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EFFECT OF CANNABIDIOL ON CYTOCHROME P-450 ISOZYMES

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Abstract—Cannabidiol (CBD) has been shown to inhibit mouse hepatic mixed-function oxidations of several drugs after acute treatment, whereas repetitive treatment resulted in the restoration of drug-metabolizing capabilities. We have found that acute CBD treatment modestly decreased cytochrome P-450 content but markedly decreased hexobarbital hydroxylase, erythromycin *N*-demethylase, and 6β-testosterone hydroxylase activities. Repetitive CBD treatment, on the other hand, resulted in the restoration of cytochrome P-450 content as well as hexobarbital hydroxylase and erythromycin *N*-demethylase activities. However, after such repeated treatments a fresh dose of CBD can once again inactivate erythromycin *N*-demethylase activity but not hexobarbital hydroxylase activity. The resistance of hexobarbital hydroxylase to re-inactivation by CBD was paralleled by stimulation of pentoxyresorufin *O*-dealkylase activity and the appearance of a 50 kD protein that was immunoreactive to an antibody raised against rat hepatic cytochrome P-450b. CBD metabolism *in vitro* by microsomes prepared from such CBD-"induced" animals, resulted in a pattern of metabolites different from that observed from comparable incubations with liver microsomes from either untreated or phenobarbital-treated animals. Thus, it appears that CBD initially inactivates at least one cytochrome P-450 isozyme, but after repetitive CBD treatment, an isozyme is induced that is resistant to further re-inactivation by CBD. This isozyme appears to be immunochemically similar to, but somewhat functionally distinct from, the isozyme induced by phenobarbital treatment in mice.

Cannabidiol (CBD)†, a major constituent of marijuana, has been shown to inhibit hepatic mixed-function oxidations [1–3] and more recently to decrease cytochrome P-450 content after acute treatment [4–7]. The mechanism for such inactivation is currently unknown but metabolic activation of CBD appears to be required [7, 8]. Although acute CBD treatment results in a decreased capacity to metabolize drugs, repetitive treatment results in the restoration of drug-metabolizing capabilities [4, 5]. For instance, while acute CBD treatment resulted in a 4-fold increase in hexobarbital-induced sleep time, repetitive CBD treatment caused no significant change in the sleep time of control animals, indicating reversal of this inhibition [5]. The mechanism of such reversal was thought to involve some functional change in microsomal enzymes because of the observed alteration in the apparent K_m of aminopyrine *N*-demethylase after repetitive CBD treatment [5]. Although the total hepatic cytochrome P-450 content is not changed, it is possible that repetitive CBD treatment results in induction of a specific cytochrome P-450 isozyme at the expense of others, resulting in no net increase in total cytochrome P-450 content. In fact, Heuman *et al.* [9] have shown that immunochemically detectable cytochrome P-450p (a cytochrome P-450 isozyme induced by glucocorticoids and macrolide antibiotics) is increased at least 10-fold by either 6α-methylprednisolone or spironolactone without any significant increase in total cytochrome P-450 content. If, in fact, CBD does induce a particular cytochrome P-450 isozyme, its identification and characterization might be of considerable clinical significance for predicting potential drug interactions when CBD is ingested either therapeutically as an anti-epileptic or illicitly in the form of marijuana.

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† Abbreviations: CBD, cannabidiol; THC, Δ^9-tetrahydrocannabinol; and PB, phenobarbital.

METHODS

Animals and treatment. Male CF-1 mice (Charles River, Portage, MI) weighing 20–30 g were used in all experiments. CBD or Δ^9-tetrahydrocannabinol (THC) was administered intraperitoneally in a Tween 80 suspension as described previously [5] at an anticonvulsant dose of 120 mg/kg in a single dose acutely (2 hr), repetitively once daily for 4 days (with the animals killed 24 hr after the last dose), or repetitively with an additional fresh dose of CBD (2 hr before sacrifice). Phenobarbital (PB) was injected intraperitoneally at a dose of 100 mg/kg daily for 4 days. Sleep time was measured from the time of the loss of the righting reflex to that of its recovery after induction of sleep by a 100 mg/kg dose of hexobarbital administered intraperitoneally.

Preparation of liver microsomes. Animals were killed by cervical dislocation, the gall bladder was removed, and livers were perfused *in situ* with ice-cold 1.15% KCl. Livers were removed and homogenized at 4° in 3 vol. of 0.1M phosphate buffer (pH 7.4) in a Potter–Elvehjem type of homogenizer with a loose fitting pestle (10 strokes) and after removal of connective tissue, a tight-fitting pestle (7

strokes). The liver homogenate was centrifuged at 9,000 *g* for 10 min in a Sorvall RC 2-B centrifuge in an SS-34 rotor. The resulting supernatant fraction was removed and centrifuged at 105,000 *g* for 60 min. The pellet was resuspended in 1.15% KCl and resedimented at 105,000 *g* for 30 min. The washed microsomal pellets were resuspended in 0.1 M phosphate buffer, pH 7.4.

Enzyme assays. Protein concentration [10] and cytochrome P-450 content [11] as well as the following mixed-function oxidase activities were determined by referenced methods: erythromycin *N*-demethylase [12], pentoxyresorufin *O*-dealkylase [13, 14], hexobarbital hydroxylase [15], and 6β- and 16α-testosterone hydroxylase [16, 17].

Western blotting. Microsomes (3 μg) were electrophoresed [18], transferred to nitrocellulose membranes, and probed with antibodies prepared against rat cytochrome P-450b or P-450p, as described previously [17]. Antibody–antigen complexes were visualized after binding of a second antibody conjugated with horseradish peroxidase and reaction with horseradish peroxidase color reagent (Bio-Rad Laboratories, Richmond, CA).

In vitro *CBD metabolism.* CBD dissolved in ethanol was added to 0.1 M phosphate buffer (pH 7.4) at a final concentration of 130 μM CBD and 0.3% (v/v) ethanol. Microsomal protein (1.5 mg) was added, and the reaction (final volume, 1 ml), was started by the addition of NADPH (1 mM). After incubation at 37° for 10 min, the reaction was terminated by the addition of ice-cold methanol (1 ml). After centrifugation, the supernatant fraction was transferred to a fresh tube, the pellet was reextracted with 0.5 ml methanol and then centrifuged, and the resultant supernatant fraction was combined with the first. This combined methanolic fraction was extracted twice with chloroform (1 and 0.5 ml respectively) and the chloroform layer evaporated under nitrogen. Aliquots were spotted onto silica TLC plates and developed with hexane–acetone (4:3, v/v) and stained as described [19].

RESULTS

In confirmation of previous reports [4, 5], acute CBD treatment of mice markedly prolonged their hexobarbital-induced sleep time, whereas repetitive CBD treatment abolished this effect (Fig. 1A). This correlates well with the effect of CBD treatment on mouse hepatic microsomal hexobarbital metabolism *in vitro*: acutely, CBD decreased hexobarbital hydroxylase activity which returned to normal values after repetitive treatment (Fig. 1B). In addition, acute CBD treatment modestly decreased hepatic cytochrome P-450 content (Fig. 2) but more markedly decreased several mixed-function oxidase activities (erythromycin *N*-demethylase, hexobarbital hydroxylase, and 16α- and 6β-testosterone hydroxylase). In contrast, *p*-chloro-*N*-methylaniline *N*-demethylase activity was not affected significantly (data not shown).

Repetitive CBD treatment, on the other hand, resulted in a slight increase in hepatic microsomal cytochrome P-450 content as compared to that found in acutely treated animals, although not significantly different from the content in untreated animals (Fig. 3). Erythromycin *N*-demethylase activity was found to return to normal by 24 hr after the last of the four repetitive CBD doses (as did 6β-testosterone hydroxylase activity, data not shown), but rechallenge with a fresh CBD dose for 2 hr could again greatly inhibit this particular activity. Hexobarbital hydroxylase activity also returned to normal after repetitive CBD treatment but, in contrast to erythromycin *N*-demethylase activity, it was not inhibited significantly by an additional CBD dose. Repetitive CBD treatment also resulted in a marked (> 35 fold) increase in pentoxyresorufin *O*-dealkylase activity, which was below the limits of detection in untreated controls. This activity, in common with the hexobarbital hydroxylase activity, also appeared to be resistant to inhibition by CBD rechallenge.

To determine if the respective inhibitory and inductive properties of such acute and repetitive treatment were specific for CBD or were a general feature of all cannabinoids, animals were treated with a comparable dose of THC. Acute THC treatment only slightly decreased the enzyme activities examined, whereas repetitive treatment with the drug failed to produce any measurable increases in pentoxyresorufin *O*-dealkylase activity, in marked contrast to those observed after CBD treatment (Fig. 4).

When hepatic microsomes from untreated mice were probed with an antibody prepared against rat hepatic cytochrome P-450b, the major isozyme induced by PB, no cross-immunoreactive protein was detected (Fig. 5), except for a slight contaminating band observed irrespective of drug treatment. This contaminating band was not observed after Western blotting of rat hepatic microsomes, and may represent a minor cross-reacting protein present in the mouse liver. When liver microsomes from an animal repetitively treated with CBD were similarly probed, an immunoreactive protein was readily observed with an apparent molecular weight of approximately 50,000 daltons. A similar immunoreactive protein was also found in mice after PB treatment. Comparable immunoblotting experiments with an antibody against rat hepatic cytochrome P-450p, the major isozyme induced by glucocorticoids and macrolide antibiotics, revealed the presence of an immunoreactive protein in untreated mice, which is somewhat induced after either CBD or PB treatment. Thus, in mice both CBD and PB appear to comparably induce hepatic microsomal proteins immunochemically similar to rat liver cytochromes P-450b and P-450p.

To further characterize the functional changes in drug metabolism induced by repetitive CBD treatment, we examined the hepatic microsomal metabolism of CBD *in vitro*. For this purpose, a metabolic profile was generated after incubation of CBD with untreated mouse liver microsomes *in vitro* (Fig. 6). Acute *in vivo* CBD treatment of mice markedly inhibited hepatic CBD metabolism *in vitro*. Repetitive *in vivo* CBD treatment, on the other hand, restored the formation of many CBD metabolites with a marked increase in two of the more non-polar metabolites (Fig. 6, arrows). In contrast, these two metabolites were not increased appreciably after PB

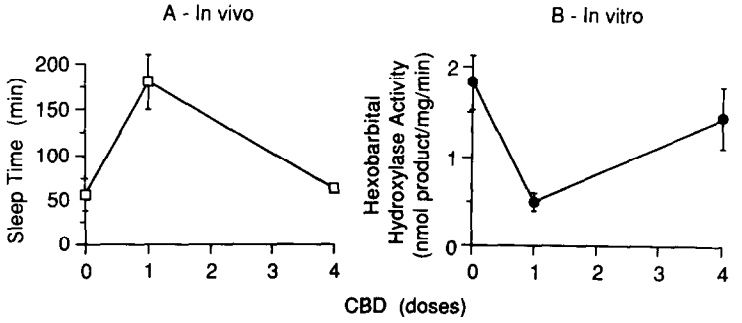

Fig. 1. Effect of CBD treatment on hexobarbital metabolism. (A) Sleep time was measured after the administration of 100 mg/kg hexobarbital, 2 hr after the administration of 120 mg/kg CBD. Animals were pretreated with either vehicle or CBD for 2 hr, or repetitively treated once daily for 4 days. Values are means ± SD of three animals. (B) Hexobarbital hydroxylase activity was measured *in vitro* with hepatic microsomes prepared from animals treated with CBD (120 mg/kg), either acutely (2 hr) or repetitively (once daily for 4 days). Values are means ± SD of three animals.

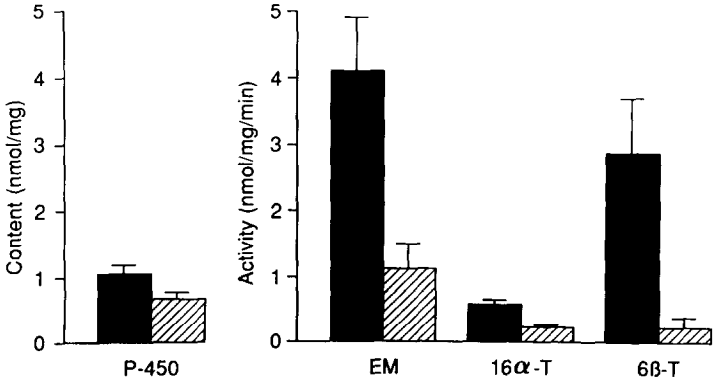

Fig. 2. Effect of acute CBD treatment on mixed-function oxidase activity. Animals were treated with CBD (120 mg/kg, striped bars) or vehicle (solid bars) for 2 hr. Hepatic microsomes were prepared and assayed for cytochrome P-450 (P-450) content, erythromycin *N*-demethylase (EM) and 16α- and 6β-testosterone hydroxylase (T) activity. Values are means ± SD of at least three animals.

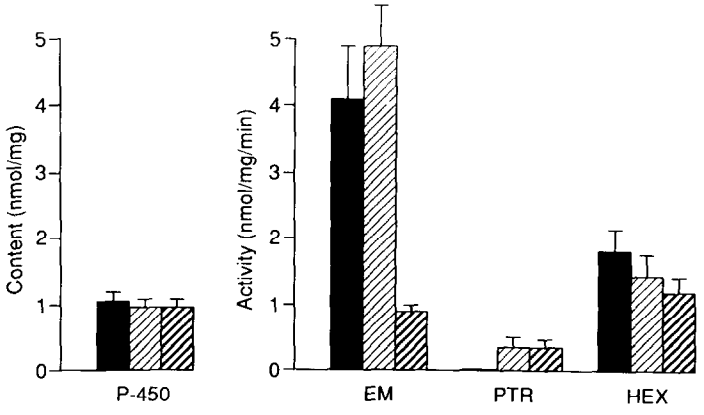

Fig. 3. Effect of repetitive CBD treatment on mixed-function oxidase activity. Animals were treated with vehicle (solid bars), or CBD (120 mg/kg) repetitively once daily for 4 days and killed 24 hr after the last dose (thin striped bars), or repetitively with an additional dose of CBD for 2 hr (bold striped bars). Hepatic microsomes were prepared and assayed for cytochrome P-450 (P-450) content and erythromycin *N*-demethylase (EM), pentoxyresorufin *O*-dealkylase (PTR), or hexobarbital hydroxylase (HEX) activities. Values are means ± SD of at least three animals.

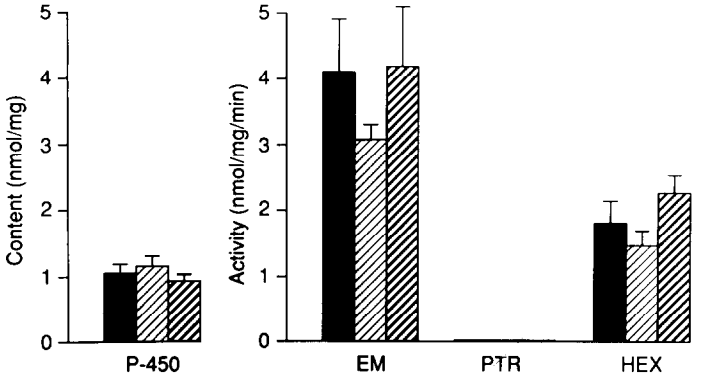

Fig. 4. Effect of THC on mixed-function oxidase activity. Animals were treated with vehicle (solid bars) or THC (120 mg/kg) either acutely (2 hr, thin striped bars) or repetitively once daily for 4 days (bold striped bars). Hepatic microsomes were prepared and assayed for cytochrome P-450 (P-450) content and erythromycin *N*-demethylase (EM), pentoxyresorufin *O*-dealkylase (PTR), or hexobarbital hydroxylase (HEX) activities. Values are means ± SD of at least three animals.

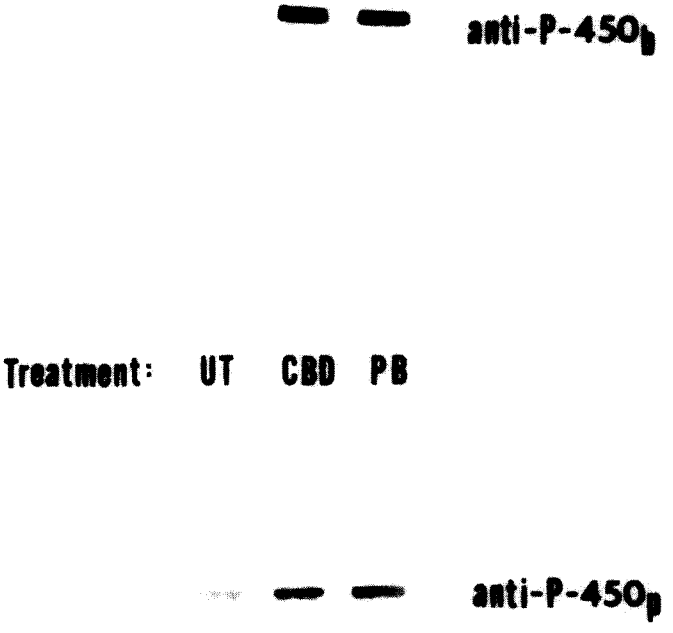

Fig. 5. Western blots of hepatic mouse microsomal cytochrome P-450s after repetitive CBD or PB treatment. Animals were untreated or treated repetitively with either CBD (120 mg/kg for 4 days) or PB (100 mg/kg for 4 days). Hepatic microsomes were prepared, and 3 μg of protein was electrophoresed, transferred to nitrocellulose membranes, and probed with antibodies prepared against rat cytochrome P-450b or P-450p as described previously [17].

induction, nor was the overall metabolism of CBD increased markedly, after such treatment.

DISCUSSION

As previously reported [1, 4, 5, 8], acute CBD treatment prolongs barbiturate-induced sleep time, a reliable measure of *in vivo* drug (barbiturate) metabolism, whereas repeated CBD treatment reverses this effect [4, 5] by a previously unknown mechanism. The present findings reveal that, although acute CBD treatment reduces the hepatic microsomal P-450 content only by 35%, it decreases certain mixed-function oxidase activities (hexobarbital hydroxylase, erythromycin *N*-demethylase, and 16α- and 6β-testosterone hydroxylase) by more than 75%, suggesting a somewhat selective inactivation of cytochrome P-450 isozymes. On the other hand, after repetitive CBD treatment, when barbiturate-induced sleep times had returned to normal, the above oxidase activities were also found to be normalized. However, subsequent acute CBD rechallenge (2 hr) reinactivated erythromycin *N*-demethylase activity but failed to affect hexobarbital metabolism as assayed either *in vivo* or *in vitro*. A possible explanation for this paradox is that acute

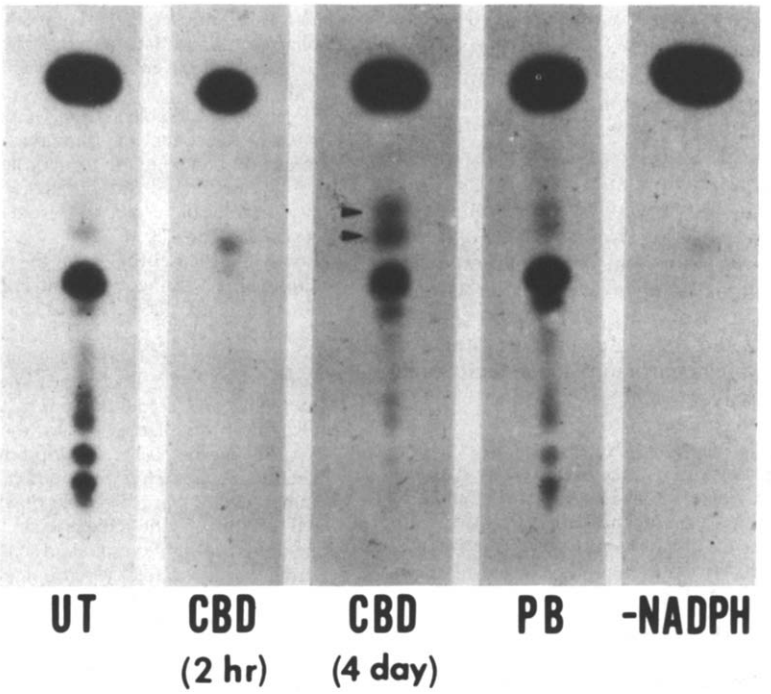

Fig. 6. Thin-layer chromatogram of CBD metabolites. Animals were treated with vehicle, CBD (120 mg/kg) acutely (2 hr) or repetitively (once daily for 4 days), or with PB (100 mg/kg for 4 days). Hepatic microsomes were prepared and incubated in the presence of CBD (130 μM) and NADPH (1 mM) at 37° for 10 min. CBD and metabolites were extracted, spotted onto silica plates, developed with hexane–acetone (4:3, v/v), and stained with 0.1% (w/v) Fast Blue B Salt.

CBD treatment inactivates a constitutive cytochrome P-450 isozyme(s) responsible for the metabolism of both erythromycin and hexobarbital, whereas repetitive CBD treatment results not only in the restoration of an erythromycin *N*-demethylase similar to that originally destroyed by CBD, but also in the induction of a different cytochrome P-450 isozyme which can metabolize hexobarbital but is refractory to CBD inactivation. Accordingly, it is not surprising that erythromycin *N*-demethylase should recover by 24 hr after the last multiple dose of CBD, but be susceptible to re-inactivation by subsequent CBD challenge. Suggestive evidence for induction of a different cytochrome P-450 isozyme not present in the untreated mouse liver is two-fold. First, pentoxyresorufin *O*-dealkylase activity, not present at detectable levels in untreated mice, was stimulated markedly after repetitive CBD treatment. Second, antibodies to rat hepatic cytochrome P-450b, the isozyme responsible for most of the pentoxyresorufin *O*-dealkylase activity in the PB-treated rat [13], recognized a protein in hepatic microsomes from mice repeatedly treated with CBD but not from untreated mice. This suggests that CBD induces a cytochrome P-450 isozyme immunochemically similar if not identical to that induced by PB. Although PB pretreatment of mice also induced an immunoreactively distinct protein and markedly stimulated pentoxyresorufin *O*-dealkylase activity, several lines of evidence suggest that the isozymes induced by these drugs may differ. For instance, although CBD and PB appeared to induce an immunoreactive protein in approximately equal amounts, the resultant pentoxyresorufin *O*-dealkylase activity was 3-fold greater after PB induction than CBD induction (1009 vs 347 pmol resorufin formed/mg/min after PB and CBD treatment, respectively). If the same immunoreactive protein were responsible for the *O*-dealkylase activity, comparable functional levels of the enzyme should have been observed, which is clearly not the case.

In addition, since cytochrome *c* reductase activity was similarly induced by both CBD and PB treatment (data not shown), it appears that the functional differences observed are probably due to differences in the cytochrome P-450 isozymes themselves. Other suggestive evidence that these compounds induce differently also may be derived from the profiles of *in vitro* CBD metabolism. CBD induction results in the formation of two non-polar CBD metabolites to a much greater extent than that observed in untreated or PB-pretreated mice. If CBD were to induce the same protein as PB but to a lesser extent (as the pentoxyresorufin *O*-dealkylase activities may be taken to suggest), then these two CBD metabolites should also have been generated to a correspondingly lesser extent than that observed after PB induction, which is also not the case. Since PB is known to induce several cytochrome P-450 isozymes (P-450s b, e, k, and p) in the rat, it is possible that CBD selectively induces only some of these isozymes, thereby explaining the similarities and differences between these two inducers. Further structural and functional characterization await purification of the specific isozymes induced by CBD and PB in mice.

It is interesting to note that both CBD and PB are effective anti-epileptic agents as well as inducers of a similar hepatic cytochrome P-450 isozyme. Phenytoin, another drug with similar anti-epileptic activity, has also been found to be a weak inducer of microsomal enzymes [20]. Since it appears to exhibit a spatial conformation very similar to that of CBD [21], it remains to be proved whether the spatial conformation which determines anti-epileptic activity of these drugs is also the conformation which determines the inducibility of a particular hepatic cytochrome P-450 isozyme. Further structure–activity studies would be useful in elucidating the mechanism of both cytochrome P-450 induction and anti-epileptic activity.

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