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Molecular and cellular changes induced by the activation of CB2 cannabinoid receptors in trabecular meshwork cells

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Purpose: To study the role of CB2 cannabinoid receptors in cellular functions of trabecular meshwork (TM) cells including cytoskeleton changes and migration and to investigate the possible signaling pathways utilized by CB2 receptor for these cellular functions.
Methods: JWH015, a selective CB2 receptor agonist, SR144528, a selective CB2 receptor antagonist, and SR141716A, a selective CB1 receptor antagonist were used on cultured porcine TM cells. In cytoskeleton studies, Alexafluor 488-labeled phalloidin staining was used to examine actin filaments and immunocytochemistry using an anti-paxillin antibody was used to detect focal adhesions on fibronectin-coated glass coverslips. Standard wound-healing assays were used to study cell migration. Rac1-GTP pull-down assays were conducted to examine the changes in the Rac1-GTPase activity. Western-blot analysis with an anti-phospho-cofilin antibody was used to measure the levels of active cofilin.
Results: JWH015 (100 nM) significantly inhibited the formation of actin stress fibers and focal adhesions in cultured TM cells. The effect of 100 nM of JWH015 on the cytoskeleton was completely blocked by 1 μM of SR144528 but not by SR141716A. The addition of 100 nM JWH015 decreased the migration of TM cells in wound-healing assays and this effect of JWH015 was blocked by 1 μM of SR144528. In contrast, SR141716A had no effect on the inhibitory effect of JWH015 on TM cell migration. In Rac1-GTP pull-down assays, treatment of TM cells with 100 nM of JWH015 decreased the activity of Rac1 GTPase activity in a time-dependent manner. Pretreatment with 1 μM SR144528, but not with SR141716A, blocked the effect of JWH015 on Rac1 activity. Western blot analysis revealed that JWH015 also diminished the level of phosphorylated cofilin in TM cells and this effect of JWH015 was antagonized by SR144528 but not by SR141716A, indicating a CB2 receptor-mediated activation of cofilin.
Conclusions: This study demonstrates that by acting through CB2 receptors, the CB2-selective cannabinoid agonist JWH015 modulates the TM cell actin's cytoskeleton and migration. This study also shows that JWH015 modulates the activities of Rac1-GTPase and cofilin, which are important signaling molecules for the cytoskeletal and migratory properties of TM cells.

Glaucoma is one of the leading causes of irreversible blindness. Since elevated intraocular pressure (IOP) is a major risk factor for glaucoma, drugs that lower IOP are used as front line treatment for glaucoma. Novel IOP-lowering drugs are needed especially those with enhanced therapeutic potency and reduced side effects. Previous studies have shown that cannabinoids are effective in reducing intraocular pressure (IOP) in animal models as well as in human subjects [1-7]. This IOP-lowering effect has been demonstrated with ligands from all four major chemical classes of cannabinoid agonists, e.g., δ9-tetrahydrocannabinol (δ9-THC) from the classic cannabinoid family, CP55940 from the non-classic, bicyclic cannabinoid family, WIN55212-2 from the aminoalkylindole family, and anandamide from endogenous fatty acid amide family [4-7].

Two major targets of cannabinoid ligands are CB1 and CB2 cannabinoid receptors [8-11]. CB1 receptors are located in the central nervous system (CNS) as well as in the periphery whereas CB2 receptors are mainly located in the peripheral tissues such as immune cells [8-11]. Earlier studies have demonstrated that CB1 receptors are located in the eye and are at least partially responsible for the IOP-lowering effects of cannabinoids [3,6,12-15]. In recent years, there is increasing evidence that functional CB2 receptors are also expressed in the eye including retina and trabecular meshwork and are involved in the functions of these ocular tissues [16,17].

Trabecular meshwork (TM), a specialized eye tissue, is a major site for regulation of the aqueous humor outflow. Malfunctioning of the trabecular meshwork is believed to be responsible for the development of glaucoma. The introduction of the perfusion organ culture model of isolated ocular anterior segments provided a valuable tool for studies of aqueous humor outflow through the trabecular meshwork [18,19]. Recently, using the perfused porcine anterior segments, we have demonstrated that the CB2 selective cannabinoid agonist, JWH015, enhances aqueous humor outflow and this effect of JWH015 is mediated through CB2 receptors in TM tissues [17]. However, currently, the cellular and molecular mechanisms underlying the CB2 receptor-mediated enhancement of outflow through TM tissues are unclear.

Understanding the mechanisms for pharmacological regulation of aqueous humor outflow through the trabecular meshwork is fundamental for developing new treatment for glaucoma. It is believed that changes in the TM cell cytoskeleton

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and cell-matrix adhesion may affect the resistance of the outflow pathway. In a number of cell types, cannabinoid ligands are known to modulate cell migration, adhesion, and cytoskeleton by acting through cannabinoid receptors [14,15,20-23]. In this study, we hypothesized that by acting through the CB2 receptors in TM cells, JWH015 may modulate the cellular properties of TM cells. To test this hypothesis, in this study, we first investigated the effects of JWH015 on the TM cell cytoskeleton and TM cell migration. Secondly, we explored the possible signaling mechanisms for JWH015-induced changes in TM cell cytoskeleton and TM cell migration by examining the effects of JWH015 on the activity of small G protein Rac1 and its downstream signaling molecule, cofilin. The involvement of CB2 receptors in JWH015-induced cellular and molecular changes of TM cells was established with the use of a selective CB2 antagonist, SR144528, and a selective CB1 antagonist, SR141716A [24,25].

METHODS

Materials: Culture plates (24-well) were purchased from Corning Costar (Cambridge, MA). JWH015 was purchased from Tocris (Baldwin, MO). SR144528 and SR141716A were obtained from the National Institute of Drug Abuse, NIH. Fatty acid-free bovine serum albumin (BSA) and fibronectin were purchased from Sigma (St. Louis, MO). Alexafluor488-conjugated phalloidin was purchased from Molecular Probes, Inc. (Eugene, OR). A polyclonal anti-paxillin antibody and a TRITC-conjugated goat anti-rabbit secondary antibody were purchased from Upstate (Lake Placid, NY). Mouse monoclonal anti-RhoA antibody, rabbit polyclonal anti-Rac1 antibody, and Rac1-GTP pull-down assay kit were purchased from Cytoskeleton (Denver, CO). Rabbit polyclonal anti-phospho-cofilin and anti-cofilin antibodies were purchased from Cell Signaling (Danvers, MA). The horseradish-peroxidase (HRP)-conjugated goat anti-rabbit antibody, sheep anti-mouse secondary antibody, and the enhanced chemiluminescence (ECL) kit were purchased from Amersham (Piscataway, NJ).

Trabecular meshwork cell culture: The trabecular meshwork (TM) was isolated from fresh porcine eyes by blunt dissection. Culture of TM cells was performed according to previously published methods [26,27]. The identity of TM cells was established by their morphology and their ability to take up acetylated low-density lipoprotein and to secrete tissue plasminogen activator.

Immunofluorescence microscopy and phalloidin staining of actin cytoskeleton: TM cells were allowed to grow to confluence on sterile glass coverslips pre-coated with 5 μg/ml fibronectin and were starved in Dulbecco's modified Eagle medium (DMEM) for 48 h. TM cells were then treated with cannabinoids for three h. Subsequently, cells were washed twice with phosphate-buffered saline (PBS), fixed with freshly prepared 4% paraformaldehyde for 15 min, washed twice with PBS, permeabilized with 0.5% Triton-X100/PBS for 10 min, washed twice again with PBS, then blocked for one h with 1% BSA/PBS. For immunohistochemistry staining, cells were incubated with a polyclonal anti-paxillin antibody for one h at 37 °C followed by an additional one h with a TRITC-conjugated goat anti-rabbit secondary antibody. For actin cytoskeleton staining, Alexa 488-conjugated phalloidin was added onto permeabilized and BSA-blocked cells at a concentration of 0.7 units/ml for one h. Finally, coverslips from immunohistochemistry and actin straining were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and viewed with a fluorescence microscope (model IX50; Olympus, Lake Success, NY).

Wound-healing assay: For the wounding-healing experiment, TM cells were seeded on fibronectin-coated (5 μg/ml) coverslips in six well plates and were allowed to grow to complete confluence. The cells were then starved in DMEM for 48 h. Subsequently, a plastic pipette tip was used to scratch the cell monolayer to create a cleared area and the wounded TM cell layer was washed with a fresh medium to remove loose cells. The cells were then refed with fresh DMEM medium without fetal bovine serum (FBS) and were treated with or without JWH015, SR144528, and SR141716A. Immediately following scratch wounding (0 h) and after incubation of cells at 37 °C for 24 h, phase-contrast images (10 x fields) of the wound-healing process were photographed digitally with an inverted microscope (Olympus IX50). The distances of the wound areas were measured on the images, set at 100% for 0 h, and the mean percentages of the total distances of the wound areas were calculated.

Western blot analysis: TM cell lysates were prepared using 20 mM Tris buffer, pH 7.4, containing 1 mM sodium orthovanadate, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 M NaCl, 50 mM NaF, 25 μg/ml aprotinin, and 25 μg/ml leupeptin. The protein concentrations of the lysates were determined by the Bradford method. Equal amounts of proteins (50 μg/lane) were separated by SDS-PAGE (10% acrylamide) followed by electrophoretic transfer of resolved proteins to polyvinylidene difluoride (PVDF) membranes. Membranes were then probed using antibodies specifically directed against RhoA, Rac1, phospho-cofilin, and cofilin followed by incubation with a HRP-conjugated secondary antibody. Detection of immunoreactivity was carried out by an enhanced chemiluminescence (ECL) kit according to manufacturer's recommendations.

Rac 1-GTP pull-down assay: Rac 1-GTP pull-down assay was performed according to the protocol from the assay kit. Briefly, after TM cells were serum-starved for 48 h, the cells were stimulated with cannabinoid ligands and immediately lysed with lysis buffer supplied in the kit. Subsequently, the lysates were centrifuged at 14,000x g for three min. A portion of supernatant from each tube was diluted in SDS-PAGE sample buffer for detection of total Rac1 GTPase. The remaining supernatant fractions were incubated with the PAK-PBD-agarose for one h at 4 °C followed by washing twice with lysis and wash buffers. Proteins bound to the beads were eluted in 2X SDS-PAGE sample buffers and subjected to western blot analysis.

Data analysis: For the wound-healing assays, images from six independent experiments were analyzed and mean percentages of the total distances of the wound areas are presented. For western blot analysis, the bands on x-ray films

were scanned (Personal Densitometer SI; Molecular Dynamics, Sunnyvale, CA) and were quantified (ImageQuant; Molecular Dynamics, Sunnyvale, CA). The bar graphs were generated with the use of GraphPad Prizm software and the data presented in figures represent mean±S.E.M. ANOVA with Neuman-Keuls post-test was used to compare the data of different treatment groups. The level of significance was chosen as $p<0.05$.

RESULTS

The effects of JWH015 on trabecular meshwork cell actin cytoskeleton: TM cells were treated with JWH015 to investigate whether the actin cytoskeleton of TM cells can be modulated by this selective CB2 receptor agonist. As shown in Figure 1A, vehicle-treated (control) TM cells kept in serum-free medium exhibited a well-organized actin cytoskeleton with F-actin fibers crossing the body of the cells and forming a dense filamentous network. Treatment of TM cells with JWH015 for three h induced alterations in Alexafluor 488-labeled phalloidin staining patterns for F-actin. Cells treated with JWH015 showed a significant reduction in the amount of stress fibers and a different pattern of these stress fibers, i.e. they are distributed more in the periphery of the cells (Figure 1B). SR144528, a CB2 selective antagonist, and SR141716A, a CB1 selective antagonist, were used to test if JWH015 attenuation of TM cell actin cytoskeleton involves CB2 or CB1 receptors. SR144528 blocked the changes brought about by JWH015, i.e., the cells exhibited most of the cytoskeletal features associated with control cells (Figure 1C). On the other hand, the treatment of the cells with SR141716A exerted no effect on the JWH015-evoked change of actin cytoskeleton (Figure 1E). TM cells treated with SR144528 or SR141716A alone had no changes on the distribution of actin stress fibers (Figure 1D,F) as compared to control TM cells. These data indicate that JWH015 treatment remodels the TM cell actin cytoskeleton through a CB2 receptor-mediated mechanism.

The effects of JWH015 on trabecular meshwork cell focal adhesion structures: Figure 2 shows the effect of JWH015 on the focal adhesions of TM cells (visualized by immunofluorescence labeling with an anti-paxillin antibody). As shown in Figure 2A, vehicle-treated (control) cells contained abundant focal adhesions that were evenly distributed toward the lower surface of the cell. Paxillin staining was diminished

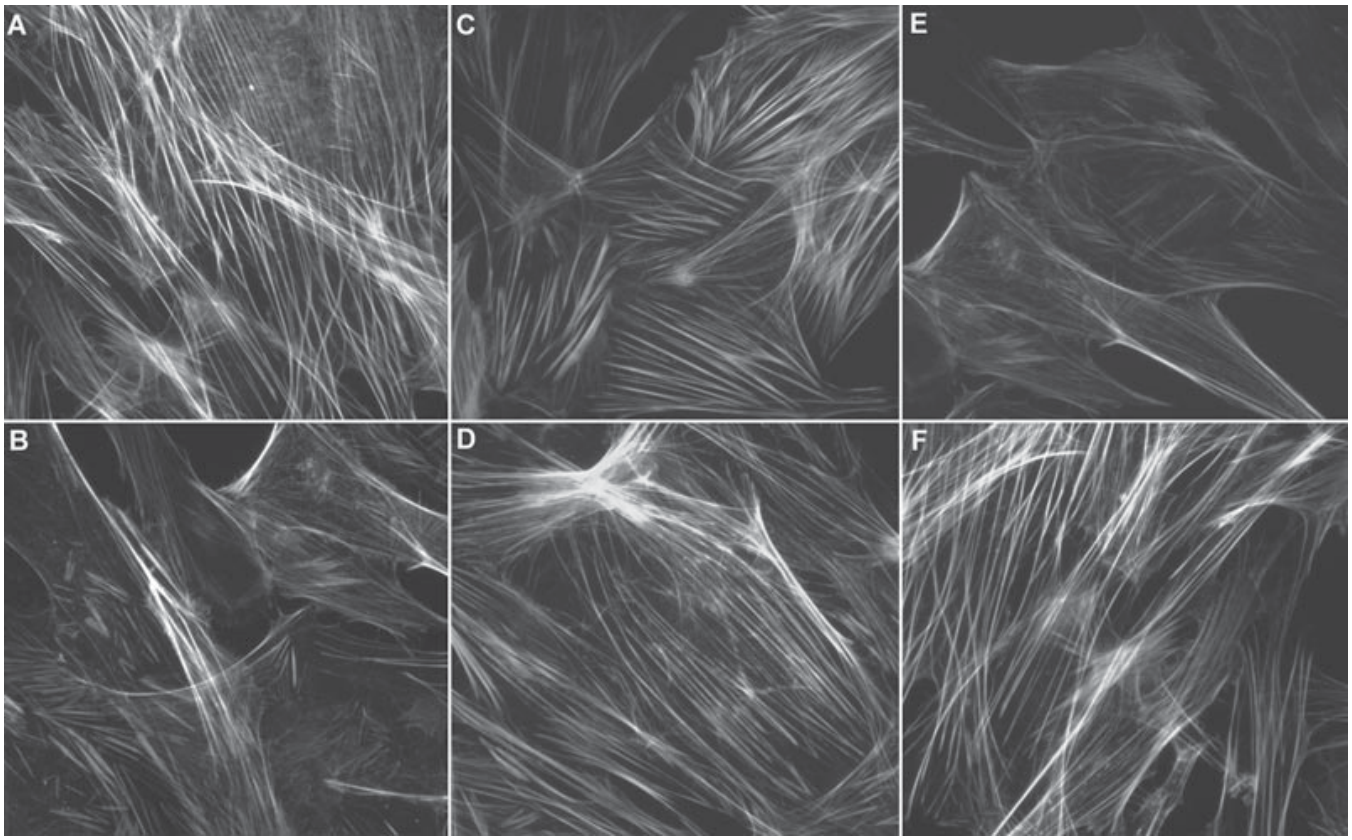


Figure 1. The effects of JWH015 on the trabecular meshwork cell actin cytoskeleton. TM cells were plated on fibronectin-coated (5 μg/ml) coverslips and grown to confluence. Cells were starved in DMEM for 48 h then treated for three h with vehicle (**A**), 100 nM JWH015 (**B**), 100 nM JWH015 plus 1 μM SR144528 (**C**), 1 μM SR144528 (**D**), 100 nM JWH015 plus 1 μM SR141716A (**E**), or 1 μM SR141716A (**F**). The TM cells were then fixed with paraformaldehyde and stained with Alexafluor 488-labeled phalloidin as detailed in the Methods section. JWH015 caused a significant decrease in staining for actin stress fibers, compared with untreated control. SR144528, but not SR141716A, blocked the changes brought about by JWH015.

centrally in cells treated with JWH015 but the staining remained at the periphery of the cells (Figure 2B). These JWH015-induced changes of focal adhesion structures were blocked by SR144528 (Figure 2C) whereas SR141716A had no effect on JWH015-induced inhibition of TM cell adhesion (Figure 2E). SR144528 or SR141716A alone had no significant effect on either the number or the distribution of anti-paxillin antibody stained focal adhesion structures (Figure 2D,F). These data demonstrated that JWH015 has a modulatory effect on the focal adhesion structures of TM cells and this effect of JWH015 is mediated through the CB2 receptors.

The effects of JWH015 on trabecular meshwork cell migration (wound healing): We investigated the effect of JWH015 on TM cell migration with an in vitro model of wound healing based on a monolayer of TM cells. The data for the wound-healing assays are shown in Figure 3. TM cells in confluent cultures were scratched with a pipette tip to create a clear cell-free wound. Immediately after scratching (0 h), the distance between the edges of the exposed region is measured and defined as 100%. At 24 h after scratching, the distance between the edges of exposed region was again measured to be 11.0±1.3, 37.9±1.5, 14.4±1.1, 9.1±1.9%, 35.8±1.2%, and 10.6±1.4% (mean ±S.E.M.) for vehicle control, 100 nM JWH015 alone, 100 nM JWH015 +1 μM SR144528, 1 μM SR144528 alone, 100 nM JWH015 +1 μM SR141716A, and 1 μM SR141716A alone, respectively (Figure 3). These results showed that an application of JWH015 causes a significant decrease in wound healing (migration) of TM cells and this effect of JWH015 is CB2 receptor dependent.

The effects of JWH015 on Rac1 GTPase activity in trabecular meshwork cells: Rho families of GTPases including RhoA and Rac1 are key signal transducers that regulate a number of cytoskeleton-dependent cell functions [28]. To explore the molecular mechanism of JWH015-induced cellular changes

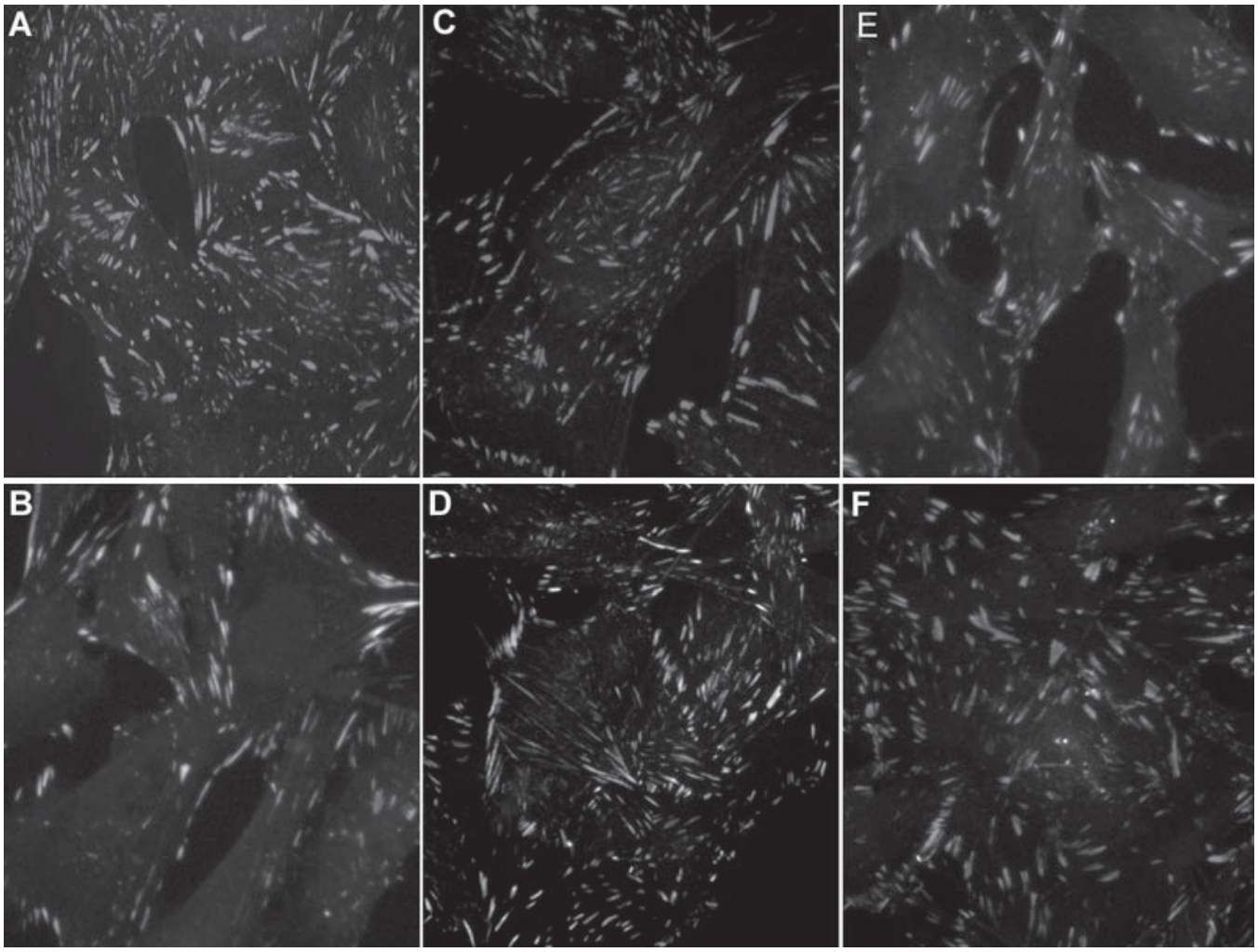
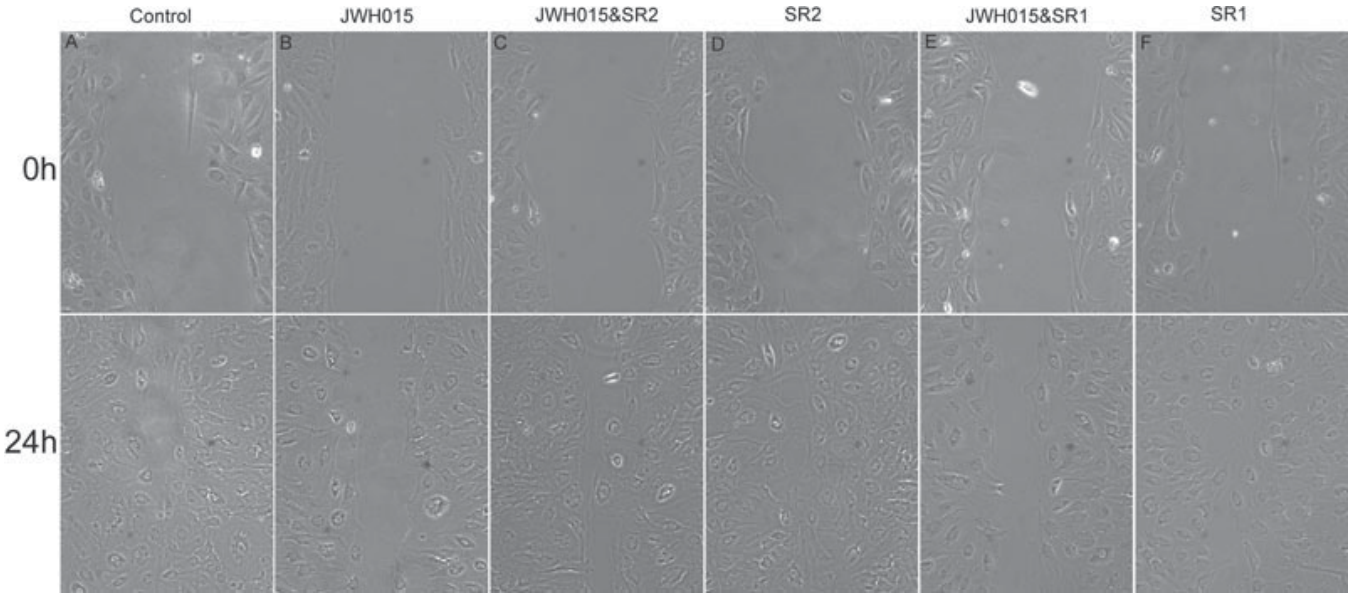


Figure 2. The effects of JWH015 on trabecular meshwork cell focal adhesion structures. Cultures of TM cells on fibronectin-coated (5 μg/ml) coverslicps were starved in DMEM for 48 h then treated for three h with vehicle (**A**), 100 nM JWH015 (**B**), 100 nM JWH015 plus 1 μM SR144528 (**C**), 1 μM of SR144528 (**D**), 100 nM JWH015 plus 1 μM SR141716A (**E**), or 1 μM SR141716A (**F**). TM cells were then fixed with paraformaldehyde and stained with an anti-paxillin antibody and a TRITC-conjugated secondary antibody as detailed in the Methods section. JWH015 treatment resulted in a significant reduction in the number of focal adhesion structures detected with anti-paxillin antibody, compared with untreated control. This effect of JWH015 was antagonized by SR144528 but not SR141716A.

of TM cells, we first examined the expression profiles of RhoA and Rac1 by western blot analysis of TM cell lysates. As shown in Figure 4, in porcine TM cell lysates containing equal amount of proteins, there are substantial amounts of Rac1 expressed. However, the expression of RhoA is much lower than that of Rac1 and barely detectable by western blot analysis. Secondly, we investigated the possible effect of JWH015 on Rac1 activity using the PAK-PBD-GST pull-down assay for Rac1-GTP. As shown in Figure 5A, in TM cells, JWH015 treatment led to a transient decrease in Rac1 activity, as shown by the lowered level of GTP bound form of Rac1 GTPase pulled down by the PAK-PBD-GST beads. The Rac1 activity decreased to a minimum after treatment with JWH015 for five min. At 15 min of JWH015 stimulation, the activity of Rac1 was recovered. Figure 5B demonstrated that pretreatment with SR144528 markedly inhibited the JWH015-induced inactivation of Rac1 whereas pretreatment with SR141716A had no significant effect on the JWH015-induced inhibition of Rac1 activity. SR144528 or SR141716A alone exerted no detectable effect on the Rac1 activity of TM cells. These results indicated that JWH015 is able to regulate Rac1 activity through the CB2 receptor in TM cells.

The effects of JWH015 on phosphorylation of cofilin in trabecular meshwork cells: Downstream effectors of the Rho GTPase are various actin-associated proteins. Cofilin, a downstream component of the Rac1 cascade, is a ubiquitously expressed actin-binding protein. Previously, it has been reported that the dephosphorylated (active) form of cofilin leads to depolymerization of actin filaments and results in the remodeling of actin cytoskeleton [29,30]. In an effort to further elucidate mechanisms related to CB2 receptor-mediated TM cell cytoskeletal remodeling and cell migratory activities, we studied JWH015-induced changes of phosphorylated forms of cofilin. Our western blot analysis showed that JWH015 treatment decreases the level of phosphorylated cofilin in a time-dependent manner (Figure 6A) whereas the level of total cofilin remains unaffected. Pretreatment with SR144528 markedly inhibited JWH015-induced dephosphorylation (activation) of



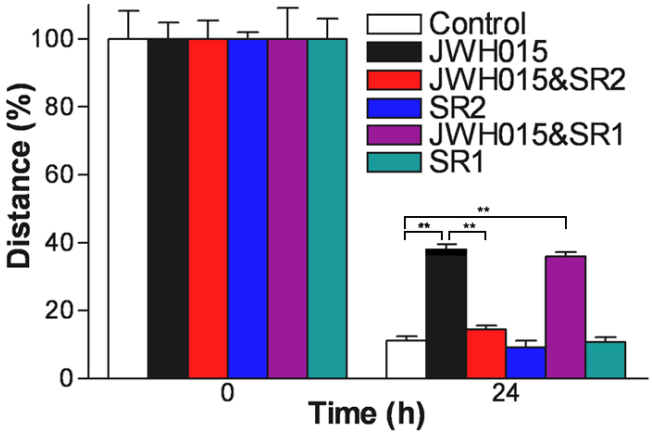


Figure 3. The effects of JWH015 on trabecular meshwork cell migration (wound healing). TM cells grown to confluence were starved for 48 h and scratched with a yellow pipette tip to create a cell-free line. The medium was replaced with fresh medium containing vehicle (**A**), 100 nM JWH015 (**B**), 100 nM JWH015 plus 1 μM SR144528 (SR2; **C**), 1 μM of SR144528 (SR2; **D**), 100 nM JWH015 plus 1 μM SR141716A (SR1; **E**), or 1 μM SR141716A (SR1; **F**). The phase-contrast images (10X field) of the wound healing process were photographed digitally with an inverted microscope at 0 h (right after the scratching wound) and after incubation of cells at 37 °C for 24 h. The distances of the wound areas were measured on the images, set at 100% for 0 h, and the mean percentages of the total distances of the wound areas were calculated. The data are presented as mean ± S.E.M. Asterisks denote that data is significantly different from the vehicle control ($p<0.05$; one-way ANOVA with Neuman-Keuls post test). JWH015 caused a significant decrease in migration of TM cells to the wound area, compared to untreated control. This inhibitory effect of JWH015 was antagonized by SR144528 but not SR141716A.

cofilin whereas pretreatment with SR141716A had no significant effect on the JWH015-induced activation of cofilin. SR144528 or SR141716A by themselves had no effect on the activities of cofilin activity (Figure 6B). These results revealed that the treatment of TM cells with JWH015 results in the activation of cofilin through a CB2 receptor-mediated mechanism.

DISCUSSION

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 conventional (trabecular meshwork) and uveoscleral route. In a previous study, we have found that the application of JWH015 enhances aqueous humor outflow facility in the perfused porcine anterior segment and this effect of JWH015 involves CB2 cannabinoid receptors in the trabecular meshwork [17]. To better understand the mechanisms underlying the CB2 receptor-mediated aqueous humor outflow-enhancing effects, we investigated here the effects of selective CB2 agonist, JWH015, on the cellular and molecular signaling properties of TM cells.

Trabecular meshwork is an avascular, multilaminar tissue located circumferentially in the anterior chamber angle of the eye. It is known that the state of TM cell actin cytoskeleton is one of the important determinants of aqueous fluid outflow through the trabecular meshwork [31,32]. In order to explore the possible mechanism by which JWH015 produces an increase in outflow facility, we examined the effects of

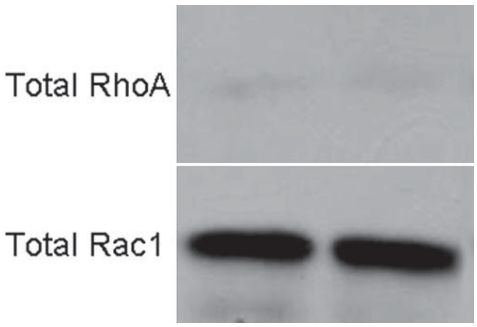


Figure 4. Expression of RhoA and Rac1 in trabecular meshwork cells. TM cell lysates containing equal amounts of total proteins were subjected to western blot analysis with anti-RhoA and anti-Rac1 antibodies, respectively. The blots were representatives of three independent experiments. The expression level of Rac1 was found to be much higher than RhoA in porcine TM cells.

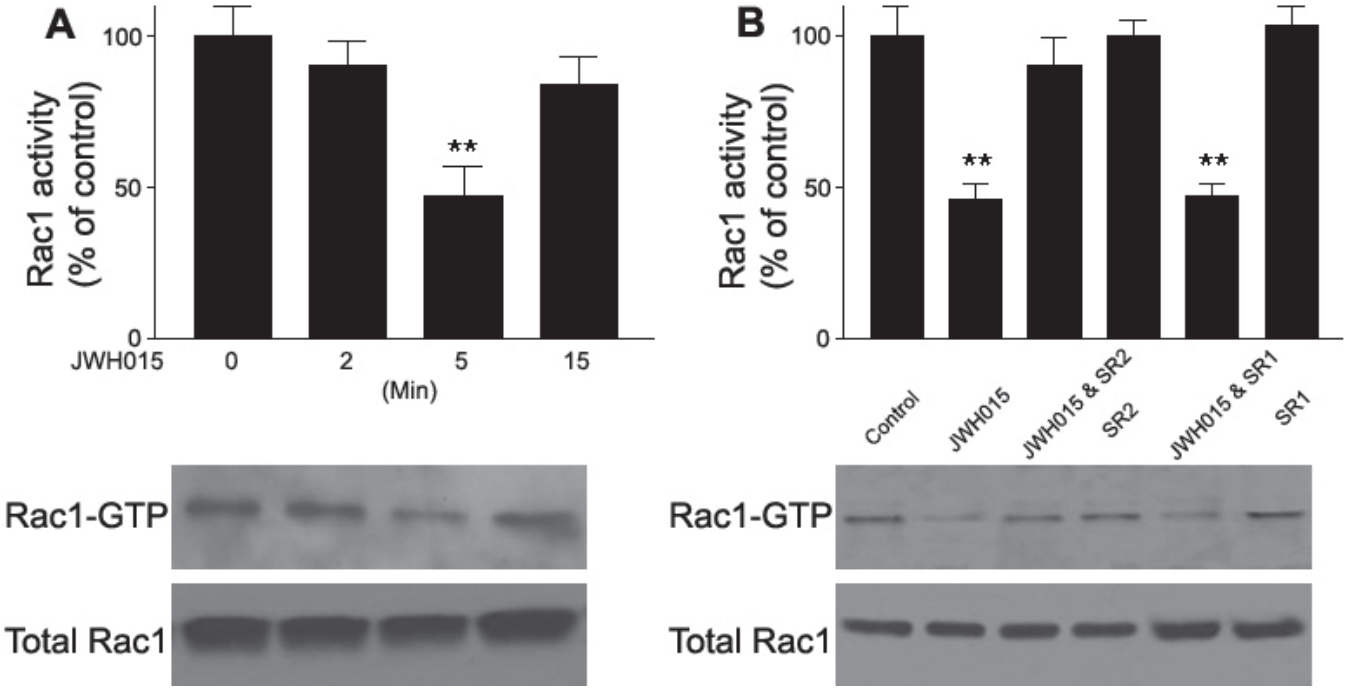


Figure 5. The effects of JWH015 on Rac1 GTPase activity in trabecular meshwork cells. **A**: Time course of the effects of JWH015 on Rac1 GTPase activity in TM cells. Cells were serum-starved for 48 h, then stimulated with 100 nM of JWH015 for the indicated times. Rac1 GTPase activity in TM cells was assessed by PAK-PBD-GST pull-down assays. Blots shown are representative of three independent experiments. In the bar graphs, results are normalized to the density of the total Rac1 bands in the corresponding samples (n=3). **B**: The effect of SR144528 and SR141716A on JWH015-induced Rac1 GTPase activity in TM cells. TM cells were serum-starved for 48 h and pretreated with 1 μM SR144528 (SR2), 1 μM SR141716A (SR1), or vehicle for 30 min followed by stimulation with 100 nM of JWH015 for five min. The Rac1 GTPase activity was measured by PAK-PBD-GST pull-down assays. Blots shown are representative of three independent experiments. In the bar graphs, results are normalized to the density of the total Rac1 bands in the corresponding samples (n=3). JWH015 treatment led to a transient decrease in Rac1 activity. SR144528, but not SR141716A markedly inhibited the JWH015-induced inactivation of Rac1.

JWH015 on actin cytoskeleton. TM cells treated with JWH015 showed a significant reduction in the amount of stress fibers and a redistribution of these stress fibers to the periphery of the cells. CB2 receptor antagonist, SR144528, blocked these JWH015-induced changes of actin cytoskeleton whereas CB1 receptor antagonist, SR141716A, had no significant effect on this JWH015-induced response. Previous studies have demonstrated that pharmacological agents that affect the cytoskeletal networks can significantly alter aqueous humor outflow facility [31,32]. Therefore, it is not unreasonable to infer from our previous publication and current data that in vivo, JWH015 may act on CB2 cannabinoid receptors to increase aqueous humor outflow through a mechanism involving actin cytoskeleton changes. The disintegration of the actin skeleton induced by JWH015 possibly result in a relaxation of the TM cells that could contribute to a more flexible architecture and in turn, facilitate the passage of the aqueous humor through the TM tissues.

Focal adhesions, which consist of a number of cytoskeletal proteins including paxillin, serve as anchoring points between actomyosin filaments and the extracellular matrix. In our experiments, paxillin staining was used to characterize the organization of TM cell-matrix adhesions. JWH015 treatment led to a reduction of the numbers of paxillin-stained TM cell focal adhesions to the fibronectin-coated surface. This effect of JWH015 was blocked by the CB2 antagonist, SR144528, but not by the CB1 antagonist, SR141716A, indicating that this is a CB2 receptor-mediated event. Fibronectin, an extracellular glycoprotein, is known to play an important role in cell-matrix interactions. Previous studies have demonstrated that excess fibronectin deposition in the trabecular meshwork occurs in primary open-angle glaucoma, corticosteroid-induced glaucoma, and in aging eyes [33-35]. These reports suggest that reduced outflow facility in glaucoma might be caused, at least in part, by excess synthesis and accumulation of extracellular matrix components such as fibronectin. Previous studies have shown that aqueous humor outflow facility can be enhanced by changing cell-cell and cell-extracellular matrix adhesions. In the context of these earlier experimental findings, our current data on JWH015-induced reduction of TM cell focal adhesion structures on fibronectin surfaces suggest that in vivo, the CB2 agonist may lead to an increase in outflow facility by modulating the interactions between TM cells and the extracellular components such as fibronectin.

The modulatory effects of cannabinoids on cell migration have been shown previously in a number of cell types.

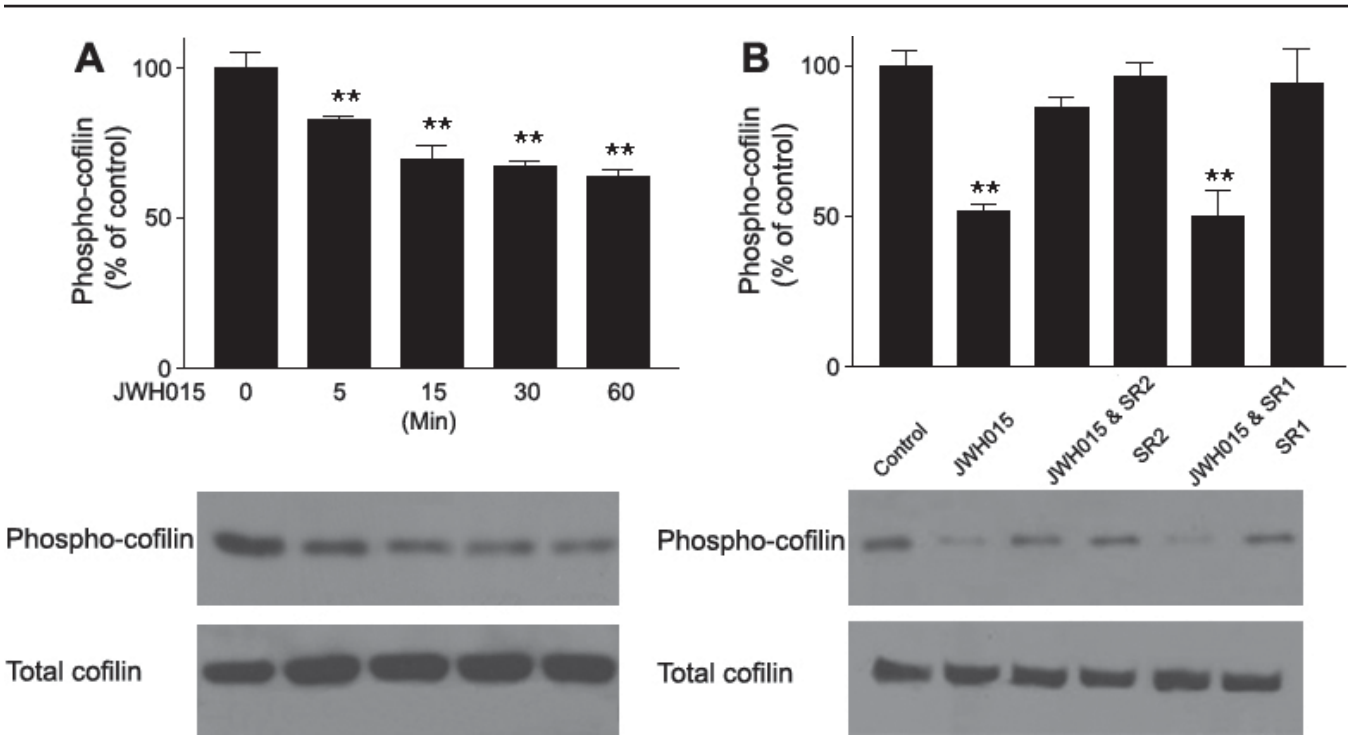


Figure 6. The effects of JWH015 on phosphorylation of cofilin in trabecular meshwork cells. (**A**) Time course of the effects of JWH015 on phosphorylation of cofilin in TM cells. Cells were serum-starved for 48 h, and then treated with 100 nM JWH015 for the indicated times. TM cell lysates were prepared and subjected to western blot analysis using anti-phospho-cofilin and anti-cofilin antibodies. Blots shown are representative of three independent experiments. In the bar graphs, results are normalized to the density of total cofilin bands in the corresponding samples (n=3). (**B**) The effects of SR144528 and SR141716A on JWH015-induced phosphrylation of cofilin in TM cells. Cells were serum-starved for 48 h and pretreated with 1 μM SR144528 (SR2), 1 μM SR141716A (SR1), or vehicle for 30 min followed by stimulation with 100 nM of JWH015 for 30 min. TM cell lysates were prepared and subjected to western blot analysis using anti-phospho-cofilin and anti-cofilin antibodies. Blots shown are representative of three independent experiments. In the bar graphs, results are normalized to the density of total cofilin bands in the corresponding samples (n=3). JWH015 treatment decreased the level of phosphorylated cofilin in a time-dependent manner. Pretreatment with SR144528, but not SR141716A, markedly inhibited JWH015-induced dephosphorylation (activation) of cofilin.

Depending on the type of cells examined, the effects of cannabinoids on cell migration can be stimulatory or inhibitory [21,22]. TM cells are known to possess contractile apparatus and are migratory. In the current study, using the wound-healing assays, JWH015 treatment led to a significant decrease in the migration of TM cells to the wound area and the CB2 antagonist, SR144528, and not the CB1 antagonist, SR141716A, blocked this inhibitory effect of JWH015 on TM cell migration. Cell migration is a complex process that requires the participation of actin cytoskeleton and is regulated through cell-substratum interactions. The decrease of TM cell migration induced by JWH015 is consistent with its negative effects on actin cytoskeleton and focal adhesion structures.

The Rho family of small GTP-binding proteins including RhoA and Rac1 regulate a number of important cytoskeleton-dependent cellular functions [28]. Previous studies performed with human TM cells have shown that RhoA plays an important role in regulating aqueous humor outflow [36,37]. Given the well-established roles of the Rho family of small G proteins in regulating actin cytoskeleton, cell adhesion, and cell migration, we hypothesized that the members of Rho-GTPase family might play an important role in the signaling cascade by which JWH015 modulates TM cell properties and aqueous humor outflow. To test this hypothesis in this study, we first examined the expression of RhoA and Rac1 in cultured TM cells. Our western blot results revealed that the expression of Rac1 is much higher than that of RhoA in porcine TM cells. Based on this result, we then investigated the effects of JWH015 on TM cell Rac1 GTPase activity. Our experiments demonstrated that treatment of TM cells with JWH015 results in a transient decrease of Rac1-GTP in the pull-down assay, indicating a reduction of Rac1 GTPase activity. In addition, the involvement of CB2 receptor was demonstrated by the fact that JWH015-induced decrease of Rac1 GTPase activity is blocked by selective CB2 antagonist, SR144528, and not by CB1 antagonist, SR141716A. Based on these data, it is reasonable to infer that in TM cells, JWH015 might produce its inhibitory effects on actin stress fiber and focal adhesion formation as well as on cell migration through CB2 receptor-mediated inactivation of Rac1 GTPase in TM cells.

Downstream effectors of the Rho GTPase are various actin-associated proteins. In this study, we examined the effects of JWH015 on cofilin, a downstream component of Rac1 cascade. Cofilin is a ubiquitous actin-binding protein and is a major mediator of Rac1-dependent actin remodeling [30]. Only unphosphorylated cofilin binds actin and severs/depolymerizes filamentous actin. In this current study, JWH015 induced a decrease in the phosphorylation levels of cofilin and this effect of JWH015 was blocked by CB2 antagonist, SR144528, but not by CB1 antagonist, SR141716A. Taken together, our data on JWH015-induced, CB2 receptor-mediated deactivation of Rac1 GTPase activity and activation of cofilin activity indicate that these may be the molecular mechanisms by which JWH015 produces its effect on TM cell actin-cytoskeleton, cell focal adhesion, and migration.

In conclusion, we have found for the first time that by acting on CB2 cannabinoid receptors, JWH015 modulates TM cell actin cytoskeleton, focal adhesion, and migration. Additionally, we have also found for the first time that CB2 receptor activation in the TM cells leads to the inactivation of Rac1 GTPase and the activation of cofilin. Overall, our data support the idea that a JWH015-induced increase in aqueous humor outflow probably involves CB2 receptor-mediated changes of TM cell Rho GTPase and cofilin activity, leading to a remodeling of the TM cytoskeleton.

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