicotine Dose-Response Study

Dose-response studies included 9 groups of rats (n=3/group), injected s.c. once per day (in the morning), for 7 consecutive days with either 0, 0.001, 0.003, 0.005, 0.01, 0.02, 0.03, 0.1 and 1.0 mg/kg nicotine. 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 behavioural 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 behavioural effects such as tolerance (Perry *et al.*, 1999; Rowell and Li, 1997). This range also includes doses that rats will self-administer (0.03 and 0.06 mg/kg per infusion) for total nicotine doses in a 2 hour session of approximately 0.3 and 0.6 mg/kg (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).

### 2.3.4 CYP2E1 mRNA Study

Rats (n=4/group) were injected s.c., once per day, for 7 days with 0 or 1.0 mg/kg/day nicotine. Saline and ethanol at a dose of 3.0 g/kg of body weight (n=4/group) were administered by gavage to food-deprived (2-4 h, to facilitate easier absorption) rats once daily for 7 days. Rats were sacrificed 4 hours after the last drug treatment.

### 2.3.5 Cotinine Study

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

## 2.4 Microsomal Preparation

Liver microsomes were prepared using the method described by Ghersi-Egea *et al.*, (1993) whose methodology results in minimal cross-contamination between mitochondrial and microsomal membranes. Fragments of liver were homogenized manually in 100 mM Tris (pH 7.4) with 0.1 mM EDTA, 0.1mM dithiothreitol (DTT) and 0.32 M sucrose (homogenizing solution) on ice. The resulting homogenates were centrifuged twice at 4000g for 3 minutes to remove cellular and nuclear debris. The supernatant fraction was transferred into an ultracentrifuge tube and was centrifuged at 11500g (Sorvall RC2-B Combi Plus Ultraspeed centrifuge, Sorvall, Newton, CT) for 20 minutes at 4°C. The resulting supernatant was further centrifuged at 110000g for 90 minutes at 4°C for a microsomal pellet. The pellet (microsomes) was loosened, transferred to a glass grinder, resuspended in a storage solution consisting of 100 mM Tris (pH 7.4), 0.1 mM EDTA, 0.1 mM DTT, 1.15% w/v KCl and 20% v/v glycerol, aliquoted into small volumes and stored at -80°C until use.

## 2.5 Western Blotting

The protein content of each sample was measured by the method described by Bradford, (1976) using bovine serum albumin (BSA) as the protein standard. To determine the linear range of detection for the assay, saline treated rat liver microsomes were serially diluted and used to construct standard curves. Proteins (1.5 μg/liver sample) were separated by SDS-polyacrylamide gel electrophoresis (4% stacking and 10% separating gels) and were then transferred overnight onto a nitrocellulose membrane. Microsomes from rat CYP2E1-expressed 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 preincubated for 1 hour in a blocking solution containing 0.5% skim milk powder (w/v), 0.1% BSA (w/v) in TBST (50mM Tris pH 7.4, 150 mM NaCl, 0.1% (v/v) Triton X-100). Membranes were then probed for 1hr with a rabbit polyclonal anti-rat CYP2E1 antibody (1:4000 dilution in TBST containing 0.1% BSA w/v), which has been characterized previously (Hansson *et al.*, 1990). Following 30 minutes of reblocking, the membranes were incubated for 1 hour with a peroxidase conjugated secondary antibody (dilution 1:25000) in blocking solution. Membranes were washed in TBST (3 x 5 minutes) after each antibody incubation step. Control blots were processed without primary antibody. Immunoreactive protein bands were detected by chemiluminescence. Membranes were exposed to Ultident film 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.

## 2.6 RNA Slot Blotting

Total RNA was isolated from rat livers using a StrataPrep Total RNA kit as described by the supplier. RNA quantity and quality were analyzed by the ratio of optical density (260/280 UV wavelength) and by gel electrophoresis in 1.2% agarose gel. Initial experiments indicated that the addition of yeast tRNA to total RNA reduced background and improved the linearity of the system. Therefore, yeast tRNA was added to all samples (9.5 μg) and cDNA standards (10 μg). Total liver RNA (2.5 μg), serially diluted full-length CYP2E1 cDNA (0.63-20 pg, to ensure linearity of the assay and as positive control), and cDNAs for CYP2B6, CYP2A6, CYP2D6 and CYP3A4 (1.0 and 10.0 pg each, as negative controls) were applied directly to nylon membrane

under vacuum and denaturing conditions by using a Bio-Dot microfiltration apparatus according to the instructions of the manufacturer. Membranes were preincubated for at least 60 minutes at 43°C in pre-hybridization buffer [50% formamide, 120 mM $Na_2HPO_4$ (pH 7.2), 7% SDS and 250 mM NaCl], and then hybridized for 16 hours with either 1.4 X $10^7$ cpm/ml [$\alpha$-$^{32}$P]dCTP random-primed full-length human CYP2E1 cDNA or with two end-labeled rat oligonucleotide hybridization probes (each 2.0 X $10^7$ cmp/ml [$\gamma$-$^{32}$P]dATP) in the same buffer. The rat oligonucleotides that were used as probes were the reverse complementary sequences to 5'-TTTATTTCAGACACATTTTTC-3' and 5'-GAAGTTTTCATTGAACAAACT-3' that correspond to positions 721 to 740 and 613 to 633 of CYP2E1 mRNA, respectively; these sequences were specific for CYP2E1 mRNA. In addition, blots were probed with a 1.3 X $10^7$ cpm/ml [$\alpha$-$^{32}$P]dCTP random-labeled 517-base pair PCR product of β-actin (a loading control). The β-actin probe was made by PCR using the forward primer 5'-CACCACAGCTGAGAGGGAAATCGTGCGTGA-3', the reverse primer 5'-ATTTGCGGTGCACGATGGAGGGGCCGGACT-3', and rat brain 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 loaded onto the membrane and probed to verify the linearity of our detection system. Blots were washed at room temperature (25°C) sequentially in 2X then 0.5X saline sodium citrate with 0.1% SDS for 15 minutes each and exposed to Kodak OMAT-XR film for 1 to 8 days at -80°C. Digital images of films were analyzed using MCID Elite software (Imaging Research Inc.). The relative density of each band was corrected for background and expressed as arbitrary density units.

## 2.7 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 until use. Nicotine and cotinine plasma concentrations were measured by standard HPLC techniques using 5-methylnicotine as the internal standard (Goodz and Tyndale, 2002; 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.

## 2.8 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 our previous studies investigating the induction of CYP2E1 we observed relatively low inter-individual variation in our rat model. Based on these findings, we calculated that a relatively small sample size (3 animals per treatment group) was sufficient for comparison of CYP2E1 protein levels among different treatment groups. 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) followed by Duncan's Multiple Range test was used for comparisons of multiple group means. In all cases, statistically significant differences were determined at the 5% level ($p<0.05$).

# 3. Results

An immunoblotting assay was developed to measure hepatic CYP2E1. Detection of serially diluted CYP2E1 indicated that immunoblotting signal was linear up to 3.0 μg of protein from saline treated liver microsomes (Fig. 3.1A). No signal was detected in the absence of primary antibody. The specificity of the rabbit antibody for rat CYP2E1 has been previously demonstrated by other investigators (Hansson *et al.*, 1990). We have reconfirmed this finding as no cross-reactivity was observed with expressed rat CYP2C11, CYP2B1, CYP3A2, CYP1A1, CYP2A1 or CYP2A2 isozymes under immunoblotting conditions used in this study (Fig. 3.1B). The immunoreactive band from expressed rat CYP2E1 microsomes comigrated with CYP2E1 liver microsomes from untreated rats (Fig. 3.1B).

## 3.1 CYP2E1 Recovery Time-Course

The induction of CYP2E1 was assessed as a function of time over 24 hours, after a 7 day treatment with 1.0 mg/kg Nic. Results are expressed as a ratio of density units from nicotine treated animals relative to saline treated animals. Western blot analyses revealed a significant increase 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. 3.2A). The higher levels of CYP2E1 in nicotine treated rats returned to the levels found in saline treated animals by 18 hours post treatment. Despite the variability (SD) observed within this study, for all times where the mean CYP2E1 ratios of nicotine/saline treated rats were greater than 1, there was sufficient statistical power with the group size of three animals.

In this study, the concentration of nicotine and its metabolite, cotinine, were determined as a function of sacrifice time after the last drug treatment in rat plasma. The plasma level of nicotine

was highest at 30 minutes and declined by 4 hours post-injection (Fig. 3.2B). The level of cotinine peaked by 4 hours and then slowly declined to near control levels by 24 hours post-treatment (Fig. 3.2B). An interesting observation is that maximal increases in CYP2E1 protein were observed at 4 hours post-treatment, a time when nicotine levels had diminished, whereas cotinine levels were at their peak. Neither nicotine nor cotinine were detected in saline treated animals.

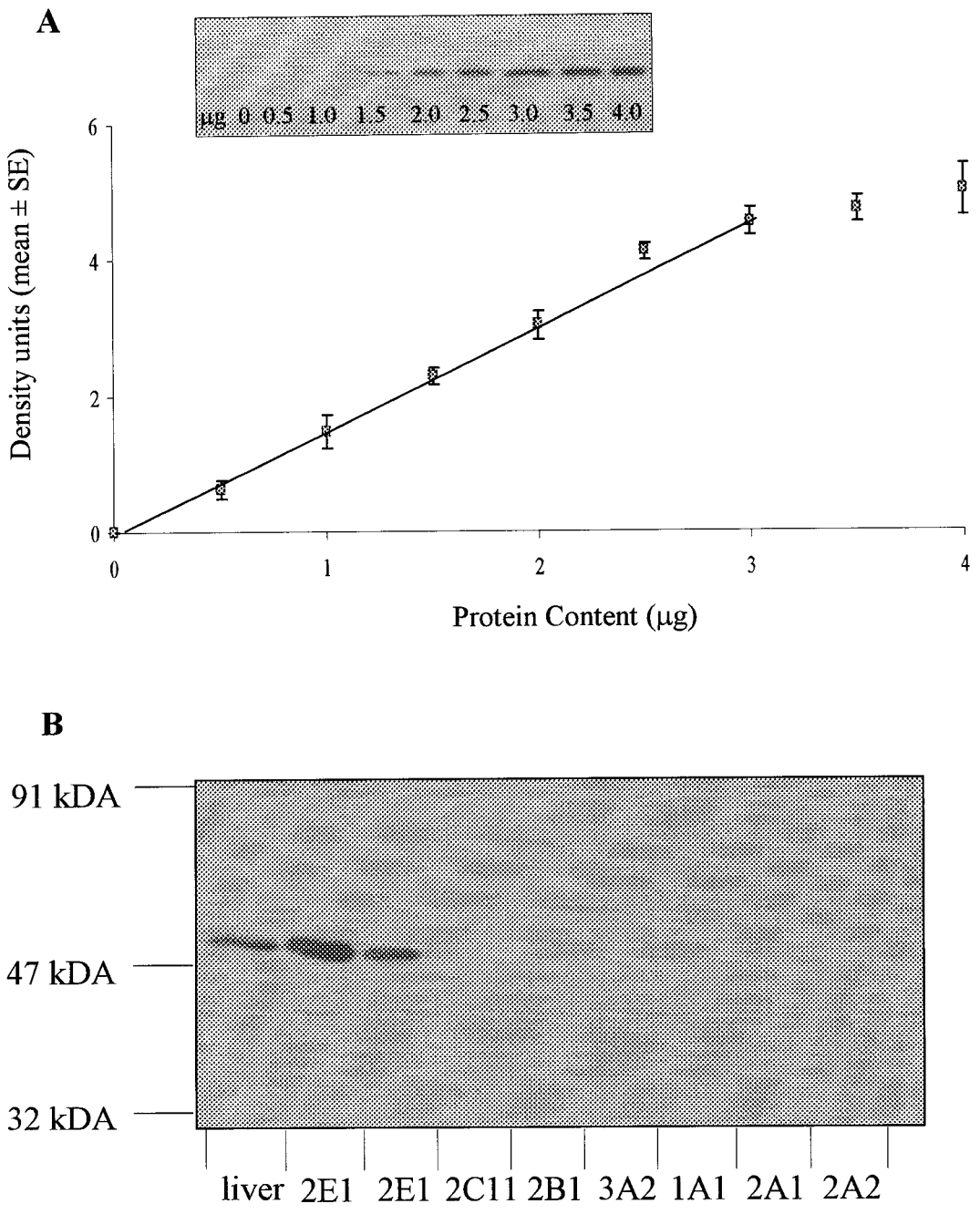


**Fig. 3.1**. Western blotting of hepatic CYP2E1. (A) Dilution curve of saline treated rat hepatic CYP2E1 (± S.E.M., three experiments); inset is a representative immunoblot. (B) Immunodetectable bands from expressed CYP2E1 (0.184 and 0.092 pmol) comigrated with 2.0 µg of untreated rat liver microsome preparation. No cross reactivity of rabbit anti-rat polyclonal antibody with 0.184 pmol of rat expressed CYP2C11, CYP2B1, CYP3A2, CYP1A1, CYP2A1 and CYP2A2 was observed.

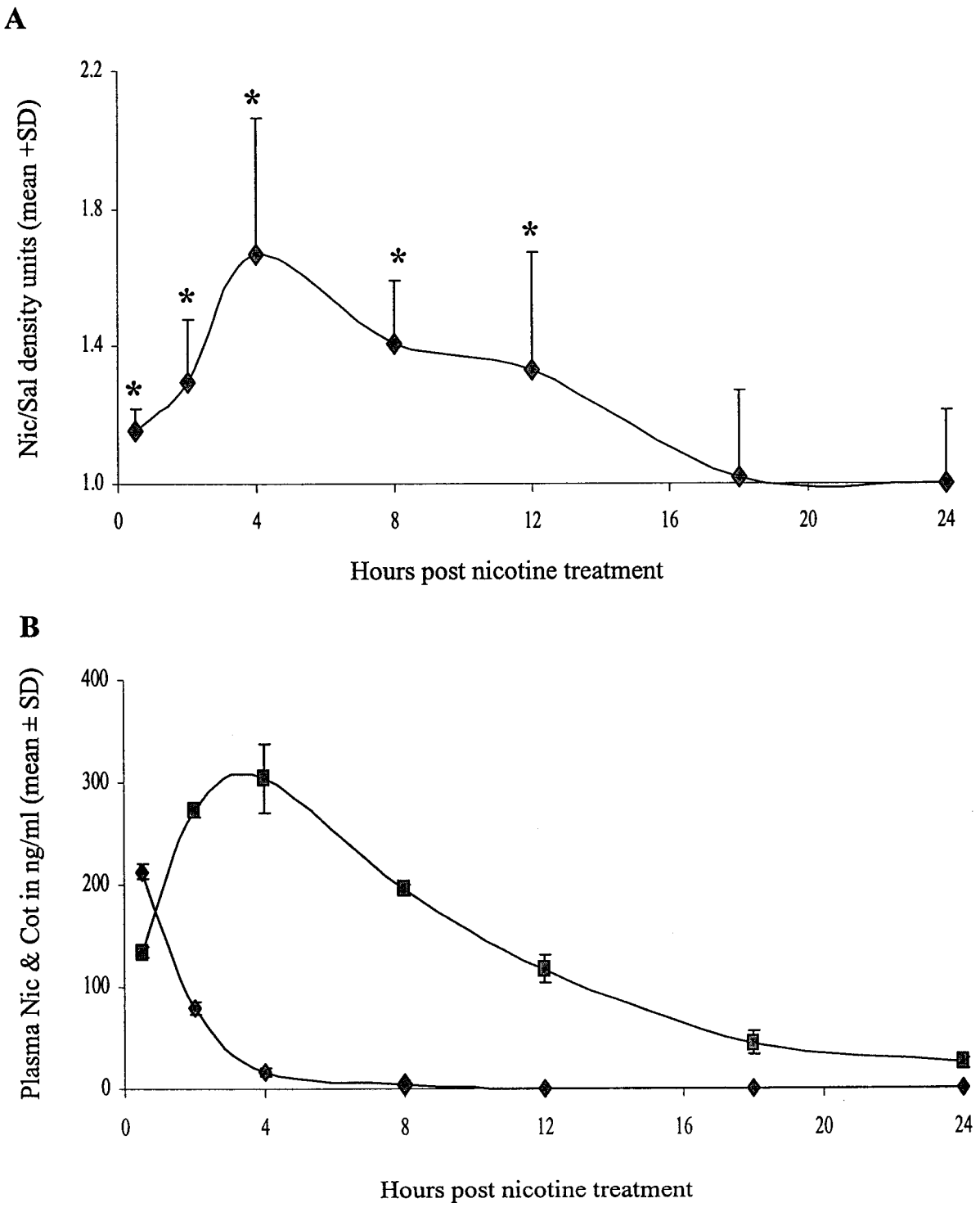


**Fig. 3.2** Recovery time-course of hepatic CYP2E1 induction by 1.0 mg/kg s.c nicotine. (A) Fold-induction in CYP2E1 levels relative to saline (Sal) treated animals over 24 hours post-treatment. Each point represents the mean + SD of at least three different experiments (n=3 rats). Significance from saline is indicated by * ($p<0.05$). (B) Corresponding plasma nicotine (◆ Nic) and cotinine ( ■ Cot) levels over 24 hours post-treatment.

## 3.2 Acute vs Chronic Effect of Nicotine

Time-course studies revealed that CYP2E1 induction by nicotine returned to basal levels by 18-24 hours post last treatment. 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. 3.3B) 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. 3.3A). However rats treated chronically with the same dose for 7 consecutive days resulted in a 1.7-fold ($p<0.01$) increase in CYP2E1 protein level.

## 3.3 Dose-Dependent Induction of CYP2E1 by Nicotine

Previous studies conducted in our laboratory have demonstrated that treatment of rats with nicotine at doses of 0.1, 0.3 and 1.0 mg/kg/day for 7 consecutive days resulted in increased CYP2E1 protein (1.4, 1.8 and 1.5-fold respectively) and activity. The apparent saturation of the level of CYP2E1 induction at the doses tested in this initial study 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. 3.4). The $ED_{50}$ value for the observed increase in CYP2E1 protein level was estimated to be approximately 0.01 mg/kg.

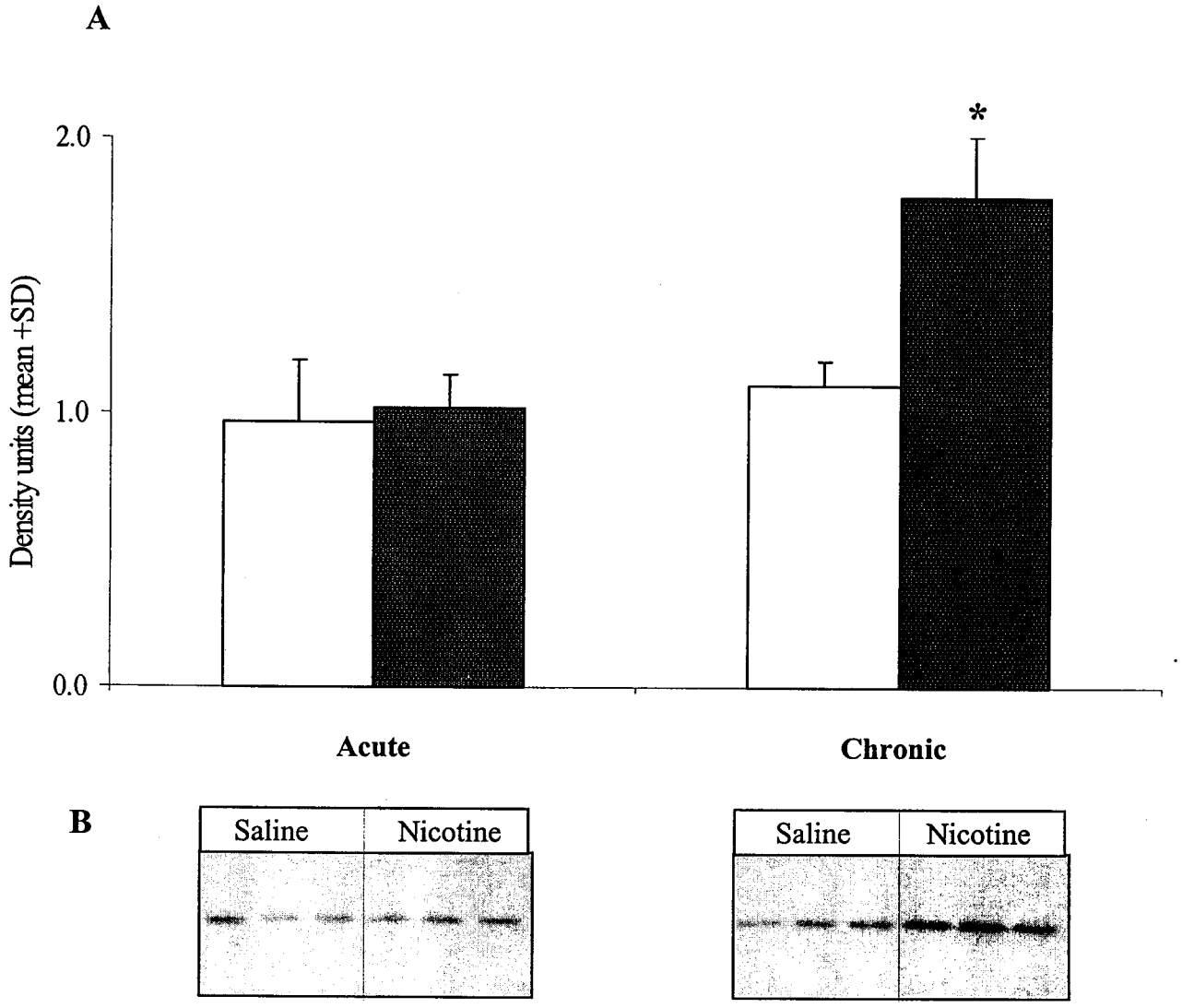


**Fig. 3.3** Acute vs Chronic effects of nicotine on CYP2E1 levels. (A) No significant difference was observed in rats treated with a single dose of 1.0 mg/kg nicotine when compared with their saline controls, whereas chronic treatment for 7 days results in a significant increase in CYP2E1 levels. Significance from saline is indicated by *($p<0.05$). (B) Representative immunoblot of 3 animals per treatment group.

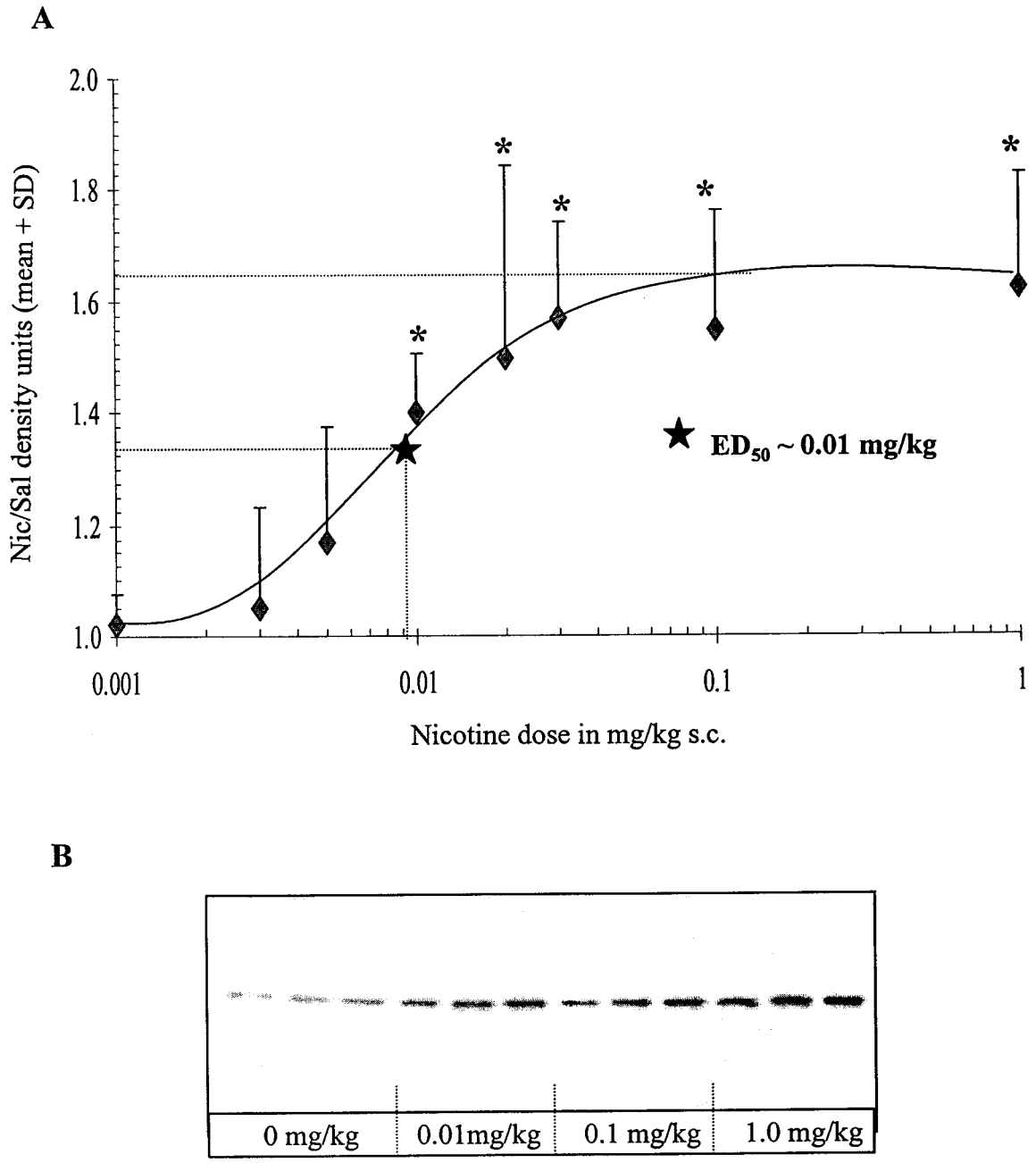


**Fig. 3.4** Dose-dependent induction of hepatic CYP2E1 by nicotine. (A) Dose-response curve of CYP2E1 induction as a function of nicotine (Nic) relative to saline (Sal) treated animals. Each point represents mean Nic/Sal of three animals/group + SD. Significance from saline is indicated by * ($p<0.05$). (B) Representative immunoblot of rats treated with 0, 0.01, 0.1 and 1.0 mg/kg Nic; three animals per treatment group.

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 as 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. In addition, plasma nicotine and cotinine levels may be useful for comparing our dosing regime in rats with plasma levels reported in smokers or in non-smokers exposed to nicotine (see section 4.3 for more detail).

**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 |

## 3.4 Effects of Nicotine on CYP2E1 mRNA Levels

The mechanism by which the majority of CYP2E1 inducers, including ethanol and low-molecular weight ligands, increase CYP2E1 is by protein stabilization. However, well established types of stabilization experiments (Barmada *et al.*, 1995; Huan and Koop, 1999) conducted in collaboration with Dennis Koop's research team (Oregon Health Sciences University) suggest that nicotine does not increase CYP2E1 protein by this mechanism. In brief, Chinese hamster ovary cells (CHO-K1) that constitutively express rabbit CYP2E1 protein were treated with various concentrations of nicotine (0.5 to 0.00005 μM). Following an overnight incubation there was no change in chlorzoxazone metabolism and no change in the level of immunodetectable CYP2E1 protein. These results suggest that nicotine does not inhibit the degradation of CYP2E1, and that in vivo the induction of CYP2E1 by nicotine may not occur via stabilization of the enzyme. An alternative mechanism of induction of CYP2E1 is by transcriptional regulation (Badger *et al.*, 1993; Lieber, 1999). The present study was conducted to examine whether nicotine treatment results in increased CYP2E1 mRNA levels. Total RNA was extracted from rat livers and slot blot analysis was used to compare CYP2E1 mRNA between different treatment groups. Ethanol 3g/kg induces hepatic CYP2E1 to a similar level to that observed with nicotine (Howard *et al.*, 2001). At these relatively low doses, ethanol does not increase CYP2E1 by transcriptional regulation (Ronis *et al.*, 1993). Therefore, hepatic CYP2E1 total RNA from ethanol treated rats was included in this study as a negative control. A serial dilution of CYP2E1 cDNA was used to determine the linear range of the assay and all samples fell within this range (Fig. 3.5A). No signal was detected from 1 and 10 pg of cDNA of CYP2B6, CYP2A6, CYP2D6, and CYP3A4 indicating the specificity of the CYP2E1 probes (Fig. 3.5B).

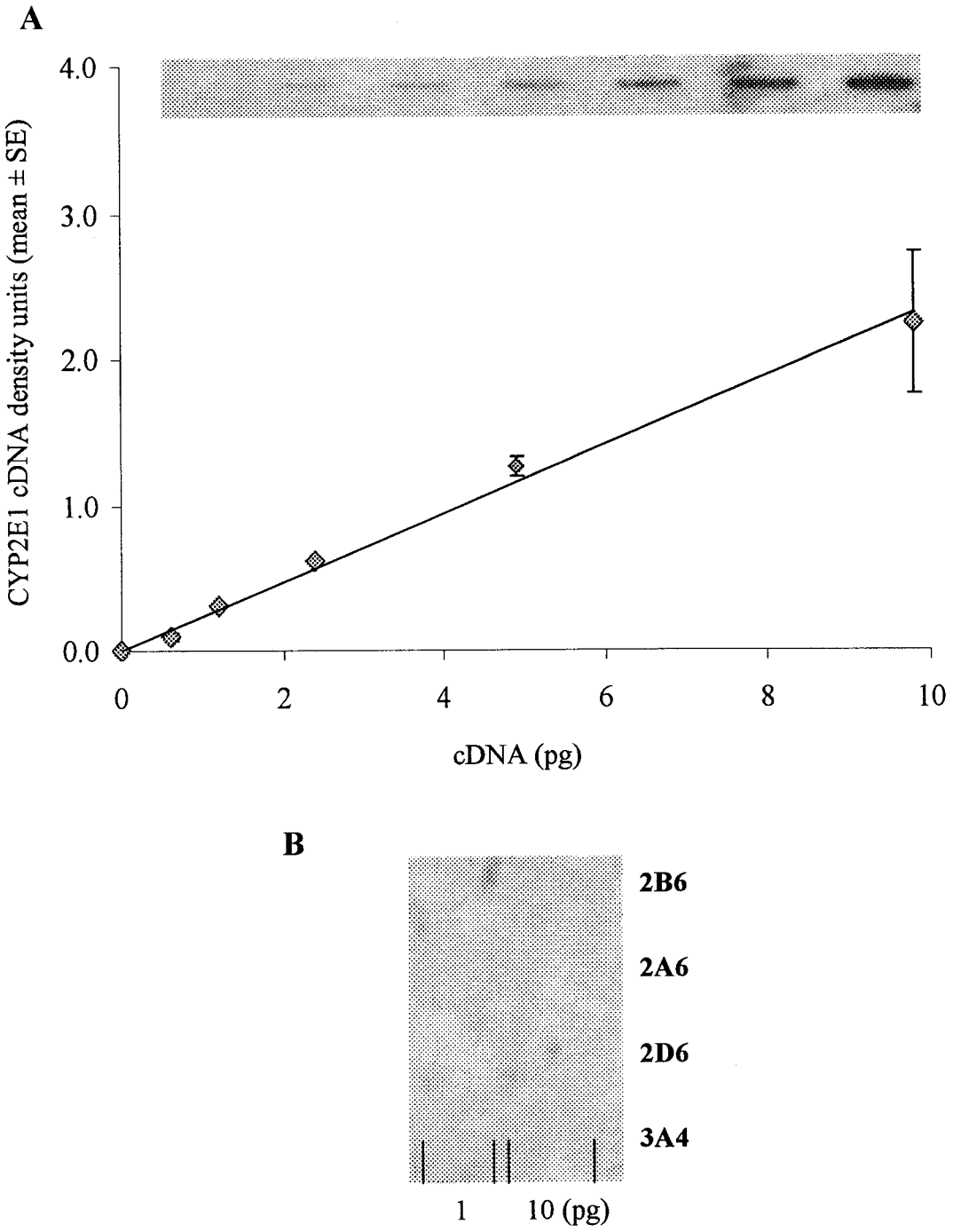


**Fig. 3.5**. Slot blotting of total RNA from livers of treated animals. (A) Detection of a serial dilution of CYP2E1 cDNA was linear from 0 to 9.8 pg; all samples fell within this range. The inset contains a representative immunoblot. (B) No cross-reactive signal from cDNA of CYP2B6, CYP2A6, CYP2D6 and CYP3A4 when loaded at 1 and 10 pg.

No significant difference in CYP2E1 mRNA levels was observed between livers from ethanol and saline-treated controls (2.24 +/- 0.16 and 2.15 +/- 0.22) (p=0.56) or between nicotine-treated animals compared to their saline controls (2.28 +/- 0.30 and 2.19 +/- 0.14) (p=0.59) (Fig. 3.6). Similar results were observed with blots hybridized with rat CYP2E1 oligonucleotide probes (data not shown). The β-actin levels between livers from ethanol and saline-treated animals (0.78 +/- 0.11 and 1.01 +/- 0.26) (p=0.18) or between livers from nicotine and saline-treated animals (1.21 +/- 0.14 and 1.32 +/- 0.30) (p=0.55) were also not significantly different; likewise, no significant differences were observed in the levels of CYP2E1/β-actin between ethanol and nicotine and their respective control groups. These results suggest that nicotine does not regulate CYP2E1 by transcriptional mechanisms, since CYP2E1 protein levels were increased in the absence of significant alterations in CYP2E1 mRNA levels.

## 3.5 Effects of Cotinine on CYP2E1 Protein Levels

The mechanistic studies described above suggest that nicotine does not increase CYP2E1 protein by two common induction mechanisms, specifically transcriptional regulation and protein stabilization (Badger *et al.*, 1993; Koop and Tierney, 1990; Lieber, 1999)). 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.

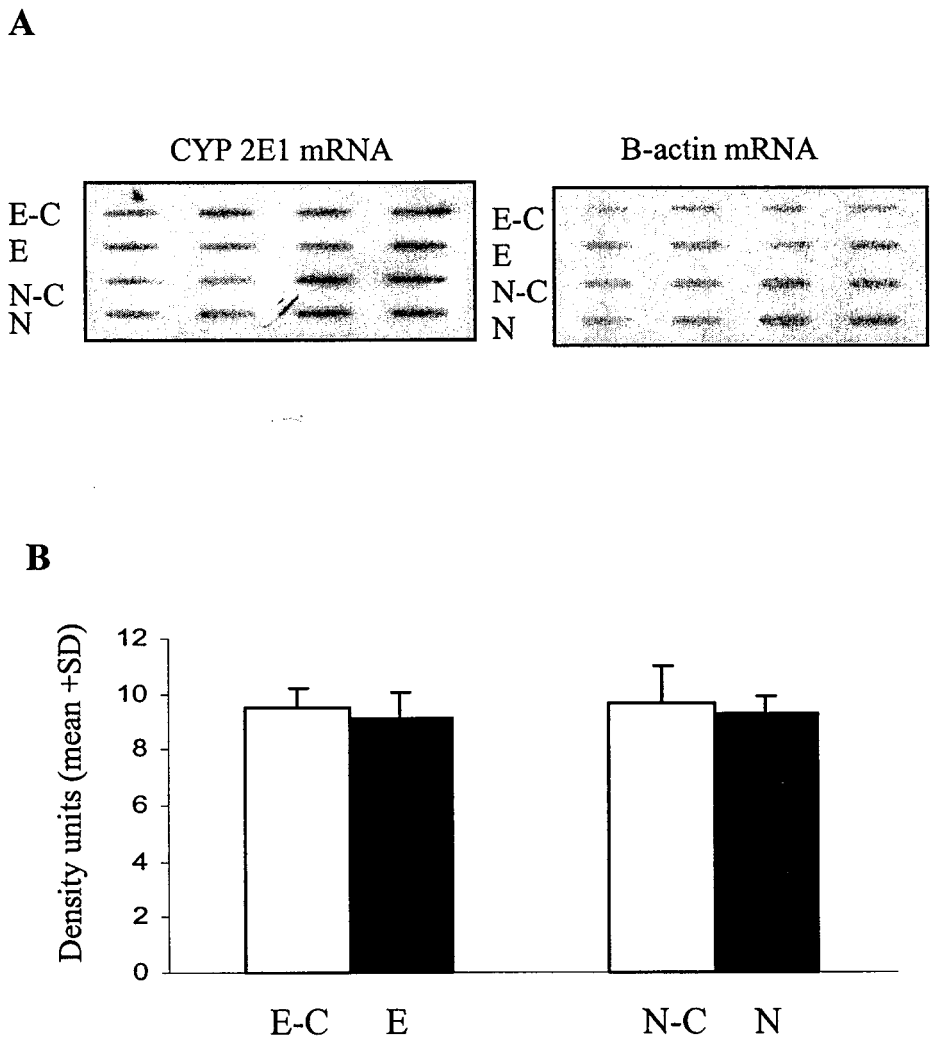


**Fig. 3.6** Effects of nicotine and ethanol on hepatic CYP2E1 mRNA levels. (A) Representative blots of CYP2E1 and β-actin mRNA levels in ethanol-control (E-C) ethanol- (E), nicotine-control (N-C), and nicotine (N) treatment groups. (B) No significant differences in CYP2E1 mRNA levels observed between E and N treated animals compared to their saline controls; mean of four animals/group (+SD).

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. 3.7B). Despite the high plasma cotinine levels achieved with this treatment at 0.5 hours post injection we observed no significant increase in CYP2E1 protein levels (Fig. 3.7, A and B). At 4 and 8 hours post treatment cotinine levels (Fig. 3.7B) were similar to those found at 4 hours post nicotine (1.0 mg/kg) treatment (Fig. 3.2B), when maximal CYP2E1 induction was observed in nicotine treated rats (Fig. 3.2A). However, CYP2E1 levels were not elevated in cotinine treated rats at 4 or at 8 hours after the last injection (Fig. 3.7A). The apparent modest increase in CYP2E1 levels of 1.2-fold at 8 hours post-treatment was not statistically significant ($p=0.30$). These results suggest that cotinine is not capable of inducing CYP2E1.

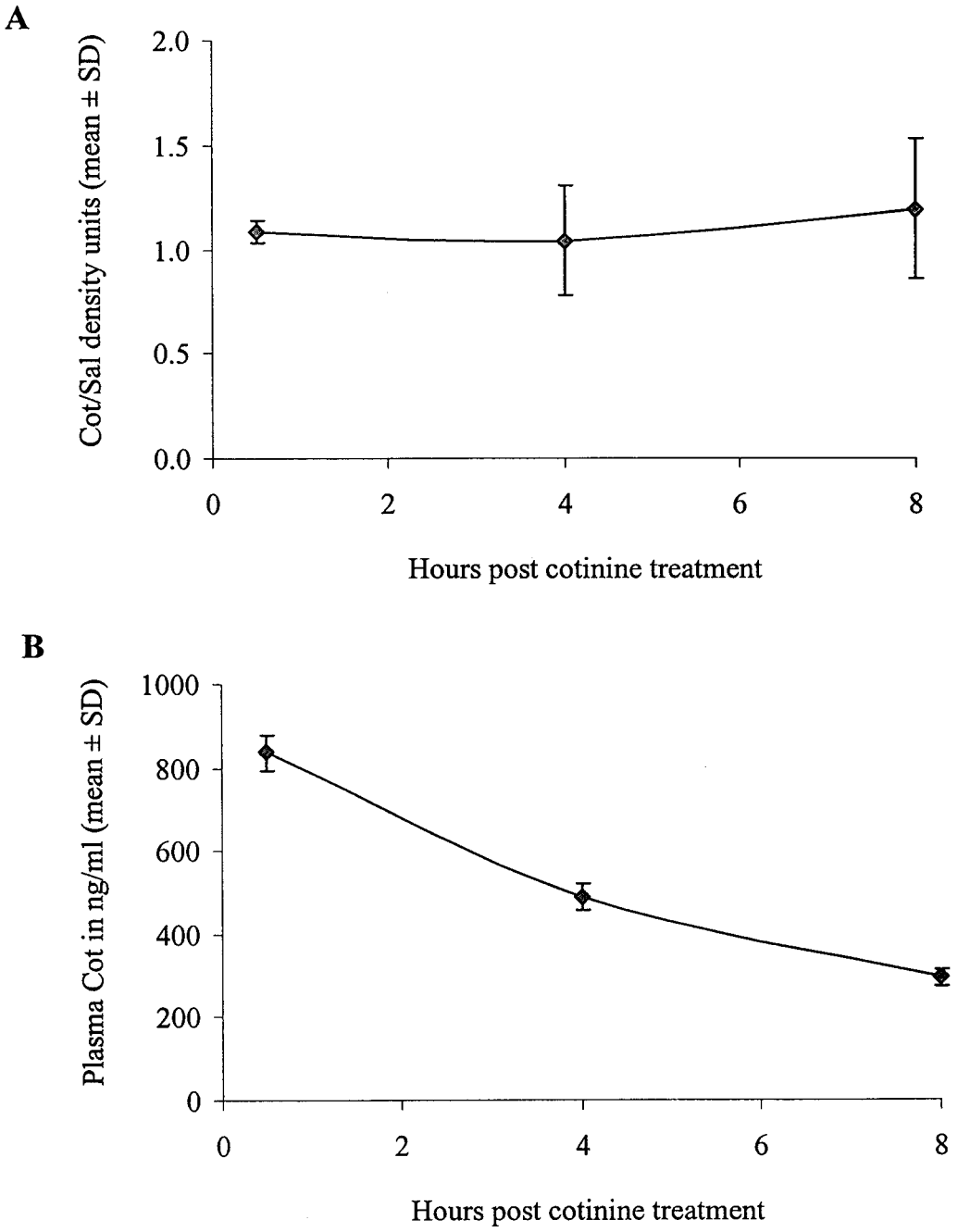


**Fig. 3.7** Effects of cotinine (Cot) treatment on CYP2E1 protein level. (A) No significant increase was observed in CYP2E1 levels at 0.5, 4 and 8 hours post cotinine treatment relative to saline (Sal) treated animals. (B) Plasma cotinine levels over time. Each point represents the mean ± SD of 3 animals/group.

# 4. Discussion of Experimental Results

Previous studies conducted in our laboratory have demonstrated that relatively low doses of nicotine induce CYP2E1 protein and activity in the rat liver (Howard *et al.*, 2001). The overall objective of the current study was to further characterize this effect in terms of the full dose-response relationship, the recovery time-course of induction, and to investigate possible mechanisms for CYP2E1 regulation by nicotine. We have also investigated the potential involvement of nicotine metabolites in the observed induction of CYP2E1.

## 4.1 Recovery-time Course of Nicotine-induced CYP2E1

We have previously demonstrated that treatment of rats with nicotine for 7 days, results in increased CYP2E1 protein and activity in the rat liver (Fig. 1.1, 1.2) (Howard *et al.*, 2001). In this initial study rats were sacrificed at 4 hours following the last nicotine injection, however, the CYP2E1 levels were not determined at earlier or later time points after nicotine treatment. Therefore, the objective of this study was to assess the induction of CYP2E1 by nicotine over 24 hours following the last injection of nicotine in order to determine how long this effect persists and when it peaks. Based on our findings we can characterize the induction of hepatic CYP2E1 by nicotine as rapid, as evidenced by the significant elevation in protein levels within 0.5 hours of nicotine treatment (Fig 3.2A). This induction is also best characterized as short-lasting, because the elevated levels of CYP2E1 in nicotine treated rats had returned to near control levels by 18-24 hours after the last nicotine injection (Fig. 3.2A). We have also established that CYP2E1 levels peak at 4 hours after nicotine treatment. This confirmed that 4 hours post-nicotine treatment is the optimal sacrifice time for examining the induction of CYP2E1, and was used as standard in all the other studies presented in this thesis. These findings also suggest that

the highest risk in terms of CYP2E1 associated toxicity is at approximately 4 hours following nicotine exposure at which time the production of CYP2E1-mediated toxic metabolites is expected to be at its peak.

## 4.2 Acute Effects of Nicotine on CYP2E1

We have established that chronic (7 days) nicotine treatment in rats results in hepatic CYP2E1 induction, however, the effect of acute nicotine treatment on hepatic CYP2E1 had not been investigated. Other 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. Moreover, our time-course study revealed that CYP2E1 induction by nicotine returns to basal levels by 24 hours. Therefore, it became of interest to assess the expression of CYP2E1 protein upon acute nicotine exposure. In contrast to chronic nicotine exposure, treatment of rats with a single dose of nicotine failed to alter CYP2E1 protein levels in the rat liver (Fig 3.3). This finding implies that the necessary changes leading to induction require more than one exposure to nicotine. We can also postulate that acute exposure to nicotine may be insignificant in potentiating CYP2E1-associated hepatotoxicity at least at the dose (1 mg/kg s.c), and using the paradigm, that we have tested.

## 4.3 Dose-dependent Induction of CYP2E1 by Nicotine

Initial studies conducted in our laboratory demonstrated that treatment of rats with nicotine at doses of 0.1, 0.3 and 1.0 mg/kg s.c. for 7 days results in increased hepatic CYP2E1 protein and activity (Howard *et al.*, 2001). However, the inductive effect of nicotine was saturated at these doses (Fig. 1.1), suggesting that lower doses may also induce CYP2E1 protein.

In the current study we have demonstrated that CYP2E1 induction by nicotine is dose-dependent, and that induction of CYP2E1 is very sensitive to 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 (London *et al.*, 1990; Pratt *et al.*, 1983), subcutaneous nicotine administration to rats leads to peak nicotine plasma levels after 30-60 minutes. However, for the dose-response studies we have measured nicotine and cotinine plasma levels only 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. 3.2) where we found plasma nicotine of approximately 210 ng/ml and cotinine of 120 ng/ml. Based on the information available we can speculate 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 1.2 - 3.8 ng/ml. Interestingly, plasma cotinine in non-smoking adults living with a smoking vs 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) vs 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 NRTs, individuals exposed to ETS may also have increased CYP2E1 levels and increased risk of CYP2E1-related toxicity. This may be most relevant to the millions of children that are exposed to ETS in the household. There is growing evidence that young children are more vulnerable than adults to genetic damage and other effects of carcinogens, and that carcinogenic exposure during early development can increase the risk of cancer in later life (Perera, 1997; Tang *et al.*, 1999). In addition, it has been estimated that 17% of lung cancer cases in non-smokers are attributed to high levels of ETS exposure during early childhood and adolescence (Janerich *et al.*, 1990). Moreover, several studies have indicated that exposure to ETS from spouses who smoke is associated with an increased risk of lung cancer among non-smoking women (Fontham *et al.*, 1994; Hackshaw *et al.*, 1997; Hirayama, 2000). Therefore we can postulate that nicotine-induced CYP2E1 in children and adults may lead to increased activation of tobacco-smoke and other procarcinogens increasing the risk for cancer development in non-smokers exposed long-term to ETS. Nevertheless, while smokers have higher levels of CYP2E1 in liver (Benowitz, 1999b) and brain (Howard *et al.*, 2003) it remains to be determined whether nicotine can induce CYP2E1 in humans, and whether this induction is associated with clinical and toxicological outcomes in passive and active smokers.

## 4.4 Mechanisms of CYP2E1 Induction by Nicotine

Because CYP2E1 plays a key role in the metabolism and bioactivation of a variety of toxins and carcinogens it is important to understand the regulation of this enzyme by nicotine, an inducer used by millions of people. Regulation of CYP2E1 expression by its various inducers

has been studied extensively in rats and other laboratory animals and is thought to involve transcriptional, post-transcriptional and post-transcriptional mechanisms (Lieber, 1999).

Some CYP2E1 inducers (including ethanol at high doses) regulate the expression of CYP2E1 by transcriptional mechanisms (Badger *et al.*, 1993; Lieber, 1999), which is detected experimentally by an increase in both protein and mRNA levels. In order to determine whether nicotine also regulates CYP2E1 by transcriptional mechanisms, we measured mRNA levels in the livers of the same rats in which we had observed increased CYP2E1 protein following nicotine and ethanol treatment (Howard *et al.*, 2001). However our RNA slot blot analyses revealed no differences in mRNA levels between livers from saline- or nicotine-treated animals, similar to the results following ethanol (low dose, used as negative control) treatment (Fig. 3.6 B). These results suggest that nicotine does not transcriptionally regulate CYP2E1, since CYP2E1 protein levels in treated rats were increased in the absence of significant alterations in mRNA levels.

An alternative mechanism of CYP2E1 induction is protein stabilization, a process whereby many CYP2E1 inducers including ethanol (at low doses), and low molecular weight compounds such as acetone and pyrazole, increase CYP2E1 protein levels in the absence of alterations in CYP2E1 mRNA levels (Ronis *et al.*, 1993; Song *et al.*, 1989; Winters and Cederbaum, 1992). These compounds 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, 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've conducted studies in collaboration with Dennis Koop's research team, to examine whether nicotine can induce CYP2E1 by protein stabilization. Using CHO cells that constitutively express rabbit CYP2E1 we detected no change in chlorzoxazone metabolism and no elevation in CYP2E1 protein levels following nicotine exposure. 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. 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 cell line. This would however distinguish the induction mechanism of nicotine from that of ethanol which has been shown to stabilize CYP2E1 in this experimental model (Koop and Tierney, 1990). 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.

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. Throughout these studies we have postulated that the observed induction of CYP2E1 is due to nicotine itself as this was the administered drug. However, nicotine is readily metabolized to cotinine which exhibits a longer elimination half-life than its precursor nicotine (Benowitz, 1983; Sastry *et al.*, 1995). 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 was 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. 3.7A). It would be of interest to investigate whether other nicotine metabolites such as nicotine-N'-oxide may be involved in CYP2E1 induction.

Therefore, further investigations are necessary in order to elucidate the mechanism involved in CYP2E1 induction by nicotine. Based on our findings, the regulation of CYP2E1 by nicotine is unlikely to occur by transcriptional mechanisms or by protein stabilization. CYP2E1 inducers, such as pyridine are thought to increase CYP2E1 protein by enhancing CYP2E1 mRNA translational efficiency (Kim and Novak, 1990; Lieber, 1999). Investigations have demonstrated increased CYP2E1 protein synthesis in the absence of any change in CYP2E1 mRNA levels in pyridine treated rats (Kim and Novak, 1990). Furthermore, pyridine administration in rats resulted in a shift in CYP2E1 mRNA toward larger polyribosomes, which indicates enhanced translational efficiency through increased loading of ribosomes on CYP2E1 mRNA (Kim et al., 1990). Although the precise mechanisms of increased efficiency of translation remain to be elucidated, xenobiotic-induced CYP2E1 translation may occur in response to evoked alterations in the activity of one or more components of the translation machinery. Since nicotine bears structural similarity to pyridine, it is possible that translational activation of CYP2E1 may be the mechanism underlying nicotine's induction of this enzyme.

# 5. General Discussion

## 5.1 Induction of CYP2E1 by Nicotine and Tobacco Smoke

Several studies have shown that cigarette smoke exposure in both humans and rodents leads to increased CYP2E1 protein and/or activity. Villard *et al.*, (1998) have demonstrated that exposure of mice to cigarette smoke in vivo results in increased levels of CYP2E1 activity and hepatic protein (Villard *et al.*, 1998). Studies with higher doses, and longer durations of nicotine administration in rats, showed increase p-nitrophenol hydroxylase activity in the liver, an enzyme activity which has been attributed to CYP2E1 (Bhagwat *et al.*, 1998). Another animal study showed that either orally administered tobacco extract or nicotine at concentrations that model oral tobacco consumption increased aniline hydroxylation (Kaur and Ali, 1982); this activity is thought to be predominantly mediated by CYP2E1. Moreover, cigarette smoking also significantly enhanced the metabolism of chlorzoxazone in humans (Benowitz, 1999b), reflecting increased CYP2E1 activity (Lucas *et al.*, 1999). The inductive effect on CYP2E1 could be attributed to the presence, in cigarette smoke, of components such as pyridine, acetone and benzene which are known CYP2E1 inducers (Gut *et al.*, 1993; Kim and Novak, 1990; Song *et al.*, 1989). The relative contribution of these inducers is difficult to determine based on the current knowledge with most of these studies conducted in a rodent model. Data for human studies are scarce and require further investigation. However, we have demonstrated that among other cigarette smoke constituents, nicotine, which is found at the highest concentration of these putative inducers, can induce CYP2E1 protein and activity in the rat liver. Moreover, an initial pilot study conducted in our laboratory suggests that nicotine can induce CYP2E1 protein (1.3-fold) in a primate model using african-green monkeys. This indicates that nicotine is also able to induce hepatic CYP2E1 in a non-human primate model and is therefore likely to increase

CYP2E1 in humans as well. Therefore, we can postulate that nicotine may contribute to the CYP2E1 induction that has been observed in rodents (Villard *et al.*, 1998) and in humans (Benowitz, 1999b) upon exposure to cigarette smoke. Although CYP2E1 isoforms show remarkable conservation among species in terms of catalytic and regulatory specificities (Paine, 1995; Tanaka *et al.*, 2000)) it remains to be determined whether nicotine can induce CYP2E1 in humans. Therefore, the effects of chronic and acute nicotine exposure in humans need to be assessed in well-designed pharmacokinetic studies that minimize the effects of potential confounding variables (eg. alcohol, drugs and dietary constituents that may modulate CYP2E1 activity). In addition, induction of CYP2E1 by nicotine is important as many people are being treated with nicotine in the absence of cigarette smoke exposure (Benowitz, 1999a).

## 5.2 Nicotine-induced CYP2E1: Possible Contribution to Increased Ethanol Consumption and Metabolism

It is well established that induction of drug-metabolizing enzymes results in accelerated rates of xenobiotic biotransformation which can lead to the subsequent development of pharmacokinetic tolerance (larger doses of a drug must be administered to achieve desired plasma levels and therapeutic effect). Therefore by inducing the level and activity of CYP2E1, nicotine may contribute to increased metabolism and development of tolerance to various CYP2E1 substrates such as ethanol.

A common observation in both animal and humans studies is that nicotine/tobacco smoke exposure results in increased ethanol consumption. It has been reported that between 80-95% of alcoholics smoke (DiFranza and Guerrera, 1990; Patten *et al.*, 1996) and that the incidence of alcoholism is 10 times more likely in smokers than nonsmokers (Batel *et al.*, 1995). Among non-alcoholics, smokers report drinking levels of alcohol that are approximately twice that of

non-smokers (Carmody *et al.*, 1985), and twin studies indicate, that for equal ethanol consumption, heavy smokers have higher ethanol elimination rates compared with non-smokers (Kopun and Propping, 1977).

Several studies have used animal models to examine the effects of tobacco availability, or exposure to nicotine, on alcohol intake. Subcutaneous injections of nicotine (Blomqvist *et al.*, 1996), as well as s.c. implantation of a nicotine pellet (Potthoff *et al.*, 1983) in experienced ethanol drinking rats, resulted in increased ethanol intake. More recently it has been shown that s.c. injections of nicotine at similar doses to those used in our studies (0.2 or 0.4 mg/kg nicotine base) increased acquisition of ethanol drinking behavior in rats using a limited ethanol access procedure (Smith *et al.*, 1999). Nicotine effects on central nicotinic receptors, as well as on 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). Another possible mechanism which could account for the nicotine-induced increase in ethanol consumption may be related to a possible alteration in ethanol metabolism upon nicotine exposure. In our current studies we have demonstrated that nicotine treatment in rats leads to increased levels and activity of the ethanol metabolizing enzyme CYP2E1. Increased CYP2E1 activity has also been observed in both rodent and human subjects upon cigarette smoke exposure (Benowitz, 1999b; Villard *et al.*, 1998). Therefore, nicotine may increase ethanol metabolism (metabolic tolerance) requiring alcohol dependent individuals to drink more ethanol for an equal effect. This 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 CYP in rats, were induced by the same relatively low doses of ethanol (Schoedel *et al.*, 2001) which we have shown increases CYP2E1 (Howard *et al.*, 2001). These findings provide

evidence that the induction of ethanol and nicotine metabolic pathways may synergistically contribute to the development of cross-tolerance between these two commonly co-used and co-abused drugs (DiFranza and Guerrera, 1990; Patten *et al.*, 1996). Since ethanol can also induce its own metabolism via CYP2E1 induction, it would be interesting to design combination experiments of nicotine and ethanol to test whether their inductive effects are additive or synergistic.

## 5.3 Potential Pathogenic Roles for Nicotine via Induction of CYP2E1

In addition to a more rapid hepatic elimination of xenobiotics induction of drug metabolizing enzymes including CYPs leads to a faster and more pronounced formation of metabolites. If such metabolites are still biologically active and/or are of toxicological concern, induction of CYPs may increase the risk of adverse effects. This is particularly true for CYP2E1, which has a remarkable capacity to bioactivate many hepatotoxins (e.g. ethanol, acetominophen, carbon tetrachloride, halothane, isoniazid) and procarcinogens (e.g. NDMA, NNK, benzene) to highly toxic metabolites. Therefore, exposure to such compounds in individuals with higher levels of CYP2E1 will result in greater formation of toxic and/or reactive metabolites, which may increase the risk of organ toxicity and carcinogenicity. We can thus postulate that by increasing the level and activity of CYP2E1 nicotine could potentially contribute and further exacerbate toxicity and disease related to CYP2E1.

### 5.3.1 Potential Contribution of Nicotine-induced CYP2E1 to Cancer Development

Tobacco smoke exposure is associated with increased cancer susceptibility in active smokers as well as in non-smokers chronically exposed to ETS (Chao *et al.*, 2002; Hackshaw *et*

*al.*, 1997; Thun *et al.*, 2002). Among the many factors that have been implicated the effect of cigarette smoke constituents on CYP-dependent activation of procarcinogens to carcinogens is considered to be of major importance (Anttila *et al.*, 1991; Badawi *et al.*, 1996; Zevin and Benowitz, 1999). Procarcinogen bioactivation by CYPs leads to the formation of electrophilic intermediates (Gonzalez and Gelboin, 1994; Guengerich *et al.*, 1991) which can produce various DNA lesions thereby increasing the risk for carcinogenic tumor formation (Pfeifer *et al.*, 2002).

The association between smoking-related cancers and CYP2E1 has not been well established to date. However, CYP2E1 activates procarcinogenic N-nitrosamines and many other small carcinogenic compounds (e.g. benzene) and several studies support a role for CYP2E1 in cancer development (Lieber, 1997; Woodcroft and Novak, 1998). A recent study investigated the expression of CYP2E1 in liver biopsies of hepatocellular carcinomas (HCC) and in other liver lesions thought to be involved in the multistep progression of HCC (Hirose *et al.*, 2002). Increased CYP2E1 expression was found in hepatocytes of tumour and peri-tumour regions as compared to non-peri-tumour regions. Liver lesions including cirrhotic nodes, adenomatous hyperplasia and atypical adenomatous hyperplasia, which are thought to be intermediates that eventually progress to HCC, also expressed increased levels of CYP2E1. These findings provide evidence for the potential involvement of CYP2E1 in the multistep carcinogenesis process of HCC. In addition, CYP2E1 has been implicated in benzene-induced hematological malignancies in humans (Rothman *et al.*, 1997)and nitrosodimethylamine-induced tumorigenesis in animals (Lieber, 1997). Moreover, CYP2E1 has been proposed as a mediator of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced chromosomal aberrations (Abdel-Rahman *et al.*, 2000). Furthermore, in vivo cigarette smoke exposure induced CYP2E1 in mice which was associated with a concomitant increase in cigarette smoke-induced DNA strand

breaks (Villard *et al.*, 1998), further implicating CYP2E1 in tobacco-related cancers. Tobacco smoke has been shown to induce CYP2E1 activity in animal models and in humans (Benowitz, 1999b; Villard *et al.*, 1998) and we have demonstrated that this effect may be partially attributed to nicotine. Therefore, nicotine-induced CYP2E1 may increase the risk of tobacco-related cancers by enhancing the metabolic activation of procarcinogens.

In addition nicotine may act in synergy with other CYP2E1 inducers to increase the risk of cancer development. Epidemiological findings have established an interactive influence of ethanol consumption and cigarette smoking on cancer development. The risks for cancer of the mouth, throat, or esophagus for the smoking drinker are more than the sum of the risks from cigarette smoking or ethanol drinking alone (Blot, 1992; Patten *et al.*, 1996). These findings could be partly attributed to the induction of CYP2E1 by both ethanol and nicotine, leading to enhanced activation of tobacco-smoke carcinogens and procarcinogens from other sources, e.g. diet (Sachse *et al.*, 2002) and environmental pollutants (Calle *et al.*, 2002).

### 5.3.2 Potential Contribution of Nicotine-induced CYP2E1 to Alcoholic-related and Non-alcoholic Liver Diseases

CYP2E1 has been implicated in the development of alcohol and non-alcohol related liver diseases via mechanisms which involve the production of ROS and the activation of hepatotoxins (Lieber, 1997; 1999). Induction of CYP2E1 is thus expected to result in enhanced generation of reactive oxygen intermediates and toxic metabolites increasing the risk for hepatotoxicity. This effect is best exemplified by the CYP2E1 inducer ethanol, and its contribution to alcoholic liver disease development (Albano, 2002; Lieber, 2000; Morgan *et al.*, 2002).

5.3.2.1 Reactive Oxygen Species Related Hepatotoxicity

Acute and chronic ethanol treatment in rats, as well as alcohol consumption in humans, results in enhanced generation of hydroxyethyl radicals, superoxide anion ($O_2^-$), $H_2O_2$, and acetaldehyde by CYP2E1 (Albano *et al.*, 1996; Aleynik *et al.*, 1998). These reactive intermediates are known to exert their hepatotoxic effect by initiating lipid peroxidation, oxidative stress, and Kupffer cell activation, thereby propagating cellular injury (Jarvelainen *et al.*, 2000). Furthermore, CYP2E1 induction correlates with lipid peroxidation and pathological severity during chronic ethanol exposure (Jarvelainen *et al.*, 2000); this effect is blocked by inhibitors of CYP2E1 (Jaeschke *et al.*, 2002). Of interest, alcoholics without clinical symptoms of liver disease exhibit lower chlorzoxazone metabolism compared with alcoholic patients with alcoholic liver disease (ALD), further implicating CYP2E1 in the development of ALD (Albano *et al.*, 1999).

Similarly, by inducing CYP2E1, nicotine may increase the risk for oxidative-stress related organ damage in passive and active smokers. In support of this hypothesis, Bhagwat *et al.*, (1998) have demonstrated that in vivo nicotine treatment results in increased production of reactive oxygen species and a concomitant increase in CYP2E1 activity in rat liver, lung and brain. Another study, has found a 25 to 40% concomitant increase in reactive oxygen species generation and lipid peroxidation in tissues from nicotine-treated rats (Wetscher *et al.*, 1995), possibly mediated by nicotine-induction of CYP2E1. Moreover, chronic treatment with 0.6 mg/kg nicotine was shown to increase lipoperoxides, hydroperoxides, conjugated dienes, and free fatty acids in liver, lungs, and heart compared with control rats (Ashakumary and Vijayammal, 1996). Ethanol and nicotine can produce an additive enhancement of lipid peroxidation and depletion of antioxidants (Ashakumary and Vijayammal, 1996). Therefore,

based on these studies, we can interpret our data as evidence that nicotine-induced CYP2E1 may contribute to nicotine-induced oxidative stress and free radical formation leading to cellular and tissue damage.

5.3.2.2 CYP2E1 Activation of Hepatotoxins and Related Liver Damage

In addition to the production of ROS, CYP2E1 has a large capacity to activate many xenobiotics to highly hepatotoxic compounds. These include commonly used drugs such as the analgesic acetominophen (Dai and Cederbaum, 1995), anesthetics such as halothane (Eliasson *et al.*, 1998; Njoku *et al.*, 1997) and industrial solvents such as carbon tetrachloride (Raucy *et al.*, 1993). Interestingly, CYP2E1 activity strongly correlates with the degree of tissue injury induced by these toxins (Lieber, 1997; Woodcroft and Novak, 1998). Therefore induction of CYP2E1 by agents such as ethanol would be expected to result in enhanced formation of toxic metabolites and increased liver damage in people exposed to these protoxins. This effect is exemplified by the observed increases in $CCl_4$- or acetominophen-induced hepatotoxicity as a result of ethanol consumption (Manno *et al.*, 1996; Zimmerman and Maddrey, 1995). CYP2E1 can convert $CCl_4$ to its highly active metabolite trichloromethyl radical, and ethanol pretreatment remarkably stimulates the toxicity of $CCl_4$ with perivenular predominance; this can be explained by the selective presence and induction of CYP2E1 in this zone of the liver lobule (Tsutsumi *et al.*, 1989). A similar mechanism of hepatotoxicity applies to acetominophen which is known to cause severe hepatic injury in alcoholics even at therapeutic doses of acetominophen (Seeff *et al.*, 1986). The enhanced hepatoxicity of acetominophen after chronic ethanol consumption is attributed, at least in part, to increased production of the highly hepatotoxic metabolite N-acetyl-benzoquinone imine by ethanol-induced CYP2E1 (Corcoran *et al.*, 1980). We have

demonstrated that nicotine can induce CYP2E1. Therefore passive and active smokers, as well as others exposed therapeutically to nicotine (see section 1.2.11), may have increased levels of CYP2E1, which may predispose them to hepatic injury associated with these various therapeutic and industrial agents.

## 5.4 Summary and Concluding Remarks

In conclusion we have demonstrated that very low doses of nicotine can induce hepatic CYP2E1 in the rat liver. This inductive effect peaks at 4 hours after the last nicotine treatment and requires multiple exposures to nicotine. The mechanisms involved in CYP2E1 induction by nicotine remain to be elucidated, however nicotine is unlikely to regulate CYP2E1 expression by transcriptional mechanisms or by protein stabilization. Cotinine, the main nicotine metabolite is not involved in CYP2E1 induction in rats, however the potential involvement of other nicotine metabolites requires further investigation. Induction of CYP2E1 results in increased oxidative stress, activation of tobacco smoke, other procarcinogens and hepatototoxins, which may increase the risk for organ damage and cancer development. Therefore, our data suggest that nicotine may increase CYP2E1 related toxicity and ethanol cross-tolerance in smokers, passive-smokers, and people treated with nicotine (e.g. smokers, patient's with Alzheimer's disease, ulcerative colitis and neuropsychiatric motor disorders).

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