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INDUCTION AND GENETIC REGULATION OF MOUSE HEPATIC CYTOCHROME P450 BY CANNABIDIOL

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Abstract—Cannabidiol (CBD) has been shown to be a selective inactivator of cytochromes P450 (P450s) 2C and 3A in the mouse and, like many P450 inactivators, it can also induce P450s after repeated administration. The inductive effects of CBD on mouse hepatic P450s 2B, 3A, and 2C were determined using cDNA probes, polyclonal antibodies, and specific functional markers. P450 2B10 mRNA was increased markedly after repeated CBD administration and correlated well with increased P450 2B immunoquantified content and functional activity. On the other hand, although the 2-fold increase in P450 3A mRNA detected after repeated CBD administration was consistent with the increased immunoquantified P450 3A protein content, the lack of an observable increase in P450 3A-specific functional activity suggested subsequent inactivation of the induced P450 3A. Repeated CBD treatment increased P450 2C mRNA content 2-fold, but did not increase either the P450 2C immunoquantified content or its functional activity. The effect of CBD treatment on the ability of tetrahydrocannabinol (THC) to induce P450 2B was also determined. A THC dose that did not induce P450 2B significantly was administered alone or in combination with a CBD dose that markedly inactivated P450s 2C- and 3A but submaximally increased P450 2B functional activity. The combination of THC and CBD did not increase P450 2B-catalyzed activity significantly over that observed after CBD treatment alone. Thus, prior CBD-mediated P450 inactivation does not appear to increase the ability of THC to induce P450 2B. To further characterize the relationship between P450 inactivation and induction, several structurally diverse CBD analogs with varying P450 inactivating potentials were tested for their ability to induce P450 2B. At least one CBD analog that is an effective P450 inactivator failed to induce P450 2B, while at least one CBD analog that is incapable of inactivating P450 was found to be a very good P450 2B inducer. It therefore appears that inherent structural features of the CBD molecule rather than its ability to inactivate P450 determine P450 2B inducibility. The complex effects of CBD treatment on P450 inactivation and induction have the potential to influence the pharmacological action of many clinically important drugs known to be metabolized by these various P450s. The mechanism of CBD-mediated P450 induction remains to be elucidated but does not appear to be related to CBD-mediated P450 inactivation.

Key words: cannabidiol; tetrahydrocannabinol; cytochrome P450; induction; inactivation; genetic regulation

Cytochromes P450 comprise a family of related hemoproteins, collectively termed P450† and consisting of at least 12 gene families and 22 subfamilies in mammals [1]. P450s are involved in the oxidative metabolism of many drugs, fatty acids, steroids, and environmental toxins [2, 3], and although such metabolism usually results in detoxification, bioactivation of certain compounds can also occur [4]. Because P450s catalyze the metabolism of many substrates, competitive inhibition can result from compounds competing for the same substrate binding site. Noncompetitive inhibition can result from irreversible or quasi-irreversible P450 inactivation occurring during metabolism of certain substrates. Many compounds that inhibit or inactivate P450(s) after acute treatment, can also induce P450(s) upon long-term exposure [5–7]. Although this biphasic response is not always observed with all P450 inactivators [8], it is possible that these two processes are related, such that P450 induction is a physiologic compensatory response to P450 inactivation.

CBD, a major constituent of marijuana and a potent inhibitor of mammalian drug metabolism [9–11], has been shown to exert its effects by competitive inhibition in the rat [12] and P450 inactivation in the mouse [13, 14]. Based on limited amino-terminal sequence data, it appears that CBD selectively inactivates P450s 2C and 3A in the mouse [13, 14] and like many P450 mechanism-based inactivators, it can also induce P450 content after repetitive treatment [11]. P450 2B is found to be induced after repetitive CBD treatment but is resistant to CBD-mediated inactivation [15]. THC, the psychoactive constituent of marijuana, although structurally similar to CBD does not inactivate or induce P450s [11].

The following studies are an attempt to characterize more fully the CBD-mediated P450 induction and to determine if there is a relationship between P450

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† Abbreviations: P450, cytochromes P450; CBD, cannabidiol; THC, tetrahydrocannabinol; PB, phenobarbital; PROD, pentoxyresorufin *O*-dealkylase; ABN, *abnormal*-CBD; and MM, monomethyl-CBD.

inactivation and induction. Several CBD analogs with differing potentials to inactivate P450 were synthesized and tested for their ability to induce P450. In addition, because prior CBD-mediated P450 inactivation would increase the persistence of THC in the liver by decreasing its metabolism, the influence of CBD treatment on the ability of THC to induce P450 2B was determined.

MATERIALS AND METHODS

Materials. CBD and THC were supplied by the National Institute on Drug Abuse. All cannabinoids were prepared for intraperitoneal injection in a Tween 80 suspension as previously described [16].

6α-, and 6-keto-CBD [17], MM [18, 19] and ABN [20] were prepared as described previously [21]. Spectra obtained after gas chromatography/mass spectrometric and NMR analyses of synthesized compounds were similar to those reported previously [17–20].

Animal treatment. Animals were treated with cannabinoids (120 mg/kg once daily for 4 days or as described in the figure legends) before being killed by cervical dislocation 24 hr after the last dose. Liver microsomes were prepared as described [11]. Sodium PB in saline was administered for 4 days (80 mg/kg, i.p.).

Analytical procedures. P450 concentrations were determined with an SLM Aminco DW 2000 spectrophotometer 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. PROD activity was determined as described [24].

Determination of THC hydroxylase activity Microsomal protein (0.1 mg) was preincubated at 37° for 3 min, in a buffer containing 0.1 M potassium phosphate, pH 7.4, diethylenetriaminepentaacetic acid (1 mM) and THC (130 μM, final concentration, added in 3 μL of ethanol) in a total volume of 1 mL. The incubation, metabolite extraction and quantitation were carried out as described previously [14].

Immunochemical procedures. Hepatic microsomes were solubilized, electrophoresed, transferred to nitrocellulose, and probed with polyclonal antibodies raised against purified mouse P450s 2B [15], 2C [14] and 3A [13] as described. Immunoblots were quantitated by densitometric analysis on a Hoefer scanning densitometer as described [13].

Isolation and sequencing of mouse P450 3A and 2C cDNA. A mouse (C57 Black/6) liver cDNA library in the lambda gt10 vector (Stratagene, La Jolla, CA) was screened according to the recommended protocol with rat P450 3A1 [25] and 2C7 [26] cDNA probes (provided by Dr. M. Lechner, Oeiras, Portugal). cDNA inserts (~1 kb) were obtained from positive clones after digestion with *Hind* III (3A) or *Eco*RI (2C), and subcloned into pBluescript SK+ phagemids (Stratagene). XL1-Blue (Stratagene) *Escherichia coli* cells were transformed by electroporation with a Gene Pulser Apparatus (Bio-Rad, Richmond, CA). Both strands

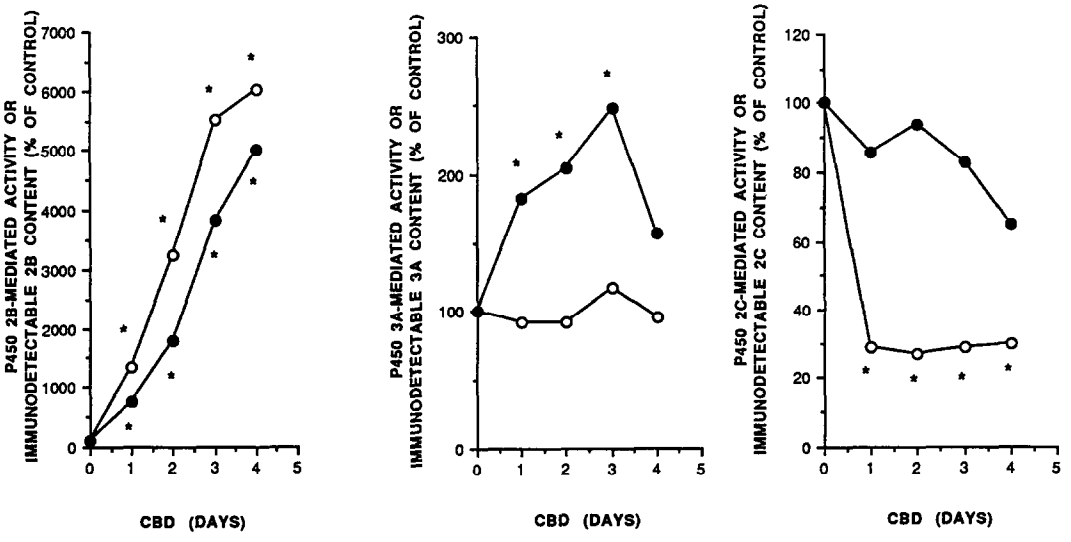

Fig. 1. Effect of repetitive daily CBD doses on hepatic microsomal P450 2B, 3A and 2C immunoquantified content and functional activity. Mice were treated with CBD (120 mg/kg) once daily for the indicated number of days. Hepatic microsomes were prepared and assayed for specific content (●) and activities catalyzed by P450s 2B, 3A and 2C (○). Values are expressed as percent of control values and were determined from the average of at least five different microsomal preparations. Basal activities in hepatic microsomes from untreated mice were <10 pmol/mg/min, 0.21 ± 0.12 nmol/mg/min, and 1.54 ± 0.22 nmol/mg/min for P450 2B-catalyzed PROD, P450 3A-catalyzed 6-keto-THC hydroxylase, and P450 2C-catalyzed 6α-THC hydroxylase activities, respectively. P450 2B, 3A and 2C protein content was determined by relative densitometric analysis. Values significantly different ($P < 0.05$) from control values are marked by an asterisk (*).

were completely sequenced by the dideoxynucleotide method [27] using a Sequenase 2.0 kit (US Biochemicals, Cleveland, OH) or the *fmol* DNA Sequencing System (Promega, Madison, WI) for the mouse hepatic P450 3A and 2C clones, respectively.

RNA blot analysis. Total hepatic RNA was prepared [28] from either untreated mice or mice treated with CBD or PB for 4 days. Poly(A)+ RNA was isolated by oligo(dT) cellulose chromatography [29], and 2 μg samples were denatured and electrophoresed in 2.2 M formaldehyde–0.8% agarose gels with subsequent transfer to Hybond-N$^+$ nylon filters (Amersham Corp., Arlington Heights, IL) by capillary blotting with 20 × SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0). RNA was fixed to the membrane by UV-irradiation, and the membranes were prehybridized in 50% formamide, 5 × SSPE (0.9 M sodium chloride, 0.05 M sodium phosphate, 5 mM EDTA, pH 7.7), 5 × Denhardt's solution, 0.5% SDS, and 250 μg/mL fragmented salmon sperm DNA at 42° for 2 hr before the addition of a denatured probe (~1 kb cDNA inserts described above) radiolabeled with a random primer DNA labeling system (GIBCO BRL, Gaithersburg, MD) for P450s 2C and 3A, or with an oligonucleotide radiolabeled by polynucleotide T4 kinase (Promega) for P450 2B10 mRNA. The sequence of the oligonucleotide probe for P450 2B10 mRNA, 5′-GAATCACACCATATTCCTTGAAGGTTGGC-TCAACGA-3′ corresponding to the reverse complement of the nucleotide sequence 279–314 from the coding region [30] was synthesized by Dr. D. Julius (University of California, San Francisco). After overnight hybridization, the filters were washed sequentially with 6 × SSC, 2 × SSC, 0.5 × SSC and 0.1 × SSC at 50° and then autoradiographed overnight at −70° for P450 2C and 3A mRNA determination or washed sequentially with 6 × SSC, 2 × SSC, and 0.5 × SSC at 42° and then autoradiographed for 72 hr at −70° for the P450 2B10 mRNA determination. All probes were found to hybridize to only one band (~2 kb) on the filters. Autoradiograms were quantitated densitometrically with a Hoefer scanning densitometer as described [13] and normalized according to the γ-actin content, determined with a γ-actin cRNA probe radiolabeled during synthesis with bacteriophage SP6 RNA polymerase [31]. The γ-actin cDNA [32] and cRNA probe were provided by Dr. Raja M. Kaikaus (University of California, San Francisco). Blot densities (mean ± SD) of hepatic mRNA from untreated mice were 34,148 ± 18,608 and 20,629 ± 4466 arbitrary area units for P450 3A and 2C mRNAs, respectively, but undetectable (<500 arbitrary area units) for P450 2B10 mRNA. The blot density (mean ± SD) of hepatic γ-actin mRNA was 31,604 ± 7340 arbitrary area units for all samples applied.

Statistical analysis. Statistical significance was determined by Student's *t*-test.

RESULTS

*Effect of repeated CBD administration on P*450s *2B, 3A and 2C.* P450 2B is found to be induced markedly after repetitive CBD (120 mg/kg) treatment [11, 15]. To more fully characterize P450 induction by CBD, various doses of CBD for different lengths of time were administered to mice,

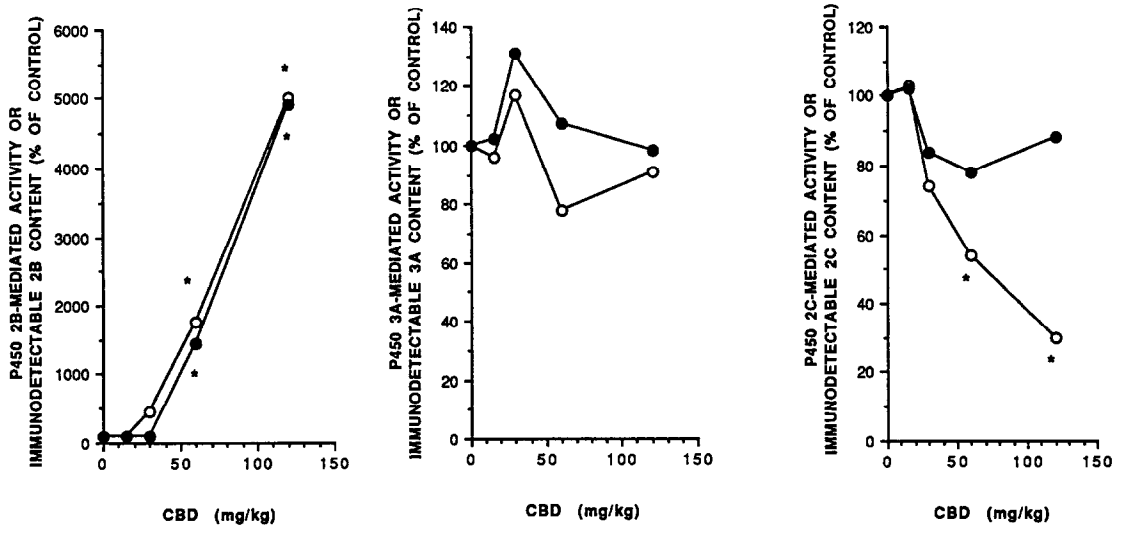

Fig. 2. Effect of CBD dose on hepatic microsomal P450 2B, 3A, and 2C immunoquantified content and functional activity. Mice were treated with CBD once daily for 4 days at the indicated doses. Hepatic microsomes were prepared and assayed for specific content (●) and activities catalyzed (○) by P450s 2B, 3A and 2C. Values are expressed as percent of control values and were determined from the average of at least five different microsomal preparations. Basal activities in hepatic microsomes from untreated mice were the same as described in the legend to Fig. 1. Values significantly different ($P < 0.05$) from control values are marked by an asterisk (*).

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1/1                                                    31/11
TTA AGA TTT GAT TTT TTT GAT CCT TTG CTC TTC TCA GTA GTA CTT TTT CCA TTC CTG ACA
leu arg phe asp phe phe asp pro leu leu phe ser val val leu phe pro phe leu thr
61/21                                                  91/31
CCA GTA TAT GAG ATG TTA AAT ATC TGC ATG TTC CCA AAG GAT TCA ATA GAA TTT TTC AAA
pro val tyr glu met leu asn ile cys met phe pro lys asp ser ile glu phe phe lys
121/41                                                 151/51
AAA TTT GTG GAC AGA ATG AAG GAA AGC CGC CTG GAT TCT AAG CAG AAG CAC CGA GTG GAT
lys phe val asp arg met lys glu ser arg leu asp ser lys gln lys his arg val asp
181/61                                                 211/71
TTT CTT CAG CTG ATG ATG AAT TCT CAT AAT AAT TCC AAA GAC AAA GTC TCT CAT AAA GCC
phe leu gln leu met met asn ser his asn asn ser lys asp lys val ser his lys ala
241/81                                                 271/91
CTT TCT GAC ATG GAG ATC ACA GCC CAG TCA ATT ATC TTT ATT TTT GCT GGG TAT GAA ACC
leu ser asp met glu ile thr ala gln ser ile ile phe ile phe ala gly tyr glu thr
301/101                                                331/111
ACC AGT AGC ACA CTT TCC TTC ACC CTG CAT TCC TTG GCC ACT CAC CCT GAT ATC CAG AAA
thr ser ser thr leu ser phe thr leu his ser leu ala thr his pro asp ile gln lys
361/121                                                391/131
AAA CTG CAG GAT GAG ATC GAT GAG GCT CTG CCC AAC AAG GCA CCT CCC ACG TAT GAT ACT
lys leu gln asp glu ile asp glu ala leu pro asn lys ala pro pro thr tyr asp thr
421/141                                                451/151
GTG ATG GAG ATG GAA TAC CTG GAT ATG GTG CTT AAT GAA ACC CTC AGA TTA TAT CCC ATT
val met glu met glu tyr leu asp met val leu asn glu thr leu arg leu tyr pro ile
481/161                                                511/171
GCT AAT AGA CTT GAG AGA GTC TGT AAG AAA GAT GTT GAA CTC AAT GGT GTG TAT ATC CCC
ala asn arg leu glu arg val cys lys lys asp val glu leu asn gly val tyr ile pro
541/181                                                571/191
AAA GGG TCA ACA GTG ATG ATT CCA TCT TAT GCT CTT CAC CAT GAC CCA CAG CAC TGG TCA
lys gly ser thr val met ile pro ser tyr ala leu his his asp pro gln his trp ser
601/201                                                631/211
GAG CCT GAA GAA TTC CAA CCT GAA AGG TTC AGC AAG GAG AAC AAG GGC AGC ATT GAT CCT
glu pro glu glu phe gln pro glu arg phe ser lys glu asn lys gly ser ile asp pro
661/221                                                691/231
TAT GTA TAT CTG CCC TTT GGG AAT GGA CCC AGG AAC TGC CTT GGC ATG AGG TTT GCT CTC
tyr val tyr leu pro phe gly asn gly pro arg asn cys leu gly met arg phe ala leu
721/241                                                751/251
ATG AAT ATG AAA CTT GCT CTC ACT AAA ATT ATG CAG AAC TTC TCC TTC CAG CCT TGT AAG
met asn met lys leu ala leu thr lys ile met gln asn phe ser phe gln pro cys lys
781/261                                                811/271
GAA ACA CAG ATA CCT CTG AAA TTA AGC AGA CAA GGA CTT CTT CAA CCA GAA AAA CCC ATT
glu thr gln ile pro leu lys leu ser arg gln gly leu leu gln pro glu lys pro ile
841/281                                                871                    901
GTT CGG AAT TCA GCT TGG ACT TAACCAGGCTGAACTTGCTCAAAAGGTGGGACTACCCAGCAGTCTATAGAG
val arg asn ser ala trp thr
                          931                                  961
CAGCTCGAAAACGGTAAAACTAAGCGACCACGACTTTTTACCAGAACTTGCGTCAGCTCTTGGCGTAAGTGTTGACTGGC
991                                   1021                                 1051
TGCTCAATGGCACCTCTGATTCGAATGTTAGATTTGTTGGGCACGTTGAGCCAAAGGGAAATATCCATTGATTAGCATG
                1081
GTTAGAGCTGGTTCGTGGTGTG
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Fig. 3. Nucleotide and deduced amino acid sequence of a mouse liver P450 3A cDNA. The nucleotide sequence was determined by the dideoxy method and compared with that of *Cyp*3a-11 reported by Yanagimoto *et al.* [33]. Underlined sequences denote differences from *Cyp*3a-11.

and P450 2B, 3A or 2C content in hepatic microsomes was measured by immunoblot analysis. In addition, the functional activities of these P450s were determined using enzyme selective substrates [14, 24]. PROD activity and 6-keto- and 6α-hydroxy-THC formation were used as selective markers for P450 2B-, 3A-, and 2C-functional activities, respectively. P450 2B was found to be induced by CBD treatment in a time- and dose-dependent manner (Figs. 1 and 2). Both immunoquantified P450 2B content and P450 2B-selective PROD activity, undetectable in untreated mice, were increased markedly after CBD treatment. Although P450 3A apoprotein content was increased sig-

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1/1                                                    31/11
TTG GAT GTT ACA ATT CCT CGG GAC TTT ATT GAT TAT TTC CTA ATT AAT GGA GGC CAG GAA
leu asp val thr ile pro arg asp phe ile asp tyr phe leu ile asn gly gly gln glu
61/21                                                  91/31
AAC GGC AAC TAT CCA*TTG AAA AAT AGG CTT GAA CAC CTG GCA ATA ACA GTG ACT GAT CTT
asn gly asn tyr pro  leu lys asn arg leu glu his leu ala ile thr val thr asp leu
121/41                                                 151/51
TTT TCT GCT GGA ACA GAG ACA ACA AGC ACA ACA CTG AGA TAT GCT CTC CTA CTC CTG TTG
phe ser ala gly thr glu thr thr ser thr thr leu arg tyr ala leu leu leu leu leu
181/61                                                 211/71
AAG TAC CCA CAT GTC ACA GCT AAA GTC CAG GAA GAA ATT GAG CAT GTG ATC GGC AAG CAC
lys tyr pro his val thr ala lys val gln glu glu ile glu his val ile gly lys his
241/81                                                 271/91
CGA AGA CCA TGC ATG CAG GAC AGG AGC CAC ATG CCC TAT ACT GAT GCC ATG ATT CAT GAG
arg arg pro cys met gln asp arg ser his met pro tyr thr asp ala met ile his glu
301/101                                                331/111
GTC CAG AGA TTC ATT GAC CTT GTC CCC AAC AGC CTG CCC CAT GAA GTG ACC TGT GAC ATT
val gln arg phe ile asp leu val pro asn ser leu pro his glu val thr cys asp ile
361/121                                                391/131
AAA TTC AGG AAC TAC TTC ATC CCC AAG GGG ACA AAT GTA ATA ACA TCA CTG TCA TCA GTG
lys phe arg asn tyr phe ile pro lys gly thr asn val ile thr ser leu ser ser val
421/141                                                451/151
CTG CGT GAT AGC AAA GAG TTC CCC AAC CCA GAG AAG TTT GAC CCT GGG CAC TTT CTA GAT
leu arg asp ser lys glu phe pro asn pro glu lys phe asp pro gly his phe leu asp
481/161                                                511/171
GAG AAC GGA AAG TTT AAG AAA AGT GAC TAC TTC ATG CCC TTC TCA ACA GGA AAA CGG ATA
glu asn gly lys phe lys lys ser asp tyr phe met pro phe ser thr gly lys arg ile
541/181                                                571/191
TGT GCA GGA GAA GGC CTG GCA CGC ATG GAG CTG TTC CTA TTC CTA ACC AGC ATT TTA CAG
cys ala gly glu gly leu ala arg met glu leu phe leu phe leu thr ser ile leu gln
601/201                                                631/211
AAC TTC AAC CTG AAA CCT CTG GTT CAC CCA AAG GAC ATA GAT GTA ACC CCA ATG CTC ATT
asn phe asn leu lys pro leu val his pro lys asp ile asp val thr pro met leu ile
661/221                                                691/231                721
GGA TTG GCC TCA GTG CCT CCT GCT TTC CAG CTC TGC TTC ATT CCT TCC TGAAAATCAAATGCCT
gly leu ala ser val pro pro ala phe gln leu cys phe ile pro ser
                              751                                      781
GGCTCCAGCTGGGACATCTTCTGTGATCACCCTGAGGCTTTAATTCACTACTTTCCACACTGGACACACTGCTTTTTATC
      811                                    841                                871
CATTTCTTTTGCCTGCTCATAATTTCATAAGGTTCTATGAACATACATTTACCAATTTGCAGATCCTGCACAAATATTTC
                  901                                    931                              961
TGACTGGTCCTATTTTCTGAAACACTGTTACCATATGCTGTGATGATATGGACCTGATTCCAAGTTAAATATACTCTCAT
                                    991                                    1021
AAAATTGAAATTACTATTGTAAGATAATTCACTGTCGCTTCTCTTCTGCATTTTCTTAATAATAAATCTTTTGGTATAAC
      1051
TAAAAAAAAAAAAAAAAA
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Fig. 4. Nucleotide and deduced amino acid sequence of a mouse liver P450 2C cDNA. The nucleotide sequence was determined by the dideoxy method and compared with that of the coding region of rat P450 2C6 reported by Kimura *et al.* [34]. Underlined sequences denote differences from rat P450 2C6. Bold sequence and asterisk denote inserted and deleted codons, respectively, as compared with P450 2C6.

nificantly after repetitive CBD treatment (120 mg/kg) for 1–3 days, P450 3A-catalyzed 6-keto-THC formation was not (Fig. 1). On the other hand, while P450 2C content was not affected significantly by CBD treatment, its dependent 6α-hydroxy-THC formation was decreased in a time- and dose-dependent manner (Figs. 1 and 2).

The lack of correlation between the CBD-increased immunodetectable P450 3A protein content and its unchanged functional activity may be rationalized either by an accumulation of CBD-inactivated P450 3A protein or by a CBD-mediated increase in P450 3A expression with subsequent inactivation. To determine if the increased amount of immunoquantitated P450 3A protein was due to increased P450 3A expression, P450 3A mRNA

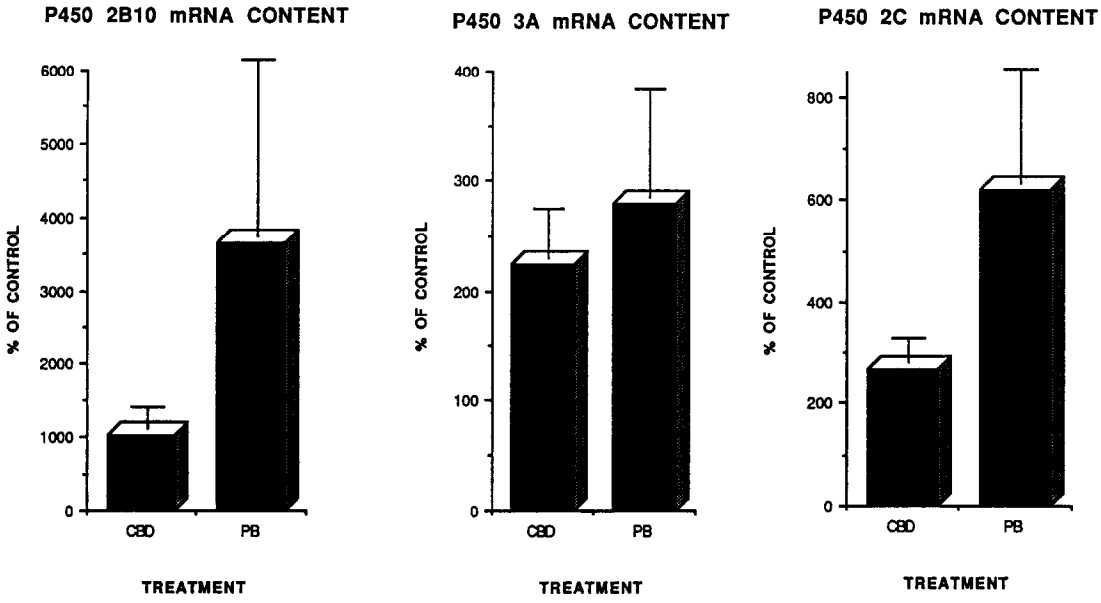

Fig. 5. Effects of CBD and PB treatment on mouse hepatic P450 2B10, 3A and 2C mRNA content. Mice were treated for 4 days with CBD (120 mg/kg) or PB (80 mg/kg) before being killed, and hepatic RNA was isolated. Poly(A)+ RNA samples were electrophoresed and transferred to nylon filters for the determination of P450 2B10, 2C and 3A mRNA content, normalized according to γ-actin content. Values (means ± SD) are expressed as percent of control values and represent the average of at least four different hepatic mRNA preparations. The blot densities (mean ± SD) of hepatic mRNA from untreated mice were 34,148 ± 18,608 and 20,629 ± 4466 arbitrary area units for P450s 3A and 2C mRNA, respectively, but undetectable (<500 arbitrary area units) for P450 2B10 mRNA. All values were significantly different ($P < 0.05$) from control values.

content was determined using a 1089 bp P450 3A probe isolated from a mouse liver cDNA library (Fig. 3). This sequence is nearly identical to the one reported for a P450 3A cDNA isolated from a liver library prepared from dexamethasone-treated mice and designated *Cyp*3a-11 [33]. In addition, the effect of CBD treatment on hepatic P450 2C and 2B10 mRNA content was determined using a 1062 bp P450 2C probe (Fig. 4) isolated from a mouse liver cDNA library and a 36-mer oligonucleotide probe specific for P450 2B10 [30], respectively.

Mice were treated repetitively with either CBD or PB (a known inducer of P450s 2B1/2, 3A1/2, and 2C6/7 in rats) once daily for 4 days, and their hepatic mRNA content was determined 24 hr after the last administered dose (Fig. 5). P450 2B10 mRNA was undetectable in the livers from untreated mice, but was readily detected in mice treated with either CBD or PB (>10- and >30-fold increases relative to untreated controls). PB treatment increased P450 2B10 mRNA content approximately 3-fold higher than CBD treatment, consistent with the corresponding increases of P450 2B-selective PROD activity [15]. P450 3A mRNA was increased 2- to 3-fold by both CBD and PB treatment relative to that in untreated controls. Thus, the CBD-mediated increase in P450 3A expression may contribute to the immunodetectably elevated P450 3A content found after CBD treatment (Figs. 1 and 2). On the other hand, P450 2C mRNA content was increased >2-fold by CBD treatment and >6-fold by PB treatment. Thus, repeated CBD administration increased the hepatic mRNA content of all P450s determined, although not to the same extent as PB.

Effect of CBD on the ability of THC to induce P450 2B10. Although repeated CBD administration induces P450 2B10, similar treatment with THC did not correspondingly result in its induction [11], suggesting that P450 2B10 induction is not a common property of all cannabinoids. Because CBD and THC also markedly differed in their ability to acutely inactivate P450 [11], the possibility existed that P450 inactivation was involved in cannabinoid-mediated P450 2B10 induction. To determine if prior CBD-mediated P450 inactivation could influence the ability of THC to induce P450 2B10, mice were first treated with CBD (30 mg/kg), which markedly inhibits P450s 2C- and 3A-dependent THC hydroxylase activity by 50 and 80%, respectively [21], and submaximally increased P450 2B10-catalyzed PROD activity to only 16% of that observed after a 120 mg/kg CBD dose (Fig. 6). Two hours after CBD treatment, a 120 mg/kg dose of THC (which normally does not induce P450 2B10 significantly) was administered, and this combined treatment was repeated once daily for 4 days. The combination of THC and CBD did not increase significantly P450 2B10-catalyzed PROD activity over that observed

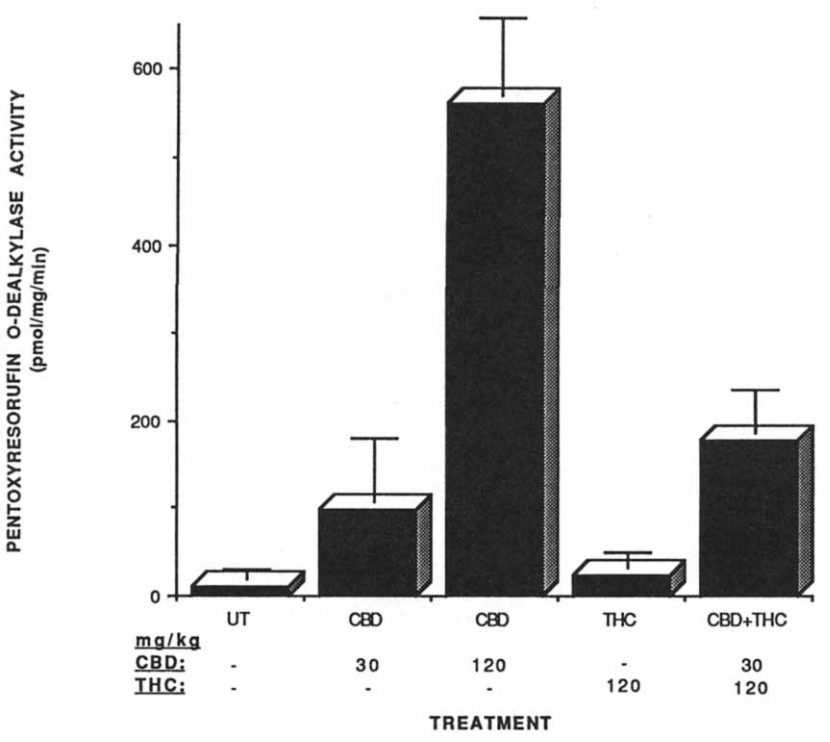

Fig. 6. Effect of CBD pretreatment on THC-mediated P450 2B10 induction. Mice were treated with vehicle, 30 or 120 mg/kg CBD, or 120 mg/kg THC daily for 4 days or with 30 mg/kg CBD 2 hr before receiving 120 mg/kg THC and this dual dosing regimen was repeated once daily for 4 days. Hepatic microsomes were prepared 24 hr after the last injection, and P450 2B10-catalyzed PROD activity was determined. Values are the means ± SD of at least three different microsomal preparations.

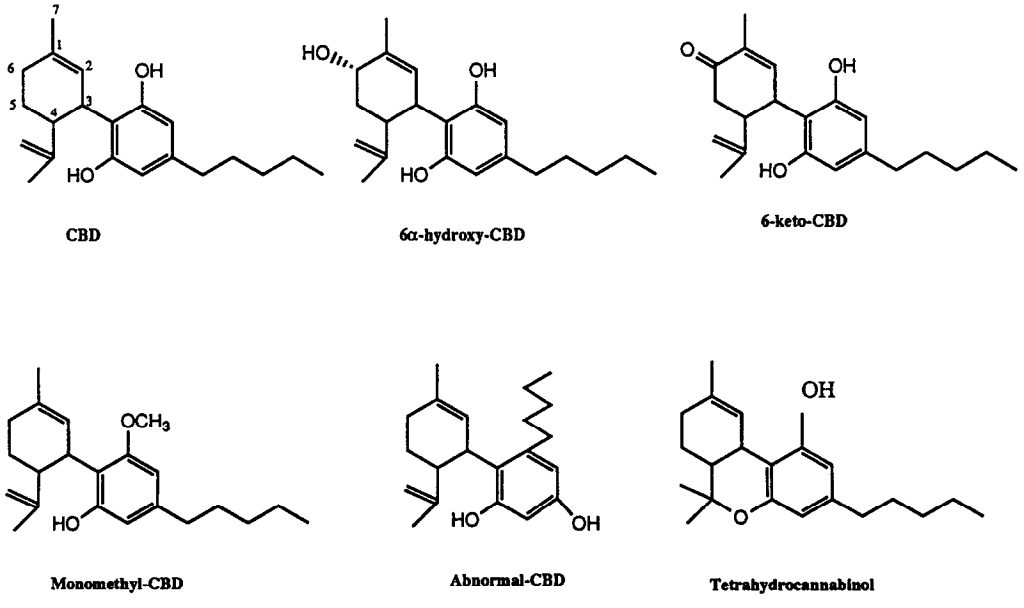

Fig. 7. Structures of CBD and other analogs.

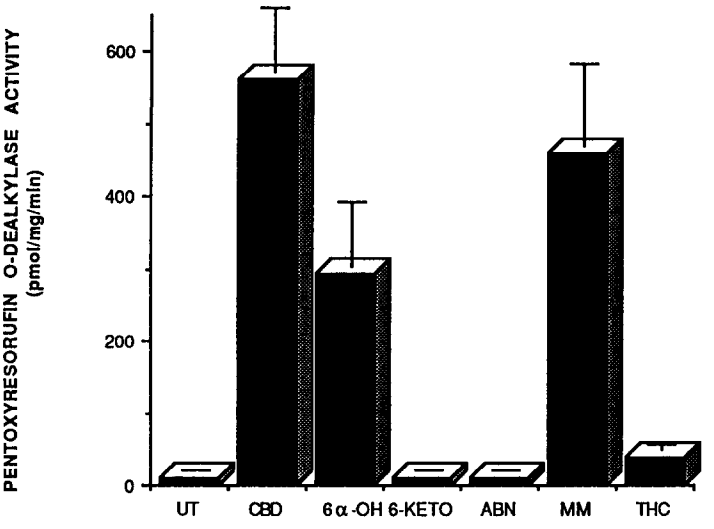

Fig. 8. Effect of CBD analogs on P450 2B10-catalyzed PROD activity. Mice were treated with vehicle or 120 mg/kg cannabinoid daily for 4 days. Hepatic microsomes were prepared 24 hr after the last injection, and P450 2B10-catalyzed PROD activity was determined. Values are the means ± SD of at least three different microsomal preparations.

after CBD treatment alone (Fig. 6). Thus, prior CBD-mediated P450 inactivation does not appear to enhance the ability of THC to induce P450 2B10.

Effect of CBD analogs on P450 inactivation/induction. To characterize further the features necessary for cannabinoid-mediated P450 2B induction, several structurally different CBD analogs with different abilities to inactivate P450 [21] were tested (Fig. 7). 6α-Hydroxy-CBD and 6-keto-CBD, primary CBD metabolites produced by mouse hepatic microsomal P450s 2C and 3A, respectively [14], were capable of acutely inactivating P450s 2C and 3A *in vivo*, as does CBD [21]. When these compounds were administered daily for 4 days, 6α-hydroxy-CBD markedly increased P450 2B10-catalyzed PROD activity but 6-keto-CBD had no effect (Fig. 8). ABN, a positional isomer of CBD which was a very good inactivator of P450s 2C and 3A [21], also failed to increase P450 2B10-catalyzed PROD activity. In contrast, MM, which did not inactivate P450s 2C and 3A after an acute 120 mg/kg dose [21], markedly increased P450 2B10-catalyzed PROD activity. In confirmation of previous results [11], THC administration for 4 days only weakly increased P450 2B10-catalyzed PROD activity.

DISCUSSION

Effect of repeated CBD administration on P450 2B. Acute CBD treatment of mice has been shown to inhibit barbiturate metabolism, as demonstrated by a marked increase in hexobarbital-induced sleep-time [16, 35], and to impair the metabolism of many other drugs [9, 36]. In mice, this effect of CBD on hepatic drug metabolism results from the inactivation of P450s 2C and 3A, most likely through alkylation of their apoproteins by a reactive CBD metabolite [21]. Refractoriness to a CBD-mediated increase in hexobarbital-induced sleep-time develops after repetitive CBD treatment [37], which correlates with induction of P450 2B10, an enzyme resistant to CBD-mediated inactivation and not immunochemically or functionally detectable in constitutive mouse liver microsomes [11]. Our results in mice clearly demonstrated that repetitive CBD treatment markedly increased immunodetectable hepatic P450 2B10 protein and P450 2B10-dependent PROD activity in a dose- and time-dependent manner (Figs. 1 and 2), consistent with increased hepatic P450 2B10 mRNA expression (Fig. 5). The 30-fold induction of P450 2B10 mRNA by PB treatment is also consistent with previous findings [38].

It has been reported recently that repetitive CBD treatment of rats does not induce P450 2B1/2 mRNA by itself but can potentiate the effect of PB on P450 2B1/2 mRNA [39]. Although the failure of CBD to induce P450 2B1/2 mRNA in rats may be species related, it is likely that the CBD dose used in the rat studies (25 mg/kg) was insufficient to induce P450 2B1/2, since our studies show little or no P450 2B10 induction in mice at CBD doses up to 30 mg/kg (Fig. 2). In fact, we have found that rats treated with a considerably higher dose of CBD (120 mg/kg daily, for 4 days) had a 9-fold increase in immunoquantified P450 2B1/2 content and a 7-fold increase in P450 2B1/2-catalyzed PROD activity (unpublished results).

Effect of repeated CBD administration on P450s 3A and 2C. In addition to determining the effect of CBD on the genetic regulation of P450 2B10, the effect of CBD on the regulation of P450s 3A and 2C syntheses was also examined. Both mouse hepatic P450 3A and 2C mRNA contents were significantly

increased over 2-fold in response to repeated CBD administration relative to untreated control values. Although the increased P450 3A mRNA correlated with significantly increased P450 3A protein content, no corresponding increase in P450 2C protein content was found to match the increased P450 2C mRNA content (Fig. 1). Despite the increased mRNA content, P450 3A- and 2C-mediated functional activities were not increased significantly (Fig. 1), probably because of continued P450 3A and 2C inactivation after repeated CBD treatment. The lack of increased functional activity cannot be attributed to any competitive effects of CBD on P450 3A-and 2C-catalyzed THC hydroxylase activities since CBD is eliminated rapidly from the liver [40] and was present in hepatic microsomal incubations at concentrations (<2 μM) insufficient to competitively inhibit THC (130 μM) hydroxylase activity. PB treatment, on the other hand, which caused even larger (3- to 6-fold) increases in mouse hepatic P450 3A and 2C mRNA contents (Fig. 5), increased, the corresponding proteins and functional activities 50–200% over untreated controls. These findings are consistent with previous reports of PB induction of rat 2C6 [41] and 2C7 [39], as well as of a P450 2C6-related mRNA in mice [38]. Thus, it appears that in mice repeated CBD administration is capable of inducing P450 2B10, 3A, and 2C mRNAs, although not to the same extent as PB.

Characterization of the P450 3A and 2C probes. The P450 3A clone isolated from a mouse liver cDNA library was nearly identical to *Cyp*3a-11 isolated from a liver library prepared from dexamethasone-treated mice [33]. Nucleotides 1–843 of this probe are identical to nucleotides 705–1548 of *Cyp*3a-11 and code for amino acids 201–491. However, the following 144 nucleotides (844–987) share only 26% identity with the corresponding region of *Cyp*3a-11. The translated region of this segment codes for 13 amino acids in *Cyp*3a-11 and 6 amino acids in the P450 3A cDNA probe used in this study. In addition, the 3′-untranslated region of this probe also extends 107 nucleotides downstream from the 3′-end of the *Cyp*3a-11 cDNA. Whether these differences represent allelic variants or truly distinct gene products of a multi-member P450 3A subfamily in mice remains to be determined. The *Cyp*3a-11 cDNA was isolated from male ddY mice treated with dexamethasone for 4 days, and thus may be related to an mRNA encoding a steroid-inducible P450 3A [33]. However, the P450 3A cDNA isolated in the present study was isolated from untreated C57 Black/6 female mice and may be related to an mRNA encoding a constitutive P450 3A. A more extensive comparison with a full-length clone will be the subject of future work. However, because the longest homologous segment between male rat constitutive P450 3A2 and steroid-inducible P450 3A1 is 425 nucleotides long [42], it is likely that the 844 homologous nucleotides of *Cyp*3a-11 and our P450 3A do not represent different genes but most probably a splice variant as reported for P450 2C6 and its pseudogene 2C6P [43]. The deduced NH_2-terminal amino acid sequence of *Cyp*3a-11 is identical to the NH_2-terminal amino acid sequence of a constitutive P450 3A purified from hepatic microsomes from dexamethasone-treated mice [13]. However, since it is also identical to the NH_2-terminal amino acid sequence of a steroid inducible P450 3A purified from hepatic microsomes from dexamethasone-treated mice [44], it is not clear whether *Cyp*3a-11 cDNA and/or our P450 3A cDNA code(s) for the constitutive or steroid-inducible mouse P450 3A isozymes. Our P450 3A cDNA probably encodes for the constitutive P450 3A, since it was isolated from a cDNA library from untreated mice, although it would probably cross-hybridize to mRNA for the steroid-inducible P450 3A, since in the rat these cDNAs share 90% homology [42]. Thus, it is not presently possible to precisely characterize the CBD-induced mRNA as encoding a constitutive or steroid-inducible P450 3A. However, since CBD- and PB-inducible P450s 3A are inactivated almost completely by subsequent acute CBD treatment [13], whereas the steroid-inducible P450 3A is resistant to CBD-mediated inactivation [13], it is therefore likely that the constitutive rather than the steroid-inducible P450 3A form is induced after repeated CBD or PB treatment. Thus, the increased P450 3A mRNA in livers from mice repetitively treated with CBD or PB (Fig. 5) probably encodes the constitutive P450 3A form and contributes to its induction (Fig. 1).

The cDNA probe used for determining mouse P450 2C mRNA content exhibited the greatest homology to coding sequences of rat P450 2C6 [34], and was 82 and 79% identical to the corresponding nucleotide and deduced amino acid sequence, respectively. However, because the deduced NH_2-terminal amino acid sequence could not be determined from the mouse cDNA clone (since it corresponded to amino acids present in its carboxy-terminus and not to those in the NH_2-terminus), it is unclear whether it encodes for the P450 2C previously purified [14] and responsible for the formation of the major THC metabolites 6α- and 7-hydroxy-THC.

Relationship between P450 inactivation and induction. To determine if CBD-mediated P450 induction is a general consequence of cannabinoid exposure, the ability of THC and other cannabinoids to induce P450 was also examined. Although THC, the major psychotropic component of marijuana, does not appear to induce P450 2B10 to any great extent [11], it was conceivable that in combination with CBD (which is also present in significant amounts in marijuana) its ability to induce P450 2B10 could be enhanced. Because CBD markedly inhibits the hepatic microsomal metabolism of THC, CBD treatment could be expected to raise THC levels in the liver sufficiently high for P450 induction. At a CBD dose that acutely inhibited P450 3A- and 2C-catalyzed THC metabolism [21], but submaximally increased P450 2B-catalyzed PROD activity (Fig. 6), THC given concomitantly failed to increase significantly P450 2B-catalyzed PROD activity over that produced by CBD alone. Because CBD is eliminated rapidly from the liver [40], it is unlikely that the inability of THC to induce P450 2B10 results from any competitive effects with CBD in the induction process. Thus, it appears that increasing THC persistence did not enhance

THC-mediated P450 2B induction and that THC, alone or in combination with CBD, is not a very effective P450 inducer. Most likely, THC does not possess the structural features required for P450 2B induction.

Furthermore, the ability of the CBD structural analogs that do not inactivate P450s 3A and 2C to induce P450 2B10, and vice versa, further demonstrates the dissociation between the processes of P450 inactivation and P450 induction. Several P450 inactivators [21] failed to induce P450 2B10, while at least one CBD analog (MM) that is incapable of inactivating P450 was a very good P450 2B10 inducer (Fig. 8). It therefore appears that CBD-mediated P450 2B10 induction is probably determined by specific structural features of the CBD molecule (possibly through a receptor-mediated process), rather than by its ability to inactivate P450. Due to the limited number of analogs tested, it is not clear at this time which particular structural features are involved in CBD-mediated P450 induction.

In summary, CBD treatment of mice resulted in the initial inactivation of P450s 3A and 2C with a subsequent increase in mRNA encoding P450s 3A, 2C, and 2B10. The induction of P450 2B10 was not related to the inactivation of P450s 3A and 2C and probably was unrelated to the increased cannabinoid accumulation that results from P450 inactivation. Although there was increased P450 3A expression after repetitive CBD treatment, the induced P450 3A was susceptible to CBD-mediated inactivation and thus no increase in P450 3A-catalyzed activity was observed. These complex effects on P450 may affect the metabolism of many clinically important drugs known to be metabolized by these P450 subfamilies.

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REFERENCES

1. Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, Gonzalez FJ, Coon MJ, Gunsalus IC, Gotoh O, Okuda K and Nebert DW, The P450 superfamily: Update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol* **12**: 1–51, 1993.
2. Conney AH, Pharmacological implications of microsomal enzyme induction. *Pharmacol Rev* **19**: 317–366, 1967.
3. Gillette J, Davis DC and Sasame HA, Cytochrome P-450 and its role in drug metabolism. *Annu Rev Pharmacol* **12**: 57–84, 1972.
4. Ortiz de Montellano PR and Reich NO, Inhibition of cytochrome P-450 enzymes. In: *Cytochrome P-450: Structure, Mechanism, and Biochemistry* (Ed. Ortiz de Montellano PR), pp. 273–314. Plenum Press, New York, 1986.
5. De Matteis F, Loss of heme in rat liver caused by the porphyrogenic agent 2-allyl-2-isopropylacetamide. *Biochem J* **124**: 767–777, 1971.
6. Ryan DE, Ramanathan L, Iida S, Thomas RE, Haniu M, Shively JE, Lieber CS and Levin W, Characterization of a major form of rat hepatic microsomal cytochrome P-450 induced by isoniazid. *J Biol Chem* **260**: 6385–6393, 1985.
7. Pessayre D, Descatoire V, Konstantinova-Mitcheva M, Wandscheer JC, Cobert B, Level R, Benhamou JP, Jaouen M and Mansuy D, Self-induction by triacetyloleandomycin of its own transformation into a metabolite forming a stable 457 nm-absorbing complex with cytochrome P-450. *Biochem Pharmacol* **30**: 553–558, 1981.
8. Ortiz de Montellano PR and Costa AK, Dissociation of cytochrome P-450 inactivation and induction. *Arch Biochem Biophys* **251**: 514–524, 1986.
9. Siemens AJ, Kalant H, Khanna JM, Marshman J and Ho G, Effect of cannabis on pentobarbital-induced sleeping time and pentobarbital metabolism in the rat. *Biochem Pharmacol* **23**: 477–488, 1974.
10. Bornheim LM, Effect of cannabidiol on drug metabolism. In: *Biochemistry and Physiology of Substance Abuse* (Ed. Watson RR), Vol. 1, pp. 21–35. CRC Press, Boca Raton, 1989.
11. Bornheim LM and Correia MA, Effect of cannabidiol on cytochrome P-450 isozymes. *Biochem Pharmacol* **38**: 2789–2794, 1989.
12. Narimatsu S, Watanabe K, Yamamoto I and Yoshimura H, Mechanism for inhibitory effect of cannabidiol on microsomal testosterone oxidation in male rat liver. *Drug Metab Dispos* **16**: 880–889, 1988.
13. Bornheim LM and Correia MA, Selective inactivation of mouse liver cytochrome P-450IIIA by cannabidiol. *Mol Pharmacol* **38**: 319–326, 1990.
14. Bornheim LM and Correia MA, Purification and characterization of the major hepatic cannabinoid hydroxylase in the mouse: A possible member of the cytochrome P-450IIC subfamily. *Mol Pharmacol* **40**: 228–234, 1991.
15. Bornheim LM and Correia MA, Purification and characterization of a mouse liver cytochrome P-450 isozyme induced by cannabidiol. *Mol Pharmacol* **36**: 377–383, 1989.
16. Bornheim LM, Borys HK and Karler R, Effect of cannabidiol on cytochrome P-450 and hexobarbital sleep time. *Biochem Pharmacol* **30**: 503–507, 1981.
17. Lander N, Ben-Zvi Z, Mechoulam R, Martin B, Nordqvist M and Agurell S, Total synthesis of cannabidiol and Δ^1-tetrahydrocannabinol metabolites. *J Chem Soc Perkin Trans I* 8–16, 1976.
18. Mechoulam R and Gaoni Y, A total synthesis of Δ^1-tetrahydrocannabinol, the active constituent of hashish. *J Am Chem Soc* **87**: 3273–3275, 1965.
19. Watanabe K, Arai M, Narimatsu S, Yamamoto I and Yoshimura H, Self-catalyzed inactivation of cytochrome P-450 during microsomal metabolism of cannabidiol. *Biochem Pharmacol* **36**: 3371–3377, 1987.
20. Razdan RK, Dalzell HC and Handrick GR, Hashish. A simple one-step synthesis of (-)-Δ^1-tetrahydrocannabinol (THC) from *p*-mentha-2,8-dian-1-ol and olivetol. *J Am Chem Soc* **96**: 5860–5865, 1974.
21. Bornheim LM, Everhart ET and Correia MA, Characterization of cannabidiol-mediated cytochrome P-450 inactivation. *Biochem Pharmacol* **45**: 1323–1331, 1993.
22. Omura T and Sato R, The carbon monoxide-binding

pigment of liver microsomes. *J Biol Chem* **239**: 2370–2378, 1964.
23. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
24. Lubet RA, Mayer RT, Cameron JW, Nims RW, Burke MD, Wolff T and Guengerich FP, Dealkylation of pentoxyresorufin: A rapid and sensitive assay for measuring induction of cytochrome(s) P-450 by phenobarbital and other xenobiotics in the rat. *Arch Biochem Biophys* **238**: 43–48, 1985.
25. Ribeiro V and Lechner MC, Cloning and characterization of a novel CYP3A1 allelic variant: Analysis of CYP3A1 and CYP3A2 sex-hormone-dependent expression reveals that the CYP3A2 gene is regulated by testosterone. *Arch Biochem Biophys* **293**: 147–152, 1992.
26. Barroso M, Dargouge O and Lechner MC, Expression of a constitutive form of cytochrome P450 during rat-liver development and sexual maturation. *Eur J Biochem* **172**: 363–369, 1988.
27. Sanger F, Nicklen S and Coulson AR, DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**: 5463–5467, 1977.
28. Chomczynski P and Sacchi N, Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**: 156–159, 1987.
29. Aviv H and Leder P, Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc Natl Acad Sci USA* **69**: 1408–1412, 1972.
30. Noshiro M, Lakso M, Kawajiri K and Negishi M, Rip locus: Regulation of female-specific isozyme (I-P-$450_{16\alpha}$) of testosterone 16α-hydroxylase in mouse liver, chromosomal localization, and cloning of P-450 cDNA. *Biochemistry* **27**: 6434–6443, 1988.
31. Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K and Green MR, Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization with plasmids containing a bacteriophage SP6 promotor. *Nucleic Acids Res* **12**: 7035–7056, 1984.
32. Enoch T, Zinn K and Maniatis T, Activation of the human β-interferon gene requires an interferon-inducible factor. *Mol Cell Biol* **6**: 801–810, 1986.
33. Yanagimoto T, Itoh S, Muller-Enoch D and Kamataki T, Mouse liver cytochrome P-450 (P-450IIIAM1): Its cDNA cloning and inducibility by dexamethasone. *Biochim Biophys Acta* **1130**: 329–332, 1992.
34. Kimura H, Yoshioka H, Sogawa K, Sakai Y and Fujii-Kuriyama Y, Complementary DNA cloning of cytochrome P-450s related to P-450(M1) from the complementary DNA library of female rat livers. *J Biol Chem* **263**: 701–707, 1988.
35. Borys HK and Karler R, Cannabidiol and Δ^9-tetrahydrocannabinol metabolism. *In vitro* comparison of mouse and rat liver crude microsome preparations. *Biochem Pharmacol* **28**: 1553–1559, 1979.
36. Paton WDM and Pertwee RG, Effect of cannabis and certain of its constituents on pentobarbitone sleeping time and phenazone metabolism. *Br J Pharmacol* **44**: 250–261, 1972.
37. Borys HK, Ingall GB and Karler R, Development of tolerance to the prolongation of hexobarbitone sleeping time caused by cannabidiol. *Br J Pharmacol* **67**: 93–101, 1979.
38. Corcos L, Phenobarbital and dexamethasone induce expression of cytochrome P-450 genes from subfamilies IIB, IIC, and IIIA in mouse liver. *Drug Metab Dispos* **20**: 797–801, 1992.
39. Deutsch DG, Tombler ER, March JE, Lo SHC and Adesnik M, Potentiation of the inductive effect of phenobarbital on cytochrome P450 mRNAs by cannabidiol. *Biochem Pharmacol* **42**: 2048–2053, 1991.
40. Karler R, Sangdee P, Turkanis SA and Borys HK, The pharmacokinetic fate of cannabidiol and its relationship to barbiturate sleep time. *Biochem Pharmacol* **28**: 777–784, 1979.
41. Freidberg T, Waxman DJ, Atchinson M, Kumar A, Haaparanta T, Raphael C and Adesnik M, Isolation and characterization of cDNA clones for cytochromes P-450 immunochemically related to rat hepatic P-450 form PB-1. *Biochemistry* **25**: 7975–7983, 1986.
42. Gonzalez FJ, Song BJ and Hardwick JP, Pregnenolone 16α-carbonitrile-inducible P-450 gene family: Gene conversion and differential regulation. *Mol Cell Biol* **6**: 2969–2976, 1986.
43. Kimura H, Sogawa K, Sakai Y and Fujii-Kuriyama Y, Alternative splicing mechanism in a cytochrome P-450 (P-450PB-1) gene generates the two mRNAs coding for proteins of different functions. *J Biol Chem* **264**: 2338–2342, 1989.
44. Bornheim LM, Everhart ET and Li J, Effect of cannabidiol (CBD) on cytochrome P450 3A in the mouse. *FASEB J* **7**: A265, 1993.