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Purification and Characterization of the Major Hepatic Cannabinoid Hydroxylase in the Mouse: A Possible Member of the Cytochrome P-450IIC Subfamily

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SUMMARY

Acute cannabidiol treatment of mice inactivated hepatic microsomal cytochrome P-450IIIA (P-450IIIA) and markedly inhibited *in vitro* cannabinoid metabolism. Antibodies raised against purified P-450IIIA inhibited the microsomal formation of quantitatively minor cannabinoid metabolites but had no effect on the major metabolites. Cannabinoid hydroxylation to the major metabolites was used as a functional probe to isolate and purify a P-450 (termed P-450$_{THC}$) from hepatic microsomes of untreated mice. The purified protein had an apparent molecular weight of 47,000 and a specific content of 15.4 nmol/mg and exhibited an absorbance maximum at 452 nm for the reduced carbon monoxide complex. NH_2-terminal sequence analysis of the first 16 residues of P-450$_{THC}$ suggests that it is a member of the P-450IIC subfamily, because its sequence is 85 and 69% identical to published sequences of rat hepatic P-450IIC7 and P-450IIC6, respectively. P-450$_{THC}$ exhibited high activity for cannabinoid hydroxylation and specifically produced 6α- and 7-hydroxy-Δ^1-tetrahydrocannabinol, as well as 6α-, 7-, and 4″-hydroxycannabidiol. Unlike anti-P-450IIIA antibody, antibody raised against purified P-450$_{THC}$ markedly inhibited the microsomal formation of all major cannabinoid metabolites. Similar immunoinhibition studies also revealed the existence of orthologs of mouse P-450$_{THC}$ and P-450IIIA in human liver microsomes. Thus, cannabidiol treatment of mice resulted in the inactivation of at least two constitutive P-450 isozymes, which together account for the majority of the detected cannabinoid metabolites.

Marijuana is a complex mixture of more than 60 cannabinoids (1), which are extensively metabolized in the liver. Over 100 metabolites of THC have been isolated and characterized (2), several of which have been found to be pharmacologically active (3). CBD, a nonpsychoactive cannabinoid present in marijuana in large concentrations, has been shown to affect the *in vivo* and *in vitro* metabolism of THC, barbiturates, and other drugs in several animal species, including humans (4–14). In addition, several studies have also demonstrated that CBD alters THC-induced psychological reactions when coadministered to human subjects (15–18). Because CBD binds extremely weakly to the recently characterized cannabinoid receptor in the brain (19), such CBD-mediated alteration of THC effects probably is due to modulation of THC metabolism and is not pharmacodynamic in nature. Characterization of CBD-mediated effects on drug metabolism may allow correlations to be made for some of the psychological effects of THC and its metabolites, as well as help predict potential drug interactions when CBD is ingested.

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We have recently reported that CBD treatment of mice selectively inactivates a constitutive P-450 isozyme belonging to the P-450IIIA subfamily (20). Repetitive CBD treatment (once daily for 4 days), which results in a sustained inactivation of P-450IIIA, also induces P-450IIB, an isozyme not detected in hepatic microsomes of untreated mice (21). P-450IIB was resistant to inhibition by CBD, and its induction normalized the *in vivo* and *in vitro* hexobarbital hydroxylase activity that was acutely inhibited by CBD. In an attempt to further characterize the effects of CBD on hepatic P-450 isozymes, the specific contribution of P-450 isozymes to cannabinoid metabolism was assessed. The findings of these studies are described below.

Experimental Procedures

Materials. CBD was generously supplied by the National Institute on Drug Abuse. DEAE- and CM-cellulose were purchased from Whatman Biosystems Ltd. (Kent, England), DEAE-Sephacel, sodium cholate, and Lubrol PX from Sigma Chemical Co. (St. Louis, MO), hydroxylapatite (Bio-Gel HTP) and Biobeads SM-2 from Bio-Rad Laboratories (Richmond, CA), and octyl-Sepharose CL-4B from Pharmacia (Uppsala, Sweden).

ABBREVIATIONS: THC, Δ^1-tetrahydrocannabinol; CBD, cannabidiol; P-450, cytochrome P-450; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; -OH, -hydroxy; EHHC, 1α,2α-epoxyhexahydrocannabinol; DTT, dithiothreitol; GC, gas chromatography; MS, mass spectrometry.

Animals and treatment. Male CF-1 mice (Charles River, Portage, ME), weighing 20–30 g, were used in all mouse experiments. CBD was administered intraperitoneally in a Tween 80 suspension, as described previously (8), at an anticonvulsant dose of 120 mg/kg for 2 hr. Livers from male Hartley guinea pigs, weighing 450–500 g, and from an unidentified human source were also used for preparation of microsomes.

P-450$_{THC}$ 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 (12). Microsomes were solubilized at 4° for 1 hr, at a final protein concentration of 10 mg/ml of buffer containing 5 mM potassium phosphate, pH 7.7, 20% (v/v) glycerol, 0.5% (w/v) sodium cholate, 0.2% (v/v) Lubrol PX, 0.1 mM EDTA, 1 mM DTT, and 30 μM THC (buffer A). Solubilized microsomes were applied at room temperature to a DEAE-cellulose (DE-52) column (2.5 × 30 cm) (10 nmol of P-450/ml of gel) equilibrated with buffer A. After washing of the column with buffer A (1 column volume), approximately 15% of the applied P-450 passed through the column. Additional P-450 was eluted with a linear gradient of 0–100 mM sodium chloride in buffer A (5 column volumes). THC hydroxylase activity was determined (see below) in the column fractions after treatment with Biobeads SM-2 (0.3 g of beads/ml) for 30 min. Fractions (eluting within the void peak and at the beginning of the salt gradient) with activities greater than microsomal values and exhibiting A_{417}/A_{280} of ≥0.2 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, 0.1 mM EDTA, 1 mM DTT, and 30 μM THC (buffer B), before application to a CM-cellulose (CM-52) column (1.5 × 20 cm) (10 nmol of P-450/ml of gel) equilibrated with 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 (10 column volumes). Fractions exhibiting high THC hydroxylase activity and A_{417}/A_{280} of ≥0.6 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, 1 mM DTT, and 30 μM THC (buffer C), before application to a hydroxylapatite column (1.5 × 10 cm) (10 nmol of P-450/ml of gel). The column was washed with buffer C, buffer C containing 45 mM and 90 mM potassium phosphate, buffer C containing only 0.05% (v/v) Lubrol PX, and buffer C containing 0.1% (w/v) sodium cholate without any Lubrol PX. P-450$_{THC}$ was eluted with buffer containing 200 mM potassium phosphate, pH 7.4, 0.6% (w/v) sodium cholate, 1 M sodium chloride, 20% (v/v) glycerol, 0.1 mM EDTA, 1 mM DTT, and 30 μM THC (buffer D) and was then applied to an octyl-Sepharose CL-4B column (1.5 × 10 cm) (10 nmol of P-450/ml of gel). The column was washed with buffer D and buffer D containing 0.2% (v/v) Lubrol PX (5 column volumes of each), and P-450$_{THC}$ was eluted with a linear gradient of 0.2–0.6% (v/v) Lubrol PX in buffer D (20 column volumes). Fractions containing homogeneous P-450$_{THC}$ (judged to be >95% pure by SDS-PAGE) were pooled, and nonionic detergent was removed as described previously (21), after binding of the P-450 preparation to a small hydroxylapatite column. The P-450 was concentrated, dialyzed against 10 mM sodium phosphate, pH 7.4, 20% (v/v) glycerol, 1 mM DTT, and stored at −70°.

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 (22). Protein concentrations were determined by the method of Lowry *et al.* (23), using bovine serum albumin as the standard. Discontinuous SDS-PAGE was carried out according to the method of Laemmli (24).

Enzyme assays. Purified P-450$_{THC}$ was reconstituted in the presence of saturating amounts of rat liver microsomal NADPH-P-450 reductase (25), dilauryl phosphatidylcholine (50 μg/ml), and NADPH (1 mM). Erythromycin, ethylmorphine, and benzphetamine *N*-demethylase (12, 25) and testosterone hydroxylase (26, 27) activities were assayed as previously described. THC and CBD hydroxylase activities were determined in the presence of 130 μM substrate. Microsomes (approximately 0.25 mg of protein) or reconstituted purified P-450$_{THC}$ (50 pmol) were incubated with substrate at 37° for 10 min after the addition of 1 mM NADPH (1-ml total incubation volume). After the addition of 2 nmol of internal standard (CBD for the THC hydroxylase assay or THC for the CBD hydroxylase assay), metabolites were extracted with 2 × 1.5 ml of ethyl acetate and extracts were evaporated under nitrogen and then redissolved in 150 μl of 60% acetonitrile in

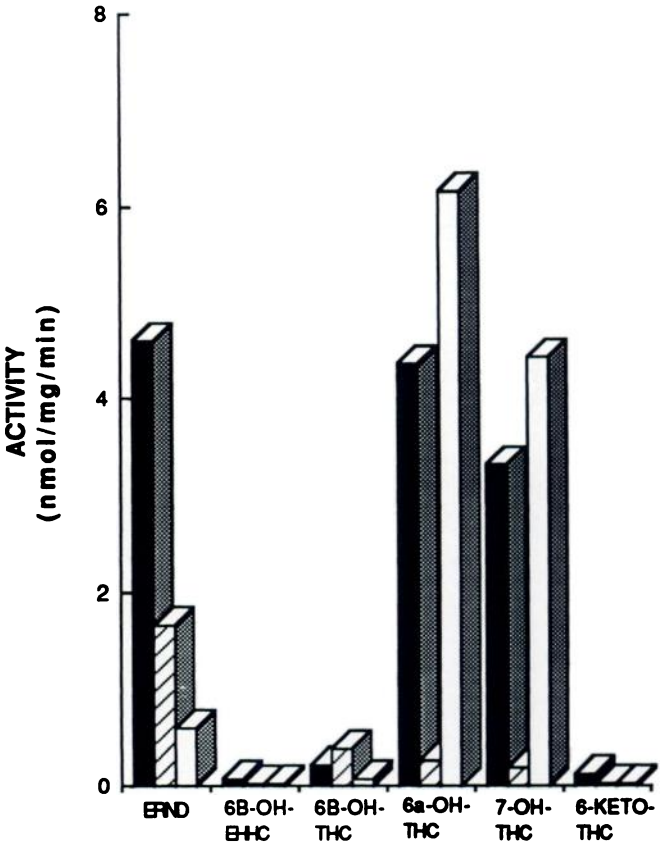

Fig. 1. Effect of CBD treatment or anti-P-450IIIA antibody on erythromycin *N*-demethylase (*ERND*) and THC hydroxylase activity. Enzyme activities were determined as described in Experimental Procedures, with hepatic microsomes obtained from animals either before (■) or after treatment with 120 mg/kg CBD for 2 hr (▩) or after preincubation of antibody raised against P-450IIIA with hepatic microsomes obtained from untreated mice (□). Comparable amounts of preimmune IgG incubated with hepatic microsomes inhibited activities <15%. Values are the average of at least three individual determinations.

TABLE 1

Chromatographic and mass spectrometric data for cannabinoid metabolites

Metabolite	HPLC retention time	GC retention time	MS peaks		
			M$^+$	Base peak	Other diagnostic ions
	min	*min*		*m/z*	
6β-OH-EHHC	6.1	22.1	490	359	143, 331
6β-OH-THC	6.6	21.9	474	343	384
6α-OH-THC	7.2	15.5	474	384	343, 459
7-OH-THC	8.0	15.9	474	371	
6-Keto-THC	9.2	21.3	400	400	343
6α-OH-CBD	4.6	11.1	546	478	337
7-OH-CBD	4.9	11.6	546	478 = 443	337, 430
4″-OH-CBD	5.0	11.7	546	478	425
6β-OH-CBD	5.4	11.2	546	478	337
3″-OH-CBD	5.5	22.1	546	478	268, 334, 425
2″-OH-CBD	5.8	11.1	546	145	

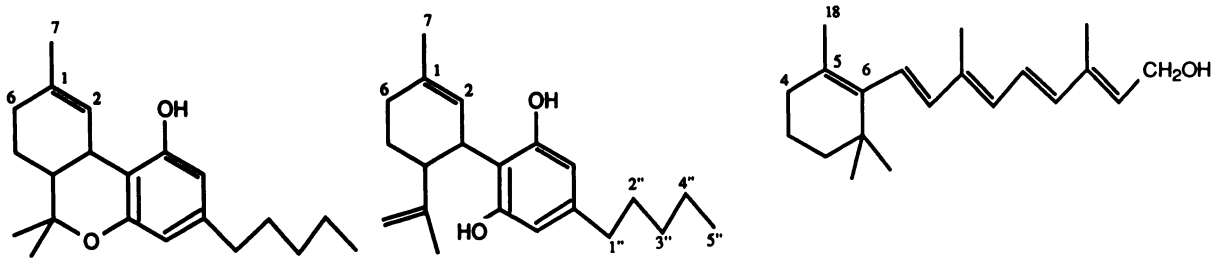

Fig. 2. Chemical structures of THC, CBD, and vitamin A. Structures are numbered at positions where oxidation has been found to occur, as discussed in the text.

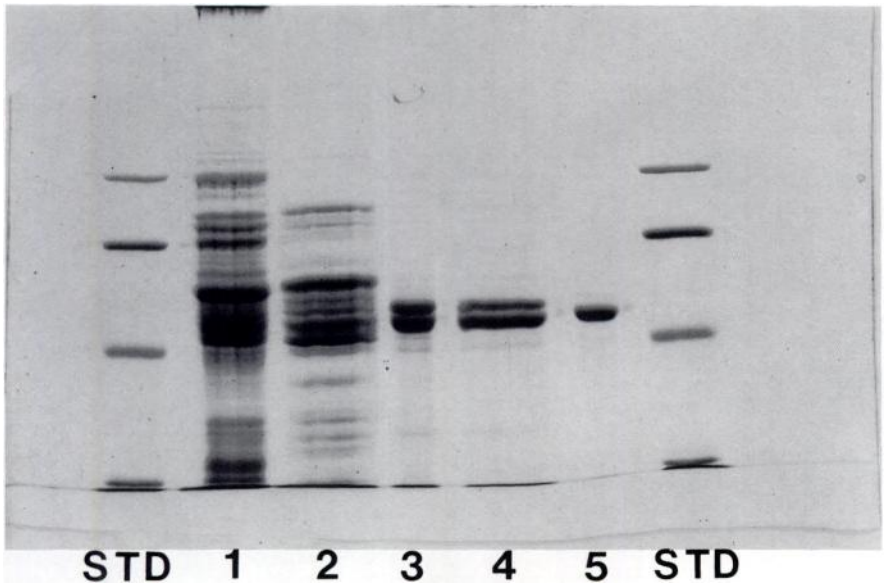

Fig. 3. SDS-PAGE of hepatic microsomes and purified P-450$_{THC}$. Microsomes or chromatographic column fractions containing P-450$_{THC}$ (10–20 pmol) were electrophoresed on a 9% acrylamide gel (0.75-mm thick) and stained with Coomassie blue. Microsomes (*lane 1*), DE-52 eluate (*lane 2*), CM-52 eluate (*lane 3*), hydroxylapatite eluate (*lane 4*), and octyl-Sepharose CL-4B eluate (*lane 5*) are shown. Protein standards (*STD*) of molecular masses 42.7, 66.2, and 97.4 kDa are given for comparison.

TABLE 2

NH_2-terminal amino acid sequence comparison of P-450$_{THC}$ with mouse and rat P-450IIC isozymes

P-450 isozyme	Residue 1	5	10	15
Mouse P-450$_{THC}$	—[a] — L V V F L A L T L S — L I L
Mouse P-450PBIII	M D L V V F L A L T L S S L I L
Rat P-450IIC7	M D L V T[b] F L V L T L S S L I L
RatP-450IIC6	M D L V M L L V L T L T S L I L

[a] —, Residues of P-450$_{THC}$ not conclusively identified.
[b] Residues of P-450 isozymes that differ from P-450$_{THC}$ are underlined.

water. Aliquots (50 μl) were subjected to HPLC on a Dynamax C_{18} column (4.6 × 250 mm; Rainin Corp.), and metabolites were monitored by absorbance changes at 215 nm. A linear gradient of 60–100% acetonitrile was applied for 10 min and 100% acetonitrile was maintained for 5 min before the column was re-equilibrated with 60% acetonitrile. All THC metabolites exhibited baseline separation, although several CBD metabolites were not fully resolved. Values for those metabolites are expressed in the text as the sum of the unresolved metabolites.

Metabolite purification. Cannabinoid metabolites were purified by HPLC from large microsomal incubations (50 ml) essentially as detailed above, except that the partially resolved CBD metabolites were rechromatographed (twice) by HPLC, using a shallower gradient (60–80% acetonitrile for 10 min) before washing and re-equilibration of the column with acetonitrile as described above. GC-MS of trimethylsilane-derivatized cannabinoid metabolites was performed as described (28), except that GC-MS data were recorded with an HP5890 mass spectrometer fitted with a DB-1 column (30 m, 1.0 μm; J & W Scientific), the trap voltage was 200 μA, the accelerating voltage was 8.0 kV, and the scan speed was 0.7 sec/decade.

Immunochemical procedures. Polyclonal antibodies were raised against the purified mouse liver isozyme in rabbits, as described previously (21). Purified IgG was prepared from rabbit sera by DEAE-Affi-Gel Blue gel chromatography, according to the method described in the Econo-Pac serum IgG purification kit (Bio-Rad Laboratories, Richmond, CA).

Immunoinhibition of microsomal enzyme activities was determined in the presence of 2 and 10 mg of antibody/mg of microsomal protein, using antibodies raised against P-450IIIA and P-450IIC, respectively. At these concentrations, IgG prepared from animals before exposure to the purified P-450 isozyme (preimmune IgG) inhibited activities only slightly (<15%). Microsomes were preincubated with antibody at room temperature for 15 min before the addition of substrate and NADPH.

Hepatic microsomes were immunoblotted as described previously (21), except that 9 μg of guinea pig microsomal protein were used.

NH_2-terminal amino acid sequence analysis. The amino acid sequence of the first 16 residues of P-450$_{THC}$ was determined at 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-

TABLE 3

Comparison of microsomal and purified P-450$_{THC}$ mixed-function oxidase activities

Activity	Metabolite	Microsomal activity	P-450 activity[a]
		nmol/nmol of P-450/min	*nmol/nmol of P-450/min*
THC hydroxylase	6β-OH-EHHC	0.11	ND[b]
	6β-OH-THC	0.37	ND
	6α-OH-THC	3.37	14.65
	7-OH-THC	2.58	11.83
	6-Keto-THC	0.18	ND
CBD hydroxylase	6α-OH-CBD	0.24	0.89
	7-OH-CBD/4″-OH-CBD	1.75	8.15
	6β-OH-CBD/3″-OH-CBD	0.60	ND
	2″-OH-CBD	0.22	ND
Benzphetamine *N*-demethylase	Formaldehyde	10.72	19.2
Ethylmorphine *N*-demethylase	Formaldehyde	17.94	26.7
Testosterone hydroxylase	16β-Hydroxytestosterone[c]	0.43	1.16

[a] Purified P-450$_{THC}$ (50 pmol) was reconstituted with saturating amounts of NADPH-P-450 reductase (1000 units), NADPH (1 mM), dilauroylphosphatidylcholine (50 μg), and cytochrome b_5 (50 pmol), in a 1-ml volume. Substrates were present at the following concentrations: cannabinoids, 130 μM; benzphetamine and ethylmorphine, 2 mM; and testosterone, 250 μM. Product formation was determined as described in Experimental Procedures.

[b] ND, not detected (<0.01 nmol/nmol of P-450/min).

[c] Only metabolic activity detected that was more than twice that observed with reconstituted P-450$_{THC}$ incubated in the absence of NADPH.

TABLE 4

Effect of cofactors on reconstituted P-450$_{THC}$ 6α-hydroxylase activity

Incubation conditions	Activity
	% of complete system
Complete system[a]	100
−P-450$_{THC}$	0
−P-450 reductase	0
−NADPH	0
−Dilauroylphosphatidylcholine	16
−Cytochrome b_5	78

[a] The complete system contained purified P-450$_{THC}$ (50 pmol), saturating amounts of NADPH-P-450 reductase (1000 units), NADPH (1 mM), dilauroylphosphatidylcholine (50 μg), cytochrome b_5 (50 pmol), and THC (130 μM), in a 1-ml volume. The catalytic activity of the complete system was 11.06 nmol of 6α-OH-THC formed/nmol of P-450$_{THC}$/min.

phase sequencer. The phenylthiohydantoin derivatives were identified and quantitated by reverse phase HPLC, using an Applied Biosystems 120A liquid chromatograph.

Results

Mouse hepatic microsomal cannabinoid metabolism: CBD-mediated inhibition and contribution of P-450IIIA. Acute CBD treatment of mice has been reported not only to inhibit *in vivo* and *in vitro* cannabinoid metabolism (4–14) but also to inactivate mouse hepatic microsomal P-450IIIA (20). To determine whether these effects were related, the effects of CBD on hepatic microsomal THC hydroxylase activity and the contribution of P-450IIIA in such metabolism were assessed. Mouse hepatic microsomes catalyzed the hydroxylation of THC to several major and minor metabolites, and CBD treatment of mice inhibited most of the hepatic microsomal

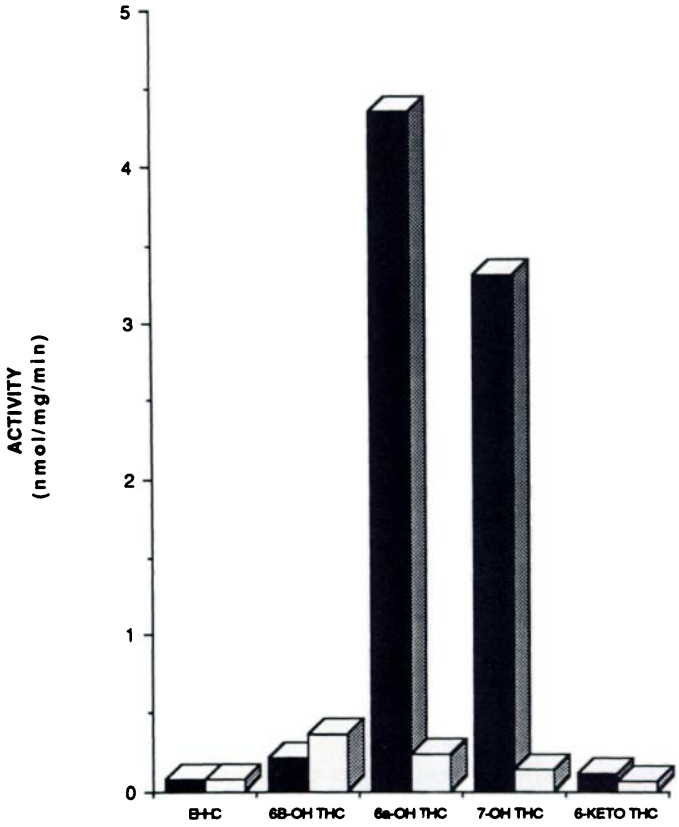

Fig. 4. Effect of anti-P-450$_{THC}$ antibody on THC hydroxylase activity. THC hydroxylase activity was determined either before (■) or after (□) preincubation of antibody with hepatic microsomes obtained from untreated mice. Comparable amounts of preimmune IgG incubated with hepatic microsomes inhibited activities <15%. Values are the average of at least three individual determinations.

THC hydroxylase activity (Fig. 1). However, antibodies raised against purified P-450IIIA, while markedly inhibiting erythromycin *N*-demethylase activity, inhibited the formation of only several minor THC metabolites and not that of the major THC metabolites. Comparable findings were obtained if CBD hydroxylation was assessed (data not shown); CBD treatment decreased the formation of all CBD metabolites, whereas anti-P-450IIIA antibodies only inhibited the formation of minor metabolites.

The cannabinoid metabolites have been tentatively identified by GC-MS, based on comparisons with published spectra (29–31), and, when possible, by HPLC, on the basis of corresponding retention times of synthesized standards (Table 1, Fig. 2). The major metabolites, identified as 6α- and 7-OH-THC and 6α-, 7-, and 4″-OH-CBD, accounted for approximately 90 and 70% of all THC and CBD metabolites measured, respectively. The minor metabolites were identified as 6β-OH-EHHC, 6β-OH-THC, and 6-keto-THC, as well as 6β-, 3″-, and 2″-OH-CBD. The lack of inhibition by the anti-P-450IIIA antibody of the formation of the major cannabinoid metabolites suggested that a different P-450 isozyme(s) was responsible for their formation.

Isolation and purification of P-450$_{THC}$. The relative THC 6α- and 7-hydroxylase activity was used as a functional probe to isolate and purify a P-450 (P-450$_{THC}$) from hepatic microsomes of untreated mice (see Experimental Procedures). The purified protein had an apparent molecular weight of

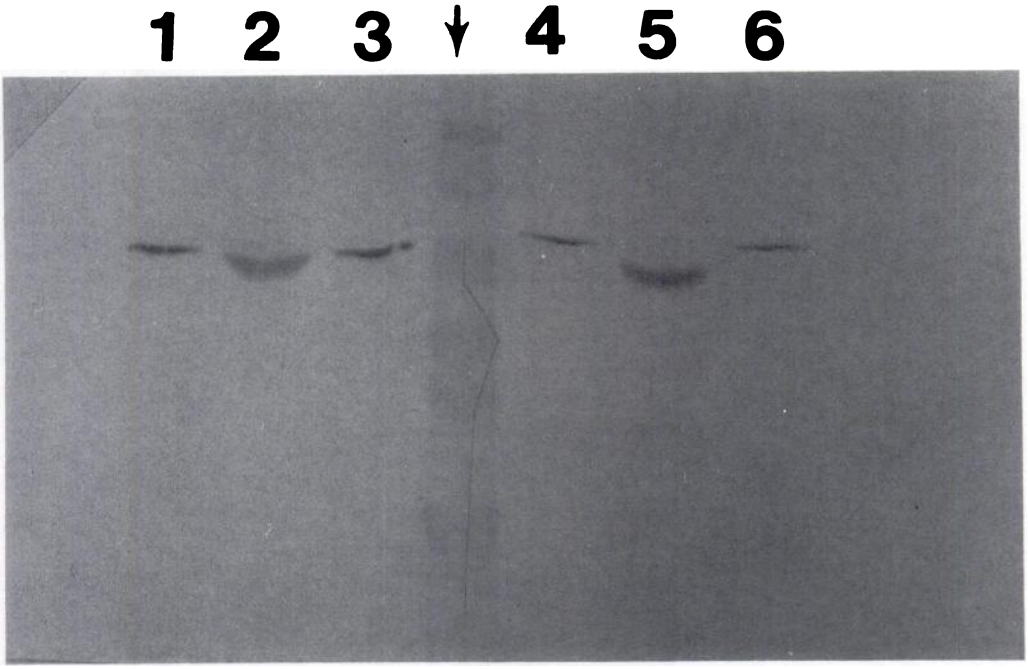

Fig. 5. Immunoblot of mouse, guinea pig, and human hepatic microsomes. Hepatic microsomes were electrophoresed, transferred to nitrocellulose membranes, and probed with antibodies prepared against purified mouse hepatic P-450IIIA or P-450$_{THC}$. *Lanes 1* and *4*, *2* and *5*, and *3* and *6* contain mouse, guinea pig, and human hepatic microsomes, respectively. *Lanes 1*, *2*, and *3* were probed with anti-mouse hepatic P-450$_{THC}$, and *lanes 4*, *5*, and *6* were probed with anti-mouse hepatic P-450IIIA. The lane marked by an *arrow* contains prestained standards with apparent molecular weights of 106,000, 80,000, 49,500, 32,500, 27,500, and 18,500.

47,000 (Fig. 3) and a specific content of 15.4 nmol/mg and exhibited an absorbance maxima at 452 nm for the reduced carbon monoxide complex. The isozyme was purified in the presence of both DTT and THC, although subsequent studies have revealed that inclusion of DTT alone was sufficient for maintaining high THC hydroxylase activity during the purification process.

NH_2-terminal amino acid sequence analysis. The NH_2-terminal sequence of 13 of the first 16 residues of P-450$_{THC}$ (Table 2) is 85 and 69% identical to published sequences of rat hepatic P-450IIC7 (32) and P-450IIC6 (33), respectively. The characterized P-450$_{THC}$ sequence is identical to a recently reported sequence of a P-450 isozyme termed P-450PBIII (34), which was purified from hepatic microsomes of phenobarbital-treated mice. The NH_2-terminal sequence of P-450$_{THC}$ appears to be most similar to those of the P-450IIC subfamily, although definitive classification of P-450$_{THC}$ within this subfamily is not possible with such limited sequence data.

Catalytic properties of P-450$_{THC}$. P-450$_{THC}$ exhibited high activity for cannabinoid hydroxylation and specifically produced 6α- and 7-OH-THC as well as 6α-, 7-, and 4″-OH-CBD (Table 3). P-450$_{THC}$ also catalyzed benzphetamine and ethylmorphine *N*-demethylation, as well as testosterone 16β-hydroxylation. THC hydroxylase activity was found to be very dependent on the presence of lipid in the reconstituted system, whereas the addition of cytochrome b_5 resulted in only a slight stimulation of activity (Table 4).

Immunoinhibition studies. Unlike the anti-P-450IIIA antibody (Fig. 1), antibody raised against purified P-450$_{THC}$ markedly inhibited the microsomal formation of the major cannabinoid metabolites (Fig. 4). 6α- and 7-OH-THC formation was significantly inhibited, to <10% of control values, whereas 6α-, 7-, and 4″-OH-CBD formation was inhibited to approximately 30% of control values.

Although mice and rats preferentially hydroxylate THC at the 6α-position, guinea pigs and humans preferentially hydroxylate THC at the 6β-position (35). Anti-mouse P-450IIIA IgG was found to immunoreact with a specific polypeptide in guinea pig and human liver microsomes that was of similar molecular weight as mouse P-450IIIA (Fig. 5). Anti-mouse P-450IIIA IgG inhibited 6β-OH-THC formation to approximately 60 and 20% of control values in guinea pig and human microsomes, respectively, but had little effect on 7-OH-THC formation (Fig. 6). In contrast, anti-P-450$_{THC}$ IgG, which was found to immunoreact with a specific polypeptide in guinea pig and human liver microsomes, decreased 7-OH-THC formation to 60% of control values in both guinea pig and human microsomes but had little effect on 6β-OH-THC formation.

Discussion

CBD treatment of mice resulted in a marked inhibition of hepatic microsomal cannabinoid metabolism. The decreased formation of several minor cannabinoid metabolites appears to be due to the previously described inactivation of P-450IIIA (20). On the other hand, formation of the major cannabinoid metabolites was catalyzed by a different isozyme, termed P-450$_{THC}$, that appears to have an NH_2-terminal amino acid sequence similar to that of members of the P-450IIC subfamily. This isozyme is responsible for most of the microsomal cannabinoid metabolism in the mouse and specifically hydroxylates THC at the 6α- and 7-positions. 7-OH-THC is the major THC metabolite observed in virtually all species studied (35) and is of particular clinical importance because its pharmacological activity is believed to be equal to or greater than that of THC itself (19). The presence of an ortholog of mouse P-450$_{THC}$ in human liver microsomes is suggested by the inhibition of 7-OH-THC formation by the anti-mouse P-450$_{THC}$ antibody (Figs. 5 and 6). The lack of complete immunoinhibition may be due to the presence of an additional isozyme involved in 7-OH-THC formation in human liver microsomes or may be related to structural differences between mouse P-450$_{THC}$ and its human ortholog, resulting in a decreased reactivity of the antibody. On the other hand, anti-mouse P-450IIIA was very effective in inhibiting THC 6β-hydroxylase activity in human

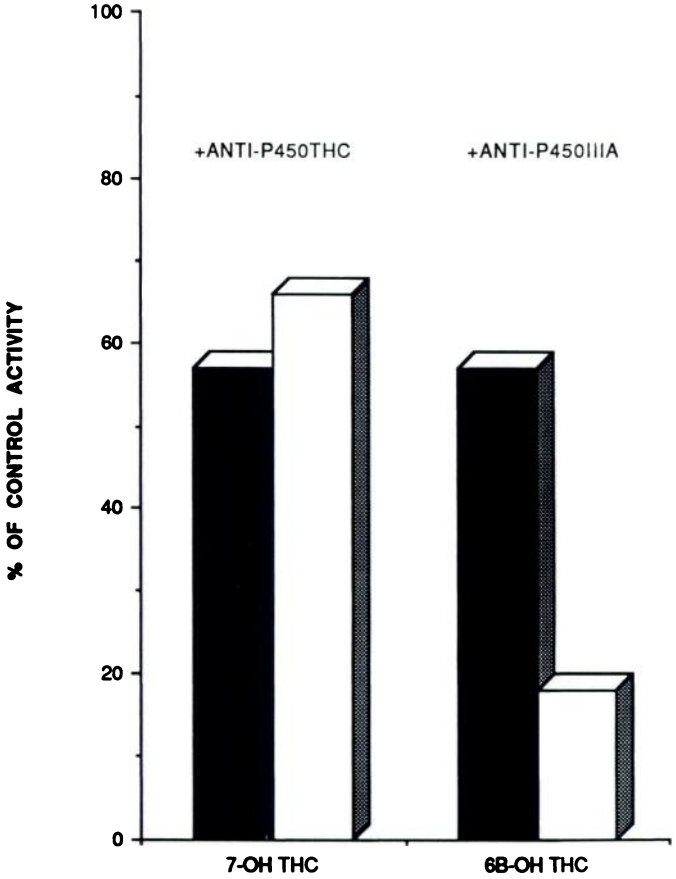

Fig. 6. Effect of anti-mouse P-450$_{THC}$ antibody on guinea pig and human THC hydroxylase activity. Microsomes prepared from guinea pig (■) or human (□) liver were preincubated with anti-mouse P-450$_{THC}$ (*left*) or anti-mouse P-450IIIA (*right*) antibody before the determination of THC hydroxylase activity, as described in Experimental Procedures. Comparable amounts of preimmune IgG incubated with hepatic microsomes inhibited activities <15%. Values for THC hydroxylase activity are expressed as the percentage of the activity in microsomes determined in the absence of antibody. Values are the average of duplicate determinations from hepatic microsomes from guinea pig (two animals) and human (one source). Metabolites eluted at identical HPLC retention times as 7- and 6β-OH-THC. Activities were 0.30 and 0.53 nmol/mg/min (7-OH-THC) and 0.35 and 1.26 nmol/mg/min (6β-OH-THC) in control guinea pig and human microsomes, respectively.

liver microsomes, suggesting that the human ortholog is immunochemically very similar to mouse P-450IIIA and that it is responsible for most of the THC 6β-hydroxylase activity of human liver microsomes.

Although *in vivo* CBD treatment was found to inactivate hepatic microsomal P-450$_{THC}$, *in vitro* incubations of CBD with purified P-450$_{THC}$ (reconstituted with appropriate cofactors) did not result in any loss of P-450 chromophore, as seen with similarly incubated purified P-450IIIA, or in irreversible functional inactivation of purified P-450$_{THC}$, as observed with microsomal P-450IIIA-dependent activities. In addition, activities attributed to P-450$_{THC}$ were also found to be resistant to CBD-mediated inhibition after preincubation of microsomes with CBD and NADPH (data not shown). Thus, CBD does not appear to inactivate P-450$_{THC}$ directly but may first require metabolic transformation to a potentially toxic metabolite via nonhepatic microsomal pathways.

Alternatively, proteolytic processes that may be negligible or nonfunctional during the limited *in vitro* incubation could be responsible for the differences observed between the *in vivo* and *in vitro* effects of CBD on P-450$_{THC}$ activity. However, increased proteolytic degradation of P-450$_{THC}$ after *in vivo* CBD-mediated inactivation does not appear likely, because in contrast to its 90% functional loss, immunochemically determined microsomal P-450$_{THC}$ content was decreased by only approximately 15%.

The NH_2-terminal amino acid sequence of P-450$_{THC}$ is identical to the sequence previously reported for P-450PBIII (34), an isozyme with benzphetamine and ethylmorphine *N*-demethylase activities comparable to those of P-450$_{THC}$. However, on the basis of immunoquantitation studies, this isozyme was reported to be markedly induced by phenobarbital (9-fold). We have found that phenobarbital treatment of mice only modestly increased microsomal P-450$_{THC}$ content and 6α- and 7-OH-THC formation (less than 2-fold), thus suggesting either that P-450$_{THC}$ is different from P-450PBIII or that P-450PBIII content was overestimated, possibly due to the well recognized cross-reactivity between structurally related isozymes (34, 36). Several members of the P-450IIC subfamily have exhibited immuno-cross-reactivity in rats (36), and it is likely that a similar cross-reactivity is also found in mice. Furthermore, it is noteworthy that phenobarbital treatment only modestly increased (<2-fold, comparable in magnitude to the increases we have observed) the reported functional activity attributed to P-450PBIII, considerably less than the 9-fold increase detected immunochemically (34).

CBD treatment of mice results in the inactivation of at least two constitutive P-450 isozymes, which together account for the majority of the detected cannabinoid metabolites. This marked inhibition of oxidative metabolism may contribute to the markedly increased glucuronidation of CBD, relative to that of THC, a major difference observed in the metabolic disposition of these cannabinoids (2). Although CBD treatment decreased the microsomal formation of most THC metabolites, the formation of the minor metabolite 6β-OH-THC was actually increased, making it the major metabolite observed under these conditions (Fig. 1). Such alterations in the metabolic profile of THC metabolism may contribute to the altered response to THC observed after CBD treatment (15–18). Because anti-P-450IIIA antibody decreased microsomal THC 6β-hydroxylase activity by only 60%, it is plausible that an additional P-450 isozyme is also responsible for 6β-OH-THC production. Its presence would explain the paradoxical increase in THC 6β-hydroxylase activity observed after *in vivo* CBD treatment.

The structural relationship of P-450$_{THC}$ to the P-450IIC subfamily is unclear. A member of the P-450IIC subfamily (P-450IIC8) has been purified from human microsomes and shown to hydroxylate retinol (vitamin A) and retinoic acid at the 4-position (37). This position is allylic to the intracyclic double bond (Fig. 2) and is equivalent to the 6-position of THC. P-450IIC8 also produced several other unidentified polar metabolites of retinol. 18-Hydroxyretinoic acid (structurally similar to 7-OH-THC) was the major metabolite identified in rat feces after a pharmacological dose of retinoic acid (38), revealing that vitamin A might be metabolized similarly to THC, i.e., predominantly at both allylic carbon positions. Because vitamin A and the cannabinoids appear to be metabolized by P-450s of the same subfamily (P-450IIC), CBD-mediated inhibition of P-450IIC function may also alter vitamin A metabolism. This interaction may be clinically important, especially when

large doses of vitamin A are therapeutically employed. Vitamin A has been proposed to be the physiological substrate of P-450IIC8 (37), and we have demonstrated that cannabinoids are extensively metabolized by the same P-450 subfamily, in a similar chemical fashion. It remains to be elucidated whether the common structural features of these two classes of compounds that determine their specific metabolism by P-450IIC also modulate their pharmacological effects. Identification and structural characterization of the endogenous ligand for the newly identified cannabinoid receptor in the brain (19) should provide important clues to this intriguing question.

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