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THE EFFECTS OF Δ^9-TETRAHYDROCANNABINOL AND CANNABIDIOL ON THE METABOLISM OF GONADAL STEROIDS IN THE RAT

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ABSTRACT

The effects of Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) on hydroxylation of estradiol and testosterone in rat liver microsomes were investigated. Acute and chronic treatment of male and female rats with either cannabinoid, 2 or 10 mg/kg, significantly elevated steroid hydroxylase activity. Acute administration of THC or CBD, 10 mg/kg, evoked no detectable changes in cytochrome P-450 levels, but a significant decrease in those of cytochrome b_5. Chronic administration of THC or CBD, 2 mg/kg, decreased levels of cytochrome P-450, whereas cytochrome b_5 levels appeared normal. Acute doses of THC or CBD at 10 mg/kg significantly depressed testosterone formation in testis microsomes. Chronic THC, 2 mg/kg, but not CBD, evoked the most dramatic decrease in testicular enzyme activity.

Recent reports indicate that prolonged use of large amounts of marijuana or its primary psychoactive constituent, Δ^9-tetrahydrocannabinol (THC),[3] can significantly depress blood plasma levels of testosterone and impair reproductive functions in man. In a group of 20 long-term cannabis users, Kolodny *et al.* (1) observed plasma testosterone levels 45% lower than those of nonuser controls. Although Mendelson *et al.* (2) reported no changes in plasma androgen levels following administration of two marijuana cigarettes per day for 21 days, a second study by Kolodny *et al.* (3) demonstrated a latent suppression of testosterone levels with higher doses. Nahas (4) also observed decreased fertility in men who smoked between two and five marijuana cigarettes daily for 2 months, as evidenced by sperm counts.

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[3] Abbreviations used are: THC, Δ^9-tetrahydrocannabinol; CBD, cannabidiol; LH, luteinizing hormone; TLC, thin-layer chromatography.

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Findings of altered gonadal function have also been reported for rodents treated chronically with cannabis constituents. Repeated injections of cannabis extract at a dose of 2 mg/kg produced complete arrest of spermatogenesis and regression of Leydig cell tissue and accessory sex glands in mice (5). Rats treated chronically with THC likewise show a decline in plasma levels of testosterone (6–8).

Evidence suggests that reduced levels of circulating testosterone result from cannabinoid blockade of the gonadotropin, luteinizing hormone (LH). Acute or chronic administration of THC, 1–10 mg/kg, dramatically reduces plasma LH levels in female (9) rats, and blocks the cyclic surge of LH which normally triggers ovulation (10). Because testosterone synthesis is dependent on gonadotropic stimulation, a blockade of LH elaboration would result in reduced levels of circulating gonadal steroids.

Despite the elegance of this proposal, studies on the metabolism of cannabinoids suggest alternative mechanisms which may also stimulate the degradation of circulating gonadal steroids. Acute and chronic administration of THC has been demonstrated to potentiate its own biotransformation (11,12) and the metabolic conversion of a narrow range of other microsomal substrates (13–15), all of which possess an aromatic ring. In contrast, THC also suppresses hepatic catabolism of select compounds, *viz.*, pentobarbital (16), aminopyrine, and morphine (17). This selectivity of stimulated activity, in

addition to the failure to demonstrate enzyme induction in hepatic microsomes by THC (18–20), suggests a qualitative rather than quantitative difference in the cytochrome P-450 population after exposure to THC. The possibility exists, therefore, that THC interacts with cytochrome P-450 in a manner that alters hepatic degradation of gonadal steroids or even gonadal biosynthesis of these steroids, which is dependent upon testicular cytochrome P-450.

To explore these possibilities, we have examined hepatic microsomal hydroxylation of testosterone and estradiol after acute and chronic pretreatment with either THC or cannabidiol (CBD). Biosynthesis of testosterone by testicular microsomes was also examined following cannabinoid pretreatment.

Materials and Methods

Animals. Female Holtzman and male Wistar rats weighing 175–200 g were maintained on Purina Lab Chow and water *ad lib.*, with a 12/12-hr light/dark schedule. Rats received ip injections not exceeding 0.33 ml of THC, CBD (both supplied by Biomedical Research Branch, National Institute on Drug Abuse, Rockville, Md.), or carrier vehicle alone [propylene glycol/Tween 80/physiological saline, 10:1:89 (v/v)]. The cannabinoids were administered either acutely or chronically, and at dosages of either 2 mg/kg or 10 mg/kg. All animals were killed between 9 and 10 A.M. to control for diurnal variation.

Hydroxylation of Gonadal Steroids. This assay is a modification of the procedure described by Conney and Klutch (21). The rats were decapitated, livers from three rats were pooled, and a 33% liver homogenate was prepared in 0.25 M sucrose solution at 0–5°C. Nuclei and mitochondria were removed by centrifugation of 15-ml aliquots of homogenate at 9000*g* for 30 min. Microsomes were obtained from the supernatant fluid by centrifugation at 105,000*g* for 60 min in a Beckman ultracentrifuge. The pellet was resuspended in ice-cold 0.1 M potassium phosphate buffer (pH 7.4) to a final volume of 7.5 ml.

Microsomal suspension (0.5 ml) was added to the reaction medium [14 μmol of NADPH, 50 μmol of glucose 6-phosphate, 12.5 μmol of $MgCl_2$, one unit of glucose 6-phosphate dehydrogenase (all from Sigma Chemical Co., St. Louis, Mo.) and 100 μmol of Tris buffer (pH 7.4) in a final volume of 5.7 ml]. After preincubation for 5 min in a shaking water bath at 37°C, the reaction media was incubated with either 500 nmol of ^{14}C-17β-estradiol or ^{14}C-testosterone (New England Nuclear, Boston, Mass.; radiochemical purity >99%, as determined by gas chromatography) for 15 and 10 min, respectively. Five milliliters of the incubation mixture were pipetted into 25 ml of distilled dichloromethane to terminate the reaction and extract the steroids. The extraction mixture was shaken for 60 min and centrifuged at 2,500 rpm for 10 min. A 20-ml portion of the organic phase was evaporated to dryness under a stream of nitrogen, and the residue then redissolved in 1.0 ml of methanol. Protein was determined as described by Sutherland *et al.* (22).

The steroids and their metabolites were separated on silica gel TLC plates previously activated by heating at 110°C for 30 min. Aliquots (20 μl) of the methanol extracts were spotted on the plates, which were developed with one of two solvent systems: testosterone extracts in chloroform/methanol (8:2), and estradiol extracts in chloroform/methanol (9:1) followed by benzene/ethanol (9:1) (all v/v). TLC plates were dried, sprayed with concentrated H_2SO_4, and heated at 110°C for 15 min to yield fluorescence of the metabolites.

Silica gel containing steroids more polar than the substrate was scraped from the plates for assay of radioactivity. Estradiol metabolites were extracted with 3 ml of acetone, shaken briefly, centrifuged at 2000 rpm for 5 min, and 2 ml of the acetone was transferred to scintillation vials with 10 ml of Bray's scintillation mixture. Silica gel containing testosterone metabolites was transferred directly to scintillation vials with 2 ml of distilled water added. Ten milliliters of scintillation fluid (5 g of 2-(4′-*tert*-butylphenyl)-5-(4′-biphenyl)-3,4-oxadiazole, 100 g of napthalene, and dioxane to make one liter) was added to these vials. Radioactivity was assayed in a Packard liquid scintillation spectrometer. The amount of metabolite formed was calculated from the radioactivity and used as a measure of steroid hydroxylation.

Cytochromes P-450 and b_5. Liver homogenates (14%) from male rats were prepared in 0.15 M KCl at 0–5°C. Microsomes were obtained as described above with the pellets resuspended in 0.1 M potassium phosphate buffer (pH 7.4) at a final protein concentration of approximately 2 mg/ml. The contents of cytochromes P-450 and b_5 were determined by the method of Omura and Sato (23) in a Cary model 14 spectrophotometer.

Androgen Biosynthesis. Microsomes were prepared from rat testes as previously described for hepatic hydroxylation of gonadal steroids. The formation of testosterone was assayed by a modification of the method of Menard and Purvis (24). The reaction was initiated by the addition of 50–125 μg of microsomal protein to the reaction media containing 0.14 μmol of [4-C^{14}]progesterone (7.8 μCi) in 1% propylene glycol, 250 μmol of $MgCl_2$, 20 μmol of NADPH, 57 μmol of glucose 6-phosphate, 200 μmol of Tris buffer (pH 7.4), and two units of glucose 6-phosphate dehydrogenase, in a total volume of 0.4 ml, and incubated aerobically in a water bath shaker at 37°C for 10 min. Enzyme activity was terminated by the addition of 1.60 ml of methanol. The mixture was shaken, refrigerated overnight, and then centrifuged to ensure complete protein precipitation. Protein was determined by the method of Sutherland *et al.* (22). Aliquots of the methanol extract

(25 μl) were chromatographed as described for testosterone extracts. Radioactivity of isolated testosterone samples was assayed as described above.

Data were analyzed by the paired t-test and the Mann-Whitney-Wilcoxon text.

Results

Hydroxylation of Gonadal Steroids. Rates of metabolite formation by hepatic microsomes are presented in tables 1 and 2. Treatment of both male and female rats with either THC or CBD significantly altered *in vitro* hydroxylation of the respective gonadal steroids. Treatment of female rats with a single dose of CBD at 2 mg/kg showed no effect, but in all other cases hydroxylase activity in males and females was significantly elevated by cannabinoid pretreatment. The magnitude of elevations were also dose-related and persisted through the periods of chronic treatment.

Cytochromes P-450 and b_5. As shown in table 3, pretreatment of male rats with either THC or CBD, 10 mg/kg, evoked no detectable change in microsomal cytochrome P-450 concentrations, but significant decreases in those of cytochrome b_5 were observed ($p < 0.05$). Repeated administration of either THC or CBD, however, decreased cytochrome P-450 levels ($p < 0.007$), whereas cytochrome b_5 levels returned to baseline.

Acute and chronic administration of either cannabinoid showed no significant changes in to-

TABLE 1

Testosterone hydroxylation by liver microsomal fractions from male rats

Male Wistar rats were treated with acute doses of 2 or 10 mg/kg and killed 6 hr later. Chronically treated rats received 2 mg/kg daily for 10 days and were killed 24 hr after the final treatment. Each datum represents mean ± SD for three experiments.

Treatment	Polar Metabolite Formation[a]	Increase
	nmol/min/mg protein	%
2 mg/kg		
Veh	103 ± 13	
THC	143 ± 04	39
CBD	173 ± 04	68
10 mg/kg		
Veh	74 ± 31	
THC	188 ± 08	154
CBD	227 ± 18	227
2 mg/kg for 10 days		
Veh	61 ± 06	
THC	89 ± 08	46
CBD	81 ± 08	33

[a] All drug-treated groups are significantly greater than the vehicle controls ($p < 0.05$).

TABLE 2

Estradiol hydroxylation by liver microsomal fractions from female rats

Female Holtzman rats were treated with acute doses of 2 or 10 mg/kg and killed 6 hr later. Chronically treated rats received 2 mg/kg daily for 7 days and were killed 24 hr after the final treatment. Each datum represents the mean ± SD for three experiments.

Treatment	Polar Metabolite Formation	Increase
	nmol/hr/mg protein	%
2 mg/kg		
Veh	237 ± 35	
THC	214 ± 16	32
CBD	233 ± 14[a]	
10 mg/kg		
Veh	283 ± 15	
THC	519 ± 21	84
CBD	508 ± 21	80
2 mg/kg for 7 days		
Veh	215 ± 24	
THC	429 ± 27	97
CBD	417 ± 30	94

[a] Shows no statistical difference. All other drug treated groups are significantly higher than the vehicle controls ($p < 0.05$).

TABLE 3

Analysis of cytochrome P-450 and cytochrome b_5 contents in rat liver microsomes

Male Wistar rats were killed 6 hr after an acute dose of 10 mg of THC or CBD per kg body weight. Male rats treated with 2 mg/kg daily for 10 days were killed 24 hr after the final treatment. Data represent means ± SD.

Treatment	Cytochrome b_5	N[a]	Cytochrome P-450	N[a]
	nmol/mg protein		*nmol/mg protein*	
10 mg/kg				
Veh	0.133 ± 0.015	5	0.239 ± 0.008	8
THC	0.113 ± 0.011[b]	5	0.236 ± 0.023	6
CBD	0.018 ± 0.010[b]	3	0.248 ± 0.002	3
2 mg/kg for 10 days				
Veh	0.139 ± 0.007	5	0.234 ± 0.005	5
THC	0.144 ± 0.006	5	0.220 ± 0.006[c]	5
CBD	0.132 ± 0.004	5	0.198 ± 0.006[c]	5

[a] N represents the number of spectra recorded from microsomal preparations obtained by pooling livers from three male rats.

[b] Significantly less than vehicle controls ($p < 0.05$).

[c] Significantly less than vehicle controls ($p < 0.007$).

TABLE 4

Androgen production in vitro by microsomal fractions of rat testes

Male Wistar rats were treated identically with those described in table 1. Each datum represents the mean ± SD for three experiments.

Treatment	Testosterone Formation	Inhibition
	nmol/min/mg protein	%
10 mg/kg		
Veh	0.937 ± 0.110	
THC	0.597 ± 0.132[a]	36
CBD	0.599 ± 0.027[a]	36
2 mg/kg for 10 days		
Veh	0.753 ± 0.104	
THC	0.389 ± 0.077[a]	48
CBD	0.607 ± 0.141	

[a] Significantly less than controls ($p < 0.05$).

tal wet liver weights or microsomal protein concentrations.

Androgen Biosynthesis. Acute doses of 10 mg/kg significantly depressed testosterone formation. Repeated THC administration evoked the most dramatic decrease in enzyme activity (see table 4).

Discussion

The evidence presented here demonstrates that THC and CBD selectively stimulate the metabolic conversion of gonadal steroids in hepatic microsomes. That microsomal protein remained unchanged and cytochrome P-450 levels diminished after chronic treatment are consistent with the observations of Berman and Bochantin (20), and further implicate a qualitative change in the nature of the terminal oxidase.

A possible explanation of our results is that THC and its congener induce a particular type of cytochrome P-450, as suggested by Franklin (25) for phenobarbital-pretreated rats, thereby increasing the overall binding specificity. The existence of multiple forms of terminal oxidase is demonstrated by selective induction of different spectral (26,27) and catalytic (27,28) species of the cytochrome by pretreatment with phenobarbital or 3-methylcholanthrene as well as by physical resolution of a number of distinguishable forms by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (29). Particularly relevant, however, is that Huang *et al.* (30), purified several forms of cytochrome P-450 from mouse liver which were selective in catalyzing the hydroxylation of testosterone at either the 6β-, 7α-, or 16α-positions. It is possible, therefore, that THC and CBD act as alternate substrates inducing or activating species of the cytochrome specific for endogenous steroids.

The accelerated rate of testosterone metabolism is compounded by an impairment of androgen biosynthesis. Acute and chronic studies here demonstrate that THC strongly inhibits testosterone production in testis microsomes. Whether this inhibition of biosynthesis *in vitro* is related to actions on LH or a direct action on testis microsomes is unknown. This issue could likely be resolved by administering exogenous LH simultaneously with THC to determine if LH is still capable of stimulating testosterone production.

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