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Anti-proliferative effect of a putative endocannabinoid, 2-arachidonylglyceryl ether in prostate carcinoma cells

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ABSTRACT

Endocannabinoids (ECs), anandamide (AEA) and 2-arachidonoylglycerol (2-AG), inhibit proliferation of carcinoma cells. Several enzymes hydrolyze ECs to reduce endogenous EC concentrations and produce eicosanoids that promote cell growth. In this study, we determined the effects of EC hydrolysis inhibitors and a putative EC, 2-arachidonylglyceryl ether (noladin ether, NE) on proliferation of prostate carcinoma (PC-3, DU-145, and LNCaP) cells. PC-3 cells had the least specific hydrolysis activity for AEA and administration of AEA effectively inhibited cell proliferation. The proliferation inhibition was blocked by SR141716A (a selective CB1R antagonist) but not SR144528 (a selective CB2R antagonist), suggesting a CB1R-mediated inhibition mechanism. On the other hand, specific hydrolysis activity for 2-AG was high and 2-AG inhibited proliferation only in the presence of EC hydrolysis inhibitors. NE inhibited proliferation in a concentration-dependent manner; however, SR141716A, SR144528 and pertussis toxin did not block the NE-inhibited proliferation, suggesting a CBR-independent mechanism of NE. A peroxisome proliferator-activated receptor gamma (PPARγ) antagonist GW9662 did not block the NE-inhibited proliferation, suggesting that PPARγ was not involved. NE also induced cell cycle arrest in G_0/G_1 phase in PC-3 cells. NE inhibited the nuclear translocation of nuclear factor-kappa B (NF-κB p65) and down-regulated the expression of cyclin D1 and cyclin E in PC-3 cells, suggesting the NF-κB/cyclin D and cyclin E pathways are involved in the arrest of G1 cell cycle and inhibition of cell growth. These results indicate therapeutic potentials of EC hydrolysis inhibitors and the enzymatically stable NE in prostate cancer.

1. Introduction

Well-characterized endocannabinoids (ECs, endogenous ligands of the cannabinoid receptors), anandamide (AEA) [1] and 2-arachidonoylglycerol (2-AG) [2,3] have therapeutic potential in various types of cancer [4–11]. For example, ECs inhibit the nerve growth factor-induced proliferation of breast carcinoma cells [12], proliferation of colorectal cancer cells [5], and colon [4] and thyroid tumor growth *in vivo* [7]. Met-F-AEA, an analog of AEA inhibits adhesion and migration of breast carcinoma cells [13,14].

AEA, through the activation of cannabinoid receptor type-1 (CB1R), inhibits the epidermal growth factor (EGF)-induced proliferation of prostate carcinoma cells by decreasing expression of the epidermal growth factor receptor (EGFR) and increasing ceramide production [15]. Interestingly, among PC-3, DU-145 and LNCaP cells, LNCaP cells are the least sensitive to AEA inhibition. AEA and 2-AG also inhibit prolactin-induced proliferation of DU-145 cells [12]. We previously demonstrated that endocannabinoids such as 2-AG, acting through CB1R, inhibit invasion of PC-3 and DU-145 cells but had little or no effect in LNCaP cells [16] due to its high hydrolysis activity in these cells [17]. AEA is hydrolyzed by fatty acid amide hydrolase (FAAH) [18–20] and 2-AG is hydrolyzed by FAAH and monoacylglycerol lipase (MGL) [21–23] to free arachidonic acid (AA). The hydrolysis has two detrimental effects: it reduces the concentrations of ECs and the free AA is further metabolized to eicosanoids. Free AA and some eicosanoids promote prostate carcinoma cell growth and motility [24–29]. Thus, it is now recognized that the inhibition of EC hydrolysis is a potential therapeutic target for cancer treatment [7,17,20,30–32].

A putative EC, 2-arachidonylglyceryl ether (noladin ether, NE), is chemically similar to 2-arachidonoylglycerol (2-AG) with the

Abbreviations: AA, arachidonic acid; AEA, anandamide (arachidonylethanolamide); 2-AG, 2-arachidonoylglycerol; CBR, cannabinoid receptor; DAK, diazomethylarachidonyl ketone; EC, endocannabinoids; FAAH, fatty acid amide hydrolase; MGL, monoacylglycerol lipase; NE, 2-arachinoylglyceryl ether (noladin ether); NF-κB, nuclear factor-kappa B; 2-OG, 2-oleoylglycerol; OTFP, 3-(octylthio)-1,1,1-trifluoropropan-2-one; PPARγ, peroxisome proliferator-activated receptor gamma; TNF-α, tumor necrosis factor-alpha.

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glycerol moiety conjugated by ether-linkage to AA [33–35]. NE is more enzymatically stable than 2-AG and AEA [36–38]. NE was first identified in porcine brain [33] and later in rat brain regions [35]; however, other studies did not detect NE in the brains of various mammalian species [39,40]. The role of NE in cancer, particularly prostate cancer is not well-understood. In this study, we investigated the effects of EC hydrolysis and the enzymatically stable NE on proliferation of prostate carcinoma cells. NE binds to CB1R [33,34,41] and has much lower affinity for CB2R [33,42]; thus, we investigated if the NE inhibited prostate carcinoma cell proliferation is CBR dependent. Since NE activates peroxisome proliferator-activated receptor gamma (PPARγ) [43] and PPARγ mediates proliferation and cell cycle arrest of prostate carcinoma cells [44–47], we investigated if the NE inhibited proliferation by activating PPARγ pathway. Furthermore, we examined the possible mechanism of NE in the regulation of nuclear factor-kappa B (NF-κB) and cell cycle regulatory proteins [48,49] that lead to the arrest of cell cycle and inhibition of growth of prostate carcinoma cells.

2. Materials and Methods

2.1. Materials

Human prostate carcinoma (PC-3, DU-145, and LNCaP) cells were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). Thymidine [methyl-^{3}H] (1 μCi/μL) was obtained from Applied Biosystems (Foster City, California). Pertussis toxin (PTX) and methyl thiazol tetrazolium (MTT) were obtained from Sigma–Aldrich Chemical (St. Louis, Missouri). Noladin ether (NE), AEA, 2-AG, AM251, and GW9662 were obtained from Cayman Chemical Co. (Ann Arbor, Michigan). SR141716A and SR144528 were obtained from Research Triangle Institute (Research Triangle Park, North Carolina). 2-Oleoyl-[$^3H_3$]-glycerol ([$^3H_3$]2-OG, 20 Ci/mmol) and anandamide [ethanolamine 1-^{3}H] ([^{3}H]AEA, 60 Ci/mmol) were obtained from American Radiolabeled Chemical (St. Louis, Missouri). SimplyBlue SafeStain and goat anti-mouse IgG-HRP were obtained from Invitrogen (Carlsbad, CA). Tumor necrosis factor-alpha (TNF-α) was obtained from EMD Chemicals, Inc. (Gibbstown, NJ). Rabbit polyclonal IgG against nuclear factor-κB (NF-κB p65) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-rabbit IgG (H + L)-FITC was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Mouse monoclonal antibody to human cyclin D1 was obtained from GenWay Biotech, Inc. (San Diego, CA). Mouse monoclonal antibody to human cyclin E was obtained from Cell Signaling Technology (Danvers, MA). (SDS-PAGE BioRad Ready Gels (10%) were obtained from BioRad (Hercules, CA). ECL Western blot detection kit and BCA protein assay kit were obtained from Thermo Scientific (Rockville, IL). 3-(Octylthio)-1,1,1-trifluoropropan-2-one (OTFP) was generously provided by Dr. Bruce D. Hammock [50,51]. Diazomethylarachidonyl ketone (DAK) was synthesized in our laboratory [52]. Poly-D-lysine was obtained from BD Biosciences (Bedford, Massachusetts). Other chemicals and reagents were analytical grade or the highest purity available from suppliers and they were used without purification. Distilled, deionized water was used for all experiments.

2.2. Cells and cell culture

Prostate carcinoma cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine (2.0 mM), streptomycin (100 μg/mL) and penicillin (100 U/mL). Cells were grown in 75-cm^2 polystyrene tissue culture flasks at 37 °C in 5% CO_2 to approximately 50–70% confluency before use.

2.3. Thymidine incorporation assay

Cell proliferation was determined by thymidine incorporation assay as previously described [53] with minor modifications. PC-3 and DU-145 cells (5.0×10^4 cells/well) and LNCaP cells (1.0×10^5 cells/well) were plated in 24-well plates coated with poly-D-lysine and incubated in 5% CO_2 for 24 h at 37 °C. Cells were washed and incubated in a serum free media for 24 h at 37 °C. Then, cells were incubated in the serum free media in the absence or presence of pharmacological agents for 24 h at 37 °C. For PTX treatment, cells were incubated with PTX overnight prior to NE treatment [54,55]. Cells in each well were then incubated with [^{3}H]thymidine (1 μCi) for additional 6 h. Cells were washed with phosphate buffered saline, fixed with cold 5% trichloroacetic acid and solubilized with 0.25 M NaOH. The solution (300 μL) was counted for radioactivity. Each experiment was performed 2–3 times with 6 wells per treatment.

2.4. Cell counting

Cell proliferation was also determined by cell counting. Cells were plated at 5×10^4 cells/well in a 24-well plate and grown to about 60% confluency and treated with pharmacological agents as described above in thymidine incorporation assay. After 24-h incubation, cells were fixed with cold ethanol:methanol on ice and stained with SimplyBlue SafeStain for 20 min to improve cell counting similar to the described protocol [56]. Stained cells were photographed using a microscope with a CCD camera (Photometrics) and counted for number of cells per field. At least 4 fields per treatment were used to average the number of cells and they were normalized to the control cells (as 100%).

2.5. MTT assay

Cells were plated in 96-well plates (2.0×10^4 cells/well) in serum-free and phenol red-free media at 37 °C for 48 h. Cell treatment and incubation were performed similar to the thymidine incorporation assay in the serum-free and phenol red-free media for 24 h. Then, methyl thiazol tetrazolium (MTT, 0.25 mg/mL) was added to each well and incubated for 6 h at 37 °C. The media was aspirated and isopropanol containing 0.35% HCl was added to lyse the cells. The absorbance was measured at 570 nm by a microplate reader (Bio-Tek Instruments Inc., Winooski, Vermont).

2.6. Determination of endocannabinoid hydrolysis

The hydrolysis of endocannabinoids (2-AG and AEA) by subcellular protein fractions (25 μg) of prostate carcinoma cells was determined by using previously described protocol [16]. 2-Oleoyl-[$^3H_3$]-glycerol ([$^3H_3$]2-OG) was used as an analog of 2-AG and anandamide [ethanolamine 1-^{3}H] ([^{3}H]AEA) was used for AEA. Incubations were carried out for 30 min at 37 °C, radioactivity in each liquid phases were separated and counted [16], and the specific hydrolysis activity was calculated.

2.7. Determination of NE by LC–ESI-MS

Concentrations of exogenously added NE in PC-3 and LNCaP cells were determined by LC–ESI-MS [16]. Cells were grown in T-75 flasks and treated with NE (18.25 μg) for 0 and 2 h at 37 °C. Then, cells were lysed, scraped, and cell lysate and media were collected. [$^2H_8$]2-AG (15 ng) was added as an internal standard into the samples and extracted by solid phase extraction. NE was determined by LC–ESI-MS [16] and the detection was made in the positive mode. The m/z 365 and 387 were used for NE and [$^2H_8$]2-AG measurements, respectively. The concentrations of NE were calculated by comparing their ratios of peak areas to the standard curves.