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Eicosapentaenoic acid decreases expression of anandamide synthesis enzyme and cannabinoid receptor 2 in osteoblast-like cells

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Abstract

Anandamide (AEA) is an endogenous agonist for the cannabinoid receptor 2 (CB2) which is expressed in osteoblasts. Arachidonic acid (AA) is the precursor for AEA and dietary n-3 polyunsaturated fatty acids (PUFA) are known to reduce the concentrations of AA in tissues and cells. Therefore, we hypothesized that n-3 PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which reduce AA in cells, could lower AEA in osteoblasts by altering enzyme expression of the endocannabinoid (EC) system. MC3T3-E1 osteoblast-like cells were grown for 6, 10, 15, 20, 25 or 30 days in osteogenic medium. Osteoblasts were treated with 10 μM of AA, EPA, DHA, oleic acid (OA) or EPA+DHA (5 μM each) for 72 h prior to their collection for measurement of mRNA and alkaline phosphatase (ALP) activity. Compared to vehicle control, osteoblasts treated with AA had higher levels of AA and n-6 PUFA while those treated with EPA and DHA had lower n-6 but higher n-3 PUFA. Independent of the fatty acid treatments, osteoblasts matured normally as evidenced by ALP activity. *N*-acyl phosphatidylethanolamine-selective phospholipase D (NAPE-PLD), fatty acid amide hydrolase (FAAH) and CB2 mRNA expression were higher at 20 days compared to 10 days. NAPE-PLD and CB2 mRNA was lower in osteoblasts treated with EPA compared to all other groups. Thus, mRNA expression for NAPE-PLD, FAAH, and CB2 increased during osteoblast maturation and EPA reduced mRNA for NAPE-PLD and CB2 receptor. In conclusion, EPA lowered mRNA levels for proteins of the EC system and mRNA for AEA synthesis/degradation is reported in osteoblasts.

Keywords: Endocannabinoids; MC3T3-E1 osteoblast-like cells; PUFA; Eicosapentaenoic acid; Arachidonic acid; Docosahexaenoic acid

1. Introduction

The endocannabinoid (EC) system is comprised of receptors, ligands, and enzymes for the synthesis and degradation of the endogenous ligands [1]. The two most studied endogenous cannabinoid ligands are synthesized from arachidonic acid (AA) by *N*-acyl phosphatidylethanolamine-selective phospholipase D (NAPE-PLD) or diacylglycerol lipases (DAGL) α and β to form *N*-arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), respectively [1]. Fatty acid amide hydrolase (FAAH) hydrolyzes AEA to yield AA and an ethanolamide. Monoacylglycerol lipase and, to some degree, FAAH [2] degrade 2-AG to release AA and glycerol [1].

Endogenous cannabinoids are found in bone compartments. AEA levels in trabecular bone are similar to that found in the brain; however, trabecular 2-AG levels are approximately one sixth of that found in brain [3]. The two isoforms of DAGL, α and β, have been identified in MC3T3-E1 osteoblast-like cells, primary calvarial osteoblasts and osteoclast-like cells [4]. The EC can be considered autocrine or paracrine ligands in bone as they act locally and are rapidly degraded. Apart from obese states [5], circulating levels of EC are typically one tenth of that found in tissues [6].

Both the bone forming cells (osteoblasts) and bone resorbing cells (osteoclasts) contain the cannabinoid receptors. Osteoblasts express cannabinoid receptor 2 (CB2) [7], while CB1 is found at barely detectable levels in differentiated osteoblasts [7,8]. During bone remodeling, signals [i.e., receptor activator of NFκB ligand, parathyroid hormone, or prostaglandin E_2 (PGE_2)] from osteoblasts activate pre-osteoclasts to mature into the adult osteoclast phenotype. Both CB1 and CB2 receptors are found in osteoclasts [8,9]. Stimulation of the CB2 receptor by synthetic agonists in either primary osteoblast cultures or the MC3T3-E1 cell line promoted increased cell

Abbreviations: AA, arachidonic acid; AEA, anandamide; 2-AG, 2-arachidonylglycerol; ALP, alkaline phosphatase; BSA, bovine serum albumin; CB1, CB2, cannabinoid receptor 1,2; EC, endocannabinoid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; FAAH, fatty acid amide hydrolase; FAME, fatty acid methyl ester; NAPE-PLD, *N*-acyl phosphatidylethanolamine-selective phospholipase D; NAE, *N*-acyl ethanolamine; OA, oleic acid; PUFA, polyunsaturated fatty acid.

Since the submission of this publication, Bruce Watkins and Yong Li have moved to the Department of Nutritional Sciences, University of Connecticut, Storrs, CT. Heather Hutchins has moved to the Center on Aging, University of Connecticut Health Center, Farmington, CT.

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proliferation as well as expression of alkaline phosphatase (ALP) activity and events associated with mineralization [7]. Moreover, since no agonist effect was observed in osteoblasts cultured from CB2 knockout mice, the CB2 receptor appears to be necessary for inducing bone formation [7]. Also, treatment of primary bone marrow cultures with 2-AG resulted in a dose-dependent increase in fibroblastic cell colony number and size as well as calcium and collagen deposition; however, when these cells were treated with a CB2 antagonist, the effects of this endocannabinoid was largely abolished [10].

Rather than a direct action of CB1 on osteoblasts, it has been suggested that the bone protective effects of CB1 activation is via attenuation of norepinephrine action in the sympathetic nerves surrounding bone from endocannabinoids (2-AG) released by osteoblasts [4]. Generally, the sympathetic nerves release norepinephrine to the β-adrenergic receptors on osteoblasts to inhibit bone formation and stimulate bone resorption [11]. Thus, CB1 activation results in inhibition of norepinephrine release by the sympathetic nerves which, in turn, diminish the catabolic effect of norepinephrine on bone [4].

The amount and type of dietary n-6 and n-3 polyunsaturated fatty acids (PUFA) can change the PUFA composition in bone compartments [12–14] and osteoblasts [15]. Feeding rats n-3 PUFA (eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA) led to an increase in n-3 PUFA concentrations in polar lipids from femur periosteum, marrow, and cortical bone and a decrease in AA concentration and the ratio of n-6/n-3 PUFA [12–14]. In mouse adipocytes compared to the vehicle control, those cultured with AA for 72 h had significantly increased 2-AG, but DHA treatment decreased both AEA and 2-AG concentrations, and EPA treatment tended to decrease 2-AG concentration in this cell culture system [16]. These findings support the premise that AA treatment of osteoblast-like cells has the potential to increase AEA and 2-AG concentrations, while long chain n-3 PUFA treatments can potentially decrease AEA and 2-AG concentrations. Moreover, the changes in substrates for EC synthesis may alter the expression of enzymes involved in the EC system. Based on the findings of Watkins et al. [17] and others [18] that gene expression in and biomarkers elaborated by MC3T3-E1 cells are changed by exposure to different amounts and types of PUFA, the cellular EC signaling system likely adapts when exposed to these nutrients. Hence, investigating the adaptation to different dietary PUFA is important to understand the EC signaling pathways of cells, tissues and organs of the body.

Therefore, we hypothesized that long chain n-3 PUFA alter EC enzyme substrate concentrations and consequently, modify EC signaling to influence osteoblast function. The objective of the study was to elucidate if long chain n-3 PUFA affects the major components of the EC system, namely the synthesis and degradation enzymes for AEA, at the molecular level, and whether the changes in the status of these enzymes will impact the differentiation and maturation of osteoblast-like cells. To test this hypothesis, long chain PUFA that are generally consumed in diets containing meat and fish (AA, EPA and DHA) were used to treat MC3T3-E1 cells to determine their effect on mRNA expression for cannabinoid receptors and AEA synthesis and degradation enzymes. Additionally, ALP activity was monitored as a surrogate marker of osteoblast maturation.

2. Materials and methods

2.1. Reagents and chemicals

Free fatty acids of AA, EPA, DHA, and oleic acid (OA) were purchased from Nu-Check-Prep (Elysian, MN, USA) and fatty acid-free bovine serum albumin (BSA) from Sigma (St. Louis, MO, USA). Fetal bovine serum and antibiotic/antimycotic were purchased from Lonza (Walkersville, MD, USA) and used in all cell culture media preparations. The β-glycerophosphate and ascorbic acid (Sigma, St. Louis, MO, USA) were added to the osteogenic media. Methanol and chloroform [high-performance liquid chromatography (HPLC) grade, Mallinckrodt Chemicals, Phillipsburg, NJ, USA], 10% boron trifluoride (Supelco, Bellefonte, PA, USA), and isooctane (HPLC grade, Fisher Scientific, Pittsburg, PA, USA) were used to extract lipids and in the procedures for the analysis of fatty acid methyl esters (FAME) by gas chromatography.

2.2. Cell cultures

The MC3T3-E1 osteoblast-like cell line (subclone 4) was purchased from ATCC (Manassas, VA, USA) and maintained in α-minimum essential media (Invitrogen/Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic at 37°C in 5% CO_2 as previously described [17]. Cells used for experiments were from passages 7-24. For the experiments, MC3T3-E1 cells were cultured in six-well plates (Corning, Corning, NY, USA) for 6, 10, 15, 20, 25 or 30 days with osteogenic medium (media changed every 2–3 days). Osteogenic medium consisted of the above supplemented maintenance media plus osteogenic additives β-glycerophosphate (10 mM) and ascorbic acid (50 μg/ml) to promote the osteoblast phenotype in culture [19]. Seventy-two hours prior to cell collection, the osteoblast-like cells were treated with 10 μM AA or 10 μM EPA+DHA (5 μM each) or 10 μM AA, EPA, DHA or OA loaded to BSA in osteogenic media. Fatty acid treatments at 10 μM were used based on our previous study that gene expression was changed in MC3T3-E1 cells [17]. These treatments were prepared by adding free fatty acids dissolved in ethanol into osteogenic media with BSA for a final molar ratio of 2:1 (fatty acid:BSA) [17]. The vehicle control consisted of the osteogenic media containing BSA at a final concentration of 5 μM BSA.

2.3. Fatty acid analysis

Cells were washed 2× with phosphate buffered saline (PBS), harvested, and the cell mass pellet frozen at -80°C until analysis. To extract lipids, cells were homogenized in a chloroform and methanol mixture (chloroform: methanol at 2:1, v/v). This mixture was then dried under nitrogen gas, treated with NaOH in methanol at 100°C. The samples were then methylated with boron trifluoride in methanol and extracted with isooctane. The resulting FAME were analyzed by gas chromatography (GC) (HP 6890 series, autosampler 7683, GC 3365 Chem Station Rev.A.08.03, Agilent Technologies, Palo Alto, CA, USA) with a DB-23 column (30 m, 0.53 mm i.d., 0.5 μm film thickness, Agilent Technologies) and equipped with a flame ionization detector [13]. The method for GC FAME analysis was programmed as follows: 100°C for 2 min, temperature increased by 4°C/min to 150°C and held for 5 min, increased temperature by 3°C/min to 165°C held for 13.5 min, 2°C/min increase to 185°C and held for 13 min and lastly, increased temperature by 10°C/min to 200°C and held for 10 min. Injector temperature was 225°C and detector temperature was 250°C. Sample peaks were identified by comparison to authentic FAME standards (Nu-Chek-Prep, Elysian, MN, USA), and the fatty acid amount was expressed as g/100 g fatty acids.

2.4. Protein assay

MC3T3-E1 cells enriched with 10 μM of AA, EPA, DHA, or OA and vehicle control for 72 h were collected in PBS, washed, harvested, and a cell mass pellet stored at −80°C until analysis. Cells were reconstituted in 800 μl PBS and protein content was determined following manufacturer instructions for the Pierce BCA protein assay kit (Thermo Scientific/Pierce Biotechnology, Rockford, IL, USA). Spectrophotometric absorbance was measured at 562 nm to determine protein concentration on a Spectra Max 190 with SOFTmax Pro 4.0 software (Molecular devices, Sunnyvale, CA, USA).

2.5. RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

MC3T3-E1 cells from all fatty acid treatments were washed 2× with cold PBS, lysed and the RNA was isolated using RNAqueous-4PCR kit (Ambion, Foster City, CA, USA) as described in the manufacturer's instructions. Briefly, the cells were lysed using the provided guanidinium thiocyanate lysis/binding solution. Cells from two wells of the six-well plate in the same treatment group were pooled. After pooling of wells, the lysed cells were washed of proteins and contaminants, and RNA was eluted from the filter system. RNA samples were then treated with DNase I (Ambion) to remove any

Table 1
Gene primers used for quantitative RT-PCR analysis

Primer	Forward	Reverse
Actin	5′ GGC TAC AGC TTC ACC ACC AC 3′	5′ TAC TCC TGC TTG CTG ATC CAC 3′
CB1	5′ CCT TGC AGA TAC AAC CTT 3′	5′ TGC CAT GTC TCC TTT GAT A 3′
CB2	5′ GGA AGG CCA GAT CTC CTC TC 3′	5′ CTG GAG CTG TCC CAG AAG AC 3′
NAPE-PLD	5′ ATG CAG AAA TGT GGC TGC GAG AAC 3′	5′ ACC ACC TTG GTT CAT AAG CTC CGA 3′
FAAH	5′ TAG CTT GCC AGT ATT GAC CTG GCT 3′	5′ AGG AAG TAA TCG GGA GGT GCC AAA 3′

CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; NAPE-PLD, *N*-acyl phosphatidylethanolamine-selective phospholipase D; FAAH, fatty acid amide hydrolase.