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Journal of Toxicology and Environmental Health, Part A
Current Issues

ISSN: 1528-7394 (Print) 1087-2620 (Online) Journal homepage: http://www.tandfonline.com/loi/uteh20

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To cite this article: S. Lee , A. L. Busby , C. Timchalk & T. S. Poet (2008) Effects of Nicotine Exposure on *In Vitro* Metabolism of Chlorpyrifos in Male Sprague-Dawley Rats, Journal of Toxicology and Environmental Health, Part A, 72:2, 74-82, DOI: 10.1080/15287390802477288

To link to this article: https://doi.org/10.1080/15287390802477288

Journal of Toxicology and Environmental Health, Part A, 72: 74–82, 2009

ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287390802477288

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Effects of Nicotine Exposure on *In Vitro* Metabolism of Chlorpyrifos in Male Sprague-Dawley Rats

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The routine use of tobacco products may modify key metabolizing systems, which will further impact the metabolism of environmental contaminants. The objective of this study was to quantify the effect of repeated *in vivo* exposures to nicotine, a major pharmacologically active component of cigarette smoke, on *in vitro* metabolism of chlorpyrifos (CPF). CPF is an organophosphorus (OP) insecticide that is metabolized by cytochrome P-450 (CYP450) to its major metabolites, chlorpyrifos-oxon (CPF-oxon) and 3,5,6-trichloro-2-pyridinol (TCP). Male Sprague-Dawley rats were dosed subcutaneously with 1 mg nicotine/kg for 1, 7, or 10 d. Rats were sacrificed 4 or 24 h after the last nicotine treatment, and liver microsomes were prepared. The microsomes were incubated with varying concentrations of CPF and the production of the metabolites CPF-oxon and TCP were measured. The metabolism of CPF to the active oxon metabolite did not show significant changes following repeated nicotine treatments, evidenced by the unchanged pseudo first-order clearance rate of V_{max}/K_{mapp}. The V_{max} describing the metabolism of CPF to the inactive metabolite, TCP was increased in 24-h postdosing groups, after both single and repeated treatments of nicotine. In contrast, the metabolism to TCP was unchanged in groups evaluated at 4 h (single or repeated) post nicotine dosing. Some basic marker substrate activities were also investigated to ensure that nicotine exerted effects on CYP450 activities. Total P450 reduced spectra were not altered by nicotine treatment, but marker substrate activities for CYP1A and CYP2E1 were increased at 24 h after the single treatment, and marker substrate activity for CYP2B was decreased 4 h after 7 d of treatment. Results of this *in vitro* study suggest that repeated nicotine exposure may result in altered metabolism of CPF. Future *in vivo* experiments based on these results need to be conducted to ascertain the impact of *in vivo* nicotine exposures on CPF metabolism in rats.

Chlorpyrifos (CPF, *O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate), a broad-spectrum organophosphorus (OP) pesticide, has been of interest and relatively well studied. Although the U.S. Environmental Protection Agency (EPA) has restricted its residential use, it is still widely use in agricultural settings (U.S. EPA 2000, 2002). In this regard, a number of biomonitoring studies have documented both occupational and nonoccupational exposures to CPF in humans (Barr & Angerer, 2006; Fenske et al., 2005; Hardt & Angerer, 2000; Whyatt et al., 2005). CPF is metabolized by CYP450s in the liver to either chlorpyrifos-oxon (CPF-oxon), or other metabolites, including 3,5,6-trichloro-2-pyridinol (TCP) and diethylthiophosphate (DETP) (Choi et al., 2006; Ma & Chambers, 1994). Coupled with the extensive use of pesticides, many agricultural workers who smoke are also exposed to a variety of chemicals from cigarette smoke. Nicotine, a major component of cigarette smoke, was shown to alter cytochromes P-450 (CYP450) protein levels and activities (Micu et al., 2003; Miksys et al., 2000; Yue et al., 2008).

The balance between bioactivation and detoxification is an important determinant of potential toxicity due to CPF. A scheme illustrating the metabolism of CPF, along with key cytochromes P-450 believed to be responsible for the metabolism of CPF in rats and humans, is presented in Figure 1. The acute toxicological effects of CPF exposures are mediated by the CPF-oxon metabolite, primarily due to the inhibition of acetylcholinesterase (AChE), resulting in an excessive acetylcholine (ACh) accumulation both at synapses and neuromuscular junctions. Therefore, any substance that alters CYP450 activities has the potential to alter the *in vivo* inhibition of AChE, which is mediated by CPF-oxon. The goal of our ongoing research program is to develop a quantitative experimental and modeling approach to evaluate the impact that smoking may have on agricultural workers exposed to OP.

Received 20 August 2008; accepted 10 September 2008.

Although the research described in this article has been funded by the Centers for Disease Control and Prevention (CDC) and CDC/NIOSH grant R01-OH003629, it has not been subject to any CDC/NIOSH review and therefore does not necessarily reflect the official views of the agency; its contents are solely the responsibility of the authors and no public endorsement should be inferred.

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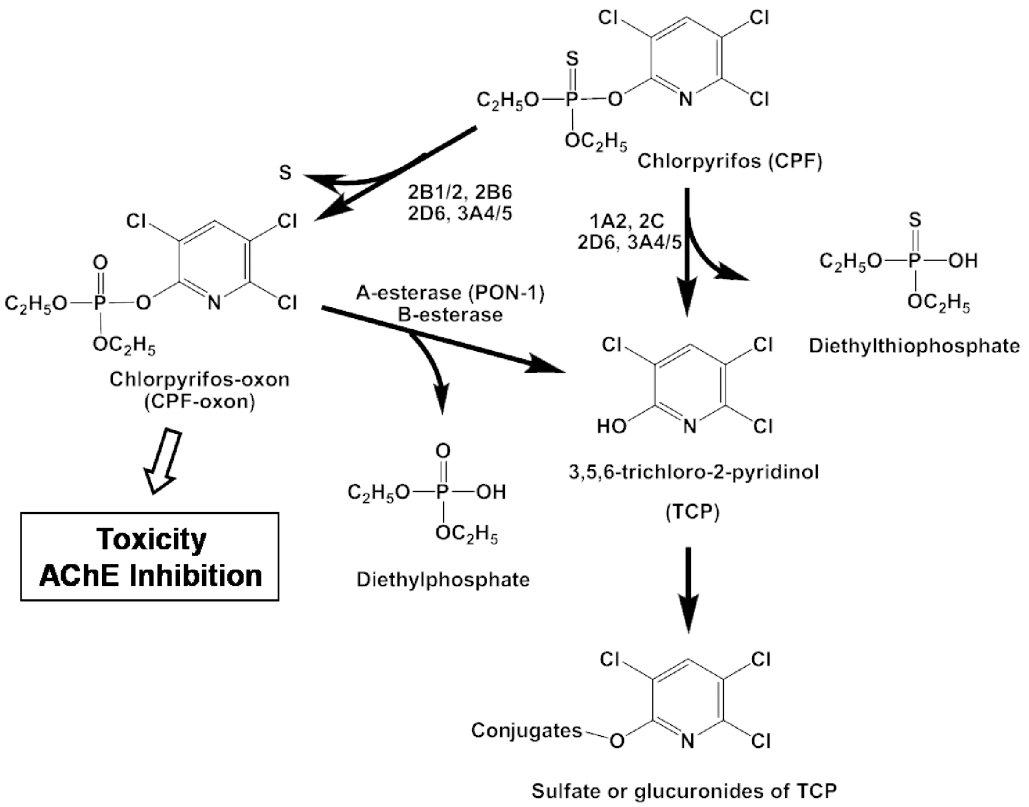

FIG. 1. Metabolic scheme of chlorpyrifos (CPF) and its major metabolites, including chlorpyrifos-oxon (CPF-oxon), 3,5,6-trichloro-2-pyridinol (TCP), diethylphosphate and diethylthiophospate, etc. The known CYP450s believed to be responsible for CPF metabolism are noted.

Nicotine, 3-(1-methyl-2-pyrrolidinyl)-pyridine, is an active constituent of tobacco smoke that has been associated with a variety of chronic adverse health effects (U.S. DHHS, 2004). Nicotine acts as a direct cholinergic agonist at the nicotinic acetylcholine receptor (nAChR) site, which may be another pharmacodynamic target for OP, including CPF (Betancourt & Carr, 2004; Slotkin, 2004; Smulders et al., 2004). Several CYP450 were identified that mediate the metabolism of nicotine to its major metabolite, cotinine (Hukkanen et al., 2005; Nakajima et al., 1996; Yamazaki et al., 1999), some of which (e.g., 2B6 in humans) also participate in the metabolism of OP, including CPF.

Studies demonstrated that nicotine induces several CYP450 *in vivo* and *in vitro* (Schoedel & Tyndale, 2003). Notably, chronic nicotine treatment induced CYP2E1 protein and activity in rat liver. Micu et al. (2003) showed that the induction of CYP2E1 protein levels peaks at 4 h post nicotine treatment (1 mg/kg sc, × 7 d), with 1.3- to 1.6-fold increases in CYP2E1 protein levels, but no change in 2E1 mRNA levels. Furthermore, Howard et al. (2001) also reported that nicotine increases CYP2E1 protein even at the low dose of 0.01 mg/kg, and cotinine does not induce CYP2E1.

Anandatheerthavarada et al. (1993a) reported that nicotine treatment resulted in increased pentoxyresorufin *O*-dealkylase (PROD) and benzyloxyresorufin *O*-dealkylase (BROD) activities in specific regions of rat brain, suggesting induction of CYP2B, which is likely to be involved with CPF metabolism (Mutch and Williams 2006). Likewise, Miksys et al. (2000) also reported that chronic nicotine treatment induces CYP2B1 protein in rat brain, but not in liver. Furthermore, smokers were found to have higher brain CYP2B6 levels than nonsmokers (Miksys et al., 2003; Miksys & Tyndale, 2006).

The objective of this study was to evaluate the *in vivo* effects of repeated nicotine exposure on the *in vitro* CPF metabolism in Sprague Dawley rats. The rationale for this approach was based on the known high exposures associated with routine use of tobacco products and the potential capability of nicotine to affect CYP450 activity levels, and thus impacting the pharmacokinetics and pharmacodynamics of CPF. The pharmacokinetics of both nicotine and CPF are well established (Hukkanen et al., 2005; Knaak et al., 2004; Timchalk et al., 2002; Tricker 2003), and the respective pharmacodynamic effects of nicotine and OP have been extensively studied (Balfour, 1982; Clegg & van Gemert, 1999a, 1999b; Slotkin, 2004; Slotkin et al., 2006; Smulders et al., 2004; Timchalk et al., 2002). A more limited number of studies showing the adverse effects of coexposures to chlorpyrifos and nicotine in combination were reported (Abou-Donia et al., 2003, 2006; Qiao et al., 2005). The metabolic effects suggest that chronic nicotine administration at pharmacologically active

doses (≥1 mg/kg sc) may influence key enzymatic processes, modifying dosimetry and biological response following CPF exposures. The current study was therefore conducted to assess the potential changes in CPF metabolism *in vitro* after repeated nicotine treatments *in vivo*. The kinetic parameters from *in vitro* CPF metabolism were then estimated through monitoring the generation of CPF-oxon and TCP. These results along with the future *in vivo* studies will be integrated into the existing physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) models for CPF, which will then be used to quantitatively assess the health implications of preexposures to nicotine on CPF dosimetry.

MATERIALS AND METHODS

Chemicals

Chlorpyrifos (CAS: 2921-88-2, 99% pure) and TCP (CAS: 6515-38-4, 99% pure) were kindly provided by Dow AgroSciences (Indianapolis, IN); CPF-oxon (98% pure) was purchased from Chem Service, Inc. (West Chester, PA) and stored at –80°C until used. The derivatizing agent, *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA), along with other chemicals, including nicotine bitartrate, and solvents were purchased from Sigma-Aldrich (St. Louis, MO), and were reagent grade or better.

Animals

Adult male Sprague-Dawley rats (250–300 g, approximately 8 wk old) were purchased from Charles River Laboratories, Inc. (Raleigh, NC). All procedures described in the present study were conducted in accordance with the guidelines for the care and use of laboratory animals in the NIH/NRC Guide and Use of Laboratory Animals, and were approved by the Institutional Animal and Care Use Committee (IACUC) of Battelle, Pacific Northwest Division. Prior to use, animals were housed in solid-bottom cages with hardwood chips, and acclimated for 1 wk in a humidity- and temperature-controlled room with a 12-h light/dark cycle. Rodent feed (PMI Certified Rodent Diet 5002) and water were provided ad libitum.

Nicotine Treatment

Animals were randomly assigned to one of six nicotine treatments and posttreatment time points for sacrifice (saline control and groups I–V, $n = 4$ per group, Table 1). Rats were dosed subcutaneously (sc) at the nape with 1 mg of nicotine base per kg body weight, as nicotine bitartrate in sterile saline in a dose volume of 1 ml/kg body weight. The animals of groups III, IV, and V received nicotine treatment once a day for 7 or 10 d. The dose of nicotine was selected based upon previous studies (Abou-Donia et al., 2003; Howard et al., 2001; Micu et al., 2003).

TABLE 1
Different Nicotine Treatment Groups

Groups	Nicotine administration	Sacrifice time after the last administration
Saline control	None	
Group I	Single	4 h
Group II	Single	24 h
Group III	Repeat 7 d	4 h
Group IV	Repeat 7 d	24 h
Group V	Repeat 10 d	24 h

Microsomes

Rats were sacrificed by CO_2 asphyxiation at the specified postdosing time points (Table 1). While the animals of group I and III were sacrificed 4 h after the last dosing, other nicotine-treated animals were sacrificed 24 h after the last treatment. Blood exsanguinations were achieved via cardiac puncture from CO_2 anesthetized rats into heparinized syringes, and the liver was excised quickly from the animals and perfused with ice-cold 1.15% KCl. Hepatic microsomes from each animal were prepared by centrifugation at 9500 × g for 30 min and then supernatants were ultracentrifuged twice at 105,000 × g for 60 min (Guengerich, 1994). Microsomes were stored at –80°C until analysis.

CPF Incubation Assay

Different concentrations (5, 30 75, 150, or 500 μ*M*) of CPF (serially diluted in methanol and distilled water) were incubated in a total volume of 0.5 ml containing 50 m*M* HEPES buffer, 15 m*M* $MgCl_2$, and 1 m*M* ethylenediamine tetraacetic acid (EDTA; pH 7.4) with 1 mg hepatic microsomal protein. The methanol did not exceed 1% of the final incubation volume. The lowest and the highest CPF incubation concentrations were chosen to optimize the ability to quantify all the metabolites and achieve saturation of metabolism. Metabolism blanks, which were added with 250 μl NaCl-saturated 2.5 *N* acetic acid, were run for comparison for nonspecific breakdown of CPF, TCP, and/or CPF-oxon. For quantitation, the matrices for standard curves were prepared using microsomes from naive rats by spiking with a series of concentrations of CPF-oxon and TCP, and analyzed alongside each set of samples. Our previous studies verified that 1 m*M* EDTA was sufficient to block any PON1-mediated metabolism of CPF-oxon, and the metabolism blanks were identical, regardless whether the acid was added first or if NADPH was not added (Poet et al., 2003). The samples were preincubated at 37°C for 2 min and 1 m*M* NADPH was added to initiate the reaction, which was terminated after 10 min by the addition of 250 μl NaCl-saturated 2.5 *N* acetic acid. The resulting solutions were extracted (3×) with 0.75 ml methanol:hexane (1:6 v/v) mixture,

vortexed in a shaking incubator for 10 min, and centrifuged to separate layers at 1100 × g for 20 min. The three subsequent organic layers were combined, and the solvent was evaporated under a gentle stream of N_2. The residues were reconstituted in 0.15 ml toluene, followed by 1 min of mixing by vortex. Half of the reconstituted solution was transferred for the analysis of TCP; 15 µl MTBSTFA was added for the derivatization of TCP to its silylated form, then heated at 60°C for 1 h (Brzak et al., 1998). The remaining solution was transferred to the other set of glass gas chromatography (GC) vials with glass inserts for CPF-oxon analysis (see next subsection).

GC/MS Analysis

Gas chromatography/mass spectrometry (GC/MS) analyses were performed using an Agilent 5975B Inert XL EI/CI mass selective detector (MSD), interfaced with an 7683B injector, G2614A autosampler, and Agilent 6890N GC equipped with ChemStation software for programming and data analysis (Agilent Technologies, Inc., Santa Clara, CA). Spectra were collected in the electron ionization mode at 70 eV with a mass range scanned from 50 to 500 amu. Separation was achieved in splitless mode using Restek RTX 1701 column (30 m × 0.25 mm ID × 1 µm df, Restek Co., Bellefonte, PA). Helium carrier gas was used at a flow rate of 1 ml/min with a head pressure of 2.5 psi. The GC oven temperature program included an initial hold at 80°C for 1 min followed by a 30°C/min ramp to 265°C, then was held for 5 min. The temperatures of the initial inlet and the detector were 250°C. The temperatures of the quad and source of MSD were kept at 150 and 230°C, respectively. The retention times for derivatized TCP, CPF, and CPF-oxon were approximately 4.9, 6.3, and 6.8 min, respectively, with a total run time of 15 min. While the scan mode with a mass range from 50 to 500 amu at 1 µl injection volume was used for the analysis of CPF and TCP, the selected ion monitoring (SIM) mode was utilized for analysis of CPF-oxon with an injection volume of 2 µl, where increased sensitivity was required. Ions selected for monitoring CPF-oxon included m/z = 298 and 270 with dwell time of 100 ms (Brzak et al., 1998).

CYP450 Assays in Nicotine-Treated Rats

Protein concentrations were determined using the bicinchoninic acid method using BCA reagent (Pierce, Rockford, IL) with bovine serum albumin (BSA) as the standard. Total microsomal CYP450 content was determined from reduced CO difference spectra (Omura & Sato, 1964). To verify that nicotine was affecting general CYP450 activities, some basic marker substrate activities were determined. The activity of 4-nitrophenol (PNP) hydroxylase in hepatic microsomes was determined by the modified method of Reinke and Moyer (1985), and was normalized using CYP450 values in order to represent in terms of 4-nitrocatechol (4NC) formed/mg protein. The activities of ethoxyresorufin *O*-dealkylase (EROD) or PROD were assayed fluorometrically using the modified method described by Pohl and Fouts (1980). Preliminary studies to confirm linearity with respect to microsomal protein concentration and time were performed (data not shown). Each assay was linear within the duration (2 min) of the assay and at the microsomal protein concentrations used. Specific activities of EROD and PROD from each animal were calculated as nanomoles of product produced per milligram protein per minute.

Data Analysis

All incubations were conducted with hepatic microsomal samples prepared from individual animals, and means and standard deviations were calculated. The *in vitro* metabolic rate constants, V_{max} and K_{mapp} (apparent affinity constants), for CPF metabolite formation were calculated by fitting the data to the Michaelis–Menten equation with least-squares fit method using Prism 5 (GraphPad Software, Inc., San Diego, CA). The statistical differences in CYP450 marker substrate activities along with V_{max} and K_{mapp} of CPF incubation studies were tested by one-way analysis of variance (ANOVA) using Prism 5, followed by post hoc Dunnett's multiple test for the comparisons between saline controls and nicotine treatment groups, and Bonferroni's tests for comparisons among the different treatment groups; p values less than .05 were considered to be significant.

RESULTS

General Observations

Initially, the administration (d 1) of nicotine in saline resulted in cholinergic effects manifested by salivation and tremors. After repeated nicotine dosing (by d 4), however, animals adapted, showing only transient or negligible cholinergic effects.

CYP450-Mediated *In Vitro* Metabolism of Chlorpyrifos

The kinetic parameters from the *in vitro* CYP450 metabolism of CPF to CPF-oxon and/or TCP across the different nicotine-treatment groups are summarized in Table 2. For the saline-treated control group, the kinetic parameters for CPF metabolism are generally comparable to what has been reported previously (Ma & Chambers, 1994; Poet et al., 2003). As expected, V_{max} of metabolism of CPF to CPF-oxon production was substantially lower (approximately 8-fold) than for CPF metabolism to TCP (Table 2).

Nicotine preexposure resulted in modifications to CPF metabolism, and the observed impacts on metabolic rates were clearly different for the formation of TCP and CPF-oxon. In addition, the timing of analysis (4 versus 24 h post dosing) also resulted in differences in CPF metabolic activity. The metabolism of CPF to TCP in microsomes prepared from animals exposed to nicotine as either a single or repeated (7-d) dose and

TABLE 2
Comparison of the Kinetic Parameters for CYP450 Metabolism of CPF in Hepatic Microsomes After Nicotine Treatments

	TCP		CPF-oxon	
Groups	V_{max}	K_{mapp}	V_{max}	K_{mapp}
Saline control	2.04 ± 0.106	110 ± 29.9	0.271 ± 0.049	80.1 ± 28.6
Group I	1.96 ± 0.322	114 ± 32.4	0.158‡ ± 0.0011	77.5 ± 2.08
Group II	3.09* ± 0.542	96.0 ± 12.6	0.238 ± 0.070	142 ± 123
Group III	1.88 ± 0.642	81.1 ± 18.1	0.311 ± 0.069	158 ± 24.3
Group IV	3.13* ± 0.698	187*,† ± 51.9	0.342 ± 0.079	114 ± 25.6
Group V	3.75* ± 0.252	124 ± 10.1	0.354 ± 0.054	235±154.0

Note. Male Sprague-Dawley (S-D) rats were treated with saline or 1 mg nicotine/kg sc once per day. They then were sacrificed either at 4 or 24 h post last dosing on d 1, 7, or 10 (Table 1). Values (± SD) for $n = 4$ determinations are expressed as V_{max} (nmol/min/mg), or K_{mapp} (μ*M*). Asterisk indicates statistically significant differences between the saline-treated controls and the nicotine-treated groups (one-way ANOVA followed by Dunnett's post test, $p < .05$); † in group IV indicates statistical differences when compared with saline control, groups I, II, and III (one-way ANOVA followed by Bonferroni's post test, $p < .05$); ‡ in group I indicates statistical differences when compared with repeated nicotine treatment groups (groups III, IV, and V) (one-way ANOVA followed by Bonferroni's post test, $p < .05$).

evaluated at 4 h post dosing (compare groups I & III vs. controls) showed no apparent changes in TCP V_{max} (Figure 2a). In microsomes prepared from rats 24 h after nicotine treatment, however, TCP V_{max} consistently demonstrated a significant increase (>1.5-fold) relative to controls (Figure 2b and Table 2), and this was apparent after the single dose (group II) as well as after multiple daily dosing (groups IV and V). A comparison of the plot of CPF concentration versus TCP formation rates for all the treatment groups is particularly striking (see Figure 2b), and is highly suggestive that nicotine preexposure increases the rate of CPF metabolism to TCP in hepatic microsomes. Only the average K_{mapp} of TCP determined for the 7-d repeated nicotine dose (group IV) was determined to be statistically different than saline controls, but the variability was substantial (±28%). Hence this suggests that there are no consistent changes in TCP K_{mapp} that are associated with nicotine pretreatment.

A similar kinetic analysis was conducted to evaluate the impact of nicotine pretreatment on *in vitro* CPF metabolism to CPF-oxon (Figure 3 and Table 2). For the comparisons of V_{max} and K_{mapp} of CPF to CPF-oxon, there was no statistically significant difference between saline control and nicotine-treated groups. There were, however, some observable trends between treatment groups. For example, following the single dose nicotine exposures (groups I and II), the average V_{max} was decreased (relative to controls) by 42 and 12%, respectively. The decreased V_{max} of group I (at 4 h post dosing) was different from all the repeated nicotine exposure groups (groups III, IV, and V). In general, there were no statistically identified differences among K_{mapp} values of CPF-oxon from the different treatment groups and there was substantial variability in the values of CPF-oxon K_{mapp} within some treatment groups (Table 2).

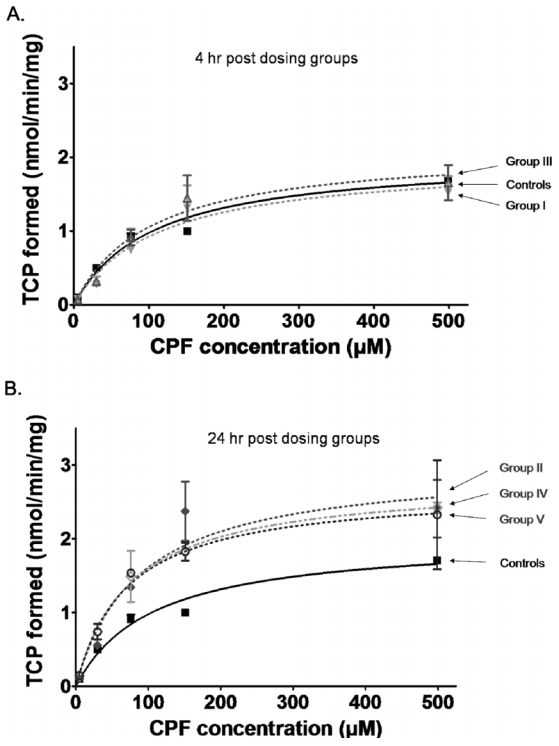

FIG. 2. The CYP-mediated *in vitro* metabolism of CPF to TCP after different nicotine treatments in hepatic microsomes of male Sprague-Dawley rats [controls: filled squares; group I: filled circles; group II: filled diamonds; group III: open triangles; group IV: open diamonds; and group V: open circles], comparing (a) 4-h post dosing groups with controls, and (b) 24-h post dosing groups with controls. Values represent means (± SD) of TCP formed (nmol/min/mg protein) per substrate concentration for $n = 4$ rats. Solid (controls) and dashed lines (nicotine-treated) denote nonlinear fits of TCP data with Michaelis–Menten kinetics for each group.

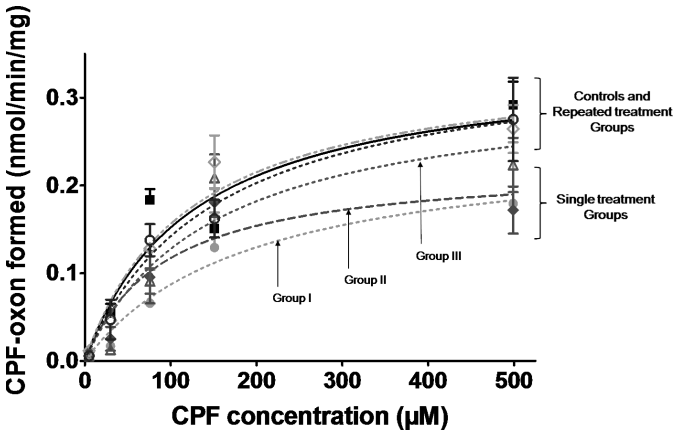

FIG. 3. The CYP-mediated *in vitro* metabolism of CPF to CPF-oxon in hepatic microsomes of male Sprague-Dawley rats after different nicotine treatments (controls: filled squares; group I: filled circles; group II: filled diamonds; group III: open triangles; group IV: open diamonds; and group V: open circles). Values represent means (± SD) of CPF-oxon formed (nmol/min/mg protein). Solid (controls) and dashed lines (nicotine-treated) denote nonlinear fits of CPF-oxon data with Michaelis–Menten kinetics for each group.

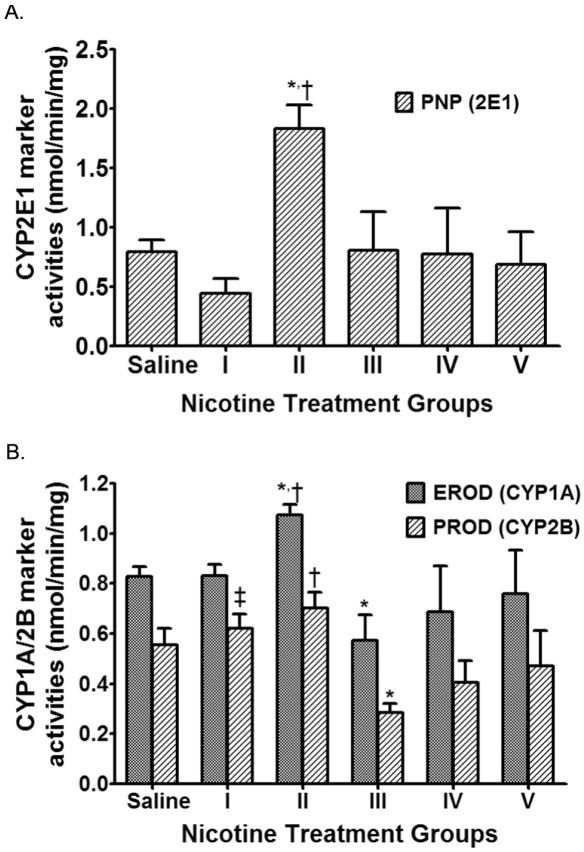

FIG. 4. Comparison of the marker substrate activities of (a) CYP2E1 and (b) CYP1A and CYP2B from hepatic microsomes prepared from the livers of male Sprague-Dawley rats, after different nicotine treatments. Bars represent means (± SD, $n = 4$) and the asterisk indicates a statistically significant difference between saline-treated controls versus nicotine treatment groups (one-way ANOVA followed by Dunnett's test, $p < .05$); † in group II indicates statistical differences when compared with groups III, IV, and V (Bonferroni's test, $p < .05$). In group I, ‡ indicates statistical differences when compared with groups III and IV (Bonferroni's test, $p < .05$).

Activities of CYP450 Marker Substrates

Total CYP450 was measured along with CY1A, CYP2B1/2, and CYP2E1 marker substrate activities to verify that nicotine pretreatment affected key CYP450 in general (Micu et al., 2003). There were no differences in the total hepatic microsomal protein or total CYP450 contents as determined by CO reduced spectra between saline and nicotine pretreatment groups (data not shown). *p*-Nitrophenol (PNP: CYP2E1 marker substrate) hydroxylase activity showed significant (2.3-fold) induction only in group II (24 h after a single nicotine dose), while there were no significant differences between saline control group and any of the other repeated nicotine treatments (Figure 4a). Like PNP, EROD activities (for CYP1A1/2) showed a statistically significant (1.3-fold) increase over that of controls in group II, while group III (4 h post dosing after 7-d repeated dosing) showed a significant decrease by 31%. At the same time, PROD activity (for CYP2B1) in group III also showed a significant decrease by 31%, 4 h post 7 d of dosing (Figure 4b). Overall, EROD and PROD activities from other groups showed no statistically significant differences over controls. However, the differences among nicotine treatment groups were noted; for example, 24-h postdosing groups after repeated nicotine treatments exhibited lower EROD and PROD activities than those groups from the same postdosing time points after the single nicotine treatment (compare groups IV with II, Figure 4b).

Although no nicotine would still be present in the microsomes after preparation, to verify that the decreases in CPF-oxon levels observed were not due to irreversible inhibition of CYP450s by nicotine, a time-dependent co-incubation with nicotine and chlorpyrifos was conducted using the methods outlined by Halpert and Neal (1981). The *in vitro* metabolism of CPF (75 μ*M*) in microsomes preincubated with 300 μ*M* nicotine (for 0, 2, 5, 10, or 20 min) did not show any time-dependent changes in the amount of CPF-oxon and/or TCP produced from CPF, indicating no mechanism-based inhibition was occurring (data not shown).

DISCUSSION

The potential impact of coexposure to chemicals and drugs to which a broad segment of the population may be exposed is of particular importance. A number of studies have investigated the impact of preexposure to chemically unrelated xenobiotics on the pharmacokinetic/ pharmacodynamic responses of OP insecticides (Ball et al., 1954; Carr et al., 2002; Chambers & Chambers, 1990; Chambers et al., 1994; Sultatos & Minor, 1987; Wu et al., 1996). For example, pretreatment with phenobarbital, a potent CYP450 inducer, protects against

the acute toxicity of CPF in mice (Sultatos, 1988). Hence the current study was designed to evaluate the impact of nicotine at a pharmacologically active dose on key metabolic processes associated with the bioactivation or detoxification of CPF. The dose of nicotine used in this study was within the range of, or only slightly higher than, the "mouth" doses estimated in a clinical study that included 74 smokers (~0.5–0.9 mg/kg/d) (St. Charles et al., 2006).

As noted in Figure 1, the metabolism of CPF to its oxon metabolite is likely to be mediated by CYP 3A4/5, 2B6 (human), 2B1/2 (rat), and 2D6 (human), whereas CYP-mediated dearylation resulting in the metabolism of CPF to TCP is likely mediated by CYP2C, 2D6, 3A4/5, and 1A2 (Buratti et al., 2003; Foxenberg et al., 2007; Mutch & Williams, 2006; Sams et al., 2000; Tang et al., 2001). In the present study, the metabolism of CPF to TCP indicated metabolic induction in microsomes prepared 24 h post *in vivo* nicotine exposure. However, metabolic activity at 4 h following a single or repeated (7-d) exposure (groups I and III) did not exhibit any metabolic induction of CPF to TCP metabolism (V_{max} similar to controls; see Figures 2 and 3 and Table 2). Coincidentally, marker substrate activities of CYP450s that were found to be significantly affected (EROD and PNP) were only increased 24 h after the last administration of nicotine and were either unchanged, or even decreased, 4 h after dosing.

Consistent with the marker substrate activities for CYP1A and CYP2B, the effects from either single or repeated nicotine treatments on the metabolism of CPF to CPF-oxon were not apparent, since V_{max} and K_{mapp} of CPF-oxon production after different nicotine treatments remained statistically unchanged, when compared with controls (Table 2). Nonetheless, the estimated K_{mapp} of CPF-oxon showed apparent increase over controls following the repeated nicotine exposures (groups III, IV, and V), which likely led to a quantitative trend of CPF-oxon V_{max} to apparently increase in those groups. Although a limited number of postdosing time points after nicotine treatments were investigated, the decrease of CPF-oxon V_{max} in group I was significantly different from those of repeated nicotine treatment groups. These results suggest that nicotine-mediated effects may be highly temporal in nature, not only on TCP formation but also on the bioactivation pathway to CPF-oxon. The current study does also confirm the time-dependent responses of CYP450 marker substrate and metabolism, observed from the microsomes prepared between 4 and 24 h after the final dose (either single or repeated exposure) of nicotine. Similarly, Micu et al. (2003) demonstrated that after 7 d of 1-mg/kg nicotine treatments, the induction of CYP2E1 protein in liver markedly changed over a 24-h period. CYP2E1 levels peaked at 4 h post nicotine dosing and returned to normal levels by about 24 h; however, mRNA levels of CYP2E1 were not affected by nicotine treatments (Howard et al., 2001).

Similar doses of nicotine were shown to lead to changes in CYP450 protein and activity levels (Anandatheerthavarada et al., 1993b; Howard et al., 2001; Joshi & Tyndale, 2006; Micu et al., 2003). Similar dynamic effects due to nicotine treatment were also found in CYP1A marker substrate activity (Figure 4b). These temporal changes in CYP1A, but not in CYP2B, from liver microsomes due to repeated nicotine treatments are consistent with the previous studies, in which nicotine was shown to induce PROD activity as well as CYP2B mRNA in rat brain, but no increases in either have been observed in rat liver (Anandatheerthavarada et al., 1993a, 1993b; Miksys et al., 2000).

The results from the current study are in line with the previously reported studies (Buratti et al., 2003; Foxenberg et al., 2007; Mutch & Williams, 2006; Sams et al., 2000; Tang et al., 2001). Mutch and Williams (2006) reported that CPF-oxon formation did not strongly correlate with any of the specific individual CYP450 markers that they investigated. Tang et al. (2001), Burrati et al. (2003), and Foxenberg et al. (2007) reported positive correlations between CPF-oxon production and CYP2B6, 2C19 and 3A4. They also showed correlations between CYP3A4/5-, 2C8-, 2C19-, and 1A2-mediated *in vitro* metabolism with TCP formation using human liver microsomes and recombinant human CYP450s.

The major hepatic CYP450 enzymes that metabolize the formation of cotinine from nicotine are CYP2A6 for humans, and CYP2B1 and CYP2C for rats (Hukkanen et al., 2005; Miksys et al., 2000; Nakayama et al., 1993; Schoedel & Tyndale, 2003). Since CPF-oxon metabolism seems not to be altered, CYP 3A4/5 activity is probably not significantly affected by nicotine pretreatment. The effects of nicotine treatments on the changes in the activities of other CYP450 enzymes, including CYP2C, need to be investigated. Since the temporal aspects of nicotine effects seem to have greater impacts on the pharmacodynamics and pharmacokinetics of CPF than the single versus multiple administration, and also it is likely that the metabolism of CPF would be affected differently from the diverse scenarios of nicotine exposures (St. Charles et al., 2006), the investigation of different doses and postdosing time points of nicotine dosing would be interesting.

In summary, the nicotine-treatment regimen employed in this study exhibited much stronger influence on the kinetic parameters of CPF metabolism to TCP than to CPF-oxon. The V_{max} and K_{mapp} of CPF-oxon production remained statistically unchanged, but the increase in TCP V_{max} 24 h after any given nicotine exposure suggest that nicotine exposures may result in dynamic and temporal changes in CPF metabolism. The current study was undertaken to investigate the potential interactions between commonly used substances, to which exposures may potentially be much higher than those exposures to the chemicals with environmentally relevant concentrations. In addition, other important chemical components within tobacco smoke could be also considered, such as polycyclic aromatic hydrocarbons, which certainly influence the metabolic systems of OP. Data demonstrated that a metabolic interaction between a behaviorally relevant dose of nicotine and chlorpyrifos is possible.

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