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Involvement of a non-CB1/CB2 cannabinoid receptor in the aqueous humor outflow-enhancing effects of abnormal-cannabidiol

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Abstract

The purpose of this study was to investigate the effects of abnormal-cannabidiol (abn-cbd), a non-psychoactive cannabinoid agonist, on aqueous humor outflow via the trabecular meshwork (TM) of porcine eye, and to examine the involvement of a non-CB1/CB2 cannabinoid receptor and the p42/44 mitogen-activated protein kinase (p42/44 MAPK) pathway. The effects of abn-cbd on aqueous humor outflow were measured using a porcine anterior segment perfused organ culture model. The activation of p42/44 MAPK by abn-cbd was determined in cultured TM cells with western blot analysis using an anti-phospho-p42/44 MAPK antibody. Administration of abn-cbd caused a concentration-dependent enhancement of aqueous humor outflow facility with a maximum effect ($155.0 \pm 11.7\%$ of basal outflow facility) after administration of 30 nM abn-cbd. Pretreatment with 1 μ M of O-1918, a cannabidiol analog that acts as a selective antagonist at the non-CB1/CB2 receptor, produced a full antagonism of 30 nM abn-cbd induced increase of aqueous humor outflow facility. Pretreatment with 1 μ M of CB1 antagonist SR141716A partially blocked, whereas pretreatment with either 1 μ M of CB1 antagonist AM251 or 1 μ M of CB2 antagonist SR144528 had no effect on abn-cbd induced enhancement of outflow facility. Treatment of TM cells with 30 nM of abn-cbd activated p42/44 MAPK, which was blocked completely by pretreatment with O-1918, and partially by pretreatment with SR141716A, but not by either AM251 or SR144528. In addition, PD98059, an inhibitor of p42/44 MAPK pathway, blocked completely the abn-cbd induced p42/44 MAPK activation and blocked partially the abn-cbd induced enhancement of outflow facility. In conclusion, the results from this study demonstrate that abn-cbd increases aqueous humor outflow through the TM pathway of the eye, and this effect is mediated by a non-CB1/CB2 cannabinoid receptor, with an involvement of p42/44 MAPK signaling pathway.

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Keywords

Abnormal-cannabidiol; Cannabinoid receptor; Intraocular pressure; Trabecular meshwork; Aqueous humor outflow; p42/44 mitogen-activated protein kinase

1. Introduction

Abnormal increase in intraocular pressure (IOP), which occurs as a result of increased resistance to drainage of aqueous humor, is a major risk factor for optic nerve damage in glaucoma (Crowston and Weinreb, 2005; Weinreb and Khaw, 2004). 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 (TM) and uveoscleral routes (Cunningham and Barry, 1986; Toris, 2010).

Four decade ago Hepler and Frank first reported the IOP-lowering effects of marijuana smoking (Hepler and Frank, 1971). Since then, the IOP-lowering effects of various cannabinoids and their potential as new anti-glaucoma agents have been studied in humans as well as in animal models (Colasanti, 1986; Green, 1998; Jarvinen et al., 2002; Tomida et al., 2004). One interesting feature for cannabinoids as potential IOP-lowering drugs is their effects to enhance aqueous humor outflow (Njie et al., 2008a; Njie et al., 2006; Njie et al., 2008b; Zhong et al., 2005).

Cannabinoids signal to cells by binding to two major cannabinoid receptors, CB1 and CB2 (Howlett, 2005; Pertwee, 2005). Previous studies, including those of ours, have shown that both CB1 (Njie et al., 2006; Stamer et al., 2001; Straiker et al., 1999) and CB2 (He and Song, 2007; Zhong et al., 2005) receptors are expressed in TM cells. In addition, we have demonstrated that administration of both CB1 and CB2 cannabinoid agonists enhance aqueous humor outflow facility (Njie et al., 2008a; Njie et al., 2006; Njie et al., 2008b; Zhong et al., 2005). It is well known that cannabinoids activate p42/44 mitogen-activated protein kinases (MAPKs), also referred to as extracellular stress-related kinase (ERK-1/ERK-2) (Howlett, 2005; Pertwee, 2005). Previously, we have shown that treatment of TM cells with noladin ether, a CB1 agonist, activated p42/44 MAPK, which was blocked by pretreatment with CB1 antagonist SR141716A (Njie et al., 2006). Similarly, treatment of TM cells with JWH015, a CB2 agonist, resulted in the activation of p42/44 MAPK, which was blocked by pretreatment with CB2 antagonist SR144528 (Zhong et al., 2005). Furthermore, CB1 and CB2 receptor-mediated activation of p42/44 MAPK in TM cells is linked to increased outflow facility induced by CB1 and CB2 agonists (Njie et al., 2008a; Njie et al., 2006; Zhong et al., 2005).

Several years ago, a US patent by Allergan Pharmaceuticals claimed that abnormal-cannabidiol (abn-cbd), a synthetic analogue of the plant-derived cannabidiol, is a potent IOP-lowering agent, with a great potential for the treatment of glaucoma (US 7,618,966). In addition, a recent publication has documented the IOP-lowering effects of abn-cbd (Szczesniak et al., 2011). However, currently the mechanisms of actions for the IOP-lowering effects of abn-cbd remain unclear. As a first step to explore the potential mechanisms of abn-cbd induced decrease of IOP, in this study we have addressed the

potential role of abn-cbd in regulating the aqueous humor outflow and the type of receptor that is involved in mediating the abn-cbd induced enhancement of outflow. Furthermore, we have studied abn-cbd induced p42/44 MAPK activation in TM cells and the involvement of p42/44 MAPK signaling pathway in abn-cbd induced enhancement of outflow facility.

2. Materials and methods

2.1. Materials

Abn-cbd, AM251 and O-1918 were purchased from Cayman Chemical (Ann Arbor, MI). The cannabinoid receptor antagonists SR141716A and SR144528 were obtained from National Institute of Drug Abuse (Rockville, MD). PD98059 was purchased from Sigma-Aldrich (St. Louis, MO). Fresh porcine eyes were obtained from Swift & Co (Louisville, KY) within 30 minutes following decapitation. Penicillin/Streptomycin mixture and Dulbecco's Modification of Eagles Medium (DMEM) were purchased from Fisher Scientific (Pittsburg, PA). The polyclonal anti-p42/44 MAPK antibody and the monoclonal anti-phospho-p42/44 (Thr202/Tyr204) MAPK antibody were purchased from Cell Signaling Technology (Beverly, MA).

2.2. Porcine anterior segment perfused organ culture model

A previously published procedure (Bradley et al., 1998) was followed for the anterior segment perfused organ culture model. Porcine anterior segment explants, comprised of the intact cornea, the undisturbed TM, and a 2–5 mm rim of sclera with the ciliary body and iris gently removed, were mounted in a standard perfusion culture apparatus and perfused with DMEM using a constant perfusion head of 10 cm (approximately 7.35 mmHg) for one day, while outflow was stabilized. Only those explants that stabilized between 1.5 and 8 μ l/min at 7.35 mmHg were used. Cultures were maintained at 37°C with 5% CO₂ and 95% air. It had been shown previously that in this model, outflow is through the TM, and that flow rates are physiologically relevant (approximately 2.75 μ l/min (Bradley et al., 1998). Abn-cbd was introduced by exchanging the perfusion chambers with drug containing medium and monitored for 5 hours; vehicle control was run in parallel. For the antagonist and inhibitor studies, the respective antagonist or inhibitor was applied to the perfusion medium 30 minutes prior to treatment with abn-cbd and was present throughout the treatment. Ten anterior eye segments were used for each treatment groups.

At the end of the perfusion study, the anterior segments were perfusion fixed at 7.35 mmHg 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 microscopy. Perfusion studies were regarded as invalid and data discarded if more than one quadrant per eye had unacceptable morphological findings, such as excessive trabecular meshwork cell loss and denudation of trabecular beams.

2.3. Culture of porcine trabecular meshwork cells

The TM was isolated from fresh porcine eyes by blunt dissection. Culture of TM cells from pooled porcine eyes was performed according to previously published methods (Polansky et al., 1979; Tripathi and Tripathi, 1982). 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 (Polansky et al., 1979; Tripathi and Tripathi, 1982). The identity of TM cells were further confirmed by phagocytosis of fluorescein-labeled polystyrene beads (Polysciences, Warrington, PA).

2.4. Western blot analysis

For analysis of MAPK activity, the TM cells were seeded into 6-well plates at a density of 2×10^5 cells per well and were grown to confluence. TM cells were maintained in serum-free medium overnight, following which the TM cells were treated with abn-cbd for 10 minutes. For antagonism experiments, the cells were pretreated with vehicle, receptor antagonists O-1918, SR141716A, AM251, SR144528, or inhibitor PD98059 for 30 minutes. The cells were then treated with abn-cbd for 10 minutes. At the end of the treatment period, cells were washed with ice cold PBS, and 100 μ l of ice-cold lysis buffer containing 50 mM β -glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM $NaVO_4$, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, and 1 μ g/ml of a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) were added. The whole cell lysate was clarified by centrifugation at 14,000 $\times g$ for 10 minutes, the supernatants were collected, and total protein concentration was measured using the Bradford protein assay reagent (Bio-Rad, Hercules, CA). 50 μ g of proteins were mixed with 4x laemmli sample buffer, and after boiling for 5 minutes, proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. Subsequently, the proteins were transferred onto a nitrocellulose membrane and the membranes were blocked with 3% non-fat milk. The blots were probed with a monoclonal anti-phospho-p42/44 MAPK (Thr202/Tyr204) antibody. Antibody binding was visualized by enhanced chemiluminescence western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). The membranes were then stripped and re-probed for total p42/44 MAPK using a rabbit polyclonal anti-p42/44 MAPK antibody.

2.5. Data analysis

For the anterior segment perfusion studies, outflow facility was calculated as the ratio of the rate of flow of perfusate (μ l/min) to the steady state perfusion pressure (mmHg). Drug effects were evaluated in each eye as the percent change of outflow facility in drug-treated eyes over pre-drug baseline outflow facility. The data was presented as mean $\pm$ SEM, and plotted as change in outflow facility versus time (in minutes) using the Prism software (Graph Pad, San Diego, CA).

For p42/44 MAPK phosphorylation assay, the bands on x-ray films were scanned (Personal Densitometer SI; Molecular Dynamics, Sunnyvale, CA) and were quantified using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The results were expressed as mean $\pm$ SEM.

For both the anterior segment perfusion and p42/44 MAPK phosphorylation studies, One-way ANOVA with Newman-Keuls post-test was used to compare the data points of the different treatment groups. The level of significance was chosen as $p < 0.05$.

3. Results

3.1. Abn-cbd induced enhancement of aqueous humor outflow facility

Outflow facility studies were performed using the porcine anterior segment perfused organ culture model. As shown in Fig. 1, the outflow facility increased in a concentration dependent manner within 1 hour of adding 3 and 30 nM of abn-cbd. The abn-cbd induced enhancement of outflow facility lasted for at least 5 hours with both 3 nM and 30 nM concentrations, with a maximum effect ($155 \pm 11.7\%$ of basal outflow facility) achieved with 30 nM of abn-cbd. In control experiments, vehicle (DMEM) alone had no significant effects on outflow facility.

3.2 Effects of cannabinoid antagonists on abn-cbd induced enhancement of aqueous humor outflow facility

We examined the antagonistic effect of O-1918, a selective abn-cbd receptor antagonist (Offertaler et al., 2003), on abn-cbd induced change of outflow facility. Following a 30 min pretreatment of perfused anterior segments with 1 μ M O-1918, there was a complete blockade of the 30 nM abn-CBD-induced enhancement of aqueous humor outflow facility (Fig. 2A). On the other hand, in control experiments, O-1918 by itself had no effects on outflow facility (Fig. 2A). SR141716A, a CB1 antagonist (Rinaldi-Carmona et al., 1994), partially blocked abn-cbd induced increase in outflow facility (Fig. 2B). However, neither AM251, a CB1 antagonist (Gatley et al., 1996), nor SR144528, a CB2 antagonist (Rinaldi-Carmona et al., 1998), had any significant effect on abn-cbd induced increase of outflow facility (Fig. 2C, 2D).

3.3. Abn-cbd induced activation of p42/44 mitogen-activated protein kinase in trabecular meshwork cells - involvement of a non-CB1/CB2 cannabinoid receptor

The p42/44 MAPK signaling pathway is known to be involved in both CB1 and CB2 cannabinoid receptor signaling (Howlett, 2005; Pertwee, 2005). To explore whether the non-CB1/CB2 cannabinoid receptor expressed on TM cells is functionally coupled to p42/44 MAPK signaling pathway, we tested the effect of abn-cbd on p42/44 MAPK phosphorylation in cultured TM cells. Exposure of TM cells to 30 nM abn-cbd for 10 min significantly increased phosphorylation of p42/44 MAPK, which was blocked by preincubation with 1 μ M of O-1918, an abn-cbd receptor antagonist (Fig. 3A). Pretreatment of TM cells with CB1 antagonist SR141716A partially blocked, whereas pretreatment with CB2 antagonist SR144528 had no effect on the phosphorylation of p42/44 MAPK induced by abn-cbd (Fig. 3A). Furthermore, pretreatment of TM cells with another CB1 antagonist, AM251, had no significant effect on the phosphorylation of p42/44 MAPK induced by abn-cbd (Fig. 3B).

3.4. Involvement of p42/44 mitogen-activated protein kinase pathway in abn-cbd induced enhancement of outflow facility

To further elucidate the role of the p42/44 MAPK signaling pathway in abn-cbd induced enhancement of outflow facility, PD98059, an inhibitor of the p42/44 MAPK pathway (Alessi et al., 1995), was administered to the perfused anterior segment prior to the administration of abn-cbd. As shown in Fig. 4A, pretreatment of the perfused porcine anterior segments with 30 μ M PD98059 significantly blocked the outflow-enhancing effects of 30 nM abn-cbd. PD98059 by itself had no significant effect on outflow facility (Fig. 4A). Furthermore, pretreatment of TM cells with 30 μ M PD98059 resulted in a significant inhibition of phosphorylation of p42/44 MAPK induced by abn-cbd (Fig. 4B).

4. Discussion

Increased IOP is a result of imbalance between the rate of aqueous humor secretion and outflow. The present study utilized perfused porcine anterior segment to examine the regulation of aqueous humor outflow by abn-cbd. The perfused anterior segment organ culture model provides ideal conditions for studying aqueous humor outflow as it preserves the critical architecture of the outflow pathway (Johnson and Tschumper, 1987; Pang et al., 2000). In previous studies, we have shown that both the CB1 and CB2 cannabinoid receptors are expressed on the trabecular meshwork cells (Njie et al., 2006; Zhong et al., 2005). In addition, we have reported that a variety of cannabinoid ligands, including CB1 selective agonist noladin ether (Njie et al., 2006), CB2 selective agonist JWH015 (Zhong et al., 2005), as well as endocannabinoids N-arachidonylethanolamide (Njie et al., 2008b) and 2-arachidonylglycerol (Njie et al., 2008a), increase aqueous humor outflow facility by acting on CB1 and CB2 cannabinoid receptors.

Abnormal-cannabidiol (abn-cbd) is a synthetic analogue of the plant-derived cannabidiol. Abn-cbd has been found to induce endothelium-dependent vasodilation and to lower blood pressure via a CB1/CB2-independent mechanism (Jarai et al., 1999; Offertaler et al., 2003). It is believed that abn-cbd activates a novel type of cannabinoid receptor distinct from CB1 and CB2, provisionally called the non-CB1/CB2, abn-cbd receptor (Jarai et al., 1999; Offertaler et al., 2003).

In 2004, a US patent was granted to Allergan Inc, which claims the use of abn-cbd as a novel anti-glaucoma agent to lower intraocular pressure (US 7,618,966). In addition, a recent publication has documented the IOP-lowering effects of abn-cbd (Szczesniak et al., 2011). However, the mechanisms for the IOP-lowering effects of abn-cbd have not been reported until the current study.

In the present study we have investigated the effect of abn-cbd in regulating aqueous humor outflow facility. We have demonstrated for the first time that abn-cbd increases outflow facility in perfused porcine anterior eye segments in a concentration-dependent manner. Pretreatment of anterior segment with O-1918, a selective non-CB1/CB2 cannabinoid receptor antagonist, blocked the outflow-enhancing effect of abn-cbd, indicating that a non-CB1/CB2 cannabinoid receptor is involved. Furthermore, CB1 antagonist SR141617A blocked partially, but neither CB1 antagonist AM251 nor CB2 antagonist SR144528 had any

significant effect on the abn-cbd induced enhancement of outflow facility. These data are consistent with those of previous reports on abn-cbd induced vasodilatation and lowering of blood pressure (Jarai et al., 1999; Offertaler et al., 2003). Abn-cbd caused an endothelium-dependent vasodilation in rat isolated mesenteric arteries through a G protein-coupled receptor distinct from CB1 or CB2 (Jarai et al., 1999; Offertaler et al., 2003). Both abn-cbd induced outflow-enhancing effects in the present study and abn-cbd induced vasodilatory effects in the previous studies (Jarai et al., 1999; Offertaler et al., 2003) were antagonized completely by O-1918, and to a lesser extent, by SR141716A. However, since it has been shown in ligand binding studies that neither abn-cbd nor O-1918 binds to the CB1 receptor (Jarai et al., 1999; Offertaler et al., 2003), it is clear that the abn-cbd receptor is distinct from CB1. In a recent study, the IOP-lowering effect of abn-cbd was blocked by O-1918 but was unaffected by CB1 antagonist AM251 or CB2 antagonist AM630 (Szczesniak et al., 2011). Furthermore, abn-cbd induced human umbilical vein endothelial cell migration was partially blocked by CB1 antagonist SR141716A but not by either CB1 antagonist AM251 or CB2 antagonist SR144528 (Mo et al., 2004). Since CB1 antagonist AM251 did not block the effect of abn-cbd in the current study, it is unlikely that CB1 is involved in the abn-cbd induced outflow facility. It is likely that SR141716A is able to bind to the non-CB1/CB2, abn-cbd receptor, so that it exhibits partial antagonistic activities on abn-cbd induced effects in TM cells as well as in blood vessels (Jarai et al., 1999; Mo et al., 2004).

The p42/44 MAPK pathway has also been shown to be involved in mediating the outflow-enhancing effects of several biochemical agents (Alexander and Acott, 2003; Shearer and Crosson, 2002). For example, the IOP-lowering effect of *N*⁶-cyclohexyladenosine, an adenosine A₁ agonist, is mediated in part by increasing in outflow facility (Shearer and Crosson, 2002) and involves the increased phosphorylation of p42/44 MAPK (Shearer and Crosson, 2002).

Activation of p42/44 MAPK activity is one of the well-characterized signaling pathways for the CB1 and CB2 cannabinoid receptors (Howlett, 2005; Pertwee, 2005). Our previous studies have shown that treatment of porcine TM cells with both CB1 and CB2 agonists increased phosphorylation of p42/44 MAPK (Njie et al., 2008a; Njie et al., 2006; Zhong et al., 2005). Furthermore, PD98059, an inhibitor of the p42/44 MAP kinase pathway, inhibited cannabinoid agonist-induced increase in aqueous humor outflow (Njie et al., 2008a; Njie et al., 2006; Zhong et al., 2005).

In the current study, abn-cbd was shown to activate p44/42 MAPK in cultured porcine TM cells, and this effect of abn-cbd was blocked completely by pretreatment of the cells with O-1918, partially by SR141716A, but not by either AM251 or SR144528. These data demonstrate the existence of functional abn-cbd receptors in TM cells that are coupled to p42/44 MAPK. Our data are consistent with a previous report that abn-cbd activates p42/44 MAPK in Human Umbilical Vein Endothelial Cells (HUVEC) cells by acting on the non-CB1/CB2 cannabinoid receptors (Offertaler et al., 2003). Thus, our finding of abn-cbd induced activation of p42/44 MAPK in TM cells suggests that abn-cbd induced increases of aqueous humor outflow may be mediated through the p42/44 MAPK pathway. This hypothesis was further supported by the evidence that pretreatment of the perfused anterior segments with PD90859, an inhibitor of the p42/44 MAP kinase pathway, partially blocked

abn-cbd induced enhancement of outflow facility. Since the blockade by PD90859 is partial, it is possible that other, yet to be identified signaling pathways are also involved in abn-cbd induced increases of aqueous humor outflow.

In summary, results from the present paper demonstrate for the first time that aqueous humor outflow facility can be modulated by administration of abn-cbd, a synthetic analogue of plant cannabidiol. This outflow-enhancing effect of abn-cbd is mediated through a non-CB1/CB2, abn-cbd receptor in TM cells. Furthermore, data from this study support the hypothesis that the p42/44 MAPK pathway is involved in mediating abn-cbd induced increase in outflow facility.

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Abbreviations

abn-cbd	abnormal-cannabidiol
GPCR	G protein-coupled receptor
p42/44 MAPK	p42/44 mitogen-activated protein kinase
TM	trabecular meshwork
IOP	intraocular pressure

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Highlights

- Abnormal-cannabidiol enhances aqueous humor outflow facility.
- A non-CB1/CB2 cannabinoid receptor is involved.
- The p42/44 mitogen-activated protein kinase signaling pathway is involved.

Fig. 1. Abn-cbd induced enhancement of aqueous humor outflow facility
 Two different concentrations (3 nM and 30 nM) of abn-cbd were used. Results are expressed as mean $\pm$ SE; $n=10$ eyes. *Significant differences from vehicle ($p<0.05$, ANOVA with Neuman-Keuls post-test).

Fig. 2. Effects of cannabinoid antagonists on abn-cbd induced enhancement of aqueous humor outflow facility

(A) The effect of O-1918 on abn-cbd induced increase of aqueous humor outflow facility. The anterior segments were pretreated with 1 μ M O-1918 for 30 minutes before the addition of 30 nM abn-cbd plus 1 μ M O-1918 for 5 hours. (B) The effect of CB1 antagonist SR141716A on abn-cbd induced increase of aqueous humor outflow facility. The anterior segments were pretreated with 1 μ M SR141716A for 30 minutes before the addition of 30 nM abn-cbd plus 1 μ M SR141716A for 5 hours. (C) The effect of CB1 antagonist AM251 on abn-cbd induced increase of aqueous humor outflow facility. The anterior segments were pretreated with 1 μ M AM251 for 30 minutes before the addition of 30 nM abn-cbd plus 1 μ M AM251 for 5 hours. (D) The effect of CB2 antagonist SR144528 on abn-cbd induced increase of aqueous humor outflow facility. The anterior segments were pretreated with 1 μ M SR144528 for 30 minutes before the addition of 30 nM abn-cbd plus 1 μ M SR144528 for 5 hours. Results are expressed as mean $\pm$ SE; $n=10$ eyes. *Significant differences between abn-cbd alone and abn-cbd plus antagonists ($p<0.05$, ANOVA with Neuman-Keuls post-test).

Fig. 3. Abn-cbd induced activation of p42/44 mitogen-activated protein kinase in trabecular meshwork cells

(A) Top: Western blot representative of results obtained in three experiments. Cells were serum-starved for 18 h and pretreated with vehicle, 1 μ M O-1918, 1 μ M SR141716A (SR1), a CB1 antagonist, or 1 μ M SR144528 (SR2), a CB2 antagonist, for 15 min, followed by stimulation with vehicle or 30 nM abn-cbd for 10 min. Bottom: Densitometry quantification of the phospho-p42/44 data from three experiments. (B) Top: Western blot representative of results obtained in three experiments. Cells were serum-starved for 18 h and pretreated with vehicle, 1 μ M AM251, a CB1 antagonist, for 15 min, followed by stimulation with vehicle or 30 nM abn-cbd for 10 min. Bottom: Densitometry quantification of the phospho-p42/44 data from three experiments. Results are expressed as mean $\pm$ SEM, $n = 3$ independent experiments. *Significant difference from vehicle alone ($P < 0.05$ ANOVA with Newman–Keuls post-test). **Significant differences between abn-cbd alone and abn-cbd plus antagonists ($p < 0.05$, ANOVA with Neuman–Keuls post-test).

Fig. 4. Involvement of p42/44 mitogen-activated protein kinase pathway in abn-cbd induced enhancement of outflow facility

(A) The effects of PD98059 on abn-cbd induced enhancement of aqueous humor outflow facility. The anterior segments were pretreated with 30 μ M PD98059 for 30 minutes before the addition of 30 nM abn-cbd. Results are expressed as the mean $\pm$ SE; $n=10$ eyes.

*Significant differences between abn-cbd alone and abn-cbd plus PD98059 ($P < 0.05$, ANOVA with Neuman–Keuls post-test). (B) The effects of PD98059 on abn-cbd induced phosphorylation of p42/44 MAPK in TM cells. Top: Western blot representative of results obtained in three experiments. Cells were serum-starved for 18 h and pretreated with 30 μ M PD98059 for 15 min, followed by stimulation with vehicle or 30 nM abn-cbd for 10 min. Bottom: Densitometry quantification of the phospho-p42/44 data from three experiments. Results are expressed as mean $\pm$ SEM; $n = 3$ independent experiments. *Significant difference from vehicle alone ($P < 0.05$ ANOVA with Newman–Keuls post-test).

**Significant differences between abn-cbd alone and abn-cbd plus PD98059 ($p < 0.05$, ANOVA with Neuman–Keuls post-test).