Eicosapentaenoic Acid Reduces Adipocyte Hypertrophy and Inflammation in Diet-Induced Obese Mice in an Adiposity-Independent Manner

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Abstract

Background: Obesity is associated with an overexpansion of adipose tissue, along with increases in blood pressure, glycemia, inflammation, and thrombosis. Research to develop nutritional interventions to prevent or treat obesity and its associated diseases is greatly needed. Previously, we demonstrated the ability of eicosapentaenoic acid (EPA) to prevent high-fat (HF) diet–induced obesity, insulin resistance, and inflammation in mice.

Objective: The objective of the current study was to determine the mechanisms mediating the anti-inflammatory and antilipogenic actions of EPA.

Methods: In a previous study, male C57BL/6J mice were fed a low-fat diet (10% of energy from fat), an HF diet (45% of energy from fat), or an HF diet supplemented with EPA (45% of energy from fat; 36 g/kg EPA: HF+EPA) for 11 wk or an HF diet for 6 wk and then switched to the HF+EPA diet for 5 wk. In this study, we used histology/immunohistochemistry, gene expression, and metabolomic analyses of white adipose tissue from these mice. In addition, cultured mouse 3T3-L1 adipocytes were treated with 100 μM EPA for 48 h and then used for extracellular flux assays with untreated 3T3-L1 adipocytes used as a control.

Results: Compared with the HF diet, the HF+EPA diet significantly reduced body weight, adiposity, adipocyte size, and macrophage infiltration into adipose tissue. No significant differences in overall body weight or fat pad weights were observed between HF-fed mice vs. those fed the HF+EPA diet for a short time after first inducing obesity with the HF diet. Interestingly, both histology and immunohistochemistry results showed a significantly lower mean adipocyte size and macrophage infiltration in mice fed the HF diet and then switched to the HF+EPA diet vs. those fed HF diets only. This indicated that EPA was able to prevent as well as reverse HF-diet–induced adipocyte inflammation and hypertrophy and that some of the metabolic effects of EPA were independent of body weight or adiposity. In addition, adipose tissue metabolomic data and cultured adipocyte extracellular flux bioenergetic assays indicated that EPA also regulated mitochondrial function by increasing fatty acid oxidation and oxygen consumption, respectively.

Conclusion: With the use of mice and cultured adipocytes, we showed that EPA ameliorates HF-diet effects at least in part by increasing oxygen consumption and fatty acid oxidation and reducing adipocyte size, adipogenesis, and adipose tissue inflammation, independent of obesity.

Keywords: omega-3 fatty acids, eicosapentaenoic acid, adipocyte, inflammation, diet-induced obesity

Introduction

With current estimates claiming that >78 million adults are obese in the United States (1), and the recognition of obesity as a disease (2), the urgency to understand and address this epidemic issue is increasingly felt. Obesity has been causally linked to the metabolic syndrome (MetS)5, which is classified as having at

5 Abbreviations used: Actb, beta-actin; AMPK, AMP-activated protein kinase; A/R, antimycin A and rotenone mixture; ATM, adipose tissue macrophage; Bmp4, bone morphogenetic protein 4; Celpa, CCAAT/enhancer-binding protein α; FCCP, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone; GPCR, G-protein coupled receptor; GPR-120, G-protein coupled receptor 120; HF, high-fat; HF-EPA-P, high-fat diet supplemented with EPA for 11 wk; HF-EPA-R, high-fat diet for 6 wk and then switched to an EPA-supplemented HF diet for 5 wk; IR, insulin resistance; LF, low-fat; Lep, leptin; MCP-1, monocyte chemoattractant protein 1; M1 macrophage, classically activated macrophage; M2 macrophage, alternatively activated macrophage; MetS, metabolic syndrome; qRT-PCR, quantitative real time polymerase chain reaction; Sirt2, sirtuin type 2; Slc2a4, insulin-dependent glucose transporter type 4; Sreb1f, sterol regulatory element–binding transcription factor 1; T2D, type 2 diabetes.
least 3 of the following: hypertension, hyperglycemia, abdominal obesity, elevated plasma TGs, and reduced plasma HDL cholesterol (3). MetS is a proinflammatory and prothrombotic metabolic state that increases the risk of type 2 diabetes (T2D) and cardiovascular disease (4, 5). One of the underlying causes of MetS and obesity is the disruption in adipose tissue function (4).

Adipose tissue secretes several bioactive peptides, collectively known as adipokines, which modulate cardiovascular function (blood pressure, fibrinolysis, atherogenesis), insulin sensitivity and secretion, inflammation, and fat mass (6, 7). In lean adipose tissue, high concentrations of anti-inflammatory adipokines and hormones, such as adiponectin and IL-10, are secreted. By contrast, “obese” adipose tissue displays a more proinflammatory profile, with high secretions of adipokines such as IL-6, IL-1β, monocyte chemoattractant protein 1 (MCP-1), and TNF-α (6–8). This imbalance between secretions of pro- vs. anti-inflammatory adipokines is a hallmark of obesity-associated low-grade inflammation. The latter is typically characterized by macrophage infiltration into adipose tissue as obesity develops. Adipose tissue macrophages (ATMs) consist of classically activated (M1) and alternatively activated (M2) macrophages. M1 macrophages are stimulated by IFN-γ and LPS and produce proinflammatory cytokines, whereas M2 macrophages are stimulated by IL-4 and IL-13 and express anti-inflammatory factors (5, 7, 9).

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In addition MCP-1 is thought to play a major role in ATM infiltration (10). In lean adipose tissue, there is a high M2:M1 ratio of ATMs that is then shifted when the tissue becomes “obese” (high M1:M2), resulting in a reduction in anti-inflammatory adipokines and an increase in proinflammatory ones (5). Details of mechanisms involved in this switch and its involvement in obesity and insulin resistance (IR) have been extensively reviewed elsewhere (9, 11).

Given the side effects and limitations of pharmacologic therapies, it is critical to understand mechanisms by which nutritional interventions may reduce obesity and its comorbidities. Long-chain omega-3 (ω-3) PUFAs, namely DHA (22:6n-3) and EPA (20:5n-3) are known for their anti-inflammatory, cardioprotective, and TG-lowering effects (4, 12). Although animal studies with high-fat (HF) (13, 14) or high-sucrose (15) diet–induced IR indicated that EPA and DHA are able to prevent the development of IR, studies in individuals with T2D have shown conflicting evidence. Some studies showed metabolic improvements in patients with T2D (16–18), whereas others did not find significant effects of these FAs or fish oil (19–21). This may be in part due to the variability in stage of disease, different subject populations used, age, and durations of interventions.

In addition, very little is known regarding the specific mechanisms mediating the effects of ω-3 PUFAs on energy balance and cell signaling pathways in adipose tissue. A recent study that used human adipose tissue and mature adipocytes models showed that coincubation with DHA or EPA decreased LPS-stimulated cytokine secretions, with EPA having a greater anti-inflammatory effect than DHA (22). Another study indicated that when insulin-resistant humans were given fish oil (EPA+DHA), adipose macrophages decreased, capillaries increased, and MCP-1 expression was reduced. This was also supported in vitro with a coculture of macrophages and adipocytes that yielded similar results (23).

In an earlier study (8), we found that feeding HF diets supplemented with EPA reduced IR, systemic inflammation, and proinflammatory secretion from adipose tissue. Because obesity and adipose tissue expansion are associated with both adipocyte inflammation concurrent with fat cell hypertrophy, we hypothesized that EPA reduced adipocyte inflammation as well as fat cell size and further investigated the mechanisms mediating these effects.

Methods

Mice and diets. Tissues used for this study were collected from our previous study (8) and are described briefly here. Adipose tissue was collected from male C57BL/6 mice that had been fed a low-fat (LF) diet (10%, 20%, and 70% of energy from fat, protein, and carbohydrate, respectively), an HF diet (45%, 20%, and 35% of energy from fat, protein, and carbohydrate, respectively), or an HF diet supplemented with EPA (15% of fat from the HF diet replaced by EPA ethyl ester [HF-EPA-P]) for 11 wk. A fourth group was fed an HF diet for 6 wk and then switched to an EPA-supplemented diet (HF-EPA-R) for the remaining 5 wk. Diets were custom-made by Research Diets, and the detailed diet compositions are described in reference 8. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Tennessee, Knoxville, where the original animal study was conducted.

Adipose tissue histology. Sections from the gonadal adipose tissue were fixed in 10% buffered formalin, routinely processed, sectioned at 5 μm, and then stained with hemotoxylin and eosin. Adipose sections were examined by using a light microscope. Adipocyte area was measured by using ImageJ software (24).

Immunofluorescence. Gonadal adipose tissue were fixed in 10% buffered formalin, embedded in paraffin, and then sectioned at 5 μm. Sections were then affixed to slides, de-paraffinized, and stained with galactin-3 (marker for total macrophages; BioScience). Images were taken by using an EVOS FL Auto Imaging System (Life Technologies) and then quantified by using Fiji, a distribution of Image J (25).

Metabolomics. Equal amounts of visceral and subcutaneous adipose tissues were collected and then prepared by using a proprietary series of organic and aqueous extractions from Metabolon in order to remove the protein fractions while maximizing small molecule recovery. The resulting extract samples were then split into 2 equal parts and analyzed by using LC–tandem MS (LC-MS/MS) and GC-MS. Data normalization was accounted for by using day-to-day instrument drift was performed by registering mediators to equal 1 and normalizing each data point proportionally to correct for experimental and day to day variability, as described previously (26). For sample variability, a Bradford assay was used to calculate total protein concentration by which the data were normalized. These services were provided by Metabolon.

Mitochondrial respiration assay. Mouse 3T3-L1 preadipocytes were seeded into 24-well XF cell culture microplates (Seahorse Bioscience) that had been coated with a 0.2% w/v solution of gelatin. The cells were allowed to grow to confluence and then differentiated by using DMEM media (Life Technologies) supplemented with 10% FBS (Atlas Biologicals), 1% penicillin-streptomycin-neomycin antibiotic mixture (Life Technologies), 250 mM/L dexamethasone (Sigma-Aldrich), 0.5 mM/L 3-isobutyl-1-methylxanthine (Sigma-Aldrich), and 1 μM/L insulin (Sigma-Aldrich). Once fully differentiated, cells were pretreated with either 100 μM EPA (99% ethyl ester; Nu-Chek Prep) that had been conjugated with 1% BSA (Sigma-Aldrich) or with 1% BSA alone for 2 d. Afterward, the cells were treated with LPS overnight to induce an inflammatory response. The media were then switched to the XF assay media (Seahorse Bioscience) supplemented with fresh 2 mM/L sodium pyruvate, and 2.5 mM/L glucose and placed in a non–carbon dioxide incubator at 37°C for 1 h. The XF Cell Mito Stress Test Kit (Seahorse Biosciences) was used to determine oxygen consumption rate changes in the different groups as per the manufacturer’s instructions. The working concentrations of the different compounds injected during the assay were first optimized for our assays and cell type as per the manufacturer’s instructions and are as follows: 1 μM/L oligomycin A, 0.3 μM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazide (FCCP), and 1 μM each) antimycin A/rotenone (A/R). Three measurements were taken after each injection in the following manner: mix for 3 min, wait for 2 min, and measure for 3 min. Three basal measurements were taken before injection of the compounds in a similar fashion, from which changes in oxygen consumption rate were compared as a percentage. Measurements per injection for each well were averaged together before analysis. Respiration profiles were calculated as follows: basal respiration = basal measurements – A/R measurements; proton leak = oligomycin A measurements – A/R measurements; minimum respiration = A/R measurements – FCCP measurements; maximum respiration = A/R measurements – oligomycin A measurements – FCCP measurements; control state respiration = FCCP measurements – oligomycin A measurements – A/R measurements; and state 3 respiration = FCCP measurements – oligomycin A measurements.
significant when ANOVA (F) function from the CAR package in R and were considered version 3.0.1 (27). Treatment differences were then tested by using the Statistical analysis.

Statistical analysis. Statistical analysis of the immunofluorescence data was performed after quantification of the images by Fiji. A linear model was first created by using the lm() function from the STATS package in R version 3.0.1 (27). Treatment differences were then tested by using the ANOVA () function from the CAR package in R and were considered significant when \( P < 0.05 \). Least-squares means were calculated by using the lsmeans () function from the LSMEANS package and were considered significant when \( P < 0.05 \). Mean adipocyte area was compared for between-group differences by using 1-factor ANOVA and Tukey’s post hoc test for multiple comparisons; the level of significance for all tests was \( P < 0.05 \). All data are expressed as means ± SEMs unless stated otherwise.

Analyses for the mitochondrial respiration assay were performed in a similar manner. Non-normally distributed variables and variables with heteroscedastic errors were log-transformed before analysis. Log-transformed variables were then back-transformed for graphical representation. The Kruskal-Wallis test was used for variables that were still non-normally distributed or had heteroscedastic errors after transformation. \( P < 0.05 \) was used to determine significance level.

Data from qRT-PCR and PCR array studies were analyzed by the web-based software RT² Profiler PCR Array Data Analysis, version 3.5 (28), provided by SA Biosciences, by using the \( 2^{-\Delta\Delta C T} \) method. The level of gene expression was normalized by the expression of the housekeeping gene Actb. Two-group comparisons were performed by using \( t \) tests, with \( P < 0.05 \) considered significant.

For metabolomics analyses, data for each compound were log-transformed and missing values were replaced with the minimal value observed across the entire data set (a process referred to as minimum value imputation), as described in reference 26. Statistical analysis was performed in a similar manner as the immunofluorescence data to identify metabolites that differed significantly between the experimental groups (\( P < 0.05 \)).

Results

Effects on inflammation and macrophage recruitment. As previously mentioned, a major feature of obesity is adipose and systemic inflammation. In our previous study we observed that MCP-1 expression in adipose tissue was decreased upon EPA supplementation (Table 3 in reference 8; Figure 1A), indicating a reduction in inflammation. Therefore, we next performed immunofluorescence assays on adipose tissue from all 4 groups (LF as control) to determine total macrophage recruitment after EPA supplementation. We observed that EPA was indeed able to lower macrophage recruitment and therefore alleviate inflammation. Indeed, total macrophage infiltration for both EPA groups (HF-EPA-P and HF-EPA-R) did not differ significantly from the LF group, whereas the HF group had significantly higher macrophage infiltration compared with LF and HF-EPA-P groups (Figure 1B, C).

Effects of EPA on adipocyte cellularity. Our previous study showed that although both the overall body weight (Figure 1 in reference 8) and fat pad weight between our HF and HF-EPA-R groups did not differ (Table 3 in reference 8; Figure 2A), both variables were increased in the HF-EPA-P group compared with the LF group. However, given that the HF-EPA-P and LF groups had similar macrophage concentrations (Figure 1B, C), we next wanted to study the adiposity-independent mechanisms of EPA alleviating adipose inflammation. Using hematoxylin and eosin staining we showed that EPA supplementation resulted in decreased adipocyte size overall, with the HF-EPA-P group having cells the same size as the LF group and the HF-EPA-R group being smaller than the HF group (Figure 2B, C).

**Figure 1** Effects of EPA supplementation on adipose tissue macrophage infiltration in C57BL/6J mice. (A) Epididymal adipose tissue MCP-1 concentrations from our previous study are shown. Values are means ± SEMs, \( n = 8–10 \). (B) Immunofluorescence images of paraffin-embedded epididymal adipose tissue histology sections stained with galactin-3 are shown. (C) Quantification of the mac- rophage area. Values are means ± SEMs, \( n = 3 \). (A and C) Labeled means without a common letter differ, \( P < 0.05 \). HF, high-fat diet; HF-EPA-P, HF diet supplemented with EPA for 11 wk; HF-EPA-R, HF diet for 6 wk and then switched to an EPA-supplemented HF diet for 5 wk; LF, low-fat diet; MCP-1, monocyte chemoattractant protein 1. Panel A adapted from reference 8 with permission.
Metabolite changes upon EPA supplementation. Next we wanted to study the mechanisms by which EPA prevents adipocyte hypertrophy. Because differences were most significant between the HF and HF-EPA-P mice, metabolomics experiments were limited to these 2 groups only. Overall, 222 named biochemicals were identified in the adipose tissue samples, with 22 in the subcutaneous and 17 in the visceral adipose tissues identified as being significantly different in the HF-EPA-P tissues compared with the HF tissues (P < 0.05). An additional 13 and 9 biochemicals in subcutaneous and visceral tissues, respectively, were identified as trending toward significance (0.05 < P < 0.1) for the HF-EPA-P tissue samples.

As expected, ω-3 PUFAs, such as EPA and others (Figure 3A, B), were significantly higher in the HF-EPA-P group than in the HF group. By contrast, ω-6 PUFAs (Figure 3C) and their PG metabolites (Figure 3D) were significantly reduced. Acetyl carnitine, a marker for FA oxidation, was significantly higher in HF-EPA-P visceral fat compared with the HF group (Figure 3E). In addition, a trend (P = 0.06) was observed in the polyamine content in which the spermidine content per milligram of protein was reduced in subcutaneous fat samples (Figure 3F). This is consistent with a reduced fat cell size that we observed earlier. In addition, gene expression analysis using qRT-PCR (Figure 4) revealed downregulated expression of several key genes in adipogenesis/lipogenesis (e.g., Bmp4 and Cebpα) and lipid accumulation (e.g., Lep and Srebf1) due to EPA and an increase in others (e.g., Sirt2 and Slc2a4). Overall, our metabolomic, protein, and gene expression data indicated higher β-oxidation and lower markers of adipogenesis after EPA supplementation.

EPA protects against mitochondrial stress. Finally, we wanted to determine whether EPA increases mitochondrial respiration by performing extracellular flux analysis assays in 3T3-L1 adipocytes that were pretreated with or without EPA and then stimulated with LPS. On the basis of the profiles generated from the cell mitochondrial stress test (Figure 5A), we were able to show that pretreatment with EPA was able to...
increase mitochondrial oxidation through the lowering of basal respiration while significantly increasing the maximal and spare capacity respiration (Figure 5B). This therefore confirmed our other results, which indicated a positive effect on the mitochondrial respiration as a result of EPA treatment.

Discussion

ω-3 FAs, namely EPA and DHA, have been well studied for their cardioprotective and hypolipidemic effects (4, 12, 29). Less research, however, has been conducted on their antiobesity or antidiabetic effects and, for the most part, findings were not conclusive. To further understand the potential therapeutic effects of ω-3 PUFAs (specifically EPA) on obesity, our laboratory previously conducted a study that showed that EPA prevented and/or reversed HF diet–induced inflammation, obesity, and other metabolic alterations in adipose tissue (5, 8). However, the mechanisms mediating these beneficial effects of EPA in dietary obesity were not examined and were therefore the focus of the current study.

In this study we showed that both adipocyte size and macrophage infiltration were reduced upon EPA supplementation, whereas mitochondrial oxidation increased. The greatest effects were observed in the HF-EPA-P group, possibly indicating the effects of longer duration of EPA treatment and the timing of supplementation (during the development of obesity vs. supplementing in already obese mice). Both our previous RBC FA analyses (13) and our metabolomic analyses of adipose tissue FA composition confirmed the incorporation of the dietary fats into the tissues studied. Indeed, as expected, the EPA-fed mice exhibited higher incorporation of ω-3 PUFAs and decreased ω-6 PUFAs and their metabolites in adipose tissue. Changes in these FAs and their metabolites can also indicate changes in inflammation. For example, EPA- and DHA-derived lipid mediators, such as resolvins and protectins, exert anti-inflammatory, proresolving effects and protect against tissue damage (6, 13), whereas, the ω-6 PUFA derivatives (i.e., PGE2) are primarily proinflammatory. This anti-inflammatory effect is also shown in the reduction of MCP-1 from our previous study (8) and from the decrease in adipose tissue macrophage infiltration upon EPA supplementation observed in this study. As previously described, MCP-1 is thought to be a major player in ATM infiltration (10), especially the recruitment of proinflammatory M1 macrophages. However, the immunofluorescence conducted for this study was for total macrophages and did not distinguish between M1 and M2 macrophages. Therefore, future studies should strive to distinguish between these 2 populations to further understand EPA’s anti-inflammatory effects.

The reduction in adipocyte size observed could be due in part to downregulation by EPA of several key adipose genes involved in adipogenesis/lipogenesis (e.g., Bmp4 and Cebpa) and lipid accumulation (e.g., Lep and Srebf1). Changes in adipogenesis were also seen in our metabolomics data in which spermidine was decreased in subcutaneous fat. Although the synthesis of polyamines, such as spermidine, was shown to be critical for adipogenesis (30, 31), it is not clear whether the decrease seen is the cause of the phenotype or merely an indicator of reduced
proliferation. These will be addressed in future studies. Overall, our results are consistent with another study that fed broiler chickens a fish-oil mixture for 36 d and was able to detect a reduction in adipocyte mean area as well (32).

On the other hand, FA oxidation was shown to be increased in visceral fat upon EPA supplementation. This was shown not only in the increased acetylcarnitine detected by our metabolomics analysis but also in the oxygen consumption rates detected by our extracellular flux analysis assays. This is consistent with our previous proteomics data, which indicated an increase in tricarboxylic acid (TCA) cycle enzymes (8). Also in agreement with our findings here, previous studies showed that EPA is able to upregulate mitochondrial biogenesis while inducing β-oxidation in white adipose tissue (33, 34). In addition, a recent study in which patients were given a moderately high dose of ω-3 PUFAs (3.4 g/d of an EPA and DHA mixture), an increase in FA metabolic gene expression and mitochondrial respiration was observed in myocardial tissue, despite no difference in mitochondrial content (35). This was thought to be a result of an effect of ω-3 PUFAs on the nuclear transactivation of PPAR-γ.

As mentioned previously, both PPARα and PPARγ as well as stimulation of AMP-activated protein kinase (AMPK) are thought to be some of the main mediators of EPA’s lipid-metabolizing effects (6, 13, 33). PPARα is thought to mediate the effects of EPA and DHA on adiponectin upregulation (5, 36), whereas PPARγ mediates EPA’s beneficial effects on hepatic insulin sensitivity, as evidenced by studies that used PPARα null mice where hepatic insulin sensitivity could not be restored by EPA (5, 37). However, it should be noted that when mitochondrial FA oxidation was impaired in mice, dietary supplementation of EPA was shown to increase the TG accumulation induced by the impaired oxidation (38). Therefore, future studies will involve exploring more closely the effects of EPA and mitochondrial FA oxidation on each other. In the current study, the concentration of acetylcarnitine, a marker of FA oxidation, was higher in the visceral but not in the subcutaneous adipose tissue of EPA-fed mice compared with HF diet–fed mice. This finding merits further investigation to understand depot-specific differences in these metabolic pathways in response to ω-3 FA supplementation.

In the literature overall, the effects of EPA have been shown to be controversial. A recent meta-analysis of 26 randomized controlled trials revealed that fish-oil supplementation (2–22 g/d) lowered fasting glucose in insulin-dependent patients while slightly increasing fasting glucose in patients with T2D without affecting glycated hemoglobin in either group (39). However, 2 other recent meta-analyses (18 and 23 randomized controlled trials) indicated that fish oil (0.9–18 g/d) yielded no overall effect on fasting glucose or glycated hemoglobin in patients with T2D (29, 40, 41). Therefore, more research is needed to fully understand the effects of ω-3 PUFAs supplementation on diabetes and insulin resistance.

In conclusion, we showed that supplementation with EPA in an HF diet in mice prevented and reversed diet-induced obesity and IR through amelioration of glucose homeostasis and metabolic profile. This was done at least in part by reducing adipose tissue inflammation (increased adiponectin and reduced proinflammatory cytokines and adipose tissue macrophages), possibly via reductions in reactive oxygen species, PG synthesis, and lipid accumulation and adipocyte size (via decreased lipogenesis and increasing FA oxidation; Figure 6). Some of these effects may be mediated by G-protein coupled receptor 120 (GPR-120), a G-protein coupled receptor (GPCR) shown to bind the ω-3 FAs. However, we did not find any significant differences in protein expression for this receptor (data not shown), which may indicate its regulation by EPA at the post-transcriptional level rather than at transcriptional or translational levels. Future studies will determine the cell signaling and molecular mechanisms mediating these EPA effects.

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