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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.

DNA contamination. Concentration and purity of the RNA were determined by measuring absorbance at 260 and 280 nm (Spectra Max 190 with SOFTmax Pro 4.0 software, Molecular Devices, Sunnyvale, CA, USA). Purity was recorded as the ratio of 260:280 nm with an average of 1.92. Total RNA (2 μg) was reverse transcribed to cDNA in a reaction mixture using RNA transcriptase superscript II (Invitrogen). Briefly, 9 μl of the following was combined for each sample: 4 μl 5× first-strand buffer, 2 μl 0.1 M DTT, 2.0 μl 100 mM dNTP, 1 μl 100 pmol/μL random hexamer. RNA samples were then added to the mix followed by 1 μl reverse transcriptase as the final step for a total volume of 20 μl. Samples were heated as specified for superscript RT: 25°C for 10 min, 42°C for 50 min and 70°C for 15 min. The cDNA product was then used for quantitative RT-PCR (Applied Biosystems 7300 RT-PCR; Applied Biosystems, Foster City, CA, USA). A master mix for RT-PCR was prepared with SYBR Green Master Mix Kit (Applied Biosystems). Briefly, 10 μl SYBR Green was mixed with 6 μl DEPC treated water (Ambion, Foster City, CA), 1 μl of 10 μM forward primer and 1 μl 10 μM reverse primer (Table 1). A total of 18 μl of master mix was added to wells of a 96-well plate followed by 2 μl of cDNA. A standard cDNA sample (mouse brain) was used to create a regression curve for each RT-PCR analysis with 0.5, 1, 2, and 4 μl of cDNA and 19.5, 19, 18 and 16 μl of master mix, respectively. All samples were analyzed in triplicate; fluorescence emission was detected and cycle threshold (CT) values calculated automatically. Gene CT value relative amounts were predicted from the standard curve calculations for each gene, which were then normalized to β-actin expression.

2.6. Alkaline phosphatase activity

Total ALP activity (U) was measured from media of MC3T3-E1 cells grown for 6, 20 and 30 d in osteogenic medium using the QuantiChrom Alkaline phosphatase assay kit (BioAssay Systems, Hayward, CA, USA). We chose these time points to represent early differentiation, late differentiation and mineralization stages of the osteoblast. ALP hydrolyzes *p*-nitrophenyl phosphate to *p*-nitrophenol plus phosphate; the kinetics of this reaction can be measured by spectrophotometric absorbance at 405 nm (Spectra Max 190 with SOFTmax Pro 4.0 software, Molecular Devices) in which the rate of reaction (measured at time 0 and 4 min) is directly proportional to ALP activity.

2.7. Statistical analyses

Data were analyzed using SAS 9.2 (SAS Institute, Cary, NC, USA). A one-way analysis of variance (ANOVA) analysis was used to determine treatment differences for the mRNA expression data from the AA and EPA+DHA treatments. A two-way ANOVA was used to assess differences of time in culture and fatty acid treatment on FAME data from AA and EPA+DHA enrichment at time points 10, 15, 20, 25 and 30 as well as EC gene mRNA expression and ALP activity. Tukey's range test was used for post hoc analysis with significance set at $P<.05$. A standardized difference test for the EC gene mRNA expression data was calculated to present the relative difference compared to the vehicle control. Each treatment was subtracted from the vehicle control value and then divided by the pooled standard deviation. Results are expressed as means±S.E. or S.D., where applicable.

3. Results

3.1. Fatty acid analysis of cell cultures

FAME measured in osteoblast cell cultures following 72 h of treatment with vehicle control, AA, and EPA+DHA are presented in Table 2. Both PUFA treatments significantly increased total saturated fatty acid and PUFA concentrations while monounsaturated fatty acid concentrations were decreased compared to the vehicle control in MC3T3-E1 cells cultured for 10 d (Table 2) and other time points (15, 20 and 25 days). AA treatment resulted in a significant increase in 20:4n-6, 22:4n-6, n-6 PUFA, long chain n-6 PUFA and ratios for long chain n-6/n-3 PUFA and n-6/n-3 PUFA compared to the EPA+DHA treatment and the vehicle control in the osteoblast-like cells grown for 10 days. A significant decrease was observed in n-3 PUFA with AA treatment compared to the EPA+DHA and vehicle control groups. The 1:1 combination treatment of EPA+DHA to MC3T3-E1 cells grown for 10 days significantly increased the concentrations of 20:5n-3, 22:5n-3, 22:6n-3, n-3 PUFA and long chain n-3 PUFA and decreased n-6 PUFA and the ratios of long chain n-6/n-3 and n-6/n-3 PUFA compared to the AA and vehicle control groups (Table 2).

The effects of AA treatment in MC3T3-E1 cells consistently increased 20:4n-6, long chain n-6 PUFA and the ratios of long chain n-6/n-3 and n-6/n-3 PUFA compared to the n-3 PUFA group at other time points tested (15, 20, 25 and 30 days) (data not shown).

Table 2
Fatty acid composition (g/100 g) of MC3T3-E1 cells cultured in osteogenic medium for 10 d

Fatty acid	Treatments			Pooled SD	ANOVA *P* value
	VC	AA	EPA+DHA		
14:0	0.39	0.47	0.38	0.13	.14
15:0	0.39	0.46	0.37	0.04	.14
16:0	10.49	10.58	10.62	0.23	.05
16:1t	1.63	1.50	1.50	0.07	.19
16:1n-7	4.81	4.37	4.12	0.21	.09
17:0	0.36^{b}	0.39^{a}	0.37^{b}	0.006	.02
18:0	18.72^{b}	20.27^{a}	20.15^{a}	0.33	.03
18:1t	0.71	0.00	0.56	0.64	.25
18:1n-9	25.39^{ab}	22.62^{a}	22.58^{b}	0.63	.03
18:1n7	8.06^{a}	6.87^{b}	6.64^{b}	0.22	.006
18:2n-6	0.72	0.65	0.69	0.02	.14
20:2n-6	6.89^{a}	5.46^{b}	5.52^{b}	0.11	<.0001
20:3n-6	0.36	0.31	0.34	0.02	.31
20:4n-6	7.34^{b}	12.15^{a}	7.08^{b}	0.15	<.001
20:5n-3	0.69^{b}	0.59^{b}	2.54^{a}	0.07	<.001
22:4n-6	0.73^{b}	1.14^{a}	0.66^{b}	0.02	<.001
22:5n-3	1.70^{c}	1.71^{bc}	2.53^{a}	0.03	<.001
22:6n-3	2.96^{b}	2.91^{b}	6.55^{a}	0.06	<.001
24:1n-9	0.16	0.27	0.12	0.17	.81
SAT	30.34^{b}	32.18^{a}	31.89^{a}	0.31	.008
MONO	40.76^{a}	35.63^{b}	35.52^{b}	0.47	.001
PUFA	21.39^{c}	24.93^{ab}	25.90^{a}	0.34	.0007
n-6 PUFA	16.04^{b}	19.71^{a}	14.29^{c}	0.24	.0001
n-3 PUFA	5.35^{bc}	5.22^{c}	11.62^{a}	0.13	<.001
Long chain $_{n-6}$	8.07^{b}	13.29^{a}	7.73^{b}	0.16	<.001
Long chain $_{n-3}$	5.35^{bc}	5.22^{c}	11.62^{a}	0.13	<.001
SAT/MONO	0.74^{b}	0.90^{a}	0.90^{a}	0.01	.001
MONO/PUFA	1.91^{a}	1.43^{b}	1.37^{b}	0.03	.0003
SAT/PUFA	1.42^{a}	1.29^{b}	1.23^{b}	0.03	.01
Long chain n-6/n-3	1.51^{b}	2.55^{a}	0.67^{c}	0.01	<.001
n-6/n-3	3.00^{b}	3.78^{a}	1.23^{c}	0.03	<.001

VC, vehicle control (bovine serum albumin); SAT, total saturated fatty acids; MONO, total monounsaturated fatty acids; PUFA, total polyunsaturated fatty acid; long chain n-6 PUFA, only includes 20:4n-6+22:4n-6+22:5n-6; long chain n-3 PUFA, only includes 20:5n-3+22:5n-3+22:6n-3

Mean values for each fatty acid in a row with different superscript letters are significantly different by one-way ANOVA followed by Tukey's range test ($n=2$ in all groups).

Treatments include 10 μM AA, 5 μM of EPA and DHA combined in the same treatment and vehicle control (BSA, 5 μM) for 72 h.

The EPA+DHA treatment significantly increased 20:5n-3, 22:5n-3, n-3 PUFA and long chain n-3 PUFA compared to the AA group at 15, 20, 25 and 30 d (data not shown). Moreover, the EPA+DHA treatment significantly decreased the ratios of long chain n-6/n-3 and n-6/n-3 PUFA in the osteoblast-like cells compared to the AA treatment and vehicle control. While AA treatment significantly increased the concentration of 20:4n-6 in the MC3T3-E1 cells at all time points, the EPA+DHA treatment did not always reduce the 20:4n-6 concentration when compared to the vehicle control.

Table 3
RT-PCR gene expression results for endocannabinoid proteins in MC3T3-E1 cells treated with AA, EPA+DHA and vehicle control

RT-PCR Parameters	Treatments			Pooled SD	ANOVA *P* value
	VC	AA	EPA+DHA		
CB2/actin	0.2501^{a}	0.0081^{b}	0.0157^{b}	0.1042	0.02
NAPE-PLD/actin	0.8218^{ab}	4.3864^{a}	0.0331^{b}	2.2017	0.046

VC, vehicle control; BSA, bovine serum albumin.

Mean values for each parameter in a row with different superscript letters signify a significant difference between treatments by one-way ANOVA followed by Tukey's range test, $n=3$ for CB2/actin, $n=4$ for NAPE-PLD/actin.

MC3T3-E1 cells were treated for 72 h with 10 μM of AA or EPA+DHA (5 μM each) in a 2:1 molar ratio of fatty acid:BSA.

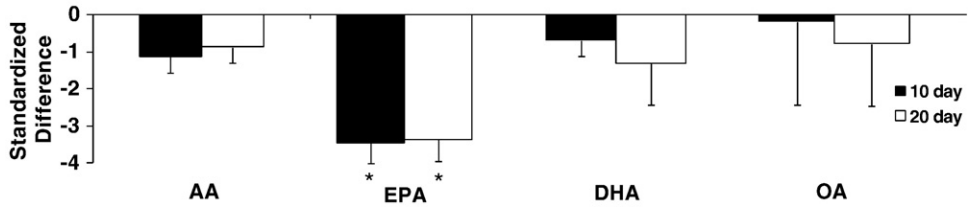

Fig. 1. Standardized difference ratio for NAPE-PLD/actin expressed per 100 μg protein from MC3T3-E1 cells cultured for 10 or 20 days in osteogenic media and treated for the last 72 h of incubation with AA, EPA, DHA and OA compared to the vehicle control (bovine serum albumin, BSA). Cells were collected and analyzed as described in materials and methods. Values are means±S.E. *Fatty acid treatment is significantly different from vehicle control ($P<.05$) by two-way ANOVA; 10 days in culture was significantly different from 20 days independent of fatty acid treatment or vehicle control. 10 μM dose for each fatty acid treatment and 5 μM BSA (fatty acid:BSA 2:1, mol/mol).

3.2. Quantitative RT-PCR

Analysis of mRNA by quantitative RT-PCR from osteoblast-like cells that were grown for 10, 25 or 30 days and treated with AA, EPA+DHA or vehicle control revealed fatty acid effects on EC system mRNA expression. This data were pooled for analysis and showed that NAPE-PLD mRNA expression was increased with AA treatment compared to the EPA+DHA treatment and vehicle control. However, both fatty acid treatments resulted in decreased CB2 mRNA levels compared to the vehicle control (Table 3).

A follow-up to the first experiment was conducted to determine individual effects of EPA and DHA on EC receptor and AEA enzyme levels in MC3T3-E1 cells. The osteoblast-like cells were cultured for 10 and 20 days; during the final 72 h of culture, cells were enriched with fatty acids (10 μM of AA, EPA, DHA, OA) or vehicle control. This analysis examined time differences (10 or 20 days in osteogenic culture) as well as fatty acid effects.

Quantitative RT-PCR analysis of NAPE-PLD (Fig. 1), FAAH (Fig. 2), and CB2 (Fig. 3) mRNA demonstrated time and fatty acid treatment effects. The mRNA levels for the cannabinoid receptor (CB2) and synthesis and degradation enzymes for AEA (NAPE-PLD and FAAH) increased over time in culture. NAPE-PLD mRNA level increased by 133% at d 20 compared to day 10; the increase for CB2 was 310% and that for FAAH was 579%. CB1 mRNA levels were below the range that could be quantified.

The MC3T3-E1 cells were also responsive to fatty acid treatments. EPA treatment significantly depressed NAPE-PLD expression compared to the vehicle control group at both 10 and 20 days of culture (Fig. 1). Data from the cells treated with both EPA+DHA also showed a lower NAPE-PLD mRNA level. Moreover, there was a fatty acid effect at both time points on CB2 mRNA levels. Cells in the EPA and OA treatment groups resulted in lower CB2 expression compared to those enriched with AA and DHA or the vehicle control (Fig. 3).

3.3. Alkaline phosphatase activity

ALP activity was measured at 6, 20 and 30 days of cell culture in osteogenic media. A two-way ANOVA revealed a significant time effect, but no fatty acid effect on osteoblast ALP activity. When ALP activity (U) was normalized relative to protein concentrations of the cells, the ALP activity appeared to decrease with increasing time in culture. ALP activity of cells was less at 30 d compared to 6 or 20 d in osteogenic culture (Fig. 4). The data suggest that from 6 to 20 d in culture, the osteoblast-like cells differentiated and entered a matrix maturation phase of the osteoblast lineage and, by 30 days, had mineralizing potential and therefore the expression of ALP was decreased.

4. Discussion

Enrichment of MC3T3-E1 osteoblast-like cells with AA resulted in an increase in n-6 PUFA and the ratio of n-6/n-3 PUFA with a decrease in n-3 PUFA. EPA+DHA treatment at a ratio of 1:1 resulted in the opposite effect of the AA treatment; there was an increase in n-3 PUFA concentrations with a decrease in n-6 PUFA levels in osteoblast-like cells. These results are in accordance with previous findings in bone compartments of rodents fed diets enriched with n-6 and n-3 PUFA [12–14]. Although the EPA+DHA treatment did not reduce the AA content of cells compared to the vehicle control, it was different from the AA group. It is possible that the AA content of fetal bovine serum in the osteogenic media resulted in a higher amount of this PUFA in cells due to the long growth period prior to enrichment with EPA +DHA. In previous studies with MC3T3–E1 osteoblast-like cells, enriching with EPA compared to AA in culture resulted in higher expression of core binding factor alpha 1 (Cbfa1) at 7 days with 1 and 10 μM fatty acids but not different at 14 (for 1 μM) and 21 days [17]. Cbfa1 is a transcription factor implicated in initiation and differentiation of osteoblasts [20]. EPA is potent in reducing PGE_2 production in the presence and absence of cyclooxygenase 2 (COX-2) inducers compared with AA treatment but had no consistent effect on COX-2 expression in these cells [17]. Thus, the current study and previous research demonstrate that long chain PUFA can mediate gene expression and cellular activity in osteoblasts.

Recent studies showed that EC concentrations in tissues and organs are responsive to the types of dietary n-6 and n-3 PUFA fed to animals. Diets containing AA promoted an increase in brain EC concentrations in piglets [21] and rats [22], while DHA diets decreased mouse brain 2-AG concentrations [23]. In peripheral tissues of rats, including the small intestine and liver, EC levels were greater after a diet with AA was consumed [24], while a fish oil diet decreased EC levels in the small intestine, liver, and visceral

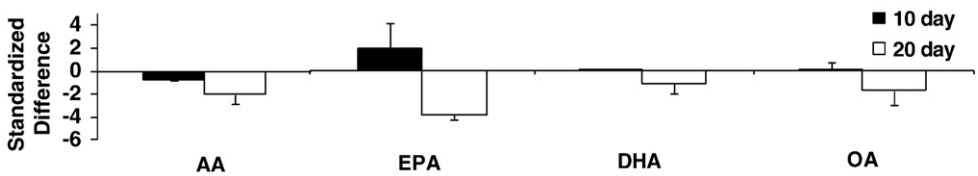

Fig. 2. Standardized difference ratio for FAAH/actin expressed per 100 μg protein from MC3T3-E1 cells cultured for 10 or 20 days in osteogenic media and treated for the last 72 h of incubation with AA, EPA, DHA and OA compared to vehicle control (bovine serum albumin, BSA); 10 μM dose for each fatty acid treatment and 5 μM BSA (fatty acid:BSA 2:1, mol/mol). Cells were collected and analyzed as described in materials and methods. Values are means±S.E.; 10 days in culture was significantly different from 20 d independent of fatty acid treatment or vehicle control.

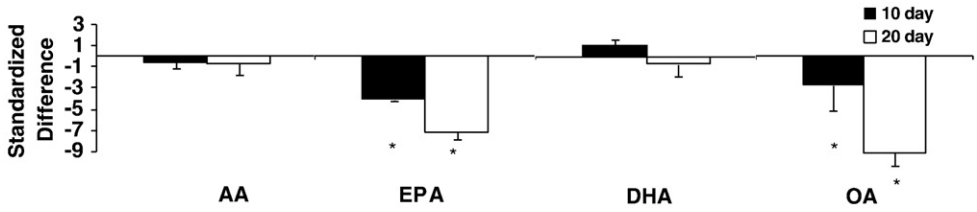

Fig. 3. Standardized difference ratio for CB2/actin expressed per 100 μg protein from MC3T3-E1 cells cultured for 10 or 20 d in osteogenic media and treated for the last 72 h of incubation with AA, EPA, DHA and OA compared to vehicle control (bovine serum albumin, BSA); 10 μM dose for each fatty acid treatment and 5 μM BSA (fatty acid:BSA 2:1, mol/mol). Cells were collected and analyzed as described in materials and methods. Values are means±S.E. *Fatty acid treatment is significantly different from vehicle control ($P<.05$) by two-way ANOVA; 10 days in culture was significantly different from 20 days independent of fatty acid treatment or vehicle control.

adipose tissue [22,24]. These studies suggest that dietary PUFA intake, which results in fatty acid incorporation into tissues, also appears to influence the EC system. Fatty acid enrichment of adipocytes in culture can also alter EC levels with some specificity. For example, Matias et al. [16] reported that AA treatment (100 μM) of 3T3-F442A adipocytes for 72 h resulted in an increase in 2-AG without any change in AEA concentration. Moreover, in the same study, DHA treatment significantly decreased both AEA and 2-AG, while EPA treatment tended to decrease 2-AG concentrations compared to the vehicle control [16]. These results support our hypothesis that dietary PUFA alter EC expression as demonstrated in experimental models from in vivo and in vitro studies.

Our findings are the first to provide evidence that MC3T3-E1 osteoblast-like cells express AEA metabolizing enzymes, NAPE-PLD and FAAH, and that their levels increase with cell maturation. Furthermore, CB2 mRNA levels increased with time in our cell culture system. These results suggest an up-regulation of the EC system with osteoblast cell maturation. Consistent with our work, Ofek et al. also observed maximal CB2 expression in MC3T3-E1 with 20 d of growth in osteogenic media [7]. The investigators found similar maximal expression of markers for osteoblast differentiation (RUNX2/Cbfa1, tissue nonspecific ALP, and parathyroid hormone [PTH]-related protein receptor-1) at 20 days, indicating a possible interaction with CB2 expression and osteoblast differentiation [7].

The mRNA level of NAPE-PLD was altered by fatty acid treatment of osteoblasts in the current study. The AA treatment, compared to EPA+DHA and vehicle control, resulted in an increased NAPE-PLD mRNA level when all time points tested (10, 25, 30 days of incubation in osteogenic media) were combined. However, when the n-3 PUFA treatments were provided individually, EPA treatment significantly decreased NAPE-PLD mRNA levels compared to vehicle control at both 10- and 20-day time points, while there was no effect of AA on NAPE-PLD levels compared to vehicle control or the other fatty acid treatments.

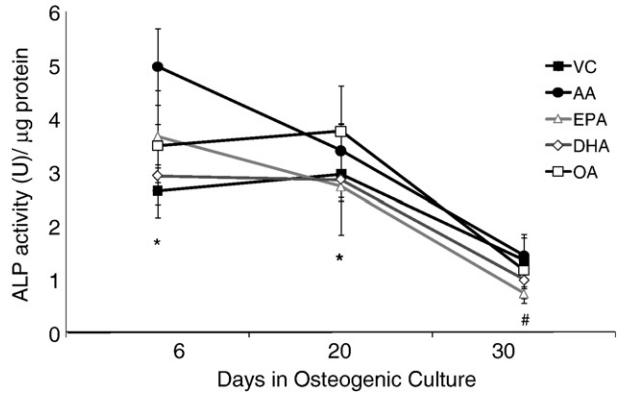

Fig. 4. ALP activity (U) expressed to protein content (1 μg) in MC3T3-E1 cells cultured in osteogenic medium for 6, 20 or 30 days and treated with AA, EPA, DHA and OA for 72 h prior to cell collection; 10 μM dose for each fatty acid treatment and 5 μM BSA (fatty acid:BSA 2:1, mol/mol). ALP activity was measured as the rate of conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol+phosphate and calculated using manufacturer's instructions to determine units (U) per ml, which was then normalized to μg protein. Data presented as means±SE, $n=3–4$. Values with different symbols are significantly different by one-way ANOVA and Tukey's range test for time ($P<.05$). No significant differences between treatment groups were observed. VC, vehicle control (BSA).

The lack of effect of EPA+DHA may have stemmed from the mixed treatment that masked the individual effects of each long chain n-3 PUFA on NAPE-PLD mRNA levels. Additionally, the formation of AEA can occur through one of three known pathways; however, NAPE-PLD is the dominant one [25]. Inactivation of the NAPE-PLD enzyme through genetic knockout in mice demonstrated no change in brain AEA [26]. It is unknown, however, if ablation of NAPE-PLD has any effect on peripheral tissue AEA concentrations. The n-3 PUFA have been found to decrease AEA concentrations in cultured adipocytes [16], which supports our finding that n-3 PUFA treatment decreased NAPE-PLD mRNA expression and may decrease AEA levels. Since we did not measure AEA directly, formation via other pathways cannot be ruled out. DHA treatment of osteoblasts in our study did not alter NAPE-PLD mRNA compared to vehicle control, suggesting that EPA and DHA may act by different mechanisms or pathways to affect AEA formation. The dose in our study was one tenth of that used by Matias et al. [16] who reported a decrease in AEA and 2-AG. We interpret the DHA effect to either be dose dependent or cell specific and that a greater dose of DHA may be necessary to reduce NAPE-PLD mRNA expression. The actions of EPA on steady state NAPE-PLD mRNA expression could be direct, involving depressed synthesis of AEA in osteoblast-like cells.

Fatty acid treatment also influenced CB2 expression. EPA and OA treatments decreased CB2 receptor expression at both 10 and 20 d time points, without an effect of AA or DHA. The effect of EPA on decreasing CB2 expression in osteoblast-like cells has potential to impact bone outcomes. CB2 activation can increase osteoblast proliferation, differentiation and mineralization [7]; therefore, EPA has the potential to diminish this effect of CB2 on osteoblasts. Ofek et al. reported an anabolic effect of CB2 activation on trabecular bone volume, bone formation rate, mineral appositional rate, and cortical bone thickness post ovariectomy in female C3H wild type mice [7]. In the ovariectomized rat, high dose EPA supplementation (1% of diet) was detrimental to bone as this treatment exacerbated bone loss compared to the ovariectomized control that was not fed any EPA or those fed a lower dose of 0.1% EPA in the diet [27]. Based on our findings that EPA decreased mRNA levels for NAPE-PLD and CB2, it is now possible to investigate the relationship between dietary PUFA and EC signaling in bone. In light of our results, investigations on dietary PUFA and bone metabolism should consider the effects of n-3 PUFA like EPA and the potential it has to lower endogenous ligands (e.g., AEA) as well as to alter EC signaling. With regard to OA, it is a substrate for oleoylethanolamide, which is also synthesized by NAPE-PLD but does not act on the cannabinoid receptors; rather, oleoylethanolamide is a ligand for peroxisome proliferator-activated receptor (PPAR) α [28]. In

summary, the finding that EPA and OA both lowered CB2 mRNA in osteoblasts is an intriguing observation. These fatty acids may influence transcription factors, such as PPAR, that are associated with genes responsible for mRNA expression of proteins involved in the metabolism of ligands and receptors for the EC system.

FAAH expression increased with time in cell culture but was not influenced by fatty acid treatment. FAAH expression was observed to be stable in heart, liver, visceral adipose tissue and subcutaneous adipose tissue from mice fed a fish oil or krill oil diet compared to the control [22]. Our results and those of other researchers suggest that dietary fatty acids can reduce AEA levels but also reduce enzymes that regulate the synthesis of AEA.

Bone specific ALP activity is utilized as a marker of bone formation *in vivo* [29] and is a marker for osteoblast differentiation and matrix maturation in primary and cell culture lines [30]. ALP activity is typically low during proliferation, increases with differentiation and matrix maturation and decreases again with mineralization. ALP activity levels during the time course used in this study suggest that the MC3T3-E1 cells followed the usual path of osteoblast lineage. Expression of the CB2 receptor has been reported to be similar to ALP expression in this cell type [7]. There was no effect of PUFA on ALP activity using 10 μM treatments; studies that did show a PUFA effect used greater dosages [17] or for longer duration [18], which may act via the EC system and CB2 receptor, in particular.

In conclusion, MC3T3-E1 osteoblast-like cells become enriched with fatty acids after 72 h of incubation and the treatment of long chain n-6 and n-3 PUFA can change the concentrations and the ratio of n-6/n-3 PUFA in osteoblasts, independent of total culture time. The increase in cellular AA content in response to 72 h of incubation with AA occurred at most all time points (except for 30 d, where there was an insignificant increase observed) and confirms that PUFA modification can potentially affect the EC system at the time points tested. These findings support the use of this model for EC system research. While ALP activity was not responsive to the fatty acid dose during the time course of this study, the ALP activity showed that the cultured MC3T3-E1 cells had a typical activity. NAPE-PLD, FAAH, and CB2 mRNA expression was higher from 10 to 20 d in osteogenic culture. At both time points, EPA treatment significantly decreased the CB2 receptor and NAPE-PLD mRNA levels compared to the vehicle control. The EC genes may be more responsive to the levels of PUFA used in this study than ALP activity. Based on our study in osteoblasts and others in adipocytes [16], n-3 PUFA appears to down-regulate the EC system. Furthermore, our results are the first to demonstrate that PUFA can alter the EC system in osteoblast-like cells and that NAPE-PLD and FAAH are present in osteoblasts and increase with cell maturation. The EC system's influence on bone cells and bone in vivo has been established [3,31,32]. Elucidation of how dietary PUFA, not traditionally associated with bone health, can impact the EC system in osteoblasts is essential to understand how these nutrients exert effects on bone modeling and remodeling processes. The findings of this study warrant further investigation into the role of PUFA on the EC system in osteoblasts and *in vivo* models of bone metabolism.

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