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Effect of (-)- Δ^9 -tetrahydrocannabinoid on the hepatic redox state of mice

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Abstract

(-)- Δ^9 -Tetrahydrocannabinol (Δ^9 -THC), a psychoactive component of marijuana, has been reported to induce oxidative damage *in vivo* and *in vitro*. In this study, we administered Δ^9 -THC to healthy C57BL/6J mice aged 15 weeks in order to determine its effect on hepatic redox state. Mice were divided into 3 groups: Δ^9 -THC (N = 10), treated with 10 mg/kg body weight Δ^9 -THC daily; VCtrl (N = 10), treated with vehicle [1:1:18, cremophor EL[®] (polyoxy 35 castor oil)/ethanol/saline]; Ctrl (N = 10), treated with saline. Animals were injected *ip* twice a day with 5 mg/kg body weight for 10 days. Lipid peroxidation, protein carbonylation and DNA oxidation were used as biomarkers of oxidative stress. The endogenous antioxidant defenses analyzed were glutathione (GSH) levels as well as enzyme activities of superoxide dismutase, catalase, glutathione S-transferase, glutathione reductase, and glutathione peroxidase (GPx) in liver homogenates. The levels of mRNA of the cannabinoid receptors CB1 and CB2 were also monitored. Treatment with Δ^9 -THC did not produce significant changes in oxidative stress markers or in mRNA levels of CB1 and CB2 receptors in the liver of mice, but attenuated the increase in the selenium-dependent GPx activity (Δ^9 -THC: 8%; VCtrl: 23% increase) and the GSH/oxidized GSH ratio (Δ^9 -THC: 61%; VCtrl: 96% increase), caused by treatment with the vehicle. Δ^9 -THC administration did not show any harmful effects on lipid peroxidation, protein carboxylation or DNA oxidation in the healthy liver of mice but attenuated unexpected effects produced by the vehicle containing ethanol/cremophor EL[®].

Key words: (-)- Δ^9 -Tetrahydrocannabinol; Oxidative stress; Liver; Cannabinoid receptors; Cremophor EL[®]

Introduction

The plant *Cannabis sativa*, also known as marijuana, is the most widely used illicit drug. Its use is common for medicinal purposes and in fact, cannabinoids, the active compounds of the plant, have been shown to possess anti-inflammatory, analgesic and antiemetic properties, among others (for a review, see Ref. 1).

In the early 1960's, the plant's most psychoactive cannabinoid was isolated and identified as (-)- Δ^9 -tetrahydrocannabinol (Δ^9 -THC), a highly lipophilic substance (2). Δ^9 -THC is now available as a prescription drug in some countries under the trade names Marinol[®] (synthetic Δ^9 -THC) and Sativex[®] (Δ^9 -THC and cannabidiol extracted from the plant). Once administered, Δ^9 -THC is rapidly absorbed by tissues and metabolized, mainly in the liver, to 11-hydroxy-tetrahydrocannabinol (3).

It is known that Δ^9 -THC non-selectively activates the cannabinoid receptors CB1 and CB2 (4). In healthy livers, these receptors are faintly or not expressed, respectively,

but are substantially up-regulated in experimental liver injury and in liver cirrhosis of various origins (5-7). The activation of CB1 and CB2 receptors has been described to induce hepatic profibrogenic (8) and antifibrogenic effects (9), respectively, in the liver, but no data are available regarding the effects of Δ^9 -THC administration on either healthy or fibrotic liver.

It has been reported that Δ^9 -THC can induce oxidative damage *in vitro* (10), and more recently *in vivo* (11), and it is known that, as a result of damaged cellular structures and oxidized macromolecules (DNA, lipids and enzymatic proteins) induced by increased oxidative stress, parenchymal liver necrosis eventually occurs (12). Therefore, in the present investigation, we studied the effect of Δ^9 -THC administration on the hepatic redox state of healthy C57BL/6J mice by determining the amount of oxidative damage produced in lipids, proteins and DNA, and the activity levels of enzymes involved in endogenous antioxidant

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defense. The levels of mRNA of each cannabinoid receptor were also monitored.

Material and Methods

Reagents

Δ^9 -THC (supplied in absolute ethanol) was purchased from LipomedAG (Switzerland). 2,4-Dinitrophenylhydrazine (DNPH) and thiobarbituric acid were purchased from Merck (Germany). Cremophor EL[®], 1,2-chloro-2-dinitrobenzene, 2-vinylpyridine, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), 8-hydroxy-2'-deoxyguanosine (8-OHdG), cumene hydroperoxide, glutathione reductase, nicotinamide adenine dinucleotide phosphate (NADPH), malonaldehyde, nitroterrazolium blue chloride, nuclease P1, oxidized glutathione (GSSG), reduced glutathione (GSH), sodium pentobarbital, xanthine, and xanthine oxidase were all from Sigma (USA); alkaline phosphatase and guanidine hydrochloride were purchased from Applichem (Germany).

Animals and treatment

C57BL/6J mice, 15 weeks of age and weighing 22-29 g, were obtained from Charles River Laboratories (Spain). The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the USA National Institutes of Health (NIH Publication #85-23, revised in 1996) and the experiments were performed according to the Portuguese law on animal welfare. Animals were kept under controlled environmental conditions (22-24°C, 12-h light/dark cycles) and provided with food and water *ad libitum* before and for the duration of the experiment.

Groups of mice (N = 10) were given two daily intraperitoneal injections, at 8 am and 8 pm, of saline (control group, Ctrl), vehicle (VCtrl, 1:1:18, cremophor EL[®]/ethanol/saline solution) or Δ^9 -THC (a total daily dose of 10 mg/kg body weight) for a period of 10 days and were killed on the morning after the last injection.

Sample preparation

Animals were anesthetized with sodium pentobarbital (60 mg/kg body weight) and perfused with ice-cold 0.9% NaCl. The liver was extracted, weighed and homogenized with 2 mL of a homogenization solution (25 mM KH₂PO₄, 30 mM Na₂HPO₄, 0.1% Triton X-100, pH 7.0) per gram of liver. The resulting homogenized liver tissue was stored at -80°C.

Protein determination

The amount of protein in each liver homogenate was determined by the method of Bradford (13) using bovine serum albumin as standard.

Evaluation of oxidative stress

The amount of lipid peroxidation was determined by the thiobarbituric acid reactive substances method (14).

Briefly, each liver homogenate was centrifuged at 16,000 g for 10 min at 4°C and the supernatant solution was mixed with 10% trichloroacetic acid (1:2) and centrifuged at 6000 g for 1 min at 4°C. The resulting supernatant solution was mixed with the same volume of 1% thiobarbituric acid and incubated at 95°C for 40 min. After cooling to room temperature, absorbance was read at 535 nm. The malonaldehyde equivalent nmol/mg protein was calculated using a malonaldehyde standard curve.

To determine the extent of protein carbonylation, the carbonyl content of each liver sample was measured using the supernatant obtained from the liver homogenate mixed with 10% trichloroacetic acid (1:2) (14). After centrifuging at 13,000 g for 2 min at 4°C, 0.5 mL DNPH (10 mM) in 2 M HCl solution was added to the resulting pellet. For the blank, only 0.5 mL 2 M HCl was added to the pellet. Samples were incubated for 1 h at room temperature and vortexed every 10 min, followed by another 15 min on ice after adding 0.5 mL 20% trichloroacetic acid. The pellet was then washed three times with ethanol-ethyl acetate (1:1) by centrifuging at 13,000 g for 2 min at 4°C and dissolved in 0.5 mL 6 M guanidine solution overnight. Insoluble material was removed by centrifuging at 3000 g for 15 min and absorbance was read at 340 nm. The carbonyl content was calculated using the extinction coefficient of DNPH of 22,000 M⁻¹ cm⁻¹ and the results are reported as carbonyl group nmol/mg protein.

DNA damage was measured by determining the amount of 8-OHdG. DNA was extracted using the Animal Tissues Genomic DNA Mini-prep Kit from V-gene[®] (Bioron, Germany), and incubated with 4 U nuclease P1, prepared in 20 mM acetate buffer, pH 5.0, for 30 min at 37°C. This was followed by 1-h incubation at 37°C with 1.3 U alkaline phosphatase prepared in 0.1 M Tris-HCl buffer, pH 7.5. Chloroform was added to the samples and these were vortexed for 10 s and centrifuged at 5000 g for 5 min, and the supernatant was collected for quantification of 8-OHdG (14). The samples were analyzed by high performance liquid chromatography (Gilson 302, Gilson Medical Electronics, France) on a 25 x 3.2 mm ID, Superspher[®] RP18-4 Endcap column (HiCHROM, Germany) and monitored for electrochemical detection (Gilson 141) at 10 V. The solvent was 50 mM KH₂PO₄/K₂HPO₄, 10% (v/v) methanol, pH 5.5, and the column was operated at a flow rate of 0.5 mL/min. 8-OHdG concentration (pmol 8-OHdG/ μ g DNA) was calculated from a calibration curve using commercial 8-OHdG as the standard.

Antioxidant enzyme activities

The content of GSH and GSSG in the liver samples was determined by the DTNB-GSSG reductase recycling assay (14). The formation of 5-thio-2-nitrobenzoic acid was measured at 405 nm for 3 min. To measure GSSG, 2-vinylpyridine was added for deactivation of GSH. The GSH content was calculated by subtracting twice the amount of

GSSG from the amount of total glutathione (GSX) measured. GSH, GSSG and GSX data are reported as nmol/mg protein.

The activity of the glutathione reductase (GR) enzyme, in U/mg (1 U GR defined as the amount of enzyme required to convert 1 nmol NADPH to NADP⁺ per min) was determined by monitoring NADPH oxidation at 340 nm for 5 min (extinction coefficient of NADP⁺: 6.22 mM⁻¹ cm⁻¹) (15).

By monitoring NADPH oxidation at 340 nm for 5 min in the presence of GR, which reduces GSSG using cumene hydroperoxide as substrate, we were able to determine the glutathione peroxidase (GPx) level of activity in the liver samples (14). The selenium-dependent GPx (Se-GPx) was assayed using H₂O₂ as substrate instead of cumene hydroperoxide. Both activities are reported as U/mg protein (1 U GPx and Se-GPx defined as the amount of enzyme required to convert 1 nmol NADPH to NADP⁺ per min).

Glutathione-S-transferase (GST) activity, in U/mg protein (1 U GST defined as the amount of enzyme required to catalyze the formation of 1 μ mol S-2,4-dinitrophenylglutathione per min), were determined by monitoring the formation of GSH-1,2-chloro-2-dinitrobenzene conjugates for 5 min at 340 nm (extinction coefficient of S-2,4-dinitrophenylglutathione: 9.6 mM⁻¹ cm⁻¹) (14).

Catalase activity, in U/mg protein (1 U catalase defined as the amount of enzyme required to hydrolyze 1 μ mol H₂O₂ per min), was determined by monitoring H₂O₂ decomposition at 240 nm for 30 s (14) (extinction coefficient of H₂O₂: 0.00394 \pm 0.0002 mM⁻¹ mm⁻¹).

Superoxide dismutase (SOD) activity was determined by the rate of inhibition of the reduction of nitrotetrazolium blue by superoxide radicals generated by a xanthine-xanthine oxidase system and monitored at 550 nm (14). SOD activity is reported as U/mg protein (1 unit was the amount of SOD inhibiting the reaction rate by 50% under the given assay conditions).

Real-time PCR

The real-time polymerase chain reaction (RT-PCR) was carried out as previously described (16). RNA from liver samples (N = 5, per group) was isolated using the PureZOL RNA isolation reagent (Bio-Rad, USA). Total RNA (1 μ g per sample) was DNase treated and reverse transcribed according to manufacturer instructions (iScriptTM cDNA Synthesis Kit, Bio-Rad). For RT-PCR, 50 μ L amplification mixture (iQTM SYBR[®] Green Supermix, Bio-Rad) was used containing 20 ng reverse transcribed RNA and specific primers (250 nM

(Sigma) for *cnr1* (sense: 5'-GGGCAAATTCCTTGTAGC A-3'; anti-sense: 5'-GGCTAACGTGACTGAGAAA-3'), *cnr2* (sense: 5'-CATCAGGATTTTGAACGACGA-3'; anti-sense: 5'-CTGTGCATGCCTGCTCTTAG-3') and β -actin (sense: 5'-CTAAGGCCAACCGTGAAAAG-3'; anti-sense: 5'-AC-CAGAGGCATACAGGGACA-3'). Reactions were run in duplicate (20 μ L) on an MJ Mini detector (Bio-Rad, USA). The cycling conditions were: 15 min polymerase activation at 95°C and 40 cycles at 95°C for 15 s, at 58°C for 30 s, and at 72°C for 30 s. Results were normalized to β -actin values and expressed as log₂ (sample/control).

Statistical analysis

Data are reported as arithmetic means \pm SEM. Significant differences between means were evaluated by ANOVA followed by the Newman-Keuls test, with the level of significance set at P < 0.05.

Results

Hepatic levels of oxidative stress biomarkers, lipid peroxidation (0.052 \pm 0.004 malonaldehyde equivalent nmol/mg protein), protein carbonylation (0.370 \pm 0.042 carbonyl group nmol/mg protein) and 8-OHdG (14.26 \pm 6.00 pmol 8-OHdG/ μ g DNA), for controls were not significantly different for mice treated with Δ^9 -THC and the vehicle (data not shown).

The activity levels of the enzymes involved in the endogenous antioxidant defense and glutathione levels are presented in Table 1. The levels of GSSG were significantly decreased in the liver of mice treated with Δ^9 -THC compared

Table 1. Endogenous oxidative defenses in liver homogenates of C57BL/6J mice: activity levels of enzymes involved in the endogenous antioxidant defenses and glutathione levels.

Endogenous oxidative defenses	Ctrl	VCtrl	Δ^9 -THC
SOD (U/mg protein)	0.430 \pm 0.012	0.425 \pm 0.018	0.418 \pm 0.009
Catalase (U/mg protein)	126.40 \pm 3.68	151.30 \pm 8.72*	149.10 \pm 8.13*
GST (U/mg protein)	0.049 \pm 0.002	0.048 \pm 0.002	0.048 \pm 0.002
GPx (U/mg protein)	0.030 \pm 0.002	0.035 \pm 0.001	0.031 \pm 0.001
Se-GPx (U/mg protein)	0.013 \pm 0.001	0.016 \pm 0.001*	0.014 \pm 0.001
GR (U/mg protein)	100.00 \pm 3.43	103.30 \pm 4.89	110.30 \pm 6.82
GSX (nmol/mg protein)	112.60 \pm 8.15	115.70 \pm 4.19	113.00 \pm 7.49
GSH (nmol/mg protein)	89.56 \pm 7.92	104.70 \pm 4.67	99.06 \pm 5.14
GSSG (nmol/mg protein)	11.54 \pm 1.54	5.51 \pm 0.89*	6.96 \pm 1.17*
GSH/GSSG ratio	10.46 \pm 1.47	20.45 \pm 2.75*	16.79 \pm 1.57

Data are reported as means \pm SEM (N = 10). Ctrl = mice treated with saline; VCtrl = mice treated with vehicle [1:1:18, cremophor EL[®] (polyoxyl 35 castor oil)/ethanol/saline]; Δ^9 -THC = mice treated with 10 mg (-)- Δ^9 -tetrahydrocannabinol/kg body weight per day for 10 days. SOD = superoxide dismutase; GST = glutathione-S-transferase; GPx = glutathione peroxidase; Se-GPx = selenium-dependent GPx; GR = glutathione reductase; GSX = total glutathione; GSH = reduced glutathione; GSSG = oxidized glutathione. *P < 0.05 compared with Ctrl (ANOVA followed by the Newman-Keuls test).

to the Ctrl group. A similar decrease was also observed in the liver of mice treated with the vehicle. Neither treatment with the vehicle nor Δ^9 -THC induced significant changes in the amount of GSX measured in the liver. The calculated amount of GSH for the liver of mice treated with Δ^9 -THC and for the liver of mice treated with the vehicle did not differ significantly from one another or when compared with the Ctrl group (although a tendency to an increase was found for both, which was higher in the vehicle group). The GSH/GSSG ratios determined were found to be significantly increased in the VCtrl group, but not in the Δ^9 -THC group, when compared to the Ctrl group.

Treatment with Δ^9 -THC produced no statistically significant changes in the enzymatic activity of the glutathione-dependent antioxidant enzymes GR and GST. Measurement of GPx, also a glutathione-dependent enzyme, tended to be increased only in the liver of mice from the VCtrl group. Se-GPx activity was only significantly increased in liver of mice from the VCtrl group.

Treatment with Δ^9 -THC did not produce statistically significant changes in the activity levels of the scavenger enzyme SOD. However, the activity level of catalase, also a scavenger enzyme, was significantly increased to a similar extent in the liver of mice treated with Δ^9 -THC and of mice treated with the vehicle when compared to the Ctrl group.

The gene expression of the cannabinoid receptors CB1 [Ctrl: 0.000 ± 0.464 ; VCtrl: 0.115 ± 0.668 ; Δ^9 -THC: $0.283 \pm 0.459 \log_2$ (sample/control)] and CB2 [Ctrl: 0.000 ± 1.351 ; VCtrl: 0.837 ± 0.504 ; Δ^9 -THC: $0.497 \pm 0.534 \log_2$ (sample/control)], as measured by RT-PCR, was not significantly changed in the liver by any of the treatments.

Discussion

In this study, we show that chronic administration of Δ^9 -THC did not produce any significant changes in the hepatic redox state of healthy mice, even though there were unexpected changes induced by administration of the vehicle containing ethanol/cremophor EL[®] alone that were attenuated by Δ^9 -THC.

Since it is known that liver injury is closely related to an increase in oxidative stress, we first assessed the level of damage to lipids, proteins and DNA in the liver of mice treated with Δ^9 -THC. The levels of these oxidative stress biomarkers in the liver were not changed by Δ^9 -THC treatment, suggesting that no damage was inflicted by either the vehicle or Δ^9 -THC administration.

Regardless of the lack of damage produced by Δ^9 -THC treatment as measured by these parameters, we found indications that some oxidative stress was unexpectedly produced by the vehicle. Catalase activity was significantly increased by 20% in the liver of mice treated with the vehicle. Similarly, in the same group of animals, Se-GPx activity was significantly increased by 23%.

Another reliable oxidative stress indicator is the level of

glutathione, a tripeptide with antioxidant capacity. A statistically significant decrease of GSSG (52%) was measured in the liver of mice treated with the vehicle. Since the GSX level measured in the liver of mice treated with the vehicle was similar to the Ctrl group, the GSH/GSSG ratio was significantly increased in the liver of mice from the VCtrl group (96%) when compared to the Ctrl group.

If the level of GSX is maintained, an increase of the GSH/GSSG ratio translates into an increased antioxidant capacity as more GSH is available (17). Although one may jump to the conclusion that the increased GSH/GSSG ratio is an indication of less oxidative stress produced by the vehicle, our results, taken together, show that some oxidative stress was induced by it: 1) catalase activity levels were increased; 2) the GSH levels were not significantly increased, but their tendency to increase supports the significantly increased Se-GPx activity found by us (otherwise this increased activity would lead to a GSH decrease), and 3) it has been shown that perfusing the liver with reagents that oxidize internal GSH to GSSG (by means of GPx) induces GSSG release into bile (17), which explains the reduction of GSSG observed by us.

Although some degree of oxidative stress was produced by the vehicle, the amount present or the time of exposure was not sufficient to cause any significant damage to the liver: we found no changes in the levels of the oxidative stress biomarkers measured.

It should be stated that the changes induced by the vehicle were unexpected since this is a very common vehicle used for Δ^9 -THC administration *in vivo*. In fact, the present study shows that care should be taken in the interpretation of the results obtained with this ethanol/cremophor EL[®] (polyoxy 35 castor oil) solution, especially in oxidative stress studies.

Even though no oxidative stress was produced by Δ^9 -THC administration, there is some evidence in our study that, in fact, Δ^9 -THC could protect the liver against oxidative stress since it decreased the effects induced by the administration of the vehicle.

Δ^9 -THC administration increased the Se-GPx activity by only 8%. Although much lower, this increase was not significantly different from the activity determined for the VCtrl group (a 23% increase compared to Ctrl). The total GPx activity measured was also increased (17%) in the VCtrl group, but again it was only increased by 3% in the liver of mice from the Δ^9 -THC group. Taken together, these results suggest attenuation by Δ^9 -THC treatment of the effect brought about by administration of the vehicle.

Similarly, when comparing the GSSG levels in the liver of Δ^9 -THC-treated mice with the GSSG levels in the liver of the Ctrl group, the measured decrease (42%) was less pronounced than that induced by treatment with the vehicle (52%). The GSH level measured tended to be increased more in the VCtrl group than in the Δ^9 -THC group. Thus, the GSH/GSSG ratio was significantly increased in the liver

of mice from the VCtrl group (96%) but not in the liver of mice from the Δ^9 -THC group (61%) when compared with the GSH/GSSG ratio determined for the liver of mice from the Ctrl group. However, the GSH/GSSG ratio increase in the Δ^9 -THC group was not significantly different from the GSH/GSSG ratio determined for the VCtrl group, again suggesting an attenuation of the effect brought about by administration of the vehicle.

Several other studies have reported that Δ^9 -THC has indeed a cannabinoid receptor-independent protective effect against increased oxidative stress conditions: 1) it prevented *in vitro* neuronal cell death induced by toxic reactive oxygen species that was unaffected by SR141716A, a CB1 receptor antagonist (18), 2) it reduced cellular oxidative stress and prevented serum-deprived cell death of human B lymphoblastoid and mouse fibroblast cells in a nonreceptor-

mediated process (19), and 3) it presented neuroprotective properties in *in vitro* oxidative stress tests in the brain tissue of CB1-deficient rats and mice (20). In fact, the gene expression of the cannabinoid receptors CB1 and CB2 measured by RT-PCR was not significantly changed, even though there were indications of some protection against oxidative stress by Δ^9 -THC administration. This protective effect was suggested to be due to the high resemblance of Δ^9 -THC to the antioxidant vitamin E (19).

Δ^9 -THC administration did not show any harmful effects on the liver of healthy mice. However, unexpected effects produced by the vehicle containing ethanol/cremophor EL[®] were attenuated by Δ^9 -THC. Further studies should be aimed at evaluating the possible protective effect of Δ^9 -THC under oxidative stress conditions.

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