METABOLIC DISPOSITION OF 8α , 9α -AND 8β , 9β -EPOXYHEXAHYDRO-CANNABINOLS IN THE MOUSE

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Metabolic disposition of 8α , 9α - and 8β , 9β -epoxyhexahydrocannabinols (EHCs) was studied using mice to clarify mechanisms which cause a difference in their pharmacological activities. At given time intervals from 0.5 to 60 min after intravenous injections of 8, 9-EHCs (10 mg/kg), levels of unchanged epoxides extracted from blood, liver and brain of mice were determined by gas chromatography. Blood levels of both epoxides declined biphasically, and the concentrations of 8α , 9α -EHC were higher than those of 8β , 9β -EHC at all the time intervals determined. Biological half-lives in the slower phase were 17 and 13 min, respectively, for 8α , 9α - and 8β , 9β -EHCs. A similar result was obtained for 8, 9-EHCs concentrations in the liver. However, no significant difference in the brain levels was found between 8α , 9α - and 8β , 9β -EHCs. Concentrations of 8α , 9β - and 8β , 9α -dihydroxyhexahydrocannabinols as well as unchanged epoxides 15 min after 8, 9-EHCs injections increased significantly in the liver of mice pretreated with SKF 525-A (25 mg/kg, *i.p.*) comparing with the control. When Δ^8 -tetrahydrocannabinol (Δ^8 -THC), 8α , 9α - or 8β , 9β -EHC was injected into mice intracerebroventricularly (25 μ g/head), pentobarbital (40 mg/kg, *i.p.*)-induced sleep prolonging effect was ranked in the following order, 8β , 9β -EHC $>$ Δ^8 -THC $>$ 8α , 9α -EHC.

These results suggest that monooxygenase system involving cytochrome P-450 and epoxide hydrolase together play important roles in the epoxides metabolism. In addition, different activities of 8α , 9α - and 8β , 9β -EHCs to the central nervous system may cause a difference in their pharmacological effects rather than metabolic factors.

Keywords — metabolic disposition; Δ^8 -tetrahydrocannabinol; 8α , 9α -epoxyhexahydrocannabinol; 8β , 9β -epoxyhexahydrocannabinol; 8α , 9β -dihydroxyhexahydrocannabinol; 8β , 9α -dihydroxyhexahydrocannabinol; cytochrome P-450; epoxide hydrolase; intracerebroventricular injection; pentobarbital-induced sleep

INTRODUCTION

Δ^8 -Tetrahydrocannabinol (THC), an active constituent of marijuana,^{1,2)} is known to be biotransformed to such active metabolites as 11-, 3'- and 4'-hydroxy- Δ^8 -THCs in various animal species.³⁻⁶⁾ We have recently found that 8α , 9α - and 8β , 9β -epoxyhexahydrocannabinols (EHCs) are formed from Δ^8 -THC in substantial

amounts by human liver microsomes and a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system.⁷⁾ In the pharmacological activity in the mouse, 8β , 9β -EHC is much more potent than Δ^8 -THC and 8α , 9α -EHC.⁸⁾ It is thus of interest to learn what mechanism contributes to the different activity between 8α , 9α - and 8β , 9β -

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EHHCs. In the present study, metabolic disposition of both epoxides was studied using mice as an initial step to search the mechanism of action.

MATERIALS AND METHODS

General — Male ddN mice (23–25 g of body weight) were obtained from Hokuriku Experimental Animals Lab. (Kanazawa, Japan). Pre-coated thin-layer chromatography (TLC) plates were purchased from Mancherey-Nagel (Düren, F. R. G.) and Fast Blue BB salt from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Pentobarbital-Na obtained from Mallinckrodt Chemical Works (St. Louis, Mo., U.S.A.) and SKF 525-A from Smith, Kline & French Lab. (Philadelphia, Pa., U.S.A.) were dissolved in isotonic saline. Heptafluorobutyric anhydride was purchased from Wako Pure Chemical Ind. (Osaka, Japan). Δ^8 -THC, 8α , 9α - and 8β , 9β -EHHCs were synthesized by the methods reported previously.^{9,10} 8α , 9β - and 8β , 9α -dihydroxyhexahydrocannabinols (diOH-HHCs) were prepared by acidic hydrolysis of 8α , 9α -EHHC. The epoxides were dissolved in isotonic saline containing 1% Tween 80. Other chemicals and solvents used were of analytical reagent grade.

Drug Treatments and Extraction of Cannabinoids — Two groups of mice (each $n=24$) were injected with 8α , 9α - or 8β , 9β -EHHC (10 mg/kg, *i.v.*), and 4 mice of each group were sacrificed 0.5, 1, 5, 15, 30 and 60 min after the injections. Blood of each mice was collected into a centrifuge tube, and extracted twice with 5 ml of acetone. The brain and liver were homogenized in 10 ml of acetone, and extracted twice with the same volume of acetone. The blood and tissue extracts were evaporated under N_2 stream, and the residues were dissolved in 0.1 ml of acetone.

Two groups of mice (each $n=8$) were administered *i.p.* with SKF 525-A (25 mg/kg) 30 min before 8α , 9α - or 8β , 9β -EHHC injection, and sacrificed 15 min after the epoxide injections. The liver excised was homogenized and extracted with acetone as described above.

Isolation and Determination of Cannabinoids

— The tissue extracts obtained in the time course experiments were spotted on pre-coated silica gel plates (SIL G-25). The samples were co-chromatographed with the synthetic epoxides spotted on both edges of the plates using a solvent system of *n*-hexane-acetone-diethylamine (20: 10: 1). On each chromatogram, a band (R_f 0.5–0.7) corresponded to a spot of synthetic standards visualized by spraying Fast Blue BB salt (1 w/v % in H_2O) was removed, and extracted twice with 8 ml of chloroform-ethyl acetate (2: 1). The tissue extracts obtained in the inhibition experiment were also submitted to preparative TLC as above, and co-chromatographed with the synthetic 8 , 9 -EHHCs and 8 , 9 -diOH-HHCs under the same conditions. On each chromatogram, two bands corresponded to 8 , 9 -EHHCs (R_f 0.5–0.7) and 8 , 9 -diOH-HHCs (R_f 0.05–0.15) were removed, and the former was extracted in the same manner as above, and the latter twice with 8 ml of chloroform-methanol (4: 1).

Then, the extracts obtained in both experiments were evaporated under N_2 stream, and cannabinoids in each residue were heptafluorobutyrylated by the method reported previously.¹¹ The heptafluorobutyryl (HFB) derivatives were submitted to gas chromatography (GC). The amounts of HFB- 8 , 9 -EHHCs and triHFB- 8 , 9 -diOH-HHCs were determined on the basis of calibration curves made up by adding various amounts of synthetic standards to homogenates of untreated mice tissues. The conditions used in GC were as follows; instrument: a Shimadzu GC-6A gas chromatograph equipped with a EC-6 electron capture detector; column: 3% OV-17 on Gas Chrom Q (60–80 mesh, 3 mm $\times$ 1 m); column temp.: 210°C (for HFB- 8 , 9 -EHHCs) and 190°C (for triHFB- 8 , 9 -diOH-HHCs); detector temp.: 275°C; carrier gas: N_2 50 ml/min; injection volume: 1 μ l. Retention time under the conditions were 6.4, 5.9, 2.1 and 4.3 min for HFB- 8α , 9α -EHHC, HFB- 8β , 9β -EHHC, triHFB- 8α , 9β -diOH-HHC and triHFB- 8β , 9α -diOH-HHC, respectively.

Pentobarbital-Induced Sleep Prolongation by Cannabinoids Injected Intracerebroventricularly (*i.c.v.*) to Mice — Three groups of mice (each $n=10$) were injected *i.c.v.* with Δ^8 -THC, 8 α , 9 α - or 8 β , 9 β -EHHC according to the method of Haley and McCormick (25 $\mu\text{g}/\text{head}$).¹²⁾ Control group ($n=10$) was administered with the vehicle. Sodium pentobarbital (40 mg/kg, *i.p.*) was injected to mice 5 min after injection of cannabinoids or vehicle, and time intervals between loss and regaining righting reflex were recorded as sleeping times.

Animal experiments were carried out in an air-conditioned room ($22 \pm 1^\circ\text{C}$). Statistical significance of the data was calculated using Student's *t*-test.

RESULTS

Blood and Tissue Levels of 8 α , 9 α - and 8 β , 9 β -EHHCs

Blood levels of 8 α , 9 α - and 8 β , 9 β -EHHCs declined biphasically after the *i.v.* injections (Fig. 1). The level of 8 α , 9 α -EHHC was higher than that of 8 β , 9 β -EHHC at every time interval determined. Biological half-lives for the epoxides were not so different each other ($t_{1/2}=15-20$ s) in the faster phase. However, a considerable difference in the half-lives was shown between 8 α , 9 α -EHHC ($t_{1/2}=17$ min) and 8 β , 9 β -EHHC ($t_{1/2}=13$ min) in the slower phase.

Table I shows the time course of brain levels

of 8 α , 9 α - and 8 β , 9 β -EHHCs after the *i.v.* injections. In the brain, the levels of the epoxides were highest 30 s after the injections, and declined gradually with time. No significant difference was found between 8 α , 9 α - and 8 β , 9 β -EHHCs in the brain levels.

Similarly to the results in the blood, however,

FIG. 1. Elimination of 8 α , 9 α - and 8 β , 9 β -EHHCs from Blood of Mice after Intravenous Injections

Each point represents the mean value $\pm$ S.E. of 4 determinations.

○ — ○ 8 α , 9 α -EHHC, ● — ● 8 β , 9 β -EHHC.

TABLE I. Brain and Liver Levels of Unchanged 8 α , 9 α - and 8 β , 9 β -EHHCs after Intravenous Injections to Mice

		Time after injections (min)					
		0.5	1	5	15	30	60
B (	8 α , 9 α -EHHC	8.09 $\pm$ 0.10	6.47 $\pm$ 0.47	3.58 $\pm$ 0.22	2.73 $\pm$ 0.20	1.19 $\pm$ 0.09	0.52 $\pm$ 0.06
	8 β , 9 β -EHHC	7.05 $\pm$ 0.49	6.08 $\pm$ 0.79	3.21 $\pm$ 0.18	2.38 $\pm$ 0.10	1.05 $\pm$ 0.15	0.46 $\pm$ 0.04
L (	8 α , 9 α -EHHC	10.18 $\pm$ 0.30	7.18 $\pm$ 0.68	2.68 $\pm$ 0.14	1.99 $\pm$ 0.31	1.27 $\pm$ 0.06	0.86 $\pm$ 0.10
	8 β , 9 β -EHHC	6.42 $\pm$ 0.60	5.02 $\pm$ 0.36	1.62 $\pm$ 0.31	0.74 $\pm$ 0.11	0.48 $\pm$ 0.04	0.21 $\pm$ 0.03

Each value represents the mean $\pm$ S.E. of 4 determinations ($\mu\text{g}/\text{g}$ tissue). a) Significantly different from the control ($p < 0.01$).

the liver levels of $8\alpha, 9\alpha$ -EHC was significantly higher than those of $8\beta, 9\beta$ -EHC at all the time points determined. The level of β -epoxide was no more than one fourth of that of α -epoxide 60 min after the injections.

Effect of SKF 525-A on $8\alpha, 9\alpha$ - and $8\beta, 9\beta$ -EHHCs Metabolism in the Liver

The effect of SKF 525-A pretreatment on the liver contents of unchanged epoxides and dihydrodiols is summarized in Table II. As compared with the control group, concentrations of $8\alpha, 9\beta$ - and $8\beta, 9\alpha$ -diOH-HHCs as well as unchanged $8, 9$ -EHHCs 15 min after the epoxide injections increased significantly in the liver of mice pretreated with SKF 525-A. That is, the inhibitor treatment increased the liver levels of $8, 9$ -diOH-HHCs up to 4 times in the mouse.

Pentobarbital-Induced Sleep Prolonging Effect

Figure 2 shows the sleeping time induced by pentobarbital in the mouse pretreated with *i.c.v.* injections of cannabinoids. The sleeping times were 67 ± 5 , 53 ± 5 and 74 ± 6 min, respectively, in Δ^8 -THC-, $8\alpha, 9\alpha$ -EHC- and $8\beta, 9\beta$ -EHC-treated groups. These values were significantly larger than that in the control group (37 ± 4 min). Further, a significant difference in the sleeping times was found between $8\alpha, 9\alpha$ -EHC- and $8\beta, 9\beta$ -EHC-treated groups.

DISCUSSION

We have demonstrated recently that the pharmacological activity of $8\beta, 9\beta$ -EHC is much higher than that of Δ^8 -THC in the mouse,

and comparable to that of 11-hydroxy- Δ^8 -THC which is a known active metabolite of Δ^8 -THC.^{3,8)} $8\beta, 9\beta$ -EHC is, therefore, one of the most pharmacologically potent cannabinoids known so far. In the present paper, a comparative study was conducted using mice on the metabolic disposition of $8\beta, 9\beta$ -EHC and its stereoisomer, $8\alpha, 9\alpha$ -EHC, whose activity is no more than one fourth of β -epoxide.⁸⁾

The blood levels of both epoxides declined biphasically after *i.v.* injections. The first phase with shorter half-lives is likely due to the distribution of the epoxides into tissues (mainly adipose tissue) as in the case of THC.¹³⁾ The blood levels of $8\alpha, 9\alpha$ -EHC were higher than those of $8\beta, 9\beta$ -EHC in the second phase. In the previous experiments using the same strain of the mouse, Δ^8 -THC levels in the blood also declined biphasically after *i.v.* injection and the biological half-life was 32 min in the second phase.¹¹⁾ This indicates that $8\alpha, 9\alpha$ -EHC ($t_{1/2}=17$ min) and $8\beta, 9\beta$ -EHC ($t_{1/2}=13$ min) were eliminated from blood 2 to 3 times faster than Δ^8 -THC. The similar result was obtained for the levels of the epoxides in the liver. In this case, α -epoxide content was several times higher than that of β -epoxide in the liver. Interestingly, the levels in the brain were not different between $8\alpha, 9\alpha$ - and $8\beta, 9\beta$ -EHHCs after the injections. Further, the levels of the epoxides in the brain quickly reached maximal (30 s after injections) and then declined gradually. The results may be caused by high lipophilicities of the epoxides.

TABLE II. *Effect of SKF 525-A on the Metabolic Disposition of $8\alpha, 9\alpha$ - and $8\beta, 9\beta$ -EHHCs in Mouse Liver*

Cannabinoids injected	Treatment	$8\alpha, 9\alpha$ -EHC	$8\beta, 9\beta$ -EHC	$8\beta, 9\alpha$ -DiOH-HHC	$8\alpha, 9\beta$ -DiOH-HHC
$8\alpha, 9\alpha$ -EHC	Control	1.42 ± 0.14		7.73 ± 0.23	0.46 ± 0.04
	SKF 525-A	$3.10 \pm 0.22^a)$		$23.95 \pm 1.65^a)$	$0.82 \pm 0.06^a)$
$8\beta, 9\beta$ -EHC	Control		0.68 ± 0.05	5.20 ± 0.88	0.79 ± 0.11
	SKF 525-A		$3.52 \pm 0.60^a)$	$22.80 \pm 3.93^a)$	$1.45 \pm 0.29^a)$

Each value represents the mean $\pm$ S.E. of 4 determinations ($\mu\text{g/g}$ tissue). a) Significantly different from the control ($p < 0.01$).

We recently found that 8α , 9α - and 8β , 9β -EHHCs were hydrolyzed stereospecifically to 8β , 9α -diOH-HHC in the mouse, and also suggested that the epoxides might be metabolized not only by epoxide hydrolase but also by monooxygenase system involving cytochrome P-450.¹⁴⁾ In the present study, unchanged epoxides and dihydrodiols formed after 8α , 9α - and 8β , 9β -EHHCs injections were determined in the liver of mice pretreated with SKF 525-A in order to get further information on the epoxides metabolism. It was found that the levels of dihydrodiols as well as unchanged epoxides were increased by the pretreatment. It is well known that SKF 525-A inhibits cytochrome P-450 in various animals *in vitro*,¹⁵⁾ and that the agent exhibits the biphasic effects on the enzymes *in vivo*, *i.e.*, inhibition followed by induction.^{16,17)} On the other hand, Thomas *et al.*¹⁸⁾ reported that the treatment of rats with SKF 525-A did not decrease the activity of epoxide hydrolase. In the

SKF 525-A-treated mice, therefore, further oxidation by cytochrome P-450 of the epoxides may be blocked, and metabolism of the epoxides may be shifted mainly to hydrolysis by epoxide hydrolase whose activity remains unaltered. On the basis of the results, it is speculated that the monooxygenase and hydrolase contribute together to the epoxides metabolism in the mouse *in vivo*.

Glutathione conjugation of the epoxides was tentatively examined using mouse liver $105\,000 \times g$ supernatant under the usual conditions in a preliminary study. When both epoxides were incubated for 60 min, 10 to 12% of each epoxide was found to be metabolized (unpublished data). Therefore, glutathione conjugation may be included as another metabolic pathway of 8α , 9α - and 8β , 9β -EHHCs. The possibility is currently under further investigation.

Considering that the brain level of β -epoxide was the same as that of α -epoxide after the injections, it is implied that their pharmacologically different potencies may be due to different activities to the central nervous system. In order to support further this hypothesis, three cannabinoids, Δ^8 -THC, 8α , 9α - and 8β , 9β -EHHCs, were injected *i.c.v.* to mice, and pentobarbital-induced sleep as index was assessed. The sleep prolonging effect was ranked in the following order, 8β , 9β -EHHC $>$ Δ^8 -THC $>$ 8α , 9α -EHHC. The order is consistent with the previous results obtained by *i.v.* injections of the cannabinoids in the mouse.⁸⁾ So far as we know, cannabinoid receptor has remained obscure although Harris *et al.*¹⁹⁾ have presented suggestive data. However, these results obtained here suggest that the different activity of the epoxides may be caused by their different affinities for the hypothetical cannabinoid receptor in the central nervous system.

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FIG. 2. Pentobarbital-Induced Sleep Prolonging Effect of Cannabinoids Administered Intracerebroventricularly in Mice

Each bar represents the mean value $\pm$ S.E. of 10 mice.

a) Significantly different from the control group ($p < 0.05$).

b) Significantly different from the control group ($p < 0.01$).

c) Significantly different from the 8α , 9α -EHHC-treated group ($p < 0.05$).

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