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THE EFFECT OF CANNABINOIDS (Δ^9-THC AND Δ^8-THC) ON HEPATIC MICROSOMAL METABOLISM OF TESTOSTERONE *IN VITRO**

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Abstract—The effects of Δ^9-THC and Δ^8-THC on testosterone metabolism by rat liver microsomal enzymes were studied *in vitro*. Δ^9-THC (25 μM) inhibit the 5α-reduction of testosterone while Δ^8-THC has no effect at double the concentration. Both Δ^9-THC and Δ^8-THC inhibit the hydroxylation of testosterone. This inhibition is dose dependent over the dose range (25–100 μM) tested. At the same molar concentration, Δ^8-THC inhibits testosterone hydroxylation to a greater extent than Δ^9-THC. The kinetic data suggest that the observed inhibition on 5α-reduction and total hydroxylation by the tetrahydrocannabinoids is of the competitive type.

The tetrahydrocannabinoids and the steroid hormones show a striking similarity in their chemical structure [1]. One would, therefore, expect the tetrahydrocannabinoids to interact with steroids at the cellular level. Recent studies have shown that tetrahydrocannabinoids affect the plasma levels of testosterone [2] and corticosterone [3], possibly via a central mechanism. In the present investigation, we have studied the interaction of cannabinoids and testosterone at the site of liver metabolism.

Our study was divided into two parts: (1) the reduction of testosterone by Δ^4-ketosteroid reductase (5α) and (2) the hydroxylation of testosterone by mixed function oxidases in the liver microsomal preparation.

MATERIALS AND METHODS

Animals. In all experiments male Sprague–Dawley rats weighing 180–220 g were used. They were fed on standard Purina rat chow and water *ad lib.* The rats were not starved before the day of the experiment.

Chemicals. Δ^9-THC and Δ^8-THC were gifts from Dr. M. C. Braude, National Institute for Drug Abuse, U.S.A. Testosterone references (6β-hydroxytestosterone, 2β-hydroxytestosterone, 7α-hydroxytesterone) were generously supplied by Professor W. Klyne and Professor D. N. Kirk from the Steroid Reference Collection, Medical Research Council, U.K. Testosterone, $NADP^+$, sodium isocitrate and isocitric dehydrogenase were purchased from Sigma Chemical Co., St. Louis, MO. [4-C^{14}]Testosterone was purchased from the Radiochemical Centre, Amersham, U.K. All organic solvents were of analytical grade and obtained from commercial sources.

Preparation of liver microsomes. Liver microsomes were prepared as described by Davis *et al.* [4]. Liver microsomal protein concentration was determined by the method of Lowry *et al.* [5] with crystalline bovine albumin as the standard.

In vitro *study of testosterone 5α reduction.* The enzyme kinetics of Δ^4-ketosteroid reductase in rat hepatic microsomal fractions was followed by incubating testosterone in a mixture containing NADPH generating system and hepatic microsomes. Since the rate of steroid reduction by microsomal preparations from different rats varies [6], the experiment was designed on a paired (related) sample basis. Thus for each rat, the microsomal testosterone reduction capacity, K_m and V_{max}, were determined with and without the addition of Δ^9-THC or Δ^8-THC. Each flask contained 500 μl 0.3 M Tris–HCl buffer (pH 7.4), 15 μl M $MgCl_2$, 6.3 mg sodium isocitrate (final conc. 8 mM), 1 mg $NADP^+$ (final conc. 0.33 mM) and 0.36 units of isocitric dehydrogenase in a final volume of 2 ml. Testosterone dissolved in absolute ethanol (0.15 m-mole/10 ml) was added to the incubation mixtures in volumes ranging from 10–35 μl (final conc. 50–175 μM). Δ^9-THC (final conc. 25 μM) or Δ^8-THC (final conc. 50 μM) in 10 μl of ethanol was added to the set of test flasks and 10 μl ethanol to the set of control flasks. Blank samples containing Δ^9-THC or Δ^8-THC but without testosterone were also prepared. The reaction was started by the addition of 1 ml of liver microsome preparation (3.0 mg protein/ml). Incubation was at 37°C with shaking for 10 min. The reaction was terminated by the addition of 10 ml dichloromethane to the incubation mixture. Testosterone and its metabolites were extracted into the dichloromethane layer. An aliquot 5 ml of dichloromethane was dried in a stream of air and the residue was dissolved in 5 ml of ethanol. The u.v. absorption of ethanol samples was read at 240 nm (Beckman DU). The amount of testosterone that

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had not undergone ring A reduction was calculated from an extracted testosterone standard curve and the amount reduced calculated by subtraction [7]. The Michaelis constant K_m and V_{max} were determined by the method of Hanes plot, the line being fitted by the method of least squares. The amount of testosterone reduced and the ratio of unreduced–reduced testosterone were calculated from the substrate concentration of 150 μM.

In vitro *study of testosterone hydroxylation.* 130 μg testosterone (final conc. 150 μM) was used as substrate together with 0.05 μCi [4-C^{14}] testosterone (sp. act. 50 mCi/m-mole). The incubation mixture and incubation procedure were as described. The reaction was stopped by the addition of 10 ml of dichloromethane. Testosterone and its hydroxylated metabolites in the incubation mixture were extracted with dichloromethane and separated in the Bush system, toluene–methanol–water (2:1:1). The chromatograms were cut into 1 inch strips, and the radioactivity was measured by liquid scintillation spectrometry (Corumatic/200 Tracerlab). Four major peaks of radioactivity were identified which had the same R_f value as testosterone, 2β-hydroxytestosterone, 6β-hydroxytestosterone, and 7α-hydroxytestosterone. The last spot was known also to contain 16α-hydroxytestosterone [8]. The radioactivity peak in most cases corresponded to the u.v. absorption peak, except for the 6β-hydroxytestosterone where the u.v. absorption spot was too faint to be identified. The amount of hydroxylated testosterone or the amount of unhydroxylated testosterone was calculated from the radioactivity present in the various areas of the paper chromatogram and was expressed as a percentage of substrate added, taking into account the percentage of substrate loss.

Study of the dose effect of cannabinoids on testosterone hydroxylation. The substrate concentration and the incubation mixture were as described. In this case pooled microsomes from three rats were used. Δ^9-THC or Δ^8-THC was added to make incubation concentrations ranging from 25 to 100 μM. Incubation time was 20 min. The extraction, separation and identification of testosterone and its hydroxylated metabolites were as described.

All values were shown as means and mean differences. The statistical significance of mean differences between the test and control pairs was calculated by Student's paired t test. In all cases where a significance level of $P > 0.05$ or better was obtained, a similar level of significance was also indicated by the nonparametric sign test.

RESULTS

Effects on testosterone 5α reduction. The ring A reduction of testosterone was measured by the decrease in absorbance at 240 nm that occurred with the saturation of the 4–5 double bond. At a concentration of 25 μM, Δ^9-THC inhibited the 5α reduction of testosterone (Table 1). The amount of testosterone reduced was decreased ($P < 0.01$) to about 90 per cent of the control. The ratio of unreduced testosterone to 5α-hydroxytestosterone was increased ($P < 0.01$). In the presence of Δ^9-THC, the K_m for the reaction was increased from a control value of 2.7×10^{-5} M to 3.5×10^{-5} M ($P < 0.05$) while the V_{max} remained relatively unchanged. This inhibitory effect of Δ^9-THC was shown graphically using the Hanes method of plotting the Michaelis equation (Fig. 1), and it is seen that parallel lines were obtained.

Δ^8-THC, when added in concentrations up to 50 μM, had no significant effect on K_m or V_{max}. The effect of Δ^8-THC on testosterone 5α reduction is inconsistent (sign test $P < 0.18$) and in spite of the apparent large mean difference in K_m, this difference is not statistically significant. The amount of testosterone reduced in the presence of Δ^8-THC (50 μM) was comparable to the control in six separate determinations.

Table 1. The effect of Δ^9-THC and Δ^8-THC on 5α reduction of testosterone *in vitro*

	K_m ($\times 10^{-5}$ M)	V_{max} (nmole/ mg protein/ 10 min)	Amount of testerone reduced* (nmole/ mg protein)	T/R†
$N = 8$				
Control	2.66	132.12	102.67	3.06
Δ^9-THC (25 μM)	3.83	126.50	89.77	3.86
Mean Difference	1.17‡	5.62	12.90§	0.80‡
± S.D.	± 0.41	± 5.06	± 4.15	± 0.19
$N = 6$				
Control	3.74	147.30	103.43	3.71
Δ^8-THC (50 μM)	6.32	182.40	100.39	3.75
Mean Difference	2.28	35.10	3.03	0.04
± S.D.	± 0.96	± 20.44	± 3.72	± 0.25

* Incubation mixture contained 150 μM testosterone and 25 μM Δ^9-THC or 50 μM Δ^8-THC.

† Percentage of unreduced testosterone/percentage of reduced testosterone. S.D. = standard deviation of mean difference.

‡ $P < 0.05$, § $P < 0.02$ as calculated by Student's paired t test.

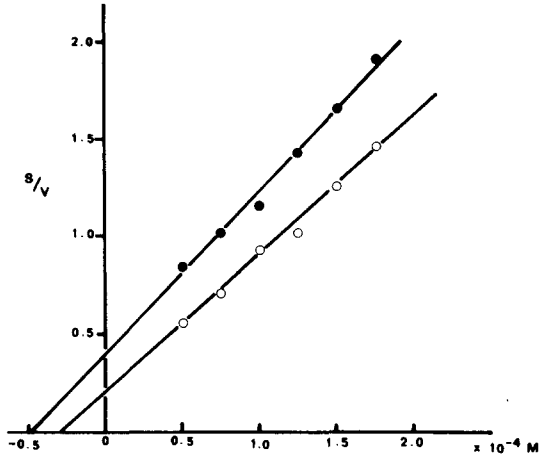

Fig. 1. Hanes plots showing the inhibition of Δ^4-3-keto-reductase (5α) activity *in vitro* by Δ^9-THC (2.5×10^{-5} M). S = molar concentration of testosterone. V = nmoles testosterone metabolized per mg protein per 10 min. Control (○——○); with Δ^9-THC (●——●).

Effects on testosterone hydroxylation. The hydroxylation of testosterone was a faster rate process than 5α reduction, and the ratio of unhydroxylated testosterone to hydroxylated testosterone was lower than that of unreduced testosterone to reduced testosterone. Both Δ^9-THC and Δ^8-THC inhibited testosterone hydroxylation. In the presence of Δ^9-THC or Δ^8-THC the amount of testosterone hydroxylated was reduced ($P < 0.01$, Table 2) and the ratio of unhydroxylated testosterone to hydroxylated testosterone was increased ($P < 0.01$). With Δ^9-THC (25 μM), the degree of inhibition of 5α reduction of testosterone was greater than that of total hydroxylation in the same microsomal preparations. No direct comparisons of the degree of inhibition by the two cannabinoids could be made as the experiments were performed on different microsomal preparations which had different hydroxylating activity. Our chromatographic data failed to show a consistent pattern of hydroxylation at the various sites which varied with the microsomal preparation. However, Δ^9-THC appeared to exert a predominantly inhibitory effect at the 2β position accounting for 60 per cent of the total inhibition, whereas with Δ^8-THC the inhibition appeared to be predominantly at the 6β position where the inhibition was over 40 per cent of the total inhibition.

The dose effect of cannabinoid on testosterone hydroxylation was investigated with pooled liver microsomes. The inhibition of testosterone hydroxylation by Δ^9-THC and Δ^8-THC was shown to be dose dependent. When the reciprocal of the amount of hydroxylated testosterone (v) was plotted against the cannabinoid concentration [i], linear lines were obtained for the concentration range of 25–100 μM (Fig. 2; Δ^9-THC, $r = 0.978$, Δ^8-THC,

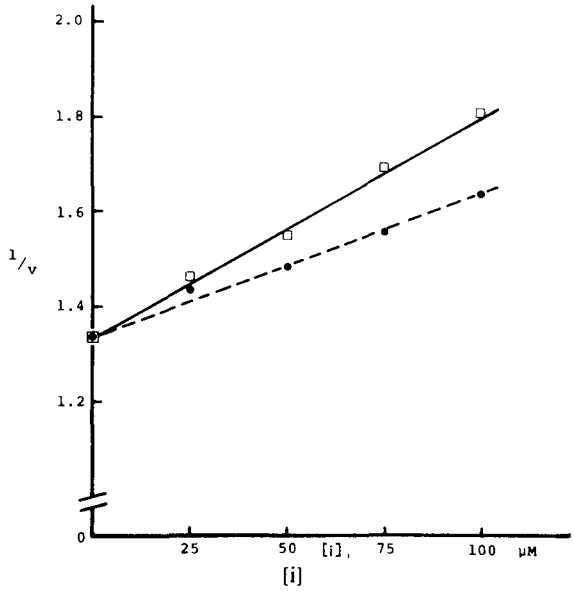

Fig. 2. The relationship between cannabinoid concentration and testosterone hydroxylation. [i] Final concentration of Δ^9-THC (●) and Δ^8-THC (□). v % of hydroxylated testosterone.

Table 2. Effect of Δ^9-THC and Δ^8-THC on testosterone hydroxylation *in vitro*

	Hydroxylation (%)				Unhydroxylated testosterone (%)	T/H*
	7α ± 16α	6β	2β	Total		
$N = 6$						
Control	19.57	5.26	14.34	39.15	45.06	1.174
Δ^9-THC (25 μM)	20.72	2.64	10.75	34.10	49.49	1.465
Mean Difference	1.22	2.61	3.29†	5.05‡	4.37†	0.262‡
± S.D.	± 0.76	± 1.04	± 1.16	± 1.36	± 1.33	± 0.044
$N = 6$						
Control	28.24	12.12	12.28	52.68	40.23	0.819
Δ^8-THC (50 μM)	26.28	9.96	11.67	47.86	46.48	1.053
Mean Difference	1.96	2.15†	0.67	4.82‡	6.25‡	0.233‡
± S.D.	± 1.01	± 0.65	± 1.08	± 0.89	± 1.52	± 0.059

* Percentage of unhydroxylated testosterone/percentage of hydroxylated testosterone.
S.D. Standard deviation of mean difference.
† $P < 0.05$, ‡ $P < 0.01$ as calculated by Student's paired *t* test.

$r = 0.998$). For the same molar concentration, Δ^8-THC exerted a greater degree of inhibition on testosterone hydroxylation than Δ^9-THC in the same microsomal preparation. Thus at a concentration of 100 μM the inhibition of testosterone hydroxylation by Δ^9-THC was 19.3 per cent, and Δ^8-THC 27.0 per cent.

DISCUSSION

Rat liver microsomes contain many Δ^4-3-ketosteroid reductases (5α) which catalyse the hydrogenation of the 4–5 double bond of various 3-ketosteroids to yield the corresponding 5α-dihydrosteroid. These reductases have been shown to be relatively specific for their substrate [9]. However, when two steroids were simultaneously incubated with microsomes together with NADPH, the hydroxylation of the larger steroid molecule was inhibited by the smaller steroid, whether or not the inhibitor was itself reduced [7]. Our data showed that the 5α reduction of testosterone was inhibited by Δ^9-THC (25 μM) but not by Δ^8-THC up to a concentration of 50 μM. This inhibition by Δ^9-THC appeared to be of the competitive type with a decrease in K_m. This shift in K_m by Δ^9-THC can be interpreted as a non-specific action of a lipid soluble drug on the penetration of substrate into the microsomes, or as a specific interference with the binding of reductase to the testosterone substrate. The fact that Δ^8-THC, which is chemically similar to Δ^9-THC except for the position of the double bond, did not inhibit the 5α reduction of testosterone indicates a fair degree of stereospecificity.

Both Δ^9-THC and Δ^8-THC react with microsomal P450 to give a type I binding spectrum [10, 11]. Testosterone also reacts with microsomal P450 to give a type I spectrum [8]. If the spectral changes are indicative of substrate reaction with cytochrome P450, which is the primary binding site of substrate for enzyme hydroxylation [12], one would expect the tetrahydrocannabinoids to interfere with testosterone hydroxylation. Our results showed that both Δ^9-THC and Δ^8-THC inhibited testosterone hydroxylation *in vitro*. These inhibitions were dose dependent over the dose range tested (25–100 μM). Again, a difference in inhibitory activity was demonstrated between Δ^9- and Δ^8-THC. Quantitatively, Δ^8-THC was shown to exert a greater inhibitory effect on testosterone hydroxylation than an equimolar concentration of Δ^9-THC, in the same microsomal preparation. Qualitatively, our chromatographic data suggested that Δ^9-THC predominantly inhibited hydroxylation at the 2β position, while Δ^8-THC predominantly inhibited hydroxylation at the 6β position. Further chromatographic purification and quantitation is needed to clarify this point, especially with regard to 7α and 16α hydroxylation. Individual steroid hydroxylases have been demonstrated to respond differently to drugs [13]. The observed differential response to Δ^9-THC and Δ^8-THC might reflect the different affinities of the tetrahydrocannabinoids for individual steroid hydroxylases. On the other hand, it might merely reflect a shift in substrate availability related to the selective activity of the cannabinoids on the reduction of testosterone at the 5α position.

On the whole, the percentage of inhibition of testosterone 5α reduction and hydroxylation by Δ^9-THC was in the range of 10–30 per cent for doses ranging from 25 to 100 μM. As the solution of cannabinoids in the incubation medium varies with the carrier and the cell fraction used, this dose-response relationship might be relative rather than absolute. Moreover, the tetrahydrocannabinoids are also substrates for microsomal hydroxylation [14]. The possibility that metabolites might be involved in this reaction cannot be excluded. The presence of metabolites and the solubility of the cannabinoids preclude quantitative evaluation of the observed inhibitory effect. The significance of this inhibition of testosterone metabolism in whole animals has not yet been studied. It might be obscured by the systemic effects of the cannabinoids on hepatic enzyme activity [15, 16].

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