Δ⁹-Tetrahydrocannabinol and Cannabidiol Differentially Regulate Intraocular Pressure

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PURPOSE. It has been known for nearly 50 years that cannabis and the psychoactive constituent Δ⁹-tetrahydrocannabinol (THC) reduce intraocular pressure (IOP). Elevated IOP remains the chief hallmark and therapeutic target for glaucoma, a major cause of blindness. THC likely acts via one of the known cannabinoid-related receptors (CB1, CB2, GPR18, GPR119, GPR55) but this has never been determined explicitly. Cannabidiol (CBD) is a second major constituent of cannabis that has been found to be without effect on IOP in most studies.

METHODS. Effects of topically applied THC and CBD were tested in living mice by using tonometry and measurements of mRNA levels. In addition the lipidomic consequences of CBD treatment were tested by using lipid analysis.

RESULTS. We now report that a single topical application of THC lowered IOP substantially (~28%) for 8 hours in male mice. This effect is due to combined activation of CB1 and GPR18 receptors each of which has been shown to lower ocular pressure when activated. We also found that the effect was sex-dependent, being stronger in male mice, and that mRNA levels of CB1 and GPR18 were higher in males. Far from inactive, CBD was found to have two opposing effects on ocular pressure, one of which involved antagonism of tonic signaling. CBD prevents THC from lowering ocular pressure.

CONCLUSIONS. We conclude that THC lowers IOP by activating two receptors—CB1 and GPR18—but in a sex-dependent manner. CBD, contrary to expectation, has two opposing effects on IOP and can interfere with the effects of THC.

Keywords: glaucoma, cannabinoid, intraocular pressure, cannabidiol, THC

Cannabis has a long and storied history tracing back thousands of years. Only recently have we begun to understand how its constituents act in the body. Δ⁹-tetrahydrocannabinol (Δ⁹-THC, THC) is understood to be the chief psychoactive ingredient of cannabis. The year 1971 marked the publication of the first work by Hepler and Frank demonstrating that cannabis inhalation has a salutary effect on intraocular pressure (IOP). This set in motion a flurry of trials as an antiepileptic in Dravet’s syndrome but CBD is also assigned many other properties, including activity at GPR18 and the cannabinoid-metabolizing enzyme FAAH (fatty acid amide hydrolase), but CBD may act as a negative allosteric modulator of CB1 signaling. This is significant because this means that CBD may antagonize THC signaling. Three of four studies that have tested CBD for effects on IOP have reported no effect, but the fourth has reported an increase in IOP. The current study was an examination of the receptor dependence of the actions of THC and CBD on IOP.

METHODS

Animals

Experiments were conducted at the Indiana University campus. All mice used for IOP experiments were handled according to

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the guidelines of the Indiana University animal care committee and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice (male and female, age 3–8 months) were kept on a 12-hour (6:00 AM–6:00 PM) light/dark cycle, and fed ad libitum. Male and female mice were used for these experiments as noted and were obtained from the colony of Ken Mackie (Indiana University, Bloomington, IN, USA). Mice were C57BL/6J (C57) strain except CB1+/− mice, which were on a CD1 strain background. We have previously shown that mice on a CD1 background see reductions in ocular pressure upon topical treatment with CB1 cannabinoid agonists WIN55212 and CP55940, which are absent in CB1 knockouts. Mice were allowed to acclimatize to the animal care facility for at least a week before their use in experiments. A total of 85 mice were used in these experiments. CB1+/− animals were kindly provided by Ken Mackie. The knockouts were global knockouts. CB1−/− animals were originally received from Catherine Ledent (Catholic University, Leuven) as heterozygotes.

Intraocular Pressure Measurements

IOP was measured to test the effects of topically applied THC and CBD in living mice by rebound tonometry, using a Tonolab (Icare Finland Oy, Helsinki, Finland). To obtain reproducible IOP measurements, mice were anesthetized with isoflurane (3% induction). The anesthetized mouse was then placed on a platform in a prone position, where anesthesia was maintained with 2% isoflurane. Baseline IOP measurements were taken in both eyes. Measurements were initiated promptly (typically within a minute) after successful induction of anesthesia. Depth of anesthesia was assessed by paw press. A “measurement” consisted of the average value of six readings. One eye was then treated with drug (dissolved in ethanol, 200 μl) and the other eye was treated with vehicle. Mice recover rapidly from isoflurane anesthesia. After an hour the animal was again anesthetized as above. IOP was then measured in the drug-treated and vehicle-treated contralateral eye. This procedure (recovery from anesthesia, return to cage, reanesthetization, measurement of IOP) was repeated at the 4- and 8-hour time points as noted and was performed with an Eppendorf RealPlex2 Mastercycler thermo-cycler (Eppendorf, Hauppauge, NT, USA).

A primer for glyceraldehyde 3-phosphate dehydrogenase was used as an internal control for each experimental condition with the threshold cycle set within the linear range (10-fold above baseline). Once the standard critical threshold (Ct) was set, the relative expression levels for genes were determined.

Lipid Extraction and LC/MS/MS Analysis and Quantification

Enucleated eyes were flash frozen in liquid nitrogen and frozen at –80°C until used for lipid analysis. Levels of ~35 cannabinoid-related lipids as well as arachidonic acid and several prostaglandin-family metabolites were measured by liquid chromatography (LC)/mass spectrometry (MS) from whole eyes as previously described. Briefly, eyes were homogenized, centrifuged at 19,000g at 24°C for 20 minutes and supernatant was collected. Compounds were isolated by using a partial purification of the 25% organic solution. C18 solid-phase extraction columns (Agilent Technologies, Santa Clara, CA, USA) were used with an elution of 100% methanol.

Samples were placed in an autosampler and held at 24°C (Agilent 1100 series autosampler; Palo Alto, CA, USA) for LC/MS/MS analysis. Ten to 20 μl of eluents were injected for each sample, which was rapidly separated with a C18 Zorbax reversed-phase analytic column (Agilent Technologies) to scan for individual compounds. Gradient elution (200 μL/min) then was accomplished under pressure (Shimadzu 10AdVP pumps; Shimadzu Scientific Instruments, Columbia, MD, USA). The electro-spray ionization was done by using an API5000 triple quadrupole mass spectrometer (Applied Biosystems/DSM Scien; Foster City, CA, USA). A multiple reaction monitoring (MRM) setting on the LC/MS/MS was used to analyze levels of each compound. Synthetic standards were used to generate optimized MRM methods and standard curves for analysis.

Individual animals in each of the treatment groups were coded and experiments were analyzed in a blinded fashion.

Materials

Δ9-THC and CBD were obtained through the National Institutes on Drug Abuse drug supply program. O-1918 and Tocrisolve were obtained from Tocris Bioscience.

RESULTS

THC Lowers IOP in a Sex-Dependent Manner

We found that THC when applied topically (5 mM) lowered IOP relative to the vehicle-treated contralateral eye in male mice. This resultant drop in IOP was quite pronounced at 8 hours, with a nearly 30% drop in IOP (Fig. 1A, IOP in THC [5 mM]–treated versus vehicle-treated contralateral eye in males; 1 and 4 hours: n = 7, 8 hours: n = 8; *P < 0.05 by 1-way ANOVA with Bonferroni post hoc test versus contralateral eye at corresponding time point). Strikingly however, in female mice given the same treatment there was a more modest effect at 4 hours than the corresponding time point in males and no effect at 8 hours (Fig. 1B, IOP in THC [5 mM]–treated versus vehicle-treated contralateral eye in females; 1 and 4 hours: n = 20, 8 hours: n = 8; *P < 0.05 by 1 way ANOVA with Bonferroni post hoc test versus contralateral eye at corresponding time point). The effect of THC was therefore sex-dependent.

Quantitative RT-PCR

Primers for selected components of the endocannabinoid system were designed by using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast, in the public domain) and the corresponding mouse gene. Primer sequences are as listed below:

**CB1 S:** 5′ CGT ATC GTG TG TGT ATC ATC TG 3′

**CB1 A:** 5′ CGT GTC TGT GGA CAC AGA CAT GGT 3′

Eyes were extracted, the lens removed, and were then immediately stored at –80°C. RNA was extracted with a Trizol reagent (Ambion, Austin, TX, USA) and genomic DNA was removed with DNase (NEB, Bethesda, MD, USA) following the manufacturer’s instructions. RT-PCR was performed by using a one-step, Sybr Green amplification process (PwrSybr; Applied Biosystems, Carlsbad, CA, USA). Quantitative PCR was performed with an Eppendorf RealPlex2 Mastercycler thermo-cycler (Eppendorf, Hauppauge, NT, USA).
THC Lowers IOP Through Combined Activation of CB1 and GPR18 Receptors

As noted in the introduction, THC lowers IOP but the mechanism by which it does this remains undetermined. A preferred hypothesis is that THC lowers IOP via CB1 receptors. We therefore tested whether the effect of THC would be absent in CB1 receptor knockout mice. Interestingly, we found that CB1 deletion only partly eliminated the effect of THC (Figs. 2A, 2B; IOP in THC [5 mM]–treated versus vehicle-treated contralateral eye in CB1 knockout (KO) males; 1 and 4 hours: \( n = 12; \ast P < 0.05 \) by paired t-test versus contralateral eye at corresponding time point). This suggests that CB1 also acts via a second receptor. One likely candidate for this is the GPR18 receptor since, as noted previously, GPR18 can lower IOP in mice and is activated by THC. We therefore tested whether the GPR18 antagonist O-1918 (5 mM) applied topically to CB1 knockout mice would prevent the effect of THC. We found that there was no effect at 1 or 4 hours under this condition (Figs. 2C, 2D; IOP in O1918 pretreated [5 mM topical] CB1 KOs: THC [5 mM]–treated versus vehicle-treated contralateral eye; 1 and 4 hours: \( n = 5; \ast P < 0.05 \) by paired t-test versus contralateral eye at corresponding time point). This argues that THC lowers IOP through a combination of CB1 and GPR18.

CBD Has Two Independent Opposing Actions on IOP

As noted above, CBD has been tested in several studies for effects on IOP. Three of those studies found no effect, while the last saw an increase in IOP. CBD has been proposed to act on a large and growing number of receptors including recent evidence that CBD is a negative allosteric modulator at CB1 receptors.\(^{25,27}\) When tested in our model, we found that CBD (5 mM) in male mice substantially raised IOP at 1 and 4 hours (Figs. 3A, 3B; IOP in CBD [5 mM]–treated versus vehicle-treated contralateral eye in wild type (WT) males; 1 hour: \( n = 13; 4 \) hours: \( n = 19; \ast P < 0.05 \) by paired t-test versus contralateral eye at corresponding time point). Female mice saw a similar rise at both time points (data not shown). Strikingly, the same experiment in CB1 knockouts resulted in a decrease in ocular pressure at 1 hour but no effect at 4 hours (Figs. 3C, 3D; IOP in CBD [5 mM]–treated versus vehicle-treated contralateral eye in CB1 KO males; 1 and 4 hours: \( n = 7; \ast P < 0.05 \) by paired t-test versus contralateral eye at corresponding time point). This indicates that CBD has two opposing effects on IOP. The first and dominant effect of raising IOP is likely CB1-dependent since the effect is absent in CB1 knockout mice and may be a consequence of cannabinoid receptor antagonism. We tested whether the IOP reduction was due to activity at GPR18 receptors, since GPR18 activation can lower IOP.\(^{13} \) CBD had no effect on IOP in animals pretreated with the GPR18 antagonist O1918 (5 mM) (Figs. 3E, 3F; IOP in O1918-pretreated animals [5 mM topical], CBD [5 mM]–treated versus vehicle-treated contralateral eye in CB1 KO males; 1 and 4 hours: \( n = 7; \) not significant (NS) by paired t-test versus contralateral eye at corresponding time point).

CBD Interferes With the IOP-Lowering Effects of THC

If CBD is raising pressure by acting as a negative allosteric modulator at CB1, then it is possible that coapplication of CBD and THC would cancel out the salutary effects of THC. To test this, we treated mice (C57Bl/6J) with combined CBD/THC (5 mM/5 mM) and found that there was no effect on IOP relative to vehicle (Fig. 4, IOP in THC and CBD [5 mM each]–treated versus vehicle-treated contralateral eye in WT males; 1 and 4 hours: \( n = 7; \) NS by paired t-test versus contralateral eye at corresponding time point).

mRNA Levels for CB1 and GPR18 Are Higher in Eyes of Male Than Female Mice

If there is a sex dependence in the effect of THC, what is the basis of that difference? Are there fewer CB1 receptors, or GPR18 receptors, or both? One way to test this is to examine the mRNA expression of these receptors in the eyes of male versus female mice. We examined mRNA expression for CB1 and GPR18 by using quantitative PCR, finding that mRNA levels of CB1 and GPR18 were lower in female mice than male mice (Fig. 5; \( n = 9 \) per condition; *\( P < 0.05 \) unpaired t-test male versus female).

Cannabinoid-Related Lipid Species Are Elevated After CBD Treatment

Though the IOP-lowering effect is blocked by a GPR18 antagonist, the action of CBD may be direct or indirect, particularly since, as noted previously, CBD has been shown to
act at the endocannabinoid-metabolizing enzyme FAAH. To explore this we measured levels of ~35 cannabinoid-related lipids as well as several prostaglandins and related lipids 1 hour after treatment with CBD. In a given animal, one eye received CBD while the contralateral eye received vehicle (N=6). As shown in the Table, the levels of most acylethanolamines rose, though not arachidonoyl-ethanolamine (AEA), an endogenous ligand for CB1/CB2 receptors. Levels of two \(N\)-acyl-gamma-aminobutyric acid (GABA) and \(N\)-acyl-taurine species were elevated. Intriguingly, levels of the GPR18 ligand N-oleoylglycerol (NOGly) and the GPR119 ligand 2-oleoylglycerol were also elevated.

**DISCUSSION**

Nearly half a century after reports of a salutary effect of cannabinoids on ocular pressure we still do not know the mechanism by which this occurs. Our chief findings in normotensive mice were that THC lowers pressure substantially and for at least 8 hours, through a combined action at two receptors, CB1 and GPR18. This effect was sex-dependent, with much stronger responses in male mice. CBD in contrast had two opposing actions on IOP: raising IOP in wild-type animals but lowering it in CB1 knockout mice likely via GPR18. Finally, at equal concentrations CBD prevented the IOP-lowering effects of THC.

Sex-dependent effects have been reported for cannabis (e.g., in the study by Cooper and Haney\(^{28}\)) but sex dependence had not been explored for cannabinoid regulation of IOP. Phytocannabinoids are not currently considered a suitable first-line therapeutic for glaucoma (e.g., American Academy of Ophthalmology [AAO] position statement 2014, www.aao.org); however, this may be based on limited evidence. The central argument is that topical THC is not effective, therefore necessitating treatment via cannabis inhalation. Cannabis inhalation, in turn, has assorted shortcomings: (1) psychoactivity, (2) short action (<4 hours), and (3) elevation of blood pressure. A key question then is whether topical THC works in humans. The negative conclusion is based on four studies, three of which pool male and female subjects. In the two 1981 studies by Merritt et al.,\(^{29,30}\) most subjects are female (4/6, 7/8). Green and Roth\(^{31}\) (1982) do not specify the makeup of their subject pool but exclude pregnant subjects, implying the presence of females, leaving only one study that includes only males.\(^{32}\) The sex dependence of THC regulation of IOP, with the robust effects in males that we report here, combined with topical THC studies in animals (e.g., Merritt et al.,\(^{29}\) Green et al.,\(^{33}\) ElSohly et al.,\(^{34}\)), suggests that the question of topical THC as a means to lower ocular pressure may merit some reconsideration.

It is notable that no less than three cannabinoid related receptors, namely, CB1, GPR18, and GPR119, each lower IOP when activated. Moreover, they all exhibit sex dependence,
FIGURE 3. CBD has two independent opposing actions on IOP. (A, B) CBD (5 mM) raises IOP at 1 and 4 hours. (C, D) CBD treatment in CB1 knockout mice unmasks a drop in IOP at 1 hour but not at 4 hours. (E, F) Pretreatment with the GPR18 antagonist O1918 in CB1 knockouts prevents the IOP-lowering effect of CBD at 1 hour. *P < 0.05, paired t-test versus vehicle-treated contralateral eye.

FIGURE 4. CBD may interfere with the IOP-lowering effects of THC. (A, B) A combined treatment of CBD and THC (5 mM each) in WT males does not result in a drop in IOP at 1 or 4 hours relative to contralateral vehicle-treated eyes. NS by paired t-test.
TABLE. Cannabinoid-Related Lipidomic Profile in Eye After CBD Treatment

| Lipid Species                          | Change With CBD (Relative to Vehicle) |
|----------------------------------------|--------------------------------------|
| N-acetyl ethanolamine                  | ↑                                    |
| N-palmityl ethanolamine                | ↑                                    |
| N-stearoyl ethanolamine                | ↑                                    |
| N-oleoyl ethanolamine                  | ↑                                    |
| N-linoleoyl ethanolamine               | ↑                                    |
| N-arachidonoyl ethanolamine            | ↑                                    |
| N-docosahexaenoyl ethanolamine         | ↑                                    |
| N-acetyl GABA                          | ↑                                    |
| N-palmityl GABA                        | ↑                                    |
| N-stearoyl GABA                        | ↑                                    |
| N-oleoyl GABA                          | ↑                                    |
| N-linoleoyl GABA                       | ↑                                    |
| N-arachidonoyl GABA                    | ↑                                    |
| N-docosahexaenoyl GABA                 | ↑                                    |
| N-acetyl glycine                       | ↑                                    |
| N-palmityl glycine                     | ↑                                    |
| N-stearoyl glycine                     | ↑                                    |
| N-oleoyl glycine                       | PAL                                  |
| N-linoleoyl glycine                    | PAL                                  |
| N-arachidonoyl glycine                 | PAL                                  |
| N-docosahexaenoyl glycine              | PAL                                  |
| N-acetyl serine                        | ↑                                    |
| N-palmityl serine                      | ↑                                    |
| N-stearoyl serine                      | ↑                                    |
| N-oleoyl serine                        | ↑                                    |
| N-linoleoyl serine                     | ↑                                    |
| N-arachidonoyl serine                  | ↑                                    |
| N-docosahexaenoyl serine               | ↑                                    |
| N-acetyl taurine                       | ↑                                    |
| N-palmityl taurine                     | ↑                                    |
| N-stearoyl taurine                     | ↑                                    |
| N-oleoyl taurine                       | ↑                                    |
| N-linoleoyl taurine                    | ↑                                    |
| N-arachidonoyl taurine                 | ↑                                    |
| N-docosahexaenoyl taurine              | ↑                                    |
| N-acetyl tyrosine                      | ↑                                    |
| N-palmityl tyrosine                    | ↑                                    |
| N-stearoyl tyrosine                    | ↑                                    |
| N-oleoyl tyrosine                      | ↑                                    |
| N-linoleoyl tyrosine                   | ↑                                    |
| N-arachidonoyl tyrosine                | ↑                                    |
| N-docosahexaenoyl tyrosine             | ↑                                    |
| 2-Acyl glycerol                        | ↑                                    |
| 2-Palmitoyl glycerol                   | ↑                                    |
| 2-Oleoyl glycerol                      | ↑                                    |
| 2-Linoleoyl glycerol                   | ↑                                    |
| 2-Arachidonoyl glycerol                | ↑                                    |
| CBD                                    | ↑                                    |
| Cannabidiol                            | ↑                                    |
| Free fatty acids                       | ↑                                    |
| Oleic acid                             | ↑                                    |
| Linoleic acid                          | ↑                                    |
| Arachidonic acid                       | ↑                                    |
| PhosphoNAEs                             | ↑                                    |
| PhosphoOEA                              | ↑                                    |
| PhosphoLEA                              | ↑                                    |
| Prostaglandins                         | ↑                                    |
| PGE2                                   | ↑                                    |
| PGF2α                                  | ↑                                    |
| 6-KetoPGF1α                             | ↑                                    |
| Sample mass                            | ↑                                    |
| Sample mass                            | ↑                                    |

Table shows lipids for which analytic standards were included for analysis. Nine of these were below detectable levels. ↑↑↑↑ denotes 3- to 9.99-fold increase; ↑↑↑ denotes 2- to 2.99-fold increase; ↑↑ denotes 1.50- to 1.99-fold increase; ↑ denotes 1- to 1.49-fold increase. BAL, below analytic limits; LEA, linoleoyl ethanolamie; NAE, N-arachidonoyl ethanolamie; PG, prostaaglandin.
CB1 directly can achieve the same outcome. Our findings for lower ocular pressure. Similarly, we have found that activating point to novel strategies to promote ocular health.

...and CBD, but also others derived from the plant, may therefore advantageous. The study of phytocannabinoids such as THC suggest that a dual CB1/GPR18 agonist may prove advantageous. Our findings for THC suggest that a dual CB1/GPR18 agonist may prove advantageous. The study of phytocannabinoids such as THC and CBD, but also others derived from the plant, may therefore point to novel strategies to promote ocular health.

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