Rat Brain CYP2B-Enzymatic Activation of Chlorpyrifos to the Oxon Mediates Cholinergic Neurotoxicity

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Chlorpyrifos is a commonly used insecticide that can be metabolically activated by CYP2B to the acetylcholinesterase inhibitor chlorpyrifos-oxon causing cholinergic overstimulation and neurotoxicity. Rat brain extracts can also activate chlorpyrifos in vitro, and the lack of circulating oxon in serum suggests that metabolic activation within the brain may be responsible for chlorpyrifos neurotoxicity. Rats received intracerebroventricular (ICV) injections of CYP2B mechanism-based inhibitors (MBI), once or repeated, followed by chlorpyrifos (62.5–250 mg/kg sc). Rats were assessed for neurochemical (acetylcholinesterase activity), physiological (temperature), and behavioral measures (e.g., gait, righting reflex, arousal, incline angles) at 4 hours 3 days after chlorpyrifos treatment. ICV CYP2B MBIs increased brain chlorpyrifos levels, decreased brain chlorpyrifos-oxon levels, and attenuated the reduction in brain acetylcholinesterase; there was no effect on serum chlorpyrifos levels or acetylcholinesterase activity reduction. Inhibition of brain chlorpyrifos metabolism by CYP2B MBIs blocked centrally mediated hypothermia but not peripherally mediated hyperthermia. A single ICV MBI treatment significantly attenuated neurotoxicity with a gradual worsening of behavioral scores through day 3, suggesting a recovery of brain CYP2B activity and an increase in local chlorpyrifos activation. Daily ICV MBI injections attenuated neurotoxicity across all test days consistent with prolonged inhibition of brain chlorpyrifos activation. Thus, rat brain CYP2B contributes significantly to chlorpyrifos's neurotoxic effects. Variable human brain CYP2B levels, influenced by genetics and environmental exposures, may contribute to interindividual differences in neurotoxicity. Therapeutic inhibition of brain CYP2B could also be explored as a treatment for exposure to CYP2B-activated neurotoxins.

Key Words: chlorpyrifos; CYP2B; neurotoxicity; inhibition; chlorpyrifos; oxon; brain.

Consistent with earlier reports of CP exposure in 96% of the U.S. population (Barr et al., 2005), a more recent study detected CP metabolites in 100% of urines tested from children in Ohio and in over 50% of food and hand wipe samples (Morgan et al., 2011). Chronic exposure to high levels of CP in pesticide applicators leads to cognitive deficits and other neurotoxicities (Steenland et al., 2000), whereas acute high-dose poisoning also results in long-lasting neurological effects (Soummer et al., 2011). CP is desulfurated by cytochromes P-450 (CYPs) to chlorpyrifos-oxon (CPO; O, O-diethyl-O-[3,5,6-trichloro-2-pyridinyl] phosphate) and through dearylation to the non-toxic 3,5,6-trichloro-2-pyridinol (TCP). CYP2B predominantly desulfurates CP to CPO, and CYP2C primarily mediates TCP production; CYP3A produces both metabolites with a lower affinity than CYP2B and CYP2C (Tang et al., 2001). CP itself does not inhibit AChE potently but leads to AChE inhibition following bioactivation to CPO (Sultatos, 1994).

CP is metabolized to CPO in the liver (Sultatos, 1988), but it is also quickly deactivated to TCP by esterases in the liver and plasma (Sultatos, 1994); CPO from the liver is unlikely to reach the target brain AChE as there is virtually no detected CPO in serum (Costa et al., 1989), but the role of central activation of CP in mediating CP neurotoxicity is unknown.

Hepatic and complementary DNA expressed CYP2B has high CP desulfuration activity, which correlates with its activity toward established CYP2B substrates, suggesting that CYP2B is likely the main enzyme involved in the activation of CP to CPO (Croom et al., 2010). CYP2B is a highly polymorphic drug-metabolizing enzyme that can metabolize a variety of clinical drugs including propofol, efavirenz, and bupropion as well as drugs of abuse such as cocaine, 3,4-methylenedioxymethamphetamine (MDMA), and nicotine (Ekins et al., 2008). It also metabolizes endogenous...
neurochemicals such as serotonin and testosterone and bioactivates tobacco-specific nitrosamines (Ekins et al., 2008). In addition to CP, CYP2B can also metabolize other organophosphorus pesticides such as methyl parathion; CYP2B in rat brain extracts can also activate methyl parathion to the neurotoxic methyl paraoxon (Albores et al., 2001). CYP2B is active in situ (Miksys and Tyndale, 2009) and contributes meaningfully to the local brain metabolism of drugs in vivo (Khokhar and Tyndale, 2011).

The expression of CYP2B in the brain, especially in areas with considerable cholinergic transmission such as the brainstem and cortex (Miksys et al., 2000; Woolf and Butcher, 1986), makes it ideally situated for localized activation of CP to CPO and inhibition of target AChE. Intracerebroventricular (ICV) injections of CYP2B mechanism-based inhibitors (MBIs) can inhibit brain, and not hepatic, CYP2B activity selectively and irreversibly throughout disparate brain regions for at least 24 h after MBI (Khokhar and Tyndale, 2011). MBIs act through metabolic activation of the MBI to a reactive metabolite that binds covalently to the enzyme. Covalent modifications of enzymes by MBIs are permanent and activity can only be recovered via synthesis of new enzyme. This makes CYP2B MBIs a useful tool for elucidating the role of brain CYP2B’s involvement in the local metabolism of substrates. This study aims to investigate the role of brain CYP2B–mediated CP activation to CPO in the cholinergic neurotoxicity resulting from CP exposure. Using a rat model of acute CP exposure at high doses, previously used in mechanistic studies to elucidate CP toxicity (Bushnell et al., 1993; Ruiz-Munoz et al., 2011), we will use CYP2B MBIs to selectively inhibit CYP2B-mediated CP desulfuration in the brain and assess its effects on CP neurotoxicity.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (200–225 g; Charles River, St-Constant, PQ, Canada) were doubly housed under a 12-h artificial light/dark cycle (lights on at 6:00 AM) and handled daily to habituate them to experimental maneuvers. All procedures were approved by the Animal Care Committee at the University of Toronto and were conducted within the guidelines of the Canadian Council on Animal Care. Five to six animals were used per treatment group in each experiment sufficient to provide statistical significance.

ICV Cannulations and MBI Treatments

As performed previously (Khokhar and Tyndale, 2011; Miksys and Tyndale, 2009), rats were anesthetized with isoflurane and placed in the stereotaxic frame. Unilateral cannulas were then implanted into their right lateral ventricle (Bregma coordinates: anterior-posterior 0.9 mm, lateral 1.4 mm, and dorsoventral 3.6 mm, Paxinos and Watson, 1986). The cannulas were held in place using dental cement and screws. The rats were allowed to recover for a week. MBIs or vehicles were delivered ICV via an injector (0.6 mm protrusion beyond the cannula) affixed to a Hamilton syringe by polyethylene tubing over 2 min, and the injector was left in place for 1 min postinjection. C8-xanthate (C8X), a specific CYP2B MBI (Yanev et al., 2000) (Toronto Research Chemicals, Toronto, ON, Canada), was given at doses of 0.625–80 µg in a 0.5–2 µl total volume of artificial cerebrospinal fluid (ACSF). To confirm that the effect of C8X was through CYP2B inhibition and not through off-target effects, another structurally distinct CYP2B MBI, 8-methoxyxuron (8MO) (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), was given at a dose of 40 µg dissolved in 0.5 µl of 75% dimethyl sulfoxide (DMSO) in ACSF (Koenigs and Trager, 1998).

Chlorpyrifos dose and treatment. Based on previous studies (Bushnell et al., 1993), the rats received 62.5, 125, or 250 mg CP/kg sc (Fluka, Sigma-Aldrich), or peanut oil vehicle, 24 h after an ICV injection of CYP2B MBI or vehicle. These doses have been previously used in mechanistic studies to model the neurotoxic behavioral and chemical changes following a single exposure to high doses of chlorpyrifos (Bushnell et al., 1993) and the possible interactions between chlorpyrifos poisoning and neurodegenerative diseases (Ruiz-Munoz et al., 2011). These doses were also found to produce neurochemical changes similar to chronic daily injections of low doses of organophosphorus pesticides, suggesting some validity as a model for continuous exposure to low levels of pesticides (Bushnell et al., 1993). No CP was detected in vehicle (peanut oil)-treated animals (n = 6 per group). Neither ICV vehicle control (ACSF) nor ICV MBI (C8X) alone altered AChE activity in brain (mean ± SD: 782 ± 140, 726 ± 294, 693 ± 279 U/g; p = 0.24) or serum (818 ± 291, 902 ± 526, 735 ± 344 U/l; p = 0.46) nor were there differences in body temperatures following the vehicle for CP (peanut oil; ACSF: 36.4 ± 0.4, C8X: 36.7 ± 0.3°C; p = 0.31). Moreover, no signs of behavioral toxicity or hypothermia/hyperthermia were observed in the ICV MBI–treated animals receiving peanut oil treatment. Animals were sacrificed for the measurement of CP and metabolites, as well as AChE activity, at 4–h day after CP treatment. For the behavioral studies, rats received either a single ICV MBI/vehicle injection 24 hr prior to CP treatment or daily repeated injections every 24 h after the first ICV injection.

Serum and brain chlorpyrifos and chlorpyrifos-oxon levels. Measurement of serum and brain concentrations of CP and CPO were modified from previously established methods (Abu-Qare et al., 2001). Chlorpyrifos (PS-674, 99.5% purity, 78.1% recovered after 279 days) and chlorpyrifos-oxon (MET-674B, 98.8% purity, 82.3% recovered after 242 days) analytical standards were purchased from ChemService Inc. (Westchester, PA). Briefly, 0.5 ml of serum or brain homogenate supernatant (half brains homogenized 1.2 wt/vol in cold 0.1M phosphate buffer, vortexed and centrifuged at 16,060 × g for 10 min at 4°C) were added to 25 ml of 10 µg/ml 2-benzoxazolinone (internal standard [IS]) and 4 ml dichloromethane. The mixture was vortexed for 20 s, mechanically shaken for 10 min, and centrifuged at 1,100 × g for 10 min. The organic layer was then transferred and evaporated to dryness under a nitrogen stream. The residue was redissolved in 110 µl of mobile phase, and 90 µl of the solution was injected into the high-performance liquid chromatography (HPLC) system consisting of an Agilent 1100 Series HPLC system (Palo Alto, CA) equipped with a degasser, quat pump, autosampler, and UV detector. Samples were separated on a ZORBAX Bonus-RP column (250 × 4.6 mm, 5 µm) using gradient elution conditions of acetonitrile and water starting at 40% acetonitrile, going up to 80% acetonitrile by 22 min, and then returning to 40% acetonitrile for 24 min for 5 min to reequilibrate. The acetonitrile gradient flow rate was 1 ml/min, and UV detection was performed at a wavelength of 280 nm for chlorpyrifos and chlorpyrifos-oxon. Retention times were 5.3, 13.6, and 22.8 min for IS, chlorpyrifos-oxon, and chlorpyrifos, respectively, with a recovery of 93.1% for CP and 85.2% for CPO. The limits of quantification were 10 ng/ml for CP in serum and 10 ng/g for CP and CPO in the brain, and the assays were linear up to 1000 ng/ml and 1000 ng/g, respectively.

Brain and Serum Cholinesterase Activity

Cholinesterase activity was measured with acetylthiocholine iodide as the substrate using a QuantiChrom AChE assay kit (BioAssay Systems, Hayward, CA). Serum samples were diluted 1:20 in the assay buffer, and supernatant from brain homogenates (half brains homogenized 1:2 wt/vol in cold 0.1M phosphate buffer, vortexed and centrifuged at 5730 × g for 15 min at 4°C) was diluted 1:10 to obtain optical density values within the assay’s linear range of detection, and 10 µl of sample was used per well with 190 µl of freshly prepared working reagent (per manufacturer’s instructions). All AChE activities are reported as a percentage reduction from AChE activity seen in animals treated with both vehicle controls, ICV ACSF and sc peanut oil.
Body Temperature Measurement

Chlorpyrifos elicits an initial hypothermic state that lasts up to 1 day followed by an elevation in core temperature that persists between 2 and 3 days after CP treatment. The initial hypothermia is mediated by the cholinergic overstimulation of heat loss pathways in central nervous system (CNS) thermoregulatory centers, whereas the fever is mediated by peripheral cholinergic and noncholinergic pathways (Gordon and Grantham, 1999). Rectal body temperatures were measured using a digital thermometer.

Behavioral Assessments

Incline plane. As previously described (Abou-Donia et al., 2003), rats were placed on a flat plane in the horizontal position, with their head facing the side of the board to be raised, and the angle at which the rat began to slip downward was recorded. The results of two trials were averaged.

Functional Observational Battery

The complete procedural details and scoring criteria for behaviors have been previously established (McDaniel and Moser, 1993). The rats were first evaluated for posture, palpebral closure, and the presence of convulsions in the home cage. The rat was removed from the cage and held briefly for the assessment of ease of removal and handling, as well as the presence of vocalizations, piloerection, and other apparent abnormalities such as lacrimation, salivation, and ptosis; no effects of ICV MBIs were seen on these measures. The rat was then placed on a clean absorbent pad-covered laboratory cart (60 × 90 cm) with a perimeter barrier (6.5 cm high). The rat was undisturbed for 3 min and observed for locomotion and the frequency of rearing responses (ability of rat to place its weight on its haunches). At the same time, gait characteristics were noted and gait, mobility, and arousal scores were assigned. The presence of tremors, convulsions, and abnormal postures were also noted. Reflex testing consisted of approach, touch, auditory, and acute pinch response measurements, with no effect of ICV MBI injections on these measures. Manipulative and physiological measures such as the aerial righting reaction (rat held supine and dropped from height of 30 cm, average of two measurements), body weight, rectal temperature, and hind limb landing foot splay (distance between hind limb foot marks after dropping from a height of 40 cm [two readings averaged]) were then measured. The entire battery of tests took approximately 6–8 min per rat. Animals were tested at 24-h intervals after administration of chlorpyrifos for 3 days by two blinded observers.

Statistical Analyses

All data analyses were performed using PASW Statistics 18 (SPSS Inc. Chicago, IL). The two MBIs and dose range experiments, as well as the within-between-treatment comparisons, were tested using a one-way ANOVA and post hoc Least Significant Difference test. Linear trends were also assessed following the one-way ANOVA using polynomial contrasts. Behavioral and temperature measurements were compared between baseline and 1–3 days, within- and between-treatment (vehicle/MBI), across time using a repeated measures ANOVA.

RESULTS

Central Injections of C8X, a CYP2B Inhibitor, Dose-Dependently Blocked Brain, but Not Serum, Reductions in AChE Activity and Attenuated Chlorpyrifos-Mediated Hypothermia

CP (250 mg/kg sc) treatment alone results in detectable levels of CP in brain and serum, increased inhibition of brain and serum AChE activities and lowered body temperatures at 4 h after CP treatment. Central injections of a CYP2B MBI (C8X), which was administered 24 h prior to CP, dose dependently increased brain (β_{rend} = 0.02) but not serum CP levels (β_{rend} = 0.24) at 4 h after CP treatment, suggesting reduced brain metabolic activation of CP to the neurotoxic CPO (Figs. 1A and B). Consistent with this, there was a dose-dependent attenuation of the percentage reduction in brain AChE activity (β_{rend} = 0.013) and body temperature (β_{rend} < 0.001) caused by CP alone, with no effect on peripheral serum AChE activity (Figs. 1C–E). An ICV dose of 40 μg MBI (C8X) was the lowest MBI dose that significantly altered brain CP concentration (β = 0.03), percentage reduction in AChE activity (p = 0.03) and body temperature (p < 0.0001), and was thus used in the subsequent experiments.

Chlorpyrifos (250 mg/kg sc) Activation to CPO, and Resulting Hypothermia, Was Attenuated by Two Structurally Distinct CYP2B MBIs

Animals pretreated with C8X or 8MOP (CYP2B MBIs) had lower CP concentrations compared with the vehicle-treated groups (ACSF or DMSO, respectively) at 4 h post-CP treatment. CPO was only detected in one of nine MBI-treated animals, whereas it was detected in five of eight ICV vehicle–treated animals (Fig. 2A; square root of the limit of quantification was used as the value for samples with CPO below levels of quantification). MBI-treated animals also had significantly higher brain, but not serum, CP concentrations (Fig. 2B, p < 0.003 compared with respective vehicles). Serum CP was not detected in any animal. Decreased brain CP activation also decreased the reduction in brain AChE activity in the ICV MBI-treated animals (Fig. 2C). Serum AChE activity was not changed by ICV MBI treatment (ACSF: 205 ± 44, C8X: 237 ± 87, DMSO: 280 ± 109, 8MOP: 285 ± 72 U/L, p = 0.21). Both CYP2B MBI pretreatments also completely blocked CPO-induced acute hypothermia (Fig. 2D, p < 0.0001 compared with respective control). Because CPO was not detected in most MBI-treated samples, Pearson’s correlations were executed using the remaining concentrations of the parent CP and not the metabolite CPO. There was a significant negative correlation between brain CP concentrations and percentage reductions in brain AChE activity (Fig. 2E, r = −0.61, p = 0.004). Body temperatures also correlated significantly with brain CP concentration (Fig. 2F, r = 0.83, p = 0.0005) and inversely with percentage reduction in AChE activity (Fig. 2G, r = −0.54, p = 0.01) but not with the percentage reduction in serum AChE activity or CP concentration (r = 0.03, p = 0.87 and R = −0.12, p = 0.39, respectively).

Chlorpyrifos (125 mg/kg sc) Neurotoxicity Was Attenuated by a Single ICV Injection of CYP2B MBI (C8X) for up to 2 Days While Daily Repeated MBI Injections Prolonged the Blockade of Neurotoxicity

To assess the behavioral effects of single and repeated daily C8X injections, an intermediate dose of CP (125 mg/kg) was used. This CP dose results in moderate levels of CP toxicity compared with both lower and higher doses of CP (Bushnell et al., 1993). In control animals (ICV ACSF), a single injection of 125 mg/kg sc CP resulted in a significant increase in CP toxicity
from baseline across all behavioral measures assessed over 3 days ($p < 0.05$, within-subject effect of time). There was also a significant overall effect of MBI treatment ($p < 0.04$, between-subject effect of treatment) with C8X-treated animals having significantly lower gait (Fig. 3A), aerial righting reflex (Fig. 3B), and mobility (not shown) scores compared with ICV ACSF (all animals received CP). Between-treatment comparisons showed significantly lower toxicity scores (within-day) in the C8X-treated animals on days 1 and 2 with the scores being similar by day 3. The C8X-treated animals also had higher arousal scores (Fig. 3C), incline plane angles (Fig. 3D), and rearing counts (not shown) for up to 2 days. This reduction in toxicity was larger on day 1, than day 2, and was gone by day 3. There were no differences between ICV treatments in brain or serum AChE activity on day 3 consistent with the lack of difference in toxicity on day 3 (Figs. 3E and F). However, with repeated daily ICV injections of C8X, there was both a reduction in toxicity (Figs. 3G–J, $p < 0.05$) and less reduction of brain AChE activity on all 3 days compared with ICV ACSF controls (Figs. 3K–L).
CYP2B Inhibition Using 8MOP Also Attenuated CP Neurotoxicity

A single ICV injection of 8MOP resulted in an attenuation of CP toxicity across all measures for 2 days after CP treatment, with no significant differences between 8MOP and ACSF on day 3 (Figs. 4A–D; \( p < 0.05 \)). Similarly, there were no differences between treatments in brain and serum AChE activities by day 3 (Figs. 4E and F). With repeated daily ICV injections of 8MOP, there was significantly less CP toxicity assessed by all measures (Figs. 4G–J; \( p < 0.05 \)) and less reduction of brain AChE activity (Figs. 4K–L) compared with ICV DMSO controls on all 3 days. These effects of single and daily ICV 8MOP treatments were also seen across other measures such as rearing and mobility (data not shown).

Both Single and Repeated ICV MBI Injections Attenuated Centrally Mediated Hypothermia but Not Peripherally Mediated CP Hyperthermia

Following CP (125 mg/kg sc), ICV C8X–treated animals (both singly and repeatedly treated) displayed no centrally mediated acute hypothermia compared with ICV ACSF controls. However, on days 2 and 3, both ICV C8X– and ACSF-treated animals showed peripherally mediated hyperthermia (Figs. 5A–B, \( p < 0.001 \)). Similarly, 8MOP-treated animals (both singly and repeatedly treated) displayed no centrally mediated hypothermia but similar peripherally mediated hyperthermia compared with DMSO-treated controls in response to CP exposure (Figs. 5C–D; \( p < 0.001 \)). No effect of ICV MBIs alone was seen on body temperature (not shown).

CP-Mediated Toxicity Is Dependent on CP Dose, and Repeated ICV MBI Treatments Blocked Neurotoxicity at All CP Doses Tested

Increasing CP doses resulted in increased gait (\( p_{\text{trend}} = 0.042 \)) and aerial righting reflex (\( p_{\text{trend}} = 0.061 \)) AUC\(_{\text{days 1–3}}\) scores, indicating worsening, in repeated ICV ACSF–treated animals suggesting that these responses are CP dose dependent. Repeated ICV C8X injections attenuated the CP toxicity reflected in significantly lower AUCs compared with ACSF–treated animals at each CP dose (Figs. 6A–B, \( p < 0.002 \) compared with respective ACSF–treated controls). CP caused a dose-dependent reduction in brain AChE activity (\( p_{\text{trend}} = 0.028 \)) in the ACSF–treated animals, which was mitigated by repeated injections ICV C8X at all CP doses; there were no
differences in the reduction of serum AChE activity between the ACSF- and C8X-treated groups consistent with no effect of central CYP2B inhibition on peripheral CP effects.

DISCUSSION

Inhibition of brain CYP2B–mediated local activation of CP to CPO increased brain CP and decreased brain CPO concentrations, reduced the inhibition of brain AChE, blocked the hypothermic effects of CP, and attenuated the behavioral deficits. Inhibition of brain CP metabolism by CYP2B had no effect on serum CP levels, AChE activity or delayed peripherally mediated CP hyperthermia, consistent with a lack of effect on peripheral CP metabolism.

Previous work on CP-mediated thermoregulation indicates that hypothermia (0–24 h) is mediated through the central cholinergic activation of hypothalamic heat loss pathways, whereas hyperthermia (48–72 h post-CP) is primarily mediated through the peripheral activation of vagal transmission as well as cyclooxygenases, through both cholinergic and noncholinergic mechanisms (Gordon and Grantham, 1999). The ability of ICV-delivered CYP2B MBIs to dose dependently block the hypothermic responses to CP, while not altering peripherally mediated hyperthermia, is in line with selective inhibition of brain CYP2B–mediated CP activation with a resulting impact on CP-mediated cholinergic toxicity.

CP dose dependently reduces brain AChE activity and behavioral toxicity (Bushnell et al., 1993) suggesting a correlation between brain CP activation, brain AChE inhibition, and behavioral deficits. The ability of a single ICV injection of a CYP2B MBI to inhibit the local production of CPO, attenuate reductions in brain AChE activity, and block CP neurotoxicity on day 1 suggests that local brain activation of CP to CPO is required for the manifestation of this cholinergic toxicity. This inhibition of CYP2B-mediated CP activation on day 1 is consistent with ICV MBI treatment irreversibly inhibiting brain CYP2B for at least 24 h after MBI in previous studies (Khokhar and Tyndale, 2011). CP has long-lasting bioavailability following acute subcutaneous poisoning in humans, with CP being detected in the serum and urine for more than 30 days (Soummer et al., 2011); both brain and serum AChE activity

FIG. 3. A single C8X ICV injection attenuated CP (125 mg/kg sc) toxicity for up to 2 days, whereas repeated C8X injections attenuated toxicity throughout the study period. Single ICV C8X–treated animals significantly lower gait (A) and aerial right reflex (B) and significantly higher arousal scores (C) and incline plane angles (D) than ACSF-treated controls on days 1 and 2. There were no differences in toxicity, or reductions in brain and serum AChE activity, between single ICV C8X– and ACSF-treated groups on day 3 (E–F). Repeated daily ICV injections of C8X reduced CP toxicity across all test days in all behavioral measures (G–J), and significantly lowered the reduction in brain, but not serum, AChE activity on day 3 (K–L); n = 4–5 per group, *p < 0.05, “a” indicates p < 0.09; arrows indicate direction of toxicity and figures depict group mean ± SD, BL indicates responses at baseline.
Activities are inhibited for at least 21 days after an acute exposure (Bushnell et al., 1993), suggesting long-lasting bioavailability of CP. After a single ICV injection of the MBI, signs of toxicity became apparent by day 2 suggesting that inhibition of brain CYP2B was short lasting, resulting in activation of CP still available in the brain after day 1. Repeated daily ICV MBI injections continued to inhibit brain CP metabolism and blocked the appearance of CP toxicity even though CP was still present in the brain. This suggests that CYP2B inhibitors given even after CP exposure may reduce the resulting toxicity. Identification of CYP2B inhibitors, which can easily cross into the brain to prevent the further activation of CP, may be useful adjuncts to the cholinesterase reactivators to be given in cases of high-dose CP poisoning.

Because CYP2B can also bioactivate other organophosphorus pesticides such as methyl parathion (Albores et al., 2001), this could also be a useful therapy for other types of organophosphate poisoning.

The low levels of both behavioral and neurochemical (AChE inhibition) toxicity still seen in animals receiving repeated daily MBI may be due to incomplete inhibition of brain CYP2B or due to local desulfuration of CP by other CYP isoforms (e.g., CYP3A) (Tang et al., 2001). Our previous investigation found that a single ICV injection of a CYP2B MBI (20 μg) could inhibit 50–75% of CYP2B activity in the brain; however, higher doses of CYP2B MBI did not further inhibit propofol metabolism, as no significant differences were seen in propofol sleep times and brain propofol concentrations between 20 and 80 μg ICV MBI (Khokhar and Tyndale, 2011). Similarly, we found no significant differences between 40 and 80 μg C8X in brain CP concentration, AChE inhibition, and body temperature (Fig. 1), suggesting that these doses may result in near maximal inhibition of CYP2B. CYP3A has the ability to desulfurate CP to CPO and could have contributed to the remaining CP toxicity in MBI-treated animals (Tang et al., 2001). Moreover, because brain CYP2B inhibition increased brain CP concentration, the parent CP could have also inhibited some AChE activity in the brain; CP can inhibit AChE activity, albeit with ~3000× less potency compared with CPO (Sultatos, 1994). CYP2B inhibition in the brain could have...
also diverted metabolism through to TCP, which has some capacity for neurotoxicity at very high levels (Zurich et al., 2004), and could have contributed to the low levels of toxicity seen.

Although studies using organophosphates such as parathion have tried to assess the role of brain activation by either hepatotectomizing the rats or blocking the descending aorta and preventing drug access to the liver (Chambers et al., 1991, 1989), we have shown that even in the presence of intact hepatic metabolism and circulation, inhibition of CP metabolizing enzymes within the brain can reduce or completely block the CNS toxicity related to CP. Pharmacokinetic and pharmacodynamic modeling suggests that localized brain CP metabolism after subcutaneous exposure can contribute to brain AChE inhibition (79 vs. 40% maximum AChE inhibition with and without brain CP metabolism, respectively). This is consistent with the greater inhibition of AChE activity in the brain after subcutaneous administration, compared with oral administration, possibly due to greater localized brain CP activation and CP sequestration in brain (Smith et al., 2009). Subcutaneous exposure to CP, documented in a case report, can produce extensive neurotoxicity (Soummer et al., 2011). Subcutaneous administration also resembles dermal absorption, which is one of the most common routes of occupational exposure (Griffin et al., 1999); dermal and subcutaneous exposures to another neurotoxin (i.e., T-2 toxin) have previously been shown to produce similar levels of brain toxicities (Chaudhary and Rao, 2010). Our findings here are an in vivo demonstration of the substantial role for local brain CYP-mediated activation of CP in AChE inhibition and CPO behavioral toxicity. Future investigations of the role of brain CYP2B in CP activation and toxicity will employ lower doses of CP, administered through either oral or dermal routes to more closely model exposure in humans.

Drugs that induce brain CYP2B might confer a greater risk for the toxic effects of CP. Smokers have higher levels of CYP2B in the brain but not liver (Hesse et al., 2004; Miksys et al., 2003), and rats receiving nicotine have prolonged induction of the enzyme in the brain (Khokhar et al., 2010) but not in the liver (Miksys et al., 2000). Rats receiving both CP and nicotine chronically had significantly increased behavioral toxicity (lower incline plane angles) compared with rats receiving either drug alone 24 h after last treatment of CP and nicotine (Abou-Donia et al., 2003), suggesting that the induced CYP2B levels in rat brain might be contributing to the increased neurotoxicity. In humans, there is substantial genetic variation in CYP2B6, resulting in a range in human brain CYP2B6 expression (Miksys et al., 2003). Therapeutic
inhibition of brain CYP2B in those who have been exposed to dangerously high levels of CP could be explored, particularly for use in smokers, people who have been exposed to other inducers of brain CYP2B and those with higher brain CYP2B6 due to genetic polymorphisms, as all these individuals might be at greater risk for CP-mediated toxicity.

Having shown a role for CYP2B-mediated metabolism in mediating neurotoxicity, other CYPs expressed in the brain may also be playing a role in mediating neurotoxicity, or conversely, neuroprotection from toxicants. A variety of other neurotoxins and drugs with neurotoxic side effects are metabolized by members of brain-expressed CYP subfamilies (e.g., CYP2B, 2D, 2E, 3A, and 4), including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, tetrahydroisoquinoline, parathion, paraquat, and 0,0-diisopropyl phosphorofluoridate (Miksys and Tyndale, 2006). Depending on whether a particular CYP activates or inactivates the neurotoxin, variation in the expression and activity of brain CYPs could confer either neuroprotection or result in greater neurotoxicity. Phenobarbital-induced hepatic and brain CYP2B can potentiate the toxicity of 9-methoxy-N-2-methyllipticinium acetate by increasing its metabolic activation (Upadhya et al., 2002). Conversely, inhibition of another closely related CYP (i.e., CYP2D6) in a human neuroblastoma line reduces the inactivation of MPP+, a Parkinsonian-causing agent, and potentiates MPP+’s toxicity (Mann and Tyndale, 2010), consistent with lower levels of CYP2D6 in the brains of Parkinson’s disease patients suggesting a protective role for CYP2D6 in inactivating Parkinson’s causing toxins (Mann et al., forthcoming). Many drugs are associated with centrally mediated or centrally occurring adverse effects (e.g., ethanol, efavirenz), and interindividual differences in the local metabolism of these drugs by brain CYPs may contribute to the variation in their toxic side effects.

This is the first finding in a living animal with intact hepatic metabolism and circulation that selective inhibition of a CYP enzyme within the brain can block central cholinergic toxicity. This indicates that local metabolic activation of CP by brain CYP2B is primarily responsible for the cholinergic CP neurotoxicity and potentially other organophosphates that are activated to their oxons. The ability of brain CYP2B to metabolize CP as well as other neurotoxic agents and drugs, in combination with the large interindividual variability seen in brain CYP expression due to genetic and environmental

FIG. 6. CP-mediated toxicity is dose-dependent, and repeated ICV MBI treatments reduced toxicity at all CP doses. CP dose dependently increased AUC<sub>days 1–3</sub> for gait and aerial righting reflex scores, and repeated daily ICV C8X injections significantly attenuated the CP toxicity across all doses (A–B). A dose-dependent reduction in day 3 brain and serum AChE activity was seen in the ICV ACSF-treated animals, and repeated ICV injections of C8X significantly decreased the percentage reductions in brain, and not serum, AChE activity across all CP doses (C–D). n = 4–5 per group, *p < 0.02; arrows indicate direction of toxicity and figures depict group mean ± SD.
factors, could contribute to the different susceptibilities of individuals to the toxicities associated with clinical drugs, drugs of abuse, and environmental toxins.

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