

Endocannabinoids May Mediate the Ability of (n-3) Fatty Acids to Reduce Ectopic Fat and Inflammatory Mediators in Obese Zucker Rats¹⁻³

Barbara Batetta,^{5,7} Mikko Griinari,⁸ Gianfranca Carta,⁴ Elisabetta Murru,⁴ Alessia Ligresti,¹⁰ Lina Cordeddu,⁴ Elena Giordano,⁷ Francesca Sanna,⁵ Tiziana Bisogno,¹⁰ Sabrina Uda,⁵ Maria Collu,⁶ Inge Bruheim,⁹ Vincenzo Di Marzo^{10*} and Sebastiano Banni^{4,7*}

⁴Dipartimento Biologia Sperimentale, ⁵Dipartimento di Scienze e Tecnologie Biomediche, and ⁶Dipartimento di Neuroscienze, Università di Cagliari, Cagliari 09100, Italy; ⁷Nutrisearch s.r.l. 09010 Pula, Italy; ⁸CLANET Ltd., 02660 Espoo, Finland; ⁹Aker Biomarine ASA, NO-0115 Oslo, Norway; and ¹⁰Endocannabinoid Research Group, Institute of Biomolecular Chemistry, Consiglio Nazionale delle Ricerche, 80078 Pozzuoli, Italy

Abstract

Dietary (n-3) long-chain PUFA [(n-3) LCPUFA] ameliorate several metabolic risk factors for cardiovascular diseases, although the mechanisms of these beneficial effects are not fully understood. In this study, we compared the effects of dietary (n-3) LCPUFA, in the form of either fish oil (FO) or krill oil (KO) balanced for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) content, with a control (C) diet containing no EPA and DHA and similar contents of oleic, linoleic, and α -linolenic acids, on ectopic fat and inflammation in Zucker rats, a model of obesity and related metabolic dysfunction. Diets were fed for 4 wk. Given the emerging evidence for an association between elevated endocannabinoid concentrations and metabolic syndrome, we also measured tissue endocannabinoid concentrations. In (n-3) LCPUFA-supplemented rats, liver triglycerides and the peritoneal macrophage response to an inflammatory stimulus were significantly lower than in rats fed the control diet, and heart triglycerides were lower, but only in KO-fed rats. These effects were associated with a lower concentration of the endocannabinoids, anandamide and 2-arachidonoylglycerol, in the visceral adipose tissue and of anandamide in the liver and heart, which, in turn, was associated with lower levels of arachidonic acid in membrane phospholipids, but not with higher activity of endocannabinoid-degrading enzymes. Our data suggest that the beneficial effects of a diet enriched with (n-3) LCPUFA are the result of changes in membrane fatty acid composition. The reduction of substrates for inflammatory molecules and endocannabinoids may account for the dampened inflammatory response and the physiological reequilibration of body fat deposition in obese rats. *J. Nutr.* 139: 1495–1501, 2009.

Introduction

Long-chain (n-3) PUFA [(n-3) LCPUFA]¹¹ are known to improve several metabolic problems associated with obesity, including

insulin resistance, liver and heart steatosis, and hypertension (1). It has been suggested that they act by reducing the biosynthesis of arachidonic acid (ARA) and its incorporation into phospholipids (PL) and by a partial replacement of ARA with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (2), thus potentially modifying the levels of PL-derived metabolites involved in inflammation (3), immune response (4,5), energy homeostasis (6), and neural activity (7). However, the exact mechanisms whereby (n-3) LCPUFA exert these effects have not yet been clarified.

In obesity, visceral adipose tissue (VAT) accumulation and its metabolic consequences might be due in part to dysregulation of the endocannabinoid system and of cannabinoid receptor of type 1 (CB₁) activity in the various depots of white adipose tissue (8,9). Endocannabinoids are ARA-containing, PL-derived mediators capable of binding to CB₁ and CB₂. The two best studied endocannabinoids are *N*-arachidonylethanolamine (AEA; anandamide) (10) and 2-arachidonoylglycerol (2-AG) (11,12). The main enzymes for degradation of AEA and 2-AG are fatty

¹ Supported by Aker Biomarine ASA, Oslo, Norway.

² Author disclosures: M. Griinari is a consultant for Aker Biomarine ASA, Oslo, Norway. I. Bruheim is employed by Aker Biomarine ASA, Oslo, Norway. B. Batetta, G. Carta, E. Murru, A. Ligresti, L. Cordeddu, E. Giordano, F. Sanna, T. Bisogno, S. Uda, M. Collu, V. Di Marzo, and S. Banni, no conflicts of interest.

³ Supplemental Tables 1–5 and Supplemental Figure 1 are available with the online posting of this paper at jn.nutrition.org.

¹¹ Abbreviations used: AEA, *N*-arachidonylethanolamine (anandamide); 2-AG, 2-arachidonoylglycerol; ALA, α -linolenic acid; ARA, arachidonic acid; C, control diet; CB₁, cannabinoid receptor of type 1; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAAH, fatty acid amide hydrolase; FO, fish oil; IL, interleukin; KO, krill oil; LA, linoleic acid; (n-3) LCPUFA, long-chain (n-3) PUFA; LPS, lipopolysaccharide; MAGL, monoacylglycerol lipase; PL, phospholipid; SAT, subcutaneous adipose tissue; TAG, triacylglycerol; TGF β , tumor growth factor- β ; TNF α , tumor necrosis factor- α ; VAT, visceral adipose tissue.

* To whom correspondence should be addressed. E-mail: banni@unica.it or vdimarzo@icmib.na.cnr.it.

acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively [see (13) for review]. In mice with high-fat diet-induced obesity, the upregulation of CB₁ receptors and/or endocannabinoid concentrations might contribute to insulin resistance, dyslipoproteinemia, and nonalcoholic fatty liver diseases (8,9,14). Endocannabinoid concentration dysregulation is due in part to alterations in the activity/expression of enzymes regulating endocannabinoid biosynthesis and degradation. Dietary (n-3) LCPUFA decrease endocannabinoid concentrations (15) and, in isolated adipocytes, they affect the amounts of ARA esterified on the *sn-1* and *sn-2*- position of PL, thus presumably reducing the amounts of endocannabinoid biosynthetic precursors (16). It was suggested that a higher or lower abundance of (n-3) and (n-6) LCPUFA precursors in high-fat diets affects AEA and 2-AG levels in a time- and tissue-specific manner (17).

The beneficial effects of (n-3) LCPUFA on metabolic disorders associated with obesity might depend on the form in which they are administered [i.e. fish oil (FO) vs. pure preparations of ethyl esters (18)]. Furthermore, some of their metabolic effects in animal models can be observed only when high doses are used (19). FO consists almost exclusively of triglyceride-bound (n-3) LCPUFA, which is different from the natural form of (n-3) LCPUFA in fish products. In salmon meat, e.g., (n-3) LCPUFA are bound to PL and triacylglycerols (TAG) in a 40:60 ratio (20). Krill oil (KO) is a novel source of (n-3) LCPUFA extracted from an Antarctic crustacean, *Euphausia superba*, with a high content of PL-, mainly phosphatidylcholine-, bound (n-3) LCPUFA. The proportion of PL in the total lipids of krill has been reported to vary between 30 and 60%, depending on krill species, age, season, and harvest time (21). Human studies have evidenced remarkable effects of dietary KO supplementation on premenstrual syndrome and dysmenorrhea in healthy women, as well as on dyslipidemia and inflammation in patients with arthritis (22–24).

In this study, our aim was to investigate the effects of relatively low doses of dietary (n-3) LCPUFA, administered as FO or KO, on lipid metabolism, ectopic fat deposition, and susceptibility to inflammation in Zucker *fafa* rats, which exhibit an inactivating mutation in the leptin receptor (25), inducing hyperphagia and resulting in visceral obesity and ectopic lipid accumulation. Fatty acid profiles and endocannabinoid concentrations were determined in different tissues to examine the possible impact of (n-3) LCPUFA on the dysregulated endocannabinoid system of Zucker rats, which were fed a diet containing 0.8% of energy (n-3) LCPUFA, a level lower than that typically used in rodent studies (19), to allow a more meaningful comparison with human studies.

Materials and Methods

Animals and diets. Eighteen male Zucker rats (Harlan) 4 wk of age, with an initial weight of 250 ± 30 g, were divided into 3 groups and fed for 4 wk a control diet (C) or diets supplemented with either FO (GC Rieber Oils) or KO (Superba, Aker BioMarine). The diets were based on the AIN-93G formulation, with substitution of soybean oil with a blend of oils (rapeseed oil, sunflower oil, coconut oil, and linseed oil). This allowed the 3 diets to be similar for total fatty acids and for oleic, linoleic (LA), and α -linolenic (ALA) acids. FO and KO diets were further balanced for EPA and DHA content (Table 1). The 3 diets were prepared by Altromin GmbH & Co. KG and stored in vacuum bags to reduce (n-3) LCPUFA oxidation. The amount of 0.5 g EPA + DHA/100 g of diet, equivalent to 0.8% of energy in the rat diet, was chosen to provide a level of (n-3) LCPUFA intake achievable in humans and corresponds to 1.8 g/d in an 8.4-MJ/d diet in humans. All experiments were performed according to the guidelines and protocols approved by the European

TABLE 1 Dietary fatty acid composition

Fatty acid	C	FO	KO
	<i>g/100 g diet</i>		
18:3(n-3)	0.26	0.26	0.29
18:4(n-3)	0.00	0.05	0.08
20:5(n-3)	0.00	0.29	0.30
22:6(n-3)	0.00	0.18	0.14
Total (n-3)	0.26	0.78	0.81
18:2(n-6)	2.07	2.23	2.22
20:4(n-6)	0.00	0.01	0.01
Total (n-6)	2.07	2.24	2.23
(n-6):(n-3)	7.85	2.86	2.76
18:1(n-9)	2.34	2.72	2.25
Total UFA	4.68	5.74	5.29
12:0	1.09	0.04	0.02
14:0	0.39	0.20	0.38
16:0	0.58	0.78	1.09
18:0	0.22	0.21	0.19
20:0	0.03	0.04	0.03
Total SFA	2.32	1.26	1.71
UFA:SFA	2.02	4.54	3.10

Union (EU Council 86/609; D.L. 27.01.1992, no. 116) and by the Animal Research Ethics Committee of the University of Cagliari, Italy.

Peritoneal macrophage and tissue collections. Rats were food deprived overnight and macrophages were isolated from their peritoneal cavity. The rats were deeply anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally; Sigma-Aldrich) before being killed. Cells were obtained by peritoneal lavage with 60 mL of cold PBS containing 5 mmol/L EDTA. The rats were subjected to a vigorous massage of the peritoneal area prior to collection of cells. Immediately after death, blood was drawn from aorta, and liver, brain, heart, subcutaneous adipose tissues (SAT), and VAT were removed and stored at -80°C .

Macrophage culture. Cells were centrifuged at $300 \times g$; 10 min and the cell pellet was washed twice with cold sterile PBS and suspended in DMEM, 10% heat-inactivated fetal calf serum, penicillin (100 kU/L), and streptomycin (100 mg/L). The cell number was determined with a Coulter Counter corrected for viability determined by trypan blue dye exclusion. The cells were then seeded at the density of 4.0×10^5 cells cm^{-2} and incubated for 2 h at 37°C and 5% CO_2 atm. After removing nonadherent cells, macrophages were cultured in DMEM with 10% fetal calf serum in the presence of lipopolysaccharide (LPS) from *Escherichia coli* 026:B6 (Sigma Aldrich) (100 $\mu\text{g/L}$) for 24 h. The incubation time was chosen based on preliminary experiments that showed no substantial difference in cytokine secretion between 24 and 48 h. At the time indicated, supernatants and cells were separated and stored at -80°C until ELISA and fatty acid analysis were performed. Sandwich ELISA tests were carried out all at the same time to avoid variations during the assay conditions and performed as described by the manufacturer.

Cytokine assay. Serum C-reactive protein (Chemicon International), tumor necrosis factor- α (TNF α), interleukin (IL)-10, and tumor growth factor- β (TGF β) (Biosource) were determined by a sandwich ELISA. Moreover, to evaluate macrophage susceptibility to inflammatory ligands, the secretion of TNF α , IL-6 (Bender MedSystem), IL-1 β , IL-10, and TGF β were assessed in culture supernatants from peritoneal macrophages activated with LPS. Media were assayed at $800 \times g$; 10 min to remove debris. Supernatants were frozen at -80°C until assayed with a sandwich ELISA (Biosource). All procedures were performed as previously reported (26) according to the manufacturer's instructions.

Measurement of fatty acid composition of tissue TAG and PL. Total lipids were extracted from tissues using chloroform:methanol 2:1

(v:v) (27). Separation of total lipids into TAG and PL was performed as previously reported (28). Aliquots were mildly saponified as previously described (28) to obtain FFA for HPLC analysis. Separation of fatty acids was conducted with a Hewlett-Packard 1100 HPLC system (Hewlett-Packard) equipped with a diode array detector as previously reported (29). Because SFA are transparent to UV, they were measured, after methylation (30), by means of a gas chromatograph (Agilent, Model 6890) equipped with split ratio of 20:1 injection port, a flame ionization detector, an autosampler (Agilent, Model 7673), a 100-m HP-88 fused capillary column (Agilent), and an Agilent ChemStation software system. The injector and detector temperatures were set at 250°C and 280°C, respectively. H₂ served as carrier gas (1 mL/min) and the flame ionization detector gases were H₂ (30 mL/min), N₂ (30 mL/min), and purified air (300 mL/min). The temperature program was as follows: initial temperature was 120°C, programmed at 10°C/min to 210°C and 5°C/min to 230°C, then programmed at 25°C/min to 250°C and held for 2 min.

Endocannabinoid analyses. AEA and 2-AG were measured as previously described (31).

Enzyme activity assays. MAGL and FAAH activities were determined in the heart, liver, VAT, and SAT from C and FO- and KO-supplemented rats. In particular, 2-AG hydrolysis (mostly by MAGL) was measured by incubating the 10,000 × g cytosolic fraction of tissues (100 μg per sample) in Tris-HCl 50 mmol/L, at pH 7.0 at 37°C for 20 min, with synthetic 2-arachidonoyl-[³H]-glycerol (40 Ci/mmol, ARC) properly diluted with 2-AG (Cayman Chemicals). After incubation, the amount of [³H]-glycerol produced was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of CHCl₃:MeOH 1:1 (v:v). AEA hydrolysis (by FAAH) was measured by incubating the 10,000 × g membrane fraction of tissues (70 μg per sample) in Tris-HCl 50 mmol/L, at pH 9.0–10.00 at 37°C for 30 min, with synthetic N-arachidonoyl-[¹⁴C]-ethanolamine (110 mCi/mmol, ARC) properly diluted with AEA (Tocris Bioscience). After incubation, the amount of [¹⁴C]-ethanolamine produced was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of CHCl₃:MeOH 1:1 (by vol.). Values in the text are means ± SD.

Statistical analysis. One-way ANOVA and the Bonferroni test for post hoc analyses were applied to evaluate statistical differences among groups. Where variances were unequal, we used Kruskal-Wallis non-parametric 1-way ANOVA.

Results

Obesity-related metabolic and inflammatory markers. Growth and food intake did not differ among the 3 groups and none of the rats exhibited adverse effects (data not shown). At the end of the 4-wk treatment, the body weight of the rats was 400 ± 35 g.

In KO-supplemented rats, and to a lesser extent in the FO group, the liver TAG concentration was significantly lower than in C (Fig. 1A). The heart TAG concentration was significantly lower than C only in KO-supplemented rats (Fig. 1B).

Rats supplemented with FO or KO had 75% lower plasma LDL cholesterol concentrations than C, whereas HDL cholesterol did not differ among the groups (Supplemental Fig. 1A). Conversely, triglyceridemia was ~30% higher than C in both (n-3) LCPUFA-supplemented groups (Supplemental Fig. 1B).

Plasma proinflammatory (TNFα, IL-6, IL-1β) and anti-inflammatory cytokines (IL-10 and TGFβ) and C-reactive protein did not differ among the experimental groups (Supplemental Table 1).

In macrophages incubated for 24 h in the presence of LPS, TNFα secretion was significantly lower in FO and KO rats compared with C (Table 2). Plasma IL-1β, IL-6, and IL-10

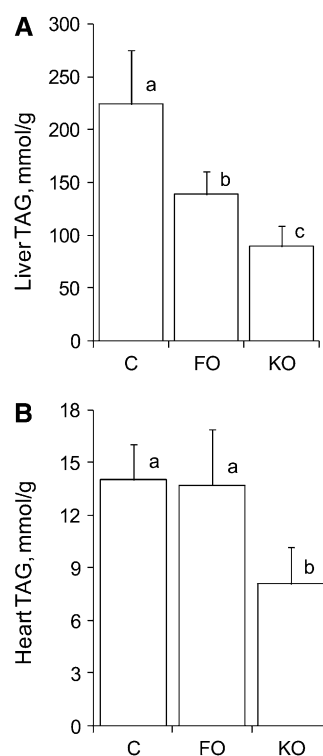


FIGURE 1 Liver (A) and heart (B) TAG concentrations of Obese Zucker rats fed C, FO, or KO diets for 4 wk. Values are means ± SD, $n = 6$. Means without a common letter differ, $P < 0.05$.

concentrations did not differ among dietary groups following LPS stimulation (data not shown).

Tissue endocannabinoid concentrations. The VAT AEA concentration was lower in the FO and KO groups than in C (Fig. 2A), whereas 2-AG was significantly lower than C only in the KO-supplemented rats (Fig. 2B). Endocannabinoid concentrations in SAT did not differ among the 3 groups (data not shown).

Liver and heart endocannabinoids were similarly affected in the KO-supplemented rats (Fig. 3). AEA concentrations were ~25% of, and 2-AG concentrations ~200% of, those of C in both tissues. In FO-fed rats, liver but not heart AEA concentrations were less than in C.

Activities of enzymes involved in endocannabinoid degradation. Changes in FAAH and MAGL activity have been observed in adipose tissues and liver of obese individuals and rodents (9,14,32–35). In this study, heart, liver, VAT, and SAT FAAH activities did not differ among the experimental groups (Table 3). Conversely, MAGL activity was significantly lower in

TABLE 2 TNFα release in LPS-treated peritoneal macrophages from Obese Zucker rats fed C, FO, or KO diets for 4 wk¹

	C	FO	KO
	<i>mg/L plasma</i>		
Basal	0.6 ± 0.1	1.9 ± 0.7	0.3 ± 0.1
LPS	68.4 ± 9.4 ^a	37.2 ± 9.7 ^b	42.2 ± 3.2 ^b

¹ Values are means ± SD, $n = 6$. Means in a row with superscripts without a common letter differ, $P < 0.05$.

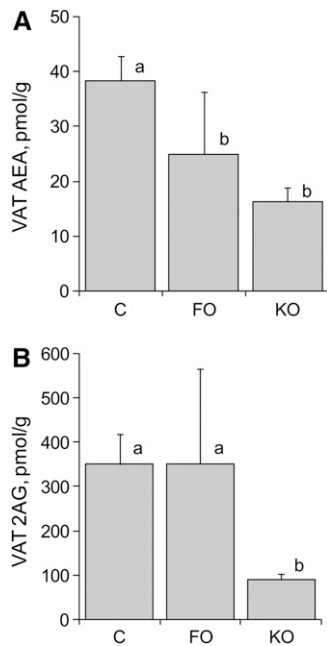


FIGURE 2 VAT AEA (A) and 2-AG (B) concentrations in Obese Zucker rats fed C, FO, or KO diets for 4 wk. Values are means \pm SD, $n = 6$. Means without a common letter differ, $P < 0.05$.

the VAT of the FO and KO groups, and in the heart tissue of the KO group, compared with C (Table 3). Liver MAGL activity tended to be lower in both groups compared with C ($P = 0.1$).

Plasma and tissue fatty acid concentrations. Plasma EPA and DHA concentrations were higher and that of ARA was lower in the FO and KO groups compared with C (Supplemental Table 2). Interestingly, the levels of ALA and LA (only in the KO group) were higher than in C despite the similar levels of these fatty acids in the diets (Table 1). Peritoneal macrophages from FO- and KO-fed rats had significantly higher EPA and DHA and lower ARA concentrations than those from the C group (Supplemental Table 3).

Dietary (n-3) LCPUFA influenced the TAG fraction of VAT and SAT with greater incorporation of EPA, DPA, and DHA in both experimental groups compared with C (Supplemental Table 4). The EPA and DHA levels in SAT were higher in FO- than in KO-supplemented rats. ARA was significantly lower than in C only in SAT of KO-supplemented rats; there was no difference among the groups in VAT. As in plasma, ALA was higher than in C in both VAT and SAT of FO and KO groups. On the contrary, LA was significantly higher only in VAT of (n-3) LCPUFA-supplemented rats compared with C. In all 3 dietary groups, remarkable differences in fatty acid profiles of the PL fraction were observed, also between VAT and SAT (Supplemental Table 4). PL ARA levels of the VAT, but not SAT, were significantly less in FO- and KO-supplemented rats than in C. Levels of EPA, DPA, and DHA were higher in the (n-3) LCPUFA-supplemented rats compared with C in VAT PL, while only EPA changed significantly in SAT PL.

In liver TAG, EPA, DPA, and DHA levels were significantly elevated in the FO and KO groups compared with C, whereas ARA levels did not differ among the groups. In liver PL, EPA and DPA concentrations were greater in both (n-3) LCPUFA-supplemented groups than in C, whereas DHA was significantly higher than C only in the KO group. LA was significantly greater in the (n-3) LCPUFA-supplemented groups than in C, whereas

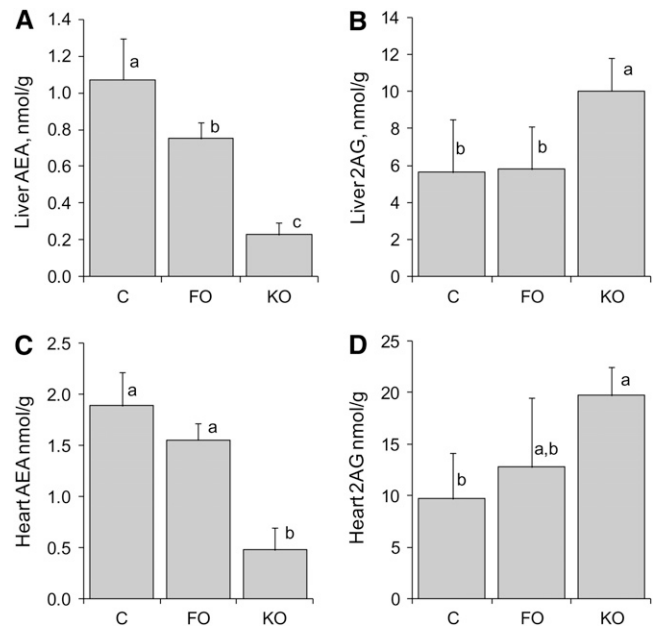


FIGURE 3 Liver (upper panels) and heart (lower panels) AEA (A,C) and 2-AG (B,D) concentrations in rats fed C, FO, or KO diets for 4 wk. Values are means \pm SD, $n = 6$. Means without a common letter differ, $P < 0.05$.

the ARA level did not differ (Supplemental Table 5). The heart TAG fatty acid profile had higher levels of EPA, DPA, and DHA and lower levels of ARA in the FO and KO groups compared with C. LA and ALA concentrations were lower than in C only in the KO-supplemented rats. In the PL fraction of the (n-3) LCPUFA-supplemented groups, concentrations of EPA, DPA, and DHA were also greater than C, with higher levels in the KO group, whereas ARA was significantly less than in C only in the FO group (Supplemental Table 5).

Discussion

In this study, we observed that relatively low doses of (n-3) LCPUFA administered to obese Zucker rats for 4 wk considerably improved both ectopic fat deposition and susceptibility to inflammation. The KO diet generally reduced both liver and heart TAG concentrations more than the FO diet, whereas both (n-3) LCPUFA diets reduced to the same extent the plasma LDL-cholesterol concentration and LPS-induced TNF α release from peritoneal macrophages. Furthermore, dietary (n-3) LCPUFA modulated endocannabinoid concentrations in the VAT, liver, and heart. This latter event, in turn, might have affected lipid metabolism in these tissues via CB₁ and/or CB₂ receptors. In fact, CB₁ receptors are involved in the control of adipocyte differentiation, proliferation and size, lipogenesis, insulin sensitivity and release, hepatic fatty acid synthesis, and oxidation [reviewed in (8)].

AEA and 2-AG concentrations in VAT, but not SAT, were lower in KO-, and to a lesser extent in FO-, fed rats. This may have reduced CB₁ receptor overactivity, which is associated with increased intra-abdominal fat in rodents and obese humans (9,32,36,37). We previously observed that lower endocannabinoid concentrations in SAT compared with VAT caused by a 14-wk high-fat diet was accompanied by VAT accumulation (9). Therefore, reduction of endocannabinoid tone in the VAT compared with SAT of obese animals by (n-3) LCPUFA might

TABLE 3 FAAH and MAGL activities in heart, liver, VAT, and SAT from Obese Zucker rats fed C, FO, or KO diets for 4 wk¹

Tissue and diet group	FAAH	MAGL
	<i>pmol/(min · mg tissue)</i>	
Heart		
C	4.8 ± 1.6	2442.7 ± 62.2 ^a
FO	4.1 ± 1.3	2169.8 ± 10.1 ^{a,b}
KO	4.0 ± 1.4	2072.6 ± 168.4 ^b
Liver		
C	208.0 ± 4.5	2075.3 ± 6.6
FO	207.6 ± 4.9	1958.6 ± 106.7
KO	208.9 ± 3.9	1938.7 ± 110.4
VAT		
C	9.7 ± 3.2	1627.5 ± 151.8 ^a
FO	7.0 ± 2.7	1257.8 ± 49.0 ^b
KO	6.2 ± 0.8	1217.4 ± 18.8 ^b
SAT		
C	29.6 ± 1.1	1279.2 ± 8.3
FO	14.1 ± 3.8	1316.3 ± 24.7
KO	50.4 ± 25.3	1277.8 ± 45.1

¹ Values are means ± SD, *n* = 4. Within a tissue, means in a column with superscripts without a common letter differ, *P* < 0.05.

lead, in the long term, to decreased fat accumulation in this adipose depot (8,37,38). We did not measure here the effect of the diets on the relative amounts of VAT and SAT, because we deemed it unlikely that a 4-wk administration of (n-3) LCPUFA would be sufficient to reduce the amount of accumulated fat in Zucker rats, particularly as we did not observe any effect on body weight. However, we did find an association between the lowering of the VAT endocannabinoid concentration and that of ectopic fat, with KO being significantly more efficacious than FO in both cases, leading to a dramatic 50% reduction of the TAG concentration in the liver. This might suggest that endocannabinoid overactivity in the VAT contributes to ectopic fat formation. Likewise, endocannabinoid overactivity in hepatocytes is associated with the development of alcoholic and nonalcoholic fatty liver disease in mice, which are fully prevented in liver-specific CB₁ null mice (39,40). In the present study, AEA, but not 2-AG, levels were reduced by dietary (n-3) LCPUFA, in agreement with the previous findings that AEA is the endocannabinoid responsible for nonalcoholic hepatic TAG deposition in rodents. Through reduction of hepatic AEA, (n-3) LCPUFA might downregulate important genes in lipogenesis (14,40). However, additional factors are likely to contribute to lower liver TAG content, such as direct activation of PPAR by (n-3) LCPUFA (41).

Although possible in theory, it is unlikely that the reduction of endocannabinoid concentrations by dietary (n-3) LCPUFA observed here is a consequence, rather than one of the causes, of the metabolic effects exerted by FO and KO. Studies in obese patients showed that only very rigorous interventions, resulting in considerable loss of body weight and intra-abdominal fat, lead to decreased peripheral endocannabinoid concentrations (42,43). Even with gastric surgery in obese rats, such changes occur only 4 wk after the intervention and 2 wk after weight loss (44), whereas in our case, dietary (n-3) LCPUFA caused decreased endocannabinoid concentrations in the absence of weight loss. Studies in Zucker rats with FO or KO in the presence of CB₁ receptor antagonists, or the use of CB₁ receptor “null” mice with high fat diet-induced obesity, might help us in

understanding whether or not the FO- and KO-induced decrease of endocannabinoid concentrations underlie the metabolic effects of the 2 diets. However, such experiments might not be easily performed. Chronic treatment of Zucker rats with the CB₁ antagonist rimonabant reduces body weight and nearly restores the metabolic profile of these animals to that of lean controls (45), whereas CB₁ receptor null mice are completely resistant to high fat diet-induced obesity and its metabolic consequences (14). Thus, a submaximal dose of rimonabant that renders Zucker rats less responsive to FO and KO should be sought to support a role of endocannabinoids in the effects of these diets. Conversely, because systemic CB₁ agonist administration affects food intake acutely, but does not cause weight gain chronically (46) or affect metabolic parameters, possibly because it cannot mimic the tissue-selective overactivation of CB₁ receptors associated with obesity (8), and causes downregulation of CB₁ receptors, such approach would likely not result in the counteraction of FO- and KO-induced metabolic changes in Zucker rats.

Rats fed (n-3) LCPUFA diets had greater plasma TAG concentrations and lower plasma LDL-cholesterol concentrations, with no changes in HDL-cholesterol. The somewhat paradoxical finding of increased plasma TAG concentrations might result from the (n-3) LCPUFA-induced increase in the rate of VLDL release from the liver under the experimental conditions used in this study. At least 1 other report showed that a massive decrease of fat deposition in the liver results in increased plasma TAG concentrations in Zucker rats (47). This phenomenon is likely to be transient and to reflect the specific metabolic state of the rats, characterized by a concentration of TAG in the liver ~5-fold higher than in lean rats, as compared with the data previously reported in the literature (48). Another possible explanation might be the relatively low dietary level of (n-3) LCPUFA used here, because the hypotriglyceridemic activity of (n-3) LCPUFA is exclusively observed with high doses in both experimental animals and humans (49,50). Future studies will be needed to investigate these hypotheses.

We investigated here the possible mechanism(s) by which dietary (n-3) LCPUFA affect endocannabinoid metabolism in the 4 tissues analyzed. It was previously shown that AEA levels in the white adipose tissue, liver, and heart during obesity can be determined by changes in the expression and activity of its degrading enzyme FAAH [reviewed in (51)], with elevations of AEA tissue concentrations usually being accompanied by reductions in FAAH activity (9,14). This is the case to some extent also for 2-AG and MAGL and for human obesity (32–35). Therefore, we analyzed here the effects of FO and KO on MAGL and FAAH. However, FAAH activity did not differ, whereas MAGL activity was slightly lower in the VAT of the FO and KO groups and in the heart of the KO group. Thus, the diet-induced decreases in AEA levels were not due to an elevation of FAAH activity. The lower activity of MAGL might partially explain the higher 2-AG levels in the heart. High intersample variability in the measured enzymatic activities might have prevented us from detecting significant differences, particularly in the SAT, where FO tended to reduce, and KO to enhance, FAAH activity. We could analyze only 4 of the 6 rats for these experiments because of the limited amount of tissue left after lipid analyses. However, even assuming that the differences in FAAH activity had been significant, the predicted effects on AEA concentrations (increased by the FO diet and decreased by the KO diet) are the opposite of what we observed in SAT. These data strengthen our hypothesis that changes in FAAH activity are not responsible for changes in AEA levels in white adipose tissue.

It was proposed that availability of ARA-containing PL, the biosynthetic precursors of endocannabinoids, can determine endocannabinoid concentrations in some tissues (9,52) and in isolated adipocytes (16). Because dietary (n-3) LCPUFA act in part by reducing the amount of ARA esterified to PL, we assessed the effects of the 2 diets on esterified ARA, EPA, and DHA levels in the 4 tissues analyzed here. Our data indicate that the diet-induced reduction of endocannabinoid concentrations in VAT is associated with lower ARA levels in the PL, but not in the TAG, fraction of the tissue. In the SAT, endocannabinoid concentrations were not altered by (n-3) LCPUFA supplementation and, consistent with a role of the tissue precursor supply in endocannabinoid biosynthesis, PL ARA content was also unaltered. Whereas in the heart and liver the decrease in AEA levels correlated with PL ARA content only in the FO group, the increase in the 2-AG levels was independent of any changes in PL or TAG ARA concentration. Therefore, whereas in the VAT, (n-3) LCPUFA might decrease endocannabinoid concentrations by decreasing the amount of ARA esterified into PL, in the liver only FO might reduce AEA levels via this mechanism. In the heart, KO might enhance 2-AG levels by reducing its degradation via MAGL. It is not clear how EPA and DHA incorporation in PL alters the biosynthetic precursors of endocannabinoids. However, it is worth noting that in all tissues exhibiting changes in endocannabinoid concentrations, EPA and DHA concentrations in the PL fraction were increased and the increase was greater in the KO- compared with FO-supplemented rats.

The effects observed here with dietary (n-3) LCPUFA were obtained with a relatively low dose of (n-3) LCPUFA, corresponding to ~2 g/d of EPA + DHA intake in humans. Several reports in the literature show that some dietary components, including (n-3) fatty acids, PL, and choline (53), reduce TAG deposition in the liver. A study involving dietary supplementation of obese rats with egg yolk phosphatidylcholine and (n-3) phosphatidylcholine (54) is also relevant to the present observations on the effects of KO. Shiourchi et al. (54) demonstrated that fish roe-derived (n-3) phosphatidylcholine reduced liver steatosis in obese OLETF rats compared with an equal amount of egg yolk-derived phosphatidylcholine.

In conclusion, we have reported that diets rich in (n-3) LCPUFA, and a KO-based diet in particular, exert beneficial effects on several metabolic dysfunctions in Zucker rats, which was associated with lower endocannabinoid concentrations in several peripheral tissues. Interestingly, some of the (n-3) LCPUFA effects, i.e. reduction of fatty liver and inflammation, were also reported in studies with Zucker rats after antagonism of endocannabinoid action at CB₁ receptors with rimonabant (45,55). Whereas CB₁ receptor antagonists dampen the over-activated endocannabinoid system by blocking the receptor, dietary (n-3) LCPUFA, and KO in particular, may reduce the activity of the endocannabinoid system by decreasing the substrate availability for endocannabinoid biosynthesis. Such a nutritional approach, if influencing the endocannabinoid system only peripherally, might avoid the adverse psychiatric effects associated with the use of CB₁ antagonists, thus potentially providing a safer alternative for “endocannabinoid reequilibration” in obese individuals.

Literature Cited

1. Carpentier YA, Portois L, Malaisse WJ. n-3 Fatty acids and the metabolic syndrome. *Am J Clin Nutr.* 2006;83:S1499–504.
2. Schmitz G, Ecker J. The opposing effects of n-3 and n-6 fatty acids. *Prog Lipid Res.* 2008;47:147–55.

3. Calder PC. n-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am J Clin Nutr.* 2006;83:S1505–19.
4. Calder PC. Immunomodulation by omega-3 fatty acids. *Prostaglandins Leukot Essent Fatty Acids.* 2007;77:327–35.
5. Fritsche K. Fatty acids as modulators of the immune response. *Annu Rev Nutr.* 2006;26:45–73.
6. Kabir M, Skurnik G, Naour N, Pechtner V, Meugnier E, Rome S, Quignard-Boulange A, Vidal H, Slama G, et al. Treatment for 2 mo with n 3 polyunsaturated fatty acids reduces adiposity and some atherogenic factors but does not improve insulin sensitivity in women with type 2 diabetes: a randomized controlled study. *Am J Clin Nutr.* 2007;86:1670–9.
7. Vancassel S, Blondeau C, Lallemand S, Cador M, Linard A, Lavielle M, Dellu-Hagedorn F. Hyperactivity in the rat is associated with spontaneous low level of n-3 polyunsaturated fatty acids in the frontal cortex. *Behav Brain Res.* 2007;180:119–26.
8. Di Marzo V. The endocannabinoid system in obesity and type 2 diabetes. *Diabetologia.* 2008;51:1356–67.
9. Starowicz KM, Cristino L, Matias I, Capasso R, Racioppi A, Izzo AA, Di Marzo V. Endocannabinoid dysregulation in the pancreas and adipose tissue of mice fed with a high-fat diet. *Obesity (Silver Spring).* 2008;16:553–65.
10. Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science.* 1992;258:1946–9.
11. Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, et al. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol.* 1995;50:83–90.
12. Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K. 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun.* 1995;215:89–97.
13. Di Marzo V, Petrosino S. Endocannabinoids and the regulation of their levels in health and disease. *Curr Opin Lipidol.* 2007;18:129–40.
14. Osei-Hyiaman D, DePetrillo M, Pacher P, Liu J, Radaeva S, Batkai S, Harvey-White J, Mackie K, Offertaler L, et al. Endocannabinoid activation at hepatic CB₁ receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *J Clin Invest.* 2005;115:1298–305.
15. Hansen HS, Artmann A. Endocannabinoids and nutrition. *J Neuroendocrinol.* 2008;20 Suppl 1:94–9.
16. Matias I, Carta G, Murru E, Petrosino S, Banni S, Di Marzo V. Effect of polyunsaturated fatty acids on endocannabinoid and N-acyl-ethanolamine levels in mouse adipocytes. *Biochim Biophys Acta.* 2008;1781:52–60.
17. Matias I, Petrosino S, Racioppi A, Capasso R, Izzo AA, Di Marzo V. Dysregulation of peripheral endocannabinoid levels in hyperglycemia and obesity: effect of high fat diets. *Mol Cell Endocrinol.* 2008;286: S66–78.
18. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. *Lancet.* 1999;354:447–55.
19. Fritsche K. Important differences exist in the dose-response relationship between diet and immune cell fatty acids in humans and rodents. *Lipids.* 2007;42:961–79.
20. Polvi SM, Ackman RG. Atlantic salmon (*Salmo salar*) muscle lipids and their response to alternative dietary fatty acid sources. *J Agric Food Chem.* 1992;40:1001–7.
21. Tou JC, Jaczynski J, Chen YC. Krill for human consumption: nutritional value and potential health benefits. *Nutr Rev.* 2007;65:63–77.
22. Bunea R, El Farrak H, Deutsch L. Evaluation of the effects of Neptune Krill Oil on the clinical course of hyperlipidemia. *Altern Med Rev.* 2004;9:420–8.
23. Deutsch L. Evaluation of the effect of Neptune Krill Oil on chronic inflammation and arthritic symptoms. *J Am Coll Nutr.* 2007;26:39–48.
24. Sampalis F, Bunea R, Pelland MF, Kowalski O, Duguet N, Dupuis S. Evaluation of the effects of Neptune Krill Oil on the management of premenstrual syndrome and dysmenorrhea. *Altern Med Rev.* 2003;8:171–9.
25. Kasiske BL, O'Donnell MP, Keane WF. The Zucker rat model of obesity, insulin resistance, hyperlipidemia, and renal injury. *Hypertension.* 1992;19:1110–5.

26. Sanna F, Bonatesta RR, Frongia B, Uda S, Banni S, Melis MP, Collu M, Madeddu C, Serpe R, et al. Production of inflammatory molecules in peripheral blood mononuclear cells from severely glucose-6-phosphate dehydrogenase-deficient subjects. *J Vasc Res.* 2007;44:253–63.
27. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem.* 1957;226:497–509.
28. Banni S, Carta G, Contini MS, Angioni E, Deiana M, Dessi MA, Melis MP, Corongiu FP. Characterization of conjugated diene fatty acids in milk, dairy products, and lamb tissues. *J Nutr Biochem.* 1996;7:150–5.
29. Melis MP, Angioni E, Carta G, Murrù E, Scanu P, Spada S, Banni S. Characterization of conjugated linoleic acid and its metabolites by RP-HPLC with diode array detector. *Eur J Lipid Sci Technol.* 2001;103:617–21.
30. Ip C, Banni S, Angioni E, Carta G, McGinley J, Thompson HJ, Barbano D, Bauman D. Conjugated linoleic acid-enriched butter fat alters mammary gland morphogenesis and reduces cancer risk in rats. *J Nutr.* 1999;129:2135–42.
31. Di Marzo V, Goparaju SK, Wang L, Liu J, Batkai S, Jarai Z, Fezza F, Miura GI, Palmiter RD, et al. Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature.* 2001;410:822–5.
32. Blüher M, Engeli S, Kloting N, Berndt J, Fasshauer M, Batkai S, Pacher P, Schon MR, Jordan J, et al. Dysregulation of the peripheral and adipose tissue endocannabinoid system in human abdominal obesity. *Diabetes.* 2006;55:3053–60.
33. Lofgren P, Sjölin E, Wahlen K, Hoffstedt J. Human adipose tissue cannabinoid receptor 1 gene expression is not related to fat cell function or adiponectin level. *J Clin Endocrinol Metab.* 2007;92:1555–9.
34. Murdolo G, Kempf K, Hammarstedt A, Herder C, Smith U, Jansson PA. Insulin differentially modulates the peripheral endocannabinoid system in human subcutaneous abdominal adipose tissue from lean and obese individuals. *J Endocrinol Invest.* 2007;30:RC17–21.
35. Pagano C, Pilon C, Calcagno A, Urbanet R, Rossato M, Milan G, Bianchi K, Rizzuto R, Bernante P, et al. The endogenous cannabinoid system stimulates glucose uptake in human fat cells via phosphatidylinositol 3-kinase and calcium-dependent mechanisms. *J Clin Endocrinol Metab.* 2007;92:4810–9.
36. Cote M, Matias I, Lemieux I, Petrosino S, Almeras N, Despres JP, Di Marzo V. Circulating endocannabinoid levels, abdominal adiposity and related cardiometabolic risk factors in obese men. *Int J Obes (Lond).* 2007;31:692–9.
37. Matias I, Gonthier MP, Orlando P, Martiadis V, De Petrocellis L, Cervino C, Petrosino S, Hoareau L, Festy F, et al. Regulation, function, and dysregulation of endocannabinoids in models of adipose and beta-pancreatic cells and in obesity and hyperglycemia. *J Clin Endocrinol Metab.* 2006;91:3171–80.
38. Bellocchio L, Cervino C, Vicennati V, Pasquali R, Pagotto U. Cannabinoid type 1 receptor: another arrow in the adipocytes' bow. *J Neuroendocrinol.* 2008;20 Suppl 1:130–8.
39. Jeong WI, Osei-Hyiaman D, Park O, Liu J, Batkai S, Mukhopadhyay P, Horiguchi N, Harvey-White J, Marsicano G, et al. Paracrine activation of hepatic CB1 receptors by stellate cell-derived endocannabinoids mediates alcoholic fatty liver. *Cell Metab.* 2008;7:227–35.
40. Osei-Hyiaman D, Liu J, Zhou L, Godlewski G, Harvey-White J, Jeong WI, Batkai S, Marsicano G, Lutz B, et al. Hepatic CB(1) receptor is required for development of diet-induced steatosis, dyslipidemia, and insulin and leptin resistance in mice. *J Clin Invest.* 2008;118:3160–9.
41. Rodriguez-Cruz M, Tovar AR, del Prado M, Torres N. [Molecular mechanisms of action and health benefits of polyunsaturated fatty acids]. *Rev Invest Clin.* 2005;57:457–72.
42. Di Marzo V, Cote M, Matias I, Lemieux I, Arsenault BJ, Cartier A, Piscitelli F, Petrosino S, Almeras N, et al. Changes in plasma endocannabinoid levels in viscerally obese men following a 1 year lifestyle modification programme and waist circumference reduction: associations with changes in metabolic risk factors. *Diabetologia.* 2009;52:213–7.
43. Engeli S, Böhnke J, Feldpausch M, Gorzelniak K, Janke J, Bätke S, Pacher P, Harvey-White J, Luft FC, et al. Activation of the peripheral endocannabinoid system in human obesity. *Diabetes.* 2005;54:2838–43.
44. Guisjarro A, Osei-Hyiaman D, Harvey-White J, Kunos G, Suzuki S, Nadochiy S, Brookes PS, Meguid MM. Sustained weight loss after Roux-en-Y gastric bypass is characterized by down regulation of endocannabinoids and mitochondrial function. *Ann Surg.* 2008;247:779–90.
45. Gary-Bobo M, Elachouri G, Gallas JF, Janiak P, Marini P, Ravinet-Trillou C, Chabbert M, Crucchioli N, Pfersdorff C, et al. Rimonabant reduces obesity-associated hepatic steatosis and features of metabolic syndrome in obese Zucker *fa/fa* rats. *Hepatology.* 2007;46:122–9.
46. Dalton VS, Wang H, Zavitsanou K. HU210-induced downregulation in cannabinoid CB1 receptor binding strongly correlates with body weight loss in the adult rat. *Neurochem Res.* 2009;34:1343–53.
47. Lanne B, Dahllöf B, Lindahl C, Ebefors K, Kanmert I, von Bahr H, Miliotis T, Nystrom AC, Arnerup G, et al. PPARalpha and PPARgamma regulation of liver and adipose proteins in obese and dyslipidemic rodents. *J Proteome Res.* 2006;5:1850–9.
48. Leonard BL, Watson RN, Loomes KM, Phillips AR, Cooper GJ. Insulin resistance in the Zucker diabetic fatty rat: a metabolic characterisation of obese and lean phenotypes. *Acta Diabetol.* 2005;42:162–70.
49. Harris WS, Bulchandani D. Why do omega-3 fatty acids lower serum triglycerides? *Curr Opin Lipidol.* 2006;17:387–93.
50. Jacobson TA. Role of n-3 fatty acids in the treatment of hypertriglyceridemia and cardiovascular disease. *Am J Clin Nutr.* 2008;87:S1981–90.
51. Fezza F, De Simone C, Amadio D, Maccarrone M. Fatty acid amide hydrolase: a gate-keeper of the endocannabinoid system. *Subcell Biochem.* 2008;49:101–32.
52. Petersen G, Sorensen C, Schmid PC, Artmann A, Tang-Christensen M, Hansen SH, Larsen PJ, Schmid HH, Hansen HS. Intestinal levels of anandamide and oleoylethanolamide in food-deprived rats are regulated through their precursors. *Biochim Biophys Acta.* 2006;1761:143–50.
53. Cohn JS, Wat E, Kamili A, Tandy S. Dietary phospholipids, hepatic lipid metabolism and cardiovascular disease. *Curr Opin Lipidol.* 2008;19:257–62.
54. Shirouchi B, Nagao K, Inoue N, Ohkubo T, Hibino H, Yanagita T. Effect of dietary omega 3 phosphatidylcholine on obesity-related disorders in obese Otsuka Long-Evans Tokushima fatty rats. *J Agric Food Chem.* 2007;55:7170–6.
55. Schafer A, Pfrang J, Neumüller J, Fiedler S, Ertl G, Bauersachs J. The cannabinoid receptor-1 antagonist rimonabant inhibits platelet activation and reduces pro-inflammatory chemokines and leukocytes in Zucker rats. *Br J Pharmacol.* 2008;154:1047–54.