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SELF-CATALYZED INACTIVATION OF CYTOCHROME P-450 DURING MICROSOMAL METABOLISM OF CANNABIDIOL

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Abstract—When cannabidiol (CBD) was incubated with hepatic microsomes of mice in the presence of an NADPH-generating system, a significant decrease of cytochrome P-450 content was observed by measuring its carbon monoxide difference spectra. The decrease of cytochrome P-450 by CBD required NADPH and molecular oxygen. The effect was partially inhibited by SKF 525-A but not by various scavengers of active oxygen species, superoxide anion, hydroxyl radical and singlet oxygen. The incubation of CBD with hepatic microsomes did not affect total heme but decreased significantly free sulfhydryl contents in the microsomes. The derivatives of CBD modified in the resorcinol moiety, CBD-monomethyl- and dimethylethers, almost lost the effect on cytochrome P-450, whereas those modified in the terpene moiety, 8,9-dihydro- and 1,2,8,9-tetrahydro-CBDs exhibited some potency to inactivate cytochrome P-450. The inactivation of cytochrome P-450 by CBD and related compounds led to the inhibition of hepatic microsomal *p*-nitroanisole *O*-demethylase and aniline hydroxylase activities. These results suggest that the resorcinol moiety of CBD plays some role in the inactivation of cytochrome P-450 by the cannabinoid.

Cannabidiol (CBD), one of the major constituents of marihuana, is known to exhibit barbiturate-induced sleep prolonging and anticonvulsant effects although this cannabinoid is devoid of psychomimetic activity [1–3]. The preclinical studies suggest that CBD is a useful drug in the treatment of epilepsy [4, 5]. Thus, understanding the mechanism of CBD to prolong barbiturate-induced sleep is of great importance. CBD has a high affinity to hepatic microsomal cytochrome P-450 and inhibits drug metabolism *in vitro* as well as *in vivo* [6–11]. This inhibition of drug metabolism by CBD is attributable at least in part to a decrease of cytochrome P-450, a key enzyme of the hepatic microsomal drug metabolizing system [11, 12]. Karler *et al.* [13, 14] suggested that the inhibitory effect of CBD on the hepatic microsomal drug metabolism might be due to inhibitory metabolites rather than CBD itself. However, there is no direct evidence for the metabolic activation of CBD at present. There has been much literature concerning the metabolic activation of Δ^9-tetrahydrocannabinol (Δ^9-THC), a psychoactive constituent of marihuana [15–18]. In contrast, limited attention has been directed to the role of metabolites in pharmacological effects of CBD. Carlini *et al.* [19] reported that some acetylated derivatives of oxygenated metabolites of CBD have anticonvulsant and barbiturate-induced sleep prolonging effects comparable to CBD. Recently, we reported that *in vitro* incubation of CBD with hepatic microsomes of mice in the presence of an NADPH-generating system led to decrease of cytochrome P-450 measured by its carbon monoxide difference spectra and this decrease was enhanced by phenobarbital-treatment [20]. The present study was undertaken to investigate the nature of enzymes which catalyze metabolic activation of CBD and the structure requirement of CBD to inactivate cytochrome P-450.

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MATERIALS AND METHODS

Animals and treatments. Male ddN mice, weighing 20–35 g were used throughout experiments. Mice were fed a normal diet (Sankyo Labo Service Co., Japan, F-2) *ad lib.* Phenobarbital was dissolved in saline containing an equivalent amount of NaOH and given intraperitoneally (i.p.) at a dose of 100 mg/kg once daily for three days.

Preparation of microsomes. Mice were killed by cervical dislocation 24 hr after the last injection of phenobarbital. The livers were perfused by ice-cold 0.9% (w/v) NaCl to remove blood. The subsequent procedures were carried out at 0–4°. The livers were removed, minced and homogenized in 3 vol. of 1.15% (w/v) KCl with a Potter–Elvehjem type homogenizer. The liver homogenate was centrifuged at 9000 *g* for 20 min with a Tomy RS-18FIII centrifuge in a 2N rotor. The supernatant fraction was further centrifuged at 105,000 *g* for 60 min with a Hitachi 65P ultracentrifuge in an RP65T rotor. The

microsomal pellets were resuspended in 1.15% KCl and used as the enzyme source.

Reagents. Catalase (bovine liver, 2890 units/mg protein), cytochrome *c* (type III), glucose-6-phosphate dehydrogenase (type V) and superoxide dismutase (bovine blood, 3200 units/mg protein) were purchased from Sigma Chemical Co. (St. Louis, MO). NAD, NADP, NADH, NADPH and glucose-6-phosphate were obtained from Boehringer Mannheim Gmb. (Darmstadt, F.R.G.) and *d*-limonene, glutathione, DTNB (5,5′-dinitro-bis-2-nitrobenzoic acid) were from Wako Pure Chemical Co. (Osaka, Japan). SKF 525-A (2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride) was supplied from Smith Kline & French Lab. (Philadelphia, PA). CBD was purified from cannabis leaves supplied by Prof. I. Nishioka of Kyushu University (Fukuoka, Japan) according to the method described previously [21]. CBD-monomethyl- and dimethylethers were prepared by the methylation of CBD with methyl iodide and sodium carbonate in ethanol. The spectral data of CBD-monomethyl- and dimethylethers synthesized were identical with those reported [22, 23]. 8,9-Dihydro- [24] and 1,2,8,9-tetrahydro-CBDs [25] were prepared by the hydrogenation of CBD with hydrogen and 10% Pd/C in ethyl acetate. All other chemicals and solvents used were of analytical reagent grade.

Enzyme assays. A typical incubation mixture consisted of CBD (30 μl in dimethylsulfoxide, 160 μM), microsomes from phenobarbital-treated mice (0.6 g liver equivalent), 0.5 mM NADP, 10 mM $MgCl_2$, 10 mM glucose-6-phosphate, 4 mM nicotinamide, 0.5 mM EDTA 2Na, 3 units of glucose-6-phosphate dehydrogenase and 100 mM potassium phosphate buffer (pH 7.4) to make a final volume of 6 ml. For the study on cofactor requirement, each pyridine nucleotide (1 mM) was added into the incubation mixture instead of the NADPH-generating system. For the study on molecular oxygen requirement and carbon monoxide inhibition, a Warburg type reaction vessel was used. After evacuation of gas in the reaction vessel by aspiration, an appropriate gas mixture was admitted into the vessel under vacuum. The above procedure was repeated three times. The reaction mixture was incubated at 37° for 20 min and then following enzyme activities and parameters were determined.

Cytochrome P-450 was determined by the carbon monoxide difference spectrum with a Union SM 401 spectrophotometer ($e = 91\ mM^{-1}$) [26]. NADPH-cytochrome *c* reductase was measured by the method of Phillips and Langdon [27]. Free sulfhydryl groups were estimated by the measurement of absorbance at 412 nm allowing microsomal suspension to react with 0.2 mM DTNB in the presence of 8 M urea [28]. Microsomal heme content was assayed using the pyridine hemochrome assay [26]. Protein concentration was determined according to Lowry *et al.* [29] using bovine serum albumin as a standard. *p*-Nitroanisole *O*-demethylase and aniline hydroxylase activities in hepatic microsomes from untreated mice were assayed by the methods described previously [30]. The concentration of CBD and related compounds added to incubation mixture was 160 μM in the inhibitory study.

Statistical significance. The statistical significance of difference was calculated using Student's *t*-test.

RESULTS

Cofactor requirement for inactivation of cytochrome P-450 *by CBD*

As seen in Table 1, NADPH was required for inactivation of cytochrome P-450 by CBD. NADH was much less effective as an electron donor than NADPH, and a synergistic effect was not observed when both pyridine nucleotides were added to the incubation mixture. Under these conditions, there was no significant effect on cytochrome P-450 in the absence of CBD regardless of with or without the NADPH-generating system. No absorption maximum at 420 nm observed indicating that CBD did not convert cytochrome P-450 to its inactive form, cytochrome P-420.

Molecular oxygen requirement and carbon monoxide inhibition

The effect of CBD on cytochrome P-450 was markedly decreased by replacement of the incubation atmosphere from air to nitrogen (Table 2). Molecular oxygen was required for the inactivation of cytochrome P-450 by CBD, indicating that CBD should be metabolized by monooxygenase to some metabolites prior to inactivate the cytochrome. Table 2 also indicates that carbon monoxide significantly

Table 1. Cofactor requirement for inactivation of cytochrome P-450 by CBD

Cofactor	nmoles cytochrome P-450 inactivated/20 min/mg protein*	% of NADPH
NAD	0.06 ± 0.05	10
NADP	0	0
NADH	0.23 ± 0.07	37
NADPH	0.62 ± 0.04	100
NADH + NADPH	0.80 ± 0.06	129

* Calculated from difference of cytochrome P-450 content in the incubation mixture between with and without CBD (160 μM).

Incubations were carried out at 37° for 20 min as described in Materials and Methods except that various pyridine nucleotides (each 1 mM) were used instead of the NADPH-generating system. The results represent the mean ± SE from triplicated incubations of the same pool of microsomes. Cytochrome P-450 content in hepatic microsomes used was 2.65 nmoles/mg protein.

Table 2. Requirement of molecular oxygen and effect of carbon monoxide on inactivation of cytochrome P-450 by CBD

Gas phase	nmoles cytochrome P-450 inactivated/20 min/mg protein	% of air
Air	0.82 ± 0.01	100
100% N_2	0.13 ± 0.05	16
O_2/CO = 1/4*	0.48 ± 0.06	59

* Dithionite difference spectra were measured for the determination of cytochrome P-450. Incubations were carried out as described in Materials and Methods except that a Warburg type vessel was used for the incubation. Cytochrome P-450 content in hepatic microsomes used was 1.92 nmoles/mg protein. The results represent the mean ± SE from triplicate incubations of the same pool of microsomes.

inhibited the effect of CBD on the cytochrome, suggesting that cytochrome P-450 is involved in the formation of the reactive metabolites of CBD.

Specificity of inactivation of cytochrome P-450 *in the microsomal electron transport system*

The effect of CBD was specific to cytochrome P-450. Table 3 demonstrates that the incubation of CBD with microsomes in the presence of the NADPH-generating system does not affect significantly another hemoprotein, cytochrome b_5, in the hepatic microsomes. NADPH-cytochrome *c* reductase activity was also not affected.

Effects of metabolic inhibitor and active oxygen scavengers on inactivation of cytochrome P-450 *by CBD*

As seen in Table 4, the addition of neither superoxide dismutase (941 units) nor catalase (1765 units) affected significantly the inactivation effect of CBD on cytochrome P-450. Neither superoxide anion nor hydrogen peroxide is significantly responsible for the metabolic activation of CBD by cytochrome P-450. In addition, the effect of CBD was not affected by cysteine, glutathione, butylhydroxytoluene, 2,5-dimethylfuran, *dl*-α-tocopherol, which are scavengers of radicals and/or singlet oxygen. SKF 525-A, a known inhibitor of cytochrome P-450, inhibited the effect of CBD on the cytochrome.

Effect of CBD on microsomal heme and sulfhydryl contents

Hepatic microsomal heme content did not decrease when incubated with CBD in the presence of the NADPH-generating system, indicating that the heme destruction is not involved in the effect of CBD on cytochrome P-450 (Table 5). Some compounds are known to be metabolized to reactive

Table 3. Effect of CBD incubation on cytochrome P-450 and b_5 contents, and NADPH-cytochrome *c* reductase activity in hepatic microsomes

Enzymes	Without CBD (control)	With CBD, 160 μM (% of control)
Cytochrome P-450 (nmoles/mg protein)	2.11 ± 0.02 (100)	1.35 ± 0.08* (64)
Cytochrome b_5 (nmoles/mg protein)	0.75 ± 0.03 (100)	0.81 ± 0.03 (108)
NADPH-cytochrome *c* reductase (nmoles/min/mg protein)	261 ± 12 (100)	254 ± 6 (97)

* Significantly different from control ($P < 0.01$).

Incubations were carried out as described in Materials and Methods. After the incubation, the reaction mixture was centrifuged at 105,000 *g* for 60 min. The resultant pellets were suspended in 1.15% KCl and amount or activity of microsomal enzyme was assayed. The results represent the mean ± SE from triplicate incubations of the same pool of microsomes.

Table 4. Effects of various inhibitors or scavengers on inactivation of cytochrome P-450 by CBD

Inhibitors or scavengers	% of control	Inhibitors or scavengers	% of control
Control	100	2,5-Dimethylfuran (1)	105
Glutathione (1)*	108	Butylhydroxytoluene (1)	95
Cysteine (1)	103	Catalase (1765 units)	108
SKF 525-A (1)	80†	Superoxide dismutase (941 units)	103
dl-α-Tocopherol (1)	99		

* Concentration of inhibitors or scavengers added (mM).

† Significantly different from control ($P < 0.01$).

Incubations were carried out as described in Materials and Methods. After incubation of microsomes with various inhibitors or scavengers, cytochrome P-450 content was measured by the method of Omura and Sato [25]. The decrease of cytochrome P-450 in control incubation (CBD, 160 μM) was 0.67 ± 0.06 nmoles/20 min/mg protein. Cytochrome P-450 content in hepatic microsomes used was 2.10 nmoles/mg protein. The results represent the mean of triplicate incubations.

Table 5. Effect of CBD on microsomal heme and free sulfhydryl contents

	Total heme content (nmoles/mg protein)	Free sulfhydryl groups/mg protein
Control (without CBD)	2.74 ± 0.11	21.9 ± 0.2
CBD	2.68 ± 0.06	19.7 ± 0.2*

* Significantly different from control ($P < 0.01$).

The methods are described in Materials and Methods. The results represent the mean ± SE of four incubations. Total heme and free sulfhydryl contents were determined after incubation for 20 min with or without CBD (160 μM) in the presence of the NADPH-generating system. The control incubation was added 30 μl of dimethylsulfoxide.

electrophiles which bind to nucleophiles such as sulfhydryl groups. In the case of CBD, neither cysteine nor glutathione prevented the inactivation of cytochrome P-450 by CBD. However, microsomal sulfhydryl content was significantly decreased during the metabolism of CBD under the conditions used, although the extent of decrease was only 10%.

Effects of CBD and related compounds on cytochrome P-450 *and the hepatic microsomal drug metabolizing enzymes*

In order to know the structure requirement of CBD to inactivate cytochrome P-450, the derivatives of CBD were prepared and their abilities to inactivate cytochrome P-450 and to inhibit the microsomal drug metabolizing enzymes examined (Table 6). CBD was the most potent in the inactivation of cytochrome P-450. CBD-monomethyl- and dimethylethers decreased the effect on the cytochrome to 37% and 11%, respectively, as compared with CBD, whereas the relative effects of 8,9-dihydro- and 1,2,8,9-tetrahydro-CBDs were 67% and 48%, respectively. Moreover, *d*-limonene, which constitutes the terpene moiety of CBD, almost lost the effect on cytochrome P-450, but olivetol which constitutes the resorcinol moiety of CBD, showed some effect on the cytochrome. Table 6 also shows the *in vitro* inhibitory effects of CBD and related compounds (160 μM) on both hepatic microsomal *p*-nitroanisole *O*-demethylase and aniline hydroxylase. Olivetol, which is known to have a high affinity to cytochrome P-450 [7], was the most potent inhibitor of the hepatic microsomal drug metabolizing enzymes among the compounds tested. The potency of the inhibitory effect was the following order: olivetol $>$ CBD = 1,2,8,9-tetrahydro-CBD $>$ 8,9-dihydro-CBD $>$ CBD-monomethylether $\gg$ *d*-limonene $\geqq$ CBD-dimethylether. The compounds having the resorcinol moiety showed relatively higher inhibitory effect than did the other compounds lacking the resorcinol moiety.

DISCUSSION

CBD has a high affinity to hepatic microsomal drug metabolizing enzymes. The present study confirms the previous finding [20] that the incubation of CBD with hepatic microsomes resulted in the decrease of cytochrome P-450 by measuring its carbon monoxide difference spectra. The effect of CBD on cytochrome P-450 required NADPH and molecular oxygen, and was partially inhibited by SKF 525-A, an inhibitor of cytochrome P-450 mediated monooxygenase. The results indicate that metabolic activation is essential for the inactivation of cytochrome P-450 by CBD, and that cytochrome P-450 catalyzes at least one of the metabolic activation steps of CBD. In the previous study, we reported that the inactivation effect of CBD on cytochrome P-450 was enhanced by phenobarbital-treatment [20], suggesting that CBD is transformed to reactive species by phenobarbital-inducible cytochrome P-450.

Phenolic compounds are known to form the corresponding semiquinone radicals which are believed to be active metabolites [31–34]. CBD has a res-

* Relative inhibitory effect was expressed as

$$\frac{\text{activity in control} - \text{activity in test compounds added}}{\text{activity in control} - \text{activity in CBD added}} \times 100.$$

All compounds (160 μM) were incubated with microsomes (2.21 nmoles cytochrome P-450/mg protein) from phenobarbital-treated mice for the experiment of cytochrome P-450 inactivation as described in Materials and Methods. The results represent the mean of triplicate incubations. Decrease of cytochrome P-450 in CBD-incubated microsomes was 0.833 ± 0.082 nmoles cytochrome P-450 decreased/20 min/mg protein. The concentration of CBD and related compounds was 160 μM in the study on inhibitory effect on hepatic microsomal *p*-nitroanisole *O*-demethylase and aniline hydroxylase. Hepatic microsomes from untreated mice were used in this experiment. The mean control values of enzyme activities presented are as follows: *p*-nitroanisole *O*-demethylase, 0.741 nmoles *p*-nitrophenol formed/min/mg protein; aniline hydroxylase, 1.119 nmoles *p*-aminophenol formed/min/mg protein. The mean values of enzyme activities in CBD added incubations are as follows: *p*-nitroanisole *O*-demethylase, 0.413 nmoles *p*-nitrophenol formed/min/mg protein; aniline hydroxylase, 0.630 nmoles *p*-aminophenol formed/min/mg protein.

Table 6. Structure requirement for the effect of CBD and related compounds on cytochrome P-450 and the hepatic microsomal drug metabolizing enzymes

	Cytochrome P-450 inactivation	Inhibitory effect*	
		p-Nitroanisole *O*-demethylase	Aniline hydroxylase
CBD	100	100	100
8,9-dihydro-CBD	67	60	96
1,2,8,9-tetrahydro-CBD	48	110	94
CBD-monomethylether	37	26	84
CBD-dimethylether	11	0	58
olivetol	59	110	169
d-limonene	11	21	66

orcinol moiety in its structure and may form phenoxy radicals by its metabolic activation. However, the lack of protection with radical scavengers, butylhydroxytoluene, glutathione and cysteine, against the inactivation of cytochrome P-450 by CBD, may rule out the above possibility. The present study indicates that none of superoxide anion, singlet oxygen, and hydroxyl radicals are mainly responsible for the metabolic activation of CBD. The addition of catalase, superoxide dismutase, 2,5-dimethylfuran and *dl*-α-tocopherol to the incubation mixture was devoid of the preventing effect on the inactivation of cytochrome P-450. Neither of other two major components of the microsomal electron transport system, a flavoprotein NADPH-cytochrome *c* reductase and, hemoprotein cytochrome b_5, were affected when CBD was incubated with microsomes in the presence of the NADPH-generating system.

A number of chemicals having unsaturated carbons are known to be suicidal substrates which can destroy cytochrome P-450 by prosthetic heme alkylation [34]. However, heme content in hepatic microsomes was not affected by the incubation of CBD, indicating that the destruction of prosthetic heme was not involved in the case of CBD for the inactivation of cytochrome P-450. On the other hand, microsomal free sulfhydryl groups decreased by 10%. Cytochrome P-450s are found to contain several sulfhydryl groups in the molecules and some of which play important role in the structure and function of the enzymes [36, 37]. The decrease of sulfhydryl content was only 10%, but it was significant as compared with that of control incubation without CBD. The data suggest that the modification of the sulfhydryl groups with CBD metabolites is one of inactivation mechanisms by CBD as described in the case of parathion [36], although cysteine and glutathione did not protect the inactivation of cytochrome P-450 by CBD. The lack of prevention with exogenous sulfhydryl compounds may be due to a high reactivity of CBD metabolites formed by cytochrome P-450 that could not leave the catalytic site of cytochrome P-450 and modifies only free sulfhydryl groups located in the neighbor of the catalytic site of the enzyme. Further studies are, however, necessary to prove the above hypothesis.

The reactive species of CBD to inactivate cytochrome P-450 are not known at present. Some olefinic compounds are known to produce epoxides as biological electrophiles by metabolic activation and to inactivate cytochrome P-450 [38]. The double bonds in CBD are not essential for the inactivation of cytochrome P-450. 8,9-Dihydro- and 1,2,8,9-tetrahydro-CBDs demonstrated a considerable effect on cytochrome P-450. The same effect of CBD derivatives reduced double bonds suggests that epoxy metabolites of CBD are not primarily involved in the inactivation of cytochrome P-450 by CBD.

The experiments in the structure requirement indicate that the resorcinol moiety is important for the inactivation of cytochrome P-450 by CBD. Thus, CBD-monomethyl- and dimethylethers decreased significantly the inactivation effect on the cytochrome as compared with CBD. Some activity observed in CBD-monomethylether seems to be its demethylation by cytochrome P-450, resulting in the formation of CBD. The considerable effect of olivetol and the marginal effect of *d*-limonene on cytochrome P-450 also suggest the importance of the resorcinol moiety, but not of the terpene moiety of CBD. The inactivation of cytochrome P-450 by CBD and related compounds leads to the inhibition of hepatic microsomal *p*-nitroanisole *O*-demethylase and aniline hydroxylase. In this study, CBD is reconfirmed as a strong inhibitor of the hepatic microsomal drug metabolizing enzymes. The importance of resorcinol moiety for the inhibition of the drug metabolism was also demonstrated, although the resorcinol moiety was not an essential requirement for the inhibition. CBD and related compounds could produce type I spectra with hepatic microsomal cytochrome P-450.* These compounds, therefore, probably act as alternate substrates for cytochrome P-450 and inhibit the enzyme activity in a different manner from the inactivation of cytochrome P-450. Thus, CBD-dimethylether and *d*-limonene, which did not significantly inactivate cytochrome P-450, inhibited aniline hydroxylase to some extent. The highest inhibitory effect of olivetol on both the enzyme activities could not be interpreted by the inactivation of cytochrome P-450. Olivetol but not CBD produced purple color products during the microsomal incubation in the present study, suggesting that quinone derivatives were formed as described in the chemical oxidation of resorcinol derivatives [39]. As mentioned above, radical intermediates are formed during the quinone formation. Therefore, olivetol may have an additional inhibitory mechanism different from the inactivation of cytochrome P-450.

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