ORIGINAL ARTICLE

Selective Cannabinoid Receptor-1 Agonists Regulate Mast Cell Activation in an Oxazolone-Induced Atopic Dermatitis Model

Gaewon Nam, Se Kyoo Jeong¹, Bu Man Park¹, Sin Hee Lee¹, Hyun Jong Kim², Seung-Phil Hong³, Beomjoon Kim⁴, Bong-Woo Kim

Department of Cosmetic Science and Technology, Seowon University, Cheongju, ¹CRID Center, NeoPharm Co., Ltd., Daejeon, ²Department of Dermatology, Atopy and Asthma Center and Seoul Medical Research Institute, Seoul Medical Center, Seoul, ³Department of Dermatology, Dankook University Medical College, Cheonan, ⁴Department of Dermatology, Chung-Ang University College of Medicine, Seoul, Korea

Background: Many inflammatory mediators, including various cytokines (e.g. interleukins and tumor necrosis factor [TNF]), inflammatory proteases, and histamine are released following mast cell activation. However, the endogenous modulators for mast cell activation and the underlying mechanism have yet to be elucidated. Endogenous cannabinoids such as palmitoylethanolamide (PEA) and N-arachidonoyl-lethanolamine (anandamide or AEA), were found in peripheral tissues and have been proposed to possess autacoid activity, implying that cannabinoids may downregulate mast cell activation and local inflammation. Objective: In order to investigate the effect of cannabinoid receptor-1 (CB1R) agonists on mast cell activation, AEA-derived compounds were newly synthesized and evaluated for their effect on mast cell activation. Methods: The effects of selected compounds on FceRI-induced histamine and β-hexosaminidase release were evaluated in a rat basophilic leukemia cell line (RBL-2H3). To further investigate the inhibitory effects of CB1R agonist in vivo, an oxazolone-induced atopic dermatitis mouse model was exploited. Results: We found that CB1R inhibited the release of inflammatory mediators without causing cytotoxicity in RBL-2H3 cells and that CB1R agonists markedly and dose-dependently suppressed mast cell proliferation indicating that CB1R plays an important role in modulating antigen-dependent immunoglobulin E (IgE)-mediated mast cell activation. We also found that topical application of CB1R agonists suppressed the recruitment of mast cells into the skin and reduced the level of blood histamine.

Conclusion: Our results indicate that CB1R agonists downregulate mast cell activation and may be used for relieving inflammatory symptoms mediated by mast cell activation, such as atopic dermatitis, psoriasis, and contact dermatitis.

Keywords- Atopic dermatitis, Cannabinoid receptor agonists, Histamine, Mast cells

INTRODUCTION

Cannabinoids are psychoactive constituents of the Cannabis plant containing over 100 C₂₁ terpenophenolic active compounds¹. Δ⁹-tetrahydrocannabinol (THC), a main psychoactive component, acts as an agonist of specific receptors, which are known as cannabinoid receptors². Two different cannabinoid receptors, cannabinoid receptor-1 (CB1R) and cannabinoid receptor-2 (CB2R) have thus far been identified and cloned from mammalian tissue³.⁴.⁵. Cannabinoid receptors are seven-transmembrane G protein coupled receptors, that couple to G₁₅ heterotrimeric G proteins⁶. CB1R is typically expressed in the central nervous system (CNS), particularly the brain and spinal cord, and to a much lesser degree in peripheral nerve ter-

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Corresponding author: Bong-Woo Kim, Department of Cosmetic Science and Technology, Seowon University, 377-3, Musimseo-ro, Seowon-gu, Cheongju 28674, Korea. Tel: 82-43-299-8493, Fax: 82-43-299-8470, E-mail: sinope530@daum.net

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CB1R is a Negative Regulator of Mast Cells

MATERIALS AND METHODS

Reagents

The cannabinoid compounds AEA and Hu-308 were purchased from Tocris Bioscience (Bristol, UK). The histamine assay kit and cAMP assay kit were purchased from Cisbio Bioassay (Bedford, MA, USA). IgE anti-2,4-dinitrophenol (DNP) antibody, DNP-bovine serum albumin (BSA), forskolin, toluidine blue, and thiazoyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). α-oleyl oleoylamine ethanolamine (α-OOE) and α-oleyl oleoylamine serinol (α-OOS) were synthesized using alkylketene dimer from oleyl chloride in triethylamine, under toluene conditions at 37°C. Interleukin (IL)-3-dependent bone marrow derived-cultured mast cells (BMCMC) were derived from the femoral bone marrow cells of BALB/c mice and were maintained in DMEM with 10% heat-inactivated FBS, 50 μM 2-mercaptoethanol, 2 mM glutamine, and 20% WEHI-3-conditioned medium containing IL-3 for 4 weeks.

CB1R- or CB2R-overexpressing stable cell lines

To establish stable cell lines expressing CBR, CHO-K1 cells were transfected with human CB1R (Cat. RC210397) and CB2R (Cat. SC118984) cDNA constructs (Origene, Rockville, MD, USA); the following day, media were replaced with F12 standard medium containing G418 (100 μg/ml) to select for stable clones. Two weeks later, single colonies were picked using an inverted light microscope and transferred to 24-well plates.

cAMP assay

CB1R- or CB2R-overexpressing CHO cells (5×10^3 cells) were seeded into 96-well plates one day prior and washed once in phosphate buffered saline (PBS). Next, the cells were incubated with serum-free F12 medium containing 10 μM forskolin and cannabinoid receptor agonists for 30 min at 37°C. Cytoplasmic cAMP was measured using the HTRF cAMP assay kit according to the manufacturer’s protocol.

Cell viability assay and toluidine blue staining

Cell viability assays were performed using a Cell Proliferation Kit I (MTT assay; Roche Diagnostics Corp., Indianapolis, IN, USA) according to the manufacturer’s instructions. For toluidine blue staining, tissue sections were stained with toluidine blue working solution for 2~3 min and then washed with distilled water. The samples were then quickly dehydrated with 95% alcohol and two washes of 100% alcohol. Next, the slides were cleared in xylene and coverslipped. Toluidine blue-stained sections
were examined under an Olympus microscope (Olympus, Tokyo, Japan) and 10 different fields of each section were counted manually by the same person.

**β-hexosaminidase and histamine release assay**

RBL-2H3 cells (10⁶/ml) were sensitized with a rat monoclonal anti-DNP-IgE antibody overnight. Cells were washed with PBS and pre-incubated with CB1R agonists prior to activation with either ionomycin (2 μM) or 100 ng/ml DNP-BSA antigen. β-hexosaminidase release from RBL-2H3 was measured by incubating 50 μl of the supernatant with 50 μl of p-NAG (Sigma-Aldrich, St. Louis, MO, USA) in a 96-well plate for 2 hr at 37°C. The reaction was stopped with 200 μl 0.2 M glycine (pH 10.6) and the resulting change in absorbance was read at 405 nm. Histamine release from RBL-2H3 was measured by incubating 10 μl of the supernatant with the HTRF histamine assay kit reagents according to the manufacturer’s protocol.

**Oxazolone-induced atopic dermatitis mouse model**

The oxazolone-induced murine model with atopic dermatitis (AD) features was developed according to a previously described method ¹⁸. All hairless mice (SKH-1) used in this study were kept at the animal facility of NeoPharm Co., Ltd. under specific pathogen-free conditions. According to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8523). In order to induce atopic dermatitis-like symptoms, each group (n = 10 for each) of mice was sensitized with a single topical treatment of 50 μl of oxazolone solution (1% in acetone) on both flanks. Seven days later, 60 μl of 0.1% oxazolone solution was applied on the same sites 10 times, once every other day. Following the 6th challenge, animals were randomly divided into 6 groups (non-treated, vehicle treated, 1% of α-OOE, 1% α-OOS, 1% AEA, and 0.01% dexamethasone) and each sample was topically applied on the same site with 0.1% oxazolone treatment twice a day for 9 days. The last treatment was performed two days af-

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![Diagram](image_url)

**Fig. 1.** Newly synthesized cannabinoids and the cAMP assay screening for cannabinoid receptor modulators. (A) The structure of α-oleoyl oleoylamine ethanolamine (α-OOE) and α-oleoyl oleoylamine serinol (α-OOS). (B, C) Synthetic compounds were screened in stable CHO cell lines overexpressing cannabinoid receptor-1 (CB1R) or CB2R. Pre-treatment with CBR agonists significantly diminished the cytosolic levels of cAMP induced by 10 μM forskolin (B). N-arachidonoyl ethanolamine (AEA) and Hu308 were used as controls for CB1R and CB2R, respectively. All data are presented as the mean ± standard error, t-test and analysis of variance. *p < 0.01.
CB1R Is a Negative Regulator of Mast Cells

RESULTS

Newly synthesized oleic acid-based ketoamines (α-OOE and α-OOS) act as selective CB1R agonists

To identify a CB1R functional modulator, we synthesized approximately forty lipophilic compounds (data not shown). These novel compounds were screened for the ability to modulate CB1R or CB2R (Fig. 1) and two compounds were selected as CBR agonists. To investigate the effect of these compounds on CB1R and CB2R activation and inhibition, we measured cAMP levels in human CB1R- or CB2R-overexpressing CHO cells following treatment with the CBR functional modulators. As shown in Fig. 1B, two compounds were identified as CB1R agonists since they reduced forskolin-induced cAMP levels in CB1R-CHO cells but did not reduce cAMP levels in CB2R-CHO cells. Of the tested compounds, α-OOE (EC$_{50}$=24.2 μM) and α-OOS (EC$_{50}$=26.8 μM) significantly attenuated cAMP production (Fig. 1B).

Effect of synthetic CB1R agonists on the release of inflammatory mediators from RBL-2H3 cells

To elucidate whether CB1R is implicated in mast cell

Fig. 2. Effects of cannabinoid receptor-1 (CB1R) agonists on the degranulation of rat basophilic leukemia cell line (RBL-2H3) mast cells. (A, B) The inhibitory effects of α-oleoyl oleoylamine ethanolamine (α-OOE), α-oleoyl oleoylamine serinol (α-OOS), and N-arachidonylethanolamine (AEA) were determined on ionomycin (2 μM)-induced histamine (A) or β-hexosaminidase release (B) from rat mast cells. (C, D) RBL-2H3 cells sensitized with anti-dinitrophenol-immunoglobulin E (anti-DNP-IgE) (0.5 μg/ml) for 12 hr were pretreated with the CB1R agonists. Histamine (C) or β-hexosaminidase release (D) from mast cells was measured. (E) Bone marrow derived-cultured mast cells from mouse bone marrow was sensitized with anti-DNP-IgE for 12 hr and histamine release from cells was measured. All data are presented as the mean±standard error, t-test and analysis of variance. NON: non-stimulated, CON: control (stimulated), IgE: Immunoglobulin E, DNP: dinitrophenol, BSA: bovine serum albumin. *p<0.01 and **p<0.05.
modulation, degranulation was in RBL-2H3 cells using ionomycin following pretreatment with the CB1R agonists, α-OOE, α-OOS, and AEA. As shown in Fig. 2A and B, the CB1R agonists decreased ionomycin-induced histamine and β-hexosaminidase secretion. To examine the modulatory effect of the CB1R agonists on IgE-induced mast cell activation, IgE-sensitized RBL-2H3 cells and BMCMC derived from mouse bone marrow were treated with α-OOE, α-OOS, and AEA at a concentration of 10^{-5} M for 12 hr. The degranulation responses in RBL-2H3 cells and BMCMC were evaluated as the release of histamine and β-hexosaminidase into the culture medium. Our results showed that the CB1R agonists significantly reduced the secretion of these inflammatory mediators, suggesting that CB1R plays a crucial role in negatively regulating antigen-dependent IgE-mediated mast cell activation (Fig. 2C~E).

**Inhibition of mast cell proliferation by CB1R agonists**

To investigate the role of the CB1R agonists in the proliferation of RBL-2H3, cells were treated with agonists (0 ~ 100 μM) and the proliferation levels were measured (Fig. 3). The MTT assay and cell counting results revealed that the CB1R agonists clearly led to a concentration-dependent decrease in proliferation levels (Fig. 3A, B, C). Compared with the controls, cell proliferation levels were significantly decreased by treatment with 25 μM α-OOE, α-OOS, or AEA, exhibiting 43% reduction in the proliferation levels of RBL-2H3 cells after 24 hr. These results indicate that the CB1R agonists modulate RBL-2H3 cell proliferation.

**Effect of synthetic CB1R agonists on mast cell infiltration and blood histamine release in oxazolone-induced atopic dermatitis**

In order to elucidate the anti-inflammatory effect of CB1R, we examined the effects of topical application of CB1R agonists on atopic dermatitis induced by repeated challenge with oxazolone in the back skin of hairless mice. The oxazolone challenge resulted in epithelial hyperplasia and an influx of inflammatory cells into the skin (Fig. 4). As shown in Fig. 4A and B, the application of CB1R agonists to the AD lesion significantly decreased skin thickness and suppressed the oxazolone-induced infiltration of mast cell infiltration and blood histamine release in oxazolone-induced atopic dermatitis.
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Fig. 4. Effects of cannabinoid receptor-1 agonists in oxazolone-induced atopic dermatitis mice models. (A) Histology of dorsal skin lesions. The dorsal skin of each mouse was removed and fixed for toluidine blue staining (×400). Images are representative of five mice. (B) Change of skin fold thickness in oxazolone model. α-oleoyl oleoylamine ethanolamine (α-OOE), α-oleoyl oleoylamine serinol (α-OOS), and N-arachidonylethanolamine (AEA) treatment prevented the increase of skin fold thickness. (C) Quantitative histomorphometry of the number of mast cells. Mast cells were counted in the toluidine blue-stained slide. (D) Total blood histamine levels in serum. The histamine concentration of each mouse was measured according to a previously described method. Each point represents the mean±standard error. Data are from five to six mice. VEH: vehicle, DEXA: dexamethasone. *p<0.01, **p<0.05 significantly different from the vehicle group (Student’s t-test).

cells, further confirming the anti-inflammatory effects of the CB1R agonists (Fig. 4C). To further investigate the effects of CB1R on the AD model, we measured the plasma histamine levels. Interestingly, the elevation of blood histamine serum concentrations was significantly inhibited by topical treatment with the CB1R agonists (Fig. 4D).

DISCUSSION

Mast cells are unique inflammatory cells that release various inflammatory mediators in response to innate and adaptive immunity signals. The mediators produced by mast cells include arachidonic acid products, serine proteases (tryptase and chymase), various cytokines (e.g. tumor necrosis factor-α, IL-3, granulocyte-macrophage colony-stimulating factor, CXC chemokine ligand 8, and CC chemokine ligand 3), and histamine. Histamine is a well-known neurotransmitter regulating immune responses including cell proliferation, differentiation, hematopoiesis, and wound healing. During the inflammatory response, histamine increases capillary permeability acting on vascular smooth muscle cells and endothelial cells.
to recruit various inflammatory cells such as T cells, eosinophils, macrophages, and mast cells to the allergic region. Little is known regarding endogenous physiological modulators of mast cell activation and histamine release, although there is growing evidence that the endocannabinoid system (ECS) acts as a key modulator of mast cell degranulation\(^{12-18}\). CB1R and CB2R are found in all organs; CB1R is expressed primarily in the CNS\(^7\), whereas CB2R is mainly localized in the immune system\(^8,9\). Interestingly, both CB1R and CB2R are expressed in mast cells and mast cell activation has been shown to be modulated by the ECS\(^16,17\). However, the role of cannabinoids and their receptors in mast cell activation and maturation is controversial. Facci et al.\(^{13}\) suggested that PEA effects modulatory activities on mast cells through CB2R, while the Tschirhart group has demonstrated that cannabinoids inhibit the release of histamine through a CB receptor-independent mechanism\(^{20,21}\). In contrast, the CB1R agonist WIN 55,212-2 reduced IgE-antigen-induced \(\beta\)-hexosaminidase release and CP55940 (CB1R/CB2R agonist) or R(+-)methanandamide (a selective CB1R agonist) suppressed IgE-antigen-induced serotonin release\(^{16}\). Recently, Sugawara et al.\(^{17}\) claimed that CB1R not only reduces mast cell degranulation but also suppressed the maturation of mast cells from resident progenitor cells in situ. These findings indicate that CB1R is essential for mast cell modulation. To investigate whether CB1R suppresses mast cell function in a dose-dependent manner, we measured the release of histamine and \(\beta\)-hexosaminidase following CB1R agonist treatment. As shown in Fig. 2, histamine and \(\beta\)-hexosaminidase levels were significantly decreased subsequent CB1R activation by various stimuli. Additionally, CB1R activation by the agonists induced the inhibition of mast cell proliferation (Fig. 3). These results suggest that CB1R is implicated in mast cell growth and activation. Recently, it was reported inhibition of CB2R by a selective antagonist (SR144528) suppressed oxazolone-induced AD in mice and reduced the infiltration of leukocytes\(^22\). Our antagonist (SR144528) suppressed oxazolone-induced AD recently, it was reported inhibition of CB2R by a selective antagonist (SR144528) suppressed oxazolone-induced AD in mice and reduced the infiltration of leukocytes\(^22\). Our antagonist (SR144528) suppressed oxazolone-induced AD in mice: an update. Br J Pharmacol 2008;153:309-318. The cannabinoid system and immune modulation. J Leukoc Biol 2003;74:486-496. Cannabinoids enhance human B-cell growth at low nanomolar concentrations. FEBS Lett 1995;369:177-182. Ständer S, Schmelz M, Metze D, Luger T, Rukwied R. Distribution of cannabinoid receptor 1 (CB1) and 2 (CB2) on sensory nerve fibers and adnexal structures in human skin. J Dermatol Sci 2005;38:177-188. Buckley NE. The peripheral cannabinoid receptor knockout mice: an update. Br J Pharmacol 2008;153:309-318. Klein TW, Newton C, Larsen K, Lu L, Perkins I, Nong L, et al. The cannabinoid system and immune modulation. J Leukoc Biol 2003;74:486-496. Deroçq JM, Séguï M, Marchand J, Le Fur G, Casellas P. Cannabinoids enhance human B-cell growth at low nanomolar concentrations. FEBS Lett 1995;369:177-182. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol 2010;125(2 Suppl 2):S73-S80. Aloe L, Leon A, Levi-Montalcini R. A proposed autacoid mechanism controlling mastocyte behaviour. Agents Actions 1993;39 Spec No:C145-C147. Facci I, Dal Toso R, Romanello S, Buriani A, Skaper SD, Leon A. Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide. Proc Natl Acad Sci U S A 1995;92:3376-3380. Sun N, Zhou C, Zhou X, Sun L, Che H. Use of a rat basophil leukemia (RBL) cell-based immunological assay for allergen identification, clinical diagnosis of allergy, and...
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