CB2 Cannabinoid Receptors in Trabecular Meshwork Cells Mediate JWH015-Induced Enhancement of Aqueous Humor Outflow Facility

Lichun Zhong, Lijun Geng, YaFatou Njie, Wenke Feng, and Zhao-Hui Song

Purpose. To study the effects of JWH015, a CB2-selective agonist, on aqueous humor outflow facility, to investigate whether functional CB2 cannabinoid receptors are expressed in trabecular meshwork cells, and to study whether these receptors are involved in the enhancement of outflow facility induced by JWH015.

Methods. A porcine anterior segment perfused organ culture model was used to measure the effects of JWH015 on aqueous humor outflow facility. Immunofluorescence microscopy, Western blot analysis, and mitogen-activated protein (MAP) kinase activity assays were used to study the expression of CB2 cannabinoid receptors on cultured porcine trabecular meshwork cells and the coupling of these receptors to p42/44 MAP kinase.

Results. The outflow facility was increased dose dependently within 1 hour after adding 10, 30, and 100 nM of JWH015, a CB2 agonist. In addition, the effect of 100 nM of JWH015 was completely blocked by SR144528, a selective CB2 antagonist. Furthermore, the outflow-enhancing effect of JWH015 was blocked by pretreatment with PD98059, an inhibitor of the p42/44 MAP kinase pathway. In immunofluorescence microscopy and Western blot studies, positive signals were detected on cultured porcine trabecular meshwork cells with an anti-CB2 antibody. In MAP kinase assays, treatment of porcine trabecular meshwork cells with 100 nM of JWH015 activated p42/44 MAP kinase activity. Pretreatment with SR144528 blocked the effect of JWH015 on p42/44 MAP kinase activity.

Conclusions. The data from this study demonstrate that the CB2-selective cannabinoid agonist JWH015 increases aqueous humor outflow facility. The results also indicate that functional CB2 cannabinoid receptors are expressed in trabecular meshwork cells, and these receptors are involved in the enhancement of outflow facility induced by JWH015. (*Invest Ophthalmol Vis Sci.* 2005;46:1988–1992) DOI:10.1167/iovs.04-0651

Previous studies have shown that cannabinoids are effective in reducing intraocular pressure (IOP) in humans, as well as in animal models.[1–4] Although cannabinoids can decrease the rate of aqueous humor formation, it has been suggested that the IOP-lowering effect of cannabinoids is primarily mediated by increasing aqueous outflow facility.[3,5] However, the exact mechanisms for cannabinoid-induced IOP reduction remain unclear. According to the current literature, it is unknown whether cannabinoids exert a direct effect on anterior chamber aqueous humor outflow and whether cannabinoid receptors on the outflow tissues are involved.

Earlier studies on the IOP-lowering effects of cannabinoids were performed before the discovery of cannabinoid receptors and involved the use of Δ^9-THC and congeners, which have only low affinity for these receptors.[1–3,5] In recent years, cannabinoid agonists with much higher affinities than Δ^9-THC have been developed.[6,7] Furthermore, selective agonists and antagonists for subtypes of cannabinoid receptors are now available.[6,7] These newer ligands are very powerful tools, and they will enable us to understand the role of cannabinoid receptors in IOP control.

Two subtypes of cannabinoid receptors, CB1 and CB2, have been cloned.[8,9] CB1 is distributed in the central nervous system as well as in peripheral tissues, whereas CB2 is distributed only in the periphery.[6,8,9] Because there is no CB2 receptor located in the brain, potential therapeutic cannabinoids that are selective for this receptor should be devoid of the psychoactive effects of marijuana.

As a first step in exploring the potentials of CB2 cannabinoid receptors as a possible target for lowering IOP, in this study we first investigated the effects of JWH015, a CB2 selective agonist, on aqueous humor outflow facility. We then investigated whether functional CB2 cannabinoid receptors are expressed in trabecular meshwork cells and whether these receptors are involved in the enhancement of outflow facility induced by JWH015.

Methods

Porcine Anterior Segment Perfused Organ Culture Model

A previously published procedure[10] was followed for the anterior segment perfused organ culture model. Fresh porcine eyes were obtained from a local slaughterhouse within 30 minutes after decapitation. Porcine anterior segment explants, comprising the intact cornea, the undisturbed trabecular meshwork, and a 2- to 5-mm rim of sclera with the ciliary body and iris were gently removed and mounted in a standard perfusion culture apparatus and perfused with culture medium (DMEM) using a constant-perfusion head of 10 cm (approximately 7.35 mm Hg) for 1 day, while outflow stabilized. Only those explants that stabilized between 1.5 and 8 μL/min at 7.35 mm Hg were used. Cultures were maintained at 37°C with 5% CO_2 and 95% air. It had already been shown that in this model, outflow is through the trabecular meshwork, and flow rates are physiological (~2.75 μL/min).[10] At the end of the perfusion study, the anterior segments were perfusion fixed at 7.35 mm Hg constant pressure with 4% paraformaldehyde for 1 hour. Anterior segments were then removed from the perfusion chamber, and 2- to 3-mm wide wedges from each quadrant containing outflow tissues were cut and immersed in 10% formalin for 1 hour and then in 70% alcohol overnight. Subsequently, tissues were embedded in paraffin and stained with hematoxylin and eosin (HE). The viability of outflow pathway tissues was evaluated by light micros-

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copy. Perfusion studies were regarded as invalid and data discarded if more than one quadrant per eye had unacceptable morphologic findings, such as excessive trabecular meshwork cell loss and denudation of trabecular beams.

JWH015 (Tocris Cookson Inc., Ellisville, MO) was applied in the perfusion medium and vehicle control was run in parallel. Outflow facility was calculated as the ratio of the rate of flow of perfusate (microliters per minute) to the steady state perfusion pressure (mm Hg). Drug effects were evaluated in each eye as the percentage change in outflow facility in drug-treated eyes over predrug baseline outflow facility.

At least six eyes were used for each group of treatment. The data were presented as mean ± SE. The data were analyzed on computer (Prism; GraphPad, San Diego, CA) software and plotted as the change in outflow facility versus time (in minutes).

Culture of Porcine Trabecular Meshwork Cells

The trabecular meshwork was isolated from fresh porcine eyes by blunt dissection. Culture of trabecular meshwork cells was performed according to previously published methods.[11,12] The identity of trabecular meshwork cells was established by their morphology and their ability to take up acetylated low-density lipoprotein and to secrete tissue plasminogen activator.

Immunofluorescent Microscopy

Cultured porcine trabecular meshwork cells were grown on cover glass (Fisher Scientific Inc., Pittsburgh, PA). Cells were washed twice with 0.1 M PBS, fixed with 4% paraformaldehyde for 15 minutes, and washed twice again with PBS. Subsequently, cells were blocked for 1 hour at room temperature in PBS containing 5% normal goat serum (NGS) and then incubated for 2 hours at room temperature with anti-CB2 antibody (Cayman Chemical, Ann Arbor, MI) at 1:1000 dilution in PBS containing 5% NGS. After they were washed three times with PBS containing 5% NGS for 10 minutes each time, the cells were incubated for 1 hour at room temperature with fluorescein isothiocyanate-conjugated anti-rabbit IgG secondary antibody (Zymed, S. San Francisco, CA). Finally, the cover glasses were washed four times with PBS, mounted with antifade medium (Vectashield; Vector Laboratories, Burlingame, CA), and viewed with a fluorescence microscope (model IX50; Olympus, Lake Success, NY).

Western Blot Analysis

Membrane samples were prepared from porcine trabecular meshwork cells according to published procedures.[13] Samples were incubated with 2× Laemmli buffer under reducing conditions at room temperature for 20 minutes, and proteins were resolved on a 10% SDS-polyacrylamide gel using a minigel electrophoresis system (Invitrogen, Carlsbad, CA). Protein bands were transferred onto a nitrocellulose membrane for immunoblotting. The nitrocellulose membranes were blocked with 5% nonfat dried milk in TBS-T buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 0.3% Tween 20) for 1 hour and then incubated overnight at 4°C with primary anti-CB2 antibody. Subsequently, the membranes were washed twice for 10 minutes each time with TBS-T buffer and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ) for 1 hour at room temperature. The membranes were then washed three times with TBS-T buffer for 10 minutes each time, and the antibody-recognized protein bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ).

MAP Kinase Activity Assay

p42/44 mitogen-activated protein (MAP) kinase activity was determined by a p42/44 MAP kinase assay kit (New England Biolabs, Inc., Beverly, MA), according to the manufacturer's instructions. The cells were grown to 80% confluence in 60-mm tissue culture dishes and then maintained in DMEM containing 0.5% fetal calf serum for 18 hours. After exposure to DMEM in the presence or absence of JWH015 for 15 minutes at 37°C, cells were washed once with ice-cold PBS containing 1 mM sodium orthovanadate and then lysed with 0.5 mL ice cold cell lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The solubilized cell extracts were clarified by centrifugation at 14,000*g* for 10 minutes. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL), and 200 μg protein was incubated overnight at 4°C with immobilized phospho-p44/42 MAP kinase (Thr202/Tyr204) monoclonal antibody. After it was washed with lysis buffer and then kinase buffer, the pellet was incubated with kinase buffer containing 200 μM adenosine triphosphate (ATP) and 2 μg Elk-1 fusion protein for 30 minutes at 30°C. The reaction was terminated by the addition of 3× SDS-PAGE sample buffer. Subsequently, the samples were heated for 5 minutes at 95°C and centrifuged for 2 minutes. The supernatants (20 μL) were separated on 10% Tris-glycine gels before being blotted onto nitrocellulose filters. To prevent nonspecific binding of antibodies, the blots were incubated in blocking buffer containing 20 mM Tris (pH 7.6), 137 mM NaCl, 0.1%Tween-20, and 5% nonfat dried milk. Subsequently, the blots were incubated in anti-phospho-Elk-1 antibody (1:1000 dilution) with gentle agitation overnight at 4°C. After an extensive washing, the blots were incubated for 1 hour at room temperature with HRP-conjugated anti-rabbit secondary antibody (1:2000 dilution). After another washing, the signals were detected with an HRP Western blot detection kit (New England Biolabs, Inc.) and autoradiography. The bands on x-ray films were scanned by a densitometer and analyzed on computer (Personal Densitometer SI with ImageQuant software; Molecular Dynamics, Sunnyvale, CA).

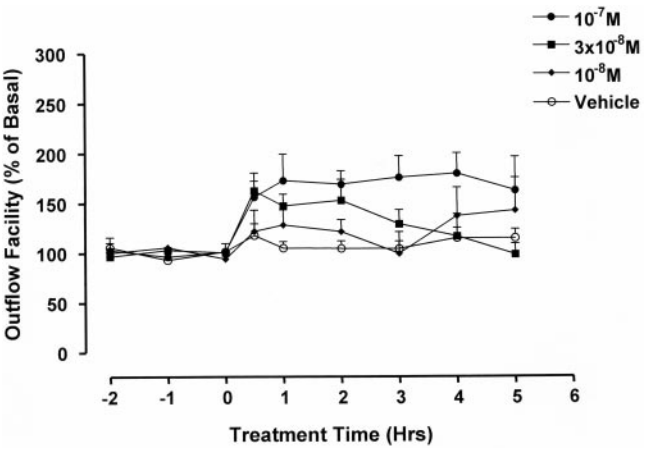

FIGURE 1. The effects of JWH015 on aqueous humor outflow facility. Different concentrations of JWH015 were used. Results are expressed as the mean ± SE.

Statistical Analyses

For anterior segment perfusion studies, unpaired two-tailed Student's *t*-tests were used to compare the data points of the treatment groups. For p42/44 MAP kinase assays, ANOVA with Newman-Keuls posttests were used. The level of significance was chosen as $P < 0.05$.

RESULTS

The Effects of JWH015 on Outflow Facility

Outflow facility studies were performed using the porcine anterior segment perfused organ culture model described herein. As shown in Figure 1, the outflow facility increased dose dependently within 1 hour after adding 10, 30, or 100 nM

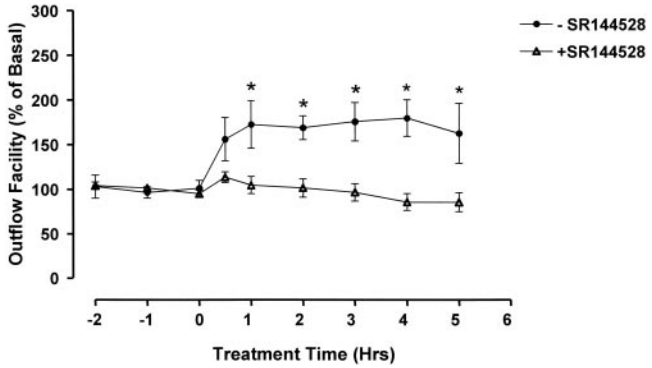

FIGURE 2. Antagonism of JWH015-induced increase of aqueous humor outflow facility by SR144528. The anterior segments were treated with 1 μM SR144528 30 minutes before the addition of 100 nM JWH015. Results are expressed as the mean ± SE. *Significant difference at each time point between JWH015 alone and JWH015+SR144528 ($P < 0.05$, *t*-test).

JWH015, a CB2 agonist. This effect lasted for at least 5 hours at 100 nM JWH015. In addition, the effect of 100 nM JWH015 was completely blocked by pretreatment with 1 μM SR144528,[14] a selective CB2 antagonist, 30 minutes before JWH015 treatment (Fig. 2). In control experiments, either medium or SR144528 alone was added. Neither the medium (Fig. 1) nor SR144528 (data not shown) had any significant effects on outflow facility.

Identification of CB2 Cannabinoid Receptors on Porcine Trabecular Meshwork Cells

Immunofluorescence microscopy and Western blot analysis were performed to determine whether CB2 cannabinoid receptors are expressed on trabecular meshwork cells. In immunofluorescence microscopy studies, positive signals were detected on porcine trabecular meshwork cells with a primary anti-CB2 antibody (Fig. 3A). In contrast, when the primary anti-CB2 antibody was preabsorbed with peptide antigen, only weak, nonspecific background signals were detected (Fig. 3B). In Western blot analysis, a protein band with a molecular weight of 44 kDa was detected with the anti-CB2 antibody, but not with the same antibody preabsorbed with peptide antigen (Fig. 3C).

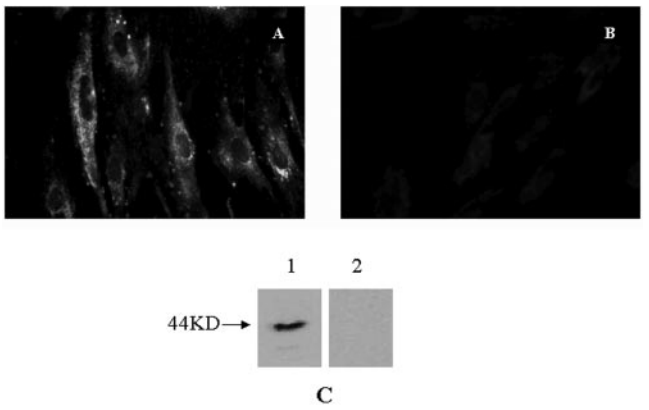

FIGURE 3. Expression of CB2 cannabinoid receptors on porcine trabecular meshwork cells. (**A**) CB2 receptor immunofluorescent activity observed with a primary anti-CB2 antibody. (**B**) Background immunofluorescence observed with the primary anti-CB2 antibody preabsorbed with the peptide antigen. (**C**) Western blot analysis. *Lane 1*: with primary anti-CB2 antibody; *lane 2*: with primary anti-CB2 antibody preabsorbed with the peptide antigen.

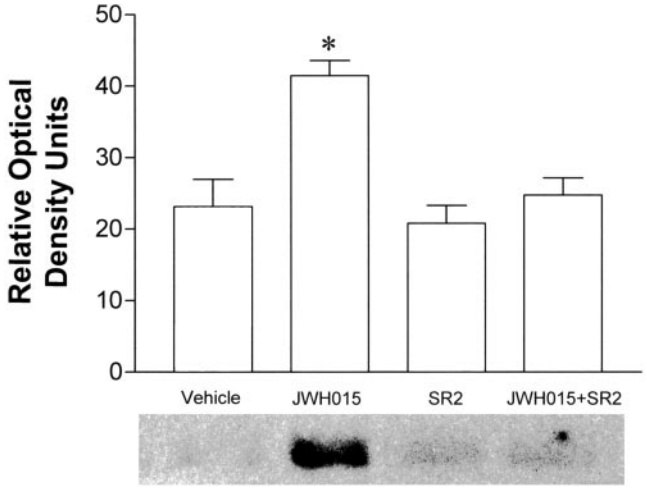

FIGURE 4. The effects of JWH015 on p42/44 MAP kinase activity in trabecular meshwork cells. *Top*: densitometry quantification of the data from three experiments. *Significant difference from vehicle alone ($P < 0.05$ ANOVA with Newman-Keuls posttest). The concentrations of JWH015 and SR144528 (SR2) were 100 nM and 1 μM, respectively. The time of JWH015 stimulation was 15 minutes. *Bottom*: a gel representative of results obtained in three experiments.

Effects of JWH015 on p42/44 MAP Kinase Activity in Trabecular Meshwork Cells

One of the signaling pathways for the CB2 cannabinoid receptor is stimulation of p42/44 MAP kinase. To explore whether the CB2 receptors expressed on trabecular meshwork cells are functionally coupled to this signaling pathway and to correlate our findings of JWH015 on outflow facility studies with the effects of this ligand in vitro, we tested the effect of JWH015 on p42/44 MAP kinase activity in cultured trabecular meshwork cells. As shown in Figure 4, treatment of porcine trabecular meshwork cells with 100 nM JWH015 for 15 minutes activated p42/44 MAP kinase activity. Pretreatment for 15 minutes with 1 μM SR144528 (Fig. 4, SR2), a selective CB2 antagonist, had no measurable effect by itself, but blocked the effect of JWH015 on p42/44 MAP kinase activity.

Effects of PD98059 on JWH015-Induced Enhancement of Outflow Facility

PD98059, an inhibitor of the p42/44 MAP kinase pathway, was used to explore the possibility that p42/44 MAP kinase may be involved in the JWH015-induced enhancement of outflow facility. As shown in Figure 5, pretreatment of the perfused porcine anterior segments with 30 μM PD98059 significantly blocked the outflow-enhancing effects of 100 nM JWH015. PD98059 by itself had no significant effect on outflow facility (data not shown).

DISCUSSION

Earlier studies on the IOP-lowering effects of cannabinoids were performed before the discovery of cannabinoid receptors, and used Δ^9THC and derivatives which have only low affinity for these receptors.[1–3,5] In recent years, newer cannabinoid ligands such as anandamide, an endogenous cannabinoid agonist; CP55940, a bicyclic cannabinoid agonist; and WIN55212-2, an aminoalkylindole cannabinoid agonist, have all been found to lower IOP after topical administration to the eyes.[15–17] However, currently, the mechanisms for the IOP-lowering effects of cannabinoids are still not clear.

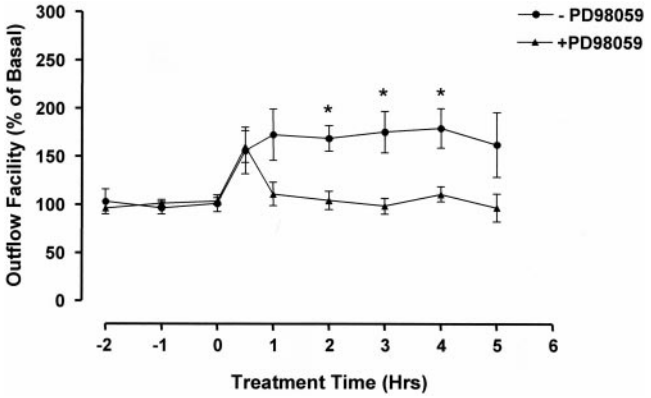

FIGURE 5. The effects of pretreatment with PD98059 on JWH015-induced enhancement of aqueous humor outflow facility. The anterior segments were treated with 30 μM PD98059 30 minutes before the addition of 100 nM JWH015. Results are expressed as the mean ± SE. *Significant difference at each time point between JWH015 alone and JWH015+PD98059 ($P < 0.05$, *t*-test).

The maintenance of IOP depends on a dynamic balance between the secretion of aqueous humor by the ciliary body and the outflow of aqueous humor through the trabecular meshwork and uveoscleral route. There are already plenty of drugs on the market to achieve the suppression of aqueous humor formation. Because cannabinoids have been found to increase outflow facility, these compounds may be useful additions to the therapeutic agents for glaucoma treatment. Using rabbits, cats, and monkeys, cannabinoids have been shown to lower IOP in vivo.[3,5] However, in the current literature it is unknown whether cannabinoids enhance outflow facility directly in the eye. Anterior segment perfused organ culture preserves the architecture of the outflow pathway and therefore is an excellent model for studying aqueous humor outflow.[18,19] In the present study, this model was successfully used to examine the responsiveness of aqueous outflow to JWH015, a selective CB2 agonist. Application of JWH015 enhanced aqueous humor outflow facility in the perfused porcine anterior segment. This outflow-enhancing effect of JWH015 was dose dependent and was blocked by pretreatment with SR144528, a selective CB2 antagonist. Thus, the data from the current anterior segment perfusion study suggest an involvement of CB2 cannabinoid receptor in JWH015-induced increase in outflow facility. It is worth mentioning that 100 nM JWH015 was needed to achieve the maximum effect in the current outflow perfusion studies, whereas the K_i of JWH015 in ligand-binding studies is 14 nM.[7] This is not unexpected, because cannabinoid ligands are notoriously hydrophobic, and therefore some loss of ligands is likely to occur as a result of nonspecific binding to surfaces of perfusion chambers and tubing. In contrast, in ligand binding studies, it is possible to minimize the loss of cannabinoid ligands by siliconizing the glassware and the addition of bovine serum albumin.[13]

Using human anterior segment sections, Straiker et al.[20] have demonstrated the presence of CB1 cannabinoid receptor in trabecular meshwork. In addition, Stamer et al.[21] recently reported the expression and activation of CB1 receptor in trabecular meshwork in both bovine and human tissues. However, the expression of CB2 cannabinoid receptor in the trabecular meshwork cells has not been reported. In the present study, with the use of both immunofluorescence microscopy and Western blot analysis, CB2 cannabinoid receptor protein was detected on porcine trabecular meshwork cells using an anti-CB2 primary antibody. These results demonstrate the presence of CB2 receptors in the trabecular meshwork and support the notion that JWH015 may act on CB2 receptors in the anterior segment perfusion model to induce an increase in outflow facility.

Activation of p44/42 MAP kinase activity is one of the well-characterized signaling pathways for the CB2 cannabinoid receptor.[22] In this study, JWH015 was shown to activate p44/42 MAP kinase activity in cultured porcine trabecular meshwork cells. This effect of JWH015 was blocked by pretreatment of the cells with SR144528, a selective CB2 antagonist. These data demonstrate the existence of functional CB2 cannabinoid receptors that are coupled to p42/44 MAP kinase in trabecular meshwork cells. It has been reported that p44/42 MAP kinase plays an important role in the cellular functions of trabecular meshwork cells.[23] Thus, our finding of JWH015-induced activation of MAP kinase in trabecular meshwork cells suggests that JWH015-induced increases of aqueous humor outflow may be mediated through the MAP kinase pathway. This hypothesis was further supported by the evidence that pretreatment of the perfused anterior segments with PD90859, an inhibitor of the p44/42 MAP kinase pathway, blocked JWH015-induced enhancement of outflow facility.

In the literature, it has been reported that JWH133, another selective agonist for CB2 cannabinoid receptor, does not lower IOP after topical application to the rabbit eye.[24] The differences between this previous negative report on the effects of JWH133 on IOP and our current positive data on the outflow-enhancing effects of JWH015 could be due to several reasons, including different species used (rabbit versus pig), different models used (intact eyes versus perfused anterior segments), the different routes by which the drugs were administered (topical versus perfusion), or the different absorption efficiencies of the two drugs.

Cannabinoids have been proposed as a new class of antiglaucoma drug. However, one of the major problems of cannabinoids as therapeutic agents is their severe psychoactive effect. The cloning of cannabinoid receptor subtypes and the development of subtype-selective cannabinoid ligands have provided us with new hope for a better separation of the therapeutic effects of cannabinoids from their undesired psychoactive side effects. Because the CB2 cannabinoid receptor is not located in the brain, cannabinoid drugs that act specifically on CB2 should not have the psychoactive effects of marijuana. The most important finding of this study is that CB2 cannabinoid receptors in the outflow pathway are involved in JWH015-induced enhancement of outflow facility. This suggests that CB2 cannabinoid receptors in the eye may be explored as a therapeutic target for developing nonpsychoactive IOP-lowering cannabinoids.

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