Mechanism of Inactivation of Human Cytochrome P450 2B6 by Phencyclidine

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The mechanism behind the observed inactivation of human P450 2B6 by phencyclidine (PCP) has been evaluated over the past 2 decades. The scope of the current investigation was to contribute to the fundamental knowledge of PCP oxidation and perhaps the mechanism behind P450 inactivation. To study the chemistry of PCP oxidation, we subjected PCP to the Fenton reagent. Under Fenton chemistry conditions, oxidation on all three PCP rings was observed by liquid chromatography/tandem mass spectrometry (LC-MS/MS). When PCP was incubated with the Fenton system in the presence of glutathione (GSH), three GSH-PCP conjugates were identified. Subsequent LC-MS/MS analysis of these conjugates revealed two species that had GSH attached to the cyclohexane ring of PCP and a third conjugate in which GSH was adducted to the piperidine ring. When PCP was incubated across a panel of P450 enzymes, several enzymes, including P450s 2D6 and 3A4, were able to catalyze the formation of the PCP iminium ion, whereas P450s 2B6 and 2C19 were exclusively able to hydroxylate secondary carbons on the cyclohexane ring of PCP. Subsequent mechanistic experiments revealed that only P450s 2B6 and 2C19 demonstrated loss of catalytic activity after preincubation with 10 μM PCP. Finally, investigation of P450 2B6 inactivation using structural analogs of PCP revealed that blocking the para-carbon atom on the cyclohexane ring of PCP from oxidation protected the P450 2B6 from inactivation, which suggests that a reactive intermediate generated during the hydroxylation of the cyclohexane ring may be linked to the mechanism of inactivation of P450 2B6 by PCP.

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The biotransformation of phencyclidine, PCP (Fig. 1), by liver proteins was shown to result in the formation of reactive metabolites leading to P450 inactivation and the formation of covalent adducts with hepatic macromolecules more than 20 years ago (Castagnoli et al., 1997). Originally, covalent binding and enzyme inactivation was thought to occur via alkylation of protein-based nucleophiles by an iminium ion metabolite, M4 (Fig. 1), that arose from the P450-dependent two-electron α-carbon oxidation of PCP (Sayre et al., 1997). This hypothesis was supported by the formation of PCP iminium-cyanide adducts and the observation that the presence of cyanide in microsomal incubations protected against the formation of covalent adducts with proteins (Ward et al., 1982b; Hoag et al., 1984). However, subsequent studies demonstrated that the P450 inactivation by the PCP iminium ion (PCP-Im) required the presence of NADPH (Hoag et al., 1984; Osaka and Coon, 1989; Sayre et al., 1991), which inferred that metabolism of PCP beyond the iminium species was required for P450 inactivation.

Later, the PCP mechanism-based inactivation of P450 2B6, using purified, recombinant human P450 enzyme, was investigated in great detail (Jushchyshyn et al., 2003). Through a series of experiments, it was shown that P450 2B6 was inactivated via covalent modification of the P450 2B6 approtein. Moreover, inclusion of cyanide in the purified P450 2B6 incubation mixture did not protect the enzyme from inactivation. This observation supported the notion that perhaps something other than the PCP iminium ion was involved in P450 2B6 inactivation. Additional evidence toward this conclusion was obtained when coincubation of PCP with glutathione (GSH) did not protect P450 2B6 from inactivation. These observations led to speculation that perhaps the chemical species that leads to nonselective microsomal protein binding (e.g., PCP iminium species) is unique from a potential short-lived, active site-bound, reactive intermediate, which results in P450 2B6 inactivation.

To investigate the probability than an alternative mechanism may be responsible for PCP inactivation of P450 2B6, we used several lines of interrogation. First, Fenton chemistry, used for the production of hydroxyl radicals, was coupled with GSH trapping to mimic the oxidation of PCP and to identify reactive intermediates in the absence of enzyme. Second, reaction phenotyping experiments allowed for the identification of unique PCP metabolites formed by P450s 2B6 and 2C19, and an inhibition screen revealed that these were the only two enzymes inhibited by PCP. Finally, the time-dependent inhibition of P450 2B6 by PCP analogs was compared. The results of these studies suggest a mechanism for the inactivation of P450 2B6 by PCP that does not involve the initial formation of the PCP iminium ion.
inactivation. In brief, two primary reaction mixtures, a control sample and an inactivation sample containing 60 pmol of P450 and 10 μM PCP in 50 mM potassium phosphate buffer (pH 7.4), for a final volume of 90 μl were equilibrated at 37°C for 5 min. The inactivation sample received 1 mM NADPH to initiate the reaction, and the control sample received an equal volume of water (4.5 μl). At 0 and 20 min after the addition of NADPH, 10 pmol of P450 was transferred from the primary reaction to a secondary reaction containing 100 μM of the marker substrate and 1 mM NADPH in 50 mM potassium phosphate buffer (pH 7.4). Secondary reaction mixtures were incubated for 10 min at 37°C before being quenched with 100 μl of acetonitrile and vortex-mixed. The final volume of the secondary reaction was 250 μl. Immediately after the 0-min transfer, the +NADPH sample received 1 mM NADPH while the control sample received an equal volume of water. Marker substrate activity of the control sample at 0 min was assigned a value of 100% activity remaining. All experiments were conducted in triplicate.

For the PCP inactivation screen, the following P450 marker substrates were used: 7-EFC (P450 2B6), 7-MFC (P450 2C9), CEC (P450 2C19), AMMC (P450 2D6), and BFC (P450 3A4). All samples (200-μl aliquots) were assayed directly for marker substrate turnover using a Safire fluorescence plate reader (Tecan, Durham, NC). The following excitation and emission wavelengths were used to detect the products of the marker substrates: for 7-EFC, 7-MFC, and BFC: λ_ex = 409 nm and λ_em = 530 nm; for CEC: λ_ex = 409 nm and λ_em = 460 nm; and for AMMC: λ_ex = 390 nm and λ_em = 460 nm. For all samples, the bandwidth was 12 nm, and the gain was set to 60 (arbitrary units).

**LC-ESI-MS/MS Analysis.** PCP metabolites and GSH adducts were chromatographically separated on a Phenomenex (Torrance, CA) C-18 column (4.6 × 150 mm, 5 μm) that had been equilibrated with 90% solvent A (100% water, 0.1% FA) and 10% solvent B (100% CH3CN, 0.1% FA) at a flow rate of 0.35 ml/min. After the injection of 20 μl of sample, solvent B was increased linearly to 60% over 15 min. Metabolites and conjugates were detected using a Thermo-Finnigan DECA-XP (Woburn, MA) mass spectrometer set in the positive ion mode. The capillary voltage and temperature were 4.5 kV and 210°C, respectively. Sheath gas and auxiliary gas were 40 and 10 (arbitrary units), respectively. The tube lens offset was set at 5 V. Data-dependent scanning was switched on, and the collision energy was 27%.

**Mechanism-Based Inactivation of P450 2B6 by PCP and PCP Ketone Analogos.** Four primary P450 mixtures were equilibrated at 37°C for 5 min before reaction initiation with 1 mM NADPH. Three mixtures contained 7.5 pmol of P450 2B6, 10 μM PCP, 10 μM 4-phenyl-4-piperidinecyclohexanone, or 10 μM 1-(1-phenylcyclohexyl)piperidine-4-one in 50 mM potassium phosphate buffer (pH 7.4) resulting in a final volume of 70 μl. The PCP ketone analog stock solutions were in acetonitrile. The final concentration of acetonitrile in each primary reaction was less than 3% (v/v). The fourth reaction mixture contained 7.5 pmol of P450 2B6 in 50 mM potassium phosphate buffer (pH 7.4) and 2 μl of acetonitrile. After the mixtures were equilibrated, 10-μl aliquots (1 pmol of P450) were transferred to secondary reaction mixtures. The secondary reaction mixtures contained 100 μM 7-EFC and 1 mM NADPH in 50 mM potassium phosphate buffer (pH 7.4) for a final volume of 250 μl. These secondary reaction mixtures were incubated at 37°C for 10 min and were quenched with 150 μl of acetonitrile. Immediately after the initial transfer to the secondary reaction mixtures, the primary reaction mixtures were initiated with the addition of 1 mM NADPH. Aliquots were transferred from the primary to the secondary reaction mixtures at the time points indicated. The secondary reaction mixtures were assayed for the oxidation of 7-EFC to HFC as described above. 7-EFC O-deethylase activity of the control sample at 0 min was assigned a value of 100% activity remaining. All experiments were performed in triplicate.

**Results**

**Oxidation of PCP by the Fenton Reagent.** When PCP was subjected to chemical oxidation by the Fenton reagent, hydroxylated products (MH+ at m/z 260) were observed (data not shown). Of the products, three (M1–M3) were identical to the microsomal derived metabolites, since they possessed identical HPLC retention times and MS fragmentation with that of authentic PCP metabolite standards. The identity of the remaining oxidative products reflected hydroxylation of the PCP phenyl ring. One oxidation product was consistent
Mechanism-Based Inactivation of P450s by PCP. Of the P450 isoforms that metabolized PCP, a select panel based upon isoform-specific metabolic profiles were assayed for potential time-dependent enzyme inactivation in the presence of 10 μM PCP over a 20-min preincubation period. Results of this study revealed that P450 2B6 lost approximately 40% catalytic activity over the 20-min preincubation period, whereas P450s 2C9, 2D6, and 3A4 exhibited no measurable loss of activity in the presence of PCP (Fig. 5). Interestingly, P450 2C19, the only other P450 that possessed M1 and M2 oxidase activity, was inactivated approximately 20% by 10 μM PCP. Control samples that did not receive NADPH showed no significant loss of marker substrate activity over the 20-min incubation period (data not shown).

Mechanism-Based Inactivation of P450 2B6 by PCP Ketone Analogs. To determine whether carbon hydroxylation on the para position of the cyclohexane or piperidine ring was required for P450 2B6 inactivation, recombinant human P450 2B6 was incubated with 10 μM PCP or two PCP ketone analogs. For the PCP analogs, the ketone moieties were located on either the para-carbon of the cyclohexane ring or the para-carbon of the piperidine ring (Fig. 6A). Figure 6B shows that over a 30-min time period, the rates of P450 2B6 inactivation by PCP and the PCP piperidine ketone analog were similar. However, the PCP cyclohexane ketone analog showed a marked decreased rate of inactivation compared with PCP. A control mixture incubated with acetonitrile in the absence of NADPH did not lose activity over the 30-min assay. In addition, when either PCP ketone analog was incubated with P450 2B6, similar amounts of the corresponding hydroxyl metabolites were detected confirming that these compounds were able to enter the P450 2B6 active site and that they were substrates for this enzyme (data not shown).

Discussion

The α-carbon oxidation of cyclic tertiary amines by cytochromes P450 to yield toxic products is a well studied metabolic pathway (Castagnoli et al., 1997). For years, researchers have postulated that P450-mediated α-carbon oxidation of the PCP piperidine ring to form the corresponding iminium ion was responsible for the observed to P450 inactivation and covalent modification of microsomal macro-molecules (Osawa et al., 1995). This belief was based upon early experimental evidence that demonstrated that cyanide, which is known to trap the PCP-Im, could attenuate the metabolism-dependent covalent binding of PCP to hepatic microsomal proteins (Ward et al., 1982b). However, subsequent studies with synthesized PCP-Im revealed that the PCP iminium intermediate was not directly involved in P450 inactivation and that further oxidation of the PCP iminium species was required for P450 inactivation (Castagnoli et al., 1997). As a consequence, a PCP dihydropyridinium species was proposed as the ultimate reactive species responsible for P450 inactivation (Castagnoli et al., 1997). However, when the mechanism-based inactivation of P450 2B6 was investigated using purified, recombinant enzyme, cyanide failed to protect the enzyme from inactivation, which suggests that PCP-Im was not involved in the inactivation of P450 2B6 (Jushchyn et al., 2003). Intrigued by the potential that an alternative explanation for P450 2B6 inactivation by PCP was possible, we sought out to reexamine the mechanism that accounts for phencyclidine inactivation of P450 2B6.

Trapping with nucleophiles (such as GSH and N-acetyl cysteine) is a commonly used technique for the identification of reactive metabolites formed as a result of P450 oxidation (Chen et al., 2002;
In many cases, the generated adduct is useful for identifying the site and chemical mechanism of a reactive intermediate (Chen et al., 2002). In the case of P450 2B6 and PCP, trapping experiments with GSH did not protect the enzyme from inactivation (Jushchyshyn et al., 2003). However, these experiments did reveal that the stoichiometry of PCP binding to inactivated P450 2B6 was reduced from 5:1 to 1:1 in the presence of GSH (Jushchyshyn et al., 2003). This result provides the basis for the current hypothesis that PCP electrophiles released from the P450 2B6 active site and trapped by GSH did not represent the ultimate reactive species responsible for P450 2B6 inactivation.

![FIG. 3. Product ion spectra for the GSH-PCP adducts obtained from the Fenton reaction. A, the product ion spectrum obtained by CID of MH$^+$ 549. B, MS/MS obtained by CID of MH$^+$ m/z 554, a d5PCP-GSH adduct. C, the product ion spectrum of the second GSH-PCP adduct having a MH$^+$ 549. The origins of the characteristic ions are shown.](image)

![FIG. 4. Chemical structure of PCP summarizing the contributions of each P450 toward formation of metabolites M1–M5. Font size and the order of the P450s reflect the relative contributions of each enzyme toward the formation of the indicated metabolite.](image)

![FIG. 5. Percent activity remaining for the indicated P450 at 0 and 20 min after incubation with 10 μM PCP and 1 mM NADPH. The activities of control samples (+PCP, −NADPH) at 0 min were assigned a value of 100% activity remaining.](image)
inactivation. In this light, although GSH may be able to trap reactive intermediates of PCP that escape from the active site, it would seem that an in vitro GSH-trapping study would provide negligible information regarding the identity of the true P450-inactivating species. To this end, we used a chemical, P450 biomimetic system that might serve to emulate the P450-catalyzed oxidative metabolism of PCP and generate reactive intermediates that may reflect species that exist only in the enzyme active site.

The Fenton reagent has been previously used to mimic the oxidative biotransformation of various P450 substrates and other molecules (Groves and McClusky, 1976; Zbaida et al., 1988; Heur et al., 1989; Zbaida and Kariv, 1990; Maldotti et al., 1996; Reis et al., 2003). Oxidation of organic compounds by Fenton’s reagent proceeds via an initial one-electron reduction of hydrogen peroxide to yield free hydroxyl radicals and subsequent hydrogen abstraction from the substrate. The resulting carbon-centered radical is then rapidly oxidized by Fe(III) (Wardman and Candeias, 1996). Inclusion of spin-trapping agents such as 5,5-dimethyl-1-pyrroline N-oxide in Fenton reactions has allowed for the unequivocal identification of secondary carbon-centered radical intermediates (O’Neill et al., 2000; Reis et al., 2003, 2004). Therefore, it was reasoned that the Fenton reagent would be a useful tool in elucidating the identities of highly reactive substrate intermediates that may not otherwise been detected in conventional microsomal experiments.

In our hands, the Fenton reagent did not oxidize the piperidine α-carbon to form the PCP iminium ion; however, it was able to catalyze the secondary carbon hydroxylation of PCP to form microsomal-like products M1, M2, and M3. In addition, hydroxylation of the phenyl ring to form M5 and a second unknown phenol metabolite also seemed to be significant routes of PCP oxidative metabolism of PCP and generate reactive intermediates that may reflect species that exist only in the enzyme active site.

When GSH was included in the Fenton reaction mixture, three GSH-PCP adducts were observed having an MH⁺ of m/z 549, which equates to a species composed of one PCP molecule plus one GSH molecule. Two conjugates had product ion spectra that indicated the GSH was adducted to the phenyl-cyclohexane ring fragment. The MS/MS of the corresponding d₅-PCP-GSH adduct narrowed down the location of the GSH molecule to the cyclohexane ring moiety. The third GSH-PCP conjugate seemed to be a species in which GSH was attached to the piperidine ring. Because the Fenton reagent system was incapable of causing the formation of PCP-Im, this adduct must have been the result of the piperidine ring hydroxylation pathway. This same would be true for the GSH adduct on the cyclohexane ring. This result suggested that in an oxidative system, the initial formation of the PCP-Im is not required for PCP to be converted to reactive species. As described previously, the hydroxylation of aliphatic compounds by the Fenton reagent proceeds via hydroxyn-abstracting, resulting in a short-lived carbon centered radical or cation intermediate. Because the GSH-PCP conjugates did not show desaturation or addition of oxygen, we postulate that GSH trapped PCP carbon-centered radical/cation intermediates.

To link metabolism to P450 2B6 inactivation, the oxidative metabolism of PCP was evaluated across a panel of eleven recombinant human P450s. From this survey, a total of seven P450 isoforms (in particular, P450 3A4) were found to form the PCP-Im (M₄), which substantiates earlier findings that this metabolite represents a significant pathway for human P450 hepatic metabolism (Ward et al., 1982a,b; Holsztynska and Domino, 1985). Moreover, P450s 2D6 and 3A4, although able to produce PCP-Im, did not exhibit inactivation when incubated with 10 μM PCP. Interestingly, only P450s 2B6 and 2C19 were able to hydroxylate secondary carbon atoms on PCP to form metabolites M₁, M₂, and M₃. In addition, these were the only two P450s that demonstrated time-dependent inactivation when incubated with 10 μM PCP. Interestingly, only P450s 2B6 and 2C19 were able to hydroxylate secondary carbon atoms on PCP to form metabolites M₁, M₂, and M₃. In addition, these were the only two P450s that demonstrated time-dependent inactivation when incubated with 10 μM PCP. These two observations when viewed in aggregate provide a compelling rationale to support the notion that PCP secondary carbon hydroxylation pathways, rather than formation of the PCP-Im, could be the mechanistic basis for P450 2B6 inactivation.

The mechanism of P450-mediated aliphatic oxidation involves initial H-abstraction from the alkane carbon to form a carbon-centered radical. This is followed by radical rebound resulting in the formation and release of the alcohol metabolite (Atkinson and Ingold, 1993;
Shaik et al., (2002). The carbon-centered radical is very short-lived and is rapidly hydroxylated and released from the active site as a metabolite much faster than the radical itself can escape from the hydrophobic enzyme active site (Heur et al., 1989; Bowry et al., 1990; Rota et al., 1997). This scenario reflects the metabolism of PCP by P450 2B6 and accounts for the partition ratio for P450 2B6 inactivation by PCP as being 45 (Jushchyshyn et al., 2003), which suggests that the majority of PCP secondary carbons are oxidized and released from the active site as hydroxylated metabolites (M1 and M2). However, a small fraction of the PCP carbon-centered radicals, as with the Fenton chemistry, may have the opportunity to undergo single-electron transfer oxidation to form a cation that could readily react with proximal nucleophilic residues at or near the active site of the enzyme, which results in covalent binding to the apoprotein and ultimately P450 2B6 inactivation (Fig. 7).

To further investigate the possibility that pathways leading to either piperidine ring and cyclohexane ring hydroxylation were responsible for P450 2B6 inactivation, we examined the time-dependent loss of P450 2B6 activity in the presence of two PCP ketone analogs. For the PCP analogs, the ketone moieties were located at either the \(\text{para}\)-carbon of the cyclohexane ring or the \(\text{para}\)-carbon on the piperidine ring, which essentially blocked these sites from P450-mediated activation. The cyclohexanone PCP analog showed a marked decrease in the rate of P450 2B6 inactivation compared with native PCP, whereas the piperidine ketone analog exhibited a similar rate of inactivation as PCP. Restated, blocking the \(\text{para}\)-carbon of the cyclohexane ring from oxidation protected P450 2B6 from inactivation and suggests that the pathway leading to C-hydroxylation at this site is involved in the inactivation of P450 2B6 as depicted in Fig. 7. A similar hypothesis was proposed for the inactivation of P450 1A by tacrine (Peng et al., 1997). This scenario reflects the metabolism of PCP by P450 1A and accounts for the partition ratio for P450 1A inactivation by PCP as being 45 (Jushchyshyn et al., 2003), which suggests that the majority of PCP secondary carbons are oxidized and released from the active site as hydroxylated metabolites (M1 and M2). However, a small fraction of the PCP carbon-centered radicals, as with the Fenton chemistry, may have the opportunity to undergo single-electron transfer oxidation to form a cation that could readily react with proximal nucleophilic residues at or near the active site of the enzyme, which results in covalent binding to the apoprotein and ultimately P450 2B6 inactivation (Fig. 7).

FIG. 7. Scheme describing the proposed mechanism for the metabolic activation of P450 leading to inactivation of P450 2B6.

References


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