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POSSIBLE ROLE OF THE CYTOCHROME P450-LINKED MONOOXYGENASE SYSTEM IN PREVENTING δ^9-TETRAHYDROCANNABINOL-INDUCED STIMULATION OF TUBEROINFUNDIBULAR DOPAMINERGIC ACTIVITY IN FEMALE RATS

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Abstract—The administration of δ^9-tetrahydrocannabinol (THC) or related cannabinoids markedly affected neurobehavioral and neuroendocrine indices in male rodents but usually failed to affect those indices in females. We examined whether inhibition of the cytochrome P450-linked monooxygenase system in female rats is able to elicit the effects of THC on one of the most characteristic targets of cannabinoid action, tuberoinfundibular dopaminergic neurons, whose activity is known to increase after cannabinoid exposure in males. It was found that the administration of THC to ovariectomized rats acutely replaced with estradiol (to discard problems derived from differences in the estrogenic status) did not affect either dopamine and L-3,4-dihydroxyphenylacetic acid (DOPAC) contents and tyrosine hydroxylase activity in the medial basal hypothalamus or the density of D2-dopaminergic receptors in the anterior pituitary. However, the administration of THC to estrogen-replaced ovariectomized rats that had been pretreated with two separately administered inhibitors of cytochrome P450, piperonyl butoxide or metyrapone, significantly increased DOPAC content in the medial basal hypothalamus, with no changes in the other parameters. Collectively, these results indicate that the metabolism of THC to inactive compounds might play a protective role in females, counteracting the effects of this cannabinoid on tuberoinfundibular dopaminergic activity because pharmacological inhibition of cytochrome P450-linked monooxygenase system elicited a significant stimulation of these neurons by THC.

Key words: δ^9-Tetrahydrocannabinol; cytochrome P450-linked monooxygenase system; piperonyl butoxide; metyrapone; tuberoinfundibular dopaminergic neurons; DOPAC

Brain dopaminergic neurons are one of the most characteristic targets of the action of the psychoactive principles of *Cannabis sativa* derivatives [1–5]. This can be concluded from the following observations: (i) THC† and related cannabinoids affect the biochemical indices of hypothalamic and extra-hypothalamic dopaminergic neurons [2–6]; (ii) cannabinoids alter DA-related behaviors [4]; and (iii) cannabinoid receptors are present in the vicinity of brain dopaminergic neurons [7, 8]. However, in spite of the large body of literature accumulated on this topic, several aspects remain to be clarified. For instance, most of these effects have been observed in male rodents [2–4, 6], the results in females being controversial [5, 9–11]. This notion is based on the following observations: (i) THC was unable to affect nigrostriatal and mesolimbic dopaminergic neurons in normal cycling rats [9, 10], whereas its effects on the activity of tuberoinfundibular dopaminergic neurons varied as a function of the estrous cycle [11]; (ii) perinatal exposure to cannabinoids profoundly affected the ontogeny of these three dopaminergic systems and their related behaviors in males, but produced only transient effects in females [1, 5]. This has led to the opinion that a "particularly sensitive protection system" exists in females that might counteract the effects of cannabinoids on dopaminergic neurons. A possible hypothesis to explain this "protection" might be that specific microsomal enzymes, such as the cytochrome P450 system, rapidly metabolize THC to inactive compounds, thus diminishing its effects on the brain. In this regard, several enzymatic activities linked to the cytochrome P450 system, which are basically located in the liver and play a role in the metabolism of xenobiotics (drugs, carcinogens and environmental chemicals) and endobiotics (prostaglandins and fatty acids) [12], have also been reported to be present in the brain [13].

The present study was designed to test this hypothesis. To this end, we examined whether the pharmacological inhibition of the cytochrome P450-linked monooxygenase system in female rats is able to elicit the effects of THC on one of the most characteristic targets of cannabinoid action, tuberoinfundibular dopaminergic neurons. The

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† Abbreviations: DA, dopamine; DOPAC, L-3,4-dihydroxyphenylacetic acid; PBO, piperonyl-butoxide; THC, δ^9-tetrahydrocannabinol; TH, tyrosine hydroxylase.

activity of these neurons usually increases after cannabinoid exposure in males [6, 14] and this stimulation underlies the cannabinoid-induced inhibition of prolactin release [6, 14, 15]. We studied the effects of a single oral dose of THC administered to ovariectomized rats treated acutely with a physiological dose of estradiol. This animal model was used for two reasons: (i) to discard the possible interference of cyclic changes in the gonadal status; and (ii) because these animals exhibit a plasma estrogen surge similar to that found during the proestrous phase [16], during which the administration of THC fails to affect tuberoinfundibular dopaminergic activity [11]. Cannabinoid administration was repeated in animals that had been pretreated with PBO or metyrapone, which inhibit the activity of the cytochrome P450-linked monooxygenase system. In all these animals, we analysed the activity of tuberoinfundibular dopaminergic neurons by measuring DA and DOPAC contents and TH activity in the medial basal hypothalamus and the density of D2 dopaminergic receptors in the anterior pituitary, their target gland. Finally, we also measured plasma THC concentrations in the animals treated with this cannabinoid and/or an inhibitor of the cytochrome P450-linked monooxygenase system (metyrapone) in order to test possible differences after the blockade of the THC metabolism.

MATERIALS AND METHODS

Animals, treatments and sampling. Female virgin rats of the Wistar strain were housed from birth in a room with controlled photoperiod (08:00–20:00 hr light) and temperature (23 ± 1°). They had free access to standard food (Panlab, Barcelona, Spain) and water. At adult age (>8 weeks of life), the animals were subjected to bilateral ovariectomy under ether anesthesia. Seven or eight days later, all the animals were subcutaneously injected with 17β-estradiol (30 ng/0.2 mL) prepared in 0.01% ethanol [16] and divided into two groups. The first group was subjected to an oral administration of THC (5 mg/kg weight) prepared in sesame oil solution as described previously [4] and the second group was administered with vehicle. One hour later, all the animals were killed. A similar treatment schedule was repeated in estrogen-replaced ovariectomized rats, but which had been pretreated with either PBO (400 mg/kg weight), prepared in sesame oil solution and administered (i.p.) 2 hr prior to cannabinoid administration, or metyrapone (40 mg/kg weight), prepared in saline–glycerol solution and administered (i.p.) 1 hr prior to cannabinoid treatment. The dose for both inhibitors of cytochrome P450 as well as the vehicle used and the time after the administration of each inhibitor at which the animals were treated with THC were chosen according to previously published reports [17, 18]. After death, brains were removed and the medial basal hypothalamus dissected out [19], weighed and immediately frozen at −70° until assayed for dopaminergic measurements. The pituitary gland was also removed, the posterior lobe discarded and the anterior pituitary gland immediately frozen at −70° until assayed for D2 receptor density. Trunk blood was also collected in all animals treated with metyrapone alone or combined with THC—but only in some animals treated with vehicle or THC—centrifuged and the plasma removed and stored frozen at −40° until assayed for plasma THC concentrations.

Tyrosine hydroxylase determination. Tyrosine hydroxylase activity was evaluated according to the method described by Nagatsu *et al.* [20]. Tissues were homogenized in 10 vol. of 0.25 M sucrose. An aliquot of homogenate corresponding to 2 mg of tissue was incubated at 37° in the presence of 0.1 M sodium acetate, 0.1 mg/mL catalase, 1 mM 6-methyl-5,6.7,8-tetrahydropterine (prepared in 1 M mercapto-ethanol solution) and 0.2 mM L-tyrosine (incubation volume: 0.1 mL). For the blank incubation, D-tyrosine was used as substrate instead of L-tyrosine. Blank tubes containing 1 μM L-dopa were also used as an internal standard for each tissue. After 30 minutes incubation, the reaction was stopped by addition of 0.2 N perchloric acid containing 0.5 mM sodium bisulfite, 0.45 mM EDTA and 3 μM dihydroxybenzylamine (as an internal standard for HPLC determination) and the tubes centrifuged. The amounts of L-dopa formed were evaluated by HPLC with electrochemical detection after a previous procedure of extraction with alumina. HPLC system consisted in a Spectra-Physics 8810 isocratic pump with a 20 μL sample loop. The column was a RP-18 (Spherisorb ODS-2; 125 mm, 4.6 mm, 5 μm particle size). The mobile phase (previously filtered and degassed) consisted of 10 mM citric acid, 5 mM sodium monohydrogen phosphate, 0.05 mM EDTA, 0.12 mM octane sulfonate and 3% methanol (pH 3) and the flow rate was 1.2–1.4 mL/min. The effluent was monitored with a Metrohm bioanalytical system amperometric detector using a glassy carbon electrode. The potential was 0.80 V relative to an Ag/AgCl reference electrode with a sensitivity of 50 nA (100 pg per sample). The signal was recorded on a Spectra-Physics 4290 integrator and the results were given as area under the peaks. Values were expressed as ng/mg of tissue/hour of incubation.

DA and DOPAC determinations. DA and DOPAC contents were analysed using HPLC with electrochemical detection. An aliquot of each homogenate for TH activity was diluted immediately after homogenization with ice-cold 0.2 N perchloric acid containing 0.5 mM sodium bisulfite and 0.45 mM EDTA. Dihydroxybenzylamine was added as an internal standard. The diluted homogenates were then centrifuged and the supernatants injected into the HPLC system. This procedure was similar to that described for the determination of L-dopa formed in the TH assay, except that: (i) electrochemical detection was done with a coulochemical detector (Coulochem II, ESA) using a procedure of oxidation/reduction (conditioning cell: +350 mV; analytical cell No. 1: +100 mV; analytical cell No. 2: −250 mV); and (ii) the mobile phase consisted of 0.15 M sodium dihydrogen phosphate, 0.5 mM octane sulfonate, 0.1 mM EDTA and 8% methanol (pH 3.8). The signal was recorded from analytical cell No. 2 with

a sensitivity of 50 nA (10 pg per sample). Values were expressed as ng/mg of tissue weight.

D2 dopaminergic binding site analysis. Owing to the amount of tissue available, a simplified method was applied for the measurement of the relative binding capacity of individual anterior pituitaries for D2-dopaminergic receptors. This procedure, described by Arita *et al.* [21], analyses total specific binding with a saturating concentration of ligand that ensures higher receptor occupancy. Tissues were thawed and homogenized for 20 sec with a Polytron at speed 2–3 in 5 mL of ice-cold 50 mM Tris–HCl buffer at pH 7.4. The homogenates were centrifuged at 40,000 *g* for 10 min at 4°. After one wash, the pellets were resuspended in a volume of the same buffer (variable as a function of the desired protein concentration) and used for the binding assay. An aliquot of membrane fraction was used to determine protein concentration by using the Lowry method [22]. This was approximately 2–3 mg/mL. The radioactive ligand was [^{3}H]spiroperidol (27.5 Ci/mmol; purchased from NEN, Boston, MA, U.S.A.). This was used at a saturating concentration (0.80 nM). (+)-Butaclamol, purchased from RBI (Natick, MA, U.S.A.), was used as displacer at a concentration of 1 μM (for the measurement of non-specific binding). Both radioactive ligand and displacer were diluted from stock solutions at the above-mentioned concentrations in incubation buffer. This consisted of 50 mM Tris–HCl (pH 7.1) containing 120 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$ and 5.7 mM ascorbic acid. The aliquot of membrane fraction was also diluted in the incubation buffer until a final protein concentration in the incubation volume of 0.2–0.3 mg/mL. The final incubation volume was 0.5 mL. Incubation was allowed for 15 min at 37° and stopped by rapid filtration through Whatman GF/C glass fiber filters, presoaked in BSA. Filters were washed twice with 5 mL of ice-cold Tris–HCl buffer (pH 7.4). Radioactivity bound to membranes was determined by liquid scintillation counting. Specific [^{3}H]-spiroperidol binding was calculated as the difference between binding in the presence or absence of butaclamol. This procedure only allows calculation of the maximum bound to the anterior pituitary membranes, expressed as fmol/mg of protein, with no indications as to affinity.

Determination of plasma THC levels. Plasma THC levels were determined using a specific radioimmunoassay kit prepared at the Research Triangle Institute (Research Triangle Park, NC, U.S.A.) and kindly furnished by NIDA. Briefly, it consisted of an antiserum generated against δ^9-THC, ^{125}I-δ^8-THC as radioligand and different standard solutions of δ^9-THC prepared in human plasma. Plasma samples and standard solutions (100 μL) were extracted with methanol prior to incubation in the presence of antiserum and radioligand. Bound radioactivity was separated by immunoprecipitation and counted. The sensitivity of the method was 2.5 ng/mL. Plasma THC levels were expressed as ng/mL.

Statistics. All data were tested for normality of distribution and accordingly assessed by one-way analysis of variance followed by the Student-Neuman–Keuls test.

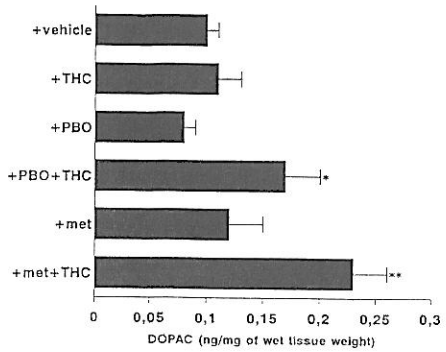

Fig. 1. DOPAC content in the medial basal hypothalamus after oral administration of THC or vehicle (oil) to ovariectomized rats acutely treated with estradiol pretreated with PBO, metyrapone or their corresponding vehicle (both vehicle-pretreated groups exhibited similar results and were combined for statistical analysis). Details in text. Values are means ± SEM of 6–10 determinations per group. Statistical differences were obtained by one-way analysis of variance followed by the Student-Neuman–Keuls test ($^*P < 0.05$, $^{**}P < 0.01$ versus the group injected with vehicle and the group injected with the corresponding inhibitor of cytochrome P450 alone).

RESULTS

The values in the different treatment groups for every dopaminergic index coupled to a normal distribution and, accordingly, were submitted to a multiple group comparison by using one-way analysis of variance. Only the hypothalamic content of DOPAC revealed the existence of statistically significant differences among the different treatment groups used in the present study [$F(5,50) = 3.706$, $P < 0.01$]. The analysis of the concrete groups that statistically differed for this parameter was done by applying an appropriate post-hoc test. Results were as follows.

THC administration to ovariectomized rats acutely replaced with estradiol did not affect either DA or DOPAC contents (Figs 1 and 2) and TH activity (Fig. 3) in the medial basal hypothalamus or the density of D2-dopaminergic receptors in the anterior pituitary (Fig. 4). However, the administration of THC to estrogen-replaced ovariectomized rats that had been pretreated with PBO, which has been reported to inhibit the activity of cytochrome P450 [17], significantly increased DOPAC content in the medial basal hypothalamus (Fig. 1), with no changes in DA contents (Fig. 2) and TH activity (Fig. 3) in the medial basal hypothalamus or in the number of anterior pituitary D2-dopaminergic receptors (Fig. 4).

A similar yet even more marked increase in DOPAC content in the medial basal hypothalamus (Fig. 1) was found after administration of THC to estrogen-replaced ovariectomized rats pretreated with metyrapone, an additional inhibitor of cyto-

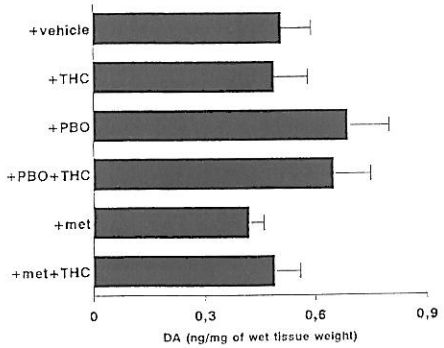

Fig. 2. DA content in the medial basal hypothalamus after oral administration of THC or vehicle (oil) to ovariectomized rats acutely treated with estradiol pretreated with PBO, metyrapone or their corresponding vehicle (both vehicle-pretreated groups exhibited similar results and were combined for statistical analysis). Details in text. Values are means ± SEM of 6–10 determinations per group. Statistical differences were obtained by one-way analysis of variance followed by the Student-Neuman–Keuls test.

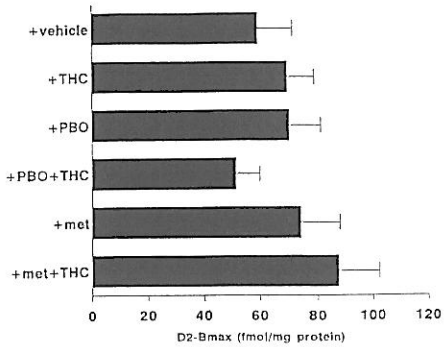

Fig. 4. Number (B_{max}) of D2 dopaminergic receptors in the anterior pituitary gland after oral administration of THC or vehicle (oil) to ovariectomized rats acutely treated with estradiol pretreated with PBO, metyrapone or their corresponding vehicle (both vehicle-pretreated groups exhibited similar results and were combined for statistical analysis). Details in text. Values are means ± SEM of 6–10 determinations per group. Statistical differences were obtained by one-way analysis of variance followed by the Student-Neuman–Keuls test.

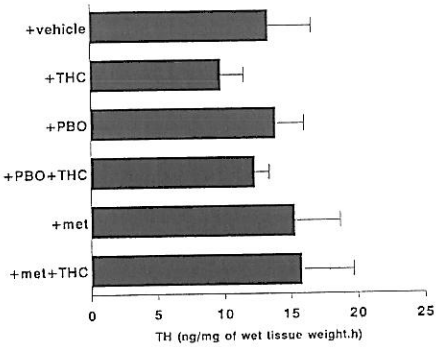

Fig. 3. TH activity in the medial basal hypothalamus after oral administration of THC or vehicle (oil) to ovariectomized rats acutely treated with estradiol pretreated with PBO, metyrapone or their corresponding vehicle (both vehicle-pretreated groups exhibited similar results and were combined for statistical analysis). Details in text. Values are means ± SEM of 6–10 determinations per group. Statistical differences were obtained by one-way analysis of variance followed by the Student-Neuman–Keuls test.

chrome P450. Moreover, as with PBO pretreatment, no changes were seen in the remaining parameters following THC administration to metyrapone-pretreated rats (Figs 2–4). The increase in DOPAC contents after the administration of THC to PBO- or metyrapone-pretreated rats was significant not only as compared with vehicle-treated animals but also versus animals pretreated with each inhibitor of cytochrome P450 alone (Fig. 1).

Finally, we also measured plasma THC levels after the administration of this cannabinoid to control or metyrapone-pretreated rats. This was done in order to elucidate whether the pharmacological blockade of the cytochrome P450-linked monooxygenase system displayed a significant increase in the presence of THC in the plasma of these animals. However, plasma THC levels observed 1 hr after administration of THC to vehicle or metyrapone-pretreated rats were similar (48.8 ± 9.1 ng/mL and 58.9 ± 8.3 ng/mL, respectively). THC was undetectable (<2.5 ng/mL) in the plasma of animals treated with vehicle or metyrapone alone.

DISCUSSION

As mentioned in the Introduction, our purpose was to test the hypothesis that the metabolism of THC to inactive compounds generated by the action of the cytochrome P450-linked monooxygenase system could be responsible for the current absence of significant modifications in hypothalamic dopaminergic neurons after administration of this cannabinoid to female rats. In view of the results, this hypothesis seems to be valid: we found that the pharmacological blockade of cytochrome P450-linked monooxygenase activity, with two different inhibitors administered individually, allowed THC to elicit a marked stimulation of the activity of tuberoinfundibular dopaminergic neurons, one of the most typical cannabinoid effects in male rats [6, 14]. Thus, the administration of THC to ovariectomized rats acutely replaced with estradiol did not affect the activity of these neurons, as revealed by the absence of changes in the hypothalamic and anterior pituitary parameters measured. This lack of effect of THC agrees with the previously reported inability of this cannabinoid to alter tuberoinfundibular dopaminergic activity in normal cycling rats during proestrus [11] because the acutely estrogen-replaced ovariectomized rats

used in the present study resembled intact animals during that ovarian phase [16]. However, the administration of THC to estrogen-replaced ovariectomized rats pretreated with either PBO or metyrapone, which inhibit the activity of the cytochrome P450-linked monooxygenase system, significantly increased DOPAC content in the medial basal hypothalamus, especially in the case of metyrapone pretreatment. We interpret this increase as indicative of a significant THC-induced stimulation of tuberoinfundibular neurons. Three interesting aspects derived from this finding are worthy of comment.

First, our results contrast with the previous suspicion that the metabolism of THC through the action of the hepatic cytochrome P450-linked monooxygenase system to produce a set of hydroxylated metabolites [23] might increase the biological activity of this cannabinoid. The present study demonstrates that THC metabolites are lacking in biological activity on hypothalamic dopaminergic neurons and that only the pharmacological blockade of the enzyme responsible for their synthesis allows cannabinoid action to become significant.

Second, this effect seems specific to tuberoinfundibular dopaminergic neurons, because it did not appear in other dopaminergic neuronal systems, such as the nigrostriatal or mesolimbic pathways, where THC was unable to alter dopaminergic indices in intact [9, 10] and PBO- or metyrapone-treated estrogen-replaced ovariectomized rats (data not shown). This might be related to the fact that, although drug metabolism mainly occurs in the liver, several activities of the cytochrome P450-linked monooxygenase system have recently been located in the brain [13] and might present a regional distribution with brain areas particularly enriched in specific activities of the cytochrome P450 family and especially able to metabolize THC as opposed to other brain areas lacking this ability. Furthermore, the possible "protection" in females against THC by the action of the cytochrome P450-linked monooxygenase system seems to be located in the activity of cytochrome P450 in specific brain areas rather than in the hepatic system. This view is supported by the fact that plasma THC levels measured 1 hr after the administration of this cannabinoid were similar in rats pretreated with metyrapone and in non-pretreated rats, although THC was only effective in increasing DOPAC content in metyrapone-pretreated animals. In our opinion, the complete absence of changes in plasma THC levels when cytochrome P450 is inhibited tentatively suggests that THC metabolism to inactive compounds might be located in the brain targets of THC action themselves rather than in the liver, and, then, with no reflection on plasma THC levels. The possibility of cross-reactivity of hydroxylated metabolites of THC in the radioimmunoanalysis for this cannabinoid (20% according to the Research Triangle Institute) seems quantitatively less important.

Finally, it would be tempting to relate the inactivation of THC through the action of cytochrome P450 to the tolerance phenomena usually found after chronic treatments with this cannabinoid. It has been well demonstrated that the extrapyramidal, limbic and neuroendocrine effects of acute THC disappear or diminish during prolonged exposure to this cannabinoid [24–26]. We have recently reported that tolerance could be related to an expected down-regulation of brain cannabinoid receptors originated by chronic treatment with their agonist THC [26], although the magnitude of the decrease in binding parameters was always significantly smaller than the degree of tolerance. This presumably suggests the existence of additional mechanisms underlying tolerance phenomena. In this regard, one possibility could be that, as with chronic nicotine [27], exposure to THC might induce brain cytochrome P450 resulting in greater P450-mediated metabolic inactivation of THC. This possibility has not been elucidated although in its support it can be argued that: (i) Deutsch *et al.* [28] have recently demonstrated that a non-psychoactive cannabinoid, cannabidiol, potentiates the inductive effect of phenobarbital on the expression of the cytochrome P450 gene; and (ii) we have recently found that the presence of THC in the blood of rats chronically treated with this cannabinoid is significantly lower than in acutely treated animals [25], presumably suggesting an enhanced THC metabolism.

In summary, our results indicate that the metabolism of THC to inactive compounds might play a protective role in females, counteracting the effects of this cannabinoid on tuberoinfundibular dopaminergic activity because pharmacological inhibition of cytochrome P450 elicited a significant stimulation of these neurons by THC.

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