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EFFECT OF CANNABIDIOL ON CYTOCHROME P-450 AND HEXOBARBITAL SLEEP TIME

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Abstract—The influences of acute and subacute cannabidiol (CBD) treatment and of subsequent drug withdrawal were investigated on hexobarbital-induced sleep time, on hepatic cytochrome P-450 concentration, on the *in vitro* formation of carbon monoxide (CO) associated with CBD metabolism, and on the kinetics of aminopyrine *N*-demethylase metabolism. In acutely treated mice, CBD prolonged sleep time, decreased cytochrome P-450 concentration, decreased the endogenous formation of CO, and increased an apparent K_m for aminopyrine *N*-demethylase activity. In subacutely treated animals, tolerance developed to the effect on sleep time but not to that on cytochrome P-450 concentration nor on the endogenous formation of CO *in vitro* nor on the K_m for the *N*-demethylase activity. Upon withdrawal from subacute treatment, tolerance to the sleep-time effect was still evident on day 14, but, by day 28, the sensitivity to CBD had returned to normal. In contrast, the cytochrome P-450 concentration returned to normal on day 14 of withdrawal, as did the K_m for the *N*-demethylase activity and the ability of CBD to induce CO synthesis *in vitro*. The comparative results lead us to conclude that the CBD effect on sleep time does not correlate with either the total amount of cytochrome P-450 or with the CBD depressant effect on the cytochrome.

Although cannabidiol (CBD) has long been recognized for its ability to prolong barbiturate sleep time [1], it is only recently that this effect has been ascribed to a blockade of the hepatic microsomal drug-metabolizing enzymes [2–5]. The mechanism of the blockade appears to involve the action of a metabolite, rather than of CBD itself [6, 7], and a decrease in the amount of hepatic microsomal cytochrome P-450. The latter effect could conceivably account for the reported decrease in rate of barbiturate metabolism caused by CBD [8]. The experiments detailed in the present paper are an extension of our previous studies and were designed to identify the mechanism by which CBD inhibits hepatic microsomal drug metabolism. An elucidation of this mechanism has toxicological significance both with respect to the illicit use of marihuana and to the potential clinical uses of the cannabinoids.

MATERIALS AND METHODS

CBD administration and hexobarbital sleep-time determination. Male, CF-1 mice (Charles River), weighing 20–30 g, were used in all the experiments. As described previously [9], CBD was prepared by adding Tween 80 to an ethanol solution of the cannabinoid, followed by thorough mixing and evaporation of the alcohol in a stream of N_2. The CBD–Tween 80 residue was dispersed by ultrasound (Branson Sonifier S-75) in an isotonic saline solution; the final drug mixture contained about 3%, by volume, Tween 80. The CBD suspension was administered intraperitoneally (0.1 ml/20 g body wt) in an anticonvulsant dose (120 mg/kg) either acutely or subacutely (once daily for 5 consecutive days). Hexobarbital was prepared for intraperitoneal injection in normal saline. Sleep time was measured from the loss to the recovery of the righting reflex, following a 100 mg/kg dose of the barbiturate. All sleep times were measured 2 hr after CBD treatment [8], both in the acute and subacute treatment groups. Sleep time was again similarly determined in subacutely treated animals, which were retested for the recovery of their sensitivity to a single dose of CBD on various days following their withdrawal. In each test, sleep times were consistently measured about noon to minimize the problem of diurnal variation.

Preparation of microsomes. Animals were killed by cervical dislocation 4 hr after administration of vehicle or 120 mg/kg CBD, and hepatic microsomes were prepared by a modification of the procedure of Franklin and Estabrook [10]. Livers were homogenized in 1.15% KCl solution (1:4, w/v) in a glass homogenizer with a motor-driven Teflon pestle. The homogenate was centrifuged at 20,000 *g* for 20 min in a Beckman L-2 centrifuge; the resulting supernatant fraction was removed and centrifuged at 100,000 *g* for 30 min. The washed pellet was suspended in 1 ml of a buffer solution containing 50 mM Tris and 0.25 sucrose, pH 7.4.

Cytochrome P-450 *and aminopyrine* N-*demethylase assays.* Cytochrome P-450 concentrations were determined by a modified method of Omura and Sato [11] with an Aminco DW-2 spectrophotometer and the use of an extinction coefficient of 91 mM^{-1} cm^{-1}. By the same procedure, cytochrome P-450 was measured in a microsomal system following the *in vitro* metabolism of CBD. In these experiments,

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microsomes were added to a 50 mM Tris–buffer solution containing 70 μM CBD, 10 mM $MgCl_2$ and 1.15% KCl, pH 7.4. The microsomal preparation was divided between two cuvettes and, after spectrophotometric balancing, NADPH (Sigma Chemical Co., St. Louis, MO) was added (final concentration: 370 μM) to each cuvette; the reaction was incubated at 25° for 10 min. Dithionite was then added to the sample cuvette, and the cytochrome P-450 was measured before and after exogenous CO was bubbled into the cuvette. The *in vitro* metabolism of CBD by a microsomal preparation results in the formation of CO [8]; it is the presence of this endogenously formed CO in the incubation medium that produces an absorbance peak at 450 nm before the usual addition of exogenous CO to the sample cuvette. Although the quantity of CO produced endogenously was not determined directly, the amount of P-450 which was measured prior to the addition of exogenous CO is a reflection of the amount of CO generated during the metabolism of CBD.

Aminopyrine *N*-demethylase activity of the 20,000 *g* supernatant fraction was measured by the method of Poland and Nebert [12] with the use of [^{14}C]aminopyrine (New England Nuclear Corp., Boston, MA); activity was calculated from the rate of formation of [^{14}C]formaldehyde in reactions containing about 3 mg/ml protein and incubated at 37° for 30 min. *N*-Demethylase activity, as well as cytochrome P-450 content, was expressed relative to the protein content, which was determined by the method of Lowry *et al.* [13].

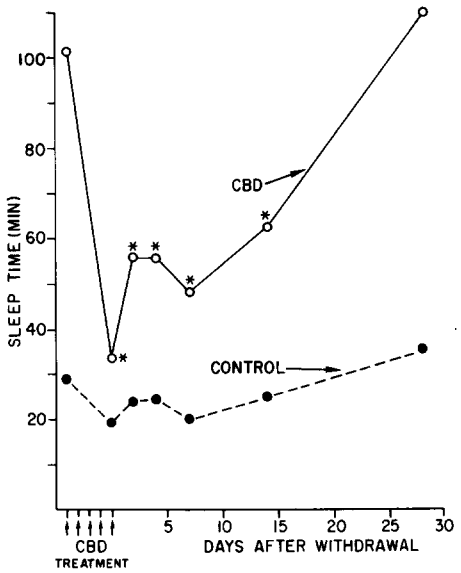

Fig. 1. Time course of the effect of CBD on hexobarbital-induced sleep time. Sleep time was measured after the i.p. administration of 100 mg/kg hexobarbital, 2 hr after the i.p. administration of 120 mg/kg CBD. Each value is the mean of groups of ten to twenty animals. All CBD-treated values are significantly different ($P < 0.05$) from their corresponding controls as determined by Student's *t*-test. An analysis of variance of the CBD-treated data and a Student–Newman–Keuls evaluation of the means for unequal sample sizes [14] indicate that the starred values are significantly different from the treated values on days 1 and 28 ($P < 0.05$), and that day 5 of CBD treatment is significantly different from the other starred values ($P < 0.05$).

RESULTS

The results shown in Fig. 1 illustrate the effect of CBD treatment on hexobarbital sleep time. On day 1 of CBD treatment, sleep time was prolonged approximately four times that of the control. After 5 days of treatment, tolerance had obviously developed, because the sleep time tended to return to the control value. Individual groups of the tolerant animals were subsequently tested for a return of their initial sensitivity to the CBD effect by administering a challenge dose of CBD on various days following withdrawal from the 5-day treatment. As shown in Fig. 1, on days 2, 4, 7 and 14 after withdrawal, the animals were still partially tolerant to CBD; sleep time was only about twice that of control. A disappearance of tolerance was found only on withdrawal day 28, when the CBD sensitivity returned to normal, as evidenced by a restoration of the initial 4-fold increase in sleep time.

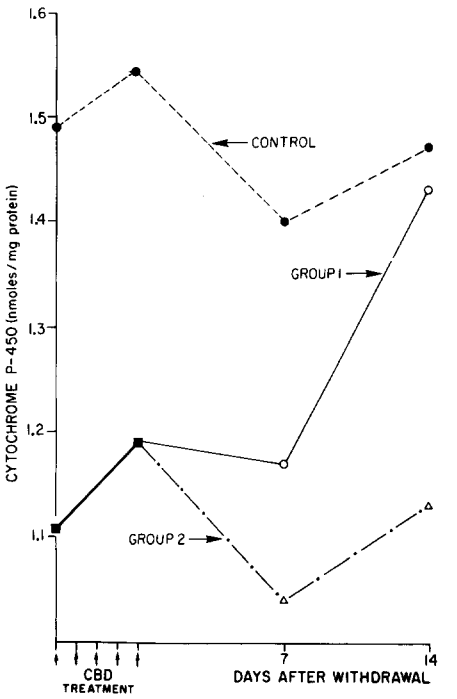

Fig. 2. Time course of the effect of CBD on cytochrome P-450 concentration. Cytochrome P-450 concentrations were measured in microsomal fractions prepared from four to six animals 4 hr after acute or subacute treatment; the subacutely treated animals were divided into two groups: Group 1, the animals were allowed to recover without any further exposure to CBD; Group 2, the animals were rechallenged with CBD on withdrawal days 7 and 14. A two-level nested analysis of variance for unequal sample sizes [14] and a many-one rank statistic of Steel [15] were applied on the control and Group 1 samples; except for day 14, all other Group 1 samples were significantly different ($P < 0.05$) from the control population. The mean P-450 concentrations of Group 1 and 2 at days 7 and 14 were compared by Student's *t*-test; only the day 14 values were found to be significantly different ($P < 0.001$).

Associated with the acute effect of CBD on sleep time was a marked decrease in the hepatic microsomal cytochrome P-450 concentration (Fig. 2). After subacute CBD treatment, however, the cytochrome P-450 concentration remained depressed; that is, unlike the effect on sleep time, there was no development of tolerance to the acute effect on the cytochrome concentration. At the end of the subacute treatment period, animals were divided (as shown in Fig. 2) into two groups: Group 1 represents animals allowed to recover without any additional exposure to CBD, whereas Group 2 animals were rechallenged with CBD on withdrawal days 7 or 14 to determine the cannabinoid sensitivity of the existing cytochrome P-450. In Group 1, 7 days after withdrawal, the P-450 remained at the previously depressed concentration, which then returned to a normal value by day 14 of recovery. In Group 2, the P-450 concentration after re-exposure to CBD on withdrawal day 7 was not significantly different from the untreated value in Group 1. At 14 days, however, the Group 2 values after CBD treatment were significantly depressed compared with the corresponding values in Group 1, which were approximately equal to the controls. The CBD-induced depression in Group 2, at 7 and 14 days, was quantitatively

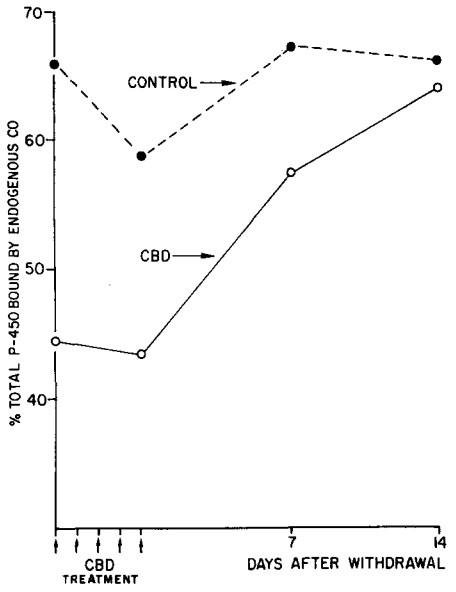

Fig. 3. Time course of the effect of CBD on the endogenous formation of CO. Microsomal suspensions were incubated with CBD and NADPH as described in Materials and Methods. Following the addition of dithionite to the sample cuvette, a measurement of P-450 could be made because of the presence of CO synthesized endogenously. The sample cuvette was then gassed with exogenous CO and the total P-450 determined (data in Fig. 2). Each value is the mean of four to six separate preparations. The statistical analyses applied were identical to those described in the legend of Fig. 2. Except for microsomal preparations from the day 14 group of CBD-treated mice, CO formation was significantly less ($P < 0.05$) in fractions prepared from CBD-treated animals than in fractions from the control population. Ordinate: the amount of P-450 measured before the addition of exogenous CO as a percentage of the total P-450 in the preparation.

indistinguishable from that observed on either day 1 or 5 of the original treatment period.

The data in Fig. 3 illustrate the influence of CBD treatment and subsequent withdrawal time on the endogenous formation of CO associated with the *in vitro* metabolism of CBD by a microsomal preparation. CBD treatment of the animals prior to the removal of their livers resulted in an initial decrease in the amount of CO formed during the *in vitro* metabolism. This effect appeared unchanged by the 5-day CBD treatment and appeared to recover and return toward the control value by day 7 and day 14 after withdrawal.

The data shown in Fig. 4 illustrate the effects of CBD treatment and subsequent withdrawal on the apparent K_m and V_{max} for the metabolism of aminopyrine. Groups of six mice were treated acutely or subacutely with vehicle or CBD, and crude microsomal suspensions were prepared at various times after the last injection. The six suspensions from each group were combined, and aminopyrine *N*-demethylase activity was measured in aliquots from the pooled supernatant fractions at 4 and 24 hr after acute treatment, and at 4, 24, 48 hr and 14 days after subacute treatment. The apparent kinetic constants were determined from the slopes and intercepts of lines fitted to the data points by linear regression analysis. As demonstrated in Fig. 4, this analysis revealed that, in control preparations, aminopyrine *N*-demethylase activity can be described by two distinct functions, as has been reported previously [12, 16]. The mean (and 95% confidence limits) of the apparent K_m and V_{max} values for the controls of the six time periods investigated were: Function 1, 3.5 (1.9 to 5.1) mM and 1.6 (1.1 to 2.0) nmoles mg protein^{-1} min^{-1}; Function 2, 0.1 (0.06 to 0.2) mM and 0.8 (0.5 to 1.0) nmoles mg protein^{-1} min^{-1}.

The results of the experiment in which aminopyrine metabolism was measured 4 hr after acute CBD administration are presented in Fig. 4A. Both Functions 1 and 2 were affected; the demethylase activity associated with Function 2 could not be detected because it had either disappeared or was depressed below the limits of sensitivity of the assay. A similar effect of CBD was also found in the 4-hr subacute experiment (not shown). The apparent V_{max} of Function 1 in the CBD-treated plot in Fig. 4A was depressed to approximately 60 per cent of the corresponding control value, but the apparent K_m values were similar: 3.8 and 3.3 mM respectively. Figure 4B represents the results of the kinetic analysis 24 hr after acute CBD treatment. In this instance, the demethylase activity in the microsomes taken from the drug-treated animals can be described by two functions. The apparent kinetic constants associated with Function 1 are essentially indistinguishable from the control; Function 2 now has an apparent K_m value which is about ten times that of the control, while the V_{max} value remains unchanged. A similar effect was noted in the 24-hr preparations from subacutely treated animals (not shown). The kinetic analyses, after a 48-hr withdrawal from the subacute treatment shown in Fig. 4C, provide similar results, which suggest that, during the 5-day treatment, tolerance to the change in aminopyrine metabolism does not develop as it does to the sleep-time effect.

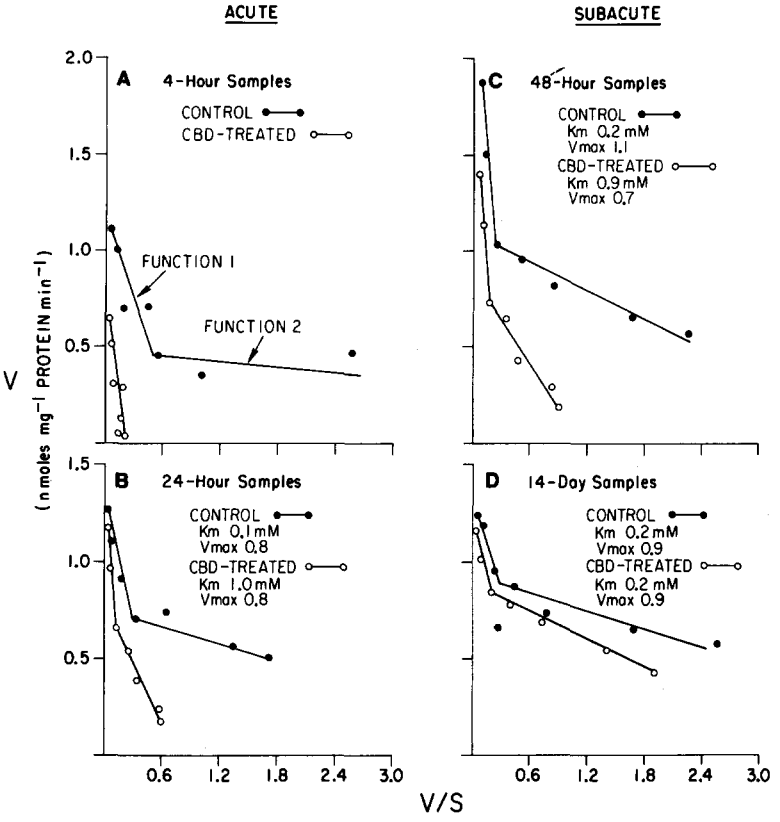

Fig. 4. Effect of CBD on the *in vitro* metabolism of aminopyrine. Metabolism of aminopyrine was measured on pooled 20,000 *g* supernatant fractions from six animals at various times after i.p. administration of 120 mg/kg CBD. Each point represents the average of duplicate determinations; the apparent K_m and V_{max} data presented in the figure are for Function 2. Apparent K_m and V_{max} values were calculated from lines fitted to the data points by linear regression analyses. Function 1: demethylase activity measured at 3.5 to 20.0 mM aminopyrine. Function 2: demethylase activity measured at 0.2 to 2.5 mM aminopyrine concentrations.

These analyses were repeated 14 days after withdrawal from the subacute treatment (Fig. 4D), and at this time the apparent K_m had returned to its control value.

DISCUSSION

The CBD-caused prolongation of barbiturate sleep time is thought to result from a CBD blockade of drug metabolism [2–5]. Conversely, the tolerance that develops to the sleep-time effect probably results from a reversal of the inhibitory action of CBD on the hepatic drug-metabolizing enzymes [8]. Neither the mechanism of such inhibition nor that of the subsequent tolerance is known; however, what *is* known about the reaction between CBD and the drug-metabolizing enzymes is that the inhibition appears to be caused by a metabolite rather than by CBD itself [6] and that, acutely, the inhibition is associated with a decrease in the total cytochrome P-450 content of the enzymes [3]. In a previous report, we postulated that the CBD-induced prolongation of sleep time may be due, in fact, to a depressant effect on the amount of cytochrome P-450 [8]. This postulate, however, is not supported by the data of the present study, because the CBD effect on sleep time does not correlate with a corresponding effect on cytochrome P-450 concentration. As shown in Figs. 1 and 2, the acute effects do correlate, but, in tolerant animals and in the withdrawal or recovery period, the two effects were clearly dissociated; indeed, after 5 days of treatment, the animals were refractory to the effect of CBD on sleep time, yet the cytochrome P-450 concentration was essentially the same on day 1 and day 5 of treatment. Again, dissociation was seen in the withdrawal period. The cytochrome recovered, both in total amount and in sensitivity to the depressant effect of CBD, after 14 days withdrawal, but the sleep-time effect did not return to normal until some time between day 14 and day 28 of recovery (Fig. 1). These data clearly indicate that the sleep-time effect does not correlate with either the total amount of cytochrome or the CBD depressant effect on the cytochrome. Nevertheless, the changes in the total amount of cytochrome P-450 did correlate with the amount of endogenously formed CO. The origin of this endogenous CO is not known, but its formation requires the oxidative metabolism of CBD [8]. Whether the decreased formation of CO is related

to a decreased rate in CBD metabolism due to a lower cytochrome P-450 level or to a CBD-induced reduction of an unknown substrate from which the CO is derived is not known.

The aminopyrine experiments were undertaken to determine whether tolerance to the inhibition of hexobarbital metabolism by CBD also develops to other Type I drugs [17]. The results of these experiments reveal that, 4 hr after CBD administration, the activity in one of the two microsomal components involved in the metabolism of aminopyrine (Function 2, Fig. 4) was depressed beyond the point where it could be measured by the radioisotopic assay procedure. The extent of the depression at 4 hr was similar in microsomal preparations derived from both acutely treated and subacutely treated (tolerant) mice. Consequently, the effect of subacute CBD treatment on aminopyrine metabolism *in vitro* indicates that the tolerance to CBD's inhibition of hexobarbital metabolism does not involve a simple recovery of hepatic enzyme activity to its normal level. Although the activity associated with Function 2 was depressed beyond the detection limits of the assay at 4 hr after the injection of CBD, activity did return by 24 hr; there was, however, a qualitative change in the enzyme, as evidenced by a marked increase in the apparent K_m associated with this function. In subacutely treated animals, the time course of the changes in the enzymatic activity of Function 2—initial depression at 4 hr, recovery with an altered apparent K_m value by 24 hr, and eventual return to normal after 2 weeks—corresponded to the time course of the depression and recovery of the cytochrome P-450 concentrations (Fig. 2). These findings are consistent with previous observations that other drugs can decrease the total P-450 concentration [18–20], which decrease, in itself, may affect the rate of drug metabolism; however, the existence of a direct connection between the observed changes in the apparent K_m of Function 2 and the decreased levels of cytochrome P-450 remains to be determined.

Our inability to detect, in tolerant animals, any change in the metabolism of aminopyrine that correlated in any obvious way to the change in the metabolism of hexobarbital indicates that further experimentation will be required before the mechanism accounting for tolerance can be understood. The data presented in the present report provide a basis for these future investigations.

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