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# Selective Inactivation of Mouse Liver Cytochrome P-450IIIA by Cannabidiol

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**SUMMARY**

Cannabidiol (CBD) inhibits hepatic drug metabolism in mice, particularly those activities known to be catalyzed by the cytochrome P-450IIIA (P-450IIIA) subfamily. CBD treatment (120 mg/kg) inhibited more than 75% of hepatic 6$\beta$-testosterone hydroxylase and erythromycin *N*-demethylase activities (functional markers of P-450IIIA) after 2 hr. An isozyme of the P-450IIIA subfamily ($M_r$ 49,960) was purified to apparent homogeneity from hepatic microsomes of untreated mice and was found to catalyze testosterone hydroxylation at the 2$\beta$-, 6$\beta$-, and 15$\beta$-positions exclusively. Incubation of this isozyme with CBD in a reconstituted system resulted in a time- and concentration-dependent inactivation, with almost complete loss of P-450 chromophore and corresponding increase in P-420 content. $NH_2$-terminal sequence analysis of the isozyme revealed an 86% similarity to the corresponding sequence of rat P-450IIIA2, a constitutive P-450 isozyme in the male rat liver. Pretreatment of mice with dexamethasone markedly (6-fold) increased the steroid-inducible P-450IIIA-dependent activities 6$\beta$-testosterone hydroxylation and erythromycin *N*-demethylation. CBD treatment of dexamethasone-pretreated animals failed to inhibit these activities, indicating that the steroid-inducible P-450IIIA was refractory to CBD-mediated inactivation. 3-Methylcholanthrene-inducible P-450IA and phenobarbital-inducible P-450IIB also appear to be refractory to CBD-mediated inactivation. On the other hand, erythromycin *N*-demethylase activity increased 4-fold after phenobarbital pretreatment and, as in untreated animals, was comparably inhibited by CBD, demonstrating its susceptibility to this drug. Thus, CBD appears to inactivate the P-450IIIA isozymes that are constitutively present in hepatic microsomes of untreated mice and/or inducible by phenobarbital pretreatment but not those that are steroid inducible.

CBD, a major constituent of marijuana, has been shown to inhibit hepatic drug metabolism after acute treatment (1–9). In mice, CBD largely inhibited ERND and 6$\beta$-testosterone hydroxylation, both known to be catalyzed predominantly by the P-450IIIA subfamily of isozymes. This family represents one of the three major hepatic xenobiotic-metabolizing enzyme families within the P-450 gene superfamily. P-450IIIA isozymes have been isolated from many species including rats (10–12), rabbits (13), and humans (14–16), and several closely related isozymes have been detected within species. Recently, three isozymes termed hPCN1-3 have been identified in adult human liver (16) and are believed to be involved in the metabolism of many clinically important drugs, such as nifedipine (15), cyclosporine (17), and 17$\alpha$-ethynylestradiol (18), as well as endogenous steroids (19) and aflatoxins (20). Three isozymes ($PCN_{a-c}$) have also been identified in rat liver (12) and appear to be differentially induced and hormonally regulated. $PCN_a$ is steroid inducible and has an $NH_2$-terminal amino acid sequence identical to the sequence deduced from a cDNA clone isolated by Gonzalez *et al.* (21), termed pP450PCN1, whose corresponding mRNA is not found constitutively in either male or female rat liver. A second clone (pP450PCN2) is 90% homologous to pP450PCN1 and is believed to encode either $PCN_b$ or $PCN_c$ which are constitutive in mature male rats and inducible by PB. $PCN_b$ and $PCN_c$ are indistinguishable except by their chromatographic behavior on DEAE-Sephacel and together will be referred to in this report as P-450IIIA2 [according to the recommended nomenclature of Nebert *et al.* (22)], when either the rat constitutive or PB-inducible isozymes of this subfamily are described. $PCN_a$ will be referred to as P-450IIIA1, when the rat steroid- or DEX-inducible isozyme is described.

The purpose of this work is to characterize the isozyme specificity of CBD-mediated P-450 inactivation, so that the effects of CBD on drug and/or steroid metabolism can be predicted when CBD is ingested either therapeutically as an antiepileptic or illicitly in marijuana. The purification and characterization of a P-450 isozyme (tentatively termed P-

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**ABBREVIATIONS:** CBD, cannabidiol; ERND, erythromycin *N*-demethylase; PB, phenobarbital; DEX, dexamethasone; 3-MC, 3-methylcholanthrene; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TAO, troleandomycin; THC, tetrahydrocannabinol; P-450, cytochrome P-450.

450IIIA$_{UT}$ but henceforth referred to simply as P-450$_{UT}$ because it was purified from the livers of untreated mice and is believed to be a member of the P-450IIIA subfamily), as well as its CBD-mediated inactivation, are described. We believe that an elucidation of the mechanism of inactivation would aid in the future design of cannabinoid analogs, several of which are currently being evaluated for their therapeutic potential (23).

## Experimental Procedures

**Materials.** CBD was generously supplied by the National Institute on Drug Abuse. DEAE- and CM-cellulose were purchased from Whatman (Kent, England), DEAE-Sephacel, sodium cholate, and Lubrol PX from Sigma (St. Louis, MO), and hydroxylapatite (Bio-Gel HTP) from Bio-Rad (Richmond, CA). All other chemicals were of reagent grade.

**Animals and treatment.** Male CF-1 mice (Charles River, Portage, ME) weighing 20–30 g were used in most experiments, although female mice of the same strain and source were used in experiments to determine sex specificity and C57BL/6 mice were used in experiments to determine the effect of CBD on 3-MC-inducible P-450. CBD was administered intraperitoneally in a Tween 80 suspension, as described previously (5), at an anticonvulsant dose of 120 mg/kg in a single acute (2-hr) dose or repetitively once daily for 4 days, and animals were killed 24 hr after the last dose. Sodium PB (in water) or DEX (in corn oil) was injected intraperitoneally at a dose of 100 mg/kg daily for 4 days, and 3-MC (in corn oil) was administered at a dose of 20 mg/kg daily for 3 days.

**P-450$_{UT}$ purification.** Animals were killed by cervical dislocation, the gall bladders were removed, and livers were perfused *in situ* with ice-cold 1.15% KCl. Livers were removed and microsomes were prepared as described previously (9). Microsomes were solubilized at 4° for 1 hr, at a final protein concentration of 10 mg/ml, in buffer containing 10 mM potassium phosphate, pH 7.4, 20% (v/v) glycerol, 0.5% (w/v) sodium cholate, 0.2% (v/v) Lubrol PX, and 0.1 mM EDTA (buffer A). Solubilized microsomes were applied at room temperature to a Whatman DEAE-cellulose (DE-52) column (10 nmol of P-450/ml of gel) equilibrated with buffer A. After the column was washed with buffer A (1 column volume), most of the P-450 was eluted with a linear gradient of 0–75 mM sodium chloride in buffer A (5 column volumes). Analysis by SDS-PAGE of the P-450-containing fractions revealed several major proteins of approximately $M_r$ 50,000, only one of which was immunoreactive after Western immunoblotting with an antibody raised against rat liver P-450$_p$ (10). Fractions containing this immunoreactive protein and exhibiting $A_{417}/A_{280} \geq 0.3$ were pooled, concentrated, and dialyzed against buffer containing 3 mM sodium phosphate, pH 6.8, 20% (v/v) glycerol, 0.2% (v/v) Lubrol PX, and 0.1 mM EDTA (buffer B), before application to a Whatman CM-cellulose (CM-52) column (10 nmol of P-450/ml of gel) equilibrated in the same buffer. After washing with buffer B (1 column volume), P-450 was eluted with a linear gradient of 0–100 mM potassium phosphate, pH 6.8, in buffer B (5 column volumes). Fractions exhibiting high anti-P450$_p$ immunoreactivity and $A_{417}/A_{280} \geq 0.8$ were pooled, concentrated, and dialyzed against buffer containing 5 mM sodium phosphate, pH 7.7, 20% (v/v) glycerol, 0.2% (v/v) Lubrol PX, and 0.1 mM EDTA (buffer C), before application to a DEAE-Sephacel column (10 nmol of P-450/ml of gel) equilibrated in the same buffer. After washing with buffer C (2 column volumes), P-450 was eluted with a linear gradient of 0–100 mM sodium acetate, pH 7.7, in buffer C (15 column volumes). Fractions with $A_{417}/A_{280} \geq 1.0$ were pooled, concentrated, and dialyzed against buffer containing 10 mM sodium phosphate, pH 7.4, 20% (v/v) glycerol, 0.2% (v/v) Lubrol PX, 0.1 mM EDTA, 5 mM dithiothreitol, and 5 μM heme (buffer D), before application to a hydroxylapatite column (10 nmol of P-450/ml of gel). The column was washed with buffer D, as well as buffer D containing 45 and 90 mM potassium phosphate (5 column volumes of each). P-450$_{UT}$ was eluted with buffer D containing 180 mM potassium phosphate and was judged to be homogeneous and >95% pure by SDS-PAGE. Nonionic detergent was removed as described previously (24), after the P-450 preparation was bound to a small hydroxylapatite column. The P-450 was concentrated, dialyzed against buffer D lacking Lubrol PX, and stored at −20°.

**Analytical procedures.** All spectral determinations were performed with an SLM Aminco DW2000 spectrophotometer. P-450 concentrations were determined according to the method of Omura and Sato (25) or of Estabrook *et al.* (26), as stated in the text. Protein concentrations were determined by the method of Lowry *et al.* (27), using bovine serum albumin as a standard. Discontinuous SDS-PAGE was carried out according to the method of Laemmli (28).

**Immunochemical procedures.** Polyclonal antibodies were raised against the purified isozyme in rabbits, as described previously (24). Western and slot blotting were performed as described previously (24).

**Enzyme assays.** Purified P-450 was reconstituted in the presence of saturating amounts of rat liver microsomal NADPH-P-450 reductase, dilauryl phosphatidylcholine (50 μg/ml), sodium cholate (0.1 mg/ml), EDTA (1.5 mM), and NADPH (1 mM). Microsomal activities of ERND (29), pentoxy- (30) and ethoxyresorufin (31) *O*-dealkylase, and testosterone hydroxylase (32, 33) and TAO complex formation (34) were assayed as previously described.

**$NH_2$-terminal sequence analyses.** The amino acid sequence of the first 24 residues of P-450$_{UT}$ was determined by the Biomolecular Resource Center at the University of California, San Francisco. Approximately 400 pmol of purified P-450 were subjected to automated Edman degradation, using an Applied Biosystems 470A gas-phase sequencer. The phenylthiohydantoin derivatives were identified and quantitated by reverse phase high pressure liquid chromatography, using an Applied Biosystems 120A liquid chromatograph.

**Densitometry.** Densitometric analyses of slot-blots were carried out with a Hoefer GS 300 scanning densitometer in the reflectance mode, as previously described (24). Peak areas were quantitated by Gaussian fit integration, using the GS 370 densitometry program, and were found to be linear with respect to protein concentration.

## Results

**Isolation and purification of P-450$_{UT}$ from mice.** CBD treatment has been shown to inhibit the metabolism of several drugs (1–9) and is particularly effective in inhibiting those activities attributed to the P-450IIIA subfamily, such as TAO complex formation, 6β-testosterone hydroxylation, and ERND activity (Fig. 1). P-450$_{UT}$ was purified (see Experimental Procedures) from hepatic microsomes of untreated mice, using an antibody raised against rat hepatic P-450$_p$/P-450IIIA1 (10) as a specific probe for the protein. The purified protein had an apparent molecular weight of 49,960 (Fig. 2) and a specific content of 13.2 nmol/mg, although a much lower specific content (<7 nmol/mg) was observed if dithiothreitol and heme were not included in the buffers employed during the final purification steps.

**$NH_2$-terminal sequence analysis.** $NH_2$-terminal sequence analysis of P-450$_{UT}$ (Table 1) suggests that it is indeed a member of the P-450IIIA subfamily, because its sequence is 86 and 82% identical to published sequences [as deduced from cDNA clones (21)] of the first 24 residues of rat hepatic PCN2 and PCN1, respectively. Although complete comparison of P-450$_{UT}$ with the rat orthologs is not possible from such limited sequence data, the $NH_2$-terminus of P-450$_{UT}$ appears to more closely resemble that of PCN2, because it too has a basic amino acid arginine at residue 24, a position at which PCN1 has a neutral glycine residue. PCN1 also differs from PCN2 at residue 18. However, this difference may be considered "conservative" in nature, because Val$_{18}$ of PCN1 is replaced with another hydrophobic residue, Ile$_{18}$, in PCN2. Unfortunately, this residue

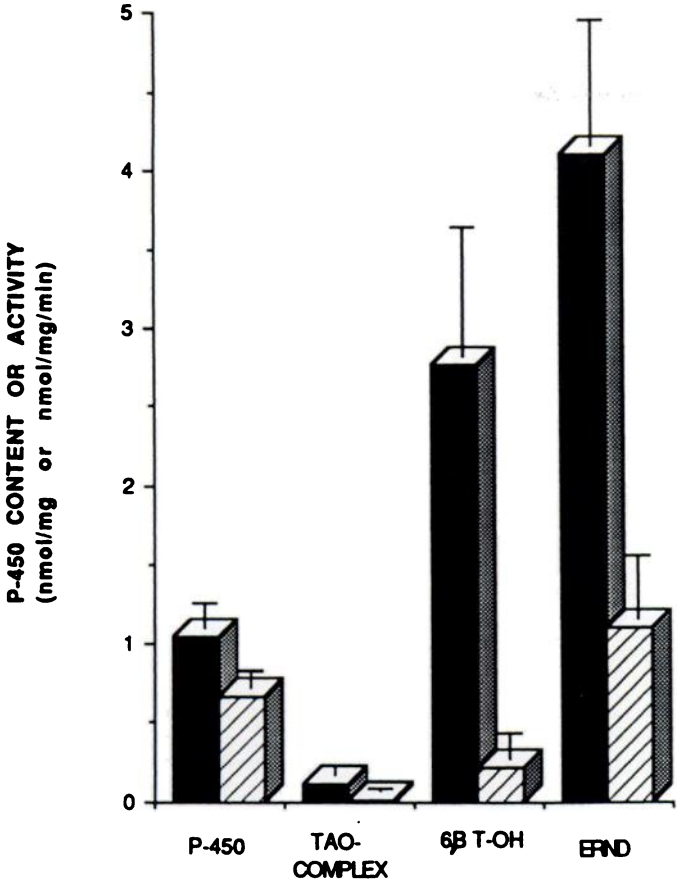


**Fig. 1.** Effect of CBD on constitutive P-450(s). Animals were treated with CBD (120 mg/kg) (▨) or vehicle (■) for 2 hr. Hepatic microsomes were assayed for total P-450 content or the amount (nmol/mg) of P-450 capable of forming a metabolic intermediate complex upon incubation with TAO *in vitro*, as well as for 6β-testosterone hydroxylase (*6β T-OH*) and ERND activities (nmol of product formed/mg of protein/min). Values are mean ± standard deviation of at least three individual determinations.

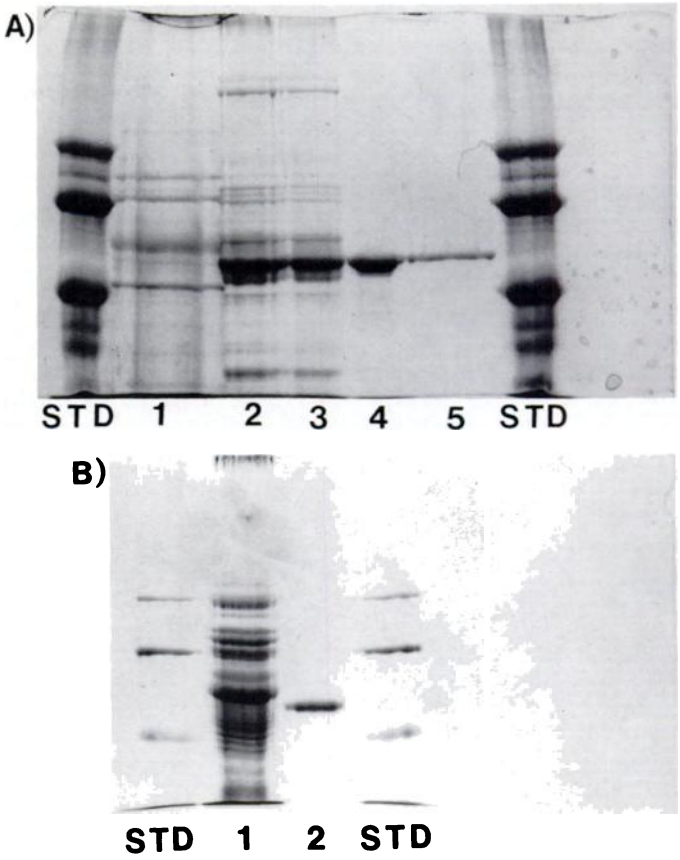


**Fig. 2.** SDS-PAGE of hepatic microsomes and purified P-450$_{UT}$. A, Microsomes or chromatographic column fractions containing P-450$_{UT}$ (10–20 pmol) were electrophoresed on a 9% acrylamide gel (0.75-mm-thick) and stained with Coomassie blue. Microsomes (*lane 1*), DE-52 eluate (*lane 2*), CM-52 eluate (*lane 3*), DEAE-Sephacel eluate (*lane 4*), and hydroxylapatite eluate (*lane 5*) are shown. Protein standards (*STD*) of molecular weights 42,700, 66,200, and 97,400 are given for comparison. B, Microsomes (*lane 1*) or P-450$_{UT}$ (*lane 2*) were electrophoresed as described above. Protein standards (*STD*) of molecular weights 42,700, 66,200, and 97,400 are given for comparison.

could not be conclusively identified in our sequence analysis of P-450$_{UT}$. The real significance of these findings is presently unclear, but more extensive comparisons will be possible when the full sequence of P-450$_{UT}$ becomes available.

**Spectral and catalytic properties of P-450$_{UT}$.** P-450$_{UT}$ exhibited an absolute oxidized absorption spectrum with maxima at 420, 532, and 568 nm, which shifted to 415 and 535 nm after reduction with dithionite. Addition of carbon monoxide after reduction shifted the maxima to 451 and 555 nm but, because of the inclusion of heme in the purification procedure, the spectrum also exhibited a large absorbance at 417 nm. However, P-450 content could be more accurately determined by the method of Estabrook *et al.* (26) (in which carbon monoxide is present in both sample and reference cuvettes and only the sample cuvette is reduced with dithionite), and the spectra so obtained suggested the presence of only small amounts of P-420.

When reconstituted with NADPH-P-450 reductase, lipid, and NADPH (see Experimental Procedures), P-450$_{UT}$ catalyzed testosterone hydroxylation specifically at the 6β-, 15β-, and 2β-positions (Table 2). Although the rate of such hydroxylation was considerably lower than that reported (35) for the purified rat constitutive liver isozyme (P-450IIIA2), such low activity is reportedly characteristic of P-450IIIA isozymes in reconstituted systems (11, 34, 36). Moreover, we found that inclusion of either cytochrome $b_5$ or the specific lipids (35) had little effect on enhancing P-450$_{UT}$ activity. Although not fully active, P-450$_{UT}$ was functional and retained its regioselectivity for testosterone hydroxylation at the positions identified with P-450IIIA by immunoinhibition studies (36).

P-450$_{UT}$ was found to be inactivated during CBD metabolism in a both time- and concentration-dependent manner (Fig. 3A). The inactivation was NADPH dependent and generated significant amounts of P-420 that were approximately equivalent to the P-450 chromophore loss (Fig. 3B). In contrast, incubation with $\Delta^9$-THC resulted in only minimal loss of P-450, indicating that P-450$_{UT}$ inactivation is not a general cannabinoid effect. In addition, incubation of CBD with a P-450 isozyme [P-450IIB, which was purified from mice repetitively treated with CBD (24) and was shown to be refractory to CBD-mediated inhibition] resulted in no loss of chromophore, thereby demonstrating the relative selectively of CBD-mediated inactivation for P-450 isozymes.

**Effect of P-450 inducers on CBD-mediated inactivation.** Because CBD inactivated the P-450IIIA isozymes, inducers of these and other hepatic isozymes were used to further characterize CBD-mediated inactivation. Thus, mice were pretreated with DEX, 3-MC, or PB to induce the P-450IIIA, IA,

TABLE 1

**Comparison of N-terminal amino acid sequences of P-450IIIA isozymes**

Only those residues of rat PCN1 and PCN2 that differ from mouse P-450$_{UT}$ have been identified and they are classified as either homologous (enclosed in parentheses) or nonhomologous (enclosed in brackets) differences. Residues of mouse P-450$_{UT}$ not conclusively identified are represented by an asterisk.

| P-450 | Residue | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | | | | | | | 10 | | | | | | | | 15 | | | | 20 | | | | 24 |
| Mouse P-450$_{UT}$ | M | D | L | V | S | A | L | S | L | E | T | W | V | L | L | A | I | * | L | V | * | L | Y | R |
| Rat PCN2 | – | – | – | (L) | – | – | – | (T) | – | – | – | – | – | – | – | – | (V) | – | – | – | – | – | – | – |
| Rat PCN1 | – | – | – | (L) | – | – | – | (T) | – | – | – | – | – | – | – | – | (V) | – | – | – | – | – | – | [G] |

TABLE 2

**Comparison of microsomal and P-450$_{UT}$ testosterone hydroxylase activities**

Catalytic activites are reported as the mean ± standard deviation of at least three separate microsomal or purified P-450$_{UT}$ preparations. Microsomes (0.1 mg of protein) or P-450$_{UT}$ (0.1 nmol) were incubated at 37° for 10 min in the presence of [$^{14}$C]testosterone (0.25 mM) and NADPH (1 mM), as described in Experimental Procdeures.

| Testosterone metabolite | Microsomal activity | P-450$_{UT}$ activity |
|---|---|---|
| | *nmol of product/nmol of P-450/min* | *nmol of product/nmol of P-450/min* |
| 6$\alpha$ | 0.13 ± 0.05 | ND[a] |
| 15$\beta$ | 0.13 ± 0.02 | 0.20 ± 0.12 |
| 15$\alpha$ | 0.17 ± 0.04 | ND |
| 7$\alpha$ | 0.20 ± 0.10 | ND |
| 6$\beta$ | 2.71 ± 0.52 | 4.67 ± 1.87 |
| 16$\alpha$ | 0.42 ± 0.17 | ND |
| 16$\beta$ | 0.42 ± 0.20 | ND |
| 2$\alpha$ | 0.04 ± 0.02 | ND |
| 2$\beta$ | 0.21 ± 0.01 | 0.43 ± 0.10 |
| Androstenedione | 0.53 ± 0.10 | ND |

[a] ND, not detected if NADPH-supplemented incubations were less than twice backround (samples incubated in the absence of NADPH).

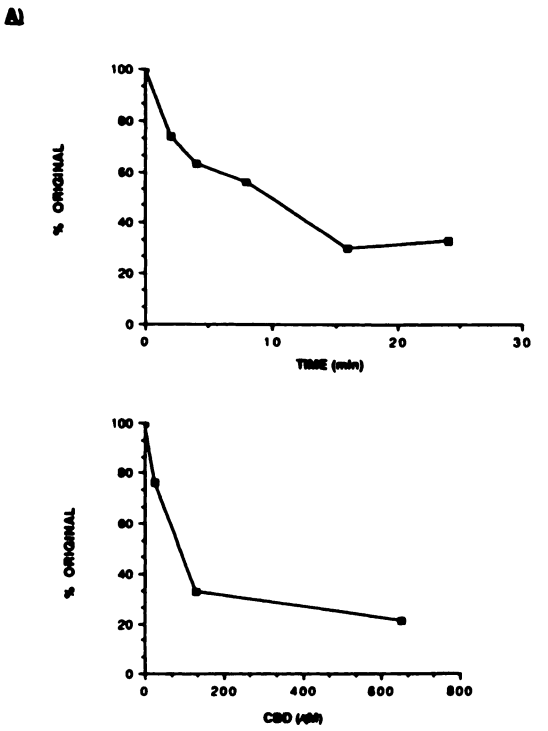


**Fig. 3.** CBD-mediated P-450$_{UT}$ inactivation. P-450$_{UT}$ (200 pmol) was reconstituted in the presence of saturating amounts of rat liver microsomal NADPH-P-450 reductase, dilauryl phosphatidyl choline (50 $\mu$g/ml), sodium cholate (200 mg/ml), and NADPH (1 mM). A, *Top*, reconstituted P-450 was incubated at 37° with CBD (130 $\mu$M) for 0 to 25 min. P-450 content was determined as described (29) and expressed relative to a nonincubated control devoid of NADPH. Loss due to incubation in the presence of either CBD or NADPH alone was less than 15% of original values. *Bottom*, determinations were performed as above except P-450$_{UT}$ was incubated at 37° for 15 min with 0 to 650 $\mu$M CBD. B, A carbon monoxide-reduced P-450 difference spectrum (400 to 500 nm) was determined (26) after incubation of P-450$_{UT}$ for 15 min at 37° in the presence (——) or absence (– – –) of CBD (130 $\mu$M), as described above.

or IIB/IIIA subfamilies, respectively. DEX pretreatment markedly increased those aforementioned activities attributed to P-450IIIA, resulting in a 6-fold increase over those observed in nonpretreated mice (Fig. 4). However, in contrast to the marked inhibition observed after CBD treatment of nonpretreated mice, only a small decrease of these activities was observed after CBD treatment. This decrease was statistically not significant but was comparable in magnitude to that observed in nonpretreated animals. Thus, 6$\beta$-testosterone hydroxylase and ERND activities, which were decreased by 2 and 3 nmol/mg/min, respectively, in nonpretreated animals after CBD treatment, were decreased by only 1 and 2 nmol/mg/min, respectively, in DEX-pretreated animals after CBD treatment, despite a 6-fold increase in these activities. Therefore, the activities attributed to P-450IIIA in nonpretreated animals appear to exhibit a much greater susceptibility to CBD-mediated inactivation than corresponding activities in DEX-pretreated mice.

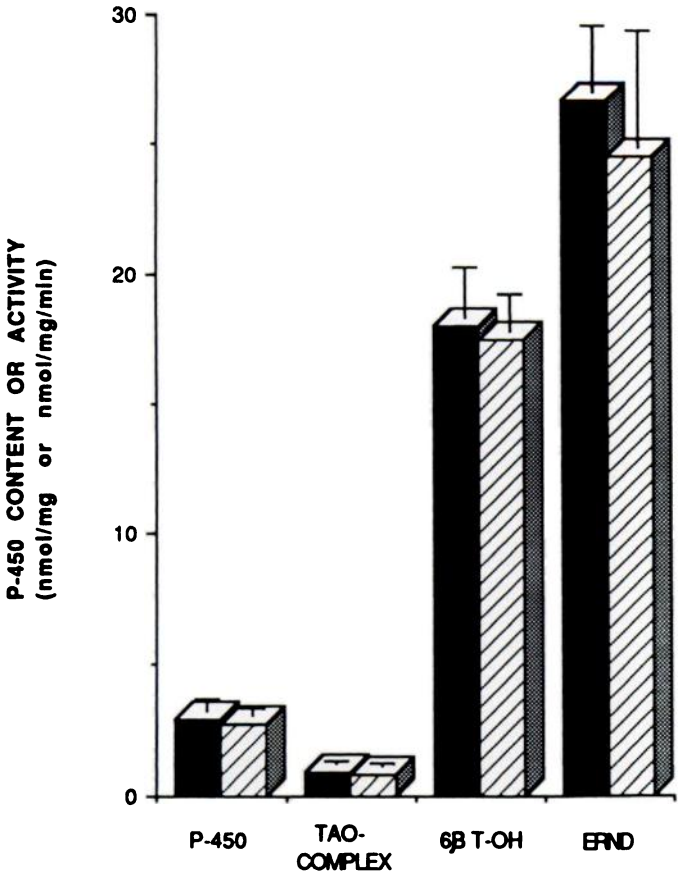


**Fig. 4.** Effect of CBD on DEX-inducible P-450(s). Animals were pretreated with DEX (100 mg/kg, daily for 4 days) before being treated with CBD (120 mg/kg) (■) or vehicle (■) for 2 hr. Hepatic microsomes were assayed for total P-450 content or the amount (nmol/mg) of P-450 capable of forming a metabolic intermediate complex upon incubation with TAO *in vitro*, as well as for 6β-testosterone hydroxylase (6β T-OH) and ERND activities (nmol of product formed/mg of protein/min). Values are mean ± standard deviation of at least three individual determinations.

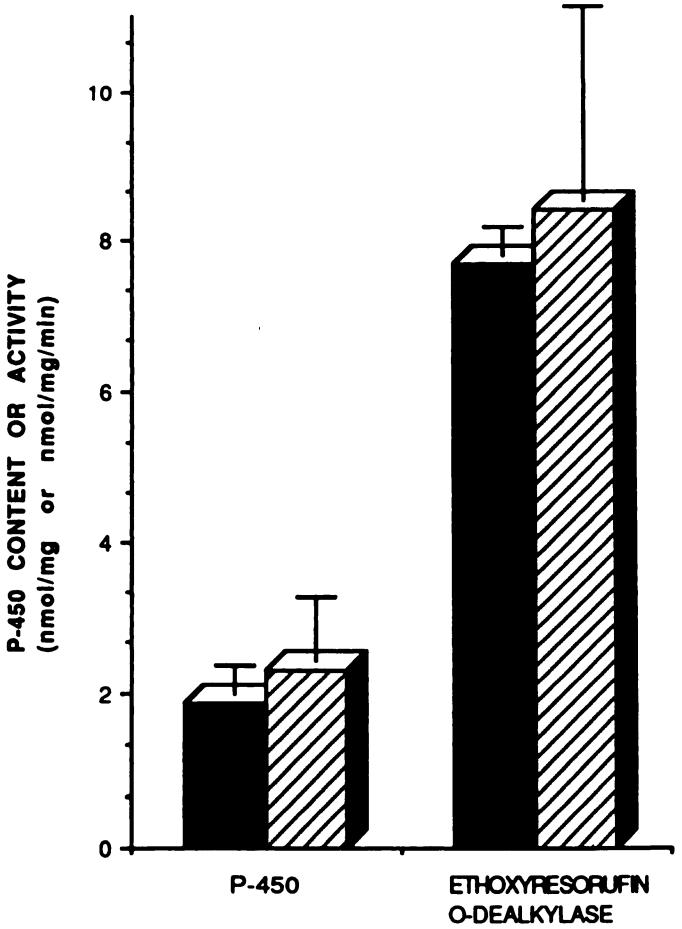


**Fig. 5.** Effect of CBD on 3-MC-inducible P-450. Animals were pretreated with 3-MC (20 mg/kg, daily for 3 days) before being treated with CBD (120 mg/kg) (■) or vehicle (■) for 2 hr. Hepatic microsomes were prepared and assayed for total P-450 content (nmol/mg) or ethoxyresorufin *O*-dealkylase activity (nmol of resorufin formed/mg of protein/min). Values are mean ± standard deviation of at least three individual determinations.

3-MC pretreatment (Fig. 5) markedly increased ethoxyresorufin *O*-dealkylase activity from 0.1 to 7.7 nmol/mg/min, but CBD treatment of these mice resulted in no decrease in this activity, suggesting that P-450IA1 was also refractory to CBD-mediated inactivation.

PB pretreatment (Fig. 6) increased pentoxyresorufin *O*-dealkylase activity (attributed to the P-450IIB subfamily) markedly and ERND activity (attributed to the P-450IIIA subfamily) modestly, resulting in >100- and 4-fold increases, respectively. CBD treatment of such animals resulted in no loss of pentoxyresorufin *O*-dealkylase activity, confirming the resistance of the P-450IIB subfamily to CBD-mediated inactivation (24). On the other hand, the increased ERND activity appeared to be very susceptible to CBD-mediated inhibition, resulting in a 3-fold greater loss after CBD treatment than that observed in nonpretreated animals (9 versus 3 nmol/mg/min). This was in marked contrast to the observed refractoriness of this particular activity to CBD-mediated inhibition in DEX-pretreated mice, suggesting that, although PB and DEX both increase ERND activity, the stimulated activities differed in their susceptibility to CBD-mediated inactivation, thereby reflecting differential induction of P-450IIIA isozymes by each inducer.

**Immunoquantitation of P-450IIIA.** Immunoquantitation of the P-450IIIA content of hepatic microsomes revealed no P-450IIIA apoprotein loss after acute (2-hr) CBD treatment of nonpretreated animals, despite an 80% decrease in ERND activity (Fig. 7). Repetitive CBD treatment (4 days) markedly increased immunochemically detectable P-450IIIA content, despite only a modest increase in ERND activity. PB pretreatment, however, which also markedly increased P-450IIIA content, proportionately increased ERND activity.

**Sex specificity of CBD-mediated inhibition.** P-450IIIA2 has been shown to be constitutively expressed in male Sprague-Dawley rats but absent in females (12). To determine whether CF-1 mice also exhibited such specificity or whether CBD-mediated inhibition in mice was sex specific, female mice were treated with CBD (2 hr) before ERND activity was determined (Fig. 8). Although ERND activity was only half of that observed in males, it appeared to be comparably susceptible to CBD-mediated inhibition, suggesting that female CF-1 mice also express a CBD-susceptible P-450IIIA isozyme.

## Discussion

Acute CBD treatment of nonpretreated mice resulted in the destruction of approximately 30% of the hepatic microsomal P-450 content. This destruction was manifested by an 80–90% loss of several activities (Fig. 1) known to be catalyzed by the

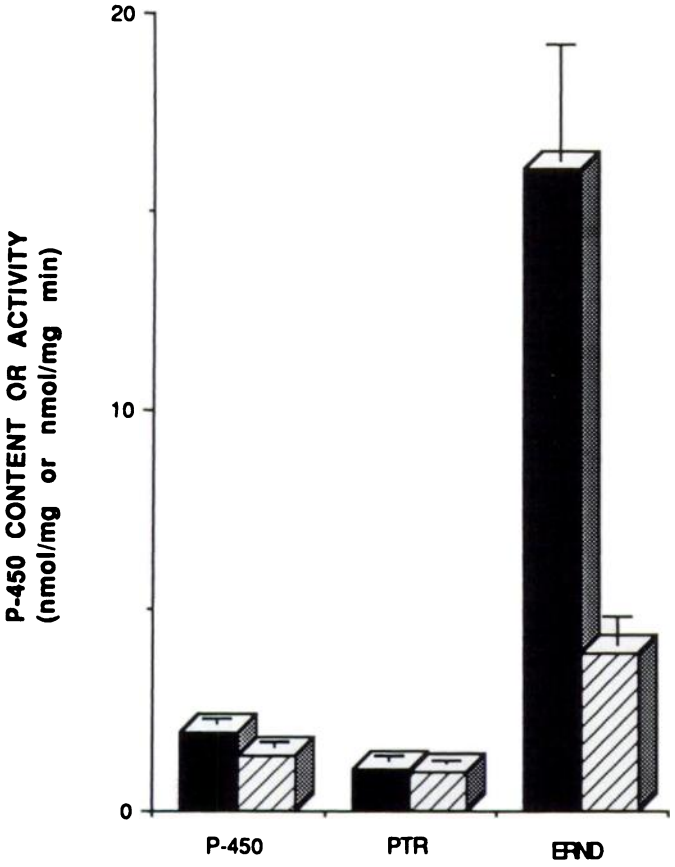


**Fig. 6.** Effect of CBD on PB-inducible P-450(s). Animals were pretreated with PB (100 mg/kg, daily for 4 days) before being treated with CBD (120 mg/kg) (▨) or vehicle (■) for 2 hr. Hepatic microsomes were prepared and assayed for total P-450 content (nmol/mg) or pentoxyresorufin *O*-dealkylase (*PTR*) and ERND activities (nmol of product formed/mg of protein/min). Values are mean ± standard deviation of at least three individual determinations.

P-450IIIA subfamily of isozymes. A P-450 isozyme was purified from hepatic microsomes of untreated animals that exhibited both structural [$NH_2$-terminal amino acid sequence (Table 1) and immunoreactivity] and functional [6$\beta$-, 2$\beta$-, and 15$\beta$-testosterone hydroxylase activity (Table 2)] characteristics previously ascribed to the P-450IIIA subfamily (36). This isozyme, termed P-450IIIA$_{UT}$ (P-450$_{UT}$), was found to be inactivated during CBD metabolism in an NADPH-, concentration-, and time-dependent process, which resulted in a loss of P-450 chromophore and a corresponding increase in P-420 content (Fig. 3). Although the mechanism of this inactivation is not yet known, the time- and concentration-dependent formation of P-420 suggests perturbation of prosthetic heme binding, with displacement of the thiolate ligand, possibly due to the binding of a reactive metabolite to the protein. It is not likely that the heme moiety itself is altered, as observed with other P-450 inactivators such as allylisopropylacetamide (29, 37), 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydopyridine (38), or spironolactone (39), because stoichiometric losses of microsomal heme and P-450 content were not detected after CBD treatment (data not shown). Because CBD treatment results in spectral P-450 loss, the inactivation process also differs from that described for chloramphenicol, wherein no loss of P-450IIB1 chromophore is detected after *in vivo* incubation with drug (40).

Inactivation of P-450IIIA by 3,5-dicarbethoxy-2,6-dimethyl-ethyl-1,4-dihydopyridine resulted in heme-alkylated apoprotein, which was found to be rapidly degraded *in vivo* (38). However, after CBD treatment (2 hr), no loss of P-450IIIA apoprotein was detected (Fig. 7B). In fact, after repetitive CBD treatment (once daily for 4 days), hepatic microsomal P-450IIIA apoprotein content increased to a much greater extent than did ERND activity. Thus, although CBD increased P-450IIIA-catalyzed activity somewhat, even greater accumulation of inactive P-450IIIA apoprotein apparently occurs, possibly due to CBD alkylation or heme-stripping of the protein.

CBD-mediated inactivation appears to be selective for the P-450IIIA isozyme subfamily, because no inhibition of 3-MC-inducible P-450IA (Fig. 5) or PB-inducible P-450IIB (Fig. 6) was detected using functional markers for these particular P-450 isozyme subfamilies. Most striking was the apparent selectivity of CBD for inactivation of isozymes within the P-450IIIA subfamily. CBD inactivated 80% of P-450$_{UT}$ *in vitro* and inhibited 80% of several P-450IIIA-catalyzed activities after *in vivo* treatment (Figs. 1 and 3). The lack of CBD-mediated inhibition

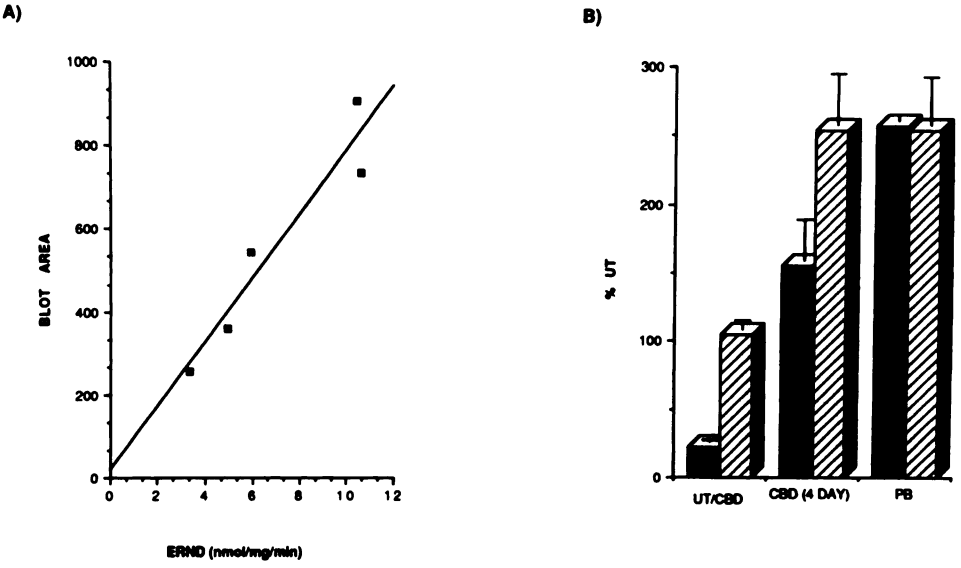


**Fig. 7.** Effect of CBD and PB on immunoquantified P-450$_{UT}$. A, Hepatic microsomes from either untreated or PB-pretreated mice were assayed for ERND activity or were applied to a nitrocellulose membrane and probed with rabbit antibody raised against P-450$_{UT}$, as described in Experimental Procedures. ERND activity was compared with blot density (found to be linear with respect to protein concentration for each of the samples) and a correlation coefficient ($r$ = 0.96) was obtained. B, Hepatic microsomes from animals treated acutely (2 hr) with CBD (UT/CBD), treated repetitively with CBD (once daily for 4 days), or treated with PB (once daily for 4 days) were assayed for ERND activity (■) or P-450$_{UT}$ content (▨), as described for A. Values for ERND and immunoblot areas are mean percentage ± standard deviation of values for untreated animals (*UT*) and were obtained from at least three individual animals.

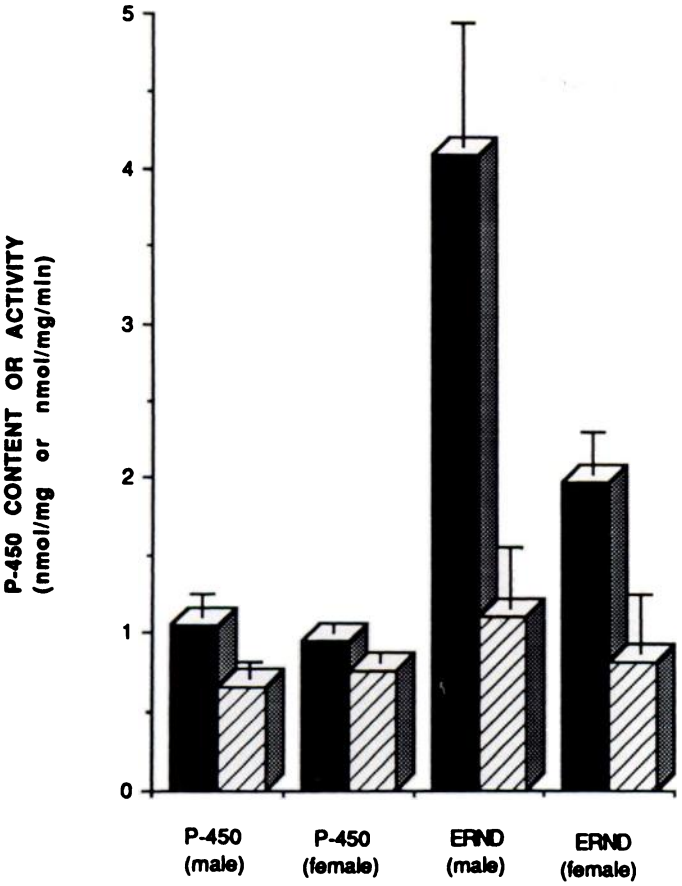


**Fig. 8.** Relative effects of CBD on P-450 content and ERND activity in male and female mice. Male or female CF-1 mice were treated with CBD (120 mg/kg) (▨) or vehicle (■) for 2 hr. Hepatic microsomes were prepared and assayed for total P-450 content (nmol/mg) or ERND activity (nmol of formaldehyde formed/mg of protein/min). Values are mean ± standard deviation of at least three individual determinations.

of P-450IIIA-catalyzed activities in DEX-pretreated mice (Fig. 4) suggests that the steroid-inducible counterpart is completely resistant to inactivation by CBD. PB pretreatment increased mouse liver microsomal P-450IIIA content (Fig. 7) and P-450IIIA-dependent activities (Fig. 6) approximately 2–3-fold over those in nonpretreated animals. In contrast to the refractoriness of the DEX-inducible isozyme, PB-inducible P-450IIIA was very susceptible to CBD-mediated inactivation (Fig. 6).

Our data in the mouse suggest that the P-450IIIA isozymes present in nonpretreated animals and in PB-pretreated animals are at least comparable in their susceptibility to CBD-mediated inactivation, whereas the corresponding steroid-inducible isozyme (in DEX-pretreated animals) appears to be resistant. This observed difference in susceptibility to CBD-mediated inactivation between the constitutive/PB-inducible P-450IIIA isozyme and the steroid-inducible isozyme is, to our knowledge, the first functional difference clearly detected within this subfamily. However, the possibility of differential isozyme inactivation within the P-450IIIA subfamily has been previously suggested (41). Thus, CBD-mediated inhibition of ERND activity represents a quick and easy functional probe for distinguishing between the steroid-inducible and constitutive/PB-inducible subfamilies in mice.

Although CBD-mediated inactivation of the constitutive P-450IIIA isozyme may account for many of the functional losses observed, P-450IIIA content as determined by TAO complex formation was only 0.12 nmol/mg of protein in nonpretreated animals, whereas total losses of spectrally detectable P-450 were approximately twice that value, implying the loss of additional isozymes. Inactivation of additional isozymes is also implicated by our previously reported decrease in 16$\alpha$-testosterone hydroxylase activity (9), known to be catalyzed by the P-450IID family in mice. Thus, constitutive/PB-inducible P-450IIIA and possibly one or more other constitutive P-450 isozyme(s) appear to be susceptible to CBD-mediated inactivation, whereas the inducible isozymes P-450IA, IIB, and steroid-inducible IIIA appear to be clearly insensitive.

Although P-450IIIA2 [determined by mRNA transcription (21) and TAO complex formation (42)] is constitutively present in male but not female rats, P-450IIIA-catalyzed ERND activity is found to be expressed in both male and female mice in this study (Fig. 8). This is in contrast to a study with DBA/2 mice, in which little if any hepatic P-450IIIA was functionally or immunologically detectable in male mice (42). In addition, both male and female CF-1 mice in our study appear to be equally susceptible to CBD-mediated inhibition, suggesting equivalent expression of similar P-450IIIA isozymes in both sexes.

CBD-mediated P-450 inactivation has been shown to markedly inhibit *in vitro* CBD metabolism (9). Such inactivation may also affect the metabolism of other cannabinoids coingested in marijuana, such as THC. Because several THC metabolites have been shown to be at least as pharmacologically active as THC itself (43), an altered production of such metabolites (through metabolic shunting) might provide an explanation for the modified physiological and psychological responses observed when THC is coingested with CBD (44–46). Thus, CBD-mediated alterations of drug metabolism may affect not only its own disposition but also that of many other drugs as well.

Furthermore, because P-450IIIA appears to represent a major fraction of human liver microsomal P-450 (14–20) and because this subfamily appears to be sensitive to CBD-mediated inactivation, our findings may have important clinical implications in the use of CBD or marijuana.

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