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Atypical cannabinoid stimulates endothelial cell migration via a G_i/G_o-coupled receptor distinct from CB_1, CB_2 or EDG-1

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Abstract

The endothelium-dependent mesenteric vasorelaxant effect of anandamide has been attributed to stimulation of a G_i/G_o-coupled receptor, for which the nonpsychoactive analog abnormal cannabidiol (abn-cbd, (−)-4-(3-3,4-*trans*-*p*-menthadien-[1,8]-yl)olivetol) is a selective agonist and the compound O-1918 ((−)-4-(3-3,4-*trans*-*p*-menthadien-(1,8)-yl)-orcinol) is a selective antagonist. In human umbilical vein endothelial cells abn-cbd was reported to increase the phosphorylation of p44/42 mitogen activated protein kinase (MAPK) and protein kinase B/Akt, and these effects could be inhibited by pertussis toxin, by phosphatidylinositol 3-kinase (PI3K) inhibitors or by O-1918 [Mol. Pharmacol. 63 (2003) 699]. In the present experiments, abn-cbd caused a concentration-dependent increase in human umbilical vein endothelial cell migration, as quantified in a transwell chamber. This effect was antagonized by O-1918, by the PI3K inhibitor wortmannin, and by pertussis toxin, but not by the cannabinoid CB_1 receptor antagonist AM251 or the cannabinoid CB_2 receptor antagonist SR144528. The EDG-1 receptor agonist sphingosine-1-phosphate also increased human umbilical vein endothelial cell migration, but this response was unaffected by O-1918. In Chinese hamster ovary cells stably transfected with the gene encoding the EDG-1 receptor, p44/42 MAPK phosphorylation was unaffected by abn-cbd, but strongly induced by sphingosine-1-phosphate. These results indicate that an abn-cbd-sensitive endothelial receptor distinct from cannabinoid CB_1, CB_2 or EDG-1 receptors mediates not only vasorelaxation but also endothelial cell migration.

Keywords: Vascular endothelium; Atypical cannabinoid receptor; Cell migration

1. Introduction

Cell migration is an integral component of numerous physiological and pathological processes such as embryogenesis, angiogenesis, tumor metastasis, inflammation, immune responses, and wound healing. It has been well established that during the process of angiogenesis, migration of endothelial cells is stimulated by mitogens such as vascular endothelial growth factor, fibroblast growth factor, and platelet-derived growth factor, each interacting with their respective receptors. Recent studies have suggested that lipid chemoattractants such as sphingosine-1-phosphate are critically important stimuli for endothelial cell migration and its corollary, angiogenesis (Wang et al., 1999; Lee et al., 1999). The effect of sphingosine-1-phosphate is mediated via its pertussis toxin-sensitive G protein-coupled receptor, the EDG-1 receptor (Lee et al., 1998).

Endogenous cannabinoids and their receptors have been also implicated in the processes of cell migration and angiogenesis. To date, two cannabinoid receptors have been identified by cloning: cannaboinoid CB_1 receptors expressed predominantly in the brain (Matsuda et al., 1990) and also in peripheral tissues including the vascular endothelium (Liu et al., 2000a,b), and cannabinoid CB_2 receptors expressed by cells of the immune (Munro et al., 1993) and hematopoetic systems (Derocq et al., 2000; Jordà et al., 2002). Both cannabinoid CB_1 and CB_2 receptors are negatively coupled to adenylate cyclase via G_i/G_o proteins (Howlett, 2002), and can also activate p44/42 mitogen activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K)/Akt pathway (Bouaboula et al., 1995; Molina-Holgado et al., 2002). The effects of cannabinoids on cell migration are complex, as both stimulation and inhibition of chemotaxis have been reported. In human embryonic kidney (HEK-293) cells stably transfected with

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the human cannabinoid CB_1 receptor gene, synthetic cannabinoids as well as the endocannabinoid anandamide potently stimulate cell migration, and these effects could be antagonized by low concentrations of the selective cannabinoid CB_1 receptor antagonist SR141716 (Song and Zhong, 2000). In contrast, activation of central cannabinoid CB_1 receptors was reported to inhibit neutrophil migration in response to inflammatory stimuli by suppressing the production of neutrophil chemoattractants (Smith et al., 2001). Stimulation of cannabinoid CB_2 receptors by the endocannabinoid 2-arachidonoylglycerol increases the migration of HL-60 cells (Jordà et al., 2002; Kishimoto et al., 2003), mouse microglia (Walter et al., 2003), human peripheral blood monocytes (Kishimoto et al., 2003) and murine myeloid leukemia cells (Jordà et al., 2002). In contrast, in rat peritoneal macrophages, cannabinoid CB_2 activation inhibited both spontaneous migration and formylated Met–Leu–Phe-induced chemotaxis (Sacerdote et al., 2000), and the selective cannabinoid CB_2 receptor agonist JWH-133 (3-(1′,1′-dimethylbutyl)-1-deoxy-Δ^8-tetrahydrocannabinol) was reported to inhibit the migration of human umbilical vein endothelial cells (Blázquez et al., 2003). Cannabinoid CB_2 receptor activation may inhibit angiogenesis indirectly via inhibition of the expression of angiogenic growth factors, such as vascular endothelial growth factor, placental growth factor and angiopoietin 3, as reported in nonmelanoma skin tumors (Casanova et al., 2003).

Our recent findings, confirmed by others, indicate that the vascular endothelium contains an as-yet-unidentified cannabinoid receptor. The endocannabinoid anandamide, but not Δ^9-tetrahydrocannabinol or potent synthetic cannabinoid CB_1 and CB_2 receptor agonists, causes endothelium-dependent mesenteric vasorelaxation susceptible to inhibition by micromolar concentrations of the cannabinoid CB_1 receptor antagonist SR141716 (Wagner et al., 1999; Járai et al., 1999). This effect is inhibited by pertussis toxin (Mukhopadhyay et al., 2002; Offertáler et al., 2003; Ho and Hiley, 2003), yet it persists in mice deficient in cannabinoid CB_1 or in both CB_1 and CB_2 receptors (Járai et al., 1999). Abnormal cannabidiol (abn-cbd, (−)-4-(3-3,4-*trans*-*p*-menthadien-[1,8]-yl)-olivetol), a structural analog of the behaviorally inactive marijuana constituent cannabidiol, is a selective agonist of this receptor, whereas the compound of O-1918 [(−)-4-(3-3,4-*trans*-*p*-menthadien-[1,8]-yl)-orcinol] is a selective, silent antagonist (Offertáler et al., 2003). Abn-cbd causes hypotension as well as endothelium-dependent mesenteric vasorelaxation, and both effects persist in mice deficient in cannabinoid CB_1 receptors (Járai et al., 1999). Additional findings in cultured human umbilical vein endothelial cells indicate that the abn-cbd-sensitive endothelial receptor is coupled through G_i/G_o to PI3K/AKT, MAPK, and BK_{Ca} channels (Begg et al., 2003).

The expression of BK_{Ca} channels in endothelial cells is increased during cell proliferation. This has been proposed to be an index of dedifferentiation (Köhler et al., 2000; Papassotiriou et al., 2000; Bychkov et al., 2002), but it could also suggest their potential involvement in angiogenesis (Kestler et al., 1998). Furthermore, it has been recently reported that cannabinoid receptor agonists promote microglial migration and that both cannabinoid CB_2 receptors and abn-cbd-sensitive receptors are involved (Walter et al., 2003). Therefore, in the present study, we examined whether activation of abn-cbd-sensitive receptors promotes the migration of human umbilical vein endothelial cells.

2. Materials and methods

2.1. Reagents

Abnormal cannabidiol and O-1918 were synthesized as described previously (Offertáler et al., 2003). Arachidonoyl ethanolamide (anandamide), pertussis toxin, wortmannin and platelet-derived growth factor were purchased from Sigma (St. Louis, MO). Sphingosine-1-phosphate was from Biomol (Plymouth Meeting, PA). HU-210 [(−)-11-*OH*-Δ^9-tetrahydrocannabinol dimethylheptyl], SR141716 (*N*-[piperidin-1-yl]-5-[4-chlorophenyl]-1-[2,4-dichlorophenyl]-4-methyl-1*H*-pyrazole-3-carboxamide HCl) and SR144528 (*N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo[2.2.1]-heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methoxybenzyl)-pyrazole-3-carboxamide) were from the National Institute on Drug Abuse, AM251 (*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methy l-1*H*-pyrazole-3-carboxamide) was from Tocris (Bristol, UK). Abnormal cannabidiol, O-1918, anandamide, SR141716A, SR144528, and AM251 were dissolved in ethanol, then diluted by adding Alkamuls EL-620 (Rhodia, Cranbury, NJ) and phosphate-buffered saline in a ratio of 1:1:8 to provide stock solutions, which were stored at −20 °C. Alkalmuls EL-620 is an emulsifier containing castor oil ethoxylates. The stock was further diluted with phosphate-buffered saline prior to treating the cells so that the final concentration of ethanol or Alkalmus EL-620 was ≤0.002% (≤0.4 mM). Vehicle was prepared and diluted in the same manner and had no significant effect on cell migration. Pertussis toxin, platelet-derived growth factor, wortmannin and sphingosine-1-phosphate were dissolved according to the manufacturers' instructions.

2.2. Cell culture

Human umbilical vein endothelial cells were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in EGM-2 BulletKit (Bio-Whittaker, Walkersville, MD) containing 2% fetal bovine serum and different growth factors at 37 °C under an atmosphere of 5% CO_2 in air. Cultures from two to five passages were used for experiments. Chinese hamster ovary cells stably transfected with the gene encoding the EDG-1 receptor were prepared as described (Lee et al., 1999).

2.3. Cell migration

The chemotactic migration of human umbilical vein endothelial cells was assayed using a transwell chamber of 10 mm in diameter and polycarbonate filters of 8 μm pore size (Nalge Nunc Intl., Naperville, IL). The lower surface of the filters was coated with 0.2% gelatin. Vehicle, agonists, antagonists or their combinations were added to M199 medium containing 1% fetal bovine serum, which was placed in the lower wells. Human umbilical vein endothelial cells were trypsinized, centrifuged and resuspended in M199 medium containing 1% fetal bovine serum in a final concentration of 1×10^6 cells/ml. In experiments where an antagonist was used, the antagonist was also added to the cell suspension 30 min before seeding to allow its equilibration with the receptor before exposure to the agonist present in the lower well. Cells were then trypsinized (0.25% trypsin, 1 mM EDTA), centrifuged and resuspended in M199 medium containing 1% FBS to a cell density of 10^6 cells/ml. Two hundred microliters of cell suspension (2×10^5 cells) were placed into an upper well and incubated at 37 °C and 5% CO_2 in air for 4 h. Nonmigrated cells that remained on the upper well were removed by wiping with a cotton swab and the wells were then rinsed twice with phosphate-buffered saline (PBS). Cells were fixed in 4% formaldehyde in phosphate-buffered saline and stained with hematoxylin. Migrated cells were quantified by counting under a microscopic field at 400× magnification. Five fields were counted for each well and duplicate samples were analyzed for each assay.

2.4. Western blotting

Phosphorylation of p44/42 MAPK was assayed by Western blotting, as described in detail previously (Offertáler et al., 2003).

2.5. Statistical analyses

Drug-induced differences in cell migration were analyzed using the paired *t*-test. For comparisons among multiple groups, one-way ANOVA followed by Bonferroni's test was used. Differences with a P value of <0.05 were considered statistically significant.

3. Results

Abn-cbd stimulated human umbilical vein endothelial cell migration, as illustrated in a representative experiment in Fig. 1. At the concentration of 30 μM, abn-cbd caused a significant increase in the number of migrated cells, whereas no such change could be observed in the presence of vehicle or abn-cbd plus 30 μM O-1918 (Fig. 1). Fig. 2 illustrates the concentration dependence of the effect of abn-cbd and its inhibition in the presence of 30 μM O-1918. The greatest increase in cell migration (>twofold) was observed at a concentration of 30 μM abn-cbd, which was earlier reported to be maximally effective for stimulating Akt phosphorylation and p44/42 MAPK activation in human umbilical vein endothelial cells (Offertáler et al., 2003). At concentrations of 50 μM or above, abn-cbd caused minor cell toxicity, as indicated by decreased cell numbers; therefore, EC_{50} values could not be determined. Overnight pretreatment of the cells with 200 ng/ml of pertussis toxin completely prevented the ability of abn-cbd to increase cell migration without affecting basal levels of migration (Fig. 3).

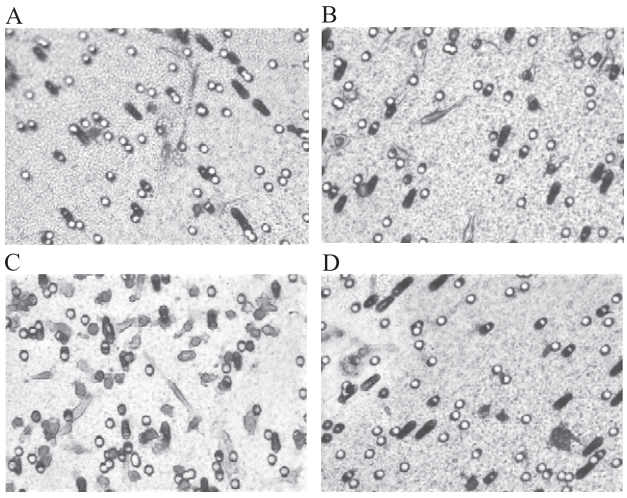

Fig. 1. Stimulation of human umbilical vein endothelial cell migration by abnormal cannabidiol. Cells were cultured and their migration assessed as described in Materials and methods. Migration across a polycarbonate membrane proceeded for 4 h toward the lower well where drugs or vehicle were placed. The membranes were then fixed, stained, and the cells counted. Representative membranes (400× magnification) are shown where the lower well contained no drug (A), vehicle only (B), 30 μM abn-cbd (C) or 30 μM abn-cbd + 30 μM O-1918 (D). Cells in Panel D were also preincubated with 30 μM O-1918 before seeding. Note the increase in cell number in Panel C compared to the other three panels.

Growth factor- and sphingosine-1-phosphate-stimulated cell migrations have been reported to be fully or partially dependent on the PI3K/Akt pathway (Morales-Ruiz et al., 2001), and we have previously reported that Akt phosphorylation by abn-cbd is PI3K dependent in human umbilical vein endothelial cells (Offertáler et al., 2003). We tested whether the ability of abn-cbd to activate the PI3K/Akt pathway is required for its effect on endothelial cell migration. Fig. 4 shows that abn-cbd (30 μM) and sphingosine-1-phosphate (100 nM) significantly increased endothelial cell migration by ~ twofold or threefold, respectively, and that these effects were absent when cells were pretreated for 30 min with 100 nM wortmannin. LY294002 (25 μM), another inhibitor of PI3K, caused similar inhibition (not shown).

Human umbilical vein endothelial cells express the cannabinoid CB_1 receptor (Liu et al., 2000a,b; Blázquez et al., 2003) as well as the abn-cbd-sensitive receptor. To distinguish their relative role in the cell migration response,

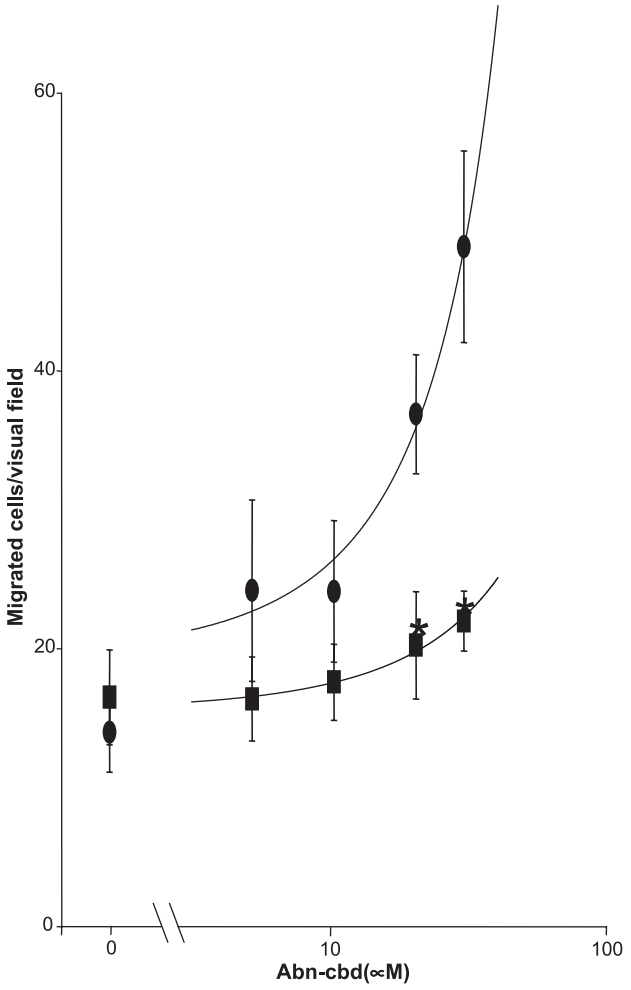

Fig. 2. Concentration-dependent increase in endothelial cell migration by abn-cbd (●) and its inhibition in the presence of 30 μM O-1918 (■). Values at 0 μM were obtained in the presence of vehicle or O-1918 only. *Significant difference from corresponding value in the presence of O-1918 ($P<0.02$). Points and bars represent means ± standard errors from five experiments.

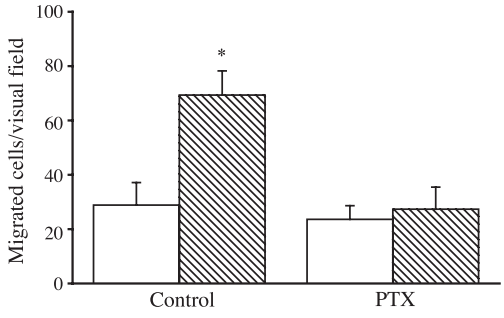

Fig. 3. Pertussis toxin inhibits abn-cbd-induced migration of human umbilical vein endothelial cells. The effect of 30 μM abn-cbd on cell migration was tested in vehicle-incubated control cells ($n=6$, open columns) and in cells preincubated overnight with 200 ng/ml pertussis toxin ($n=6$, hatched columns). *Significant difference ($P<0.01$) from value in controls.

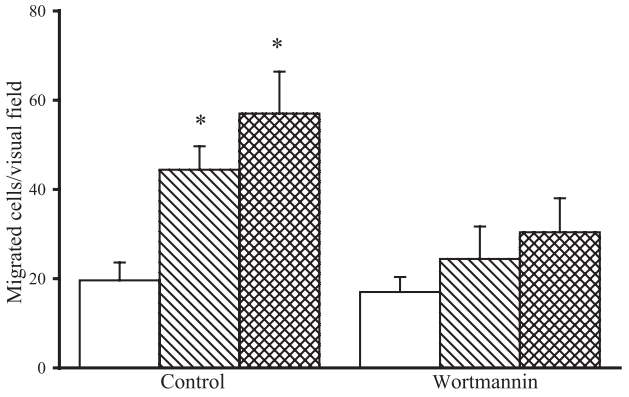

Fig. 4. Endothelial cell migration stimulated by abn-cbd and by sphingosine-1-phosphate (S1P) is PI3K dependent. Migration was assayed in control cells (group of three columns on the left) and in cells preincubated with 100 nM wortmannin (group of three columns on the right) in the presence of vehicle (open columns), 30 μM abn-cbd (hatched columns) or 100 nM sphingosine-1-phosphate (cross-hatched columns). Columns and bars indicate means ± S.E. from five experiments in each group. *Significant difference from vehicle treated cells in the same group ($P<0.01$).

we tested the effect of HU-210, a potent synthetic cannabinoid CB_1/CB_2 receptor agonist with no effect at the abn-cbd-sensitive endothelial receptor (Wagner et al., 1999; Offertáler et al., 2003). As illustrated in Fig. 5, at a concentration maximally effective at cannabinoid CB_1 and CB_2 receptors (100 nM), HU-210 caused only a marginal increase in endothelial cell migration, compared to a significant twofold increase by abn-cbd (30 μM). The effect of abn-cbd was partially inhibited by 3 μM SR141716, but was unaffected by 3 μM AM251, another cannabinoid CB_1 antagonist with subnanomolar K_d for the cannabinoid CB_1 receptor (Gatley et al., 1996). The cannabinoid CB_2 receptor antagonist SR144528 (3 μM) was similarly ineffective in inhibiting the response to abn-cbd.

Cannabinoids including abn-cbd are highly lipophilic and could possibly interact with receptors for other lipid

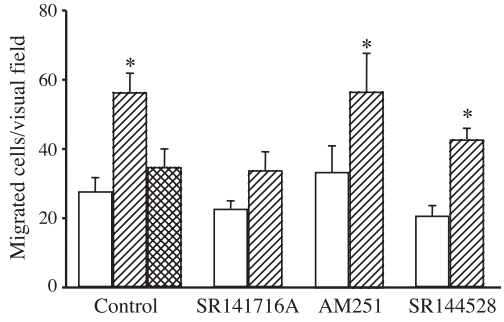

Fig. 5. Cannabinoid CB_1 or CB_2 receptors are not involved in the effect of abn-cbd on human umbilical vein endothelial cell migration. Cells were incubated with vehicle (open columns) or 30 μM abn-cbd (hatched columns) in the presence of no antagonist (control, $n=8$), 3 μM SR141716 ($n=5$), 3 μM AM251 ($n=5$) or 3 μM SR144528 ($n=5$). The effect of 100 nM HU-210 on HUVEC migration was also tested in control cells (cross-hatched column). *Significant difference from corresponding vehicle-treated value ($P<0.05$).

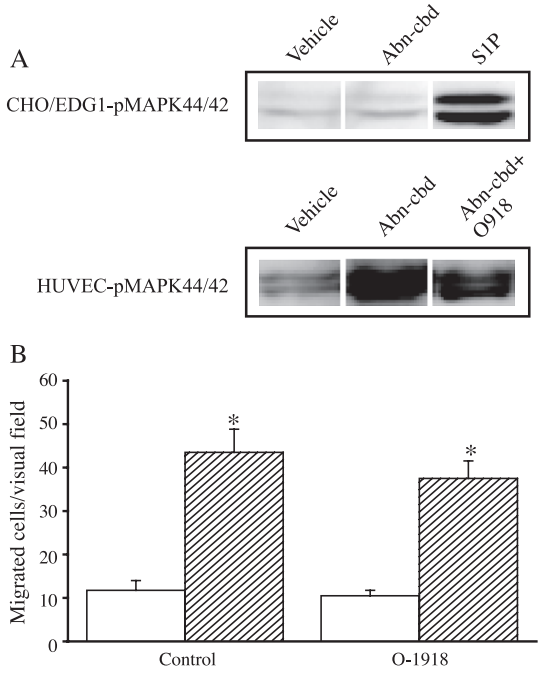

Fig. 6. The abn-cbd-sensitive receptor is distinct from the EDG-1 receptor. (A) Sphingosine-1-phosphate (100 nM), but not abn-cbd (30 μM) stimulates p44/42 MAPK phosphorylation in Chinese hamster ovary cells stably transfected with the EDG-1 receptor gene (top blot), whereas abn-cbd (30 μM) stimulates MAPK phosphorylation in human umbilical vein endothelial cells, and the effect is inhibited by 30 μM O-1918 (bottom blot). These are representative experiments, replicated two more times with similar results. (B) Human umbilical vein endothelial cell migration (vehicle: open columns) stimulated by 100 nM sphingosine-1-phosphate (hatched columns) is not inhibited in the presence of 30 μM O-1918 ($n=4$). *Significant difference ($P<0.01$) from the value in vehicle treated controls, $n=6$ for both groups. Chinese hamster ovary cells transfected with Edg-1 receptor cDNA (CHO/EDG1); human umbilical vein endothelial cells (HUVEC); phosphorylated mitogen activated protein kinase (pMAPK).

ligands such as the EDG-1 receptor, which mediates the chemotactic effect of sphingosine-1-phosphate. We therefore tested whether abn-cbd may be a ligand for the EDG-1 receptor. As illustrated in Fig. 6A, in Chinese hamster ovary cells stably transfected with the gene encoding the EDG-1 receptor, 100 nM sphingosine-1-phosphate caused a pronounced increase in p44/42 MAPK phosphorylation, whereas 30 μM abn-cbd had no effect. The same concentration of abn-cbd activated p44/42 MAPK phosphorylation, and this effect was abrogated in the presence of 30 μM O-1918. Furthermore, endothelial cell migration stimulated by 100 nM sphingosine-1-phosphate was unaffected by 30 μM O-1918 (Fig. 6B).

4. Discussion

The main finding of this study is that abn-cbd, a behaviorally inactive cannabinoid analog, stimulates the migration of vascular endothelial cells through a G_i/G_o-coupled, PI3K/Akt-dependent pathway. Previous studies have established that abn-cbd is a selective agonist and the cannabidiol analog O-1918 is a selective antagonist of an endothelial cannabinoid receptor distinct from cannabinoid CB_1 or CB_2 receptors, stimulation of which elicits vasodilation, as documented in rat mesenteric arteries (Offertáler et al., 2003), and promotes microglia migration (Walter et al., 2003). The presence of this putative endothelial receptor in human umbilical vein endothelial cells is strongly indicated by our earlier findings that abn-cbd stimulated the phosphorylation of p44/p42 MAPK and Akt, and that these effects could be inhibited by pertussis toxin, PI3K inhibitors or O-1918 (Offertáler et al., 2003). The present finding that the abn-cbd-induced migration of endothelial cells is similarly inhibited by O-1918, pertussis toxin or PI3K inhibitors strongly suggests that the same receptor is involved in promoting endothelial cell migration, although the concentrations at which abn-cbd stimulates cell migration (see Fig. 2) are somewhat higher than the concentrations required to elicit mesenteric vasorelaxation (EC_{50} ~ 3 μM, Offertáler et al., 2003).

Human umbilical vein endothelial cells express functional cannabinoid CB_1 receptors (Liu et al., 2000a,b), and the presence in these cells of cannabinoid CB_2 receptor mRNA has also been reported (Blázquez et al., 2003). However, the role of these receptors in the effects of abn-cbd can be excluded. First, abn-cbd is neurobehaviorally inactive and does not bind to cannabinoid CB_1 or CB_2 receptors at concentrations up to 100 μM (Járai et al., 1999). Second, when tested at a concentration maximally effective at cannabinoid CB_1 or CB_2 receptors, the potent cannabinoid receptor agonist HU-210, which has no significant mesenteric vasodilator activity (Wagner et al., 1999), caused only a marginal increase in endothelial cell migration. Third, the effect of abn-cbd on cell migration was unaffected by the cannabinoid CB_1 receptor antagonist AM251 or the cannabinoid CB_2 receptor antagonist SR144528, when used at concentrations several orders of magnitude higher than their respective K_d's for cannabinoid CB_1 or CB_2 receptors. Although the benchmark cannabinoid CB_1 antagonist SR141716 partially inhibited the effect of abn-cbd, earlier studies have demonstrated that micromolar concentrations of this (but not other) cannabinoid CB_1 receptor antagonist inhibits the endothelium-dependent vasodilator effect of anandamide and abn-cbd (Wagner et al., 1999; Járai et al., 1999; Mukhopadhyay et al., 2002; Ho and Hiley, 2003), and even in animals that lack cannabinoid CB_1 or both CB_1 and CB_2 receptors (Járai et al., 1999). Furthermore, in unpublished experiments, we found that AM251 completely lacks the ability of SR141716 to antagonize endotoxin-induced hypotension in rats, and published studies by others have documented a similar differential effectiveness of SR141716 vs. AM251 in inhibiting abn-cbd- (Ho and Hiley, 2003) or *N*-arachidonyldopamine-induced mesenteric vasorelaxation (O'Sullivan et al., 2004). SR141716 and AM251 are close structural analogs as they differ only in one halogen side group, a chloride in

SR141716 being replaced by an iodine in AM251. Therefore, the striking difference in their ability to inhibit the endothelial site of action of abn-cbd may be exploited to learn about structure/activity relationships of this putative receptor.

Activation of PI3K and its downstream effector protein kinase B/Akt have been implicated in angiogenic signalling by growth factors and lipid mediators (Brazil et al., 2002), including sphingosine-1-phosphate (Lee et al., 2001). The findings that PI3K inhibitors prevent the abn-cbd-induced increase in endothelial cell migration (Fig. 4) and abn-cbd increases Akt phosphorylation in a PI3K-dependent manner (Offertáler et al., 2003) indicate that the PI3K/Akt pathway is also involved in the chemotactic effect of abn-cbd. Sphingosine-1-phosphate and its cognate receptor EDG-1 play a key role in angiogenesis, best illustrated by the obligatory role of EDG-1 in embryonic vascular maturation (Liu et al., 2000a,b). Because recent findings indicate promiscuity among lipid ligands for various receptors (Lim and Dey, 2002), it was of interest to test whether the lipophilic abn-cbd may be a ligand for the EDG-1 receptor, which could mediate its effect on human umbilical vein endothelial cell migration. The results illustrated in Fig. 6 clearly exclude this possibility. Abn-cbd, at a concentration which caused O-1918-sensitive activation of p44/42 MAPK phosphorylation in the endothelial cells, had no such effect in Chinese hamster ovary cells expressing high levels of functional EDG-1 receptors, as indicated by the ability of sphingosine-1-phosphate to elicit strong MAPK phosphorylation (Fig. 6A). Furthermore, endothelial cell migration stimulated by sphingosine-1-phosphate was not influenced by O-1918 (Fig. 6B). Although the molecular identity of the abn-cbd-sensitive receptor remains unresolved, the present findings suggest that they may be involved in the physiological modulation of angiogenesis.

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