THE MECHANISM-BASED INACTIVATION OF HUMAN CYTOCHROME P450 2B6 BY PHENCYCLIDINE

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ABSTRACT:

Phencyclidine (PCP) was analyzed for its ability to inactivate human cytochrome P450 (P450) 2B6. PCP inactivated the 7-ethoxy-4-(trifluoromethyl)coumarin O-deethylation activity of P450 2B6 in a concentration-, time-, and NADPH-dependent manner and exhibited pseudo-first order kinetics. The $K_i$ was 10 μM, $k_{inact}$ was 0.01 min$^{-1}$, which corresponds to a $t_{1/2}$ of 31 min. The partition ratio was approximately 45. Spectral analysis of the heme moiety demonstrated that the heme was not modified during inactivation. Extensive dialysis of the PCP-inactivated P450 2B6 did not cause a return in catalytic activity demonstrating PCP inactivation was irreversible. Including 7-ethoxycoumarin, an alternate substrate, protected 2B6 from inactivation by PCP indicating competition of the two substrates for the active site. Exogenous nucleophiles such as glutathione (GSH) and cyanide could not protect P450 2B6 from PCP inactivation demonstrating that the reactive intermediate remained within the P450 active site. High performance liquid chromatography analysis of P450 2B6 inactivated in the presence of $^3$H-labeled PCP showed that PCP binding was specific for the P450 and not to other proteins in the reaction mixture. The stoichiometry of binding of PCP to P450 2B6 was demonstrated using $^3$H-labeled PCP. In the absence of GSH, the stoichiometry was 5.5:1 (PCP/P450). In the presence of GSH, the stoichiometry was 1:1. This stoichiometry was further supported using electrospray ionization-liquid chromatography-mass spectrometry to analyze PCP-inactivated P450 2B1, 2B4, and 2B6.

Phencyclidine (PCP) was developed in the 1950s to be used as an anesthetic but fell into disuse due to its physical and psychological side-effects. PCP eventually became a drug of abuse and although its popularity has decreased over the past decade, it is still taken recreationally. We have previously shown that PCP inactivated P450 2B1 in a time-, concentration-, and NADPH-dependent manner (Crowley and Hollenberg, 1995). Further investigations in our laboratory (Sharma et al., 1997) showed that radiolabeled PCP binds exclusively to PCP-inactivated P450 2B1 in the reconstituted system. Also, an anti-PCP antibody developed by Owens and coworkers (1988) was labeling of the active sites by mechanism-based inactivators is one such tool that has been applied. Mechanism-based inactivators are compounds that are metabolized by the target enzyme to a reactive species, which then inactivates the enzyme (Silverman, 1996). Already, this method has proven to be useful in determining the orientation of the heme in the active site as well as identifying portions of the polypeptide chain responsible for substrate binding (Kunze et al., 1983; Ortiz de Montellano et al., 1983; Yun et al., 1992 and Roberts et al., 1993). Ultimately, mechanism-based inactivators can be used to identify the critical amino acids responsible for carrying out the chemistry required for enzyme catalysis.

The P450 2B subfamily is known to be induced by phenobarbital. Rat P450 2B1 and its human homolog, P450 2B6, are members of this family. Despite comprising only a small amount of total P450 in human liver microsomes (approximately 0.2% on average), P450 2B6 metabolizes more than 3% of drugs in clinical use (Lewis et al., 1998). Because of the importance of P450 2B6 in drug metabolism, it is of great interest to identify the amino acid residues critically involved in catalysis in its active site.

Phencyclidine (PCP) was developed in the 1950s to be used as an anesthetic but fell into disuse due to its physical and psychological side-effects. PCP eventually became a drug of abuse and although its popularity has decreased over the past decade, it is still taken recreationally. We have previously shown that PCP inactivated P450 2B1 in a time-, concentration-, and NADPH-dependent manner (Crowley and Hollenberg, 1995). Further investigations in our laboratory (Sharma et al., 1997) showed that radiolabeled PCP binds exclusively to PCP-inactivated P450 2B1 in the reconstituted system. Also, an anti-PCP antibody developed by Owens and coworkers (1988) was labeling of the active sites by mechanism-based inactivators is one such tool that has been applied. Mechanism-based inactivators are compounds that are metabolized by the target enzyme to a reactive species, which then inactivates the enzyme (Silverman, 1996). Already, this method has proven to be useful in determining the orientation of the heme in the active site as well as identifying portions of the polypeptide chain responsible for substrate binding (Kunze et al., 1983; Ortiz de Montellano et al., 1983; Yun et al., 1992 and Roberts et al., 1993). Ultimately, mechanism-based inactivators can be used to identify the critical amino acids responsible for carrying out the chemistry required for enzyme catalysis.

The cytochrome P450 (P450$^1$) enzymes belong to a superfamily of heme-containing monooxygenases. P450s are found in every biological kingdom and almost every tissue in mammals (Nelson et al., 1996). Cytochrome P450s serve as detoxifying enzymes that are capable of metabolizing many endogenous and exogenous substrates, and they also play a role in activating pro-carcinogens and other toxic compounds (Ortiz de Montellano, 1996). Although the many isoforms of P450s display diverse substrate specificities, the overall chemistry of the catalytic mechanism is believed to be the same. That is, metabolism occurs via a two-electron reduction of molecular oxygen to form a reactive oxygen species and water (Porter and Coon, 1991).

Due to the membrane-bound nature of the mammalian P450s, crystal structures have been difficult to obtain. To date, only a highly modified P450 2C5 has been crystallized (Cosme and Johnson, 1991). Therefore, a variety of other tools have been employed to gain an understanding of the architecture of the P450 active sites. Covalent labeling of the active sites by mechanism-based inactivators is one such tool that has been applied. Mechanism-based inactivators are compounds that are metabolized by the target enzyme to a reactive species, which then inactivates the enzyme (Silverman, 1996). Already, this method has proven to be useful in determining the orientation of the heme in the active site as well as identifying portions of the polypeptide chain responsible for substrate binding (Kunze et al., 1983; Ortiz de Montellano et al., 1983; Yun et al., 1992 and Roberts et al., 1993). Ultimately, mechanism-based inactivators can be used to identify the critical amino acids responsible for carrying out the chemistry required for enzyme catalysis.
used to demonstrate binding of PCP to the P450 2B1 apoprotein following inactivation by PCP (Sharma et al., 1997). More recently, we observed (Chun et al., 2000) that 2-phenyl-2-[(1-piperidinyl)propane, an analog of PCP, inactivated both P450 2B1 and 2B6 in a mechanism-based manner. In all cases, inactivation by PCP or 2-phenyl-2-[(1-piperidinyl)propane appeared to be due to binding to the apoprotein and not as a result of heme modification, suggesting that PCP is a promising compound to use as a tool for identifying the amino acid residues responsible for substrate turnover.

In this report, we investigated the interactions of PCP with human P450 2B6 to validate the use of PCP as a tool to further investigate the enzyme. Inactivation of P450 2B6 by PCP exhibited time-, concentration-, and NADPH-dependence. Concurrently, inactivation appeared to be due to modification of the apoprotein versus alteration of the heme moiety. Additionally, a one to one stoichiometry of PCP to P450 was demonstrated along with supporting ESI-LC-MS analyses of the PCP-inactivated P450 2B6. Taken together, the data suggest that PCP is a good candidate for labeling critical amino acid residues and gaining more information about the active site architecture of human P450 2B6.

Materials and Methods

Materials. Dilauroyl-L-α-phosphatidylcholine (DLPC), NADPH, glutathione (GSH), DMSO, bovine serum albumin, PCP, catalase and EDTA were purchased from Sigma-Aldrich (St. Louis, MO). The potassium phosphate, glycero1, and HPLC grade acetone and methanol were obtained from Fisher Scientific Co. (Pittsburg, PA). Trifluoroacetic acid (TFA) was obtained from Pierce Chemical (Rockford, IL). 7-Ethoxy-4-(trifluoromethyl) coumarin was from Molecular Probes (Eugene, OR). 3,4-[(1H]Vinyl)piperidin phenylcoumarin and 1-[(1-phenyl-3-H)nuclear]piperidine were obtained from the National Institute on Drug Abuse (Research Triangle Park, NC). 7-Ethoxy coumarin was purchased from Aldrich Chemical Co. (Milwaukee, WI). Ultima Gold liquid scintillation cocktail (Packard, Meriden, CT) was used.

Enzyme Purification. P450 2B6 and reductase were expressed in Escherichia coli and then purified as previously described (Hanna et al., 1998, 2000; Kent et al., 1999).

Inactivation of P450 2B6 by Phencyclidine. A mixture of 0.35 nmol of P450 2B6, 0.35 nmol of reductase, and 140 μg of DLPC were reconstituted at 4°C for 30 min at which time 1200 units of catalase and 50 mM potassium phosphate buffer (pH 7.4) were added to give a final volume of 522 μl. The reaction mixture containing 7-EFC as described above for the inactivation assays. Each primary reaction mixture contained 55 pmol of P450 2B6, 1 nmol of NADPH, and 400 μM PCP, and either 10 mM cold PCP, or 10 μM cold PCP. This mixture was then divided into three separate samples. The first sample received no PCP and then was dialyzed against 0.6% tertigol Nonidet P-40 in 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 60 μl. Primary reactions were initiated by addition of 0.9 mM NADPH and incubated for 30 min at 30°C to allow the reaction to go to completion. Aliquots of 12 μl were removed at 0 and 30 min and added to 988 μl of secondary 7-EFC reaction mixture. The secondary mixture was incubated for 10 min at 30°C and quenched with 334 μl of ice-cold acetonitrile. Formation of HFC product was measured as described previously.

Irreversibility of the Inactivation. Two primary reaction mixtures contained 0.25 nmol of P450 2B6, 0.25 nmol of reductase, 100 μg of DLPC, 425 units of catalase, and 160 μM PCP in 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 220 μl were preincubated at 30°C for 5 min. The control sample received 30 μl of water while the inactivated sample received NADPH (0.9 mM). The residual 7-EFC O-deethylation activity was measured at 0 and 30 min by transferring 12 μl (14 pmol of P450 2B6) to a secondary reaction mixture containing 7-EFC as described above for the inactivation assays. The remaining samples were injected into diolysis cassettes and dialyzed extensively at 4°C against two 500-ml changes of buffer containing 2% glycerol and 0.1 mM EDTA in 100 mM phosphate buffer (pH 7.4). Fresh lipid was added to the dialyzed samples. Half of each sample also received fresh reductase (0.2 nmol). The samples were incubated at 30°C for 20 min before removing aliquots for determination of 7-EFC activity.

Protection by Alternate Substrate. Equimolar amounts of P450 2B6 and reductase (0.25 nmol) were reconstituted with catalase (835 units) and DLPC (100 μg) in 50 mM potassium phosphate buffer (pH 7.4) to a final volume of 380 μl. The control sample contained no PCP or NADPH. Three samples contained 40 μM PCP, and two of these also received the alternate substrate, ethoxy coumarin (EC), in 1:4 and 1:8 ratios of PCP to EC. The inactivation reactions were initiated by the addition of 0.9 mM NADPH. Samples were preincubated for 5 min at 30°C. Aliquots were removed and added to the secondary reaction mixture for initial activity measurement. Further aliquots were removed at 5, 10, 15, 20, 30, and 40 min for the determination of 7-EFC O-deethylation activity.

Effects of Exogenous Nucleophiles. Equimolar amounts of P450 2B6 and reductase were reconstituted with lipid, catalase, and potassium phosphate buffer as described previously in inactivation assays. Each primary reaction mixture contained 55 nmol of P450 2B6, 55 nmol of reductase, 22 μg of DLPC, and 94 units of catalase in 50 mM potassium phosphate buffer in a final volume of 80 μl. The 7-EFC O-deethylation activity of P450 2B6 incubated in the primary reaction in the presence of NADPH, 40 μM PCP, and either 10 mM GSH or 1 mM KCN was measured at the time points indicated. One primary reaction, the control, received no NADPH, PCP, GSH, or KCN. The activity of the P450 2B6 in the presence of GSH or KCN alone was also measured to determine their effect on the enzyme activity.

Specificity of Binding. Equimolar amounts (0.72 nmol) of P450 2B6 and reductase were reconstituted with 62 units of catalase in 50 mM potassium phosphate buffer (pH 7.4) to make a final volume of 468 μl. The reconstitution mixture received 1 μCi of the 3,4-[(1H]Vinyl)piperidyl PCP and 86 μCi cold PCP. This mixture was then divided into three separate samples. The first sample received no NADPH, the second received 0.9 mM NADPH, and the third sample received both NADPH and 10 mM GSH. Aliquots were removed before the reactions were initiated with NADPH to test for initial 7-EFC O-deethylation activity. The samples were incubated at 30°C for 30 min after which time, 20 μl of 10% cholate was added to each sample, and then they were dialyzed as described earlier against several changes of a solution containing 20% glycerol, 0.1 mM EDTA, and 0.2% cholate in 100 mM potassium phosphate buffer (pH 7.4). After dialysis, the samples were analyzed by reversed-phase HPLC on a C8 Vydac column (Dionex, Sunnyvale, CA) with a solvent system composed of water with 0.1% TFA (A) and 95% acetonitrile with 0.1% TFA (B). The HPLC system consisted of a Waters 490E
Determining the Stoichiometry of Binding. P450 2B6 (0.5 nmol) was reconstituted with 0.5 nmol of reductase, 134 units of catalase, 40-µg lipid in 50 mM potassium phosphate buffer (pH 7.4) to a final volume of 360 µl. Half of the reconstitution mixture was incubated with PCP (0.2 mM) without NADPH whereas the other half (160 µl) also contained 0.8 mM NADPH. The reaction mixtures were incubated at 30°C, and aliquots were removed and added to 988 µl of secondary reaction mixture to measure 7-EFC O-deethylation activity. The remaining primary reaction mixtures received 10 µl of 10% cholate each and were injected into separate Slide-A-Lyzer Dialysis cassettes (Pierce Chemical). The samples were dialyzed extensively at 4°C against several changes of 350 ml of 20% glycerol, 100 mM potassium phosphate (pH 7.4), 0.1 mM EDTA, and 0.1% cholate until the counts in the external buffer were at background. Aliquots of 20 µl from each of the three samples were removed and used for liquid scintillation counting. The stoichiometry of binding was calculated after subtracting the number of counts in the sample without NADPH and accounting for the percentage of inactivation.

ESI-IC-MS Analysis. The analyses of control and PCP-inactivated P450 2B6 were performed as described by Regal et al. (2000). P450 2B6 (0.5 nmol) was reconstituted with 0.5 nmol of reductase, 134 units of catalase, 40-µg lipid in 50 mM potassium phosphate buffer (pH 7.4) to a final volume of 360 µl. The inactivation reaction was initiated by the addition of 0.8 mM NAPDH and incubated for 20 min at 30°C. Aliquots of 12 µl were removed and added to 988 µl of secondary reaction mixture to determine the rate of inactivation. It is defined as the number of molecules of inactivator metabolized per molecule of enzyme inactivated (Silverman, 1996). The rate of inactivation, $k_{\text{inactivation}}$, was 0.01 min$^{-1}$, and the half-time for the reaction was 31 min.

Determination of Heme Retention During Inactivation. In the presence of PCP and NADPH, P450 2B6 lost approximately 60% of its activity in 40 min (Table 1). P450 2B6 incubated with PCP in the absence of NADPH did not lose activity compared with a control sample incubated without PCP or NADPH. After 40 min, the PCP-inactivated P450 2B6 retained its ability to form a reduced CO complex comparable to that with the control as did the sample incubated with PCP alone. Likewise, the inactivated P450 2B6 was able to retain 88% of its absolute absorbance spectrum at 416 nm. The sample incubated with PCP alone also retained the absolute spectrum compared with control. The ability of the PCP-inactivated sample to retain both its absolute and reduced CO absorption spectra suggest that the heme moiety was not adulterated during the inactivation process.

Partition Ratio. The partition ratio is a measure of efficiency for inactivation. It is defined as the number of molecules of inactivator metabolized per molecule of enzyme inactivated (Silverman, 1996). Reconstituted P450 2B6 was incubated with NADPH and increasing
molar ratios of PCP. EFC O-deethylation activity was measured after the inactivation reaction had gone to completion. The percent activity remaining was plotted versus the ratio of [PCP]/[P450 2B6] as shown in Fig. 2. Extrapolating the intercept of the linear regression of lower PCP concentrations and the horizontal line from saturating PCP concentrations yielded a turnover number of approximately 45.

**Irreversibility of Inactivation.** The 7-EFC O-deethylation activity of P450 2B6 was measured before and after extensive dialysis. Samples inactivated with PCP did not show a return of activity after dialysis (Table 2). However, when fresh reductase was added to half the dialyzed, PCP-treated sample, there was a 15% recovery in activity. A control sample treated with fresh reductase also showed increased activity (raw data not shown). To test if reductase was inactivated by PCP as well, a cytochrome c assay was performed. Here, reductase incubated with PCP, NADPH, and P450 2B6 was not affected in its ability to reduce cytochrome c (data not shown). This demonstrates that inactivation of P450 2B6 by PCP was irreversible by dialysis and primarily affected the P450 and not the reductase.

**Protection by an Alternate Substrate.** An alternate substrate, 7-ethoxycoumarin (7-EC), was included in the primary incubations by dialysis and primarily affected the P450 and not the reductase. This demonstrates that inactivation of P450 2B6 by PCP was irreversible by dialysis and primarily affected the P450 and not the reductase.

**Effect of Exogenous Nucleophiles on Inactivation.** In mechanism-based inactivation, the inactivation event should occur via covalent binding of a reactive intermediate to the active site and not elsewhere on the protein. To test if the reactive intermediate left the active site, a trapping agent, such as glutathione can be included in the reaction mixture. Figure 4 shows that glutathione had very little effect on the rate of inactivation. Glutathione, alone, did not reduce the activity of P450 2B6 (data not shown). It has previously been reported that CN− can trap the PCP iminium ion, a known metabolite of PCP (Ward et al., 1982). Here, CN− had no protective effect against the inactivation of P450 2B6 by PCP.

**Specificity of Binding.** Reconstituted P450 2B6 was incubated with [3H]PCP in the presence or absence of GSH. The control and inactivated samples were dialyzed against cholate-containing buffer to remove all noncovalently bound counts and eliminate nonspecific binding. The samples were subjected to HPLC separation, and fractions were collected and analyzed for radioactivity by liquid scintillation counting. Figure 5 shows that there was some radioactivity associated with the P450, however, upon inactivation, radioactivity associated with the P450 increased by just over 2-fold. Also, the lack of radioactivity associated with the other proteins demonstrates that PCP binds primarily to the P450 during inactivation. In the presence of GSH, the radio-labeled binding was attenuated, although this sample was inactivated to the same extent as the sample incubated in the absence of GSH. Similar results were observed with PCP that contained the 3H-label on the phenyl ring (data not shown).

**Stoichiometry of Binding.** The stoichiometry for the binding of a radiolabeled metabolite of PCP to P450 2B6 was determined by incubating [phenyl-3H(t)]phenylcyclohexane with P450 2B6, with or without NADPH and GSH. The samples were dialyzed extensively before removing aliquots for liquid scintillation counting. The counts remaining in each aliquot were used to calculate the stoichiometry. The control sample, which was incubated without NADPH or GSH, showed a stoichiometry of 0.3 nmol of PCP to 1 nmol of P450 2B6. Upon 50% inactivation in the absence of GSH, the stoichiometry was approximately 5.5 nmol of PCP to 1 nmol of P450 2B6 (Table 3). In the presence of GSH (50% inactivation), the stoichiometry of PCP to P450 2B6 for inactivation was reduced to essentially 1:1. This dem-

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Activity Remaining</th>
<th>% P450 Remaining</th>
<th>% Absolute Spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>40 min</td>
<td>0 min</td>
</tr>
<tr>
<td>−PCP, −NADPH</td>
<td>100</td>
<td>92 ± 11</td>
<td>100</td>
</tr>
<tr>
<td>+PCP, −NADPH</td>
<td>108 ± 10</td>
<td>91 ± 10</td>
<td>88 ± 9</td>
</tr>
<tr>
<td>+PCP, +NADPH</td>
<td>109 ± 17</td>
<td>39 ± 5</td>
<td>84 ± 4</td>
</tr>
</tbody>
</table>

**Effect of PCP on 7-EFC O-deethylation activity, P450 CO-reduced spectrum, and absolute spectrum of P450 2B6**

Reconstituted P450 2B6 was incubated in the presence or absence of PCP and NADPH as described under Materials and Methods. Each value represents the mean and S.D. of three separate experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>After dialysis</td>
<td>100</td>
</tr>
<tr>
<td>After dialysis + fresh reductase</td>
<td>100</td>
</tr>
</tbody>
</table>

*For comparison purposes, the values obtained for the control samples in each case were designated to represent 100% activity.*

**TABLE 2**

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Inactivated</td>
<td>34 ± 14</td>
</tr>
</tbody>
</table>

Each point represents the mean and S.D. from at least three experiments. The partition coefficient was estimated from the intercept of the regression line derived from the lower PCP concentrations and the straight line generated from the higher PCP concentrations.

**TABLE 3**

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Inactivated</td>
<td>100</td>
</tr>
</tbody>
</table>

Stoichiometry of Binding. The stoichiometry for the binding of a radiolabeled metabolite of PCP to P450 2B6 was determined by incubating [phenyl-3H(t)]phenylcyclohexane with P450 2B6, with or without NADPH and GSH. The samples were dialyzed extensively before removing aliquots for liquid scintillation counting. The counts remaining in each aliquot were used to calculate the stoichiometry. The control sample, which was incubated without NADPH or GSH, showed a stoichiometry of 0.3 nmol of PCP to 1 nmol of P450 2B6. Upon 50% inactivation in the absence of GSH, the stoichiometry was approximately 5.5 nmol of PCP to 1 nmol of P450 2B6 (Table 3). In the presence of GSH (50% inactivation), the stoichiometry of PCP to P450 2B6 for inactivation was reduced to essentially 1:1. This dem-

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**FIG. 2.** Loss of P450 2B6 7-EFC O-deethylation activity as a function of the ratio of PCP to P450 2B6.

Each point represents the mean and S.D. from at least three experiments. The partition coefficient was estimated from the intercept of the regression line derived from the lower PCP concentrations and the straight line generated from the higher PCP concentrations.
Reconstituted P450 2B6 was inactivated in the presence of a trace amount of [3H]PCP as described under Materials and Methods. The HPLC separation profile for a typical sample at 280 nm is shown in the solid (---) line. The short-dashed line (——) shows the radioactivity associated with the control sample (+PCP, −NADPH). The stippled line (----) depicts the radioactivity for the inactivated (+PCP, +NADPH) sample.

**Table 3**

**Stoichiometry of binding of PCP to P450 2B6**

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Inactivation</th>
<th>cpm*</th>
</tr>
</thead>
<tbody>
<tr>
<td>+PCP, −NADPH, −GSH</td>
<td>0 ± 6.8</td>
<td>0.3 ± 0.08:1</td>
</tr>
<tr>
<td>+PCP, +NADPH, −GSH</td>
<td>53.33 ± 1.53</td>
<td>5.5 ± 0.44:1</td>
</tr>
<tr>
<td>+PCP, +NADPH, +GSH</td>
<td>55.00 ± 4.36</td>
<td>1.1 ± 0.06:1</td>
</tr>
</tbody>
</table>

*Counts per minute for each sample after subtracting the number of counts for the background or control sample and accounting for percent inactivation.

**Discussion**

It has long been recognized that phencyclidine and its reactive metabolites can covalently bind to microsomal macromolecules (Law and Farquharson, 1980; Ward et al., 1982; and Hoag et al., 1984). Furthermore, Osawa and Coon (1989) showed that PCP could selectively inactivate the rabbit P450 2B isoform in a concentration-, time-, and NADPH-dependent manner. They concluded that activity loss was, in part, due to heme modification of the P450. Later studies with rat P450 2B1 reconstituted in the presence of catalase showed that the P450 heme moiety was not affected by PCP inactivation. It was concluded that inactivation of P450 2B1 was due to modification of the apoprotein (Crowley and Hollenberg, 1995; Sharma et al., 1997). These results suggested that PCP could be an efficient mechanism-based inactivator for human P450 2B6.

In this investigation, PCP inhibited the 7-EFC O-deethylation activity of P450 2B6 in a time- and concentration-dependent manner and demonstrated pseudofirst order kinetics. Furthermore, the inactivation was NADPH-dependent, showing that catalytic turnover was required for inactivation. The \( k_{inact}\), \( K_T\), \( t_{1/2}\), and partition coefficient were 0.01 min\(^{-1}\), 10 \( \mu \)M, 31 min, and 45, respectively. Spectral analysis demonstrated that there was no heme alkylation. This suggested that the inactivation was due to binding of a PCP reactive intermediate to the apoprotein and not the heme. Inactivation experiments carried out in the presence of an alternate substrate suggested that PCP inactivated P450 2B6 by reacting with the active site. In the presence of the alternate substrate, 7-EC, the rate of P450 2B6 inactivation was decreased by about half demonstrating that PCP was competing with 7-EC for metabolism.

It appeared that a metabolite of PCP was covalently bound to the...
P450 active site because extensive dialysis of the inactivated P450 2B6 did not result in the return of enzymatic activity. When fresh reductase was added to the dialyzed sample, there was a slight return of activity. However, the ability of the reductase to reduce cytochrome c was not affected by incubation in the presence of P450 2B6, NADPH, and PCP (data not shown). The small increase in P450 activity upon the addition of fresh reductase could have been due to the increase in the ratio between reductase and P450. Similar results were seen with appropriate control samples (data not shown). This demonstrates that P450 2B6 was the primary target for inactivation by PCP. The specificity of binding was further demonstrated by inactivating the P450 2B6 in the presence of a trace amount of [3 H]PCP. HPLC analysis of this sample showed that the radioactivity was associated with P450 2B6 rather than reductase or catalase. Although there was some radioactivity associated with P450 2B6 in a noninactivated sample, this experiment also showed the requirement for NADPH to obtain significant PCP binding to the protein. The inclusion of 10 mM GSH in the incubation attenuated binding of PCP to P450 2B6 even though this sample was inactivated to the same extent as the sample without GSH.

This effect of an exogenous nucleophile, GSH, and another trapping agent, CN\(^-\), on the rate of inactivation was also investigated. The rate of inactivation was not decreased when the enzyme was incubated with GSH suggesting that the reactive intermediate does not leave the active site. Furthermore, CN\(^-\) had no protective effect on the rate of inactivation. Ward et al. (1982) demonstrated that CN\(^-\) is able to trap the PCP iminium ion. Therefore, our results suggest that the iminium ion may not be the reactive intermediate responsible for inactivation. This conclusion is supported by the fact that the PCP iminium ion can only inactivate P450 2B6 when NADPH is present in the reaction mixture (data not shown). This suggests to us that the iminium ion may be a precursor to the reactive intermediate.

Sharma and coworkers (1997) have previously demonstrated that PCP fulfilled many of the requirements characteristic for a mechanism-based inactivator for rat P450 2B1. In the absence of GSH, the stoichiometry of PCP to P450 2B1 was 4:1. In the presence of GSH, radioactive PCP was not incorporated into the protein, and the rate of inactivation was decreased. It was concluded that PCP was a metabolic inactivator for P450 2B1 where the reactive intermediate could leak out of the active site to be trapped by GSH. In this investigation, we observed a 5.5:1 ratio of PCP to P450 2B6. In the presence of GSH, this stoichiometry was reduced to 1:1. However, GSH had no effect on the rate of inactivation. This suggests that in the absence of GSH, approximately 4 molecules of a PCP reactive intermediate escaped the active site and tightly associated with protein or lipid sites not critical to catalysis. In the presence of GSH, the reactive intermediates that escaped the active site became trapped by GSH whereas the one molecule of reactive intermediate that remained within the active site caused the inactivation. This argument was supported by the LC-MS results. As reported, it was difficult to consistently observe a mass adduct on P450 2B6. However, we did occasionally see an adduct of 244 Da on the P450 2B6 apoprotein. Under the high temperatures and conditions by which ionization of the protein takes place in the instrument, it is likely that the tight interactions between the enzyme and PCP molecules on the surface were dissociated. This resulted in a 1:1 stoichiometry of PCP to P450 2B6 in the absence of GSH. To confirm the size of the mass adduct on P450 2B6, PCP-inactivated P450s 2B1 and 2B4 were also analyzed by LC-MS. In
both cases, a one to one stoichiometry of PCP to P450 was seen. Once again, the reason for the reduced stoichiometry for PCP to P450 2B1 in the absence of GSH compared with what Sharma observed appears to be due to the dissociation of loosely bound intermediates under the ionization conditions. For P450 2B4, the size of the mass adduct corresponded to one PCP molecule plus a hydroxyl group (261 Da).

In conclusion, this study shows that PCP inactivates human P450 2B6 in a mechanism-based manner. We have demonstrated for the first time that there is one PCP bound per P450 2B6 molecule. This result coupled with the strong evidence that PCP modifies the apoprotein of P450 2B6 suggests that PCP can be used to further study the structure of the active site of this enzyme. The demonstration that P450 2B isoforms are located in brain may prove to be of importance with regards to PCP metabolism and enzyme modification in the brain (Tirumalai et al., 1998). Studies are underway to identify the GSH-PCP adduct and PCP metabolites that are formed during turnover to determine the identity of the reactive intermediate responsible for inactivation and to identify the peptide and the amino acid residue in P450 2B6 that is modified by PCP.

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References


