Inhibition of Hepatic Microsomal Cytochrome P450 by Cannabidiol in Adult Male Rats

Shizuo NARIMATSU,[a] Kazuhito WATANABE,[a] Tamihide MATSUNAGA,[a] Ikuo YAMAMOTO,*[,a] Susumu IMAOKA,[b] Yoshihiko FUNAE[b] and Hidetoshi YOSHIMURA[c]

Faculty of Pharmaceutical Sciences, Hokuriku University,[a] Kanagawamachi, Kanazawa 920-11, Japan, Laboratory of Chemistry, Osaka City University Medical School,[b] Asahimachi, Abeno-ku, Osaka 545, Japan and Faculty of Pharmaceutical Sciences, Kyushu University,[c] Maidashi, Higashi-ku, Fukuoka 812, Japan. *Received October 16, 1989*

The mechanism of inhibitory effect of cannabidiol (CBD) on the hepatic drug-metabolizing enzyme system was studied in adult male rats *in vivo*. Time course studies revealed that microsomal *d*-benzphetamine *N*-demethylation and testosterone 2α-, 16α- and 17-oxidation were markedly suppressed 6 to 48 h after the single administration of CBD (10 mg/kg, intraperitoneally). Decreases in activities of aniline hydroxylation and *p*-nitroanisole *O*-demethylation and in content of total cytochrome P450 were intermittent and moderate. On the other hand, no change was observed in reduced nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome c reductase activity or cytochrome b_5 content in the hepatic microsomes of the CBD-treated rats. Western blotting analysis showed a marked decrease in the male-specific cytochrome P450 UT-2 in the hepatic microsomes, especially 24 to 48 h after pretreatment with CBD. It is possible that CBD given 6 to 12 h before the sacrifice might interact with cytochrome P450 as a substrate, resulting in inhibition of the drug-metabolizing enzyme activities in the earlier stages. In the later stages from 24 to 48 h after CBD treatment, the reduction in content of the male-specific cytochrome P450 UT-2 may play a major role in the inhibitory effect of CBD on the hepatic drug-metabolizing enzyme system in the adult male rat *in vivo*.

Keywords cannabidiol; testosterone; hepatic drug-metabolizing enzyme system; oxidation; male-specific cytochrome P450; inhibition; rat

Introduction

It has been established that cannabidiol (CBD), one of the major components of marihuana, has anticonvulsant activity in various experimental animal species.[1-3] This cannabinoid is known to lack psychotropic activity but to inhibit the hepatic drug metabolism.[4-6] Carlini and Cunha[7] reported that coadministration to epileptic patients of CBD and known antiepileptics such as phenytoin and primidone showed better results than single administration. They suggested that this improved effect of CBD might be attributable not only to its anticonvulsant activity but also to its inhibitory effect on the hepatic metabolism of the antiepileptics coadministered.[7] Considering the possibility that CBD may be an antiepileptic, this information provides further reason to study the mechanism of this inhibition on the hepatic drug-metabolizing enzyme system.

We recently reported that CBD inhibited testosterone oxidation, especially at the 2α- and 16α-positions with hepatic microsomes of the male rat.[8] The results indicated that a male-specific cytochrome P450 might selectively be suppressed by CBD. The purpose of the present study was to further clarify the mechanism of the CBD inhibitory effect on the hepatic drug-metabolizing enzyme system of the adult male rat.

Experimental

Materials Chemicals and enzymes were obtained as follows: glucose-6-phosphate (G-6-P), nicotinamide adenine dinucleotide phosphate (NADP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Boehringer-Mannheim GmbH (Darmstadt, F.R.G.); G-6-P dehydrogenase (type V, EC 1.1.1.49), molecular weight marker (Dalton Mark VII) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), cytochrome c (from horse heart, type III), androstenedione, testosterone and epitestosterone were from Sigma Chemical Co. (St. Louis, MO); 2α-hydroxy-testosterone (2α-OH-T), 6β-OH-T, and 16α-OH-T were from Steraloid Inc. (Wilton, NH); anti-rabbit goat immunoglobulin G (IgG) conjugated with alkaline phosphatase was from ICN Biologicals (Lisle, IL); instrument and membranes (Clear Blot Membrane-p) for blot transfer were from Atto Co. (Tokyo, Japan); Freund's complete adjuvant was from Wako Pure Chemicals Co. (Osaka, Japan). Other reagents and solvents used were of the highest quality commercially available.

Treatment of Rats with CBD Male Spraque-Dawley rats (175—200 g body weight, Hokuriku Experimental Animals Lab., Kanazawa, Japan) were intraperitoneally (i.p.) given CBD (10 mg/kg) once a day for 1 or 3 d. The animals were killed by decapitation 24 h after the last injection, and livers were perfused with ice-cold 0.9% (w/v) NaCl. Hepatic microsomal fraction was prepared by the method reported previously.[8] In the time course experiments, rats were given CBD (10 mg/kg, i.p.) and were killed 6, 12, 24, 48, 72 h, and 1 week later.

Enzyme Assay Hepatic microsomal aniline (AN) hydroxylation,[9] *p*-nitroanisole (*p*-NA) *O*-demethylation,[10] and *d*-benzphetamine (*d*-BP) *N*-demethylation[11] were determined by the reported methods. Contents of cytochromes P450 and b_5 were measured by the methods of Omura and Sato,[12] using extinction coefficients of 91 and 185 $mM^{-1}cm^{-1}$, respectively. NADPH-cytochrome c reductase (EC 1.6.2.4) activity was assayed according to the method of Phillips and Langdon.[13] Protein concentration was determined by the method of Lowry *et al.*[14] Microsomal testosterone oxidation was assayed by high performance liquid chromatography (HPLC) using the same method as described previously[8] except for employing a combination of a guard column (Cosmosil $10C_{18}$, 4.5 × 50 mm, Nacarai Tesque, Tokyo, Japan) and a longer Zorbax ODS column (4.6 × 250 mm, DuPont, Wilmington, MA). Retention times of testosterone metabolites were 5.80 (6β-OH-T), 6.45 (16α-OH-T), 8.76 (2α-OH-T), 13.38 (androstenedione), 15.09 (testosterone) and 21.51 min (epitestosterone, internal standard). Statistical significance was assessed by Student's *t*-test.

Preparation of Antiserum against Cytochrome P450 UT-2 A male-specific cytochrome P450 UT-2[15] was purified to be homogenous on SDS-PAGE from livers of male Sprague-Dawley rats (250—300 g body weight) as previously described.[16] The enzyme preparation was mixed with Freund's complete adjuvant to make an emulsion and was given subcutaneously to adult female rabbits (Japanese white, 2.7 to 3.2 kg body weight, obtained from the same source as rats) once a week (50 μg protein of the enzyme per rabbit per week) for four weeks. Blood was collected one week after the last injection, and serum was obtained by centrifugation.

Immunoblot Analysis of Protein A portion of the microsomes was subjected to electrophoresis on 8% polyacrylamide gels according to the method of Laemmli.[17] The protein developed was then electrophoretically transferred to Clear Blot Membrane-p as described by Towbin *et al.*[18] The membrane was probed with the antiserum and alkaline phosphatase-conjugated secondary antibody by the method reported by Gonzalez *et al.*[19] Contents of cytochrome P450 UT-2 were tentatively determined by scanning the bands on the membrane in the Western blot at 750 nm of the wave length using a Shimadzu CS-920 TLC (thin layer chromatography) scanner.

Results

Effects of CBD Pretreatment on the Drug-Metabolizing Enzyme System CBD (10 mg/kg, i.p.) was given once or three times to rats, and effect of the cannabinoid on hepatic microsomal drug metabolism was assessed (Table I). Cytochrome P450 content after three CBD injections and *d*-BP *N*-demethylase activity after single and three injections of CBD were significantly lower than those of the control group which received a vehicle only (1% Tween 80–saline, once). However, no differences were seen in the content of cytochrome b_5 or activities of NADPH–cytochrome c reductase, AN hydroxylase, or *p*-NA *O*-demethylase between the CBD-treated and control groups.

Time Course of Drug-Metabolizing Enzyme Activities after CBD Treatment When male adult rats were injected with CBD (10 mg/kg, i.p.) and were killed 6 h to one week later, the inhibitory effect of CBD pretreatment on the microsomal enzymes was determined. As shown in Fig. 1, cytochrome P450 content was significantly decreased 6 and 12 h after the CBD administration. Significant decreases were also seen in AN hydroxylation and *p*-NA *O*-demethylation 6 and 48 h after the injection. The activity of *d*-BP *N*-demethylation, on the other hand, was continually suppressed from 6 to 48 h after the cannabinoid administration. These suppressed activities returned to the control levels 72 h after the CBD treatment. Activity of NADPH–cytochrome c reductase tended to increase at 6 h and to decrease 72 h after the CBD treatment, but without significance. Furthermore, cytochrome b_5 contents in hepatic microsomes of the animals treated with CBD were unchanged from those of the control group throughout the test period (Table II).

Time Course of Testosterone Oxidation after CBD Treatment Figure 2 shows the inhibitory effect of CBD pretreatment on the microsomal testosterone oxidation determined by HPLC. The activity of 6β-OH-T formation was significantly suppressed only at 6 h after the CBD injection. Other metabolite formations were continually suppressed 6 to 48 h after the administration of the cannabinoid.

Immunoblotting Analysis Western blotting analysis using rabbit antiserum against a male-specific cytochrome P450 UT-2 revealed that the protein of cytochrome P450 UT-2 was reduced by CBD pretreatment (Fig. 3). The

TABLE I. Effects of CBD Pretreatment on Hepatic Microsomal Drug-Metabolizing Enzyme System of Adult Male Rats

Treatment	Cytochrome P450[a)]	Cytochrome b_5[a)]	NADPH–cyt.c reductase[b)]	AN hydroxylase[c)]	*p*-NA *O*-demethylase[c)]	*d*-BP *N*-demethylase[c)]
Control (Vehicle)	0.59±0.05 (100)	0.39±0.04 (100)	0.12±0.01 (100)	1.03±0.08 (100)	2.21±0.16 (100)	2.72±0.14 (100)
Treated (CBD×1)	0.50±0.07 (86)	0.39±0.05 (100)	0.11±0.01 (91)	1.04±0.05 (101)	2.18±0.09 (98)	1.96±0.07[d)] (72)
Treated (CBD×3)	0.41±0.09[d)] (69)	0.36±0.04 (94)	0.12±0.01 (100)	0.95±0.09 (92)	2.18±0.08 (98)	1.54±0.15[e)] (57)

a) nmol per mg of protein. *b*) μmol per min per mg. *c*) nmol per min per mg. AN, *p*-NA and *d*-BP mean aniline, *p*-nitroanisole and *d*-benzphetamine, respectively. Each value represents the mean of four determinations. Numbers in parentheses are relative activities (%) to the control. *d*) Significantly different from the control ($p<0.05$). *e*) Significantly different from the control ($p<0.01$).

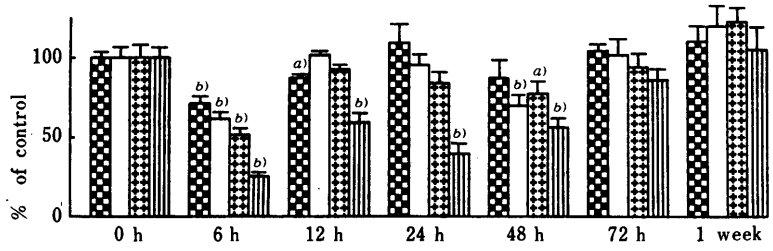

Fig. 1. Time Course of Hepatic Microsomal Drug-Metabolizing Enzyme Activities after Pretreatment of Adult Male Rats with CBD

Each column and bar represents the mean value±S.E. of four determinations. Enzyme content or activities of the control group (0 h) were: cytochrome P450, 0.625±0.019 nmol per mg of protein; AN hydroxylase, 0.83±0.03 nmol per min per mg; *p*-NA *O*-demethylase, 1.45±0.09 nmol per min per mg; *d*-BP *N*-demethylase, 4.52±0.22 nmol per min per mg. *a*) Significantly different from the control ($p<0.05$). *b*) Significantly different from the control ($p<0.01$). ▩, cytochrome P-450; □, aniline hydroxylase; ▦, *p*-nitroanisole *O*-demethylase; ▥, *d*-benzphetamine *N*-demethylase.

TABLE II. Time Course of NADPH–Cytochrome c Reductase Activity and Cytochrome b_5 Content in Hepatic Microsomes of Adult Male Rats Pretreated with CBD

	Time after CBD treatment (h)						
	0	6	12	24	48	72	1 week
NADPH–cyt.c reductase[a)]	0.147±0.010 (100)	0.139±0.019 (95)	0.174±0.030 (118)	0.142±0.021 (97)	0.162±0.018 (110)	0.151±0.009 (103)	0.155±0.012 (105)
Cyt.b_5[b)]	0.532±0.040 (100)	0.447±0.043 (85)	0.538±0.027 (103)	0.510±0.064 (98)	0.565±0.070 (108)	0.646±0.071 (124)	0.586±0.051 (112)

a) μmol per min per mg protein. *b*) nmol per mg protein. Each value represents the mean±S.E. of four determinations. Numbers in parentheses indicate relative activity or content to those of the control (0 h). None of values is significantly different from those of the control group ($p>0.05$).

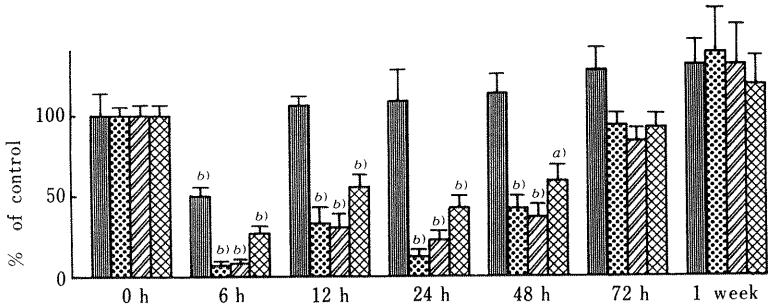

Fig. 2. Time Course of Hepatic Microsomal Testosterone Oxidation Activities after Pretreatment of Adult Male Rats with CBD

Each column and bar represents the mean value ± S.E. of four determinations. Activities of the control group (0 h) were: 6β-OH-T formation, 2.79 ± 0.14 nmol per min per mg of protein; 16α-OH-T formation, 2.31 ± 0.23 nmol per min per mg; 2α-OH-T formation, 1.57 ± 0.07 nmol per min per mg; androstenedione formation, 0.69 ± 0.07 nmol per min per mg. *a*) Significantly different from the control ($p < 0.05$). *b*) Significantly different from the control ($p < 0.01$). ■, 6β-OH-T formation; ▩, 16α-OH-T formation; ▨, 2α-OH-T formation; ▧, AD formation.

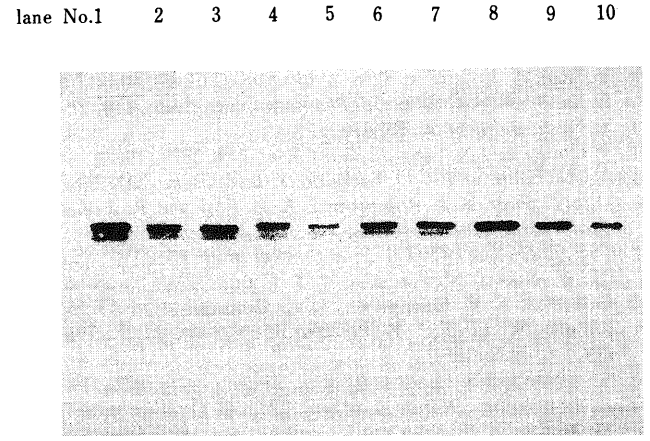

Fig. 3. Western Blotting Analysis of Cytochrome P450 UT-2 in Hepatic Microsomes of Adult Male Rats Pretreated with CBD

Three hepatic microsomes (1 ml each) were combined as one sample for SDS-PAGE. Microsomal protein (10 μg each) were subjected to SDS-PAGE, and was electrophoretically transferred to Clear Blot Membrane-p. The cathode was at the top in SDS-PAGE. The protein of Cytochrome P450 UT-2 was probed by rabbit antiserum against the isozyme and alkaline phosphatase-conjugated goat second antibody. Lanes are; 1, control (0 h); 2, 6 h; 3, 12 h; 4, 24 h; 5, 48 h; 6, 72 h; 7, 1 week after the administration of CBD. 8, 9, and 10 were cytochrome P450 UT-2 5.0, 2.5, and 1.25 pmol, respectively.

figure shows that the content of the male-specific cytochrome protein was minimum 24 to 48 h after the CBD injection, and seemed then to gradually return to the normal level. The contents of cytochrome P450 UT-2 were tentatively determined with a TLC scanner to be 0.370 (before CBD treatment, 100% as the control) and 0.109 nmol per mg of protein (48 h after CBD treatment, 29%).

Discussion

CBD has been known to inhibit the hepatic drug-metabolizing enzyme system in mammals,[20] but the mechanism has not yet been identified. Since CBD has been clinically evaluated as an antiepileptic,[7,21] the inhibitory nature of the cannabinoid should be characterized. We recently investigated in detail the *in vitro* effect of CBD on microsomal drug-metabolizing enzymes of adult male rats, and suggested that male-specific cytochrome P450 isozyme(s) was inhibited by CBD *in vitro*.[8] In order to further characterize the inhibitory nature, we examined the *in vivo* effects of CBD on the hepatic drug-metabolizing enzyme system in adult male rats in the present study. We expected that the administration of CBD to adult male rats would induce an inhibitory effect on various drug metabolisms with the liver microsomes. A single injection, however, produced a significant decrease only in *d*-BP *N*-demethylation, and no changes were seen in the contents of cytochromes P450 or b_5, or in the activities of NADPH-cytochrome c reductase, AN hydroxylase, or *p*-NA *O*-demethylase. Even three daily injections of CBD resulted only in reduced cytochrome P450 content in addition to *d*-BP *N*-demethylase.

We then examined the time course of the effect of a single CBD injection on the oxidative activities in the liver microsomes. No change was observed in the microsomal levels of NADPH–cytochrome c reductase activity or cytochrome b_5 content through the experimental period (from 6 h to 1 week after the CBD treatment). Interestingly, cytochrome P450 content and enzyme activities determined other than NADPH–cytochrome c reductase and cytochrome b_5 were all lower 6 h after the injection, but except for *d*-BP *N*-demethylase they returned to the control level 24 h after the cannabinoid injection. Furthermore, activities of AN hydroxylation and *p*-NA *O*-demethylation were suppressed again 48 h after the CBD treatment, while *d*-BP *N*-demethylation was continually suppressed from 6 to 48 h. This profile of the decrease in *d*-BP oxidation activity corresponded well with that in testosterone oxidation at the 2α-, 16α- and 17-positions. Earlier studies have proved that these oxidative activities of testosterone, especially at the 2α- and 16α-positions are mediated by a male-specific cytochrome P450 UT-2 in the adult male rat.[22,23] This isozyme is also known to have a relatively high turnover number for *d*-BP *N*-demethylation in the reconstituted system.[15,23] Therefore, we believed that CBD administration caused a decrease in the male-specific isozyme in the rat.

To confirm this speculation, we raised the polyclonal antibody against cytochrome P450 UT-2 in the rabbit, and the protein of UT-2 was probed in the hepatic microsomes of rats pretreated with CBD. Western blotting analysis revealed that the enzyme protein was markedly decreased following CBD administration and this decrease reached maximum after 24 and 48 h, which well corresponds to the decrease in activities of *d*-BP *N*-demethylation and testosterone 2α- and 16α-oxidation. Below the band of the cytochrome P450 UT-2 on the membrane in the Western blot, there appeared another band whose apparent mo-

lecular weight is 48000. This protein is assumed to be cytochrome P450 UT-5[16,24] corresponding to P450 g which is immunochemically related to P450 UT-2.[25] These results suggest that CBD may suppress the contents not only of P450 UT-2 but also of P450 UT-5 in the male rat liver *in vivo*.

It is thought that other mechanisms might cause the suppression of cytochrome P450 content and enzyme activities at earlier stages (6 and 12 h after the CBD injection). CBD is known to be converted into various oxidative metabolites by the hepatic drug-metabolizing enzyme system.[26,27] It is thus feasible that, if given 6 or 12 h before the sacrifice, CBD might interact with cytochrome P450 as a substrate, resulting in the inhibition of various activities of the drug-metabolizing system such as AN hydroxylation, *p*-NA *O*-demethylation and *d*-BP *N*-demethylation. Moreover, we have recently reported that a carbon monoxide (CO)-like complex with cytochrome P450 was formed during the incubation of CBD with mouse hepatic microsomes.[28] We observed that by incubation with CBD and NADPH, hepatic microsomes of adult male rat produced a similar complex which was indistinguishable from the CO-binding species of cytochrome P450 in spectral properties under similar conditions (unpublished data). Therefore, suppression of cytochrome P450 content and enzyme activities in the earlier stages from 6 to 12 h after CBD administration might be due, at least in part, to the CO-like complex with cytochrome P450, which would be formed during the metabolic process of CBD.

It is possible that CBD suppressed cytochrome P450 UT-2 among various constitutive P450 isozymes in the hepatic microsomes of adult male rats in the later stages of inhibition (from 24 to 48 h after administration), while total content of microsomal cytochrome P450 was not suppressed. We have not yet elucidated the mechanism of the decrease of cytochrome P450 UT-2 content. Waxman[29] reported that inducers such as phenobarbital and 3-methylcholanthrene reduced the content of constitutive cytochrome P450 isozymes as compensation for induction of other isozymes with these compounds. Aztreonum, a monobactam antibiotic, was also reported to suppress the male-specific cytochrome P450 in the rat *in vivo*.[30] Yeowell *et al.*[31] demonstrated that administration to the male rat of 3,4,5,3′,4′,5′-hexachlorobiphenyl (HCB) caused suppression in the content of the male-specific P450 UT-2, while the compound induced cytochrome P450 3 corresponding to P450 IF-3.[24] In the present study, neither cytochrome P450 content nor activities of the NADPH-dependent oxidations examined were increased by CBD pretreatment. This knowledge and results suggest that, similar to HBC, CBD might suppress cytochrome P450 UT-2 on the one hand, and induce some isozyme(s) of P450 on the other. We are further studying the mechanism responsible for the decreased level of cytochrome P450 UT-2 protein in the male rat pretreated with CBD.

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