INDUCTION AND RECOVERY TIME COURSE OF RAT BRAIN CYP2E1 AFTER NICOTINE TREATMENT

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ABSTRACT:

Cytochrome P450 2E1 (CYP2E1), the primary ethanol-metabolizing cytochrome P450, metabolizes endogenous substrates (e.g., arachidonic acid) and drugs (e.g., acetaminophen, chlorzoxazone) and bioactivates procarcinogens (e.g., tobacco-specific nitrosamines) and toxins (e.g., carbon tetrachloride). Nicotine from tobacco smoke may contribute to the enhanced hepatic CYP2E1 activity in smokers. We have previously shown that chronic nicotine treatment can increase CYP2E1 in rat liver and brain. In this study, induction of brain CYP2E1 was assessed after a single acute or a 7-day chronic treatment with saline or nicotine (1 mg/kg s.c.), with sacrifice performed at various times after the last injection. Chronic 7-day nicotine treatment showed the highest levels of CYP2E1 12 h after the last injection in frontal cortex (1.4-fold, \( p < 0.05 \)) versus 8 h in hippocampus (1.8-fold, \( p < 0.01 \)) and cerebellum (1.4-fold, \( p < 0.05 \)), returning to basal levels by 24 h. In contrast, acute nicotine treatment did not induce CYP2E1 in frontal cortex and hippocampus but increased CYP2E1 in cerebellum 8 h after treatment (1.6-fold, \( p < 0.01 \)). Brain CYP2E1 mRNA levels did not increase after chronic nicotine treatment, suggesting nontranscriptional regulation. Thus, humans exposed to nicotine may have altered CYP2E1-mediated metabolism of centrally acting drugs and toxins as well as altered toxicity because of oxidative stress caused by CYP2E1. Those affected may include current and passive smokers and people that may be treated with nicotine such as smokers and, potentially, patients with Alzheimer’s, Parkinson’s disease, or ulcerative colitis.

Cytochrome P450 2E1 (CYP2E1) is expressed in rat liver as well as in various rat brain regions (Upadhya et al., 2000; Howard et al., 2003). Besides a role in the inactivation of alcohol, CYP2E1 can metabolize drugs such as acetaminophen, chlorzoxazone, and halothane and endogenous substrates such as arachidonic acid, gluconeogenic precursors, and estrogenic metabolites (Lieber, 1999; Ohe et al., 2000). CYP2E1 bioactivates several procarcinogens (e.g., tobacco-derived nitrosamines and benzene) and cytotoxins (e.g., carbon tetrachloride) to their reactive intermediates (Lieber, 1999). CYP2E1 can also generate reactive oxygen species and substrate-derived radicals, especially when induced; these can mediate lipid peroxidation, protein inactivation, and DNA damage, leading to cellular injury (Cederbaum et al., 2003). Elevated levels of CYP2E1 in the central nervous system could thus lead to increased risk of neurotoxicity and influence the cerebral disposition and pharmacodynamic effects of centrally acting CYP2E1 substrate drugs.

Cigarette smoking accelerates chlorzoxazone metabolism in humans, most likely by induction of hepatic CYP2E1 activity (Benowitz et al., 2003). Nicotine, present in tobacco smoke, is widely used in replacement therapy for smoking cessation (Henningfield et al., 2005). Nicotine, present in tobacco smoke, is widely used in replacement therapy for smoking cessation (Henningfield et al., 2005).

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ABBREVIATIONS: P450, cytochrome P450; HNF-1, hepatic nuclear factor 1; ANOVA, analysis of variance.
Materials and Methods

Materials. Nicotine bitartrate and cotinine were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). The protein assay dye reagent concentrate, Bio-dot microfiltration apparatus, and nylon membrane were obtained from Bio-Rad (Hercules, CA). TRIZol was from Invitrogen Canada Inc. (Burlington, ON, Canada), and the NEasy kit was from Qiagen Inc. (Mississauga, ON, Canada). Prestained molecular weight protein markers were purchased from MBI Ferments (Flamborough, ON, Canada). Lymphoblastoid cDNA-expressed rat CYP2E1 and polyclonal NADPH-P450 reductase antibody were obtained from BD Biosciences (Mississauga, ON, Canada). The polyclonal rabbit anti-rat CYP2E1 antibody was generously provided by Dr. Magnus Ingelman-Sundberg (Department of Biosciences, Karolinska Institute, Stockholm, Sweden). The monoclonal Na,K-ATPase, β1 subunit antibody was from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase-conjugated goat anti-rabbit antibody was from Chemicon International Inc. (Temecula, CA). SuperSignal West Pico and Femto chemiluminescence substrate and peroxidase-conjugated goat anti-rabbit antibody were from Pierce Chemical (Rockford, IL). Peroxidase-conjugated anti-goat antibody was from Roche Diagnostics (LaVal, QC, Canada). BioTrace NT nitrocellulose membrane was from Pall Corporation (Pensacola, FL), and autoradiography film was purchased from Ultidyn Scientific (St. Laurent, PQ, Canada). All other reagents were obtained from standard commercial sources.

Animals. Adult male Wistar rats (250–300 g; Charles River, St-Constant, PQ, Canada) were housed two per cage and allowed to adapt to the novel controlled environment with a 12-h artificial light/dark cycle (light on at 6:00 AM) for 1 week. 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 University of Toronto.

Drug Treatment and Time-Course Study. Rats were injected subcutaneously with 1 mg of nicotine base per kilogram body weight, in the form of nicotine bitartrate in sterile saline, pH 7.4 (Miciu et al., 2003). Control rats were injected with saline alone. For the chronic recovery time course, rats (n = 3/group) were treated with saline or nicotine once a day for 7 days and sacrificed at 0.5, 2, 4, 8, 12, 18, and 24 h after the last drug treatment. For the acute study, rats (n = 4/group) received a single injection of saline or nicotine and were sacrificed 4, 8, or 12 h after treatment. After sacrifice by decapitation, brains were rapidly removed, dissected within 5 min, frozen immediately in liquid nitrogen, and stored at −80°C. There were no significant differences in body weights of rats administered nicotine either acutely or chronically compared with rats in the respective saline control groups.

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 stored at −20°C. Nicotine and cotinine plasma concentrations were measured by standard high-performance liquid chromatography technique using 5-methylcotinine as the internal standard (Xu et al., 2002).

Membrane Preparation for Western Blotting. Whole brain membranes were prepared because it has been shown that brain P450s are present in microsomal and mitochondrial membranes (Miksys et al., 2000). Briefly, brain regions were homogenized in 100 mM Tris (pH 7.4) with 0.1 mM EDTA, 0.32 M sucrose, and 0.1 mM dithiothreitol on ice. Homogenates were centrifuged twice at 3000g for 5 min to remove cellular and nuclear debris, and then the supernatant was centrifuged at 110,000g for 4°C for 60 min. The resulting membrane pellets were resuspended in 100 mM Tris (pH 7.4), 0.1 mM EDTA, 1.15% (w/v) KCl, and 20% (v/v) glycerol and stored in aliquots at −80°C until used. Microsomes from rat liver were prepared as described previously (Howard et al., 2001). The protein content of each sample was assayed using a Bio-Rad protein assay kit.

Western Blotting. Membrane proteins were separated by SDS-polyacrylamide gel electrophoresis (4% stacking and 10% separating gels), transferred overnight onto nitrocellulose membrane, blocked with 3% skim milk in Tris-buffered saline containing 0.1% BSA and 0.05% Triton X-100, and probed with polyclonal rabbit anti-rat CYP2E1 antibody diluted 1:1500. Blots were then incubated with peroxidase-conjugated anti-rabbit antibody (1:4000) and developed using chemiluminescent detection and autoradiography film. The antibody used is specific for CYP2E1, and cross-reactivity was not observed with other cDNA-expressed rat P450s (Howard et al., 2001). Untreated rat membranes from each brain region tested were serially diluted and used to construct standard curves to determine the linear range of detection for the immunoblotting assay. Microsomes from rat liver and cDNA-expressed rat CYP2E1 were used as positive controls. Equal amounts of membrane protein from saline- and nicotine-treated animals were loaded, and equivalence was confirmed by Comassie Blue gel staining. Poncet S staining of the transferred protein on the membrane, and reprobing membranes with NADPH-P450 reductase antibody and Na,K-ATPase antibody. After confirmation that each of these steps provided similar results, equal loading was used in subsequent experiments. Digital images of immunoblots were analyzed using MCID Elite software (Imaging Research Inc., St. Catherine’s, ON, Canada), and the relative density of each band was expressed as arbitrary density units after correcting for slot background.

RNA Isolation and Slot Blot Hybridization. Total RNA from rat brain samples was isolated using NEasy kit, and its quality assessed by electrophoresis in 1.2% agarose gel. Serially diluted CYP2E1 cDNA was used as a positive control to detect linearity of the assay. Brain RNA (5 μg) was applied directly to nylon membranes using a Bio-Dot microfiltration apparatus, and hybridization was carried out according to manufacturer’s instructions (Bio-Rad). Radiolabeled oligonucleotide probes specific for rat CYP2E1 and β-actin were made as described previously (Howard et al., 2001). Autoradiography films were analyzed using MCID Elite software.

Data Analyses. CYP2E1 protein levels were expressed as density units (mean ± S.D.), average values obtained from n animals per treatment group, from at least three separate experiments. Relative CYP2E1 levels were calculated as a ratio of CYP2E1 in nicotine-treated animals to levels in saline-treated animals. For the recovery time course, an average saline value from all time points was used to determine the CYP2E1 ratio because no significant differences between saline CYP2E1 levels were observed. For mRNA, CYP2E1 levels were an average of two experiments and were expressed relative to β-actin mRNA to control for loading efficiency and quality of the RNA. The differences between brain regions, post-treatment time points, and time of the day, within treatment groups, were tested by one-way analysis of variance (ANOVA or Kruskal-Wallis) followed by a post hoc test (Duncan’s new multiple range test). The differences between treatment groups were tested using unpaired Student’s t tests.

Results

Detection of CYP2E1 in Rat Brain Regions. A quantitative Western blot assay was developed to measure CYP2E1 in rat brain, where basal expression is low. Detection of CYP2E1 in serially diluted brain membrane protein from saline-treated animals was linear, and 30 μg of protein was used for all subsequent experiments (Fig. 1). Brain CYP2E1 protein migrated slightly faster at higher protein concentrations. cDNA-expressed CYP2E1 or rat liver microsomes was therefore added to confirm conjugation and detection of brain CYP2E1 and to ascertain the linearity of the assay at potentially higher levels of CYP2E1 in nicotine-treated rat brain (Fig. 1).

Induction of CYP2E1 in Rat Brain Regions by Chronic Nicotine Treatment. To determine whether the induction of CYP2E1 in rat brain regions was seen at a later time point than previously investigated (Howard et al., 2003), we examined CYP2E1 levels 8 h...
after the last nicotine injection. The amount of CYP2E1 from saline- and nicotine-treated animals varied among brain regions (ANOVA, \( p < 0.01 \) for both treatments). Levels of CYP2E1 in frontal cortex, hippocampus, and cerebellum after chronic nicotine treatment, compared with the corresponding saline controls, showed an increase in the frontal cortex (1.4-fold, \( p < 0.01 \)), hippocampus (1.5-fold, \( p < 0.01 \)), and cerebellum (1.4-fold, \( p < 0.05 \); Fig. 2). This prompted us to examine a wider recovery time course (30 min–24 h) in each brain region.

**Recovery Time Course of CYP2E1 in Rat Brain.** The recovery time course of CYP2E1 showed distinct patterns among the brain regions (Fig. 3), with highest levels of CYP2E1 occurring at 12 h after the last drug treatment in frontal cortex and at 8 h post-treatment in hippocampus and cerebellum relative to the saline-treated group. In the frontal cortex, ANOVA was significant (\( p < 0.05 \)), and post hoc analysis indicated that CYP2E1 levels at 12 h were significantly different from those at 2, 4, 18, and 24 h. In addition, significant increases in CYP2E1 levels of 1.4-fold were seen at 4 h (\( p < 0.05 \)), 8 h (\( p < 0.01 \)), and 12 h (\( p = 0.01 \)) post-treatment compared with their respective saline controls. In the hippocampus (\( p = 0.05 \)), post hoc analysis indicated that CYP2E1 levels at 8 h were different from levels at 0.5, 2, and 24 h. Compared with its respective saline controls at 8 h, CYP2E1 levels increased 1.8-fold (\( p < 0.01 \)) in nicotine-treated animals. In the cerebellum, CYP2E1 levels increased 1.4-fold (\( p < 0.05 \)) in nicotine-treated animals compared with its respective saline controls at 8 h and trended toward an increase of 1.4-fold (\( p = 0.07 \)) at 12 h. In all three regions, levels returned to those in salinetreated animals by 24 h.

**CYP2E1 mRNA in Rat Brain after Chronic Nicotine Treatment.** We examined whether levels of mRNA were increased in brain tissues of chronically treated rats. Detection of CYP2E1 cDNA (Fig. 4A) was linear, as was detection of CYP2E1 and \( \beta \)-actin in a serial dilution of rat liver RNA. A reliable CYP2E1 signal was detected from 5 \( \mu \)g of brain RNA from chronic saline- and nicotine-treated animals, which was within the linear range of the assay. The \( \beta \)-actin RNA levels did not differ (\( p = 0.4 \)) between treatment groups within each brain region. Levels of CYP2E1 mRNA, normalized to \( \beta \)-actin mRNA, were not increased after chronic nicotine treatment at any time tested and were significantly lower than saline levels in hippocampus (at 4 and 8 h, \( p < 0.01 \)) and in cerebellum (at 4 h, \( p < 0.05 \); Fig. 4B, Table 1).

CYP2E1 mRNA levels from saline-treated animals were different at the post-treatment sacrifice time points in cerebellum (ANOVA, \( p < 0.01 \)) and hippocampus (ANOVA, \( p < 0.05 \)). CYP2E1 levels varied with the time of the day in frontal cortex (ANOVA, \( p < 0.05 \)) and cerebellum (ANOVA, \( p < 0.01 \)), and higher levels were seen at 12 h post-treatment sacrifice time, corresponding to 6 PM in both regions (Table 1). CYP2E1 mRNA levels did not correlate with CYP2E1 protein levels in any brain region for either treatment group (0.2 < \( p < 0.9 \)).

**Induction of CYP2E1 in Rat Brain by Acute Nicotine Treatment.** To determine whether induction of brain CYP2E1 occurs after...
acute nicotine treatment, we examined the effect of a single dose of saline or nicotine on CYP2E1 levels in rat brain sacrificed 4, 8, or 12 h after treatment. CYP2E1 was induced significantly in cerebellum at 8 h (1.6-fold, \( p < 0.01 \)), and CYP2E1 levels were 1.2-fold (\( p = 0.37 \) and 1.4-fold (\( p = 0.15 \)) above saline levels at 4 and 12 h, respectively (Fig. 5). In the hippocampus, CYP2E1 levels in nicotine-treated rats were 1.2-fold (\( p = 0.54 \)) at 8 h. In addition, no significant induction was observed in the frontal cortex. Plasma nicotine and cotinine levels were 12 and 230 ng/ml at 4 h (Micu et al., 2003). Plasma nicotine levels were undetectable at 8 and 12 h, and cotinine levels were 190 ± 40 and 95 ± 15 ng/ml at 8 and 12 h, respectively.

**Discussion**

In the current study, we have characterized the recovery time course of induction of brain CYP2E1 after chronic nicotine treatment, examined CYP2E1 mRNA levels, and investigated the effect of acute nicotine treatment. We have shown that there was variation in the amount of CYP2E1 among brain regions in both saline- and nicotine-treated rats 8 h after treatment. Chronic nicotine treatment produced a 1.4- to 1.5-fold increase in CYP2E1 protein levels within 12 h, with a return to the control saline levels by 24 h in all three brain regions studied. The results presented here support a short-lived induction of CYP2E1 in the brain like that in the liver (Micu et al., 2003). However, CYP2E1 levels in the brain peaked later than the 4-h peak seen in the liver (Micu et al., 2003).

Region-specific and time-dependent changes in transcriptional response of various neural genes have been shown in rat brain after nicotine exposure (Konu et al., 2001; Li et al., 2004). We have shown that CYP2E1 mRNA levels were unchanged or decreased in frontal cortex, hippocampus, and cerebellum of nicotine-treated rats compared with saline controls. In rat liver, CYP2E1 mRNA levels were also not increased in response to nicotine treatment, whereas protein levels were (Howard et al., 2001). Despite increases in CYP2E1 protein, decreases in hepatic CYP2E1 RNA levels have been shown in mice exposed to chronic acetone treatment (Forkert et al., 1994), in
ruth treated with a single dose of pyridine (Kim and Novak, 1990), and in rabbits after imidazole and ethanol treatment (Porter et al., 1989). However, increases in hepatic CYP2E1 mRNA have been reported in hamsters after ethanol and pyrazole treatment and in rats after ethanol treatment (Kubota et al., 1988; Badger et al., 1993). These data indicate that CYP2E1 regulatory mechanisms may vary and can be influenced by species, tissue, and inducing agent as well as its mode of administration (Forkert et al., 1994). Changes were seen in CYP2E1 mRNA levels from saline-treated rats dependent on the post-treatment sacrifice time and time of the day when animals were sacrificed, but the protein levels did not vary correspondingly. Higher mRNA levels were seen at 6 PM, the beginning of the active/dark cycle. Diurnal cycles have been previously reported in P450 expression, for example, CYP7 and CYP2A (Lavery et al., 1999). Mouse hepatic CYP2A4 and CYP2A5 expression exhibits circadian rhythm, with the highest expression levels occurring in the evening, and mRNA levels showed more pronounced increases than protein levels (Lavery et al., 1999; Su and Ding, 2004).

CYP2E1 inducers, including ethanol (Petersen et al., 1982), acetone (Forkert et al., 1994), and pyridine (Kim and Novak, 1990), increase hepatic CYP2E1 protein levels after acute and chronic treatment. However, an acute dose of nicotine failed to alter rat hepatic CYP2E1 levels, whereas chronic nicotine treatment did increase hepatic CYP2E1 (Micz et al., 2003). In rat brain, at 8 h after an acute dose of nicotine, an increase in CYP2E1 protein was seen in cerebellum but not in frontal cortex or in hippocampus. Chronic nicotine did induce CYP2E1 levels in all three brain regions. Differences in acute and chronic nicotine treatment have been previously observed; for example, levels of brain-derived neurotrophic factor mRNA in rat hippocampus decreased after acute treatment but increased after chronic treatment (Kenny et al., 2000). Acute nicotine exposure in rats resulted in nicotine and cotinine plasma levels not different (p > 0.1) from those found after chronic nicotine treatment (Micz et al., 2003), indicating that the treatment regime did not alter nicotine bioavailability in rats. One possible explanation for acute induction in cerebellum but not in the liver may be related to the subcutaneous route of nicotine administration, whereby the brain may be exposed to higher nicotine concentrations than the liver.

Several mechanisms have been described for the regulation of CYP2E1 expression. Protein stabilization and increased translational efficiency have been implicated in the regulation of CYP2E1 protein expression by xenobiotics such as ethanol, acetone, and pyridine (Novak and Woodcroft, 2000). We previously reported that nicotine did not alter levels or activity of cDNA-expressed CYP2E1, suggesting that nicotine does not inhibit the degradation of CYP2E1 and that the induction of CYP2E1 by nicotine is unlikely to occur via stabilization of the enzyme (Micz et al., 2003). We found no increase in mRNA levels in the rat liver (Howard et al., 2001) or brain after nicotine treatment, suggesting nontranscriptional regulation. Increased translation efficiency may be involved in CYP2E1 induction by nicotine. Induction of CYP2E1 by pyridine involving increased protein synthesis in the absence of transcriptional activation has been reported (Kim and Novak, 1990).

Expression of brain P450s is highly localized in specific cells, and induction of these P450s may alter local substrate and metabolite concentrations of drugs (Miksys and Tyndale, 2004). Therefore, although the increase in CYP2E1 levels detected by Western blot was modest, region- and cell-specific CYP2E1 expression and induction (Howard et al., 2003) may lead to a substantial increase in CYP2E1 levels in the microenvironment. CYP2E1 has been suggested to play a role in dopamine regulation (Nissbrandt et al., 2001) and/or neuronal detoxification mechanisms (Vaglini et al., 2001, 2004) and has been detected in the neurons of substantia nigra in rats (Watts et al., 1998).

We have not investigated whether nicotine induces CYP2E1 levels in rat substantia nigra; however, we have seen an increase in CYP2E1 levels after nicotine treatment in substantia nigra in monkeys, as detected by immunohistochemistry (Joshi and Tyndale, 2005). The induction of CYP2E1 may have significant effects on local drug and metabolite concentrations. The different areas of the brain investigated in this study are known to play diverse roles in behavior and may thus be differently affected by increased CYP2E1 levels, metabolism of neuroactive substrates, and/or CYP2E1-mediated oxidative stress.

In conclusion, we have shown that acute and chronic nicotine treatment induced CYP2E1 expression in rat brain in a region-specific manner. The induction of CYP2E1 after chronic treatment was not regulated at the transcriptional level, and the recovery time course varied among brain regions with CYP2E1 levels returning to basal levels by 24 h after treatment. Induction of brain CYP2E1 may result in altered neuroactive substrate metabolism, increased oxidative stress, activation of procarcinogens and toxins, and increased risk of cell and tissue damage. Nicotine may thus increase CYP2E1-related toxicity in the central nervous system and may be of relevance in current smokers, passive smokers, and people treated with nicotine such as smokers and, potentially, patients with Alzheimer’s disease, Parkinson’s disease, or ulcerative colitis.

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References


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