In Vitro Inhibitory Effects of Cannabinoids on Progesterone 17α-Hydroxylase Activity in Rat Testis Microsomes

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The inhibitory effects of three major cannabinoids [Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN)] contained in marijuana, an abused drug, on progesterone 17α-hydroxylase activity in rat testis microsomes were investigated. Microsomal progesterone 17α-hydroxylase activity was significantly inhibited in the presence of more than 50 μM of Δ^9-THC and CBN compared with control activity, and the IC_{50} values for Δ^9-THC and CBN were estimated to be 42.8 and 32.9 μM, respectively. CBD showed less but significant inhibitory effects on 17α-hydroxylase activity at concentrations greater than 100 μM, and the IC_{50} value for the cannabinoid was estimated to be 290.9 μM. Kinetic analysis using double reciprocal plots showed that the type of inhibition by CBN was competitive, whereas that of Δ^9-THC and CBD was the mixed type. These results suggest that the inhibition may be due to metabolic interactions between each cannabinoid and 17α-hydroxylase.

Key words —— cannabinoid, 17α-hydroxylase, testis, enzyme inhibition

INTRODUCTION

Δ^9-Tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabinol (CBN), the three major cannabinoids isolated from *Cannabis Sativa* L. (Fig. 1), have been reported to exert many effects on sex hormone production and reproductive functions in mammals.[1–4] Several investigators have examined the effects of cannabinoids on the enzymes involved in steroid biosynthesis. An early *in vitro* study indicated that no change occurred in the transformation of either pregnenolone or progesterone to testosterone with Δ^9-THC 1.0 μg/ml, a dose at which both Δ^9-THC and CBN caused a greater than 60% decrease in testosterone biosynthesis in mouse Leydig cells.[5] Later, it was found that Δ^9-THC inhibited the activity of the cholesterol esterase involved in the conversion of cholesterol to pregnenolone in crude Leydig cell preparations[6] and also in the supernatants from rat adrenal homogenate.[7] Moreover, *in vitro* experiments indicated that THC can exert a variety of direct effects on the testis including inhibition of testosterone production, and that CBD and CBN, both of which are devoid of psychoactivity, are also capable of directly influencing testicular function.[8–10] Recently, we have reported that the conversion of progesterone to 17α-hydroxyprogesterone in rat testis microsomes was significantly inhibited by CBD, CBN, and Δ^9-THC.[11] A single microsomal enzyme, 17α-hydroxylase (EC 1.14.99.4)/C17,20-lyase (EC 4.1.2.30) (P450 17, CYP17) catalyzes the 17α-hydroxylation of pregnenolone and progesterone, and the subsequent cleavage of the C20,21-acetyl group to the corresponding androgens.[12] In the adult rat, this enzyme is located in both testicular and duodenal tissues,[13] but the adrenal gland contains negligible levels of the enzyme, differing from other mammals including humans.[14] The early study suggested that the concentrations of 17α-hydroxyprogesterone, androstenedione, and estradiol in mouse testis incubated with human chorionic gonadotropin were not changed by Δ^9-THC.[15] At present, it is not clear whether cannabinoids function as inhibitors of 17α-hydroxylase.

To obtain direct evidence for the inhibitory effects of cannabinoids on 17α-hydroxylase activity, we examined the effects of the three major cannabinoids on the kinetic parameters of the enzyme in rat testis microsomes.

MATERIALS AND METHODS

Chemicals —— Δ^9-THC, CBD, and CBN were isolated and purified from cannabis leaves using the method reported previously.[16] The purities of the

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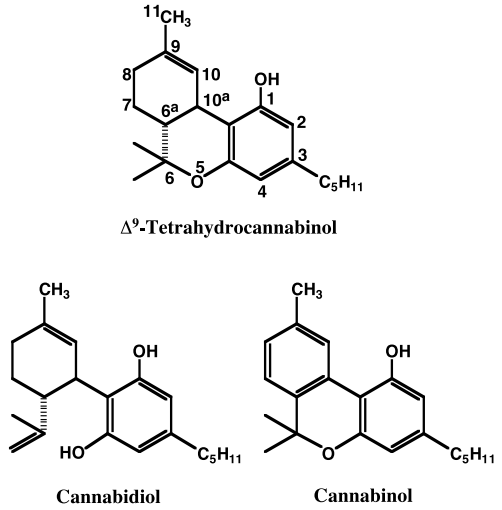

Fig. 1. Chemical Structures of Major Cannabinoids in *Cannabis Sativa* L.

cannabinoids were more than 98% in gas chromatography acccording to the previous methods.[11] Progesterone, 17α-hydroxyprogesterone, and androstenedione were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [1,2,6,7-^{3}H]Progesterone (102.1 Ci/mmol) was obtained from PerkinElmer Life Sciences, Inc. (Boston, MA, U.S.A.). $NADP^+$ and glucose-6-phosphate were bought from Roche Diagnostics GmbH (Mannheim, Germany). Glucose-6-phosphate dehydrogenase was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other chemicals and solvents were of the highest grade available from commercial sources.

Animals and Tissue Preparation —— Male Sprague-Dawley rats (8 weeks old, Sankyo Lab., Toyama, Japan) were killed by decapitation and their testes were dissected. Testes from three rats were pooled and then homogenized in 3 volumes of 50 mM of potassium phosphate buffer (pH 7.4) containing sucrose 50 mM and dithiothreitol 0.5 mM with a Potter-Elvehjem-type homogenizer. The homogenate was centrifuged at 9000 $\times$ *g* for 20 min at 4°C, and the supernatant was further centrifuged at 105000 $\times$ *g* for 1 hr to yield a microsomal pellet. The pellet was washed twice in the same buffer and suspended in 50 mM of potassium phosphate buffer (pH 7.4) containing sucrose 50 mM and 20% glycerol.

The protein concentration was determined using the method of Lowry *et al.*[17] with bovine serum albumin as a standard.

Determination of 17α-Hydroxylase Activity —— 17α-Hydroxylase activity was determined by measuring the oxidation of progesterone to 17α-hydroxyprogesterone and androstenedione. In a total volume of 0.5 ml the incubation mixture contained: 100 mM of sodium potassium phosphate buffer (pH 7.4), 0.2 μCi of [1,2,6,7-^{3}H]progesterone, an appropriate amount of nonradioactive progesterone dissolved in dimethylsulfoxide (DMSO) : water (1 : 9), rat testis microsomes, an NADPH-generating system (final concentrations: $NADP^+$ 0.5 mM, glucose 6-phosphate 10 mM, glucose-6-phosphate dehydrogenase 1 IU, $MgCl_2$ 10 mM). Cannabinoids (dissolved in DMSO) were added to the incubation mixture within the range of 0–300 μM. DMSO was added to the incubation mixture to give a final concentration of 1%. The reaction mixture was incubated at 37°C for 2 min and transferred to a glass test tube containing 3 ml of diethylether : chloroform (4 : 1), nonradioactive progesterone, 17α-hydroxyprogesterone, and androstenedione (each 10 μg) as the carrier steroids. The mixture was then vortexed vigorously to terminate the reactions and to extract the steroids. After centrifugation at 900 $\times$ *g* for 10 min, the organic phase was transferred to an alternative test tube. After the third extraction, the organic phase was evaporated under N_2 gas. The final residue was dissolved in ethanol and subjected to thin-layer chromatography in chloroform : ethyl acetate (4 : 1) using Silica gel plates (Whatman K6F Silica gel 60). The region corresponding to 17α-hydroxyprogesterone and androstenedione was scraped off under ultraviolet light and transferred to a vial containing scintillation fluid, Clear-sol (Nacalai-Tesque, Kyoto, Japan). The radioactivity was measured in a scintillation spectrometer (Aloka LSC-3500, Tokyo, Japan). After the third extraction, part of the organic phase was subjected to analysis of the radioactivity of extracted tritium. The recovery of tritium radioactivity in this reaction was more than 98% of that in the substrate. 17α-Hydroxylase activity was determined from the sum of 17α-hydroxyprogesterone and androstenedione.

Kinetic Analyses —— The kinetic parameters K_m and V_{max} values were calculated by estimating the corresponding double-reciprocal Lineweaver-Burk plots. The K_i values were obtained from the slope of each reciprocal plot against the cannabinoid concentration using linear regression analysis. The IC_{50} values were calculated using nonlinear regression analysis program from the plot of enzyme activity (%) against the log of cannabinoid concentration (Origin 6.1J, OriginLab. Co., Northampton, MA, U.S.A.). The statistical significance of difference was

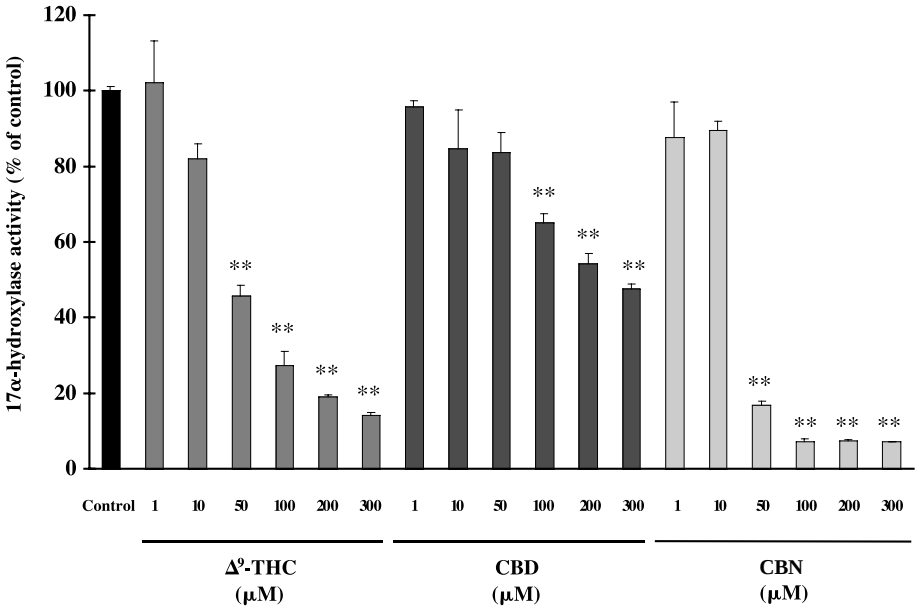

Fig. 2. Effects of Three Major Cannabinoids on Rat Testis 17α-Hydroxylase Activity

17α-Hydroxylase activity was expressed as a percentage of the control value ± S.E.M. obtained from triplicate experiments. The substrate concentration was 75 nM. The control activity was 81.9 ± 1.0 pmol/min/mg protein. **$p < 0.01$ as compared with the control group.

calculated using analysis of variance (ANOVA), followed by Bonferroni's multiple comparison tests. Differences were considered statistically significant at the $p < 0.01$ level.

RESULTS

Effects of Cannabinoids on Microsomal 17α-Hydroxylase Activity in Rat Testis

The effects of cannabinoids on rat testis microsomal 17α-hydroxylase activity are shown in Fig. 2. Δ^9-THC and CBN significantly inhibited 17α-hydroxylase activity by 19 and 7%, respectively, as compared with the control at the concentration of 300 μM. CBN is thought to act as a potent inhibitor at high concentrations. Although 17α-hydroxylase activity was also significantly inhibited by CBD at concentrations greater than 100 μM, the residual activity was reduced to only 48% compared with the control activity at 300 μM. CBD also showed comparatively weak but significant inhibition. The IC_{50} values for Δ^9-THC, CBD, and CBN 17α-hydroxylase inhibition were 42.8, 290.9, and 32.9 μM, respectively (Table 1).

Effects of Cannabinoids on Kinetic Parameters of Rat Testis Microsomal 17α-Hydroxylase

When microsomal 17α-hydroxylase activity in rat testis was expressed as the cumulative conversion of [1,2,6,7-^{3}H]progesterone 2 μM to 17α-hydroxyprogesterone and androstenedione, the reaction was found to be linear with the amount of total microsomal protein up to 300 μg after 2-min incubation. In the subsequent enzyme assays to measure the kinetic parameters of 17α-hydroxylase, the conditions were maintained by incubating 50 μg of microsomal protein for 2 min with up to 0.5 μM of progesterone.

The type of testis 17α-hydroxylase inhibition by each cannabinoid was examined in kinetic analysis using Lineweaver-Burk plots. Kinetic analyses demonstrated that CBN at the concentration of 100 μM caused an increase in the K_m value (about 30-fold) without significantly affecting the V_{max} value (Table 1). These data suggest that the observed inhibition of 17α-hydroxylase activity by CBN is the competitive type. Although Δ^9-THC caused an increase in the K_m value of 17α-hydroxylase activity, V_{max} was reduced to about 60% compared with the control activity (Table 1). Moreover, the double reciprocal plot intersected above the 1/[S]-axis (Fig. 3), suggesting that 17α-hydroxylase inhibition by Δ^9-THC is the mixed type. Similarly, CBD showed a mixed-type inhibition with an increase in the K_m and a decrease in V_{max} values, although the inhibitory effects of the cannabinoid on 17α-hydroxylase activity was very weak compared with those of Δ^9-THC

Table 1. Effects of Cannabinoids on Kinetic Parameters of Microsomal 17α-Hydroxylase in Rat Testis

	K_m (nM)	V_{max} (pmol/min/mg protein)	r^2	IC_{50}
Experiment 1				
Control	51.3	142.9	0.9941	
Δ^9-THC (50 μM)	119.2	119.2	0.9870	42.8 $\pm$ 1.1
Δ^9-THC (100 μM)	202.2	90.9	0.9977	
Experiment 2				
Control	41.4	133.3	0.9757	
CBD (50 μM)	41.7	105.3	0.9926	290.9 $\pm$ 1.2
CBD (100 μM)	51.3	92.6	0.9990	
Experiment 3				
Control	53.2	129.9	0.9951	
CBN (50 μM)	582.6	116.3	0.9987	32.9 $\pm$ 1.3
CBN (100 μM)	1657.6	172.4	0.9997	

The K_m and V_{max} values were determined from Lineweaver-Burk plots (Fig. 3). The IC_{50} values represent mean $\pm$ S.E. obtained from non-linear regression analysis mentioned in materials and methods.

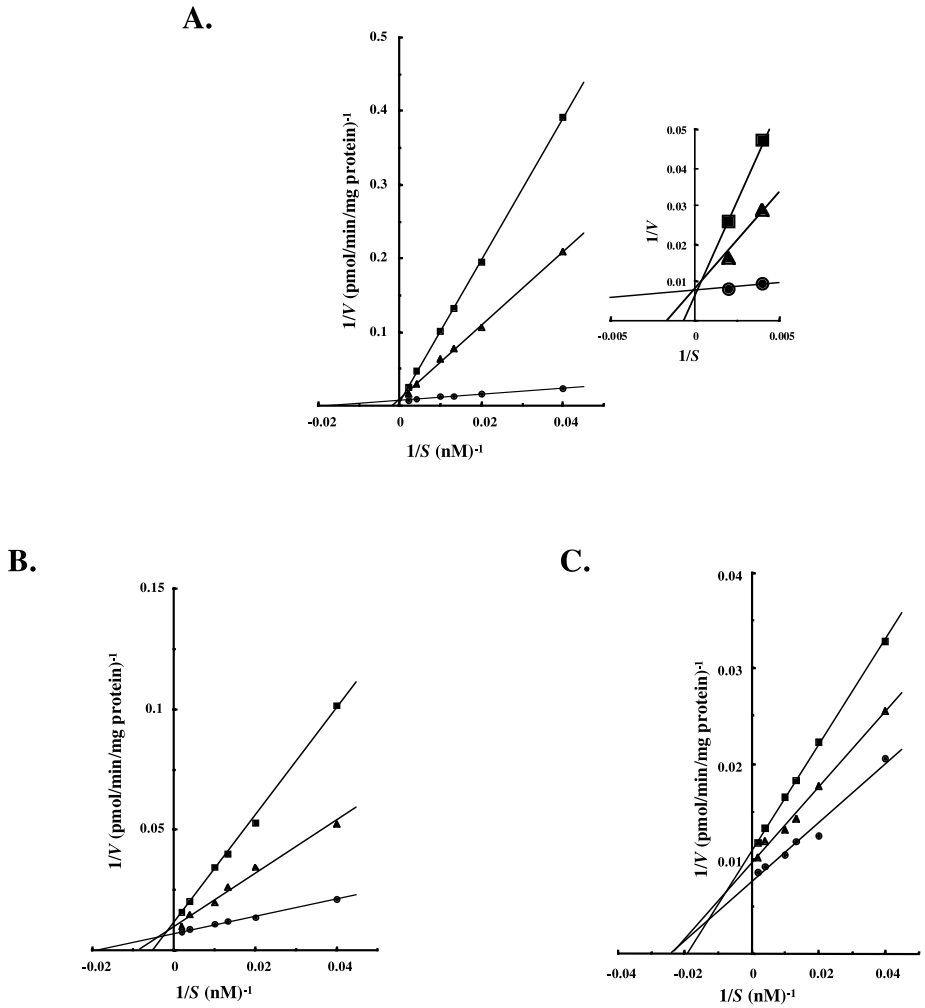

Fig. 3. Inhibition of Rat Testis 17α-Hydroxylase Activity by Cannabinoids

17α-Hydroxylase activities are shown by double reciprocal Lineweaver-Burk plots against the concentrations of progesterone in the presence of CBN (A), Δ^9-THC (B), and CBD (C). The concentrations of the cannabinoids were 0 μM (control), ●; 50 μM, ▲; and 100 μM, ■. Each point is the mean of duplicate incubations of two independent experiments. The double reciprocal plot (A) partially magnified is shown on the right side of (A).

and CBN. The inhibition constants (K_i, μM) for Δ^9-THC, CBD, and CBN were 15.9, 124.4, and 4.5, respectively.

DISCUSSION

Several studies have shown that cannabinoids have inhibitory effects on a variety of enzyme activities. In particular, CBD is known to inhibit hepatic drug-metabolizing enzymes through its interaction with cytochrome P450.[18–20] Narimatsu *et al*.[21] demonstrated that CBD, but not Δ^9-THC and CBN, competitively inhibited testosterone 2α- and 16α-hydroxylation in male rat liver microsomes and that the inhibitory effects were supported by an *in vivo* investigation. On the other hand, it was reported that not only CBD but also both Δ^9-THC and CBN showed inhibitory effects on rat hepatic microsomal enzyme activities, *N*-demethylation of aminopyrine and morphine,[22] and microsomal anandamide amidohydrolase activity in mouse brain.[23] It was also reported that the hydroxylation of the same sites on the structures of Δ^9-THC and testosterone were catalyzed by rat male-specific cytochrome P450 (CYP2C11), suggesting that the oxidation of testosterone might be inhibited competitively by the cannabinoid.[24] It is well known that the structure of Δ^9-THC is similar to those of steroids such as testosterone and estradiol.[25] However, there is no direct evidence of the binding of Δ^9-THC to the receptors of steroids such as estrogen and androgen.[11] Recently, we have shown that testosterone 6β- and 16α-hydroxylase activities in rat liver microsomes were inhibited by Δ^9-THC, CBD, and CBN as well as by crude extracts of marijuana, and that the cannabinoids inhibited progesterone 17α-hydroxylase activity in rat testis microsomes.[11] However, the inhibition of 17α-hydroxylase activity was detected at relatively higher concentrations compared with the inhibition of testosterone 6β- and 16α-hydroxylation in rat liver microsomes, and effects of the cannabinoids on kinetic parameters of rat testis 17α-hydroxylase activity were not examined. In the present study, we studied the inhibitory effects of cannabinoids on progesterone 17α-hydroxylase activity and found that the activity was significantly inhibited by cannabinoids at concentrations greater than 100 μM. On the other hand, our previous study demonstrated that progesterone 17α-hydroxylase activity was inhibited to 88, 50, and 27%, respectively, by relatively higher concentrations of CBD, Δ^9-THC, and CBN 1000 μM, while the activity was not significantly affected by concentrations of less than 100 μM of these cannabinoids.[11] There might be some reason for the difference between these results. One reason is the influence of substrate concentration added to the reaction mixture. The substrate concentration (75 nM) used in the present study was lower than that (40 μM) in the previous study.[11] The other reason may be that the method used to determine progesterone 17α-hydroxylase activity in the present study was different from that reported previously. In this study, the activity of 17α-hydroxylase was calculated as the sum of [^{3}H]17α-hydroxyprogesterone and [^{3}H]androstenedione formed from [^{3}H]progesterone, although the previous report investigated the inhibitory effects of cannabinoids on 17α-hydroxylase activity by determining only the amount of 17α-hydroxyprogesterone formed. These results suggest that these cannabinoids may have more potent inhibitory effects on 17,20-lyase than those on 17α-hydroxylase. The data therefore do not conflict with the inhibitory effects of the cannabinoids on progesterone 17α-hydroxylase activity.

Kinetic analyses showed that the inhibition by CBN was the competitive type and different from the mixed-type inhibition by Δ^9-THC and CBD. As a result, the inhibitory effects of the cannabinoids on rat testis 17α-hydroxylase activity were unrelated to their psychoactivity, because CBN, which has extremely low psychoactivity, exhibited the highest potency in the inhibition of 17α-hydroxylase activity among the cannabinoids examined. The similarity of chemical structures between the cannabinoids and progesterone was implicated in the possibility that the cannabinoids are used as substrates for rat testis 17α-hydroxylase. Although the hydroxylation of cannabinoids catalyzed by rat testis 17α-hydroxylase is not known, their metabolic interactions may be responsible for the inhibition by the cannabinoids of progesterone 17α-hydroxylase activity.

In humans, dose-related oligospermia has been observed in chronic marijuana users.[26] Similarly, a 58% decrease in sperm concentration was reported in chronic users after intensive marijuana smoking without a significant change in luteinizing hormone (LH) or testosterone levels.[27] Reversible reductions in sperm production were seen 5 to 6 weeks after the initiation of intensive smoking, suggesting an effect on sperm production.[28] In addition, abnormal sperm morphology and lower testosterone levels have been reported in chronic marijuana smokers.[26,29] However, in contrast to those findings, it has

been reported that testosterone levels showed no significant changes after a 21-day period of intense marijuana smoking.[30] Thus the results of human studies on the effects of cannabinoids on testosterone production have been conflicting.

Rat 17α-hydroxylase/17,20-lyase was cloned from testis and the deduced amino acid sequence was reported to have 69% identity with that of the human form.[31] The activity profile of rat testis 17α-hydroxylase/17,20-lyase expressed in Cos 1 cells is distinct from that of the human enzyme, which is unable to catalyze 17,20-lyase at significant rates. Alternatively, this ability of rat testis 17α-hydroxylase/17,20-lyase to convert 17α-hydroxyprogesterone to androstenedione is not shared with the human enzyme form. Further study is therefore necessary to clarify whether cannabinoids inhibit 17α-hydroxylase activity in marijuana smokers.

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