Formation of Carbon Monoxide during Mouse Hepatic Microsomal Oxidative Metabolism of Cannabidiol; Identification and Determination

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Carbon monoxide (CO) was generated in the process of hepatic microsomal oxidative metabolism of cannabidiol (CBD). After the generated CO was reduced to methane (CH₄) with a methanizer, CH₄ formed was determined by gas chromatography (GC) with a flame ionization detector. After oxidation with hopcalite, CO was also identified as CO₂ by gas chromatography/mass spectrometry (GC/MS). The reaction was NADPH-dependent and required molecular oxygen. It was inhibited by addition of some inhibitors of cytochrome P450-dependent monooxygenase. When CBD (191 μM) was incubated with hepatic microsomes of mice in the presence of an NADPH-generating system and oxygen, concentration of CO determined by GC was 4.7 ± 0.5 ppm/nmol P450 in the incubation atmosphere. Pretreatment with phenobarbital (100 mg/kg, i.p. for 3 d) but not 3-methylcholanthrene (80 mg/kg, i.p.) increased the CO formation 78%, while pretreatment with cobaltous chloride (40 mg/kg, i.p. for 3 d) decreased the formation 56%. When CBD was incubated under oxygen-18 gas, molecular oxygen was not incorporated into the CO molecule. 8,9-Dihydro- and 1,2,8,9-tetrahydro-CBDs also produced CO to some extent, whereas CBD monomethyl- and dimethylethers reduced the ability to produce CO. In addition, cannabidivarin and olivetol produced CO, although none of Δ⁹-tetrahydrocannabinol, cannabinol and *d*-limonene did. Thus, the resorcinol moiety of CBD is important for CO formation.

Key words carbon monoxide formation; cannabidiol; microsome; cytochrome P450; gas chromatography/mass spectrometry; gas chromatography

Cannabidiol (CBD), one of the major constituents of marihuana, is known to have barbiturate-induced sleep prolonging and anticonvulsant effects, although this cannabinoid is devoid of the psychomimetic activity observed in tetrahydrocannabinol (THC).¹⁾ The sleep prolongation produced by CBD is believed to be mediated by inhibition of barbiturate metabolism in the liver, because of the decreased content of hepatic microsomal cytochrome P450 (P450).²⁾ Moreover, the inhibitory effect of CBD modified the pharmacological effects of THC in marihuana.³⁾ Karler *et al.*⁴⁾ suggested that CBD effect on the hepatic microsomal drug metabolism might be due to its metabolites rather than to the cannabinoid itself. However, the inhibitory mechanism has not been fully elucidated. Our previous studies⁵⁾ demonstrated that CBD decreased hepatic microsomal P450 measured by its carbon monoxide (CO) difference spectra, when incubated with mouse hepatic microsomes in the presence of an NADPH-generating system. This suggested the requirement of metabolic activation for the effect of CBD on P450. An additional study⁶⁾ indicated that CBD induced a CO-like complex with P450 during hepatic microsomal metabolism and that phenobarbital-induced P450s mediated at least one of the metabolic steps of CBD in forming the complex. In relation to our findings, a variety of organic compounds⁷⁾ have been reported to form CO during their metabolic process, however, none of these compounds has structural similarity to CBD. As stated above, it was presumed that CO could be formed during metabolism of CBD. Therefore, we conducted a study on the mechanism of toxic manifestation by metabolic activation of CBD as a part of biochemical and toxicological research on

marihuana. The present study was undertaken to obtain direct evidence for CO formation during hepatic microsomal metabolism of CBD using gas chromatography (GC) and GC/mass spectrometry (GC/MS).

MATERIALS AND METHODS

Chemicals NADP, NADPH, NAD, NADH, glucose-6-phosphate dehydrogenase (type V, EC 1.1.1.49) and metyrapone were obtained from Sigma Chemical Co. (St. Louis, MO). *d*-Limonene, cobaltous chloride (CoCl₂) and 3-methylcholanthrene (3-MC) were from Wako Pure Chemical Ind. (Osaka, Japan). α-Naphthoflavone was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). SKF 525-A (2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride) was kindly supplied by the Smith Kline and French Lab. (Philadelphia, PA). Oxygen-18 gas (97 atom%) was purchased from Amersham International plc. (Amersham, U.K.). All other chemicals and solvents used were of analytical reagent grade.

Cannabinoids CBD and Δ⁹-tetrahydrocannabinol (Δ⁹-THC) were isolated and purified from cannabis leaves supplied by Prof. Nishioka, Kyushu University (Fukuoka, Japan) according to the method of Aramaki *et al.*⁸⁾ Cannabidivarin (CBDV) was also kindly supplied by Prof. Nishioka. CBD monomethylether, CBD dimethylether, 8,9-dihydro- and 1,2,8,9-tetrahydro-CBD were prepared by the methods described previously.^{5b)} Olivetol was prepared according to the method of Lieman *et al.*⁹⁾ The purities of these cannabinoids used were determined to be at least 98% by GC.

Animal Treatments and Preparation of Microsomes

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Male ddN mice (25–35 g) were pretreated with phenobarbital (PheB) (100 mg/kg, i.p. for 3 d), 3-MC (80 mg/kg, i.p., single injection) or CoCl_2 (40 mg/kg, i.p. for 3 d). Mice were killed by cervical dislocation 24 h (PheB and CoCl_2 treatments) or 48 h (3-MC treatment) after the last injection. Non-treated mice were not given any drug or vehicle. Hepatic microsomes were prepared as described previously.¹⁰⁾ P450 and protein in the microsomes were determined by the methods of Omura and Sato¹¹⁾ and Lowry *et al.*,¹²⁾ respectively.

Calibration Curve for CO Measurement The Calibration curve for standard CO was made by GC and GC/MS, using 10–100 ppm as a straight line.

Incubation System and CO Measurement A typical incubation mixture consisted of CBD (191 μM , 30 μl ethanol), microsomes from PheB-treated mice unless otherwise noted (0.2 g liver equivalent), 0.5 mM NADP, 10 mM glucose-6-phosphate, 1 mM disodium ethylenediaminetetraacetic acid, 10 mM MgCl_2 , 2 units glucose-6-phosphate dehydrogenase and 100 mM potassium phosphate buffer (pH 7.4) to make a final volume of 5 ml in a test tube with a volume of 11 ml. The reaction mixture was incubated at 25 °C for 20 min in a sealed test tube, and then added to 2 ml of a solution mixture of saponin (50 mg) and $\text{K}_3[\text{Fe}(\text{CN})_6]$ (200 mg) in distilled water (1 ml). The gas phase (2 ml) was withdrawn with a gas-tight syringe and injected to GC and GC/MS. In all cases, control incubation was carried out identically as described above with the exception that the NADPH-generating system was omitted. For the experiment on the cofactor requirement, the NADPH-generating system was replaced by NAD, NADP, NADH, NADPH (each 1 mM) or cumene hydroperoxide (1 mM). For the experiment on molecular oxygen requirement, the reaction mixture was placed on a Warburg type reaction vessel and

equilibrated with an appropriate gas phase. After evacuation and admittance of appropriate gas phase ($\text{N}_2:\text{O}_2=2:1$, 4:1 or 8:1), the reaction mixture was incubated at 25 °C for 20 min, and treated as mentioned above. GC/MS analysis was carried out immediately after the oxidation of CO to CO_2 with hopcalite. The GC/MS of the sample was also recorded to confirm the incorporation of oxygen-18 into CO from molecular oxygen. GC was performed with a Shimadzu GC-16A gas chromatograph equipped with a hydrogen flame ionization detector and a Shimadzu Methanizer MTN-1. The conditions were as follows: column, molecular sieve 13X on Chromosorb W (60–80 mesh, 2 mm $\times$ 2 m); column temperature, 100 °C; carrier gas, N_2 50 ml/min; Methanizer temperature, 400 °C. GC/MS was carried out at 70 eV with a JEOL GCG-06 gas chromatograph coupled with a JEOL JMS-DX-300 mass spectrometer and a JEOL JMA-DA 5000 mass data system. The conditions were as follows: column, molecular sieve 13X on Chromosorb W (60–80 mesh, 3 mm $\times$ 2 m); column temperature, 40 °C; carrier gas, He 50 ml/min; ionizing current, 300 μA .

RESULTS

Determination of CO by GC and GC/MS Calibration curve for standard CO was made by GC and GC/MS (data not shown). Figure 1 shows GC data on the incubation atmosphere of the CBD incubation. The peak around 2.3 min on the chromatogram of GC (CO was reduced to CH_4) was identified as CH_4 originated from CO by comparison with standard CO. In this case, CBD induced CO formation when incubated with the mouse hepatic microsomes in the presence of an NADPH-generating system. The peak was small and negligible

Fig. 1. Identification of CO Generated during Hepatic Microsomal Metabolism of CBD by GC-Methanizer

CBD (191 μM) was incubated with hepatic microsomes (content of P450: 2.25 nmol/mg protein) of mice in the presence of an NADPH-generating system at 25 °C for 20 min. Arrows show the peak of CH_4 derived from CO. Retention time is shown by each arrow.

Fig. 2. Identification of CO Generated during Hepatic Microsomal Metabolism of CBD by GC/MS

CBD (191 μ M) was incubated with hepatic microsomes (content of P450: 1.57 nmol/mg protein) of mice in the presence of an NADPH-generating system at 25 °C for 20 min. Arrows show the peak of CH₄ derived from CO. TIC, total ion chromatogram.

when either CBD or the NADPH-generating system was omitted from the incubation mixture, and the concentration of CO was 1.8 ppm/nmol P450 (– CBD) or 1.5 ppm/nmol P450 (– NADPH-generating system). In the presence of CBD and the cofactor, CO was markedly increased to the concentration of 5.5 ppm/nmol P450. Mass chromatograms and mass spectrum of the atmosphere incubated CBD with microsomes are shown in Fig. 2. A peak around 5.6 min in the mass chromatogram of GC/MS (CO was oxidized to CO₂) was confirmed to be CO₂ by its mass spectrum showing a molecular ion at m/z 44 (M^+) (Fig. 2). CO was detected only in the atmosphere incubated CBD with microsomes in the presence of the NADPH-generating system.

Cofactor and Molecular Oxygen Requirement for the CO Formation As shown in Table 1—Exp. I, the CO formation required NADPH. NADH was much less effective as an electron donor and showed no synergistic effect when added with NADPH. Cumene hydroperoxide, which is known to support the oxidation by P450,¹³ was

also effective for the CO formation. The reaction required molecular oxygen, and was inhibited to 27% under 100% nitrogen atmosphere (Table 1—Exp. II).

Effects of pH, Protein Concentration, Incubation Temperature, Incubation Time, and Substrate Concentration on the CO Formation The CO formation reached maximum with protein concentration at 2.12 mg protein/ml (Fig. 3) and decreased with higher concentration. The reason for the decrease is not clear at present. The optimal conditions for the reaction were observed in the range of pH 6.0 to 10.0, and at around 25–40 °C of the incubation temperature (Fig. 3). Further, the reaction was constant over 91.5 μ M of CBD concentration and was linear for 20 min (Fig. 3).

Effects of Inducers, Inhibitors and Scavengers on the CO Formation Pretreatment of mice with PheB increased the CO formation to 188% as compared with Non-treatment, whereas that with CoCl₂ lowered the reaction (Table 2). 3-MC treatment did not significantly affect the CO formation. The reaction was not significantly affected by

Table 1. Cofactor Requirement and Effect of Incubation Atmosphere on CO Formation from CBD

	Cofactor	Concentration of CO ppm/nmol P450
Experiment I ^{a)}	–Cofactor	3.2±0.3 (27) ^{c)}
	NAD	4.1±0.9 (34)
	NADH	6.4±0.4 (54)**
	NADP	3.8±0.5 (32)
	NADPH	11.1±0.5 (93)**
	NADPH-generating system	11.9±0.5 (100)**
	Cumene hydroperoxide	13.4±0.9 (113)**

	Atmosphere	Concentration of CO ppm/nmol P450
Experiment II ^{b)}	Air	5.5±0.7 (100) ^{d)}
	N ₂	1.5±0.2 (27)**
	N ₂ :O ₂ =2:1	11.1±2.8 (202)**
	N ₂ :O ₂ =4:1	7.8±1.4 (142)**
	N ₂ :O ₂ =8:1	4.2±4.2 (76)

a) Cofactor requirement on CO formation from CBD. b) Effect of incubation atmosphere on CO formation from CBD. c) Figures in parentheses represent percent of the value with an NADPH-generating system. d) Figures in parentheses represent percent of the value with air. CBD (191 μM) was incubated with hepatic microsomes (content of P450: a, 1.53 nmol/mg protein; b, 1.44 nmol/mg protein) in the presence of an NADPH-generating system at 25°C for 20 min. The measurement of CO was carried out by GC. Concentration of cofactors added was 1 mM. The results represent the mean±S.E. of three determinations. ** Significantly different from –cofactor or air ($p<0.01$).

Table 2. Effects of Inducers and Inhibitor on CO Formation

Inducers and inhibitor	Content of P450 nmol/mg protein	Concentration of CO	
		ppm/mg protein	ppm/nmol P450
Non-treated	1.15±0.05	8.8±1.2	8.3±1.2
PheB	2.39±0.09**	16.5±1.5**	6.9±0.5
3-MC	1.34±0.18	10.2±1.2	7.7±0.6
CoCl ₂	1.11±0.06	4.4±0.5	4.0±0.5**

CBD (191 μM) was incubated with hepatic microsomes in the presence of an NADPH-generating system at 25°C for 20 min. The measurement of CO was carried out by GC. The results represent the mean±S.E. of three determinations. ** Significantly different from non-treated ($p<0.01$).

Table 3. Effects of Various Inhibitors or Scavengers on CO Formation by CBD

Inhibitor or scavenger ^{a)}	Concentration of CO ppm/nmol P450
Control (1 mM) ^{a)}	4.3 (100) ^{b)}
Ascorbic acid (1 mM)	4.7 (109)
Glutathione (1 mM)	4.8 (112)
SKF 525-A (1 mM)	1.7 (40)
Metirapone (1 mM)	2.9 (67)
α-Naphthoflavone (0.5 mM)	1.5 (35)

a) Figures in parentheses represent concentration of inhibitor or scavenger. b) Figures in parentheses represent percent of control value. CBD (191 μM) was incubated with hepatic microsomes (content of P450: 1.74 nmol/mg protein) in the presence of an NADPH-generating system at 25°C for 20 min. The measurement of CO was carried out by GC. The data represent the mean of duplicate incubations.

Fig. 3. Effects of pH, Protein Concentration, Incubation Temperature, Incubation Time and CBD Concentration on CO Formation

The incubations and analyses were carried out as described in Materials and Methods except that the parameters were varied as indicated in each segment. Content of P450 in hepatic microsomes used was as follows: effect of pH, 2.64 nmol/mg protein; effect of incubation temperature, 3.75 nmol/mg protein; effect of protein concentration, 1.72 nmol/mg protein; effect of CBD concentration, 2.33 nmol/mg protein; effect of incubation time, 2.17 nmol/mg protein. CBD (191 μM) was incubated with hepatic microsomes in the presence of an NADPH-generating system at 25°C for 10 min with the exception of incubation temperature and time. The data represent the mean of duplicate incubations. ●, Na-K phosphate buffer; ▲, Tris-HCl buffer; ■, glycine-NaOH buffer.

Fig. 4. Mass Chromatograms and Mass Spectrum of CO Generated from by CBD under Oxygen-18 Gas

CBD (191 μM) was incubated with hepatic microsomes (content of P450: 3.32 nmol/mg protein) of mice in the presence of an NADPH-generating system at 25 $^{\circ}\text{C}$ for 20 min. The arrow shows the peak of CO_2 derived from CO .

addition (1 mM each) of glutathione or ascorbic acid, both of which are known as scavengers of radicals (Table 3). The CO formation was significantly inhibited by three known inhibitors of P450, metyrapone (1 mM), α -naphthoflavone (0.5 mM) and SKF 525-A (1 mM).

Lack of Incorporation of Molecular Oxygen into CO It is important to determine whether a molecular oxygen is incorporated into the CO formed in order to understand the formation mechanism. After CBD was incubated with mouse hepatic microsomes under oxygen-18 gas, the CO formed was determined by GC/MS (Fig. 4). A molecular ion of CO_2 derived from CO formed under conditions containing oxygen-18 gas was seen at m/z 44 (M^+), indicating that molecular oxygen was not significantly incorporated into the CO molecule.

Structural Requirement for CO Formation The structural requirement for CO formation is shown in Table 4. 8,9-Dihydro- and 1,2,8,9-tetrahydro-CBDs, in which the double bonds in the terpene moiety are reduced, generated CO to some extent. On the other hand, CBD monomethyl- and dimethylethers, hydroxy groups of which were blocked with methyl group, significantly decreased the ability to generate CO, indicating the importance of the resorcinol moiety but not the terpene moiety of CBD in the CO formation. The ability of CBDV was comparable to that of CBD, although Δ^9 -THC and cannabiniol (CBN) markedly decreased the ability to generate CO as well as CBD monomethylether. Olivetol but not *d*-limonene formed CO, supporting the hypothesis that the resorcinol moiety of CBD plays an important role in the CO formation.

DISCUSSION

We previously reported that CBD decreased the content of hepatic microsomal P450 when incubated with NADPH-generating system based on measurement of the CO difference spectra.⁵⁾ Moreover, CBD induced the CO-like complex with P450 under similar experimental conditions to those used in the above studies.^{5b)} The present study confirmed qualitatively that CBD produced CO when incubated with an NADPH-generating system and hepatic microsomal P450. This indicates that the CO formation results in a decrease of spectrally detectable P450. The effect of CBD on the content of P450 can be partially explained by the CO formation demonstrated in the present study as well as that described previously.⁵⁾ The CO formation from CBD required NADPH and molecular oxygen, and was inhibited by metyrapone, α -naphthoflavone and SKF 525-A, which are known inhibitors of P450. The results indicate that the CO formation requires oxidative metabolism of CBD which is catalyzed by P450. Increase in the CO formation by PheB-treatment also suggests that PheB-inducible P450s mediate at least one of the metabolic steps of CBD to form CO. Decrease in the CO formation in terms of P450 by CoCl_2 , which is known to decrease P450 content,¹⁴⁾ suggests that a particular form of P450 is responsible for the CO formation by CBD, although P450 content was not significantly decreased by the treatment. Negligible change in the CO formation by 3-MC treatment suggests that 3-MC does not cause induction of the P450s responsible for the CO formation. Lack of the effect of radical scavengers such as glutathione and ascorbic acid

Table 4. Comparison of CO Formation from CBD and Related Compounds

Substrate	Structure	CO Concentration ppm/nmol P450
CBD		8.6 ± 0.5 (100) ^{a)}
CBD monomethylether		3.9 ± 0.4 (45)
CBD dimethylether		2.6 ± 0.2 (30)
8,9-Dihydro-CBD		4.9 ± 0.3 (57)
1,2,8,9-Tetrahydro-CBD		6.2 ± 0.6 (72)
CBDV		10.3 ± 0.2 (120)
CBN		1.2 ± 0.1 (14)
Δ^9 -THC		2.2 ± 0.4 (26)
Olivetol		2.6 ± 0.1 (30)
d-Limonene		1.4 ± 0.1 (16)

a) Figures in parentheses represent percent of the value with CBD. CBD and related compounds ($191 \mu\text{M}$) were incubated with hepatic microsomes (content of P450: $2.79 \text{ nmol/mg protein}$) in the presence of an NADPH-generating system at 25°C for 20 min. The measurement of CO was carried out by GC. The results represent the mean $\pm$ S.E. of three determinations.

indicates that radical intermediates are not significantly responsible for the CO formation.

The source of CO which is generated during the microsomal metabolism of CBD is not clear at present.

We previously suggested the importance of the resorcinol moiety in the decrease of P450 by CBD *in vitro* and in the inhibitory effect of CBD on the hepatic microsomal drug-metabolizing enzymes.^{5b)} Moreover, we recently reported that the resorcinol moiety of CBD plays an important role in the CO-like complex formation.⁶⁾ More recently, Bornheim *et al.*¹⁵⁾ reported the importance of the resorcinol moiety of CBD to inactivate P450. Thus, 8,9-dihydro- and 1,2,8,9-tetrahydro-CBDs, in which the double bonds in the terpene moiety of CBD are reduced, induced the CO formation, suggesting that epoxy metabolites of CBD in the terpene moiety are not involved in the formation. On the contrary, CBD monomethyl- and dimethylethers, in which hydroxy groups were blocked with methyl group, almost lost the ability to produce CO. The production ability of CBDV, which has a resorcinol structure, was comparable to that of CBD, although Δ^9 -THC and CBN, which are monophenols, markedly decreased the ability to generate CO as well as CBD monomethylether. The effect of CBD on barbiturate-induced sleep was believed to be due to inhibition of barbiturate metabolism in the liver, which was caused by the reduction in content of hepatic microsomal P450 and inactivation of P450 by CO complex formation.

The present study confirmed that CO was generated during hepatic microsomal metabolism of CBD and suggested that CO-P450 complex formation played a role in the pharmacological and toxicological effects of marijuana components. The resorcinol moiety of CBD, especially the phenolic hydroxy groups, appeared to be important in the CO formation. Further studies are necessary to determine the source of CO in the CBD molecule with ^{14}C -labelled compound of carbon atom and the toxicological significance of the CO formation.

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