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CB1 cannabinoid receptor-mediated changes of trabecular meshwork cellular properties

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Purpose: To evaluate the roles of CB1 cannabinoid receptors in cellular functions of trabecular meshwork (TM) cells, including cell migration, adhesion, morphology and cytoskeleton changes.
Methods: Noladin ether, a selective CB1 receptor agonist, and SR141716A, a selective CB1 receptor antagonist, were used to characterize the cellular functions of cultured porcine TM cells. Fluorescence assisted transmigration invasion and motility assays (FATIMA) were conducted to study TM cell migration using soluble fibronectin as a chemoattractant. Wound healing assays were used to further study TM cell migration. Standard cell adhesion assays of TM cells were performed on fibronectin-coated plates. In morphological studies, Alexafluor 488-labeled phalloidin staining was used to examine actin filaments, and immunocytochemistry using anti-paxillin antibodies was used to detect focal adhesions.
Results: In cell migration assays, CB1 agonist noladin ether at nanomolar ranges led to a concentration-dependent inhibition of migration of TM cells toward soluble fibronectin. CB1 antagonist SR141716A antagonized noladin ether-induced inhibition of migration of TM cells. In addition, noladin ether caused a delay in wound healing of confluent trabecular meshwork monolayers and this effect of noladin ether was antagonized by SR141716A. In cell adhesion assays, noladin ether treatment led to a moderate, but significant decrease of adhesion of TM cells to fibronectin-coated surface. This effect of noladin ether was concentration-dependent, and was antagonized by SR141716A. In morphological studies, noladin ether treatment caused rounding of TM cells in contrast to well-spread control TM cells. In addition, there was a reduction and fragmentation of actin stress fibers stained with Alexafluor 488-labeled phalloidin and a decrease of focal adhesions detected with an anti-paxillin antibody.
Conclusions: Noladin ether modulates the migration, adhesion, morphology, and actin cytoskeleton of TM cells. These effects of noladin ether are mediated through TM cell CB1 cannabinoid receptors.

Cannabinoids are well known for their psychotropic properties and for their therapeutic potentials. The major targets for cannabinoid ligands are CB1 and CB2 cannabinoid receptors [1,2]. CB1 receptors are located in the CNS and periphery [3], whereas CB2 receptors are mainly distributed in the periphery tissues, such as immune cells [4]. There are two well-recognized endocannabinoids, arachidonylethanolamide (anandamide) [5] and arachidonylglycerol (2-AG) [6,7]. Both of these endogenous cannabinoid ligands are unstable and both of them act on CB1 and CB2 receptors.

The synthesis of an ether-linked, metabolically stable analog of 2-AG was first reported by Sugiura et al. [8]. After it was isolated from porcine brain, the 2-AG ether was suggested to be the third endocannabinoid agonist, and was given the name noladin ether [9]. Noladin ether has been shown to be highly selective for the CB1 receptors. Thus this stable CB1 agonist is a useful reagent for studying the function of CB1 receptors.

Increased intraocular pressure (IOP) is a major risk factor for glaucoma. Numerous studies have demonstrated that cannabinoid administration lowers IOP [10,11]. Recent studies have shown that CB1 receptors are at least partially involved in the IOP-lowering effects of cannabinoids [12,13]. TM is a major site for aqueous humor outflow; therefore it is critical for regulating IOP. It has been reported recently that CB1 receptors are located on TM cells [14,15]. However, the roles of these receptors in the cellular functions of TM cells are currently unknown.

In a number of cell types, cannabinoid ligands are known to modulate cell migration, adhesion, morphology, and cytoskeleton, possibly by acting through cannabinoid receptors [16-19]. In this study, we hypothesized that CB1 agonist may modulate these cellular properties of TM cells through CB1 receptors. To test this hypothesis, we investigated the effects of CB1 selective agonist noladin ether on the TM cell migration, adhesion, morphology, and actin cytoskeleton architecture. The involvements of CB1 receptors in noladin-induced cellular changes of TM cells were further investigated with the use of a selective CB1 antagonist, SR141716A and a selective CB2 antagonist SR144528 [20,21].

METHODS

Materials: Noladin ether was purchased from Tocris (Balwin, MO). SR141716A and SR144528 were obtained from the National Institute of Drug Abuse, NIH. Fatty acid free BSA and fibronectin were purchased from Sigma (St. Louis, MO). Twentyfour-well HTS FluoroBlok systems with polycarbonate membranes of 8 μm pore size were purchased from Becton,

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Dickinson and Company (Franklin Lakes, NJ). DiI and Alexafluor488-conjugated phalloidin were purchased from Molecular Probes, Inc. (Eugene, OR). A polyclonal anti-paxillin antibody raised in rabbit and a fluorescein-conjugated goat anti-rabbit secondary antibody was purchased from Upstate (Lake Pacid, NY).

Trabecular meshwork (TM) cell culture: The TM was isolated from fresh porcine eyes by blunt dissection. Culture of TM cells was performed according to previously published methods [22,23]. 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.

Cell migration assay: TM cell migration was monitored with the fluorescence-assisted transmigration invasion and motility assay (FATIMA) [24]. This assay monitors the migration of fluorescently labeled cells from the upper chamber to the lower chamber of the HTS FluoroBlok system, which consisting of a fluorescence-shielding porous PET membrane with 8 μm pores. Briefly, TM cells were trypsinized, pelleted by centrifugation, and resuspended in DMEM/0.1% BSA. Subsequently, the cells were fluorescently labeled with DiI, a lipophilic fluorescent dye at a concentration of 50 μg/ml for 30 min at 37 °C. The cells were then washed three times with DMEM/0.1% BSA. To induce cell migration, 20 μg/ml of soluble fibronectin was added to the lower chamber. To study the effects of cannabinoid on cell migration, cannabinoid ligands were added to both the upper of lower chambers. TM cell suspension ($1x10^5$ cells) was loaded into the upper chamber of the HTS FluoroBlok system, and incubated for 3 h at 37 °C. The migrated cells were quantified by using a TECAN GENios Pro plate reader (Durham, NC), using excitation and emission filters of 535 and 590 nm, respectively.

Wound healing assay: For the wounding healing experiment, TM cells were seeded on fibronectin (10 μg/ml) coated cover slips in 6 well plates and TM cells were allowed to grow to complete confluence. The cells were then starved in low serum (0.5%) for five days. 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 fresh medium to remove loose cells. The cells were then re-fed with fresh medium containing 0.5% FBS and were treated with or without noladin ether and SR141716A. Immediately following scratch wounding (0 h) and after incubation of cells at 37 °C for 24 h, phase-contrast images (10x field) of the wound healing process were photographed digitally with an inverted microscope (Olympus IX50). The distance of the wound areas were measured on the images, set at 100% for 0 h, and the mean percentage of the total distances of the wound areas was calculated.

Cell adhesion assay: The wells of a 96 well plate were pre-coated with 10 μg/ml fibronectin for 3 h, washed once

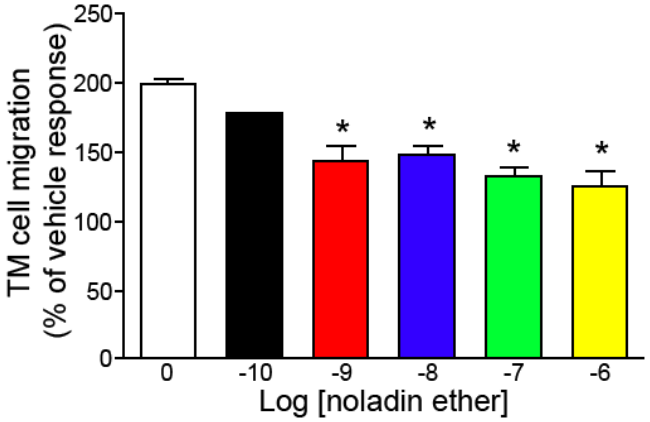

Figure 1. Effects of different concentrations of noladin ether on fibronectin-induced migration of trabecular meshwork cells. Soluble fibronectin (20 μg/ml) was added to the lower chamber to induce TM cell migration. Different concentrations of noladin ether were added to both the upper and the lower chamber. TM cells ($1x10^5$) were loaded to the upper chamber and cells migrating to the lower chamber were quantified by fluorenscence readings three h later as detailed in the Methods. Results were expressed as percent of vehicle response, which was cell migration when only vehicle (no fibronectin) was added. Data from four different experiments are presented. Error bars represent the SEM. Asterisks denote data is significantly different from the control (fibronectin alone; $p<0.05$; ANOVA with Neuman-Keuls post hoc test).

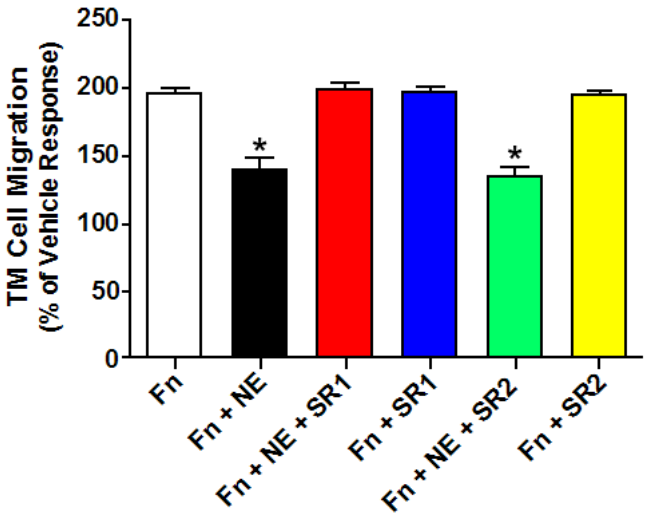

Figure 2. Involvement of CB1 cannabinoid receptors in inhibition of trabecular meshwork cell migration caused by noladin ether treatment. Soluble fibronectin (Fn; 20 μg/ml) was added to the lower chamber to induce TM cell migration. Noladin ether (NE; 100 nM) alone, 100 nM NE plus one μM of SR141716A (SR1), one μM of SR1, 100 nM NE plus one μM of SR144528 (SR2), or one μM of SR2 alone were added to both the upper and the lower chamber. TM cells ($1x10^5$) were loaded to the upper chamber and cells migrating to the lower chamber were quantified by fluorescence readings three h later, as detailed in the Methods. Results are expressed as percent of vehicle response, which was cell migration when only vehicle (no fibronectin) was added. Data from four different experiments are presented. Error bars represent the SEM. Asterisks denote data is significantly different from the control (fibronectin alone; $p<0.05$; ANOVA with Neuman-Keuls post hoc test).

with phosphate-buffered saline (PBS), blocked with 1% fatty acid free BSA for 2 h, and rewashed once with PBS. A confluent plate of TM cells was harvested by brief trypsinization, and trypsin activity was stopped using soybean trypsin inhibitor in PBS. Subsequently, TM cells were washed once with DMEM and plated onto the fibronectin-coated 96 well plates at a density of $3x10^4$ cells/well. Cannabinoid ligands were added to each well, and incubated with TM cells for 3 h at 37 °C. The non-adherent cells were then removed, the wells were washed with PBS and remaining adhered cells were fixed with 10% methanol for 1 h. The cells were then washed with PBS and stained with 0.1% crystal violet for 1 h, following which the cells were washed with PBS, air-dried and lysed with 2% SDS with agitation for 15 min. The adherent cells in each well were quantified by monitoring optical density at 595 nm using the TECAN GENios Pro plate reader.

Immunofluorescence microscopy and phalloidin staining of actin cytoskeleton: TM cells were allowed to grown to confluence on sterile glass cover slips pre-coated with 10 μg/ml fibronectin, and were starved in DMEM for 24 h. TM cells were then treated with cannabinoids for 3 h. Subsequently, cells were washed twice with PBS, fixed with freshly prepared 4% paraformaldehyde for 15 min, washed twice with PBS, permeabilized with 0.1% Triton-X100/PBS for 10 min, washed twice again with PBS, and blocked for 1 h with 1% BSA/PBS. For immunohistochemistry staining, cells were incubated with a rabbit anti-human paxillin antibody for 1 h at 37 °C, followed by an additional 1 h with an FITC-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For actin cytoskeleton staining, Alexa 488-conjugated phalloidin (Invitrogen Corporation, Carlsbad, CA) was added onto permeabilized and BSA blocked cells at a concentration of 0.7 units/ml for 1 h. Finally, cover slips from immunohistochemistry and actin stainings were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and viewed with a fluorescence microscope (Olympus IX50).

Data analysis: Results from migration assays are expressed as percent of vehicle response, which was cell migration when only vehicle (no fibronectin) was added. Results from adhesion assays are expressed as absorbance at 590 nm. Data from the migration and adhesion assays were presented

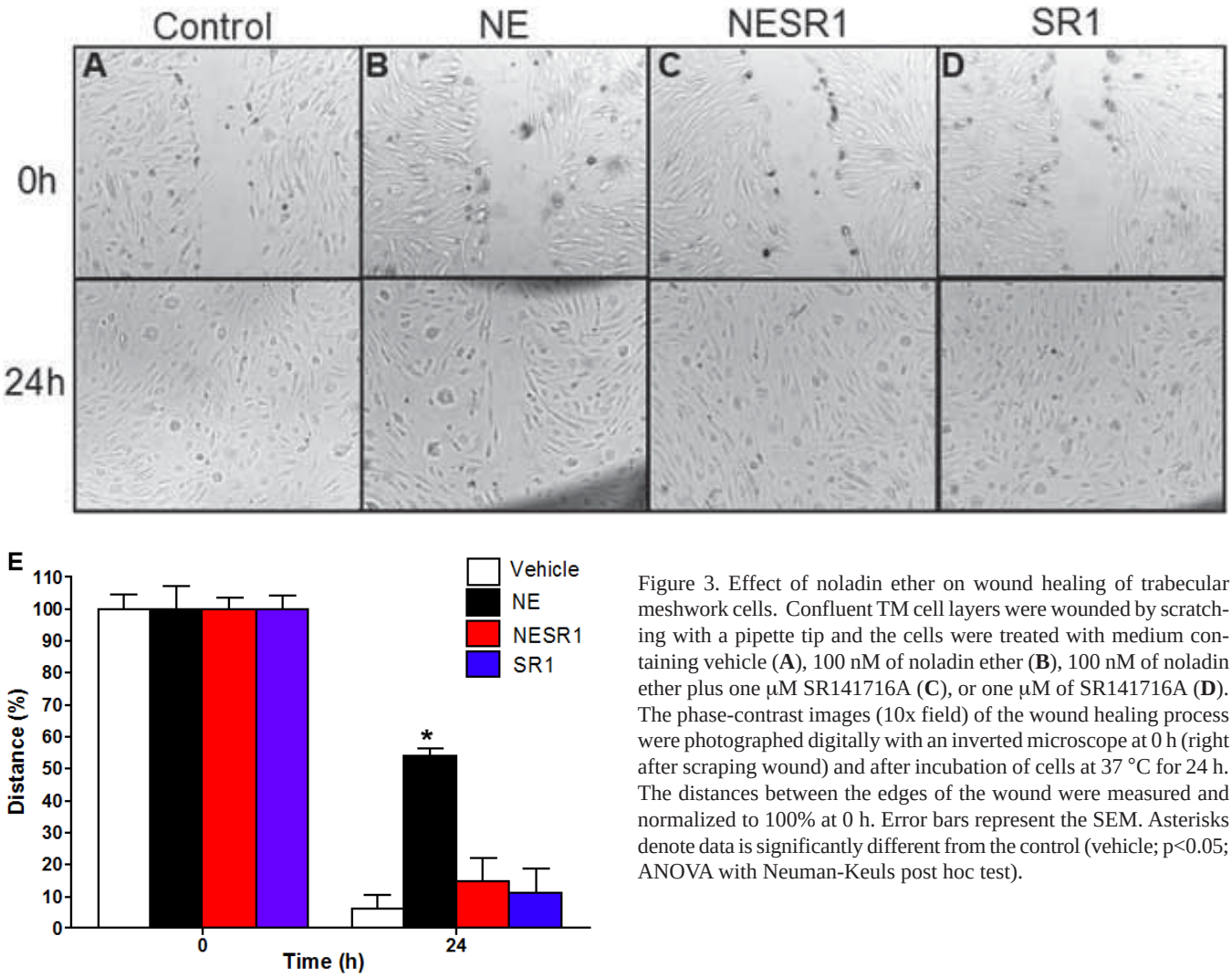

Figure 3. Effect of noladin ether on wound healing of trabecular meshwork cells. Confluent TM cell layers were wounded by scratching with a pipette tip and the cells were treated with medium containing vehicle (**A**), 100 nM of noladin ether (**B**), 100 nM of noladin ether plus one μM SR141716A (**C**), or one μM of SR141716A (**D**). The phase-contrast images (10x field) of the wound healing process were photographed digitally with an inverted microscope at 0 h (right after scraping wound) and after incubation of cells at 37 °C for 24 h. The distances between the edges of the wound were measured and normalized to 100% at 0 h. Error bars represent the SEM. Asterisks denote data is significantly different from the control (vehicle; $p<0.05$; ANOVA with Neuman-Keuls post hoc test).

as mean±SEM of values obtained from four independent experiments. For the wound healing assay, images from six independent experiments were analyzed and mean percentage of the total distances of the wound areas was presented. Statistical analyses between different treatments were determined by one-way ANOVA and with Neuman-Keuls post hoc test, using the Prism software from GraphPad. A p value of <0.05 was considered to be statistically significant.

RESULTS

Effects of noladin ether on cell migration: To address the possibility of noladin ether, a selective CB1 agonist, or SR141716A and SR144528, a selective CB1 and a selective CB2 antagonist, respectively, in regulating the cell migration, these drugs were added individually to the lower chamber (to test chemotactic effects) or both the higher and lower chambers (to test chemokenitic effects) of the HTS FluoroBlok, and the migration of fluorescently labeled TM cells through the membrane in response to these cannabinoid ligands was quantified. Adding noladin ether, SR141716A, or SR144528 to the lower chamber or both the higher and lower chamber had no significant effect on the migration of TM cells, indicating that none of these ligands by themselves had any chemotactic or chemokinetic effects on TM cells (data not shown).

Soluble fibronectin is known to induce TM cell chemotaxis. To test if noladin ether can modulate TM cell chemotaxis in response to soluble fibronectin, different concentrations of noladin ether was added to both the upper and lower chambers, and soluble fibronectin was added to the lower chamber of the HTS FluoroBlok. As shown in Figure 1, TM cell migration in response to fibronectin was inhibited by noladin ether in a concentration-dependent manner. The effect of noladin ether reached a plateau at 10^{-7} M of noladin ether, with a maximum inhibition of approximately 30% of fibronectin-induced TM cell migration.

SR141716A, a selective CB1 antagonist, and SR144528, a selective CB2 antagonist, were used to test if the inhibition of TM cell migration caused by noladin ether involves CB1 or CB2 receptors. The addition of SR141716A and SR144528 by themselves had no significant effect on fibronectin-induced TM cell migration (Figure 2). However, the inhibitory effect of noladin ether on TM cell migration was completely blocked by SR141716A. In contrast, SR144528 had no effect on inhibitory effect of noladin ether on TM cell migration (Figure 2).

Effects of noladin ether in wound healing assay: The effect of noladin ether on cell migration was further investigated using the wound-healing assay. The confluent TM cell monolayers were scratched using a pipet tip to create a clear wound area. Immediately after scratching (0 h), the distance between the edges of the wound area is measured and defined as 100%. At 24 h after scratching, the distance between the edges of the wound region was again measured and presented as a percentage of the distance between the edges of wound area at 0 h (Figure 3). These percentages were calculated to be 9.8±6.9, 46.8±10.3, 18.8±7.1, and 17.4±6.7%, respectively, for vehicle control, 10 nM noladin alone, 10 nM noladin + 100 nM SR141716A, and 100 nM SR141716A alone. The 10 nM noladin ether alone treated group has a significantly higher

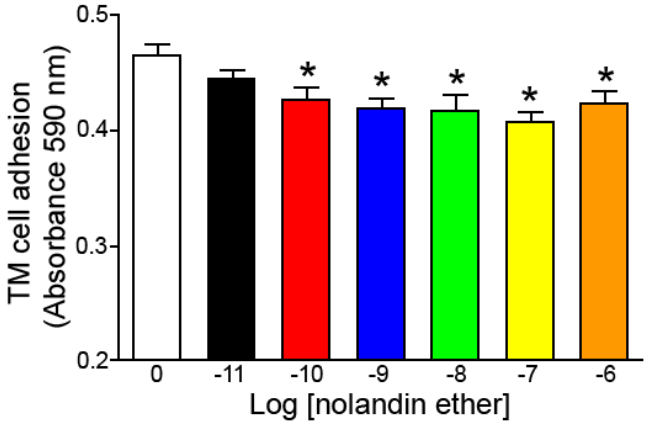

Figure 4. Effects of different concentrations of noladin ether on adhesion of trabecular meshwork cells to fibronectin-coated surface. In the presence of different concentrations of noladin ether, TM cells were allowed to attach for three h to wells pre-coated with 10 μg/ml fibronectin. Adhered TM cells were quantified by crystal violet staining as detailed in the Methods and results are expressed as absorbance at 590 nm. Data from four different experiments are presented. Error bars represent the SEM. Asterisks denote data is significantly different from the control (vehicle; p<0.05; ANOVA with Neuman-Keuls post hoc test).

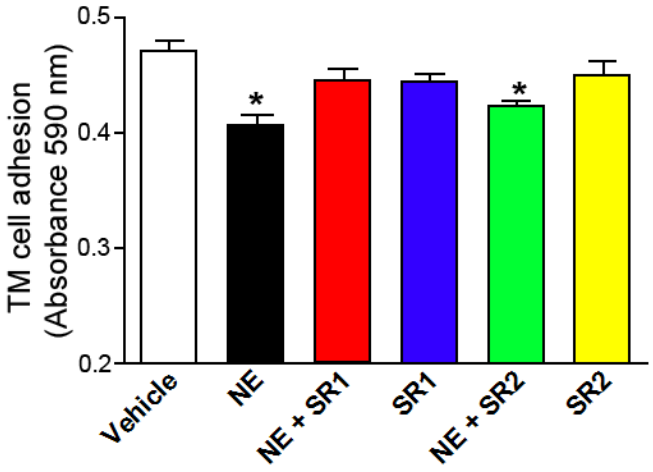

Figure 5. Involvement of CB1 cannabinoid receptors in inhibition of trabecular meshwork cell adhesion to fibronectin-coated surface caused by noladin ether treatment. In the presence of 100 nM of Noladin ether (NE) alone, 100 nM NE plus one μM of SR141716A (SR1), one μM of SR1 alone, 100 nM NE plus one μM of SR144528 (SR2), or one μM of SR2 alone, TM cells were allowed to attach for three h to wells pre-coated with 10 μg/ml fibronectin. Adhered TM cells were quantified by crystal violet staining as detailed in the Methods and results are expressed as absorbance at 590 nm. Data from four different experiments are presented. Error bars represent the SEM. Asterisks denote data is significantly different from the control (vehicle; p<0.05; ANOVA with Neuman-Keuls post hoc test).

percentage than the other three groups ($p<0.05$), whereas the other three groups are not significantly different from each other. These results demonstrate that by acting through the CB1 receptor, noladin ether caused a significant decrease in migration of TM cells to the wound area.

Effects of noladin ether on cell adhesion: TM cells were treated with different concentrations of noladin ether to investigate whether the adhesive properties of TM cells can be modulated by noladin ether. As shown in Figure 4, noladin ether treatment led to a concentration-dependent decrease of TM cell adhesion to the fibronectin-coated surface. SR141716A, a CB1 selective antagonist, and SR144528, a CB2 selective antagonist, were used to test if noladin ether-induced inhibition of TM cell adhesion involves CB1 or CB2 receptors. While neither SR141716A SR144528 by itself had any significant effect on cell adhesion, SR141716A blocked the inhibitory effect of noladin ether on TM cell adhesion to fibronectin-coated surface (Figure 5). In contrast, SR144528 had no effect on the noladin ether-induced inhibition of TM cell adhesion (Figure 5).

Effects of noladin ether on actin stress fibers: As shown in Figure 6A, the control TM cells appeared elongated and well spread. In these cells the actin stress fibers were prominently visible and are distributed along the long axis of the cells. In contrast, the noladin ether treatment caused rounding of TM cells and disruption of actin stress fibers. In noladin ether treated cells, actin filaments became fragmented in the center of the cells, although elongated actin stress fibers remained in the periphery of the cells (Figure 6B). CB1 receptor antagonist SR141716A blocked the changes brought about by noladin ether (i.e., the cells exhibited most of the morphological features associated with control cells; Figure 6C). TM cells treated with SR141716A alone had no changes on the morphology and the distribution of actin stress fibers (Figure 6D), as compared to control TM cells.

Effects of noladin ether on focal adhesion structures: As shown in Figure 7A, abundant anti-paxillin antibody-stained focal adhesion structures were distributed throughout control TM cells. Noladin ether treatment resulted in a reduction of the number of focal adhesion structures, and the remaining focal adhesion structures were localized at the periphery of the cells (Figure 7B). These noladin ether-induced changes of focal adhesion structures were blocked by treatment with SR141716A (Figure 7C), whereas SR141716A alone had no effect on the number and distribution of ant-paxillin antibody-stained focal adhesion structures (Figure 7D).

DISCUSSION

Earlier studies have demonstrated that CB1 cannabinoid receptors are at least partially involved in cannabinoid-induced IOP-lowering effects [12,13]. In addition, strong-to-moderate levels of CB1 receptor immunoreactivity have been detected in trabecular meshwork cells [14,15]. Recently, the effect noladin ether in the eye has been investigated, and it has been shown to lower IOP through a CB1 receptor mediated mechanism [25,26]. In addition, we have found that by acting through TM cell CB1 cannabinoid receptors, noladin ether enhances the aqueous humor outflow facility [27]. The current study was performed to explore, at the cellular level, the possible CB1 receptor-mediated effects of noladin ether on TM cells.

Previously, the modulatory effects of cannabinoids on cell migration have been demonstrated in a number of cell types. Depending on the type of cells tested, the effects of cannabinoids on cell migration can be stimulatory or inhibitory

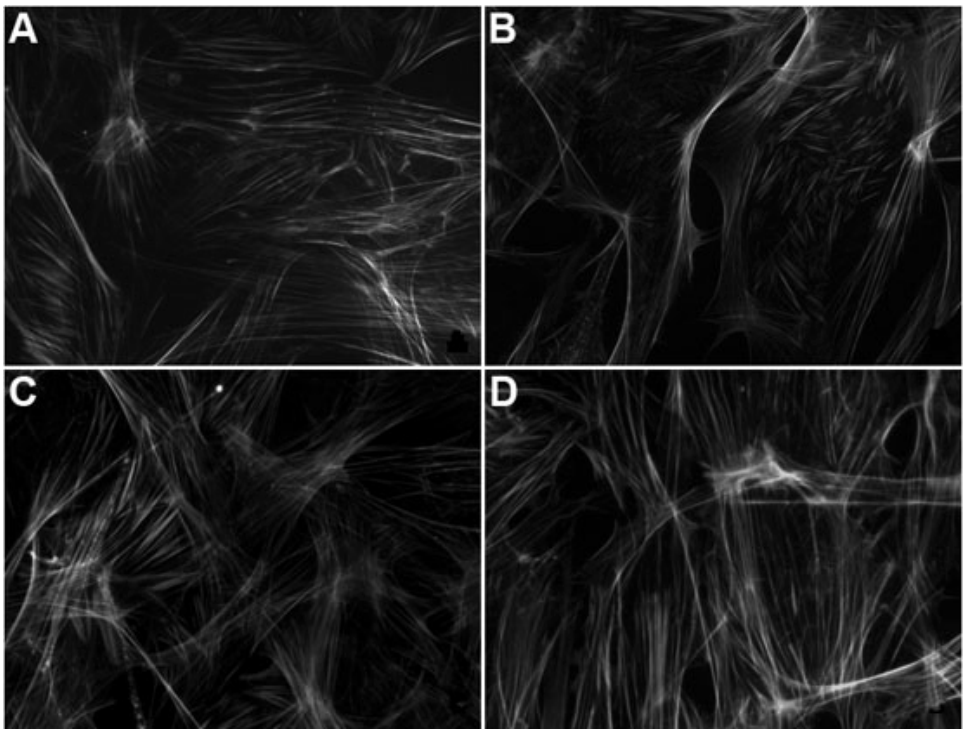

Figure 6. Effects of noladin ether on trabecular meshwork cell actin cytoskeleton. Trabecular meshwork cells were plated on fibronectin (10 μg/ml)-coated cover glasses and grown to confluence. Cells were starved in DMEM for 24 h, and then treated for 3 h with vehicle (**A**), 100 nM of noladin ether (**B**), 100 nM of noladin ether plus 1 μM SR141716A (**C**), or 1 μM of SR141716A (**D**). The trabecular meshwork cells were then fixed and stained with Alexafluor 488-labeled phalloidin as detailed in the Methods.

[17,18]. In this study we have found that treatment of TM cells with noladin ether at nanomolar range leads to a concentration-dependent inhibition of migration of TM cells induced by fibronectin. The CB1 antagonist SR141716A antagonized the inhibition of migration caused by noladin ether, whereas CB2 antagonist SR144528 had no effect on noladin-induced inhibition of TM cell migration. These data clearly indicate that noladin ether acts on CB1 receptors to inhibit fibronectin-induced TM cell migration.

To further confirm the inhibitory effect of noladin ether on TM cell migration, a wound-healing assay was used in this study. Comparing with migration assay with dissociated TM cells, the wound-healing assay on confluent, non-dividing monolayers of TM cells may be more physiologically relevant, because TM cells are considered quiescent in the adult eye. In this assay, noladin ether caused a significant decrease in migration of TM cells to the wound area, and CB1 antagonist SR141716A blocked this effect of noladin ether. These data obtained from wound-healing assays are consistent with our data using dissociated TM cells.

In a previous study, aqueous humor has been shown to be a chemoattractant for TM cells in vitro [28]. The major chemoattractant in the aqueous humor was found to be fibronectin, because the chemotactic effects of aqueous humor can be partially neutralized by the addition of antibody against fibronectin [28]. In a later study, it was found that aqueous humor from glaucomatous patients appears to be better migratory stimulant than nonglaucomatous aqueous humor in vitro, and the major chemoattractant constituent of aqueous humor from the glaucomatous patients was again found to be fibronectin [29]. With regards to the clinical relevance of these findings from in vitro migration assays, the authors suggested that in vivo, fibronectin in the aqueous humor might cause the loss of TM cells in the aging TM and possibly some of the extra loss in primary open-angle glaucoma [29]. Cannbinoids have been proposed to be a new class of anti-glaucoma agents. In the context of the previous findings of Hogg et al. [28,29] on fibronectin-induced chemotaxis of TM cells, our current data regarding inhibition of fibronectin-induced TM cell migration by noladin ether suggests that as a potential anti-glaucoma agent, cannabinoid agonist may prevent the loss of TM cells in aging eye, and may be beneficial for the treatment of glaucoma.

In the current study, noladin ether treatment led to a moderate, but significant decrease of adhesion of TM cells to fibronectin-coated surface. This effect of noladin ether was concentration-dependent, and was antagonized by SR141716A but not SR144528, indicating that CB1 receptors play an important role in the inhibitory effects of noladin ether on adhesion of TM cells.

Cells within the TM interact with each other and with the extracellular matrix substratum. These interactions play crucial roles in regulating the resistance within the conventional aqueous humor outflow pathway. Earlier studies have demonstrated that excess fibronectin deposition in the trabecular meshwork occurs in primary open-angle glaucoma, corticosteroid-induced glaucoma, and in aging eyes [30-32]. 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. In the context of these earlier experimental findings, our current data on noladin ether-induced decrease of cell adhesion on fibronectin surface suggest that in vivo, cannabinoids may lead to an increase in outflow facility by modulating the inter-

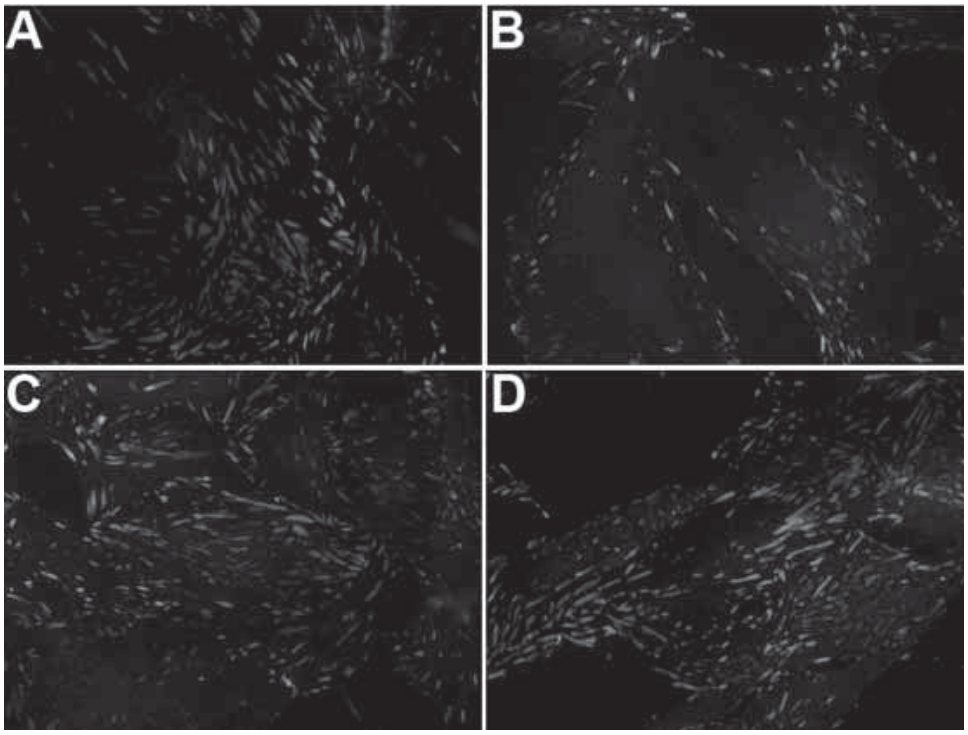

Figure 7. Effects of noladin ether on trabecular meshwork cell focal adhesion structures detected with an anti-paxillin antibody. Trabecular meshwork cells were plated on fibronectin (10 μg/ml)-coated cover glasses and grown to confluence. Cells were starved in DMEM for 24 h, and then treated for three h with vehicle (**A**), 100 nM of noladin ether (**B**), 100 nM of noladin ether plus one μM SR141716A (**C**), or one μM of SR141716A (**D**). Trabecular meshwork cells were then fixed and stained with an anti-paxillin antibody as detailed in the Methods.

action between TM cells and the extracellular components such as fibronectin.

In a previous study of a neuronal cell line, we have shown that cannabinoid agonist treatment can alter cell morphology and cytoskeleton by acting through CB1 cannabinoid receptors [19]. Consistent with this previous report, in the present study treatment with noladin ether resulted in rounding of TM cells, fragmentation, and reduction of actin stress fibers, and decrease of focal adhesion structures. Previous studies have demonstrated that the TM cell cytoskeleton is involved in the regulation IOP and several cytoskeleton-modulating drugs enhances aqueous humor outflow [33,34]. Therefore, it is not unreasonable to infer from our current data that in vivo, noladin ether may act on CB1 cannabinoid receptors to increase aqueous humor outflow through a mechanism involving TM cell morphology and actin cytoskeleton changes.

In summary, in this study we have found, for the first time, that noladin ether modulates TM cell migration, adhesion, morphology, and actin cytoskeleton. These effects of noladin ether are mediated through TM cell CB1 cannabinoid receptors.

ACKNOWLEDGEMENTS

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