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Phencyclidine Continuous Dosing Produces a Treatment Time-Dependent Regulation of Rat CYP2C11 Function, Protein Expression and mRNA Levels[1]

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ABSTRACT

These studies determined the effects of continuous phencyclidine (PCP) administration on cytochrome P_{450} 2C11 (CYP2C11) function, protein expression and mRNA levels. Male Sprague-Dawley rats received s.c. PCP infusions (18 mg/kg/day) for 1, 3, 10 or 20 days ($n = 4$ per group). Control animals received saline infusions for 3 or 20 days. Livers were collected 24 hr postinfusion, a time when PCP was completely cleared from the animals. In microsomes from the 1- and 3-day PCP infusions, there was a significant decrease ($P < .05$) in CYP2C11 protein expression (61 and 46% of control values, respectively) and in CYP2C11-mediated metabolism of PCP to a reactive metabolite (36 and 41% of control values). Both protein expression and PCP metabolite formation had returned to normal by 10 days of continuous PCP infusion. CYP2C11 function (as measured by 2α-OH testosterone formation) was decreased in the 1-, 3- and 10-day infused rats to 46, 28 and 45% of control values ($P < .05$). CYP2C11 function, expression and reactive PCP metabolite formation returned to normal after 20 days of PCP infusion. In contrast, CYP2C11 mRNA levels were decreased ($P < .05$) in liver tissue in PCP-treated rats from 1 to 20 days (43, 31, 37 and 47%, respectively). These data suggest that continuous PCP infusions initially decrease CYP2C11 function and protein expression by a pretranslational mechanism, but continued exposure to PCP leads to metabolic adaptation without the recovery of mRNA levels. Thus, chronic exposure to PCP can produce time-dependent regulation of CYP2C11-mediated metabolism of endogenous and exogenous compounds.

PCP is metabolized by CYP enzymes to at least six major metabolites in mammals (Holsztynska and Domino, 1983). These metabolites include a highly reactive metabolite that can be detected in rat tissues as covalent adducts after *in vivo* administration of radiolabeled PCP (Law, 1981). Although the number and identity of all the enzymes that metabolize PCP are not known, several different CYP isoforms have been implicated in the formation of the reactive metabolite. In livers from normal male Sprague-Dawley rats, the CYP2C11 isoform is involved in the formation of this metabolite (Shelnutt *et al*., 1996), but this metabolite is not found in female rats because of the absence of the male-specific CYP2C11 isoform. In liver microsomes from phenobarbital-induced rats and rabbits, the nonconstitutive CYP2B1 and CYP2B4 isoforms, respectively, are the major liver enzymes involved in the formation of reactive metabolites. Previous studies suggest this unidentified metabolite produces a mechanism-based inactivation of CYP2B isoforms (*e.g.,* Hoag *et al.*, 1987; Brady *et al.*, 1987; Osawa and Coon, 1989; Crowley and Hollenberg, 1995). Thus, the formation of these PCP metabolite-protein adducts is affected by the sex of the animal and the presence or absence of a metabolic inducer.

Chronic s.c. infusions of PCP for up to 20 days in normal male Sprague-Dawley rats produce complex time- and dose-dependent changes in the *in vitro* metabolism of PCP (Owens *et al.*, 1993). These studies show that the *in vitro* formation of several PCP metabolites (including the irreversibly bound metabolite) is significantly decreased after 1 to 4 days of chronic dosing, but the formation of all of these metabolites is essentially back to normal levels after 20 days of continuous PCP infusion. These data indicate an apparent recovery of CYP function with continued PCP administration. Although a selective mechanism-based inactivation of the major phe-

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ABBREVIATIONS: CYP, cytochrome P_{450}; MK-801, (+)-methyl-10,11-dihydro-5H-dibenzo[a, d]cyclohepten-5,10-imine; NMDA, N-methyl-D-aspartate; PCP, phencyclidine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; T_3, triiodothyronine; TLC, thin-layer chromatography; 2α-OH, 4-androsten-2α,17β-diol-3-one; 6α-OH, 4-androsten-6α,17β-diol-3-one.

nobarbital inducible CYP isoform by PCP in rat and rabbits has been reported (Hoag *et al*., 1984, 1987; Brady *et al.*, 1987; Osawa and Coon, 1989), the restoration of CYP function after continuous PCP exposure in normal rats (Owens *et al*., 1993) suggests PCP could alter CYP function in a manner that is distinct from that of inactivation by a reactive metabolite. For example, PCP could affect CYP isoforms by altering CYP protein function and/or expression.

It has also been determined that chronic PCP infusion can lead to the development of behavioral tolerance (Wessinger and Owens, 1991b). In these studies, PCP infusions in male rats for 10 days (at rates of 10 and 17.8 mg/kg/day) produced significant decreases in the operant behavior for the first several days, but after this initial time period the animal's behavior returned to normal. These time-dependent changes in behavior, and the development of tolerance, could not be explained by PCP pharmacokinetics, because PCP serum steady-state concentrations from day 1 (when steady-state was achieved) until day 10 were constant. In a later study, Owens *et al*. (1993) showed changes in liver function in chronically infused rats (as measured by *in vitro* PCP metabolism) seemed to parallel the time course of PCP-induced behavioral effects and tolerance.

Therefore, our studies were conducted to examine mechanisms underlying the previously reported time-dependent changes in PCP metabolism (Owens *et al.*, 1993). Because CYP2C11 is known to be directly involved in the formation of a PCP reactive metabolite in male rats (Shelnutt *et al*., 1996), we chose to study changes in CYP2C11 function, protein expression and mRNA levels in livers collected from male Sprague-Dawley rats infused with PCP for 1 to 20 days. In addition, we also wondered if a better understanding of the time-dependent changes in metabolism could provide some insight into the time-dependent changes in PCP-induced behavioral effects in chronically treated rats (Wessinger and Owens, 1991b).

Materials and Methods

Materials. Phencyclidine hydrochloride and [^{3}H]PCP (1-{1-[phenyl-3-^{3}H(n)]cyclohexyl}piperidine, 15.69 Ci/mmol) were supplied by the National Institute on Drug Abuse (Rockville, MD). All chemicals were obtained from Fisher Scientific (Springfield, NJ), unless otherwise noted. D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase, β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), ethylenediaminetetraacetic acid (EDTA), a goat anti-mouse IgG (alkaline phosphate conjugate), T_3, testosterone, 4-androstene-3,17-dione, 5α-androstane-3β,17β-diol and 5α-androstan-17β-ol-3-one were obtained from Sigma Chemical Co. (St. Louis, MO). Trichloroacetic acid (10%) was obtained from Baxter Scientific Products (Grand Prairie, TX). Ecoscint A scintillation cocktail was purchased from National Diagnostics Inc. (Atlanta, GA). The GF/B filters and filtration device (model M24R) were obtained from Brandel Laboratories (Gaithersburg, MD). Osmotic pumps for s.c. implantation were obtained from ALZA Corp. (Palo Alto, CA). The pumps used were models 2ML2 (capable of up to 14 days of infusion) and 2ML1 (capable of up to 7 days of infusion). The testosterone metabolites 4-androsten-7α,17β-diol-3-one, 4-androsten-16α,17β-diol-3-one, 4-androsten-6α,17β-diol-3-one, 4-androsten-6β,17β-diol-3-one (6β-OH), 4-androsten-2β,17β-diol-3-one and 4-androsten-2α,17β-diol-3-one (2α-OH) were purchased from Steraloids Inc. (Wilton, NH). TLC plates were Whatman high performance glass-backed, prechanneled silica plates with a preadsorbent strip (Fisher Scientific).

Polyacrylamide and N, N′-methylene-bis-acrylamide were purchased from U.S. Biochemical Corporation (Cleveland, OH). SDS-PAGE was performed using a Mighty Small II SE 250 Vertical Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, CA). Alkaline phosphatase color development reagents 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt, p-nitro blue tetrazolium chloride and molecular weight markers were obtained from Bio-Rad Laboratories (Hercules, CA). The Mini Trans-Blot Electrophoretic transfer cell was purchased from Bio-Rad. A monoclonal anti-rat CYP2C11 antibody (IgG isotype) for Western blot analysis was obtained from Oxford Biomedical Research, Inc. (Oxford, MI).

Northern and slot blots were probed with a CYP2C11 oligonucleotide that was complementary to nucleotides 925–954 of the CYP2C11 coding sequence (Waxman, 1991a) and a full-length chicken β-actin cDNA. The CYP2C11 oligonucleotide and the β-actin cDNA were radiolabeled with [γ-^{32}P]ATP (specific activity 6000 Ci/mmol) and [α-^{32}P]dATP (specific activity 6,000 Ci/mmol), respectively, obtained from Du Pont NEN Research Products (Boston, MA).

Animals. Adult male Sprague-Dawley rats (approximately 360 g) were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN), and were allowed to acclimate to their new environment for at least 1 wk. All animal experiments in these studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

PCP dosing. Osmotic pumps for drug or saline (control) infusions were implanted s.c. according to the manufacturer's directions while under ether anesthesia as previously described (Owens *et al*., 1993). A dose of 18 mg/kg/day of PCP was used for all treatment groups. This is a pharmacologically active dose that produces steady-state serum levels of approximately 180 ng/ml. This dose was chosen because it produces moderate suppression of spontaneous motor activity and other behavioral effects for 3 to 4 days in male Sprague-Dawley rats (Wessinger and Owens, 1991b), and because it is the same dose used in our previous studies of time- and dose-dependent metabolic effects of PCP (Owens *et al*., 1993).

Rats from the treatment groups were infused with PCP for either 1, 3, 10 or 20 days. Control rats for the 1- and 3-day PCP-infused animals were given a 3-day infusion of saline. Control rats for the 10- and 20-day PCP-infused rats were given a 20-day infusion of saline (n = 4 for all groups).

Seven-day osmotic pumps were implanted in the animals that received a 1- or 3-day infusion. Fourteen-day osmotic pumps were implanted in animals that received a 10- or 20-day infusion. Animals that received a 20-day infusion were implanted with one 14-day pump, which was then replaced with another 14-day pump after 10 days of infusion. The new pump was inserted at a different location on the back of the rat. All pumps were surgically implanted and removed (after the predetermined infusion period) at the same time of day. Animals were killed and livers removed for microsome and RNA preparation 24 hr after pump removal. Because the half-life of PCP is 3.9 hr in male Sprague-Dawley rats (Valentine *et al.*, 1994), the 24-hr waiting period allowed time for essentially complete elimination of PCP from the animals (*i.e.,* more than six half-lives of the drug). The absence of PCP in serum samples collected at the time of microsome preparation (24 hr after removal of the pumps) was shown in our previous studies (Owens *et al*., 1993).

Preparation of liver microsomes and PCP metabolite irreversible binding. Liver microsomes were prepared from liver sections immediately after the animals were killed using standard methods described elsewhere (Owens *et al.*, 1993). The microsomes were stored frozen at -80°C until needed. CYP content was determined by the method of Omura and Sato (1964) as modified by Johannesen and DePierce (1978). The Pierce Coomassie Protein Reagent Assay (Rockville, IL) was used to determine microsomal protein concentrations, with bovine serum albumin as the protein standard. PCP irreversible binding was determined as previously described (Shelnutt *et al*., 1996). Briefly, irreversible binding was assessed by incubating liver microsomal proteins (at 2 mg/ml) with 1

μM PCP and [^{3}H]PCP as a tracer (approximately 1×10^6 dpm) for 20 min at 37°C in a complete NADPH regenerating system (or without $NADP^+$ to determine nonspecific binding). Microsomal proteins were precipitated by the addition of 1 ml of ice cold 10% trichloroacetic acid. The proteins were then filtered through glass fiber filters using a Brandel cell harvester, and washed once with 10% trichloroacetic acid followed by repeated washings with 40% ethanol, until no further radioactivity was removed. The radioactive decays per min remaining on the filter were determined in a liquid scintillation counter using the instruments external standards correction procedure (Packard Instruments, Downers Grove, IL).

Function of the CYP2C11 and CYP3A2 isoforms. CYP2C11 and CYP3A2 isozyme function was determined using the method of Waxman (1991a, b). The formation of the 2α-OH testosterone metabolite was used to assess CYP2C11 function and the formation of the 6β-OH testosterone metabolite was used to assess CYP3A2 function. Briefly, 9.5 nmol of testosterone (containing 8.36 nmol of unlabeled testosterone plus 1.14 nmol of 4-[^{14}C]testosterone, or ~200,000 dpm) were incubated with 0.2 mg/ml of microsomal protein and a complete NADPH regenerating system in a total volume of 190 μl. At the end of a 10-min incubation period, the reaction was stopped by adding ethyl acetate, vortexing and placing the tube in an ice bath. The ethyl acetate layer was transferred to a clean test tube. The samples were taken to dryness and resuspended in 25 μl of ethyl acetate. The samples were spotted on TLC plates and allowed to dry. The TLC plates were then run twice in a solvent system consisting of methylene chloride and acetone (80:20, v/v). The plates were exposed to a storage phosphor screen for 2 hr and then were scanned with the laser optical imager to locate areas of radioactivity. Radioactivity in areas corresponding to the migration of testosterone and the 2α-OH and 6β-OH metabolite standards were quantitated and expressed as a percentage of the total radioactivity in that lane. Authentic standards of testosterone, 4-androstene-3,17-dione, 5α-androstane-3β,17β-diol, 5α-androstan-17β-ol-3-one, 2α-OH, 6α-OH, 7α-OH (4-androsten-7α,17β-diol-3-one), 16α-OH (4-androsten-16α,17β-diol-3-one), 2β-OH (4-androsten-2β,17β-diol-3-one), and 6β-OH, (4-androsten-6β,17β-diol-3-one) were also spotted at the origin along with the samples to ensure complete chromatographic separation and to allow identification of radioactive metabolites.

Western blots. Microsomal proteins (1 μg) were subjected to SDS-PAGE on a 10% polyacrylamide gel for 1.5 hr at 120V. The proteins were transferred to nitrocellulose filters at 85V for 45 min at 4°C using a prechilled 25 mM Tris, 192 mM glycine, 20% methanol (v/v, pH 8.3) transfer buffer. Anti-CYP2C11 antibody and a goat anti-mouse IgG (alkaline phosphatase conjugate) were used to detect CYP2C11 protein on the filters, according to the method of Waxman (1991a). The protein band was then detected with Bio-Rad color development reagents following the manufacturer's directions. Approximate protein molecular weights were determined based on their relative electrophoretic mobility in comparison with pre-stained molecular weight markers. The relative intensity (or amount) of the anti-CYP2C11 protein in each lane was measured using a Bio-Rad GS-670 Imaging Densitometer. In preliminary assay validation experiments, the anti-CYP2C11 antibody detected only one band of immunoreactivity at the correct molecular size for CYP2C11, and there was a linear relationship between the relative protein band intensity and the amount of rat liver microsomal protein applied to the gel (microsomal protein range of 0.2 to 1 μg/lane, $r = 0.99$).

mRNA analysis. Total cellular RNA was prepared from livers using TriReagent 228 (Molecular Research Center, Cincinnati, OH) according to the manufacturer's directions. For Northern blot analysis, RNA was subjected to electrophoresis on 1% agarose/6% formaldehyde gels followed by transfer to Magna NT nylon membranes (Micron Separations, Inc., Westboro, MA) by capillary blotting with sodium chloride/sodium citrate buffer (3 M NaCl, 0.3 M Na_3 citrate · 2 H_2O (88 g/liter) adjusted to pH 7.0 with 1 M HCl) as previously described (McGehee *et al*., 1990). mRNA sizes were estimated by comparison with the migration of a 0.24- to 9.5-kb RNA ladder (Betheseda Research Laboratories, Gaithersburg, MD) treated in parallel with RNA samples. For slot blot analysis, RNA was diluted in 50% deionized formamide/6% formaldehyde and was applied to Magna NT nylon membranes (Micron Separations, Inc., Westboro, MA) using a MilliBlot-S slot blot system (Millipore Corp., Bedford, MA). Membranes were baked under vacuum at 80°C for 1 hr and were probed with a CYP2C11 oligonucleotide (5′ATCCACGTGTTTCAGCAGCAGCAGGAGTCC-3′) as previously described (Shimada *et al*., 1989; Waxman, 1991a; Kraner *et al*., 1994). For sequential hybridization with the β-actin cDNA, radioactivity was removed from membranes by boiling for 20 min in 250 mM Tris (pH 8.0), 100 mM $Na_4P_2O_7$, 10 mM EDTA and Denhardt solution (0.1 g Ficoll 400, 0.1 g polyvinylpyrrolidone, 0.1 g bovine serum albumin in 500 ml H_2O). mRNA species were visualized either by a PhosphoImager (Molecular Dynamics, Sunnyvale, CA) after exposure of membranes to storage phosphor screens or by autoradiography using Kodak X-OMAT film and intensifying screens. For quantitation, the densitometric volume of bands obtained on the PhosphorImager were calculated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Testosterone and T_3 serum levels. Sera from treated and control animals were obtained for analysis at the time of liver collection and samples were stored frozen at -20°C until needed. Testosterone and T_3 concentrations in serum were determined using the testosterone RIA kit and the total T_3 Coat-a-Count RIA kit, respectively (Diagnostic Products Corp., Los Angeles, CA). A standard curve for T_3 analysis was prepared according to the manufacturer's directions using charcoal stripped rat sera.

Statistics and data analysis. All values are reported as the mean ± S.D. In all statistical analyses, the 1- and 3-day PCP-infused rats were compared with the short-term infusion control group (3 day saline-infused rats), and the 10- and 20-day PCP-infused rats were compared with the long-term infusion control group (20-day saline-infused rats). It should be noted that the results of the 3- and 20-day saline-infused rats were not significantly different from each other in any experiment. Statistical analysis was performed using a one-way analysis of variance. If a significant difference was found, a *post hoc* Dunnett's test was used to determine which groups differed from controls. Statistical significance of the slopes of lines determined by linear regression analysis were performed using a *t* test. Significance was determined at the $P < .05$ level in all experiments. The statistical software package SigmaStat (Jandel Corp., San Rafael, CA) was used for all statistical analyses.

Results

Effects of PCP infusions on liver CYP content and PCP metabolite irreversible binding. The total CYP content for the 1-, 3- and 10-day PCP-infused groups was significantly decreased compared to control values ($P < .05$, table 1). The formation of the reactive PCP metabolite in the 1- and 3-day PCP-infused rats decreased to 36 ± 17 and 41 ± 21% of the 3-day saline-infused control animals, respectively ($P <$

TABLE 1

CYP content in PCP-infused male rats

All values are mean (±S.D.).

Control Groups	Treatment Groups	CYP Content (nmol CYP/mg Protein)
3-day saline infusion		1.3 ± (0.1)
	1-day PCP infusion	0.8 ± (0.11)[a]
	3-day PCP infusion	0.71 ± (0.04)[a]
20-day saline infusion		1.2 ± (0.23)
	10-day PCP infusion	0.73 ± (0.08)[b]
	20-day PCP infusion	1.02 ± (0.15)

[a] $P < .05$, compared to 3-day saline-infused rats.
[b] $P < .05$, compared to 20-day saline-infused rats.

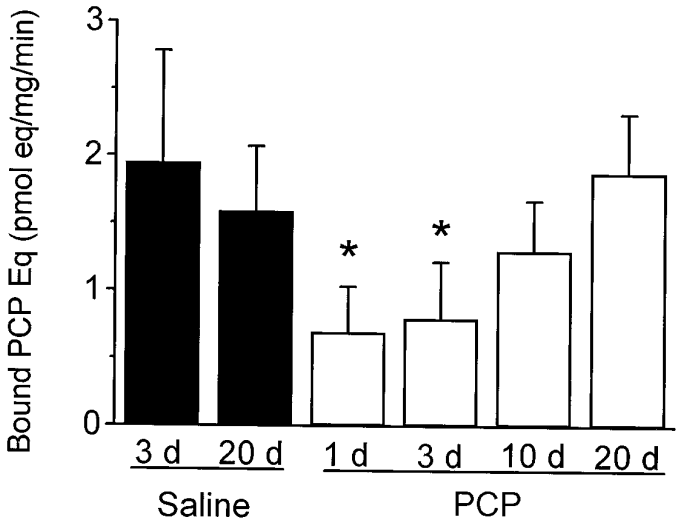

Fig. 1. Formation of irreversibly bound PCP metabolites in liver microsomes prepared from PCP-infused male rats. Male rats received a 1-, 3-, 10- or 20-day infusion of PCP (abbreviated as 1d PCP, 3d PCP, 10d PCP and 20d PCP, respectively). Control rats received saline for 3 or 20 days (abbreviated as 3d Sal and 20d Sal). Microsomes were incubated with PCP and the formation of the metabolite-protein adducts was quantitated. Statistical analysis was performed using a one-way analysis of variance followed by Dunnett's test (*P $<$ .05). Data are presented as the mean $\pm$ S.D. (n = 4 per group).

.05, fig. 1). In contrast, the amount of the reactive metabolite formed in the rats given PCP infusions for 10 and 20 days did not differ from the 20-day saline-infused control values.

The constant dosing protocol in our study was designed to reproduce the experimental conditions of a previous study in our laboratory (Owens *et al.*, 1993). For comparison of results, we first calculated the percentage of control binding by dividing the amount of PCP metabolite irreversible binding (in pmol eq/mg/min) in PCP-treated groups by the binding in matched saline control groups. A linear regression analysis of these percent of control data showed a coefficient of determination (r^2) of 0.99 for data from the two studies. This indicated a highly reproducible experimental result. The only significant experimental difference in the two studies was the use of 1 μM PCP for *in vitro* metabolism studies in our study, compared to 100 μM in the previous study (Owens *et al.*, 1993). Nevertheless, this experimental difference in the dose did not affect the comparison because the metabolite irreversible binding is linear over an extended range of doses with a K_m value of 180 μM (Owens *et al.*, 1993). We used the lower dose in our study to match the predicted blood concentrations of PCP at an infusion rate of 18 mg/kg/day (Wessinger and Owens, 1991a). However, more recent studies of liver concentrations of PCP after i.v. bolus doses of PCP suggest that concentrations in the liver are significantly greater than blood concentrations (Valentine and Owens, 1996). Therefore, *in vitro* doses of PCP over the range of 1 to 100 μM would reflect the full range of liver concentration found after pharmacologically active doses of PCP.

Changes in CYP isoform function after PCP infusions. Continuous infusions of PCP also produced significant infusion time-dependent effects on the function of CYP. CYP2C11 function (assessed by 2α-OH formation) in microsomes prepared from animals infused with PCP for 1, 3 or 10 days decreased significantly to 46 $\pm$ 18, 28 $\pm$ 18 and 45 $\pm$ 5%, respectively, when compared to the values in matched control animals (P $<$ .05, fig. 2). However, CYP2C11 function in microsomes prepared from animals infused with PCP for 20 days was not different from the 20-day saline-infused control animals.

The testosterone metabolism assay used in these studies allowed for the simultaneous quantitation of nine metabolites. Although these studies were originally designed to evaluate the effects of PCP on just CYP2C11 function (by quantitating the formation of 2α-OH), a visual inspection of the metabolite TLC spots after PhosphorImager analysis showed that the formation of 6β-OH (catalyzed by CYP3A2) also changed after the PCP infusions. Therefore this metabolite was included in the analysis. The changes in CYP3A2 function followed a different pattern than the changes in CYP2C11 function. CYP3A2 function appeared almost nonexistent in the rats given a 3-day PCP infusion, because there was no significant formation of 6β-OH (P $<$ .05, fig. 3). However, CYP3A2 function was not different from control values in the rats given a 1-, 10- or 20-day infusion of PCP.

Western blot analysis of CYP2C11 protein expression in microsomes from PCP-infused animals. Liver microsomes from three animals randomly selected from each treatment and control group were analyzed for CYP2C11 content by Western blot analysis. The amount of CYP2C11 protein decreased significantly to 61 $\pm$ 3.7 and 46 $\pm$ 15% of 3-day saline-infused control values in microsomes prepared from 1- and 3-day PCP-infused rats, respectively (P $<$ .05, fig. 4). The amount of CYP2C11 protein in microsomes prepared from rats administered 10- and 20-day PCP infusions did not differ from 20-day saline-infused control values. A linear regression analysis of the relationship between CYP2C11 protein expression and CYP2C11 function (as measured by 2α-OH formation) showed the slope was significantly different from zero (P $<$ .05) with an r value of 0.74.

Northern and slot blot analysis of CYP2C11 mRNA in livers from PCP-infused animals. Total cellular RNA iso-

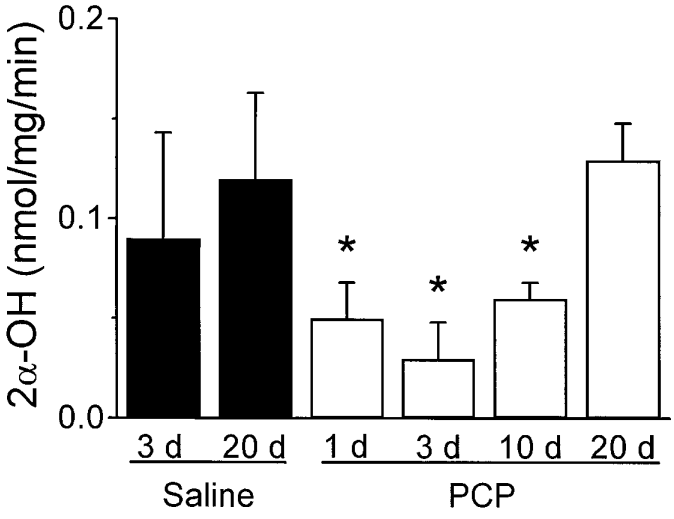

Fig. 2. Formation of 2α-OH testosterone (catalyzed by CYP2C11) in microsomes prepared from control and PCP-infused male rats. Microsomes were incubated with testosterone and the formation of 2α-OH was assessed by a TLC method. Statistical analysis was performed using a one-way analysis of variance followed by Dunnett's test (*P $<$ .05). Data are presented as the mean $\pm$ S.D. n = 4 for all groups except for the 10- and 20-day PCP groups, where n = 3 because of a technical error.

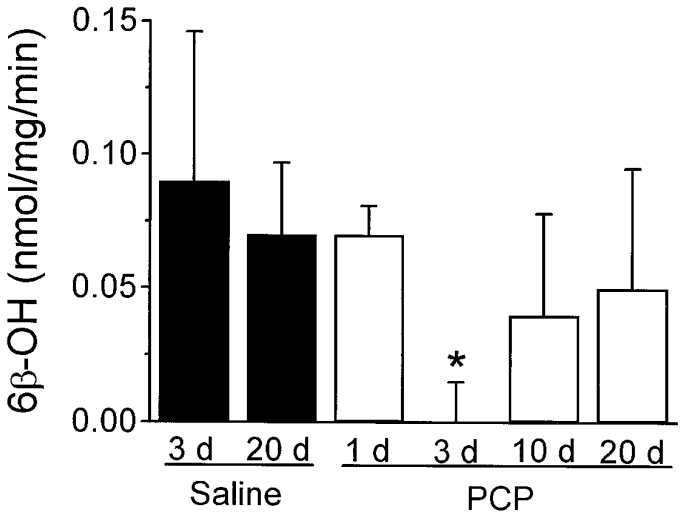

Fig. 3. Formation of 6β-OH testosterone (catalyzed by CYP3A2) in liver microsomes prepared from control and PCP-infused male rats. Microsomes were incubated with testosterone and the formation of 6β-OH was assessed by a TLC method. Data are presented as the mean ± S.D., *P < .05. n = 4 for all groups except for the 10- and 20-day PCP groups, where n = 3 because of a technical error.

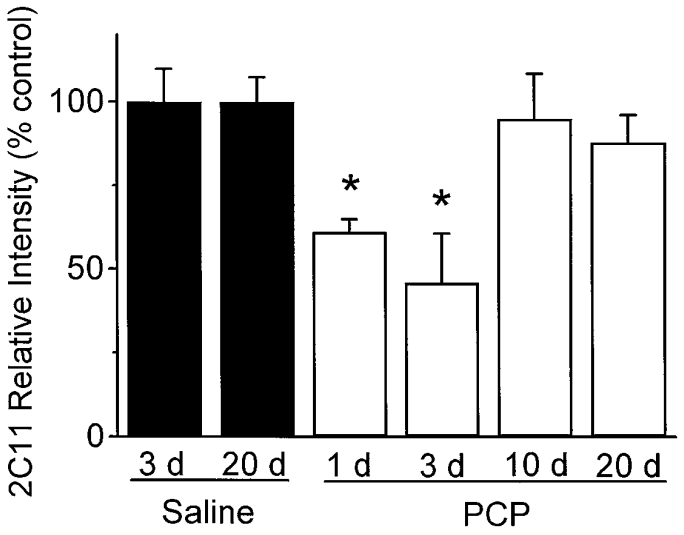

Fig. 4. Western blot analysis of microsomal CYP2C11 protein content. The Western blots were performed using liver microsomal proteins from three randomly selected animals from all groups. The relative intensity of the CYP2C11 band in microsomes from 1- and 3-day PCP-infused rats was calculated as a percentage of the levels in the 3-day saline-infused rats. The relative intensity of the CYP2C11 band in microsomes from the 10- and 20-day PCP-infused rats was calculated as a percentage of the levels in the 20-day saline-infused rats. Data are presented as the mean ± S.D., *P < .05.

lated from livers of rats given either a 1-, 3-, 10- or 20-day PCP infusion and rats given saline infusions for either 3 or 20 days was subjected to both Northern and slot blot analysis to determine CYP2C11 mRNA levels. In a representative Northern blot probed with the CYP2C11 nucleotide, a single 2.0-kb mRNA was observed (fig. 5), the expected size of the primary transcript from the CYP2C11 gene (Janeczko *et al.*, 1990). The same blot, stripped and reprobed with the β-actin cDNA, resulted in a single 2.2-kb band (fig. 5).

To quantitate CYP2C11 mRNA levels for statistical analysis of treatment groups, total cellular RNA isolated from

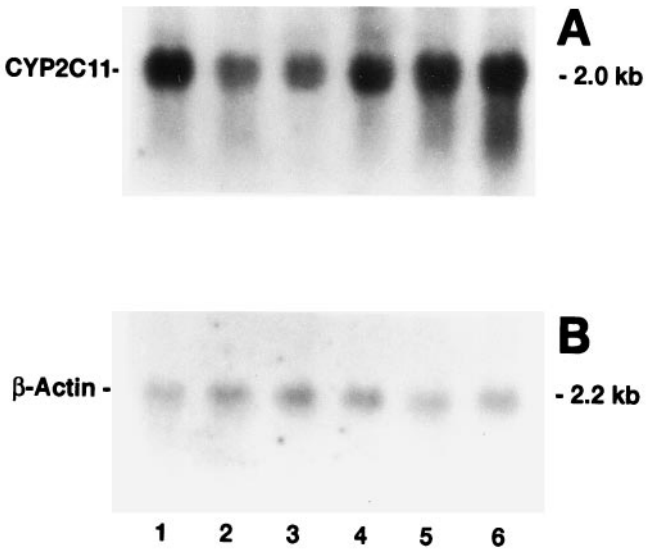

Fig. 5. Effect of continuous PCP infusion on rat liver CYP2C11 mRNA levels. Total cellular RNA was isolated from livers of rats that had been infused with either saline for 3 days (lane 1) and 20 days (lane 6) or PCP for 1 day (lane 2), 3 days (lane 3), 10 days (lane 4) and 20 days (lane 5) and then killed 24 hr after the removal of the minipumps. Blots were hybridized sequentially with probes for CYP2C11 (A) and β-actin (B) mRNA. This figure is representative of four experiments which showed similar results. These Northern blots were performed to confirm the integrity of the RNA sample. Slot blot analysis (fig. 6) was performed to quantitate CYP2C11 mRNA levels for statistical analysis of treatment groups.

saline- and PCP-infused rats was analyzed by slot blot analysis followed by densitometric scanning of bands. Steady-state levels of hepatic CYP2C11 mRNA were significantly (P < .05) reduced to 43.3 ± 19.5, 30.6 ± 12.2, 36.8 ± 18.9 and 47.4 ± 10.2 of the control values in rats given a 1-, 3-, 10- or 20-day PCP infusion, respectively (fig. 6).

Serum T_3 and testosterone concentrations. T_3 and testosterone serum concentrations were measured in all animals to determine if levels of these hormones were altered by chronic PCP infusions. T_3 levels averaged 172 ± 23 ng/dl, and there were no significant differences between groups. Testosterone levels averaged 0.66 ± 0.53 ng/ml, and were also not different between groups. It should be noted that the testosterone assay is designed for use with human serum samples, and the results obtained with rat sera should be considered semiquantitative.

Discussion

These studies were designed to better understand the mechanisms underlying time-dependent changes in PCP liver metabolism after continuous dosing. These data showed that shorter duration infusions (1 and 3 day) lead to a significant reduction in PCP metabolite irreversible binding (fig. 1), along with significant reductions in CYP2C11 function (fig. 2), protein expression (fig. 4) and mRNA levels (fig. 6). Longer duration infusions (10 to 20 days) resulted in the eventual full recovery of CYP2C11 function and protein expression, but not CYP2C11 mRNA levels.

The recovery of PCP metabolism (as measured by PCP metabolite irreversible binding) and CYP2C11 function and protein expression with continued PCP exposure indicated an adaptive change. The significant fluctuations in CYP2C11

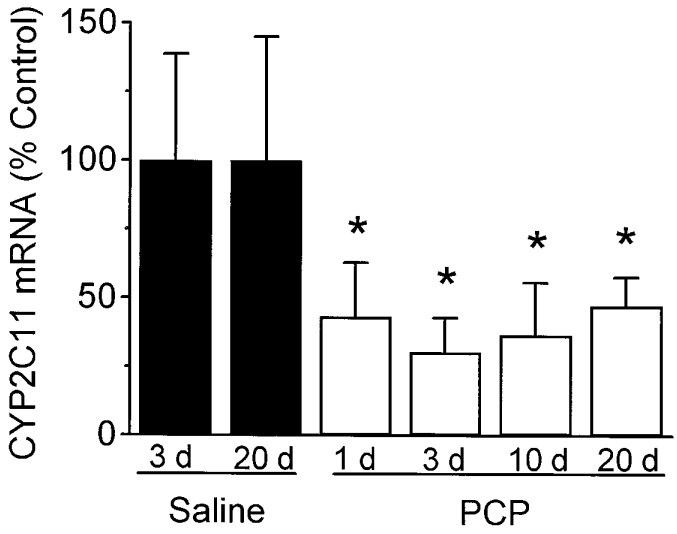

Fig. 6. Effect of continuous PCP infusion on rat liver CYP2C11 mRNA levels. Total cellular RNA was isolated from livers of rats that had been infused with either saline or PCP for the indicated periods and then killed 24 hr after the removal of the minipumps. After Northern blot analysis to assume the integrity of the mRNA, slot blot analyses were performed to produce intensity values (in arbitrary units) for quantitation. Blots were hybridized sequentially with probes for CYP2C11 and β-actin mRNA. The values for CYP2C11 mRNA were normalized with the values for β-actin mRNA and are presented as a percentage of the respective control animals (3-day saline-infused for the 1- and 3-day PCP-infused rats and 20-day saline-infused for the 10- and 20-day PCP-infused rats). Statistical analysis of the data was by a one-way analysis of variance comparing the treatment groups to each other (n = 4 per group, $P < .05$).

function and expression occurred even though PCP pharmacokinetics during continuous infusions are known to be first-order (Wessinger and Owens, 1991a, b). Indeed, detailed pharmacokinetic studies in male Sprague-Dawley rats show steady-state PCP serum concentrations and systemic clearance values are constant (181 ng/ml and 77 ml/min/kg, respectively) from 1 to 10 days after the start of a continuous infusion via s.c. implanted pumps (Owens *et al*., 1993). Furthermore, 24 hr after the pumps are removed (as in this experiment) the serum concentrations are <1 ng/ml. Although PCP and total metabolite elimination are essentially complete in the blood and urine in <24 hr (Valentine *et al*., 1994), the presence of a very minor radiolabeled compound (probably a metabolite of PCP or a [^{3}H]PCP breakdown product) can be detected for an extended period of time. The $t_{1/2}$ of this compound(s) is about 100 hr.

Regardless of the stability of PCP serum pharmacokinetics, significant time-dependent metabolic changes are occurring during the first several days of continuous dosing. In addition to the changes in PCP metabolite irreversible binding (fig. 1), *in vitro* formation of at least three major mono or dihydroxylated PCP metabolites show a similar time-dependent pattern of change (Owens *et al*., 1993). These metabolites are trans-1-(1-phenyl-4-hydroxycyclohexyl)piperidine; trans-4-(4′-hydroxypiperidino)-4-phenylcyclohexanol and cis-4-(4′-hydroxypiperidino)-4-phenylcyclohexanol.

The changes in CYP2C11 function (figs. 1 and 2) and expression (fig. 4) could help to explain the effects of PCP dosing on the *in vitro* metabolism of other chemicals and drugs. For instance, Hiratsuka *et al.* (1995) pretreated adult male Sprague-Dawley rats with PCP (25 mg/kg/day, i.p.) for 2 days, killed the animals at 3 and 16 hr after the last dose and prepared liver microsomes. The authors showed *in vitro* metabolism of several drugs was decreased, possibly due to changes in CYP2C11 and/or CYP2D isoforms. They also found that at the 16-hr time point, the *in vitro* metabolism of PCP was increased by 240%, even though the formation of five major metabolites of PCP were decreased. These results suggest PCP may induce CYP isoforms that contribute to its metabolism, as well as inhibit or inactivate other CYP isoforms. These data also illustrate the complex actions of PCP and the effect of different dosing schedules on CYP function and expression.

Although we did not originally plan to study changes in other CYP isoforms, we noticed that the formation of another testosterone metabolite appeared to be affected by PCP dosing. The formation of 6β-OH (catalyzed by CYP3A2) was also greatly affected. In fact, 6β-OH formation was almost undetected in microsomes prepared after the 3-day PCP infusion (fig. 3). In contrast, 6β-OH formation did not differ significantly from control values in the other treatment groups. Because the loss of CYP2C11 and CYP3A2 function occurred at different times (compare figs. 2 and 3) and the CYP3A2 isoform does not appear to be involved in the formation of the reactive metabolite (Shelnutt *et al*., 1996), these data suggest there could be a different mechanism involved in the temporal loss of function for these two isoforms. This is not unprecedented, as chloramphenicol causes changes in the function of CYP isoforms through a mechanism-based inactivation of CYP2C11, CYP2C6, CYP2B1/2 and CYP3A1/2, and by changing hormone levels that alter the expression of CYP2C11 (Halpert *et al.*, 1985; Halpert *et al.*, 1988; Kraner *et al*., 1994). Unlike chloramphenicol, PCP infusions in the current study did not affect testosterone or T_3 serum concentrations.

Chronic PCP infusion decreased the total CYP content of the microsomes (table 1). This finding was not unexpected for several reasons. First, CYP2C11 accounts for approximately 20 to 30% of the total amount of CYP in male rat livers (Yamazoe *et al*., 1986). Because the amount of CYP2C11 protein was decreased to approximately one-half after 1 and 3 days of PCP exposure (fig. 4), this could account for some of the decrease in total CYP content. Second, *in vitro* incubations of PCP with liver microsomes from phenobarbital-induced animals results in a 20 to 30% decrease in microsomal CYP content (Hoag *et al.*, 1984; Osawa and Coon, 1989). Osawa and Coon (1989) attributed the loss in CYP content to the binding of the reactive metabolite to the heme moiety of the isoforms, because modified heme molecules were isolated from the *in vitro* reaction mixtures. Although studies of *in vitro* metabolism of PCP with microsomes from phenobarbital-induced rats is not necessarily pertinent to the results from normal animals (as in our studies), if the reactive PCP metabolite binds to the heme moiety of CYP in PCP-infused rat, this could lead to decreased detection by spectrophotometric analysis. A loss of total CYP content was not detected in our previous study of chronically infused PCP rats (Owens *et al*., 1993). The reason for this is not known, but total CYP content is not a good predictor of changes in individual CYP isoforms.

In vivo administration of PCP to rats could affect CYP2C11 function by a mechanism-based inactivation, or it could decrease CYP2C11 function by altering its expression at a pretranslational step, or it could act by both mechanisms.

These data showed there were decreases in CYP2C11 mRNA levels along with decreases in CYP2C11 function and protein expression (see composite graph, fig. 7). This indicates PCP affects CYP2C11 function at a pretranslational step. Based on the CYP2C11-mediated formation of 2α-OH testosterone in microsomes collected on day 11 (after a 10-day PCP infusion; see figs. 2 and 7), the amount of CYP2C11 protein was greater than expected. If the reactive PCP metabolite binds to and inactivates CYP2C11, the inactivated protein would most likely still be detected on Western blot analysis. This could account for the discrepancy between the amount of CYP2C11 protein detected and the amount of 2α-OH formed, but it does not explain the good correlation between the amount of CYP2C11 protein and the amount of PCP irreversible binding from days 1 to 20. Consequently, if a mechanism-based inactivation of CYP2C11 is occurring, it is not a major factor. Indeed, these data suggest that PCP does not produce an *in vivo* mechanism-based inactivation of CYP2C11.

Even if the reactive metabolite inactivates CYP2C11 by forming a CYP2C11 adduct, the question still remains as to why CYP2C11 function (as measured by 2α-OH formation) was completely restored after 20 days of a PCP infusion (fig. 2). A clue to the answer might be obtained by evaluating the time course of CYP2C11 recovery at several time points after stopping the infusion (fig. 7). For example, there might be a faster turnover of PCP-CYP2C11 adducts after 20 days of PCP infusion than after 3 days of PCP infusion. The normal turnover time for CYP2C11 is about 20 hr (Correia, 1991). An increase in turnover time could cause CYP2C11 function to be restored at 24 hr after a 20-day infusion, but to be still depressed at 24 hr after a 3-day infusion. A change in CYP turnover time after xenobiotic administration is not unprecedented. Phenobarbital increases the half-life of CYP2C11 from 20 to 35 hr, even though phenobarbital does not induce CYP2C11 expression (Shiraki and Guengerich, 1984).

Regardless of the importance, or the lack of importance, of *in vivo* PCP covalent binding in normal male rats, these studies showed PCP significantly alters *in vivo* CPY2C11 function, expression and mRNA levels in a time-dependent fashion, without significantly affecting its own systemic clearance. Because CYP2C11 accounts for 20 to 30% of the CYP liver enzymes in male rats, the down-regulation of CYP2C11 could have a profound impact on the clearance of other drugs and endogenous compounds (such as steroids) that are metabolized by this important isoform (*i.e.,* a drug interaction).

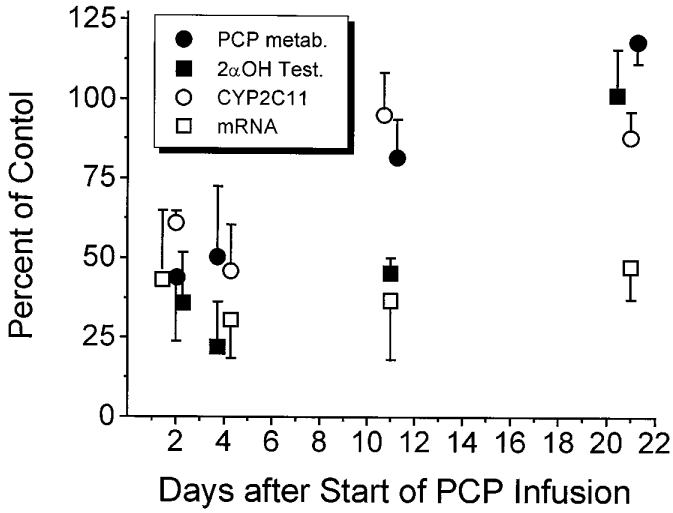

Fig. 7. Composite cluster graph of the data from figures 1, 2, 4 and 6. These data show the overall time-course of the effects of continuous PCP infusions on *in vitro* PCP reactive metabolite formation, CYP2C11 function and protein expression, and CYP2C11 mRNA levels 1 day after completion of the 1-, 3-, 10- or 20-day PCP infusions at 18 mg/kg/day (*i.e.,* days 2, 4, 11 or 21). Data are presented as the mean ± S.D. Data points at each day are spread out to allow visualization of error bars.

Another important finding of these studies is that the time course of changes in CYP2C11 follows the same time course and dose dependency as PCP-induced behavioral effects and tolerance (Wessinger and Owens, 1991b). CYP2C11 liver expression in the male rat is primarily controlled by the pulsatile central nervous system-mediated release of growth hormone (see Shelnutt *et al*., 1996). It is also known that MK-801 ((+)-methyl-10,11-dihydro-5H-dibenzo[a, d]cycloheptene-5,10-imine) can inhibit the release of growth hormone (Cocilovo *et al*., 1992), presumably through its effects on the central nervous system NMDA receptor. Because PCP is a noncompetitive antagonist of the NMDA receptor (Lodge *et al.*, 1983), as is MK-801, PCP may modulate CYP2C11 expression through central nervous system control of growth hormone release. To test this hypothesis, we conducted a preliminary study of the effects of chronic PCP dosing on the pulsatile release of growth hormone in four male rats (two saline infused animals and two PCP-treated animals, results not shown). Compared to the matched saline-infused control animal, growth hormone concentrations were decreased in PCP-treated rats after a 3- and 10-day PCP infusion. If our hypothesis is correct, we also suspect that PCP-induced changes in the pulsatile release of growth hormone could be a clue to mechanism(s) responsible for the time- and dose-dependent changes in behavior and tolerance (Wessinger and Owens, 1991b).

In conclusion, these studies represent a useful step toward understanding the consequences of chronic PCP use. However, we do not think that covalent binding is part of the mechanism of the adverse *in vivo* effects; rather it is a useful *in vitro* marker that parallels the up- and down-regulation of the expression of CYP2C11. There are many reports in the literature concerning the importance of PCP covalent binding (*e.g.,* Law, 1981; Hoag *et al.*, 1987; Brady *et al.*, 1987; Osawa and Coon, 1989). We think our studies may add a new perspective and interpretation to the actual *in vivo* relevance of this metabolic pathway.

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