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Cannabinoid CB₁ receptor inhibition decreases vascular smooth muscle migration and proliferation

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Abstract

Vascular smooth muscle proliferation and migration triggered by inflammatory stimuli and chemoattractants such as platelet-derived growth factor (PDGF) are key events in the development and progression of atherosclerosis and restenosis. Cannabinoids may modulate cell proliferation and migration in various cell types through cannabinoid receptors. Here we investigated the effects of CB₁ receptor antagonist rimonabant (SR141716A), which has recently been shown to have anti-atherosclerotic effects both in mice and humans on PDGF-induced proliferation, migration, and signal transduction of human coronary artery smooth muscle cells (HCASMCs). PDGF induced Ras and ERK 1/2 activation, while increasing proliferation and migration of HCASMCs, which were dose-dependently attenuated by CB₁ antagonist, rimonabant. These findings suggest that in addition to improving plasma lipid alterations and decreasing inflammatory cell migration and inflammatory response, CB₁ antagonists may exert beneficial effects in atherosclerosis and restenosis by decreasing vascular smooth muscle proliferation and migration.

Keywords

Cannabinoid receptors; vascular smooth muscle; proliferation; migration

Introduction

Vascular smooth muscle proliferation and migration are crucial events in the pathogenesis of atherosclerosis and are directly implicated in the failure of many clinical interventions (e.g. percutaneous transluminal angioplasty) aiming to treat patients with coronary heart disease. Vascular smooth muscle cell is the main cell type in both atherosclerotic and restenotic lesions [1] [2], which are formed as the result of numerous pathological processes involving generation of reactive oxygen and nitrogen species [3], and the accumulation of inflammatory cells and the release of cytokines, chemokines such as platelet-derived growth factor (PDGF) [4] [5]. Platelet-derived growth factor plays a central role in the onset and development of various vascular disorders, and is one of the most potent mitogens and chemoattractants for vascular smooth muscle cells [6] [7] [8].

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The modulation of the endocannabinoid system comprising of the G protein-coupled CB₁ and CB₂ receptors, their endogenous ligands termed endocannabinoids (generated virtually in all tissues, which elicit a broad range of biological effects), and the enzymes and membrane transporter(s) involved in the biosynthesis/degradation and cellular uptake/release of these lipid mediators, emerges as one of the most promising novel strategies to treat pain, various inflammatory, neurodegenerative, metabolic, liver and cardiovascular disorders [9] [10] [11] [12] [13]. Tonic activation of CB₁ receptors by endocannabinoids may also contribute to cardiovascular risk factors in patients with obesity/metabolic syndrome and diabetes, such as plasma lipid alterations, abdominal obesity, hepatic steatosis, and insulin and leptin resistance [9] [12]. In addition, CB₁ antagonist rimonabant (SR141716A) has recently been shown to reduce the percent of atheroma volume in patients [14] and attenuated the atherosclerosis development in mice [15]. To further explore the beneficial effects of CB₁ blockade in atherosclerosis, we aimed to evaluate the effects of rimonabant on PDGF-induced proliferation, migration and signal transduction of human coronary artery smooth muscle cells (HCASMCs). Considering that the vascular smooth muscle proliferation and migration triggered by PDGF is a pivotal event in the pathogenesis and progression of atherosclerosis and restenosis, our findings may have important clinical implications.

Materials and Methods

Reagents

Human recombinant PDGF-BB was purchased from R&D systems (Minneapolis, MN). N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR141716A; designated as SR1) was synthesized and obtained from Research Triangle Institute (Research triangle park, NC). Sources of other reagents/kits are mentioned in the text wherever appropriate.

Cell Culture

Human coronary arterial smooth muscle cells (HCASMC) and growth medium were purchased from Cascade Biologicals/Invitrogen (Carlsbad, CA). Cells were used within passages 3 to 7. Prior to treatment, cells were conditioned in smooth muscle cell basal medium containing 2% FBS (Invitrogen, CA) for 6 hrs and then used for the experiments.

Detection of apoptosis by flow cytometry

HCASMC were grown in 12 well tissue culture plates and then treated with SR1 (1.0 μ M-10.0 μ M) for 6 hrs. Subsequently, apoptosis was determined by flow cytometry using the annexin-V –APC binding/sytox green assay (Invitrogen, CA) as described previously [16].

Determination of CB₁ expression by Western Immunoblot Assay

HCASMC were grown to confluence in 100 mm culture dishes coated with 0.2% gelatin and cell lysates were prepared using lysis buffer (Pierce Biotechnology, IL) supplemented with protease inhibitors (Roche, GmbH). Lysates were prepared by sonication (15k for 20s) on ice. Then the lysates were clarified to remove the cellular debris by centrifuging at 10,000 rpm for 15 min at 4 °C. Protein content in the lysates was determined using the Lowry assay (Bio-Rad, CA). 20 μ g of protein was resolved in 12% SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). The membrane was blocked for 2 hr at room temperature (RT) with 5% non-fat skimmed milk powder prepared in PBS containing 0.1% Tween-20 (Sigma). After washing with PBST, membranes were probed with either rabbit polyclonal CB₁ [Cayman chemical; 1:1000 dilution, C-terminal] overnight at 4°C. After subsequent washing with phosphate buffered saline 0.1% Tween20 (PBST), the secondary antibody - goat anti-rabbit HRP (Perkin Elmer, MA) was used and incubated at RT for 1 hr. Then the membranes were

developed using chemiluminescence detection kit (Super signal -west pico substrate, Pierce). To confirm uniform loading, membranes were stripped and re-probed with β -actin (Chemicon, CA).

Proliferation assay

HCASMC (5×10^3) cells were suspended in 100 μ l of growth factor free medium containing 2 % FBS and then treated with either PDGF-BB 25 ng/ml alone or with SR1 and seeded on to 96 well plates and allowed to proliferate for 24 – 36 hrs. Then BrdU labeling solution was added and incubated further for 12 hrs. After the incubation, the effect of CB₁ receptor antagonist SR1 on PDGF induced proliferation of the smooth muscle cells were determined by the extent of BrdU incorporation using ELISA kit following the protocol supplied by the manufacturer (Roche Diagnostics, IN) and described previously [17]. In brief, after the incubation of cells with BrdU labeling solution, the cells were fixed and incubated with anti-BrdU antibody. After washing, the cells were incubated with secondary antibody conjugated with horse radish peroxidase. Finally, the extent of BrdU incorporation was determined colorimetrically by determining the absorbance at 450nm. The treatments were performed in triplicate and the experiment was repeated at least three times.

Migration assay

The migration assays were performed in a 24-well modified Boyden chamber as described earlier [18] [17]. In brief, 8 μ m cell culture inserts (BD Biosciences) coated with 0.2 % gelatin (Sigma) were placed over the bottom chamber with or without 20 ng/ml PDGF-BB. Then HCASMC (3×10^4 cells) were suspended in 150 μ l in growth factor free medium containing 1% FBS. Then the cells were treated with SR1 for 90 min 37°C in 5% CO₂ incubator. Then treated cell were added to the upper chamber. After 8 hrs of incubation at 37°C, the non-migrated cells on the upper surface of the filter were removed with cotton swab. The cells that had migrated to the lower side of the chamber were fixed with 100 % methanol for 15 min at RT After complete drying; the inserts containing migrated cells were stained with 0.5 % Giemsa solution (Sigma). 3 fields per insert were counted using 10X objective using Olympus IX81 microscope. The assays were performed in duplicate and the experiments were repeated three times.

Ras activation assay

Ras activation in HCASMCs was determined using commercially available kit (Pierce, IL). In brief, Ras activation assay involved the use of GTP-fusion protein containing Ras-binding domain (RBD) of Raf1, which specifically binds to and pulls-down active Ras. The active pulled-down active Ras was detected by Western blot analysis using anti-Ras antibody supplied with the kit (Pierce).

Western blot analysis of PDGFR- β and ERK1/2 activation

HCASMC were grown in 100 mm dishes and after treatment with PDGF-BB $\pm$ CB₁ antagonist (SR1), cell lysates were prepared using RIPA lysis buffer (Pierce). 25 μ g of protein was resolved in 12% SDS-PAGE and transferred to nitrocellulose membrane (GE Biosciences, NJ) and the blots were probed with either anti-human, rabbit ERK1/2, or phospho-ERK1/2 (Thr202/Tyr204), PDGFR- β or Phospho-PDGFR- β (Tyr 751) at 1:1000 dilution respectively (Cell signaling technology, MA) overnight at 4°C, and the blots were developed using anti-rabbit HRP with a chemiluminescence detection kit (Super Signal-West Pico Substrate, Pierce). Blots were scanned and quantified using the Quantity-One software (BioRad, CA).

Statistical analysis

Values are represented as mean $\pm$ S.E.M. The statistical significance of the data was analyzed using one way Student's *t* test or ANOVA, followed by post-hoc Student-Newman-Keuls if required. $P < 0.05$ was considered as significant.

Results

CB₁ receptor expression and the effect of SR 1 on apoptosis in HCASMC

As shown in Fig. 1A, consistently with our previous observations [17], Western immublot assay revealed cannabinoid receptor 1 (CB₁) expression in human coronary artery smooth muscle cells (HCASMC). Furthermore, the CB₁ antagonist SR1 in the concentrations studied did not influence cell death in HCASMCs (Fig. 1B).

SR1 inhibits PDGF-BB induced proliferation of HCASMC

As shown in Fig. 2 PDGF-BB (20 ng/ml) markedly induced proliferation of smooth muscle cells (~7 fold vs. control), which was dose-dependently inhibited by SR1 treatment. Importantly, SR1 (5.0 μ M) alone did not induce proliferation of HCASMC (Fig. 2).

SR1 mitigates PDGF-BB induced migration of HCASMC

As shown in Fig. 3, PDGF-BB (20 ng/ml) treatment resulted in enhanced migration of smooth muscle cells (~6 fold vs. control), which was inhibited by SR1 in dose-dependent fashion (Fig. 3A,B). SR1 by itself did not elicit chemotactic activity on HCASMC.

SR1 did not inhibit PDGFR- β activation

Earlier studies have suggested that treatment of vascular smooth muscle cells with PDGF-BB (5-20 ng/ml) results in rapid activation of platelet derived growth factor receptor- β (PDGFR- β) within 30 min of treatment [19]. Therefore, we treated HCASMC with PDGF-BB (20 ng/ml) alone for 25 min or first treated with SR1 at the indicated concentration for one hr, followed by stimulation with PDGF-BB for 25 min. Then activation of PDGFR- β was evaluated by determining its phosphorylation by western blot analysis. As shown in Fig. 4A, PDGF-BB treatment significantly stimulated the phosphorylation PDGFR- β , which was not inhibited with the treatment of SR1.

SR1 attenuates PDGF-BB induced Ras and ERK1/2 activation in HCASMC

HCASMCs were treated with PDGF-BB $\pm$ SR1 to determine the activation of Ras and ERK1/2. As shown in Fig. 4B and C, PDGF-BB treatment resulted in marked activation of Ras and ERK1/2 respectively, which was dose-dependently attenuated by SR1.

Discussion

In this study we demonstrate that a selective CB₁ receptor antagonist attenuates PDGF-induced proliferation and migration of human coronary artery smooth muscle cells and the activation of various interrelated signaling pathways (Ras and ERK 1/2).

Previous studies have attributed pivotal role for platelet-derived growth factor (one of the main triggers involved in promoting vascular smooth muscle proliferation and migration) in the development of various vascular disorders such as atherosclerosis and restenosis following vascular injury and/or percutaneous transluminal angioplasty [6] [7] [8]. Recent studies have reported that CB₁ inhibition in human peripheral blood mononuclear, human breast cancer cells, and NIH3T3 fibroblasts results in inhibition of the cell proliferation and migration and/or apoptosis mediated via inhibition of ERK1/2 and interrelated signaling pathways [20] [21]

[22] [23]. CB₁ inhibition also attenuates doxorubicin-induced cell death in cardiomyocytes, which may also involve ERK1/2 activation [24]. Furthermore CB₁ antagonist rimonabant also inhibits inflammatory cell migration and attenuates the development of atherosclerosis in a mouse model of disease [15], in addition to the reduction of the percent of atheroma volume in patients [14]. On the basis of the above mentioned studies and the accumulating evidence suggesting that CB₁ activation involves MAPK signaling (e.g. ERK1/2 activation) in various cell types, we hypothesized that CB₁ receptor blockade in vascular smooth muscle cells may attenuate PDGF-induced cell migration and proliferation. Indeed, as shown in Figures 2 and 4, CB₁ receptor inhibition with rimonabant concentration-dependently attenuated PDGF-induced, but not basal coronary artery vascular smooth muscle cell proliferation (measured by BrdU incorporation), as well as cell migration, without affecting PDGF-induced PDGFR- β activation. CB₁ antagonist also attenuated the activation of the PDGF-induced signaling cascade (ERK1/2 and Ras activation) involved in vascular smooth muscle cell proliferation and migration (Figure 4).

Collectively, our results demonstrate that CB₁ inhibition with rimonabant attenuates PDGF-induced Ras and ERK 1/2 activation, while decreasing proliferation and migration of HCASMCs. These findings forecast that in addition to improving plasma lipid alterations and decreasing inflammatory cell migration and inflammatory response, CB₁ antagonists may exert beneficial effects in atherosclerosis and restenosis by decreasing vascular smooth muscle proliferation and migration.

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Fig.1. Expression of CB₁ receptor and the effect of rimonabant (SR1) on cell death in HCASMCs
 (A) Western blot reveals expression of CB₁ in HCASMCs. Lanes 1-4 indicate lysates prepared from various batches of HCASMCs. (B) Shown are the representative images of scatter plots from three independent experiments demonstrating that SR1 at various concentrations had no and effect on apoptosis/necrosis in HCASMCs determined by flow cytometry.

Fig.2. Effect of rimonabant (SR1) on PDGF-BB induced proliferation of HCASMCs

Cells were treated as indicated and the effect of SR1 on PDGF-BB induced proliferation of human vascular smooth muscle cells was determined by measuring the rate of BrdU incorporation employing the commercially available kit. SR1 (5.0 μM) by itself had no any effect of proliferation of HCASMCs. *P< 0.01 vs. control/SR1; #< 0.05 or 0.01 vs. PDGF-BB (20 ng/ml) alone, n=9.

A **B**

Fig.3. Effect of rimonabant (SR1) on PDGF-BB induced migration of HCASMCs

Cells were treated as indicated and migration assays were performed as described in the methods section. (A) Shown are the representative images of smooth muscle cells migrated as per the treatments indicated. (B) Depicts the quantification data for the cells that had migrated in response to the treatment. * $P < 0.01$ vs. control/SR1; # $P < 0.05$ or 0.01 vs. PDGF-BB (20 ng/ml) alone, $n=6$.

Fig.4. Effect of rimonabant (SR1) on PDGFR-β, Ras and ERK1/2 activation in HCASMCs

(A) PDGF-BB activation of PDGFR-β was not inhibited by treatment with SR1. Shown is the representative image of PDGFR-β activation by PDGF-BB (20 ng/ml) for 25 min and the effect of SR1. * $P < 0.01$ vs. control; $n=3$. SR1 (5.0 μM) by itself had no effect on PDGFR-β phosphorylation. (B) Representative immunoblot depicts the concentration-dependent inhibitory effect of SR1 on PDGF-BB-induced stimulation of Ras activation in smooth muscle cells. * $P < 0.01$ vs. control; # $P < 0.05/0.01$ vs. PDGF, $n=3$. (C) Shown is the representative blot of PDGF-BB-induced activation of ERK1/2 and the concentration-dependent suppression of it by SR1. * $P < 0.01$ vs. control; # $P < 0.05/0.01$ vs. PDGF, $n=3$.