Drug and Alcohol Dependence 112 (2010) 126–133

Contents lists available at ScienceDirect

Drug and Alcohol Dependence

journal homepage: www.elsevier.com/locate/drugalcdep

Pharmacological evaluation of the natural constituent of *Cannabis sativa*, cannabichromene and its modulation by Δ^9-tetrahydrocannabinol☆

Gerald T. DeLong[a,1], Carl E. Wolf[b], Alphonse Poklis[b], Aron H. Lichtman[a,*]

[a] Department of Pharmacology and Toxicology, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA 23298, USA
[b] Department of Pathology, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA 23298, USA

ARTICLE INFO

Article history:
Received 12 February 2010
Received in revised form 25 May 2010
Accepted 31 May 2010

Keywords:
Cannabis sativa
Marijuana
Phytocannabinoid
Cannabichromene
Delta-9-tetrahydrocannabinol (THC)
CB_1 cannabinoid receptor
CB_2 cannabinoid receptor
Anti-inflammatory

ABSTRACT

In contrast to the numerous reports on the pharmacological effects of Δ^9-tetrahydrocannabinol (THC), the pharmacological activity of another substituent of *Cannabis sativa*, cannabichromene (CBC) remains comparatively unknown. In the present study, we investigated whether CBC elicits cannabinoid activity in the tetrad assay, which consists of the following four endpoints: hypomotility, antinociception, catalepsy, and hypothermia. Because cannabinoids are well documented to possess anti-inflammatory properties, we examined CBC, THC, and combination of both phytocannabinoids in the lipopolysaccharide (LPS) paw edema assay. CBC elicited activity in the tetrad that was not blocked by the CB_1 receptor antagonist, rimonabant. Moreover, a behaviorally inactive dose of THC augmented the effects of CBC in the tetrad that was associated with an increase in THC brain concentrations. Both CBC and THC elicited dose-dependent anti-inflammatory effects in the LPS-induced paw edema model. The CB_2 receptor, SR144528 blocked the anti-edematous actions of THC, but not those produced by CBC. Isobolographic analysis revealed that the anti-edematous effects of these cannabinoids in combination were additive. Although CBC produced pharmacological effects, unlike THC, its underlying mechanism of action did not involve CB_1 or CB_2 receptors. In addition, there was evidence of a possible pharmacokinetic component in which CBC dose-dependently increased THC brain levels following an i.v. injection of 0.3 mg/kg THC. In conclusion, CBC produced a subset of behavioral activity in the tetrad assay and reduced LPS-induced paw edema through a noncannabinoid receptor mechanism of action. These effects were augmented when CBC and THC were co-administered.

1. Introduction

While Δ^9-tetrahydrocannabinol (THC) is recognized as the primary psychoactive constituent in *Cannabis sativa* (Gaoni and Mechoulam, 1964), the issue of whether other constituents in this plant contribute to its pharmacological effects, have pharmacological effects of their own, or modulate the effects of THC remains of interest from a drug abuse perspective as well as for the development of cannabinoid-based medications. One particular class of constituents in marijuana, the phytocannabinoids (of which THC is the prototypical type), has received considerable attention for their contribution to the pharmacological effects of marijuana. Of the approximately 70 identified phytocannabinoids in marijuana, many are present in trace amounts, and some of these compounds can be quantitatively abundant in the plant (McPartland and Russo, 2001; ElSohly and Slade, 2005). For example, the respective percentages of THC and cannabidiol (CBD), a non-psychoactive phytocannabinoid, from confiscated cannabis preparations are 8.8% and 0.4% (Mehmedic et al., 2010). CBD is documented to possess anxiolytic-like, antinociceptive, anti-psychotic-like, anti-inflammatory, and anti-oxidant effects (McPartland and Russo, 2001; Mechoulam et al., 2007).

The pharmacological actions of another phytocannabinoid, cannabichromene (CBC) (Mechoulam et al., 1968), present at relatively high concentrations in certain strains of marijuana remain to be extensively investigated. Turner et al. (1980) and Holley et al. (1975) noted that "drug type" marijuana primarily contained higher concentrations of CBC than CBD, while "fiber type" marijuana contained higher concentrations of CBD than CBC. This classification of "drug type" or "fiber type" marijuana is related to some strains of marijuana that have more potent psychotropic effects compared to other strains that cultivated as hemp for their fiber. While CBC is the second most abundant cannabinoid in some

☆ A supplementary data figure is available with the online version of this article at doi:10.1016/j.drugalcdep.2010.05.019. Please see Appendix A.

* Corresponding author at: Virginia Commonwealth University, Department of Pharmacology & Toxicology, 410 N. 12th Street, Smith Building P.O. Box 980613, Richmond, VA 23298-0613, USA. Tel.: +1 804 828 8480; fax: +1 804 828 2117.
E-mail addresses: alichtma@vcu.edu, alichtma@vcu.edu (A.H. Lichtman).
[1] Now at Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA.

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doi:10.1016/j.drugalcdep.2010.05.019

strains of marijuana growing in the United States (Brown and Harvey, 1990), it represents 0.3% of the constituents from confiscated cannabis preparations (Mehmedic et al., 2010).

Despite the presence of CBC in certain marijuana strains, relatively few studies have investigated the pharmacological effects of this compound. CBC was shown to have analgesic properties and potentiated the analgesic effects of THC in the mouse tail-flick assay (Davis and Hatoum, 1983). It also was found to prolong hexabarbital-induced hypnosis (Hatoum et al., 1981). In addition, it potentiated bradycardia caused by THC, though it did not induce bradycardia on its own (O'Neil et al., 1979). CBC was reported to induce sedation and ataxia in canines (Gaoni and Mechoulam, 1966); however, subsequent studies failed to replicate these effects (Gaoni and Mechoulam, 1971). Of relevance to the present study, CBC and its analogs have been reported to have anti-inflammatory properties *in vivo* and *in vitro* (Turner and ElSohly, 1981; Wirth et al., 1980a,b).

THC, other naturally occurring psychoactive cannabinoids, and synthetic cannabinoids produce their pharmacological effects predominantly through CB_1 and CB_2 receptors, with the former receptor largely responsible for behavioral and metabolic actions of these drugs, and latter receptor generally associated with the modulation of immune responses. This class of drugs produces a myriad of effects, including locomotor suppression, catalepsy, antinociception, and hypothermia, which collectively are known as the tetrad assay and is used to screen cannabinoid activity (Fride, 2006; Martin et al., 1991). The pharmacological effects of cannabinoids in each of these four in vivo tests yield a high positive correlation with their affinity for the CB_1 receptor (Compton et al., 1993). Importantly, CB_1 receptor binding affinity also positively correlates with drug discrimination studies in rats and psychoactivity in humans. Accordingly, the tetrad assay has had great utility because of its high predictive value in identifying compounds that possess marijuana-like effects. However, there are currently no published reports that evaluated CBC in the tetrad.

Phytocannabinoids, such as THC, have been effective anti-inflammatory agents in a variety of inflammatory models and there is substantial *in vivo* and *in vitro* evidence that both cannabinoid receptors (CB_1 and CB_2) are involved in anti-inflammatory processes (Zurier, 2003). Although both cannabinoid receptors are found on various populations of immune cells, CB_2 receptors are far more abundant than CB_1 receptors (Croxford and Yamamura, 2005). Many studies have shown that stimulation of CB_2 receptors inhibits pro-inflammatory cytokine release triggered by inflammatory agents, such as lipopolysaccharide (LPS), a constituent of the outer membrane of the cell wall of most gram negative bacteria (Puffenbarger et al., 2000). Intraplantar administration of LPS elicits paw edema that has been utilized to investigate the anti-inflammatory effects of various classes of anti-inflammatory compounds including steroidal and non-steroidal anti-inflammatory drugs (Kanaan et al., 1997), as well as cannabinoid receptor agonists (Naidu et al., 2010). However, there are presently no published reports on the possible anti-inflammatory effects of THC or CBC in this LPS-induced inflammatory paw model.

There were five objectives of the present study. The first goal was to determine whether CBC produces cannabinoid activity in the tetrad (locomotor suppression, catalepsy, antinociception, and hypothermia). Second, we evaluated whether this phytocannabinoid would elicit anti-edematous effects in the LPS-induced paw edema model. The third aim was to determine whether CBC produces its pharmacological effects in the aforementioned assays through CB_1 or CB_2 receptor mechanisms of action, using the respective receptor antagonists, rimonabant and SR144528. The fourth goal was to investigate the ability of CBC to modulate the pharmacological effects of THC in the tetrad and the LPS-induced paw edema model. The final goal was to examine whether co-administration of THC and CBC alters blood and brain levels of each phytocannabinoid.

2. Materials and methods

2.1. Animals

Studies utilized male ICR mice (Harlan Laboratories, Dublin, VA) weighing 20–30 g that were housed six to a cage in the animal care facility maintained at 22 ± 2 °C on a 12 h light/dark cycle. Food and water were available *ad libitum* and mice were given 24 h to acclimate to the test environment (22 ± 2 °C) before analysis; animals were housed in the test environment until the termination of experimental procedures for LPS-induced inflammation studies. All animal studies were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University in accordance with the *National Institute of Health Guide for the Care and Use of Laboratory Animals*.

2.2. Drugs

CBC was synthesized by Organix Inc. (Woburn, MA), and THC, rimonabant, and SR144528 were supplied by the National Institute on Drug Abuse (Bethesda, MD). All compounds were dissolved in a vehicle consisting of absolute ethanol, alkamuls-620 (Rhone-Poulenc, Princeton, NJ), and saline in a ratio of 1:1:18. For tetrad experiments, all injections were administered intravenously in a volume of 0.1 ml per 10 g of body weight. For LPS-induced inflammation studies, drugs were administered intraperitoneally in a volume of 0.1 ml per 10 g of body weight. LPS from *Escherichia coli* 026:B6 (Sigma–Aldrich, St. Louis) was suspended in 0.9% saline for paw administration.

2.3. Tetrad procedure

Pretreatment baseline tail-flick response to radiant heat (D'Amour and Smith, 1941) and rectal temperature were recorded. The intensity of the radiant heat stimulus was held constant to yield control latencies of 2–4 s with an automated cut-off of 10 s to prevent tissue damage to the tail. Rectal temperatures were taken with a digital thermometer (Fisher Scientific, Pittsburgh, PA) at a depth of 2 cm and recorded to the nearest 0.1 °C. Locomotor suppression, antinociception, hypothermia, and catalepsy were evaluated (in that respective order) after the mice received drug treatment. Five-min post-drug administration, mice were placed in individual activity chambers (plastic mouse cages in a dark cabinet) surrounded by photocell beams (Digiscan Animal Activity Monitor; Omnitech Electronics, Inc., Columbus, OH). Locomotor suppression was measured for 10 min and the data were expressed as the total number of photocell beam breaks. Tail-flick latency was measured at 20 min after drug administration. Antinociception was calculated as a percent of maximum possible effect (%MPE) by the following formula: $\%MPE = [(\text{test latency} - \text{pretreatment latency})/(10 - \text{pretreatment latency})] \times 100\}$. At 30 min, rectal temperature was measured and data were expressed as the difference between pretreatment rectal temperature and post-injection rectal temperature. At 40 min, mice were evaluated for catalepsy in a 5 min ring immobility test (Gill et al., 1970; Pertwee, 1972) in which each subject was placed on a metal ring (5.5 cm diameter) that was suspended 16 cm from the benchtop with a black background board attached (for contrast with the albino mice). The total duration of time spent motionless (no voluntary movement) was timed using a stopwatch.

2.4. Extraction procedure, GC/MS analysis

Mice were decapitated at two different time points (discussed in Section 3) and the blood was collected in heparinized tubes (Fisher Scientific, Pennsylvania). Whole brains were harvested and stored on ice until homogenized. Brain tissue was homogenized less than 2 h following the harvest in a 2:1 ratio (vol/wt) of saline to brain tissue. Aliquots (0.5 ml) were prepared from heparinized mouse whole blood or homogenized brain tissue. Calibrators were prepared by adding drug standards to naïve mouse blood and brain tissue. Fifty microliters of internal standard (THC-d_3; Cerilliant, Round Rock, TX) was added to all calibrators and samples (minus blank samples). All tubes were equilibrated overnight at 5 °C.

"Ice cold" acetonitrile (2 ml) was added dropwise while vortexing and the mixture was centrifuged at 3000 rpm for 10 min. The mixture was stored overnight at −30 °C to assist in further separation of the layers. The top layer (acetonitrile layer) was isolated and 2 ml of 0.2N NaOH was added while vortexing the tubes. Four milliliters of organic solvent (9:1, hexane:ethyl acetate) was added and the tubes were rotated at 30 rpm for 30 min. The tubes were then centrifuged at 3000 rpm for 10 min. The organic layer was isolated and evaporated to dryness in a Savant AES1000. Fifty microliters of derivatizing agent (RC3; Regis Technologies, Inc., Morton Grove, IL) was added to each tube and samples were heated at 70 °C for 20 min. Samples were then transferred to microvials for GC/MS analysis.

Microvials were loaded on a Shimadzu QP2010 GC/MS utilizing a splitless injection port and DB-5 column (30 m × 0.25 mm I.D. × 25 μm film thickness). The GC/MS was operated under the following temperatures: initial, 190 °C; ramp, 30 °C/min to 330 °C then hold 1 min; injection port, 250 °C; transfer line, 280 °C; and ion source, 260 °C. Ions monitored in SIM mode: 315, 343, 386 *m/z* for THC; 303, 246, 371 *m/z*