Increased Adiponectin Secretion by Highly Purified Eicosapentaenoic Acid in Rodent Models of Obesity and Human Obese Subjects

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Objectives—Fish oil rich in n-3 polyunsaturated fatty acids (PUFAs) or n-3 PUFAs have been shown to reduce the incidence of coronary heart disease. Here we investigated the effect of highly purified eicosapentaenoic acid (EPA) on production of adiponectin, the only established antiatherogenic and antiinflammatory adipocytokine, in rodent models of obesity and human obese subjects.

Methods and Results—We demonstrated that EPA increases adiponectin secretion in genetically obese ob/ob mice and high-fat diet–induced obese mice. In the in vitro coculture of adipocytes and macrophages, EPA reversed the coculture-induced decrease in adiponectin secretion at least in part through downregulation of tumor necrosis factor-α in macrophages. We also showed significant increase in plasma adiponectin concentrations in human obese subjects after a 3-month treatment with EPA (1.8 g daily). Multivariate regression analysis revealed that EPA treatment is the only independent determinant of plasma adiponectin concentrations.

Conclusion—This study demonstrates that EPA increases adiponectin secretion in rodent models of obesity and human obese subjects, possibly through the improvement of the inflammatory changes in obese adipose tissue. Because EPA has reduced the risk of major coronary events in a large-scale, prospective, randomized clinical trial, this study provides important insight into its therapeutic implication in obesity-related metabolic sequelae. (Arterioscler Thromb Vasc Biol. 2007;27:1918-1925.)

Key Words: adipocytes ■ adiponectin ■ EPA ■ macrophages ■ obesity

The adipose tissue has a high capacity to secrete many biologically active substances (or adipocytokines) such as leptin and tumor necrosis factor-α (TNFα).1 Dysregulation of pro- and antiinflammatory adipocytokine production is associated with the metabolic syndrome, suggesting that inflammatory changes within obese adipose tissue may critically contribute to the development of many aspects of the metabolic syndrome and results in diabetes and atherosclerosis. Among numerous adipocytokines, adiponectin is unique in that it is the only established adipocytokine with antiatherogenic and antiinflammatory properties.2,3 It also increases tissue fat oxidation, leading to reduced levels of fatty acids (FAs) and tissue triglyceride content, thus enhancing insulin sensitivity in the liver and skeletal muscle.2,4,5 Because plasma adiponectin concentrations are decreased in obese subjects,1,2 extensive researches have been aimed at the upregulation of adiponectin and its cognate receptors (AdipoR1 and AdipoR2) for the treatment of obesity-related metabolic sequelae.6

Previous studies showed that the adipose tissue is markedly infiltrated by macrophages in several models of rodent obesities and human obese subjects,6,7 suggesting that macrophages participate in the inflammatory pathways that are activated in obese adipose tissue. Using in vitro coculture system composed of adipocytes and macrophages, we have demonstrated that a paracrine loop involving saturated FAs and TNFα derived from adipocytes and macrophages, respectively, establishes a vicious cycle that augments the inflammatory changes; ie, marked upregulation of proinflammatory adipocytokines such as monocyte chemoattractant protein-1 (MCAMP)}
(MCP-1) and TNFα and downregulation of adiponectin. These findings led us to speculate that macrophages, when infiltrated, induce the release of saturated FAs from adipocytes via lipolysis, which, in turn, may serve as a proinflammatory adipocytokine in the adipose tissue. Interestingly, n-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) are unable to activate macrophages or even antagonize the proinflammatory effect of saturated FAs, suggesting the structural specificity of FAs in the induction of inflammatory changes.

In epidemiological and clinical trials, fish oil rich in n-3 PUFAs or n-3 PUFAs have reduced the incidence of coronary heart disease. Given the pleiotropic effect of n-3 PUFAs, it is tempting to speculate that changes in adipocytokine production. There are a couple of recent reports showing that fish oil or n-3 PUFAs increase adiponectin mRNA expression and/or secretion in several models of rodent obesity. However, it is still unknown whether EPA, the only class of n-3 PUFAs used clinically to treat hyperlipidemia, increases adiponectin production in obesity, and if so, how it does in obese adipose tissue remains to be elucidated. Furthermore, there has been no report showing the direct effect of EPA on adiponectin secretion in human obese subjects.

Here we report that serum adiponectin concentrations are increased by EPA in several models of rodent obesity and in human obese subjects. Using the in vitro coculture of adipocytes and macrophages, we also show that EPA reverses the coculture-induced decrease in adiponectin secretion at least in part through downregulation of TNFα in macrophages. Because EPA has reduced the risk of major coronary events in a large-scale, prospective, randomized clinical trial, this study provides important insight into its therapeutic implication of obesity-related metabolic sequelae and thus the metabolic syndrome.

**Methods**

**Materials**

Preparation and characterization of highly purified eicosapentaenoic acid (EPA) ethyl ester (purity: >98%, Mochida Pharmaceutical Co Ltd) used in animal and clinical studies was reported elsewhere. Other materials are described in supplemental Methods (available online at http://atvb.ahajournals.org.).

**Animals**

Male C57BL/6J ob/ob mice and their wild-type (WT) littermates were purchased from Charles River Japan (Tsukuba, Ibaraki, Japan). The animals were housed in individual cages in a temperature-, humidity-, and light-controlled room (12-hour light and 12-hour dark cycle) and allowed free access to water and fish meal–free diet (fish meal–free F1; 362 kcal/100 g, 4.4% energy as fat; Funabashi Farm, Chiba, Japan). All animal experiments were conducted in accordance with the guidelines of Tokyo Medical and Dental University Committee on Animal Research (No. 0060026).

**Administration of EPA in ob/ob Mice**

Six-week-old male ob/ob mice and WT littermates had unrestricted access to the fish meal–free diet (control group) or fish meal–free diet supplemented with 5% EPA (wt/wt) (EPA-treated group) for 4 weeks (n = 10 to 14). In the short-term administration protocol, 8-week-old male ob/ob mice were treated with EPA for 2 weeks (n = 7 to 8). All diets were changed every day and served with nonmetallic feeder to prevent oxidation of fatty acids. At the end of the experiments, mice were euthanized after 5-hour starvation under intraperitoneal pentobarbital anesthesia (30 mg/kg). Blood glucose and serum concentrations of triglyceride (TG) and free fatty acid (FFA) were measured as previously described. Serum EPA concentrations were measured by gas chromatography.

**Histological Analysis**

The epididymal white adipose tissue (WAT) was fixed with neutral-buffered formalin and embedded in paraffin. Five μm–thick sections were stained with hematoxylin and eosin and studied under ×200 magnification to compare the adipocyte cell size using the software Win Roof (Mitani Co Ltd). More than 200 cells were counted per each section. The presence of F4/80-positive macrophages in WAT was detected immunohistochemically using the rat monoclonal anti-mouse F4/80 antibody described elsewhere. The number of F4/80-positive cells was counted in more than 10 μm² area of each section and expressed as the mean number/μm².

**Coculture of Adipocytes and Macrophages**

Details are described in supplemental Methods.

**Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was extracted from mouse epididymal WAT and cultured cells using TRIzol reagent (Invitrogen) and quantitative real-time polymerase chain reaction was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems) as described. Primers used in this study were described elsewhere. Levels of mRNA were normalized to those of 36B4 mRNA.

**Measurement of Adiponectin Concentrations**

Serum adiponectin concentrations in ob/ob mice and diet-induced obese mice were determined by a commercially available enzyme-linked immunoassorbent assay (ELISA) kit (Otsuka Pharmaceutical Assaypro). Adiponectin concentrations in culture supernatants were also determined by an ELISA kit (Otsuka Pharmaceutical).

**Human Study**

Details are described in supplemental Methods.

**Statistical Analysis**

Data are presented as mean±SE, and P<0.05 was considered statistically significant. In cell culture experiments and animal studies, statistical analysis was performed using analysis of variance followed by Scheffe test.

**Results**

**Administration of EPA in ob/ob Mice**

Increased Serum Adiponectin Concentrations in ob/ob Mice Treated With EPA

To elucidate the effect of EPA on adiponectin production in obesity, we performed 4-week administration of EPA in ob/ob mice. Body weight gain did not change in ob/ob and WT mice by EPA treatment throughout the experiment (Figure 1A). There was no appreciable difference in the weights of the adipose tissue and liver between EPA-treated and control WT mice, whereas the weights of the epididymal and subcutaneous WATs and liver were significantly reduced in EPA-treated ob/ob mice relative to control ob/ob mice (epididymal WAT, P<0.01; subcutaneous WAT and liver, P<0.05; Figure 1B). Blood glucose concentrations did not change in ob/ob and WT mice by EPA treatment, whereas serum TG and FFA concentrations were significantly decreased in EPA-treated WT mice (Figure 1C). Histological analysis revealed that hepatic steatosis is markedly improved in...
ob/ob mice by EPA treatment (supplemental Figure IA). Both EPA-treated ob/ob and WT mice exhibited significant increase in serum EPA concentrations (WT, 5.30 ± 0.35 versus 260.78 ± 16.39; ob/ob, 16.23 ± 0.78 versus 422.43 ± 18.11 μg/mL, n = 3 to 4, P < 0.01). Serum adiponectin concentrations tended to be increased in WT mice and were significantly increased in ob/ob mice by EPA treatment (P < 0.05; Figure 1D). These observations indicate that administration of EPA increases serum adiponectin concentrations in ob/ob mice in parallel with the reduction of adipose tissue weight.

Effect of EPA Treatment on Adipose Tissue Histology and mRNA Expression

Histological examination of the epididymal WAT revealed that adipocyte cell size is significantly reduced by EPA treatment in both ob/ob and WT mice (mean cell diameters: WT, 44.6 ± 0.4 versus 37.4 ± 0.3 μm; ob/ob, 99.5 ± 1.2 versus 85.5 ± 1.0 μm; P < 0.01; Figure 2A and 2C). We next examined mRNA expression in the epididymal WAT. As reported previously, adiponectin mRNA expression was markedly reduced in ob/ob mice relative to WT mice (Figure 2E). On the contrary to the increment of serum adiponectin concen-

Figure 1. Body weight, adipose tissue and liver weight, and serum parameters in mice treated with EPA for 4 weeks. A, Growth curve of ob/ob and WT mice on either control fish meal–free diet (EPA (−)) or 5% EPA-supplemented diet (EPA (+)). Open triangle, WT-EPA (−) (n = 14); closed triangle, WT-EPA (+) (n = 10); open circle, ob/ob-EPA (−) (n = 10); closed circle, ob/ob-EPA (+) (n = 10). B, Weight of the epididymal (epi), mesenteric (mes), and subcutaneous (sub) WATs and liver in ob/ob and WT mice on either EPA (−) or EPA (+) diet. Lane 1, WT-EPA (−); lane 2, WT-EPA (+); lane 3, ob/ob-EPA (−); lane 4, ob/ob-EPA (+). C, Blood glucose levels and serum concentrations of TG and FFA. D, Serum adiponectin concentrations in ob/ob and WT mice. *P < 0.05; **P < 0.01. n = 10 to 14.

Figure 2. Histological analysis and mRNA expression in epididymal WAT from ob/ob and WT mice treated with EPA for 4 weeks. A, Hematoxylin and eosin staining of the epididymal WAT from ob/ob and WT mice. B, F4/80 staining of the epididymal WAT from ob/ob mice. Original magnification, ×200. Scale bars, 100 μm. C, Histogram of diameters of adipocytes in the epididymal WAT. D, Cell count of F4/80-positive cells in the epididymal WAT. E, Expression of adiponectin and TNFα mRNAs in the epididymal WAT. n.s., not significant. **P < 0.01. n = 10 to 14.
trations, there was no significant change in adiponectin mRNA expression by EPA treatment (Figure 2E). Although F4/80-positive macrophage accumulation in the adipose tissue was significantly increased in EPA-treated ob/ob mice relative to control group (Figure 2B and 2D, \( P<0.01 \)), there was no significant difference in mRNA expression of TNF\(\alpha\), a proinflammatory cytokine expressed predominantly in macrophages (Figure 2E). Plasminogen activator inhibitor (PAI)-1 and heparin-binding epidermal growth factor–like growth factor (HB-EGF) mRNA expression was significantly decreased in EPA-treated ob/ob mice \( (P<0.01) \), although there was no significant change in MCP-1, interleukin (IL)-6, and resistin mRNA expression (supplemental Figure II). These observations indicate that EPA treatment increases serum adiponectin concentrations in ob/ob mice without affecting its mRNA expression, and attenuates inflammatory changes in the adipose tissue.

**Effect of a Short-Term EPA Treatment in ob/ob Mice**

To further examine whether the reduction of adipocyte cell size is related to the increased serum adiponectin concentrations by EPA treatment, we examined the metabolic phenotypes of ob/ob mice that received a short-term (or 2-week) treatment with EPA. There were no significant differences in body weight and adipose tissue weight between EPA-treated and control groups at the end of the experiment (supplemental Table I). The liver weight was significantly reduced \( (P<0.01) \) and hepatic steatosis was histologically improved in EPA-treated group relative to control group (supplemental Figure IB). There was no significant difference in adipocyte cell size (mean cell diameters: \( 97.5\pm0.9 \) versus \( 94.1\pm0.9 \mu m \)) and mRNA expression of F4/80 and TNF\(\alpha\) in the epididymal WAT between EPA-treated and control groups (Figure 3A and 3B). PAI-1, HB-EGF, and resistin mRNA expression was significantly reduced with 2-week EPA administration \( (P<0.05; \text{supplemental Figure III}) \). mRNA expression of IL-10 and arginase, which is specific for antiinflammatory M2-polarized macrophages, tended to be increased, and that of inducible nitric oxide synthase (iNOS), which is specific for proinflammatory M1-polarized macrophages, tended to be decreased with EPA treatment (supplemental Figure IV). There was no significant change in mRNA expression of M1-specific CD11c (supplemental Figure IV). In this study, adiponectin mRNA expression was reduced in the epididymal WAT by the 2-week EPA treatment (Figure 3C). In this setting, serum adiponectin concentrations were significantly increased \( (P<0.01; \text{Figure 3D}) \), which is comparable to those attained by the 4-week EPA treatment. These observations indicate that EPA treatment increases serum adiponectin concentrations independently of adipocyte cell size.

**Administration of EPA in Mice With High-Fat Diet–Induced Obesity**

We next examined the effect of EPA on adiponectin production in mice with diet-induced obesity. Ten-week-old male C57BL/6J mice were fed high-fat diet, with or without EPA by daily oral administration for 8 weeks. The mice on high-fat diet weighed 14% more than those on standard diet at the end of the experiment (supplemental Figure VA). By histological analysis, adipocyte hypertrophy and macrophage accumulation in high-fat diet–induced obese mice were much milder than those in ob/ob mice (data not shown), suggesting that mice with diet-induced obesity and ob/ob mice used in this study represent models of the early and advanced stages of obesity, respectively. There was no significant difference in body weight between the groups fed high-fat diet. Plasma adiponectin concentrations in obese mice with EPA treatment were significantly increased relative to those without EPA treatment as early as 2 weeks after the treatment \( (P<0.01; \text{supplemental Figure VB}) \), whereas there was no significant upregulation of adiponectin mRNA levels (supplemental Figure VC). These observations, taken together, indicate that EPA treatment effectively increases plasma adiponectin concentrations without increase in adiponectin mRNA expression at the early stage of obesity as well as at the advanced stage of obesity.

**Effect of EPA in the Coculture of Adipocytes and Macrophages**

To explore the molecular mechanism for the EPA-induced increase in adiponectin secretion in vivo, we examined the effect of EPA on adiponectin production in the in vitro coculture of 3T3-L1 adipocytes and RAW264 macrophages. As we reported previously, adiponectin mRNA expression and secretion to the media were significantly reduced in...
Figure 4. Effect of EPA on adiponectin secretion and mRNA expression in the coculture of adipocytes and macrophages. A, Effect of EPA on adiponectin secretion and mRNA expression in 3T3-L1 adipocytes cocultured with RAW264 macrophages. B, Effect of EPA on the palmitate-induced increase in TNFα mRNA expression and NF-κB activation in RAW264 macrophages. xB-luc, a luciferase reporter vector for NF-κB; pGL3, a promoterless luciferase reporter vector; RLA, relative luciferase activity. C, Effect of EPA on the LPS-induced increase in TNFα mRNA expression and NF-κB activation in RAW264 macrophages. EPA 50 to 200 µmol/L; pal, palmitate 200 µmol/L; LPS 1 ng/mL. n.s., not significant. *P<0.05, **P<0.01. n=4 to 6.

3T3-L1 adipocytes cocultured with RAW264 macrophages (P<0.01; Figure 4A). Treatment with EPA reversed significantly the coculture-induced decrease in adiponectin secretion in a dose-dependent manner (P<0.05), whereas adiponectin mRNA expression was unchanged (Figure 4A). In this study, EPA did not affect adiponectin secretion and mRNA expression in 3T3-L1 adipocytes alone (or in the control culture; Figure 4A). We found that treatment with EPA at doses of 100 and 200 µmol/L suppresses significantly the coculture-induced increase in TNFα mRNA expression (P<0.05; supplemental Figure VIA), which occurs predominantly in RAW264 macrophages in the coculture system.8 Furthermore, blockade of TNFα using an anti-TNFα neutralizing antibody at a dose that can abolish the effect of recombinant TNFα reversed significantly the coculture-induced decrease in adiponectin secretion (P<0.05; supplemental Figure VIB). We also observed that adiponectin secretion is reduced significantly in 3T3-L1 adipocytes treated with recombinant TNFα at a dose of 200 pg/mL (P<0.05; supplemental Figure VIC), which is roughly comparable to those detected in the coculture system.8 In this study, adiponectin mRNA expression was unaffected, although both adiponectin mRNA expression and secretion are shown to be suppressed in vitro when treated with TNFα at much higher doses.22

Because saturated FAs is an important adipocyte-derived paracrine signal to induce TNFα mRNA expression and NF-κB activation in macrophages,8,9 we also examined the effect of EPA on saturated FA-induced increase in the inflammatory changes in RAW264 macrophages. The basal NF-κB activity in RAW264 macrophages transfected with pGL3 vector as a promoterless control was approximately 1/20 relative to those transfected with the luciferase reporter vector for NF-κB activity, and EPA tended to decrease TNFα mRNA expression in RAW264 macrophages (Figure 4B). Treatment with palmitate, a major saturated FA released from 3T3-L1 adipocytes,8 induced significantly TNFα mRNA expression in RAW264 macrophages (P<0.01), which were effectively reversed by EPA (P<0.01; Figure 4B). Furthermore, a luciferase reporter assay showed that EPA suppresses the palmitate-induced activation of NF-κB in RAW264 macrophages (Figure 4B). Because saturated FAs act as a proinflammatory adipocytokine through toll-like receptor 4 (TLR4),8,10 a bona fide receptor for the recognition of lipopolysaccharide (LPS), we also examined the effect of EPA on the LPS-induced increase in TNFα mRNA expression and NF-κB activation. In this study, EPA was capable of reversing dose-dependently the LPS-induced increase in TNFα mRNA expression and NF-κB activation (Figure 4C). Moreover, EPA significantly reduced FFA release from 3T3-L1 adipocytes cocultured with RAW264 macrophages (P<0.05; supplemental Figure VII). These observations, taken together, suggest that EPA reverses the coculture-induced decrease in adiponectin secretion at least in part through the reduction of the inflammatory changes induced by the interaction between adipocytes and macrophages.

Treatment With EPA in Human Obese Subjects

We also examined whether EPA increases adiponectin secretion in human obese subjects. There were no significant differences between EPA-treated and control groups in all measured variables before EPA treatment (Table 1). After the 3-month EPA treatment, plasma concentrations of EPA were significantly increased in EPA-treated group relative to control group (P<0.01). In EPA-treated group, plasma TG concentrations were significantly decreased (P<0.05 relative to control group, although there were no changes in BMI, waist circumference, systolic blood pressure (SBP), fasting plasma glucose (FPG), total cholesterol, high density lipoprotein cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C) between both groups. Plasma adiponectin concentrations were increased after the treatment with EPA (P<0.01), but unchanged in control group. To determine the risk factors independently influencing the changes of plasma adiponectin, multivariate regression analysis was performed using the variables shown in supplemental Table II. Only the treatment with EPA was an independent determinant of plasma adiponectin concentrations in obese subjects.
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Clinical Characteristics and Metabolic Parameters Before and After the Follow-Up Period

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Data are expressed as the mean±SE.
*P<0.05; †P<0.01 vs before determined by 2-tailed paired t test; ‡P<0.01 vs control determined by Student t test.

(β=0.378, P=0.018). By stepwise multivariate regression analysis (r²=0.170), plasma adiponectin concentrations were significantly correlated only with the treatment with EPA (F=9.24, P=0.004).

Discussion

In a large-scale, prospective, randomized clinical trial, highly purified EPA has reduced the risk of major coronary events via cholesterol-independent mechanisms.12 However, the detailed mechanism is still poorly understood. Here we examined the effect of EPA on adiponectin production in rodent models of obesity and obese human subjects. Using the in vitro coculture system composed of 3T3-L1 adipocytes and RAW264 macrophages,8 we also examined how EPA increases adiponectin production in the interaction between adipocytes and macrophages in obese adipose tissue.

This study demonstrates that EPA is capable of increasing serum adiponectin concentrations in genetically obese ob/ob mice at the late stage of obesity, when macrophages are markedly infiltrated into the adipose tissue. The effect was observed as early as 2 weeks and persisted up to 4 weeks after the administration. In this study, there was no appreciable increase in adiponectin mRNA expression in the adipose tissue, indicating that EPA increases adiponectin secretion rather than its mRNA expression. Using an in vitro coculture of adipocytes and macrophages,8 we demonstrated that treatment with EPA reverses the coculture-induced decrease in adiponectin secretion, with no effect on its mRNA expression. These observations indicate that EPA increases adiponectin secretion rather than its biosynthesis in vitro. We also found that EPA reduces the coculture-induced increase in TNFα mRNA expression, which is considered to reflect the inflammatory state of macrophages. Importantly, blockade of the TNFα action effectively reversed the coculture-induced decrease in adiponectin secretion. These observations, taken together, suggest that EPA reverses the coculture-induced decrease in adiponectin secretion possibly through the attenuation of inflammatory changes in macrophages. We have recently demonstrated that saturated FAs, which are released via the macrophage-induced adipocyte lipolysis, serve as a naturally occurring ligand for TLR4, thereby inducing the inflammatory changes in macrophages through NF-κB activation.9 In this study, EPA suppressed the palmitate- and LPS-induced increase in TNFα mRNA expression and NF-κB activation in macrophages. It is, therefore, likely that EPA acts directly on macrophages, thereby interrupting the vicious cycle created by the interaction between adipocytes and macrophages in obese adipose tissue.

Histological analysis revealed significant reduction of adipocyte cell size in ob/ob mice treated with EPA relative to control group, which is consistent with previous reports.23,24 In this study, the adipose tissue weights tended to be reduced by 2-week administration of EPA, and were significantly reduced after 4-week or longer-term administration. The reduction of adipose tissue weights and serum FFA concentrations may be secondary to improved lipid metabolism, as n-3 PUFAs are shown to act as peroxisome proliferator-activated receptor-α (PPARα) ligands, thereby inducing the upregulation of genes involved in fatty acid oxidation in the liver.25 Although it is largely unknown about the effect of n-3 PUFAs on the regulation of FFA production in the adipose tissue, we have observed that EPA significantly suppresses the coculture-induced increase in FFA release from 3T3-L1 adipocytes. Flachs et al also reported that n-3 PUFAs increase mRNA expression of PPARγ coactivator 1-α and nuclear respiratory factor-1, which in turn stimulate β-oxidation and mitochondrial biogenesis in the adipose tissue and 3T3-L1 adipocytes.26 We also observed increased adiponectin secretion in ob/ob mice during the 2-week administration of EPA, with no change in adipocyte cell size. These observations, taken together, suggest that the positive effect of EPA on adiponectin secretion is not related to the adipocyte cell size.

In this study, it is interesting to note that macrophage accumulation is apparently increased in the adipose tissue from ob/ob mice treated with EPA for 4 weeks, although TNFα mRNA expression is not increased in parallel with the number of macrophages. Because we used fish meal–free diet (control group) and fish meal–free diet supplemented with EPA, the difference in fat intake between EPA-treated and control groups (4.4 versus 9.4% [wt/wt], respectively) may influence macrophage infiltration. We and others have recently demonstrated that TLR4-deficient or mutant mice rendered obese by high-fat diet feeding show decrease in expression of inflammatory markers and increase in serum adiponectin concentrations relative to wild-type mice, with no changes in the number of macrophages infiltrated.27,28 Moreover, Lumeng et al recently reported that adipose tissue macrophages isolated from lean and obese mice are in different activation state like M1 or “classically activated” (proinflammatory) and M2 or “alternatively activated” (anti-inflammatory) polarization, suggesting the implication of macrophage subpopulation.21 These findings suggest that not only the number but also the activation state of macrophages in the adipose tissue affects adipocytokine production. In-
deed, n-3 PUFAs are known to suppress expression of inflammatory markers such as TNFα and IL-1β in macrophages in vitro. Collectively, we speculate that macrophages infiltrated into the adipose tissue of ob/ob mice treated with EPA are not activated to induce adipose tissue inflammation. Further studies are needed to evaluate the activation state of macrophages in vivo.

In this study, we confirmed that EPA increases serum adiponectin concentrations in mice rendered mildly obese by a short-term high-fat diet at the early stage of obesity, when macrophages are not apparently infiltrated. These observations suggest that EPA acts directly on adipocytes in the adipose tissue, where it increases adiponectin secretion. Interestingly, there was no significant increase in adiponectin mRNA expression in these animals. In this regard, Nishizawa et al demonstrated that testosterone inhibits adiponectin mRNA expression in 3T3-L1 adipocytes. There may be unknown mechanisms involved in the regulation of adiponectin secretion in adipocytes, whose activity is modulated by EPA. Indeed, Xie et al recently reported that intracellular trafficking and secretion of adiponectin are uniquely mediated by specific coated vesicles formed at the trans-Golgi network of adipocytes, whereas those of another adipocytokine, leptin are not. EPA is known to be taken into cell membrane phospholipids, and it may affect adiponectin secretion through modulation of fatty acid composition of adipocyte cell membrane. On the other hand, Neschen et al reported that fish oil increases both adiponectin mRNA expression and secretion in mice on a short-term (or 2-week) high-fat diet. This may be because of the potential difference in antiinflammatory effect between EPA used in this study and fish oil or the doses of administration. In addition, we and others observed that n-3 PUFAs do not affect adiponectin mRNA expression and secretion in 3T3-L1 adipocytes, suggesting the difficulty to examine the direct effect of n-3 PUFAs on adiponectin production in adipocytes in vitro. Further studies are needed to elucidate how EPA regulates adiponectin mRNA expression or secretion in mildly obese animals in vivo.

The limitation to this study includes the difference between the in vivo and in vitro data as to the mechanism by which EPA increases adiponectin secretion. To get insight into the effect of EPA on the inflammatory changes in obese adipose tissue, we used the unique in vitro coculture system composed of adipocytes and macrophages, by which we have reproduced the proinflammatory gene expression profile found in obese adipose tissue. In this study, we found that EPA reverses the coculture-induced reduction of adiponectin secretion possibly through the attenuation of macrophage activation. However, although EPA reduces the coculture-induced increase in TNFα production in vitro, TNFα mRNA expression is not significantly reduced in the epidymal WAT from EPA-treated ob/ob mice. The difference between the in vivo and in vitro settings may be because the adipose tissue shows dramatic changes in cellular population during the development of obesity. Otherwise, 3T3-L1 adipocytes may not respond to EPA in vitro. Nonetheless, we found that treatment with EPA for 2 and 4 weeks reduces mRNA expression of several proinflammatory adipocytokines including PAI-1 and HB-EGF, whereas there was no apparent change in that of MCP-1, IL-6, and resistin in this study. These observations suggest that EPA reduces the inflammatory changes in obese adipose tissue in vivo and those induced by the interaction between adipocytes and macrophages in vitro, thereby leading to increased adiponectin secretion.

We also demonstrated increased plasma adiponectin concentrations in obese human subjects after the treatment with EPA. Multivariate regression analysis revealed that EPA administration is only the independent determinant of plasma adiponectin concentrations, suggesting the direct involvement of EPA in adiponectin secretion. It was reported that weight loss improves inflammation in the adipose tissue and that n-3 PUFAs has additive effect on plasma adiponectin concentrations in combination with weight loss. In this study, there was no significant change in body weight of our study subjects, suggesting that EPA itself increases adiponectin secretion without body weight change. Although we did not obtain the adipose tissue samples used for histological examination, it has been reported that there exists macrophage infiltration in the adipose tissue from obese subjects with BMI of approximately 30. We speculate that EPA increases adiponectin secretion at least partly by interrupting the vicious cycle created by adipocytes and macrophages in human obese subjects as in the in vitro coculture experiments.

In conclusion, this study demonstrates that EPA increases adiponectin secretion in rodent models of obesity and human obese subjects. Given that hypoadiponectinemia has been shown to increase the risk of coronary heart disease, the beneficial effect of EPA may be attributable at least in part to the modulation of the inflammatory changes in obese adipose tissue and increased adiponectin secretion. Because EPA is the only class of n-3 PUFAs, which has proved to reduce the risk of major coronary events, the data of this study provide important insight into its therapeutic implication in obesity-related metabolic sequelae.

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Disclosures
None.
References


Increased Adiponectin Secretion by Highly Purified Eicosapentaenoic Acid in Rodent Models of Obesity and Human Obese Subjects

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Methods

Materials

Palmitate and eicosapentaenoic acid (EPA) used in vitro were purchased from Sigma (St. Louis, MO), solubilized in ethanol, and combined with fatty acid- and immunoglobulin-free bovine serum albumin (Sigma) in low serum medium. Recombinant mouse tumor necrosis factor-α (TNFα) and anti-TNFα neutralizing antibody were obtained from R&D systems (Minneapolis, MN). All other reagents were purchased from Sigma and Nacalai Tesque (Kyoto, Japan) unless otherwise described.

Administration of EPA in Diet-induced Obese Mice

Ten-week old male C57BL/6J mice (CLEA Japan, Tokyo, Japan) were assigned to fish meal-free diet group or high-fat diet group (D12492; 524 kcal/100 g, 60% energy as fat; Research Diets, New Brunswick, NJ) supplemented with or without EPA (daily oral administration, 1000 mg/kg·wt/day). Blood samples were collected via the retro-orbital sinus of mice every 2 weeks to determine plasma adiponectin concentrations. Mice were sacrificed after 8-week administration of EPA.

Co-culture of Adipocytes and Macrophages

RAW264 macrophage cell line (RIKEN BioResource Center, Tsukuba, Japan) and 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium (Nacalai Tesque) containing 10% fetal bovine serum (FBS; BioWest, Miami, FL). Differentiation of 3T3-L1 preadipocytes to mature adipocytes was performed as described and used as differentiated 3T3-L1 adipocytes at day 8 after the induction of differentiation. Co-culture of adipocytes and macrophages was performed as described with slight modifications. In brief, serum starved 3T3-L1 adipocytes were
cultured in a 6-well plate and 1.0 x 10^5 cells of RAW264 macrophages were plated onto 3T3-L1 adipocytes. The cells were cultured for 48 h with contact each other and harvested. As a control, adipocytes and macrophages, the numbers of which were equal to those in the co-culture, were cultured separately and mixed after harvest.

**Transient Transfection and Luciferase Reporter Assay**

NF-κB activity was determined by a luciferase reporter assay as previously described.\(^1\)\(^,\)\(^2\) In brief, RAW264 macrophages were transiently transfected by electroporation (Nucleofector system; Amaxa, Gaithersburg, MD) with a luciferase reporter vector for NF-κB activity or the pGL3 basic vector (Promega, Madison, WI) as a promoterless control, together with the phRL-SV40 vector (Promega) as an internal control for transfection efficiency. After incubation for 24 h, cells were serum starved for another 24 h and used for the experiments. The luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega).

**Human Study**

A total of 52 Japanese obese subjects were recruited in our clinics to examine the effect of EPA on adiponectin secretion in human obese subjects. All the participants in this study satisfied the metabolic syndrome criteria in Japan (waist circumference \(\geq 85\) cm for men and \(\geq 90\) cm for women, and two or more of the following risk factors: 1) triglyceride (TG) \(\geq 150\) mg/dl or high density lipoprotein cholesterol (HDL-C) < 40 mg/dl; 2) systolic blood pressure (SBP) \(\geq 130\) mmHg or diastolic blood pressure \(\geq 85\) mmHg; 3) fasting plasma glucose (FPG) \(\geq 110\) mg/dl).\(^3\) Cardio- and cerebro- vascular diseases were excluded. The study protocol was approved by the ethical committee on human research of Kyoto Medical Center and Medical Research Institute, Tokyo Medical and Dental University, and all participants gave written informed consent. Patients were assigned to one of the following treatment groups (a single-blind and a run-in period randomization, which patients received): treated for 3 months
with either diet alone (control group) or diet plus EPA (1.8 g daily) (EPA-treated group). Diet therapy consisted of 25 kcal/kg of ideal body weight per day. At the beginning and the end of the study, we measured body mass index (BMI), waist circumference, SBP, FPG, plasma concentrations of total cholesterol, TG, HDL-C, low density lipoprotein cholesterol (LDL-C), and EPA according to the standard procedures. Plasma concentrations of adiponectin were determined using a radioimmunoassay kit (Linco Research, St. Charles, MO). There were no differences between EPA-treated and control groups in all the parameters measured at the beginning of this study. Lipid lowering medications such as statins and fibrates were excluded. Several subjects in both groups had medication with oral antidiabetic agents (sulfonylureas or α-glucosidase inhibitors) and antihypertensive agents (calcium channel blockers) and there were no changes in medication during this study.

**Statistical analysis**

In the human study, repeated measures ANOVA (control and EPA-treated groups x before and after the treatment) was used to access the comparative effect of EPA treatment on the measured variables. A two-tailed, paired t-test was applied for the evaluation of changes from baseline conditions to those at 3 months. Comparisons of the means between the two groups at baseline or post-treatment were performed by Student’s t-test. Changes from baseline conditions to those at 3 months were abbreviated as Δ. Stepwise multivariate regression analysis was performed using a model in which the dependent variable was Δadiponectin, with the following explanatory variables: sex, age, Δwaist circumference, ΔSBP, ΔFPG, ΔTG, ΔHDL-C, ΔLDL-C, and the treatment with EPA. All statistical analyses were performed using the Stat View program version 5.0 for Windows (SAS Institute Inc.).
References


Legends to Supplemental Figures

Figure I. Effect of EPA on hepatic steatosis in ob/ob mice. (A) Periodic-acid Schiff staining of liver from ob/ob and wildtype (WT) mice after a 4-week (A) and a 2-week (B) administration of EPA. Original magnification, x 100. Scale bars, 100 µm.

Figure II. Effect of 4-week EPA treatment on mRNA expression in epididymal white adipose tissue (WAT) from ob/ob and WT mice. mRNA expression of plasminogen activator inhibitor-1 (PAI-1), heparin-binding epidermal growth factor-like growth factor (HB-EGF), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) and resistin. n.s., not significant, * P < 0.05, ** P < 0.01. n = 10-14.

Figure III. Effect of 2-week EPA treatment on mRNA expression in epididymal WAT from ob/ob mice. mRNA expression of PAI-1, HB-EGF, MCP-1, IL-6 and resistin. n.s., not significant, * P < 0.05. n = 7-8.

Figure IV. Effect of 2-week EPA treatment on mRNA expression of M1 and M2 macrophage markers in epididymal WAT from ob/ob mice. mRNA expression of IL-10 and arginase as M2 macrophage markers, and iNOS and CD11c as M1 macrophage markers. n = 7-8.

Figure V. Effect of EPA in mice with diet-induced obesity. (A) Growth curve of mice on fish meal-free control diet (open triangles), high fat diet (HFD, open circles), or HFD supplemented with EPA (HFD + EPA, closed circles). **P < 0.01 vs. control. (B) Change in plasma adiponectin concentrations during the EPA treatment. # P < 0.05 vs. control. * P < 0.05, ** P < 0.01 vs. HFD. (C) Adiponectin, TNFα and F4/80 mRNA levels in the epididymal WAT. n.s., not significant. n = 9-10.

Figure VI. Role of TNFα in adiponectin secretion and mRNA expression. (A) Effect of EPA on TNFα mRNA expression in the co-culture of 3T3-L1 adipocytes and RAW264
macrophages. (B) Effect of an anti-TNFα neutralizing antibody on adiponectin secretion in the co-culture system. (C) Effect of mouse recombinant TNFα on adiponectin secretion and mRNA expression in 3T3-L1 adipocytes. EPA 50-200 µmol/l; Ab, anti-TNFα neutralizing antibody, 0.5 µg/ml; IgG, non-immune IgG, 0.5 µg/ml; TNFα, 200 pg/ml. *P < 0.05, **P < 0.01. n = 4-6.

Figure VII. Effect of EPA on FFA release in media from 3T3-L1 adipocytes co-cultured with RAW264 macrophages. Differentiated 3T3-L1 adipocytes were co-cultured with RAW264 macrophages (1 x 10^5 cells/well) for 48 h in the presence or absence of EPA (50-200 µmol/l). * P < 0.05, ** P < 0.01. n = 4-5.
Table I. Effect of EPA on body weight, tissue weight, glucose and lipid metabolism after 2-week administration of EPA in ob/ob mice.

<table>
<thead>
<tr>
<th></th>
<th>EPA (-)</th>
<th>EPA (+)</th>
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<tbody>
<tr>
<td>Body weight (before)</td>
<td>42.8 ± 1.0</td>
<td>42.3 ± 0.7</td>
</tr>
<tr>
<td>Body weight (after)</td>
<td>48.4 ± 0.6</td>
<td>47.8 ± 0.5</td>
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<tr>
<td>Epididymal fat (g)</td>
<td>3.69 ± 0.11</td>
<td>3.67 ± 0.11</td>
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<td>Mesenteric fat (g)</td>
<td>0.86 ± 0.05</td>
<td>0.98 ± 0.04</td>
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<tr>
<td>Subcutaneous fat (g)</td>
<td>2.58 ± 0.09</td>
<td>2.42 ± 0.07</td>
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<tr>
<td>Liver (g)</td>
<td>2.42 ± 0.10</td>
<td>2.02 ± 0.06**</td>
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<tr>
<td>Blood glucose (mg/dl)</td>
<td>154 ± 10</td>
<td>142 ± 8</td>
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<tr>
<td>TG (mg/dl)</td>
<td>49.9 ± 6.0</td>
<td>58.0 ± 5.1</td>
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<tr>
<td>FFA (mEq/l)</td>
<td>0.49 ± 0.06</td>
<td>0.30 ± 0.03*</td>
</tr>
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</table>

Data are expressed as the mean ± SE. *P < 0.05, **P < 0.01 vs. EPA (-).
Table II. Multivariate regression analysis for changes in plasma adiponectin concentrations after EPA treatment.

<table>
<thead>
<tr>
<th>Dependent variable</th>
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<tr>
<td></td>
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<td>$P$ value</td>
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<tr>
<td></td>
<td>coefficient</td>
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</tr>
<tr>
<td>∆ Adiponectin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>0.059</td>
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<td>∆ Waist circumference</td>
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<td>∆ SBP</td>
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<td>∆ FPG</td>
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<td>∆ TG</td>
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<td>∆ HDL-C</td>
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<td>∆ LDL-C</td>
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<tr>
<td>EPA treatment</td>
<td>0.378</td>
<td>0.018</td>
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</table>
Figure I
**Figure II**

The figure shows the mRNA levels of various genes under different conditions. The graphs display the levels of PAI-1, HB-EGF, MCP-1, IL-6, and Resistin in WT and ob/ob mice with or without EPA treatment.

- **PAI-1**: The graph shows a significant increase in mRNA levels with EPA treatment in both WT and ob/ob mice. The difference between WT and ob/ob is not statistically significant in EPA-treated mice.
- **HB-EGF**: EPA treatment significantly increases mRNA levels in WT mice and ob/ob mice, with no significant difference between the strains.
- **MCP-1**: EPA treatment significantly increases mRNA levels in WT mice, but not in ob/ob mice. There is no significant difference between the strains in EPA-treated mice.
- **IL-6**: EPA treatment increases mRNA levels in WT mice, but not in ob/ob mice. There is no significant difference between the strains in EPA-treated mice.
- **Resistin**: EPA treatment significantly increases mRNA levels in WT mice and ob/ob mice, with no significant difference between the strains.

The figure indicates that EPA treatment has a significant impact on the mRNA levels of these genes, with differences observed between WT and ob/ob mice. **n.s.** indicates no statistically significant difference.
Figure III

- **PAI-1**
  - mRNA levels (arbitrary unit)
  - EPA (-) vs EPA (+)
  - * indicates statistical significance

- **HB-EGF**
  - mRNA levels (arbitrary unit)
  - EPA (-) vs EPA (+)
  - * indicates statistical significance

- **MCP-1**
  - mRNA levels (arbitrary unit)
  - EPA (-) vs EPA (+)
  - n.s. indicates no significant difference

- **IL-6**
  - mRNA levels (arbitrary unit)
  - EPA (-) vs EPA (+)
  - n.s. indicates no significant difference

- **Resistin**
  - mRNA levels (arbitrary unit)
  - EPA (-) vs EPA (+)
  - * indicates statistical significance

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Figure IV
Figure V

A. Graph showing body weight (g) over weeks of age for different groups: HFD + EPA, HFD control, EPA control, and EPA HFD.

B. Graph showing serum levels (µg/ml) over weeks of age for the same groups.

C. Bar charts showing mRNA levels (arbitrary unit) for Adiponectin, TNFα, and F4/80 for the control and HFD groups with and without EPA treatment.

Legend:
- n.s.: not significant
- **: p < 0.01
- *: p < 0.05
- #: p < 0.1
Figure VI

A) TNFα mRNA levels

B) Adiponectin secretion

C) Adiponectin secretion and mRNA levels

- veh = vehicle
- TNFα = tumor necrosis factor alpha
- EPA = eicosapentaenoic acid
- IgG Ab = immunoglobulin G antibody
- n.s. = not significant
Figure VII