Indirect sympatholytic actions at β-adrenoceptors account for the ocular hypotensive actions of cannabinoid receptor agonists.

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Cannabinoids and IOP

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Abbreviations:

AH aqueous humor
ARs adrenoceptors
CP CP 55,940
NE norepinephrine
IOP intraocular pressure
ISO isoprenaline
TH tyrosine hydroxylase
WIN WIN 55,212-2
Abstract

Intraocular pressure (IOP) is the primary risk factor for glaucoma, a blinding eye disease. Cannabinoid agonists have long been known to decrease IOP, suggesting they may be useful in glaucoma treatment. However, the specific mechanism by which cannabinoids generate this ocular hypotensive effect remains unknown. The current evidence suggests the cannabinoids reduce IOP through actions at CB1 receptors within the eye, and that adrenergic receptors may also contribute to this action of cannabinoids. Considering this, the present study aimed to elucidate the mechanism behind the ocular hypotensive properties of cannabinoids through the use of mice genetically lacking either cannabinoid receptors or β-adrenergic receptors (βARs). Cannabinoid agonists, βAR antagonists, and βAR agonists decreased IOP in wild type mice and CB2−/− mice. In contrast, none of these compounds were found to reduce IOP in βAR−/− or CB1−/− mice. Desensitization of the βARs and depletion of catecholamines in wild type mice also eliminated the ability of the cannabinoid agonist, WIN 55,212-2 (WIN), to reduce IOP, strongly implicating a role for both βARs and catecholamines in the ocular hypotensive properties of cannabinoids. Finally, CB1 cannabinoid receptors were shown to co-localize with tyrosine hydroxylase, a marker for adrenergic neurons. Taken together, these findings suggest that βARs are required for the ocular hypotensive properties of cannabinoids, and that cannabinoids reduce IOP by acting as indirect sympatholytics and inhibiting norepinephrine (NE) release within the eye.
Introduction

Cannabinoid agonists and antagonists have received significant interest for use as therapeutics in the treatment of a variety of conditions including: obesity and diabetes, pain, neurodegenerative disorders, and immune dysfunction among many others (Piomelli et al., 2000). One property of cannabinoid agonists that has received attention is their ability to reduce intraocular pressure (IOP) (Hepler and Frank, 1971). IOP is the primary risk factor for glaucoma, a blinding neurodegenerative eye disease, and drugs that reduce IOP, so-called ocular hypotensives, are used in the treatment of glaucoma. While the IOP-lowering ability of cannabinoid agonists is well-established (Chien et al., 2003; Hepler and Frank, 1971; Liu and Dacus, 1987; Szczesniak et al., 2006), none have been developed for the treatment of glaucoma; largely due to a limited understanding of the mechanism(s) by which cannabinoids produce their effect on IOP.

IOP is maintained in the eye by the balance between aqueous humor (AH) secretion and outflow. AH is secreted by the cells of the ciliary body (Civan and Macknight, 2004), while outflow occurs through one of two distinct pathways: 1) the trabecular meshwork pathway (Ferrer, 2006), and 2) the uveoscleral pathway (Alm and Nilsson, 2009). Ocular hypotensive actions of cannabinoids may include effects on both AH production and outflow, as CB₁ cannabinoid receptors are expressed in the ciliary body, as well on trabecular meshwork cells (Straiker et al., 1999). There is also in vitro and in vivo evidence suggesting that cannabinoids have effects on both AH secretion (Chien et al., 2003) and outflow, specifically through the trabecular meshwork outflow pathway (McIntosh et al., 2007; Stumpff et al., 2005).
While the specific site(s) of the cannabinoid-mediated reduction in IOP may still be unclear, there is general consensus that the effects are not mediated via CNS receptors, but instead by CB₁ receptors present within the eye (Liu and Dacus, 1987; Pate et al., 1998).

Drugs targeting ocular adrenoceptors (ARs) also reduce IOP and βAR antagonists are first-line therapeutics in the treatment of glaucoma, due to their ability to reduce AH secretion (Watanabe and Chiou, 1983). βAR agonists somewhat paradoxically also reduce IOP, however, they do so by increasing AH outflow through the uveoscleral and trabecular meshwork pathways (Alvarado et al., 1998). In addition to the βARs, α₂AR agonists are also ocular hypotensive, reducing AH secretion, while at the same time increasing uveoscleral outflow (Woodward and Gil, 2004).

Given the importance of the various ARs in IOP regulation, it is not surprising that several lines of evidence suggest adrenergic receptors contribute to the ocular hypotensive actions of cannabinoids (Green, 1979). In several studies where βAR antagonists have been co-administered with cannabinoid agonists, additive effects on IOP were not observed (Green et al., 1977; Oltmanns et al., 2008). This lack of additive effect suggests that there may be an overlap in the IOP lowering mechanism(s) for these two drug classes. Further support for the involvement of ARs in the ocular hypotensive properties of cannabinoids comes from the observation that when rabbits were gangliectomized to eliminate sympathetic input, cannabinoids agonists had reduced ability to lower IOP (Green and Kim, 1976; Green et al., 1977; Green, 1979), although this appears to be species dependent as
similar findings were not observed in gangliectomized cats (Colasanti and Powell, 1985). To date, no study has definitively examined whether ARs are involved in the ocular hypotensive properties of cannabinoids in mice, or considered the specific mechanism by which individual AR subtypes may be involved in the cannabinoid-mediated reduction in IOP.

In several other tissues presynaptic CB₁ receptors inhibit norepinephrine (NE) release (Ishac et al., 1996). If this were also the case in the eye, it suggests that cannabinoid agonists could reduce IOP by inhibiting NE release in the ciliary body, thus acting as indirect sympatholytics. The present study is the first to examine this possibility through the use of βAR⁻/⁻, CB₁⁻/⁻ and CB₂⁻/⁻ mice. We demonstrate that the ocular hypotensive properties of cannabinoids are absent in mice genetically lacking either CB₁ or the βARs, but remain intact in mice lacking CB₂. In addition, we demonstrate that treating wild-type mice with the catecholamine-depleting agent, reserpine, also eliminates the ability of cannabinoid agonists to reduce IOP. These findings indicate that cannabinoids reduce IOP through activation of the CB₁ receptor, in a mechanism that is also dependent upon the presence of both the βARs and catecholamines.

**Methods**

**Animals**

All C57BL6 mice used for IOP experiments were handled according to the Canadian Council for Animal Care guidelines (http://www.ccac.ca/), and all experimental procedures were approved by the Dalhousie University Committee on
Laboratory Animals. Mice were kept on a 12 h (07:00-19:00) light dark cycle, and fed *ad libitum*. C57BL/6J mice were obtained from Charles River Laboratories International Inc. (Wilmington, MA). C57BL/6J mice were obtained at 6-8 weeks of age and allowed to acclimatize to the animal care facility for at least a week prior to their use in experiments. Mice genetically lacking both β1AR and β2AR (βAR-/-) were obtained from The Jackson Laboratory (Bar Harbor, ME, Stock number 003810) and bred in homozygous breeding pairs to produce all animals used in this study. Mice genetically lacking the CB1 receptor (CB1-/-) were kindly provided by Dr. Carl Lupica (National Institute on Drug Abuse, Bethesda, MD). Heterozygous mice were obtained and bred with the resulting offspring genotyped by PCR using a CB1 forward primer: 5′-GTACCACACCAGCCTCTCTC-3′, in combination with a CB1 wild type reverse primer: 5′-GGATTCAGAGTTATGAAATCCGC-3′, or a CB1 KO reverse primer: 5′-AAGAAGGATCTAGACGCTCTA-3′.

CD1 strain animals (WT and CB1-/-) used for immunohistochemistry and IOP experiments were kindly provided by Dr. Ken Mackie (Indiana University, Bloomington IN) and were handled according to the Guidelines of the National Institutes of Health on the Care and Use of Animals; all procedures used in this study were approved by the Animal Care Committee of Indiana University, Bloomington Campus. Adult mice (CD1 strain, from breeding colony) were housed under a 12/12 hour day/night cycle then killed (during the light cycle) by rapid cervical dislocation for immunohistochemistry.

*Intraocular pressure measurements*
IOP was measured in mice by rebound tonometry, using a Tonolab (Icare Finland Oy, Helsinki, Finland). This instrument involves a light plastic tipped probe briefly making contact with the cornea; after the probe hits the eye the instrument measures the speed at which it rebounds in order to calculate IOP (Cervino, 2006).

To obtain reproducible IOP measurements, mice were anesthetized with isoflurane (4% induction). The anesthetized mouse was then placed on a platform in a prone position, where anesthesia was maintained with isoflurane (2% maintenance). IOP measurements were then made with at least six individual pressure readings taken from each eye. The pressure from each eye was then recorded as the average of these measurements.

For diurnal IOP experiments, IOP was measured on the same day and from the same animal both early in the light cycle, between 09:00 and 09:30 (reported as 09:00), or early in the dark cycle, between 21:00-21:30 (reported as 21:00). Statistical analysis of these data was carried for each eye independently by matched-pair t-test, comparing the 09:00 and 21:00 measurements.

All IOP measurements following drug administration were recorded between 16:00 and 18:00 in order to reduce any variability in IOP resulting from diurnal changes. Drugs were applied topically to one randomly assigned eye, while the other eye received the appropriate vehicle. IOP measurements following drug administration were analyzed by match-pair t-tests comparing the drug-treated eye to the vehicle-treated eye of the same animal.

In the chronic isoprenaline (ISO) desensitization experiments, either ISO (2.5%), or saline vehicle was administered twice daily for the 1 week experiment
(09:00 and 21:00), or three times daily (5%) for the 2 week experiment, to both eyes of the animal. After seven or fourteen days, the ability of the βAR antagonist, timolol, to reduce IOP was tested by applying timolol to the right eye and saline vehicle to the left eye and comparing the IOP from the two eyes. ISO treatment was again resumed for an additional three days, before the ability of WIN to reduce IOP in these animals was examined.

**Immunohistochemistry**

After animals were killed, their eyes were removed, and the anterior or posterior eye section cut away to form a posterior or anterior eyecup; the eyecup was fixed in 4% paraformaldehyde followed by a 30% sucrose immersion for 24-72 hours at 4°C. Tissue was then frozen in OCT compound and sectioned (15-25 μm) using a Leica CM1850 cryostat. Tissue sections were mounted onto Superfrost-plus slides, washed, treated with a detergent (Triton-X100, 0.3% or saponin, 0.1%) and milk (5%), followed by primary antibodies overnight at 4 °C. Secondary antibodies (Alexa 594 or Alexa 488, 1:500, Invitrogen, Inc., Carlsbad, CA) were subsequently applied at room temperature for 1.5 hours. The tyrosine hydroxylase (TH) antibody (Sigma-Aldrich, St. Louis, MO; 1:500) is well-characterized (Whitney et al., 2009). TH retinal staining was limited to a characteristic population of amacrine cells and their processes in the distal inner plexiform layer (data not shown). The CB₁ antibody was the generous gift of Dr. Ken Mackie (Indiana University). The specificity of the CB₁ antibody (1:300) was characterized by use of knockout mouse models (Hu et al., 2010), including anterior eye (data not shown). Images were
acquired with a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) using Leica LAS AF software and a 63X oil objective. Images were processed using ImageJ (available at http://rsbweb.nih.gov/ij/) and/or Photoshop (Adobe Inc., San Jose, CA). Images were modified only in terms of brightness and contrast.

Reserpine Depletion of Catecholamines

Reserpine treatment was used to deplete catecholamines in mice, according to the previously described protocol (Olfe et al., 2010). Briefly, reserpine (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) was dissolved in 5% glacial acetic acid at 2 μg/μl. The dose given was μg/g body weight and the resulting solution was combined with 100ul of saline. Reserpine solution, or saline vehicle, was then injected s.c. 24 hours prior to experimental manipulation. After 24 h, animals were anesthetized using pentobarbital at 60 mg/kg for induction and 5 mg/kg for maintenance of anesthesia if needed. Baseline IOP measurements were then taken by rebound tonometry (Tonolab) prior to any further treatment to determine basal IOP under pentobarbital anesthesia. Following basal IOP measurement, one eye was treated topically with drug, while the other was treated with the appropriate vehicle. IOP was then measured 20 min following the drug administration.

Drugs
Timolol (S-(-)1-(t-Butylamino)-3-[(4-morpholino-1,2,5-thiadiazol-3-yl)oxy]-2-propanol), isoprenaline (1-[(3’,4’-Dihydroxyphenyl)-2-isoproplaminoethanol hydrochloride, N-Isopropyl-DL-noradrenaline) (IS0) and metoprolol ((±)1-(Isopropylamino)-3-[p-(β-methoxyethyl)phenoxy]-2-propanol) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) and prepared in 0.9% sterile saline for topical applications. WIN55,212-2 ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethane mesylate) (WIN); AM281 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide); AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone) and ACEA (N-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide) were obtained from Tocris Bioscience (Ellisville, MO) and were prepared in Tocrisolve™ 100 (Tocris Bioscience, Ellisville, MO) for topical application. Latanoprost (isopropyl (Z)-7-[(1R,2R,3R,5S)-3,5-dihydroxy-2-[3R]-hydroxy-5-phenylpentyl]-cyclopentyl]hept-5-enoate) was obtained as an ophthalmic solution (Xalatan, Pfizer).

**Results**

**Diurnal Variations in IOP**

Diurnal variation in the IOP of rodents is well established, such that IOP is lowest early in the light cycle, and highest at the beginning of the dark cycle (Savinova et al., 2001). Therefore, in order to validate our experimental method of
measuring the IOP in the mouse eye, diurnal changes in IOP were measured first in wild type mice (Figure 1A), and then second in \( \beta AR^{--} \), C57BL6-CB\(_1^{--} \) and CB\(_2^{--} \) mice to determine what effects genetic disruption of these receptors has on the diurnal regulation of IOP in mice (Figure 1B, 1C and 1D).

In wild type mice, the expected diurnal variation in IOP was observed with significantly higher IOP (0.95 ±0.20 mmHg) recorded at 21:00 compared with that recorded at 09:00 (Figure 1A; \( p<0.001 \)). Similarly, significantly higher IOPs were measured from \( \beta AR^{--} \) mice at 21:00 compared with 09:00 (Figure 1B; 1.62 ±0.28 mmHg higher; \( p<0.001 \)); C57BL6-CB\(_1^{--} \) mice (Figure 1C; 1.10 ±0.26 mmHg higher at 21:00; \( p<0.001 \)); and the CB\(_2^{--} \) mice (Figure 1D; 0.74 ±0.14 mmHg higher at 21:00; \( p<0.001 \)). These findings demonstrate our ability to measure changes in IOP in each of the strains of mice tested, and that the diurnal regulation of IOP remains intact in mice genetically lacking the \( \beta ARs \), the CB\(_1 \) cannabinoid receptor, or the CB\(_2 \) cannabinoid receptor. Interestingly, there did appear to be significant differences in the basal IOPs (measured at 09:00) across the four different mouse genotypes, specifically with each of the \( \beta AR^{--} \) (10.0 ±0.12 mmHg), C57BL6-CB\(_1^{--} \) (10.1 ±0.21 mmHg) and CB\(_2^{--} \) (10.1 ±0.09 mmHg) mice having significantly lower (\( p<0.001 \)) IOP at 09:00 compared to the wild type mice (11.0 ±0.13 mmHg).

**\( \beta AR \) antagonists and agonists reduce IOP in C57BL6 mice**

Before examining interactions between the cannabinoid and adrenergic systems in their regulation of IOP, the ability of various \( \beta AR \) ligands to lower IOP was first examined. The non-selective \( \beta AR \) antagonist timolol, a \( \beta AR \) antagonist...
commonly used clinically to reduce IOP, was first examined (Figure 2A). The baseline IOP from the vehicle-treated control eye displayed some variation among animals, with control IOP values ranging from 10.40 to 11.33 mmHg (Mean 10.88 ±0.31 mmHg). However, in each animal tested the IOP measured from timolol-treated eye was less than that of the vehicle-treated eye of the same animal. Based on this, all subsequent statistical analyses of drug treatments were carried out using matched-pairs t tests, comparing the drug treated eye to the vehicle-treated eye of the same animal.

To establish the role of specific βARs in the ocular hypotensive effects of βAR antagonists, the IOP-lowering effect was compared between the non-selective antagonist, timolol, and the β1AR selective antagonist, metoprolol (Figure 2B). In these experiments, both timolol (0.5%) and metoprolol (1.0%) produced statistically significant reductions in IOP (p<0.001 and p<0.05 respectively). However, the magnitude of IOP decrease for timolol treatment (1.056 ±0.18 mmHg) appeared to be greater than for metoprolol treatment (0.62 ±024 mmHg). This greater efficacy of the non-selective antagonist suggests that the IOP-lowering effect of βAR antagonists is mediated in part by both the β1AR and the β2AR. Finally, the effect of the βAR agonist, isoprenaline (ISO), on the IOP of C57BL6 mice was also considered (Figure 2C). In these experiments, ISO (1.0%) resulted in a significant reduction in IOP of 0.98 ± 0.23 mmHg (p<0.01).

*Cannabinoid agonist mediated reduction in IOP is additive with the prostaglandin analog latanoprost, but not with the βAR antagonist timolol.*
The ability of the non-selective cannabinoid agonists, WIN 55,212-2 (WIN), and CP 55,940 (CP) to reduce IOP in in C57BL6 mice was next determined (Figure 3A). Treatment with either cannabinoid agonist resulted in a significant decrease in IOP, with WIN (1.0%) producing a 0.86 ±0.23 mmHg (p<0.01) drop in pressure, while CP (1.0%) treatment resulted in a drop in IOP of 1.02 ±0.21 mmHg (p<0.001). In order to establish the ideal concentration of WIN to use for subsequent experiments, a WIN dose response experiment was conducted (Figure 3B). WIN significantly reduced IOP at concentrations of 0.5, 1.0 and 2.0% and the maximum response was achieved with concentrations equal or greater than 1.0%. Therefore, 1.0% WIN was selected as the optimal concentration to use in all subsequent experiments.

It was next tested whether WIN has an additive effect on IOP when given in combination with a ΔBAR antagonist, or the prostaglandin analog, latanoprost, another agent that is commonly used clinically to reduce IOP (Figure 3C and 3D). Treatment with all drug combinations resulted in significant reductions in IOP: 0.86 ±0.39 mmHg for WIN alone (p<0.01); 0.47 ±0.13 mmHg for WIN and timolol (p<0.001); and 1.45 ±0.34 mmHg for WIN and latanoprost (p<0.01). Statistical analysis by one-way ANOVA of these results demonstrates that the magnitude of IOP-reduction is significantly greater when WIN is given in combination with latanoprost (p<0.05) compared with WIN treatment alone. In contrast, the combination of WIN and timolol produced an IOP reduction that was not significantly different from WIN treatment alone (p>0.05). These data demonstrate that cannabinoid agonists reduce IOP and that the effect of WIN on IOP is additive.
with the prostaglandin analog, latanoprost, but is not additive with the βAR antagonist, timolol.

**CB1 Colocalizes with a Marker of Adrenergic Neurons in the Anterior Chamber of the Eye**

To examine the possibility that CB1 lowers IOP by inhibiting NE release in the anterior chamber of the eye, thus acting as an indirect adrenergic antagonist, we first examined whether CB1 receptors co-localize with the catecholamine synthetic enzyme, tyrosine hydroxylase (TH). We have previously shown that CB1 is prominently expressed throughout the anterior chamber of the human eye (Straiker et al., 1999) including the ciliary epithelium and the angle, sites consistent with a role in regulation of IOP. Therefore, if CB1 is affecting IOP by inhibiting NE release, it should be expected that some level of colocalization will be seen between TH and CB1 in this tissue. To test this hypothesis, we used immunohistochemistry to co-stain for these two proteins in frozen sections of anterior murine eye. We found that CB1 costained with TH in portions of the ciliary epithelium, the angle, the iris and the cornea. In the ciliary epithelium TH and CB1 overlap near presumed blood vessels (Figure 4A, arrows). We also observed double-labeling in presumed neuronal projections in the angle (Figure 4B, arrow). CB1 also colocalized with TH in cornea sections; co-staining was restricted to the corneal periphery (Figure 4C). Lastly, CB1 and TH both prominently stained the murine iris, with some overlap (Figure 4D).
The ability of βAR agonists and antagonists to reduce IOP in mice genetically lacking both βARs (βAR−/−) was next considered (Figure 5A). For these experiments, mice lacking both β1AR and β2AR were chosen because our previous data using the non-selective βAR antagonist, timolol, and the β1AR selective antagonist, metoprolol (Figure 2B), indicates that both β1ARs and β2ARs are involved in the ocular hypotensive properties of βAR antagonists. Not surprisingly, in the βAR−/− mice, neither the βAR antagonists, timolol (0.5%) and metoprolol (1.0%), nor the βAR agonist, ISO (1.0%), significantly changed IOP (p>0.05). Interestingly, ISO treatment actually produced a statistically significant (p<0.05) small increase in IOP of 0.42 ±0.26 mmHg, suggesting that ISO may also act on other targets in addition to β1AR and β2AR. To verify that pharmacological manipulations of IOP could still be measured in βAR−/− mice, the effect of the prostaglandin analog, latanoprost, on the IOP of these mice was examined (Figure 5B). In these experiments, as expected, latanoprost was able to produce a significant 1.00 ±0.45 mmHg reduction in IOP (p<0.05), demonstrating that IOP can be manipulated by βAR-independent pathways in βAR−/− mice.

To establish whether the βARs were also involved in the ocular hypotensive effects of cannabinoid agonists, the ability of various cannabinoids to lower IOP in βAR−/− mice was examined (Figure 5C). In these experiments, neither the non-selective cannabinoid agonists, WIN (1.0%) and CP (1.0%), nor a CB1-selective cannabinoid agonist, ACEA (1.0%), were able to significantly affect IOP in βAR−/−
mice (p>0.05). In order to further confirm that cannabinoids did not significantly affect the IOP of βAR−/− mice, additional experiments were carried out with both shorter (30 min) and longer (2 h) WIN treatments (Figure 5D), none of which resulted in significant changes in the IOP of these animals (p>0.05). These findings demonstrate that βAR antagonists and agonists, as well as cannabinoid agonists all reduce IOP through mechanisms that are dependent on the presence of βARs.

*Cannabinoid agonists and βAR ligands do not reduce IOP in CB1−/− mice that are on a C57BL6 genetic background*

Having examined the effect on IOP of cannabinoid agonists on βAR−/− mice, these compounds were then examined in CB1−/− and CB2−/− mice, each on a C57BL6 genetic background, to establish which cannabinoid receptor was responsible for their IOP-lowering effects. First, the effect of WIN on the IOP of CB1−/− mice was compared to its effect on CB1+/+ littermates (Figure 6A). In these experiments, WIN significantly decreased IOP in the CB1+/+ mice (p<0.05); but was found to increase IOP in the CB1−/− mice (p<0.05). These findings demonstrate that not only is the IOP-lowering effect of WIN mediated by the CB1 receptor, but also that WIN may have an additional non-CB1 site of action that increases IOP.

The βAR antagonists, timolol (0.5%) and metoprolol (1.0%), as well as the βAR agonist, ISO (1.0%), were then examined in CB1−/− mice (Figure 6B). Interestingly, none of these βAR ligands produced a significant reduction in IOP when administered to CB1−/− mice (p<0.05). Latanoprost was then tested in these mice to verify that pharmacological reductions in IOP could still be measured in CB1−/−...
Latanoprost still reduced IOP in CB<sup>1</sup>−/− mice, producing a drop in IOP of 0.78 ±0.68 mmHg, although it did not quite achieve statistical significance (p=0.058).

To verify that the IOP-lowering effect of cannabinoid agonists are mediated by CB<sup>1</sup> cannabinoid receptors, the IOP-lowering effect of WIN was also evaluated in CB<sup>2</sup>−/− mice (Figure 6C). As expected, WIN significantly reduced IOP in the CB<sup>2</sup>−/− mice (0.65 ±0.10 mmHg reduction; p<0.001), an effect that was blocked by pre-treatment with the CB<sup>1</sup> antagonist, AM281. Timolol was also effective at reducing IOP in the CB<sup>2</sup>−/− mice, producing a 0.98 ±0.25 mmHg reduction (p<0.01). Together these findings demonstrate that CB<sup>2</sup> does not appear to play a role in the ocular hypotensive properties of either cannabinoid or βAR ligands.

The effect of cannabinoid agonists and βAR antagonists on CB<sup>1</sup>−/− mice on a CD1 genetic background.

We observed several unexpected findings in the C57BL6-CB<sup>1</sup>−/− mice, specifically that WIN increased IOP in these mice, and that timolol had no effect on IOP. Therefore, in order to rule out that these observations may be mouse-strain dependent, we next tested these compounds in independently-generated CB<sup>1</sup>−/− mice on a CD1 genetic background (Ledent et al., 1999). For this, we first demonstrated that timolol (0.5%), WIN (1.0%) and CP (1.0%) all significantly decreased IOP in wild-type CD1 mice (p<0.001; Figure 7A), producing decreases of 0.82 ± 0.20, 0.80 ± 0.30 and 0.96 ± 0.20, respectively. In contrast, as was observed in the C57BL6-CB<sup>1</sup>−/− mice, neither timolol nor WIN reduced IOP in the CD1-CB<sup>1</sup>−/− mice, while WIN again produced a small increase in IOP (p<0.05; Figure 7B).
IOP could be reduced in these mice using an unrelated ocular hypotensive agent, dorzolamide (Figure 7B). This agent was chosen as the positive control in the CD1 background mice instead of latanoprost, which was used as in the C57BL6 mice, because latanoprost was found to have a lesser effect on IOP in wild-type CD1 mice than it had on wild-type C57BL6 mice (data not shown).

Since WIN is a non-selective CB₁/CB₂ agonist, in order to determine whether the WIN mediated increase in IOP is mediated by CB₂ we attempted to block the effect by pre-treatment with AM630 (Figure 7C). Somewhat surprisingly treatment with AM630 appeared to decrease the basal IOP in these animals (0.74 mmHg ± 0.29, p<0.05); however, despite this, AM630 had no statistically significant effect on the WIN-mediated increase in IOP. Specifically, WIN increased IOP by 1.38 ± 0.31 mmHg in the absence of AM630, compared to increasing IOP by 1.14 ± 0.26 mmHg in the presence of AM630. Next to establish whether the effect was specific to WIN, we tested another non-selective cannabinoid agonist, CP, for its effect on the IOP of CD1-CB₁⁻/⁻ mice. This compound did not significantly alter the IOP of these mice (p>0.05), suggesting that the WIN-mediated increase in IOP observed in CB₁⁻/⁻ mice may be specific to this particular class of cannabinoid agonist.

Chronic treatment with the βAR agonist ISO desensitizes the IOP responses to both timolol and WIN

The lack of IOP response to βAR agonists and antagonists in the CB₁⁻/⁻ mice may result from a compensatory increase in basal βAR tone due to the elimination of presynaptic CB₁ receptors; this in turn could lead to the desensitization of βARs,
thus reducing the ability of adrenergic ligands to affect IOP. As a proof of principle experiment to demonstrate that increased adrenergic tone could result in βAR desensitization, wild type C57BL6 mice were treated chronically with the βAR agonist, ISO, for one week, after which the ability of timolol to reduce IOP in these mice was assessed (Figure 8A). In this experiment, timolol significantly reduced IOP in both the saline control (p<0.001) and ISO-treated animals (p<0.001); however, although not significant the magnitude of the timolol IOP-decrease appeared to be somewhat diminished in the ISO-treated (0.78 ±0.13 mmHg) compared with the saline control-treated (1.06 ±0.17 mmHg) animals. Based on this, a subsequent experiment was carried out where ISO treatment was carried out for two weeks before the effect of timolol on IOP was determined (Figure 8B). In this case, after two weeks of ISO treatment, timolol no longer produced a significant reduction in IOP (p>0.05), while still reducing IOP in the saline control animals (1.14 ±0.63 mmHg reduction; p<0.05). These results indicate that chronic adrenergic activation by ISO results in desensitization of βARs.

The fact that chronic ISO treatment in C57BL6 mice resulted in a desensitization of βARs, provided an additional means to demonstrate the dependence of the IOP-lowering effects of the cannabinoid agonist, WIN, on βARs. To test this, the effect of WIN on C57BL6 mice was examined in mice that were treated for 2 weeks with either saline control or ISO (Figure 8B). In the saline control mice, WIN produced a significant 1.10 ±0.49 mmHg (p<0.001) reduction in IOP. In contrast, in the ISO-treated mice, WIN had no significant effect on the measured IOP (p>0.05). These observations demonstrate a proof of principle that
chronic activation of the βARs leads to the desensitization of these receptors, and that following this desensitization, the βAR antagonist, timolol, and the cannabinoid agonist, WIN, are no longer able to reduce IOP.

*Depletion of catecholamines with reserpine eliminates the IOP-lowering effects of both timolol and WIN*

Finally, in order to directly demonstrate the dependence on catecholamines of the IOP-lowering effects of both timolol and WIN, C57BL6 mice were pretreated with the catecholamine-depleting agent, reserpine. For this, it was first established that reserpine treatment did not significantly affect the baseline IOP of these mice (*Figure 9A*). Then, since timolol is a βAR antagonist and, therefore, produces its effect by blocking activation of βARs by catecholamines, the effect of timolol treatment was assessed in reserpine- and saline control-treated mice in order to demonstrate catecholamine depletion (*Figure 9B*). Timolol significantly reduced IOP in saline control-treated mice (*p*<0.05), but did not significantly affect IOP in the reserpine-treated animals (*p*>0.05). Similarly, WIN treatment was found to reduce IOP in the saline control animals (*p*<0.01), but did not reduce IOP in reserpine-treated animals (*Figure 9C; p*>0.05). These findings demonstrate that catecholamines are required for the IOP-lowering effects of both the βAR antagonist, timolol, as well as the cannabinoid agonist, WIN.

*Discussion*
Cannabinoid agonists have long been known to lower IOP (Hepler and Frank, 1971; Merritt et al., 1980). While the mechanism(s) underlying this ocular hypotensive effect has remained unclear, several previous studies have suggested some involvement of the adrenergic nervous system (Green and Kim, 1976; Green et al., 1977; Oltmanns et al., 2008). The present study, using genetic receptor knock-out models and pharmacology, demonstrates that cannabinoids reduce IOP primarily by acting as indirect sympatholytic agents. This mechanism involves CB$_1$ receptors and is dependent on the presence of both catecholamines and βARs. Together with evidence that CB$_1$ receptors colocalized with adrenergic inputs in the anterior eye, our findings indicate that cannabinoids produce their primary ocular hypotensive effect in the mouse eye by inhibiting NE release.

Intraocular pressure is maintained in the eye through the balance of AH secretion from the ciliary body, and outflow through either the trabecular meshwork or the uveoscleral outflow pathways. Since both CB$_1$ cannabinoid receptors and βARs are expressed in the tissues involved in each of these processes (Watanabe and Chiu, 1983; Alvarado et al., 1998; Straiker et al., 1999), the overall effect on IOP of drugs acting on these receptors are likely the result of the combination of their effects on each of these tissues. For example, both βAR antagonists and agonists can reduce IOP, βAR antagonists decrease AH inflow from the ciliary body (Watanabe and Chiu, 1983), while βAR agonists increase AH outflow. (Alvarado et al., 1998). Given that CB$_1$ receptors are expressed in tissues of both inflow and outflow pathways, it is also likely that these compounds, acting
either indirectly or directly, have more than one site of action affecting IOP (Chien et al., 2003; McIntosh et al., 2007; Stumpf et al., 2005).

The results of this study are consistent with previous work demonstrating that when cannabinoid agonists and βAR antagonists are given in combination they do not produce an additive effect (Green et al., 1977; Oltmanns et al., 2008). This observation is noteworthy in that IOP-lowering agents acting through independent mechanisms are typically found to have additive effects both in rodents and humans (Akaishi et al., 2009). While previous studies have suggested this lack of additive effect may indicate that that the ocular hypotensive actions of cannabinoids involve βARs (Green et al., 1977), the present study provides the first evidence that cannabinoid agonists do not reduce IOP in βAR-/- mice, directly demonstrating for the first time the involvement of βARs in the ocular hypotensive actions of cannabinoids.

The most plausible mechanism accounting for this observation is that CB₁ receptor agonists lower IOP primarily by activating pre-synaptic CB₁ receptors to inhibit norepinephrine release, as has been shown in other tissues (Ishac et al., 1996), and which is consistent with our observation that CB₁ colocalizes with adrenergic inputs in the anterior eye. This pre-synaptic effect should decrease AH secretion from the ciliary epithelium, leading to reduce IOP. However, given that activation of βARs in the trabecular meshwork increases outflow, this same pre-synaptic effect of cannabinoids could conceivably also increase IOP, by decreasing trabecular outflow. Indeed the net adrenergic-dependent effect of cannabinoids on IOP is likely to result from the balance of these two actions. Considering the fact that
there is substantially more adrenergic innervation to the subepithelial layer of ciliary body than there is to the trabecular meshwork (Akagi et al., 1976), it is not surprising that the IOP-lowering effect on AH secretion predominates. Indeed, such a mechanism is consistent with previous work demonstrating that, at least in primates, that cannabinoids reduce IOP through actions on AH secretion (Chien et al., 2001). However, it should be noted that although CB₁ was found to colocalize with adrenergic inputs in the anterior eye, the bulk of CB₁ staining did not overlap with the inputs, suggesting that CB₁ may have additional post-synaptic (or non-synaptic) functions. Recent work has demonstrated that CB₁ receptors heterodimerize with β₂AR receptors (Hudson et al., 2010), and since both of these receptors are expressed within the ciliary body epithelium (Crider and Sharif, 2002; Stamer et al., 2001; Straiker et al., 1999), the possibility that CB₁/β₂AR heterodimerization within the ciliary epithelial cells contributes to the ocular hypotensive properties of cannabinoids cannot be completely ruled out.

In addition to a possible action at the ciliary epithelium on AH inflow, CB₁/β₂AR heterodimerization could also affect AH outflow. Previous work has suggested cannabinoids may increase trabecular outflow (Stumpff et al., 2005) and CB₁/β₂AR heterodimerization modulates the function of β₂ARs within trabecular meshwork cells (Hudson et al., 2010). Therefore this heterodimer, at least theoretically, could be one mechanism that explains the fact that cannabinoid and βAR agonists did not reduce IOP in CB₁⁻/⁻ and βAR⁻/⁻ mice. However, the pharmacological properties of the CB₁/β₂AR heterodimer are such that this interaction appears to have more of a modulatory effect on CB₁ and β₂AR signaling
(Hudson et al., 2010), and thus is not likely to account for the complete loss of ocular hypotensive function observed in the knockout mice used in the present study.

Our findings in both C57BL6 and CD1 genetic background CB\(_1\)/-/- mice confirm the involvement of CB\(_1\) receptors in the ocular hypotensive actions of cannabinoids. However, the fact that WIN not only failed to reduce the IOP of CB\(_1\)/-/- mice, but instead actually produced a small increase in IOP was surprising. Pharmacological evidence in rodents has reported that WIN mediates its IOP effects through the CB\(_1\) receptor (Oltmanns et al., 2008; Pate et al., 1998; Song and Slowey, 2000), however this has never been directly tested in mice genetically lacking this receptor. Considering our findings, it appears that there is at least one additional, non-CB\(_1\), ocular target for WIN. As the WIN-mediated IOP increase was not blocked by the CB\(_2\) antagonist AM630, nor mimicked by a chemically distinct cannabinoid agonist, CP55,940, the effect is likely specific to WIN, or potentially to the aminoalkylindole class of cannabinoid agonists to which WIN belongs. The chemical structures of aminoalkylindoles are quite distinct from all other CB\(_1\) agonist classes (Howlett et al., 2002), so it is perhaps not surprising that this class would have actions at additional non-CB\(_1\)/CB\(_2\) targets.

More difficult to account for, however, is the lack of IOP response to either timolol or ISO in the CB\(_1\)/-/- mice. The fact that timolol and ISO reduce IOP through completely independent mechanisms of action (Alvarado et al., 1998; Watanabe and Chiou, 1983), suggests that the only commonality shared by these ligands is that they both produce their effects through actions on the βARs. Based on this, the lack of effect for both timolol and ISO in CB\(_1\)/-/- mice could likely be explained by a
compensatory desensitization of βARs that occurs in the CB$_{1}^{-/-}$ mice. Such a desensitization would not be unexpected, since the elimination of inhibitory presynaptic CB$_{1}$ receptors would increase basal noradrenergic tone, an effect seen in other tissues of CB$_{1}^{-/-}$ mice (Schlicker et al., 2003), which in turn could produce desensitization of postsynaptic βARs. This hypothesis may also be supported by our observation that basal IOP at 09:00 was decreased in both CB$_{1}^{-/-}$ and β$_{2}$AR$^{-/-}$ mice compared with wild-type mice. Indeed, one might predict that desensitization or genetic elimination of βARs in the ciliary body could lead to decreased basal levels of AH secretion, and as a result lower IOP. As a proof of principle, our demonstration that chronic ISO treatment does indeed desensitize the timolol IOP response, indicates that even after only two weeks of receptor over-activation, desensitization of βARs can be observed in mice.

The overall findings of this study demonstrate that cannabinoid agonists reduce IOP through activation of the CB$_{1}$ receptor, which in turn affects IOP through a mechanism that is dependent on the presence of both catecholamines and βARs. Considering this mechanism of action, it may be expected that cannabinoid agonists acting on this pathway are unlikely to produce better IOP-lowering agents than currently available βAR antagonists. Given this, the results presented in this study indicate CB$_{1}$-targeting cannabinoid agonists may not to be useful therapeutics when used for their ocular hypotensive abilities alone. In addition, the results of the present study suggest that the aminoalkylindole class of cannabinoid agonists in particular are the least likely to be useful therapeutics, since they appear to produce additional non-CB$_{1}$/CB$_{2}$ effects on AH dynamics, which are likely to reduce their
overall ability to lower IOP. Despite these limitations, cannabinoid compounds may still be of interest in the treatment of glaucoma, based on their neuroprotective properties (Jarvinen et al., 2002; Nucci et al., 2008). Recent evidence has also suggested that cannabinoid compounds that act at receptor targets independent of CB₁ and CB₂ may also reduce IOP (Colasanti et al., 1984; Szczensiak et al., 2011), and therefore may represent alternative clinically useful drugs in the treatment of glaucoma. Based on the findings of the present study, we suggest that it is these non-CB₁ cannabinoid targets, as well as the neuroprotective actions of cannabinoids that are likely to be the most successful in producing therapeutically useful cannabinoids for the treatment of glaucoma.

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Authorship contributions

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act at novel cannabinoid receptors to reduce intraocular pressure. J Ocul Pharmacol Ther (In Press)


Footnotes

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Figure Legends

Figure 1. Diurnal variation in the IOP of C57BL6, βAR−/−, CB1−/− and CB2−/− mice. A. Mean IOP data recorded from wild type male C57BL6 mice at 09:00 and 21:00 of the same day. ***p<0.001; n=9. B. Mean IOP data recorded from male βAR−/− mice at 09:00 and 21:00 of the same day. ***p<0.001; n=8. C. Mean IOP measured from C57BL6-CB1−/− mice at 09:00 and 21:00 of the same day. **p<0.01; n=6. D. IOP measured from CB2−/− mice at 09:00 and 21:00 of the same day. ***p<0.001; n=8.

Figure 2. Topical application of βAR antagonists and agonists both reduce IOP in C57BL6 mice. A. IOP data from wild type C57BL6 mice treated topically with either vehicle or 0.5% timolol. Data are plotted as individual points showing the difference between vehicle and treated eyes of the same animal. ***p<0.001. B. Mean IOP data recorded from wild type male C57BL6 mice treated with: vehicle, the non-selective βAR antagonist timolol (0.5%; n=9), or the β1AR selective antagonist metoprolol (1.0%; n=5). ***p<0.001, **p<0.01 compared with the appropriate vehicle control. C. IOP measured from wild type male C57BL6 mice treated with vehicle or the non-selective βAR agonist ISO (1.0%; n=5). **p<0.01.

Figure 3. Cannabinoid agonists reduce IOP in C57BL6 mice, but are not additive with βAR antagonists. A. Mean IOP data recorded from wild type male C57BL6 mice treated for 1 h with: vehicle or WIN (1.0%; n=6), or with vehicle or CP (1.0%; n=6). **p<0.01, ***p<0.001 compared with appropriate vehicle control. B. Mean change in
IOP between vehicle and WIN treated (0.25, 0.5, 1.0 and 2.0%) eyes of the same animal. Statistically significant changes in IOP between vehicle and treated eyes are indicated as **p<0.01 and ***p<0.001 respectively; n=5. **C. Mean IOP measured from wild type male C57BL6 mice treated with Vehicle or WIN (1%; solid bars; n=6) or with a combination of WIN (1%) and: timolol (0.5%; dark grey bars; n=6), or latanoprost (0.005%; open bars; n=5). ***p<0.001, **p<0.01, *p<0.05 compared with the appropriate vehicle control. **D. Mean change in IOP following treatment with WIN, WIN + timolol (+timolol), and WIN + latanoprost (+Latano). *p<0.05.

**Figure 4.** CB$_1$ colocalizes with tyrosine hydroxylase in the murine anterior eye. **A)** Left and center panels show tyrosine hydroxylase (TH) and CB$_1$ staining, respectively, in the ciliary epithelium (CilEp). Right panel shows TH (green) and CB$_1$ (red), with overlapping staining in yellow (arrows). **B)** Overview of ciliary epithelium (CilEp) including the angle shows TH and CB$_1$ staining in the angle (arrow). **C)** In the cornea, CB$_1$ is present in corneal epithelium (CEP), stroma (Str) and corneal endothelium (CEnd) and colocalizes with TH in the peripheral distal stroma (arrows). **D)** CB$_1$ exhibits partial overlap with TH in the iris (arrows). Scale bars: 25 μm.

**Figure 5.** βAR antagonists, βAR agonists and cannabinoid agonists do not reduce the IOP of βAR$^{-/-}$ mice. **A.** Mean IOP data recorded from βAR$^{-/-}$ mice following the topical administration of vehicle with the βAR antagonists, timolol (0.5%; solid bars; n=5) and metoprolol (1.0%; grey bars; n=5); or the βAR agonist ISO (1.0%; open bars;
n=5). *p<0.05 compared with appropriate vehicle. B. Mean IOP data recorded from βAR−/− mice following the topical administration of either vehicle or latanoprost. *p<0.05; n=6. C. Mean IOP data obtained from βAR−/− mice following 1 h topical administration of vehicle or: the non-selective cannabinoid agonists, WIN (1.0%; n=13) and CP (1.0%; n=5); or the CB1 selective agonist, ACEA (1%; n=4). D. Time course for change in IOP in βAR−/− mice following administration of WIN 55,212-2 (1.0%; n=5).

Figure 6. The effect of cannabinoid and βAR ligands on IOP in CB1−/− and CB2−/− mice on a C57BL6 Genetic Background. A. Mean IOP data recorded from either C57BL6 CB1−/− (n=3) mice or CB1+/+ (n=5) littermates following the topical application of either vehicle or WIN (1%). *p<0.05 compared with the appropriate vehicle control. B. Mean IOP measured in CB1−/− mice after treatment with vehicle as well as: timolol (0.5%; n=4), ISO (1%; n=3), or latanoprost (0.005%; n=4). C. IOP measured in CB2−/− mice following 1 h topical administration of WIN (1.0%; solid bars) or timolol (0.5%; open bars). ***p<0.001, **p<0.01 compared with appropriate vehicle control.

Figure 7. The effect of cannabinoid and βAR ligands on IOP in CB1−/− mice on a CD1 genetic background. A. Mean IOP measured from male wild type CD1 mice following topical application of the appropriate vehicle, or timolol (0.5%), WIN (1.0%), or CP (1.0%). ***p<0.001 compared with appropriate vehicle control. B. Mean IOP measured in male CD1-CB1−/− mice after treatment with vehicle as well as: timolol (0.5%; n=8), WIN (1%; n=8), or dorzolamide (1.0 %; n=8). ***p<0.001, *p<0.05
compared with the appropriate vehicle control. C. IOP measured from male CD1-CB1−/− mice following topical treatment with vehicle, WIN (1.0%), or CP (1.0%) in the absence, or presence of pre-treatment with the CB2 antagonist AM630. ***p<0.001 compared with the appropriate vehicle control. φ p<0.05 compared with non-AM630 treated vehicle.

Figure 8. Chronic topical application of the βAR agonist ISO, desensitizes the IOP response to timolol in C57BL6 mice. A. Mean IOPs measured from wild type C57BL6 mice treated for one week with saline vehicle (solid bars; n=5) or ISO (2.5%; open bars; n=5), followed by topical application of either vehicle or timolol (1%). ***p<0.001 compared with the appropriate vehicle control. B. Mean IOPs measured from wild type C57BL6 mice treated for two weeks with saline vehicle (solid bars; n=5) or ISO (2.5%; open bars; n=4), followed by topical application of vehicle, timolol (0.05%) or WIN (1%). **p<0.01, *p<0.05 compared with the appropriate vehicle control.

Figure 9. Pre-treatment with reserpine to deplete catecholamines eliminates the ability of either timolol and WIN to reduce IOP. A. Mean IOP recorded from each eye in C57BL6 mice pre-treated with saline vehicle or with reserpine to deplete catecholamines. B. Mean IOP data for either vehicle or timolol treatment in mice pre-treated with either saline (solid bars; n=3), or reserpine (open bars; n=5) to deplete catecholamines. *p<0.05 compared with the appropriate vehicle control.
Mean IOP data measured following topical application of either vehicle or WIN from mice pre-treated with either saline (solid bars; n=4), or reserpine (open bars; n=6) to deplete catecholamines. **p<0.01 compared with the appropriate vehicle control.
Figure 1

A

C57BL6

IOP (mmHg)

09:00 21:00

B

βAR−/−

IOP (mmHg)

09:00 21:00

C

CB1−/−

IOP (mmHg)

09:00 21:00

D

CB2−/−

IOP (mmHg)

09:00 21:00

** ***
Figure 5

A

IOP (mmHg)

Vehicle  Timolol  Vehicle  ISO  Vehicle  Metoprolol

B

IOP (mmHg)

Vehicle  Latanoprost

C

IOP (mmHg)

Vehicle  WIN  Vehicle  CP  Vehicle  ACEA

D

Change in IOP (mmHg)

Length of WIN Treatment (h)
Figure 7

A

CD1-Wt

IOP (mmHg)

Vehicle Timolol Vehicle WIN Vehicle CP

9 10 11 12

B

CD1-CB₁⁻⁻

IOP (mmHg)

Vehicle Timolol Vehicle WIN Vehicle Dorzolamide

8 9 10 11 12

C

IOP (mmHg)

Vehicle WIN AM630 + Vehicle AM630 + WIN Vehicle CP

9 10 11 12

** Stars indicate statistical significance.