IN VITRO AND IN VIVO INTERACTIONS OF Δ^8 -TETRAHYDROCANNABINOL AND ITS METABOLITES WITH HEPATIC MICROSOMAL DRUG METABOLIZING ENZYME SYSTEMS OF MICE

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Interactions of Δ^8 -tetrahydrocannabinol (Δ^8 -THC) and its metabolites, 11-hydroxy- Δ^8 -THC, 11-oxo- Δ^8 -THC and Δ^8 -THC-11-oic acid, with hepatic microsomal drug metabolizing enzyme systems were studied *in vitro* and *in vivo* using mice. All the cannabinoids used were shown to produce a type I spectral change of cytochrome P-450 in hepatic microsomes. The apparent binding affinities (K_s) for Δ^8 -THC, 11-hydroxy- Δ^8 -THC, 11-oxo- Δ^8 -THC and Δ^8 -THC-11-oic acid were 5.2, 169.6, 33.2 and 148.8 μM , respectively. Δ^8 -THC, 11-oxo- Δ^8 -THC and Δ^8 -THC-11-oic acid (100 μM) caused a significant stimulation of NADPH oxidation *in vitro* with microsomes. In addition, Δ^8 -THC (20, 40 and 80 μM) markedly inhibited *p*-nitroanisole O-demethylase and aniline hydroxylase in microsomes. 11-Hydroxy- Δ^8 -THC and 11-oxo- Δ^8 -THC also showed the inhibitory effect, but to lesser extents.

Furthermore, single pretreatments with Δ^8 -THC and 11-oxo- Δ^8 -THC (30 mg/kg, *i.v.*) significantly decreased activities of *p*-nitroanisole O-demethylase and aniline hydroxylase in hepatic microsomes, accompanying a decrease of cytochrome P-450 content in case of Δ^8 -THC. On the other hand, all these pretreatments except for that with Δ^8 -THC-11-oic acid showed a stimulatory effect on *p*-nitrophenol glucuronidation with hepatic microsomes, in contrast to an inhibitory effect *in vitro*.

Keywords — Δ^8 -tetrahydrocannabinol; 11-hydroxy- Δ^8 -tetrahydrocannabinol; 11-oxo- Δ^8 -tetrahydrocannabinol; Δ^8 -tetrahydrocannabinol-11-oic acid; microsomal drug metabolism; spectral interaction; NADPH oxidation; *p*-nitroanisole O-demethylase; aniline hydroxylase; *p*-nitrophenol glucuronidation

Δ^8 -Tetrahydrocannabinol (Δ^8 -THC) and Δ^9 -THC, which are active constituents of marijuana and possess high lipid solubilities, are known to interact with hepatic microsomal drug metabolizing systems, producing a type I spectral change.¹⁻⁴ It is also established that Δ^9 -THC is a good substrate of above enzyme system.⁵ Furthermore, Cohen *et al.*¹) and Fernandes *et al.*⁴) reported that Δ^9 -THC inhibited microsomal drug oxidations in rats *in vitro*. Mitra *et al.*⁶) also reported the inhibitory effect of Δ^9 -THC on hepatic microsomal enzymes of rats *in vivo* as well as *in vitro*. Paton and Pertwee⁷) found that

cannabis extract, Δ^9 -THC and cannabidiol (CBD), prolonged pentobarbital-induced sleeping time and proposed that CBD prolongs sleeping time of pentobarbital by inhibiting its metabolism. In addition, Karler *et al.*⁸) suggested that the inhibition of hepatic metabolism of hexobarbital is caused by polar metabolites of CBD rather than CBD. In spite of above findings, however, there is a report that Δ^8 -THC did not affect the *in vivo* metabolism of hexobarbital.⁹ In addition to these inhibitory effects, Witschi and Saint-Francois¹⁰) reported an inductive effect of Δ^8 -THC on hepatic and pulmonary

microsomal benzo[a]pyrene hydroxylase in rats. Ho *et al.*¹¹⁾ further reported that a chronic administration of Δ^9 -THC enhanced metabolism of Δ^9 -THC itself.

Recently, we found that 11-hydroxy- Δ^8 -THC (11-OH- Δ^8 -THC) and 11-oxo- Δ^8 -THC, which are the metabolites of Δ^8 -THC, exhibited greater prolonging effect on pentobarbital-induced sleeping time in mice than Δ^8 -THC.¹²⁾ The question has thus been arisen as to whether the greater prolonging effect of these metabolites is attributed to the metabolic inhibition of pentobarbital or to the direct action to the central nervous system of the metabolites. The interactions of these metabolites with hepatic microsomal drug metabolizing enzymes, however, is not well established at present.

The present study was thus undertaken to investigate the *in vitro* and *in vivo* interactions of Δ^8 -THC and its metabolites, 11-OH- Δ^8 -THC, 11-oxo- Δ^8 -THC and Δ^8 -THC-11-oic acid, with hepatic microsomal enzyme systems of mice.

MATERIALS AND METHODS

Male ddN mice weighing 20–25 g were used. Δ^8 -THC, 11-OH- Δ^8 -THC, 11-oxo- Δ^8 -THC and Δ^8 -THC-11-oic acid were prepared by a previously reported methods.¹²⁾ Glucose-6-phosphate, glucose-6-phosphate dehydrogenase (Type V from bakers yeast), NADP, NADPH and uridine-5'-diphosphoglucuronate (UDPGA) were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Difference spectra produced by the interaction of cannabinoids with cytochrome P-450 were measured by the method of earlier report¹³⁾ using a Union SM-401 spectrophotometer. The spectral dissociation constant (K_s) was determined using a double reciprocal Lineweaver-Burk plot of the absorbance difference between a peak and a trough against the concentration of each cannabinoid (5–300 μ M).

Microsomal NADPH oxidase activity was assayed by the method of Jansson *et al.*¹⁴⁾ The reaction mixture contained in a total volume of 3 ml, 50 mM Tris-HCl buffer (pH 7.4), 0.66 mM

MgCl₂, 0.05 mM NADPH, hepatic microsomes (1 mg protein) and 100 μ M of each cannabinoid, and was incubated at 25°C.

In assaying activity of aniline hydroxylase or *p*-nitroanisole O-demethylase, the substrate, aniline (2 μ mol) or *p*-nitroanisole (3 μ mol) was incubated at 37°C for 10 min in a reaction mixture consisting of 10 mM MgCl₂, 4 mM nicotinamide (only for *p*-nitroanisole O-demethylase), 0.5 mM NADP, 10 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, microsomal suspension (0.2 g liver equivalent) and 100 mM Tris-HCl buffer, pH 7.4 (for aniline hydroxylase) or 100 mM potassium phosphate buffer, pH 7.4 (for *p*-nitroanisole O-demethylase) in final volume of 2 ml. After termination of the reaction by the addition of trichloroacetic acid (TCA), *p*-aminophenol or *p*-nitrophenol formed was determined by the method of Imai *et al.*¹⁵⁾ or of Kato and Gillette,¹⁶⁾ respectively. Microsomal protein was measured by the method of Lowry *et al.*,¹⁷⁾ using bovine serum albumin as a standard. Hepatic microsomal cytochrome P-450 was determined by the method of Omura and Sato using an extinction coefficient of 91 mM⁻¹ cm⁻¹.¹⁸⁾

For assaying UDP-glucuronyltransferase activity, *p*-nitrophenol (0.25 mM) was incubated at 37°C for 20 min in a final volume of 1 ml containing 100 mM potassium phosphate buffer (pH 7.4), 10 mM MgCl₂, 2 mM UDPGA and microsomal suspension corresponding to 0.2 g of liver. The reaction was terminated by the addition of 1 ml of 2 N KOH to the supernatant, the color development was read at 400 nm.

In vitro metabolism of cannabinoids was investigated under the same condition as described for the assay of *p*-nitroanisole O-demethylase, except the final volume (1 ml) and the amount of substrate (20 μ g of each cannabinoid). The reaction was terminated by addition of 0.2 M HCl-KCl, and cannabinoids were extracted with 5 ml of ethylacetate. An aliquot of the extract was submitted to determination of the cannabinoid remaining unchanged by ECD-gas chroma-

tographic method.¹⁹⁾ For an inhibition study, Δ^8 -THC, 11-OH- Δ^8 -THC, 11-oxo- Δ^8 -THC and Δ^8 -THC-11-oic acid were added to a reaction mixture (final concentrations were 20, 40 and 80 μM , respectively).

For *in vivo* study, cannabinoids were suspended in saline solution containing 1% Tween 80 and intravenously injected to each group consisting of 10 mice, at a dose of 5 or 30 mg/kg. The animals were then sacrificed 24 h after the injection, and microsomes were prepared from 2 mice. In these treatments, 2 mice were omitted from the experiments since they were dead within 24 h by the injection of Δ^8 -THC (30 mg/kg).

RESULTS

Type I spectral change, binding affinities (K_s) and maximal type I spectral change per nmol cytochrome P-450 produced by these cannabinoids are listed in Table I. The apparent K_s values for Δ^8 -THC, 11-OH- Δ^8 -THC, 11-oxo- Δ^8 -THC and Δ^8 -THC-11-oic acid were determined graphically to be 5.2, 169.6, 33.2 and 148.8 μM , respectively. The binding affinity of Δ^8 -THC was, therefore, 6–33 times higher than those of the metabolites although a maximal spectral change produced by Δ^8 -THC was smaller than that produced by Δ^8 -THC-11-oic acid.

Δ^8 -THC, 11-oxo- Δ^8 -THC and Δ^8 -THC-11-

TABLE I. K_s and Maximal Spectral Change of Δ^8 -THC and Its Metabolites with Hepatic Microsomes of Mice

Cannabinoids	K_s (μM)	Maximal spectral change ($\Delta\text{O.D.}$)/nmol cytochrome P-450	λ max (nm)	λ min (nm)
Δ^8 -THC	5.2 ± 0.2	0.038 ± 0.002	390	420
11-OH- Δ^8 -THC	169.6 ± 15.1	0.020 ± 0.002	395	428
11-oxo- Δ^8 -THC	33.2 ± 0.8	0.033 ± 0.001	390	423
Δ^8 -THC-11-oic acid	148.8 ± 21.2	0.047 ± 0.001	385	420

Values represent the mean $\pm$ S.E. of four experiments.

The protein concentration of microsomal suspension used was 1.3 mg/ml.

TABLE II. Effects of Δ^8 -THC and Its Metabolites on NADPH Oxidase Activity in Hepatic Microsomes of Mice

Additions	NADPH oxidation (nmol/min/mg protein)	% of control
Control	4.8 ± 0.2	100
Δ^8 -THC	11.6 ± 0.1 ^{b)}	242
11-OH- Δ^8 -THC	6.4 ± 0.5	133
11-Oxo- Δ^8 -THC	11.1 ± 0.6 ^{b)}	231
Δ^8 -THC-11-oic acid	7.3 ± 0.7 ^{a)}	152

Values represent the mean $\pm$ S.E. of three experiments.

The rates of NADPH oxidation in the presence or absence of cannabinoids were carried out as described in Materials and Methods.

a) Significantly different from the control value, $p < 0.05$.

b) Significantly different from the control value, $p < 0.01$.

TABLE III. Metabolism of Δ⁸-THC and Its Metabolites with Hepatic Microsomes of Mice

Substrates	Metabolic rate (nmol/min/mg protein)
Δ ⁸ -THC	1.23 ± 0.02
11-OH-Δ ⁸ -THC	0.70 ± 0.07
11-oxo-Δ ⁸ -THC	0.93 ± 0.02
Δ ⁸ -THC-11-oic acid	0.08 ± 0.01

Values are the mean ± S.E. of four experiments. Reaction conditions are described in Materials and Methods.

oic acid significantly stimulated NADPH oxidation in hepatic microsomes as summarized in Table II. NADPH oxidation, however, was not significantly affected by the addition of 11-OH-Δ⁸-THC under the same condition. Table III indicates that the metabolic rate of Δ⁸-THC is greater than that of the metabolites. Δ⁸-THC-11-oic acid was metabolized at the lowest rate among cannabinoids tested. Figure 1 shows the *in vitro* effects of Δ⁸-THC and its metabolites on activities of aniline hydroxylation, *p*-nitroanisole O-demethylation

FIG. 1. Effects of Δ⁸-THC and Its Metabolites on Drug Metabolizing Enzymes in Vitro Values represent the mean (expressed as percent of control) ± S.E. of 4 incubations from 8 mice. Reaction conditions are described in Materials and Methods. The mean control values of enzyme activities presented are as follows: *p*-nitroanisole O-demethylase, 0.762 nmol *p*-nitrophenol formed/ min/ mg protein; aniline hydroxylase, 0.694 nmol *p*-aminophenol formed/ min/ mg protein; *p*-nitrophenol glucuronidation, 1.015 nmol *p*-nitrophenol conjugated/ min/ mg protein. * Significantly different from the control value, *p* < 0.05. ** Significantly different from the control value, *p* < 0.01.

and *p*-nitrophenol glucuronidation in hepatic microsomes. Δ^8 -THC and 11-OH- Δ^8 -THC caused a significant inhibition to both *p*-nitroanisole O-demethylase and aniline hydroxylase activities at the concentrations of 40 and 80 μ M, whereas 11-oxo- Δ^8 -THC significantly inhibited aniline hydroxylase only, and Δ^8 -THC-11-oic acid did not inhibit these activities under the same condition. In a lower concentration (20 μ M), only Δ^8 -THC significantly inhibited *p*-nitroanisole O-demethylase activity, but three cannabinoids (Δ^8 -THC, 11-OH- Δ^8 -THC and 11-oxo- Δ^8 -THC) inhibited aniline hydroxylase. On the other hand, all the cannabinoids inhibited *p*-nitrophenol glucuronidation with hepatic microsomes.

Figure 2 shows the *in vivo* effects of Δ^8 -THC and its metabolites on microsomal drug metabolizing enzymes at 24 h after intravenous injection

(30 mg/kg). The pretreatments with Δ^8 -THC and 11-oxo- Δ^8 -THC markedly decreased both *p*-nitroanisole O-demethylase and aniline hydroxylase, and Δ^8 -THC pretreatment also decreased cytochrome P-450 content in microsomes. No inhibitory effect, however, was found after the pretreatments with 11-OH- Δ^8 -THC and Δ^8 -THC-11-oic acid. On the other hand, all the cannabinoids did not show any inhibitory effects at a dose of 5 mg/kg *i.v.* (data not shown). Contrary to the *in vitro* effect on microsomal glucuronidation, all the cannabinoids except for Δ^8 -THC-11-oic acid exhibited a significant enhancement of *p*-nitrophenol glucuronidation *in vivo*.

DISCUSSION

The present study demonstrated that not only Δ^8 -THC but also its metabolites oxidized at

FIG. 2. Effects of Δ^8 -THC and Its Metabolites on Drug Metabolizing Enzymes in Vivo

Values represent the mean (expressed as percent of control) $\pm$ S.E. of 4 to 5 incubations from 8–10 mice. Reaction conditions are described in Materials and Methods. Cannabinoids were administered at a dose of 30 mg/kg *i.v.* The mean control values of enzyme activities and of content are as follows: *p*-nitroanisole O-demethylase, 0.891 nmol *p*-nitrophenol formed/min/mg protein; aniline hydroxylase, 0.941 nmol *p*-aminophenol formed/min/mg protein; *p*-nitrophenol glucuronidation, 0.969 nmol *p*-nitrophenol conjugated/min/mg protein; cytochrome P-450 content, 0.714 nmol/mg protein.

* Significantly different from the control value, $p < 0.05$.

** Significantly different from the control value, $p < 0.01$.

11-position (11-OH- Δ^8 -THC, 11-oxo- Δ^8 -THC and Δ^8 -THC-11-oic acid) produced type I spectral change of cytochrome P-450 in hepatic microsomes of mice. It is well known that binding affinity of a compound to produce a type I spectral change primarily depends on its lipophilicity.²⁰⁾ The binding affinity of Δ^8 -THC, which seems to possess the highest lipophilicity among cannabinoids tested, was 6–33 times higher than those of its metabolites. The magnitude of type I spectral change produced by these cannabinoids, however, were not correlated to these binding affinities. Al-Gailany *et al.*²¹⁾ also described that there was no correlation between the maximal spectral changes and K_s values of type I compounds. As a possible explanation for this disagreement, it can be considered that these cannabinoids interact with different species of cytochrome P-450 or the same cytochrome but located in different environment of microsomal membrane.

NADPH oxidation was highly stimulated by addition of all the cannabinoids except for 11-OH- Δ^8 -THC. In relation to this stimulation, Gigon *et al.*²²⁾ have also found that type I compounds enhance NADPH oxidation with hepatic microsomes of rats and have suggested that the increase is due to their effects on various components of the NADPH-dependent electron transport system, being partially utilized for its metabolism in hepatic microsomes. Such a stimulation was not significant in 11-OH- Δ^8 -THC, this may be caused by the property of the agent, namely, 11-OH- Δ^8 -THC is not a pure type I substrate but a partially modified type II substrate as reported with barbiturates.¹⁴⁾ Teschke *et al.*²³⁾ reported that ethanol, which is a modified type II substrate,²⁴⁾ did not stimulate NADPH oxidation with hepatic microsomes of rats.

Δ^8 -THC and 11-OH- Δ^8 -THC inhibited the metabolism of *p*-nitroanisole and aniline *in vitro* at the concentration used, whereas 11-oxo- Δ^8 -THC inhibited only aniline hydroxylase and Δ^8 -THC-11-oic acid has no effect on these enzymes at the same concentrations. The inhibitory effects were roughly correlated to these K_s

values for cytochrome P-450 except for 11-OH- Δ^8 -THC, which revealed a potent inhibition in spite of its high K_s value. This suggests that 11-OH- Δ^8 -THC interacts with microsomal drug metabolizing enzyme systems by different manner as those of the others. In the *in vivo* study only Δ^8 -THC and 11-oxo- Δ^8 -THC (30 mg/kg, *i.v.*) exhibited an inhibitory effect on drug metabolizing enzymes, the latter being less active than the former. Both cannabinoids, however, did not show any inhibitory effects at a dose of 5 mg/kg *i.v.* The mechanism for this inhibition is not completely clear at the moment although it seems that the inhibition is not caused by cannabinoids themselves because Δ^8 -THC and 11-oxo- Δ^8 -THC are known to be rapidly metabolized in liver of mice,^{19,25)} and the decrease of cytochrome P-450 content is partially attributable.

An interesting result obtained is the difference between the *in vitro* and *in vivo* effects of Δ^8 -THC and its metabolites on *p*-nitrophenol glucuronidation. All the cannabinoids tested inhibited the *in vitro* glucuronidation at the concentration of 160 μ M. On the contrary, administration (30 mg/kg, *i.v.*) of Δ^8 -THC, 11-OH- Δ^8 -THC and 11-oxo- Δ^8 -THC significantly increased *p*-nitrophenol glucuronidation. Glucuronyltransferase is tightly bound to microsomal membrane and can be activated by a modification of membrane environment.²⁶⁾ In connection with this knowledge, Sarker and Ghosh²⁷⁾ reported that acute and chronic administrations of Δ^9 -THC resulted in the change of membranous lipid components. Because of their high lipid solubilities, it appears that Δ^8 -THC and the metabolites are concentrated in a lipid-rich microsomes due to changes in membranous environment, resulting in an apparent activation of UDP-glucuronyltransferase. The *in vitro* inhibitory effect of THC on *p*-nitrophenol glucuronidation has also been reported by Dingell *et al.*²⁸⁾

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