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Hind limb suspension and long-chain omega-3 PUFA increase mRNA endocannabinoid system levels in skeletal muscle

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Abstract

Muscle disuse has numerous physiological consequences that end up with significant catabolic metabolism and ultimately tissue atrophy. What is not known is how muscle atrophy affects the endocannabinoid (EC) system. Arachidonic acid (AA) is the substrate for anandamide (AEA) and 2-arachidonylgycerol (2-AG), which act as agonists for cannabinoid receptors CB1 and CB2 found in muscle. Diets with n-3 polyunsaturated fatty acids (PUFA) have been shown to reduce tissue levels of AA, AEA and 2-AG. Therefore, we hypothesized that hind limb suspension (HS)-induced muscle atrophy and intake of n-3 PUFA will change mRNA levels of the EC system. Mice were randomized and assigned to a moderate n-3 PUFA [11.7 g/kg eicosapentaenoic acid (EPA)+docosahexaenoic acid (DHA)], high n-3 PUFA (17.6 g/kg EPA+DHA) or control diets for 12 days and then subjected to HS or continued weight bearing (WB) for 14 days. HS resulted in body weight, epididymal fat pad and quadriceps muscle loss compared to WB. Compared to WB, HS had greater mRNA levels of AEA and 2-AG synthesis enzymes and CB2 in the atrophied quadriceps muscle. The high n-3 PUFA diet resulted in greater mRNA levels of EC synthesis enzymes, and CB1 and CB2. The higher mRNA levels for EC with HS and dietary n-3 PUFA suggest that muscle disuse and diet induce changes in the EC system to sensitize muscle in response to metabolic and physiological consequences of atrophy.

Keywords: Endocannabinoids; Mice; Omega-3; Omega-6 PUFA; Eicosapentaenoic acid; Docosahexaenoic acid; Hind limb suspension; Cannabinoid receptors

1. Introduction

The cannabinoid receptors CB1 and CB2 are seven-transmembrane $G_{i/o}$-protein-coupled receptors that are activated by cannabinoids and pharmaceutical receptor agonists or inhibited by receptor antagonists [1]. Activation of the CB1 receptor stimulates appetite to promote body weight gain in wasted states and weight loss when antagonized in obesity [2]. Therefore, the endocannabinoid (EC) system can be considered a homeostatic modulator of systemic energy status [3].

Endogenous cannabinoids *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonylglycerol (2-AG) are biosynthesized from arachidonic acid (AA) in the phospholipid membrane. Dietary sources of n-3 polyunsaturated fatty acids (PUFA) are known to reduce the concentrations of AA in blood and other tissues, but more recently to reduce the levels of AEA and 2-AG in plasma and brain [4].

CB1 is involved in not only central regulation of food intake but also glucose and fatty acid oxidation pathways in peripheral tissues as evidenced in obesity and excessive energy intake [5]. Both CB1 and CB2 expressions have been confirmed in skeletal muscle of rodents and humans [6]. Soleus muscle from mice fed a high-fat diet had a greater concentration of CB1 mRNA compared to chow-fed mice [7]. However, there was no difference observed in skeletal muscle CB1 expression between lean and obese humans [8]. Skeletal muscle from obese subjects and leptin-deficient obese mice had reduced glucose uptake and fatty acid oxidation, which can be reversed by CB1 antagonism [8,9].

In adipocytes, CB1 activation promotes glucose uptake to the same degree as insulin stimulation [10], suggesting that CB1 activation in adipose and skeletal muscle would promote energy storage in adipose rather than systemic energy utilization by skeletal muscle. CB1 is up-regulated in abdominal, subcutaneous and visceral adipose tissues of obese compared to lean individuals [10], and blood levels of 2-AG and

Abbreviations: 2-AG, 2-arachidonylgycerol; AA, arachidonic acid; AEA, anandamide; AMPK, AMP-activated protein kinase; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; DAGL, diacylglycerol; DHA, docosahexaenoic acid; DXA, dual-energy X-ray absorptiometry; EC, endocannabinoid; EPA, eicosapentaenoic acid; FAAH, fatty acid amide hydrolase; FAME, fatty acid methyl ester; MAGL, monoacylglycerol lipase; MONO, monounsaturated fatty acids; NAPE-PLD, *n*-acyl phosphatidylethanolamine selective phospholipase D; NFκB, nuclear factor kappa B; PPAR, peroxisome proliferator-activated receptors; PUFA, polyunsaturated fatty acids; SAT, saturated fatty acid; WB, Weight bearing.

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AEA are elevated in obese compared to lean individuals [11,12]. These changes in obesity relative to a lean body composition are referred to as dysregulation of the EC system [13]. Moreover, treatment with AEA was found to decrease fatty acid oxidation pathways in skeletal muscle of obese individuals [8], and CB1 antagonism was reported to promote glucose uptake in cultured mouse myotubes [14] and soleus muscle in mice [9]. As an example of CB1 antagonism, treatment with SR141716 of the soleus muscle from leptin-deficient obese mice resulted in increased glucose uptake as 2-deoxyglucose [9]. The response with the CB1 antagonist SR141716 occurred in the presence of very low insulin indicative of increased GLUT1-mediated glucose uptake [9].

The n-3 PUFA, eicosapentaenoic acid (EPA), has also been shown to increase both fatty acid and glucose uptake in cultured human skeletal muscle cells from healthy human subjects, and GLUT1 expression was increased 2.5-fold in the EPA-treated muscle cells compared to the vehicle control [15]. The increase in glucose uptake from n-3 PUFA treatment was similar to the action of CB1 antagonism in muscle. Much of the focus on the cannabinoid system in skeletal muscle has been to explain EC dysregulation associated with obesity. However, there is a lack of information on the action and expression of the EC system in peripheral tissue during catabolic states such as disuse or disease. The catabolic metabolism associated with muscle atrophy occurs in cancer cachexia, sepsis, muscle disuse or aging and is defined as an unintentional loss of 5%–10% of muscle mass [16]. Rodent hind limb suspension (HS) is a model used to simulate muscle disuse and atrophy in spaceflight [17].

A novel aspect of the rodent HS model is that muscle disuse results in a metabolic shift towards utilization of glucose away from fat as evidenced by increased gene expression of proteins involved in glycolysis and decreased expression of proteins involved in β-oxidation in hind limb atrophied soleus muscle [17]. Previous work by Grinchko and colleagues found an increase in glycogen use by both type I (slow) and II (fast) muscle fibers in rats; the slow type I fibers moved to a substrate profile that resembled the type II fibers by increased ATP and phosphocreatine [18]. Also in the soleus muscle of leptin-deficient mice, CB1 antagonists promote glucose uptake [9], therefore suggesting that CB1 expression may be involved in the glycolytic profile of atrophied soleus muscle. The result of muscle disuse and altered macronutrient utilization is also evident in the liver of HS rats where an increase in gluconeogenic and lipogenic gene expression is observed [19]. The shift in energy metabolism towards glucose in atrophied skeletal muscle is also observed with aging rats [20] and bed rest in humans [21], two conditions associated with muscle disuse and loss of mass.

Cancer cachexia and AIDS in humans are also states of overt muscle wasting in which cannabinoids or cannabinoid-derived drugs have been utilized as a treatment modality to attenuate pain and stimulate appetite [2]. However, EC system expression and subsequent signaling cascades in peripheral tissues such as skeletal muscle have not been extensively studied. EPA supplementation in rodent models of induced cancer cachexia and muscle wasting was shown to attenuate muscle protein degradation [22,23]. Since some evidence indicates that muscle wasting in cachexia can be attenuated with n-3 PUFA and the use of cannabinoid drugs can stimulate appetite, investigations to explore nutritional approaches to redirect actions of the EC system during aging and disease with n-3 PUFA are warranted.

The actions of the EC system on intermediary metabolism for glucose and fatty acids demonstrate a dysregulation in obesity that can be improved with CB1 antagonism. One approach to study this signaling system is to measure mRNA for EC receptors and enzymes for the synthesis and degradation of EC. For example, mRNA expression for the CB1 receptor and enzymes for the synthesis and degradation of 2-AG and AEA are increased in abdominal subcutaneous fat depots of obese compared to lean individuals [10]. In contrast, exercise reduced adipocyte CB1 expression in high-fat-diet-induced obese rodents [24], indicating that the EC receptors and enzyme expression can be altered during physiological condition. The shift in glucose utilization that occurs with HS, the increased glucose uptake by skeletal muscle with CB1 antagonism and findings that EC system mRNA levels can be changed by exercise led us to speculate that the mRNA for EC system proteins can change by HS and dietary n-3 PUFA.

Manipulation of the diet, particularly n-3 and n-6 PUFA, in rodents and piglets demonstrates that PUFA can alter EC concentrations. When feeding n-3 PUFA, the EC concentrations were decreased in the brain [4,25–27], plasma [4], small intestine [26], liver [26,28], adipose tissue and heart [28]. Specific n-3 PUFA are also known to activate transcription factors, particularly the family of nuclear receptors, peroxisome proliferator-activated receptors (PPARs), to mediate gene expression [29]. In a recent cell culture study of osteoblast-like cells, EPA enrichment resulted in lower mRNA levels of the AEA synthesis enzyme *n*-acyl phosphatidylethanolamine selective phospholipase D (NAPE-PLD) and CB2 receptor compared to control [30], demonstrating that n-3 PUFA can mediate EC system mRNA changes. At this point, the levels of mRNA for the EC system have not been examined in atrophied skeletal muscle. Therefore, we hypothesize that induced muscle atrophy with the associated alterations in glucose and fatty acid metabolism will change mRNA levels of the EC system. To test our hypothesis, the mouse HS model was used to induce muscle atrophy for measurement of differences in mRNA levels of the cannabinoid receptors CB1 and CB2, synthesis enzymes NAPE-PLD and diacylglycerol (DAGL) α and β isoforms, and degradation enzymes monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH). In addition, our study determined the effects of dietary n-3 PUFA in mice fed semipurified diets on these mRNA levels of the EC system.

2. Materials and methods

2.1. Animals, dietary treatments and experimental design

Sixty-seven male ND4 Swiss Webster mice (6 weeks old) were fed pelleted, semipurified diets (modified AIN-93G, 25% of energy from fat) that varied only in their source of PUFA. Diets included control (AIN-93G with safflower oil), a moderate n-3 PUFA (EPA+DHA 11.7 g/kg) diet and a high n-3 PUFA (EPA+DHA 17.6 g/kg) diet (Table 1). The source of EPA+DHA was fish oil comprised of 40% EPA and 20% DHA. Total diet fatty acid analysis revealed that the moderate n-3 PUFA diet contained 7.0% EPA and 3.7% DHA of total fatty acids, while the high n-3 diet contained 11.6% EPA and 6.0% DHA of total fatty acids. Food intake and body weights were recorded every 2–3 days for the duration of the study. After 12 days of diet treatment, mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine/xylazine. The mice were then subjected to dual-energy X-ray absorptiometry (DXA) to measure lean mass (muscle mass density) and fat mass as well as bone mineral density (BMD) and bone mineral content (BMC) (pDEXA Sabre; Norland Medical Systems Inc., Fort Atkinson, WI, USA). Following the DXA scan at day 12, HS was initiated in half of the mice within each dietary treatment group in a randomized fashion. Mice continued on their respective diets during HS or continued to be weight bearing (WB) for an additional 14 days. On day 26 of feeding, all mice were anesthetized with an i.p. injection of ketamine/xylazine and subjected to a repeat DXA analysis. The mice were then euthanized, and epididymal fat pads, quadriceps and gastrocnemius muscles were harvested and weighed on an analytical balance (Sartorius GMBH, Gottingen, Germany). The time line for mice dietary and unloading treatments is shown in Fig. 1. Tissues to be used for RNA isolation were immediately frozen in liquid nitrogen. Tissues for fatty acid determination were placed in a −80°C freezer until analysis. Animal care protocols were approved by and met all Purdue Animal Care and Use Committee procedures and guidelines.

2.2. Fatty acid analysis

Fatty acid composition was measured in quadriceps, gastrocnemius and cerebrum. Lipids were extracted from tissue samples with chloroform:methanol (2:1, vol/vol, Mallinckrodt Chemicals, Phillipsburg, NJ, USA), and tissue samples were treated in the solvent system by repeated sonicating and vortexing. The solvent was recovered from the mixture using a Pasteur pipette and dried under a stream of nitrogen gas, NaOH in methanol (0.5 N) was added, and the samples were subjected to heating at 100°C for 5 min. The lipid samples were methylated with 10% boron trifluoride in methanol (Supelco, Bellefonte, PA, USA) at 100°C 5 min followed by extraction with isooctane (HPLC grade, Fisher Scientific, Pittsburg, PA, USA). The resulting fatty acid methyl esters

Table 1
Fatty acid composition (area%) and ingredient of diets fed to mice for 28 days[a,b,c,d]

Fatty acid	Control	Moderate n-3	High n-3
14:0	0.19	0.28	0.33
16:0	6.81	5.99	5.48
16:1n-7	0.09	0.19	0.26
17:0	0.04	n.d.	n.d.
18:0	2.83	2.81	2.78
18:1n-9	15.74	14.16	13.12
18:1n-7	0.61	0.86	1.01
18:2n-6	71.63	60.05	52.67
18:3n-3	0.15	0.22	0.27
20:0	0.37	0.43	0.46
20:1n-9	0.20	0.79	1.01
20:2n-6	0.03	n.d.	0.16
20:4n-6	n.d.	0.50	0.83
20:5n-3	n.d.	7.16	11.55
22:0	0.35	0.34	0.32
22:1n-9	0.28	0.37	0.37
22:5n-3	n.d.	0.51	0.83
22:6n-3	n.d.	3.73	5.95
24:1n-9	0.16	0.23	0.27
SAT	10.59	9.85	9.38
MONO	17.07	16.60	16.04
n-6 PUFA	71.66	60.55	53.65
n-3 PUFA	0.27	11.62	18.60
n-6/n-3	262.86	5.21	2.89

SAT, total saturated fatty acids; MONO, total monounsaturated fatty acids; PUFA, total polyunsaturated fatty acids, n.d., not detected.

[a] The semipurified basal diet contained the following (g/kg): casein, 200; corn starch, 367.076; DYETROSE, 122; sucrose, 100; cellulose, 50; L-lysine, 3; choline bitartrate, 2.5; salt mix, 35; vitamin mix, 10.

[b] Salt mix provided (mg/kg diet): $CaCO_3$, 12495; K_2HPO_4, 6860; $C_6H_5O_7K_3 \cdot H_2O$, 2477; NaCl, 2590; K_2SO_4, 1631; MgO, 840; $C_6H_5O_7Fe$, U.S.P., 212.1; $ZnCO_3$, 57.75; $MnCO_3$, 22.05; $CuCO_3$, 10.5; KIO_3, 0.35; Na_2SeO_4, 0.359; $(NH_4)_2MoO_4 \cdot H_2O$, 0.278; $Na_2O_3Si \cdot 9H_2O$, 50.75; $CrK(SO_4)_2 \cdot 12H_2O$, 9.625; LiCl, 0.609; H_3BO_3, 2.853; NaF, 2.223; $NiCO_3$, 1.113; NH_4VO_3, 0.231.

[c] Vitamin mix provided (mg/kg diet): thiamine HCl, 6; riboflavin, 6; pyridoxine HCl, 17; niacin, 30; calcium pantothenate, 16; folic acid, 2; biotin, 0.2; cyanocobalamin (B_{12}) (0.1%), 25; vitamin A palmitate (500,000 IU/g), 8; vitamin E acetate (500 IU/g), 150; vitamin D_3, 2.5; vitamin K_1, 0.75.

[d] Dietary fat treatments included safflower oil (control), 82.85% safflower oil+17.15% fish oil (Ocean Nutrition) (low n-3 group) or 72.38% safflower oil+27.62% fish oil (Ocean Nutrition) (high n-3 group). Total fat content in each diet was 110.4 g/kg of diet.

(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, Santa Clara, CA, USA) and a flame ionization detector [31]. For FAME analysis, the GC was programmed as follows: initial at 100°C for 2 min, temperature increased by 4°C/min to 150°C and held for 5 min, increased by 3°C/min to 165°C and held for 13.5 min, 2°C/min to 185°C and held for 13 min, and increased by 10°C/min to 200°C and held for 10 min. Injector and detector temperatures were 225°C and 250°C, respectively. Sample peaks were identified by comparison to authentic FAME standards (Nu-Chek-Prep Inc., Elysian, MN, USA). The fatty acid composition was reported as area percentages of total fatty acids.

2.3. RNA isolation and polymerase chain reaction (PCR)

Mouse quadriceps RNA was isolated using a TRIzol/chloroform/isopropanol method followed by the removal of supernatants [32]. The RNA pellet was then dissolved in diethylpyrocarbonate-treated RNase-free water (Ambion, Austin, TX, USA). DNA was removed using the DNase I kit (RNAqueous-4 PCR kit, Ambion), and the RNA samples were checked for concentration and purity (260:280 nm absorbency). Total RNA (2 μg) was reverse transcribed to a cDNA library in a reaction mixture using RNA transcriptase superscript II (Invitrogen, Carlsbad, CA, USA). The cDNA library was then used for quantitative, real-time (RT) PCR (Applied Biosystems 7300 Real Time PCR, Applied Biosystems, Foster City, CA, USA). SYBR Green was used for fluorescence detection. A master mix was prepared with SYBR Green Master Mix Kit (Applied Biosystems) including 1 μl of 10-μM forward primer and 1 μl of 10-μM reverse primers. Primers include NAPE-PLD (forward) 5′ ATG CAG AAA TGT GGC TGC GAG AAC 3′, (reverse) 5′ ACC ACC TTG GTT CAT AAG CTC CGA 3′; DAGLα (forward) 5′ AAT TTG CGG ACT TAC AAC CTG CGG 3′, (reverse) 5′ TCC CAG ACA GGA AAG CCA AGA TGT 3′; DAGLβ (forward) 5′ TGT GTG TCA GCA TGA GAG GAA CCA 3′, (reverse) 5′ ACC ATG GTG GCA GCA ATG ACA ATC 3′; FAAH (forward) 5′ TAG CTT GCC AGT ATT GAC CTG GCT 3′, (reverse) 5′ AGG AAG TAA TCG GGA GGT GCC AAA 3′; MAGL (forward) 5′ TGG CAT GGT CCT GAT TTC ACC TCT 3′, (reverse) 5′ TTC AGC AGC TGT ATG CCA AAG CAC 3′; CB1 (forward) 5′ CCT TGC AGA TAC AAC CTT 3′, (reverse) 5′ TGC CAT GTC TCC TTT GAT A 3′; CB2 (forward) 5′ GGA AGG CCA GAT CTC CTC TC 3′, (reverse) 5′ CTG GAG CTG TCC CAG AAG AC 3′; 18S (forward) 5′ CGG CTA CCA CAT CCA AGG AA 3′, (reverse) 5′ GCT GGA ATT ACC GCG GCT 3′. Eighteen microliters of master mix was added to wells of a 96-well plate followed by 2 μl of cDNA. In these analyses, a standard curve quantification that used a comparative cDNA sample (mouse brain tissue) was used for all sets of primers in each 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 run in triplicate, fluorescence emission was detected, and cycle threshold values were calculated automatically. The results from standard curve quantification were then reported using relative quantification that compared the genes of interest to 18S, the housekeeping gene.

2.4. Statistical analyses

Differences among treatment groups were analyzed for significance by two-way analysis of variance (ANOVA) followed by post hoc analysis using Tukey's Studentized Range Test (SAS for Windows version 9.2, SAS Institute Inc., Cary, NC, USA). Data in tables are presented as means ± pooled S.D. Significance level was defined as $P<.05$.

3. Results

3.1. Body and organ weights and DXA analysis of mice

Food intake and body weight were measured every 2–3 days throughout the course of the study. All mice gained weight during the first 12 days of the feeding study. HS induced significant body weight loss compared to WB, independent of the diet group (Table 2). No change in the gastrocnemius muscle wet weight was observed between groups; however, quadriceps muscle and epididymal fat pad weights were decreased in the HS groups compared to WB without a diet effect (Table 2).

Whole-body DXA scan was done for BMD, BMC, lean mass and fat mass at initiation of HS or continued WB and at the end of the study. After 14 days of suspension, the BMD and BMC values were lower in the HS mice compared to the WB mice (0.061 vs. 0.065 g/cm^2 for BMD

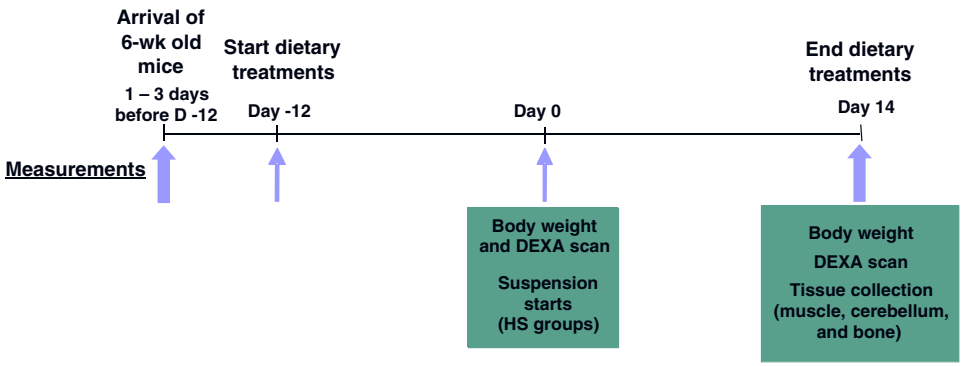

Fig. 1. Experimental time line depicting mouse groups, dietary treatments and weight bearing or unloading.

Table 2
Body and tissue wet weights

	Control		Moderate n-3		High n-3		Pooled S.D.	ANOVA *P* value		
	WB	HS	WB	HS	WB	HS		Diet	HS	Inter.
Body weight(g)	34.8^{x}	28.8^{y}	34.9^{x}	29.6^{y}	35.3^{x}	30.0^{y}	2.7	.8	<.0001	.9
Epididymal (g)	1.1378^{x}	0.5340^{y}	0.9917^{x}	0.5250^{y}	1.0765^{x}	0.7629^{y}	0.3039	.3	<.0001	.3
Right quad (g)	0.2035^{x}	0.2000^{y}	0.2012^{x}	0.1892^{y}	0.2152^{x}	0.1965^{y}	0.0161	.1	.009	.3
Right gast (g)	0.1306	0.1316	0.1457	0.1356	0.1424	0.1326	0.0136	.07	.1	.3

WB, weight bearing; HS, hind limb suspension; Inter., interaction effect of diet and suspension; Quad, quadriceps muscle; Gast, gastrocnemius muscle; epididymal, epididymal fat pad. Data represented as means, $n=13$ for the control groups, $n=11$ for both n-3 HS groups, $n=10$ for the moderate n-3 WB group and $n=9$ for the high n-3 WB group. Two-way ANOVA with Tukey's test; x and y for suspension effect.

and 0.834 vs. 1.005 g for BMC, $P<.0001$) irrespective of the dietary treatments. Lean mass was not different across all groups for HS and WB and control and n-3 PUFA diets (lean mass averaged 19.26 g for HS group vs. 18.60 g for WB group, $P=.3$). In contrast, fat mass (8.37 vs. 14.21 g, $P<.0001$) was reduced in all HS mice compared to their respective WB counterparts. The total fat mass determined by DXA is consistent with the results on epididymal fat pad weights in mice.

3.2. mRNA levels for EC enzymes and receptors in quadriceps muscle

Suspension (HS vs. WB) and diet (control vs. high n-3 PUFA) effects were observed for EC enzymes and receptors mRNA levels normalized to the housekeeping gene 18S. The mRNA values for the AEA synthesis enzyme NAPE-PLD and the 2-AG synthesis enzyme DAGLα were higher in HS mice compared to WB mice (Table 3). The mRNA levels for NAPE-PLD and DAGLα and β isoforms were greater in mice fed the high n-3 PUFA diets compared to control-fed mice (Table 3).

The FAAH mRNA levels were lowest in the HS control diet group compared to all other groups, while HS mice in the high n-3 PUFA group had the highest concentration of FAAH mRNA (Table 3); there was no difference, however, between the diet groups for the WB mice. No suspension or diet effects were observed for the 2-AG degradation enzyme MAGL.

The quadriceps muscle from mice in the high n-3 PUFA group had greater mRNA levels for both CB1 and CB2 compared to values in the control diet group. CB2 mRNA was higher in HS mice fed the high n-3 PUFA diet compared to all other groups, whereas there was no difference for CB2 mRNA between the HS and WB for the control group of mice. Within the control and the high n-3 diet groups, there was no difference for CB1 mRNA between HS and WB mice; however, those fed the high n-3 diet had greater CB1 mRNA compared to the control.

Table 3
RT-PCR results in mouse quadriceps muscle for EC system synthesis and degradation enzymes and receptors expressed as a ratio normalized to the housekeeping gene (18S)

Parameter	Control		High n-3		Pooled S.D.	ANOVA *P* value		
	WB	HS	WB	HS		Diet	HS	Inter.
Synthesis								
NAPE:18S	0.29^{by}	0.53^{bx}	0.64^{ay}	1.06^{ax}	0.25	.0004	.0048	.38
DAGLα:18S	0.12^{by}	0.16^{bx}	0.17^{ay}	0.30^{ax}	0.07	.0043	.012	.20
DAGLβ:18S	0.26^{b}	0.25^{b}	0.40^{a}	0.58^{a}	0.15	.0014	.20	.16
Degradation								
FAAH:18S	0.03^{AB}	0.01^{B}	0.03^{AB}	0.05^{A}	0.02	.035	.49	.041
MAGL:18S	0.35	0.24	0.40	0.56	0.16	.11	.71	.065
Receptor								
CB1:18S	0.06^{b}	0.06^{b}	0.10^{a}	0.10^{a}	0.04	.026	.93	.85
CB2:18S	0.30^{C}	0.17^{C}	0.53^{B}	1.06^{A}	0.14	<.0001	.0023	<.0001

WB, weight bearing; HS, hind limb suspension; Inter., interaction effect of diet and suspension; NAPE-PLD, *N*-acyl phosphatidylethanolamine-selective phospholipase D; DAGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; CB1, CB2, cannabinoid receptors 1 and 2. Data presented as means, $n=6$. Superscript letters signify significant differences: diet effects, a, b; suspension effects, x, y; interaction effects, A, B, C.

3.3. Fatty acid composition of quadriceps muscle

The n-3 PUFA diets resulted in several changes in the fatty acid composition of quadriceps muscle compared to the control diet (Table 4). The control-fed mice had greater levels of 18:1n-9, 18:1n-7, 18:2n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6, 22:5n-6, total monounsaturated fatty acids (MONO), n-6 PUFA and ratio of n-6/n-3 PUFA compared to both the moderate and high n-3 PUFA diet groups. As the dietary amount of total n-3 PUFA increased, so did the level of 20:5n-3 in quadriceps muscle. Both the moderate and high n-3 PUFA diet groups had significantly greater concentrations of 16:0, 17:0, 20:5n-3, 22:5n-3, 22:6n-3, saturated fatty acid (SAT), n-3 PUFA and ratio of SAT/MONO, SAT/PUFA and n-6/n-3 PUFA in quadriceps muscle compared to the values for mice in the control-diet group. The high n-3 PUFA diet group had a significantly greater concentration of 18:3n-3 compared to the moderate n-3 PUFA and control-diet groups.

Hind limb suspension also resulted in changes of the fatty acid composition of quadriceps muscle compared to WB mice. The amounts of 14:0, 16:1n-7, 18:3n-3, MONO and the ratio of MONO/PUFA were lower in HS mice, whereas concentrations of 18:0, 20:1n-9, 20:2n-6, 20:4n-6, 22:5n-6, 22:5n-3, 22:6n-3, PUFA, n-3 PUFA, n-6 PUFA and the ratio of SAT/MONO were significantly greater in HS mice compared to WB (Table 4).

In general, the fatty acid composition of gastrocnemius muscle of mice revealed changes similar to those found in the quadriceps for hind limb suspension and n-3 dietary PUFA treatments (data not shown). Moreover, feeding n-3 PUFA resulted in lower amounts of AA and total n-6 PUFA but higher amounts of EPA, DHA and total n-3 PUFA in gastrocnemius muscle.

Interestingly, suspension in mice resulted in higher levels of EPA and DHA, as well as total n-3 PUFA in the brain (left cerebrum). AA was not affected by suspension, although its level was lower in mice fed n-3 PUFA, as was the ratio of n-6/n-3 PUFA (data not shown).

4. Discussion

In the current study, we observed that both HS and feeding the high n-3 PUFA diet resulted in higher values for mRNA levels of EC system enzymes and receptors in skeletal muscle. In addition, mice fed the two dietary levels of n-3 PUFA demonstrated greater amounts of EPA and DHA in muscle compared to the controls. Mice subjected to HS demonstrated reduction in body weight, atrophy in quadriceps muscle and loss of epididymal fat compared to their respective weight-bearing counterparts. Similar to the loss of epididymal fat, the DXA scan revealed a lower total body fat mass in all HS mice compared to the WB groups. The effects of HS and n-3 PUFA on quadriceps muscle EC system expression are novel and provide *in vivo* support for genetic regulation of the EC system in skeletal muscle. A major finding of HS alone was the increase in the mRNA for NAPE

Table 4
Left quadriceps muscle total fatty acid composition (area%)

Fatty acid	Control		Moderate n-3		High n-3		Pooled S.D.	ANOVA *P* value		
	WB	HS	WB	HS	WB	HS		Diet	HS	Inter.
14:0	$0.85^{b,x}$	$0.68^{b,y}$	$0.77^{b,x}$	$0.69^{b,y}$	$0.98^{a,x}$	$0.88^{a,y}$	0.16	.0007	.0041	.61
15:0	0.03	0.01	0.04	0.01	0.03	0.03	0.04	.53	.053	.61
16:0	18.78^{c}	18.16^{c}	20.57^{b}	20.21^{b}	22.21^{a}	22.07^{a}	1.10	<.0001	.15	.77
16:1t	0.47	0.46	0.48	0.45	0.44	0.42	0.05	.04	.11	.6
16:1n-7	$5.25^{a,x}$	$2.92^{a,y}$	$3.34^{b,x}$	$2.32^{b,y}$	$4.19^{ab,x}$	$3.26^{ab,y}$	1.36	.011	<.0001	.16
17:0	0.13^{b}	0.14^{b}	0.18^{a}	0.20^{a}	0.17^{a}	0.19^{a}	0.04	<.0001	.11	.69
18:0	6.45^{y}	8.20^{x}	7.31^{y}	8.45^{x}	6.71^{y}	7.46^{x}	1.22	.12	.0001	.39
18:1n-9	$16.78^{a,x}$	$15.03^{a,y}$	$14.20^{b,x}$	$13.38^{b,y}$	$14.85^{b,x}$	$13.44^{b,y}$	2.06	.0017	.01	.75
18:1n-7	2.05^{a}	1.97^{a}	1.79^{b}	1.78^{b}	1.77^{b}	1.74^{b}	0.14	<.0001	.24	.66
18:2n-6	29.94^{a}	29.44^{a}	25.14^{b}	24.31^{b}	23.36^{b}	22.59^{b}	2.55	<.0001	.28	.97
18:3n-6	0.06	0.03	0.05	0.03	n.d.	0.02	0.06	.13	.46	.3
18:3n-3	$0.27^{b,x}$	$0.15^{b,y}$	$0.27^{b,x}$	$0.19^{b,y}$	$0.35^{a,x}$	$0.34^{a,y}$	0.10	<.0001	.0021	.2
20:0	0.11	0.08	0.05	n.d.	0.13	0.11	0.17	.17	.46	.96
20:1n-9	0.21^{y}	0.29^{x}	0.28^{y}	0.35^{x}	0.25^{y}	0.31^{x}	0.09	.061	.0017	.95
20:2n-6	$0.42^{a,y}$	$0.48^{a,x}$	$0.30^{b,y}$	$0.33^{b,x}$	$0.24^{c,y}$	$0.26^{c,x}$	0.06	<.0001	.0061	.3
20:3n-6	1.00^{a}	1.10^{a}	0.46^{b}	0.44^{b}	0.31^{b}	0.31^{b}	0.20	<.0001	.49	.52
20:4n-6	$6.47^{a,y}$	$7.93^{a,x}$	$2.75^{b,y}$	$3.24^{b,x}$	$2.16^{b,y}$	$2.55^{b,x}$	1.16	<.0001	.0055	.23
20:5n-3	n.d.^{c}	n.d.^{c}	1.23^{b}	1.15^{b}	1.91^{a}	1.83^{a}	0.19	<.0001	.31	.72
22:4n-6	1.28^{a}	1.47^{a}	0.21^{b}	0.23^{b}	0.16^{b}	0.14^{b}	0.22	<.0001	.17	.25
22:5n-6	$2.69^{a,y}$	$3.28^{a,x}$	$0.32^{b,y}$	$0.36^{b,x}$	$0.28^{b,y}$	$0.35^{b,x}$	0.48	<.0001	.037	.099
22:5n-3	$0.51^{b,y}$	$0.58^{b,x}$	$2.45^{a,y}$	$2.60^{a,x}$	$2.28^{a,y}$	$2.54^{a,x}$	0.30	<.0001	.042	.6
22:6n-3	$4.75^{b,y}$	$6.07^{b,x}$	$16.18^{a,y}$	$17.65^{a,x}$	$15.56^{a,y}$	$17.60^{a,x}$	2.59	<.0001	.016	.89
SAT	$26.36^{c,y}$	$27.26^{c,x}$	$28.92^{b,y}$	$29.56^{b,x}$	$30.22^{a,y}$	$30.74^{a,x}$	1.17	<.0001	.018	.85
MONO	$24.75^{a,x}$	$21.01^{a,y}$	$20.08^{b,x}$	$18.27^{b,y}$	$21.50^{b,x}$	$19.17^{b,y}$	3.11	.0005	.0008	.55
PUFA	$47.39^{ab,y}$	$50.54^{ab,x}$	$49.36^{a,y}$	$50.51^{a,x}$	$46.61^{b,y}$	$48.53^{b,x}$	2.88	.045	.0038	.5
n-6 PUFA	41.87^{a}	43.74^{a}	29.22^{b}	28.92^{b}	26.51^{c}	26.22^{c}	1.92	<.0001	.27	.094
n-3 PUFA	$5.52^{b,y}$	$6.80^{b,x}$	$20.13^{a,y}$	$21.59^{a,x}$	$20.10^{a,y}$	$22.31^{a,x}$	2.83	<.0001	.024	.85
LCn-6	$10.45^{a,y}$	$12.68^{a,x}$	$3.28^{b,y}$	$3.82^{b,x}$	$2.60^{b,y}$	$3.04^{b,x}$	1.78	<.0001	.011	.17
LCn-3	$5.25^{b,y}$	$6.65^{b,x}$	$19.86^{a,y}$	$21.40^{a,x}$	$19.75^{a,y}$	$21.97^{a,x}$	2.88	<.0001	.021	.89
SAT/MONO	$1.11^{b,y}$	$1.31^{b,x}$	$1.47^{a,y}$	$1.68^{a,x}$	$1.42^{a,y}$	$1.63^{a,x}$	0.25	<.0001	.0011	.998
MONO/PUFA	$0.53^{a,x}$	$0.42^{a,y}$	$0.41^{b,x}$	$0.37^{b,y}$	$0.47^{ab,x}$	$0.40^{ab,y}$	0.09	.0098	.0008	.43
SAT/PUFA	0.56^{c}	0.54^{c}	0.59^{b}	0.59^{b}	0.65^{a}	0.63^{a}	0.05	<.0001	.23	.79
LCn-6/n-3	2.03^{a}	1.94^{a}	0.17^{b}	0.18^{b}	0.13^{b}	0.14^{b}	0.14	<.0001	.46	.43
n-6/n-3	8.26^{a}	6.85^{a}	1.50^{b}	1.40^{b}	1.33^{b}	1.21^{b}	1.43	<.0001	.092	.21

WB, weight bearing; HS, hind limb suspension; Inter., interaction effect of diet and suspension. $n=12$ for control WB group, $n=13$ for control HS group, $n=11$ for both fish oil HS groups, $n=10$ for the moderate n-3 WB group and $n=9$ for the high n-3 WB group. Data were analyzed by two-way ANOVA with Tukey's test. Lowercase superscript letters indicate main effect of diet (a, b and c) and suspension (x and y), and uppercase letters indicate the interaction effect. n.d., not detected at the integration condition applied on these data; long-chain (LC) n-6 PUFA only include 20:4n-6+22:4n-6+22:5n-6; LC n-3 PUFA only include 20:5n-3+22:5n-3+22:6n-3.

and DAGLα, enzymes for the synthesis of AEA and 2-AG, respectively. Feeding mice n-3 PUFA also resulted in higher mRNA for the aforementioned enzymes as well as DAGLβ and CB1. An interaction effect of HS and n-3 PUFA feeding was observed for FAAH (degradation enzyme for AEA and 2-AG) and CB2.

A system closely related to the EC system, the PPAR is an example of a nuclear signaling system that is also influenced by both HS and PUFA. PPARδ mRNA expression has been shown to be greater with acute HS, while PPARα and γ are decreased in HS rodents compared to WB [33]. PPARδ is involved in muscle fiber type switching and represents a molecular adaptation to HS related to energy utilization; the decrease in PPARγ is indicative of adipocyte loss, as PPARγ is integral for adipogenesis. EC and PPAR are closely related since PUFA metabolites, such as AEA, can activate both system's receptors [34] and mediate genetic regulation to increase expression of PPARγ2, CB1, FAAH and cyclooxygenase-2 but decrease CB2 expression in adipocytes [35].

Apart from the therapeutic use of cannabinoids to induce food intake during catabolic states, EC levels and EC gene regulation during catabolism are less well understood, especially in peripheral tissues. The HS mouse model was utilized to characterize how skeletal muscle atrophy would impact EC system expression when fed a high n-3 PUFA diet. In the HS model, muscle atrophy was induced in quadriceps without alteration in gastrocnemius muscle wet weight. The quadriceps muscles are extensor muscles composed of more type II muscle fibers than type I oxidative fibers. Antigravity promotes atrophy in extensor muscles and loss of peak force at a faster rate than flexor muscles, such as the gastrocnemius [36]; this can be caused by changes in peripheral neuron excitement that would favor flexors over extensors, or related to the fibers with larger diameter in quadriceps that demonstrate greater initial atrophy than smaller fibers [36]. Therefore, atrophied quadriceps muscle was used to measure mRNA levels and fatty acid concentrations by quantitative RT-PCR and FAME, respectively, to determine the impact of HS-induced atrophy on the EC system.

HS resulted in greater mRNA levels of synthesis enzymes and the CB2 receptor compared to WB. The increase in synthesis enzymes without a corresponding increase in degradation enzymes indicates the potential for greater AEA and 2-AG levels to act as agonists on the cannabinoid receptors to up-regulate EC signaling and actions in the skeletal muscle. The CB1 receptor in skeletal muscle is intimately involved in metabolic processes related to glycolytic and oxidative flux for energy uptake and utilization. The skeletal muscle must be able to metabolize both glucose and fatty acids and switch between the two substrates to maintain metabolic flexibility. During HS and muscle disuse, a glycolytic switch has been described in the soleus muscle [17,18,37] and liver [19,37]. CB1 activation and antagonism have opposing effects on skeletal muscle glucose uptake. AEA has been shown to decrease and CB1 antagonism to increase glucose uptake [38]. Similar actions are also reported in the regulations of metabolic regulators PPARγ coactivator 1α and pyruvate dehydrogenase kinase 4 mRNA levels from primary myotube cultures of lean subjects [8]. In addition, CB1 antagonism promotes an increase, while AEA promotes a decrease, in the master regulator of metabolic flux

and AMP-activated protein kinase α1 (AMPKα1) mRNA level [8]. Liver samples from mice treated with a cannabinoid agonist, delta-9-tetrahydrocannabinol, also resulted in decreased AMPKα1 and α2 activity [39]. These regulatory effects demonstrate a role for CB1 and AEA in fatty acid oxidation and glucose flux in skeletal muscle.

The CB1 receptor has been the focus of much of EC and metabolic research to date, primarily stemming from the discovery of the CB1 antagonist SR141716. HS, however, did not alter CB1 expression. CB2 expression, on the other hand, was greater with HS compared to WB, suggesting that CB2 may play a role in the metabolic switch that occurs in skeletal muscle. The potential increase in AEA with HS and increase in CB2 mRNA indicate the potential for heightened CB2 activation and signaling. CB2 has been reported to be involved in whole-body glucose tolerance. Activation of the CB2 receptor by specific agonists and CB1 antagonism improved whole-body glucose homeostasis after a glucose load in rodents, whereas CB2 antagonism or CB1 specific agonists had the opposite effect; the cannabinoid receptor nonspecific agonist, 2-AG, did not influence glucose tolerance [40]. This finding suggests that CB1 and CB2 have opposing effects in relation to glucose utilization and the increase in CB2 that we observed in quadriceps muscle of HS mice may be involved in the glycolytic shift observed with muscle disuse. Further research in this area is warranted to determine the influence of CB2 on glucose flux in skeletal muscle.

Alternatively, n-3 PUFA have been shown to promote metabolic flexibility [41], a state when there is the capacity for a tissue to metabolize glucose or fatty acid interchangeably [42]. EPA treatment in cell culture has been shown to increase muscle glucose uptake and utilization accompanied with an increase in fatty acid oxidation [15]. At the molecular level, EPA promoted an increase in GLUT1 and CD36/FAT mRNA expression and decreased PPARγ mRNA expression compared to vehicle control [15], all of which are involved in skeletal muscle metabolic processing. The increase in EC synthesis enzymes observed with HS and n-3 PUFA intake in this model suggests that the EC system is involved in the skeletal muscle response to HS. While there was no effect of HS on CB1, a dramatic increase in CB2 mRNA was observed with HS. The greatest increase was observed in the HS mice fed a high n-3 PUFA diet on CB2 mRNA levels and suggests an additive effect on CB2 that may be linked to metabolic and physiological regulation in the atrophied skeletal muscle.

CB2 expression in skeletal muscle has been confirmed [6], but its actions are largely unknown. Cavuoto and colleagues have postulated that CB2 expression is constitutive in skeletal muscle but can be up-regulated with physiological stimulations or pathological insults [6], such as HS. Our findings that HS promoted greater CB2 mRNA levels support the concept of CB2 up-regulation as a result of physiological stress.

The increase in CB2 mRNA levels may also be related to an inflammatory stress response. Muscle injury and repair involve an inflammatory response with tissue invasion by neutrophils and macrophages [43]. Skeletal muscle disuse and atrophy have been associated with macrophage infiltration upon reloading [44,45]. Neutrophils, macrophages and skeletal muscle express CB2; therefore, the increase in CB2 mRNA level may represent skeletal muscle infiltration of immune cells and the inflammatory response to injury induced by HS. Increases in macrophages and neutrophils were not noted until 1 day post-HS [44], suggesting they were involved in the initiation of recovery processes.

Skeletal muscle atrophy is a result of increased protein degradation without the compensatory protein synthesis for proper turnover [46]. The macrophages in skeletal muscle are involved in both damage via inflammatory cytokine release [47] and regeneration to stimulate muscle fiber growth [48,49]. The CB2 receptor is associated with anti-inflammatory and antioxidative regulation in macrophages [50,51]. In the case of HS, the greater expression of CB2 in skeletal muscle may represent an anti-inflammatory action that is enhanced by the high n-3 PUFA diet. The skeletal muscle itself produces inflammatory signals that have been linked to loss of muscle mass [52,53]. In particular, there is an increase in nuclear factor kappa B (NFκB) activity in atrophied soleus muscle, and NFκB knockout mice are partially protected from HS-induced atrophy [54]. The n-3 PUFA are known to be less inflammatory than their n-6 PUFA counterparts [55] and have been implicated as an inhibitor of NFκB [56–58]. The promotion of CB2 expression by n-3 PUFA may be one way in which n-3 PUFA exhibit anti-inflammatory actions in the atrophied skeletal muscle.

The n-3 PUFA can also alter EC levels compared to other fatty acids, particularly n-6 PUFA. In the present study, we demonstrate that peripheral tissue mRNA levels of fatty acid and EC system enzymes and receptors are responsive to dietary fat intake. Although the mRNA levels of the synthesis enzymes for AEA and 2-AG were greater in mice from the high n-3 PUFA group, this does not mean an increase in EC levels in the tissue since the n-3 PUFA diet diminished the concentration of the primary EC substrate, AA, in mouse quadriceps. Rodents fed diets containing EPA and DHA have previously been shown to have lower levels of EC in plasma [4], small intestine [26], liver [26,28], adipose tissue and heart [28]. The findings by others that n-3 PUFA can depress EC levels and our findings of lower AA levels in the quadriceps of n-3 PUFA fed mice suggest that EPA and DHA in the diet can decrease skeletal muscle EC concentrations.

The greater level of mRNA synthesis enzymes with the high n-3 PUFA diet may be an attempt by the quadriceps muscle to maintain basal AEA and 2-AG levels when the substrate AA is limited. However, the increase in synthesis enzyme mRNA expression (NAPE-PLD and DAGL) that was observed with the high n-3 PUFA diet group and with HS may or may not actually increase AEA and 2-AG concentrations for reasons other than diminished substrate with the n-3 PUFA diet. Changes in mRNA expression do not always equate to similar changes in protein levels; posttranslational modifications can occur. In this study, the degradation enzyme FAAH was also altered (FAAH levels from HS, n-3 PUFA mice were greater compared to HS, control mice), and AEA and 2-AG concentrations were not directly measured. Nonetheless, we observed an increased response in EC synthesis with the n-3 PUFA diet, which could be a direct result of EPA and/or DHA signaling to the gene response element, such as PPAR [59], or indirectly due to the decreased substrate levels. In an osteoblast-like cell model, we previously reported a decrease in NAPE-PLD mRNA with EPA treatment without affecting the AA substrate level [30]. However, no effect was observed on NAPE-PLD mRNA with a combined EPA and DHA (1:1) treatment or DHA alone. The differences in these results compared to the present *in vivo* findings in skeletal muscle may be treatment (EPA vs. EPA and DHA), cell type or model (*in vivo* vs. *in vitro*) specific.

The fact that high n-3 PUFA intake increased the expression of NAPE-PLD, DAGLα, FAAH and CB2 mRNA coupled with PUFA preservation during hind limb suspension in the quadriceps muscle suggests these factors may work in concert during catabolic atrophy to conserve muscle mass. The n-3 PUFA have been shown to protect skeletal muscle metabolic flexibility [41]. Both n-3 PUFA [55–58] and CB2 activation [50,51,60] promote anti-inflammatory processes. Signaling pathways involved in the catabolic process of disuse atrophy were not measured in the present study, but previous work suggests that n-3 PUFA interact with inflammatory signals to attenuate muscle loss in cachectic states [22,23,61]. One of the pathways involved in the increased protein degradation with atrophy is activation of NFκB. As stated previously, n-3 PUFA are known to inhibit this activation [56–58]. AEA has recently also been show to inhibit NFκB activation in a CB2-mediated manner from primary murine microglial cells [60]. Therefore, the greater NAPE-PLD and DAGLα mRNA levels that were observed in quadriceps muscle from mice fed the high n-3 PUFA diet and in the HS mice are a way in which n-3 PUFA can indirectly mediate anti-inflammatory actions via potential synthesis of AEA and CB2 activation.

In conclusion, the greater mRNA level of EC synthesis enzymes and the CB2 receptor with HS reveals molecular modification of a system involved in energy homeostasis and inflammation. The increase in EC system mRNA from HS mice compared to WB may represent either adaptive changes to HS or that the EC system contributes to the physiological alterations that occur with unloading of skeletal muscle, such as adaptation to disuse-induced inflammation or the altered glycolytic flux. The increase in EC synthesis and degradation enzymes and cannabinoid receptors that we observed with the high n-3 PUFA diet compared to the control diet demonstrates a novel pathway that EPA and DHA may act upon to modulate inflammatory and metabolic homeostasis via the EC system.

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