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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 ± 11.7% of basal outflow facility) after administration of 30 nM abn-cbd. Pretreatment with 1 μM 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 μM of CB1 antagonist SR141716A partially blocked, whereas pretreatment with either 1 μM of CB1 antagonist AM251 or 1 μM 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.

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., 2006, 2008a, 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., 2006, 2008a, 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

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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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., 2006, 2008a; Zhong et al., 2005).

Several years ago, a US patent by Allergan Pharmaceuticals claimed that abnormal-cannabidiol (abn-cbd), a synthetic analog 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 min following decapitation. Penicillin/Streptomycin mixture and Dulbeco's Modification of Eagles Medium (DMEM) were purchased from Fisher Scientific (Pittsburgh, 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 μl/min at 7.35 mmHg were used. Cultures were maintained at 37 °C with 5% CO_2 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 μl/min) (Bradley et al., 1998). Abn-cbd was introduced by exchanging the perfusion chambers with drug containing medium and monitored for 5 h; vehicle control was run in parallel. For the antagonist and inhibitor studies, the respective antagonist or inhibitor was applied to the perfusion medium 30 min 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 h. 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 h 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 min. For antagonism experiments, the cells were pretreated with vehicle, receptor antagonists O-1918, SR141716A, AM251, SR144528, or inhibitor PD98059 for 30 min. The cells were then treated with abn-cbd for 10 min. At the end of the treatment period, cells were washed with ice-cold PBS, and 100 μl 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 μg/ml of a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) were added. The whole cell lysate was clarified by centrifugation at 14,000 × *g* for 10 min, the supernatants were collected, and total protein concentration was measured using the Bradford protein assay reagent (Bio-Rad, Hercules, CA). 50 μg of proteins were mixed with 4× laemmli sample buffer, and after boiling for 5 min, 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 (μl/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 ± 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