Fish Oil Increases Cholesterol Storage in White Adipose Tissue with Concomitant Decreases in Inflammation, Hepatic Steatosis, and Atherosclerosis in Mice

Viswanathan Saraswathi, Ling Gao, Jason D. Morrow, Alan Chait, Kevin D. Niswender, and Alyssa H. Hasty

Abstract

Although fish oil has hypolipidemic and antiatherosclerotic properties, the potential for white adipose tissue (WAT) to mediate these effects has not been studied. LDL-receptor deficient (LDLR<sup>−/−</sup>) mice were fed high fat, olive oil–containing diets supplemented with additional olive oil or with fish oil for 12 wk. Fish oil feeding significantly reduced plasma lipid levels. In contrast, lipid storage in WAT was increased in fish oil–fed mice as evidenced by increased total fat (P<0.05) and perigonadal WAT mass (P<0.05), increased cholesterol storage (P<0.001), and adipocyte hypertrophy. Despite increased adipose tissue mass, WAT-specific inflammation and insulin sensitivity were improved (P<0.05), concomitant with reduced macrophage infiltration. Furthermore, fish oil increased WAT and plasma levels of adiponectin. In addition, fish oil feeding decreased the formation of proinflammatory F<sub>2</sub>-isoprostanes, markers of oxidative stress (P<0.05). The increased WAT lipid storage in fish oil–fed mice was associated with reduced lipid accumulation in liver (P<0.05) and decreased atherosclerotic lesion area (P<0.05). Taken together, these data highlight the specific role of WAT in regulating dietary fish oil–mediated improvement in systemic lipid homeostasis and atherosclerosis. J. Nutr. 137: 1776–1782, 2007.

Introduction

Obesity is a growing worldwide epidemic and is one of the key features of the metabolic syndrome. Although increased adiposity represents a major risk factor for the development of type 2 diabetes and its associated cardiovascular complications, an emerging paradigm supports the view that adipose tissue dysregulation, characterized by impaired lipid storage and inflammatory cytokine production, may play a crucial role in the pathogenesis of insulin resistance and atherosclerosis (1). The atheroprotective effects of different therapeutic or dietary interventions due to their direct effects on WAT have not been extensively studied.

Fish oil, containing (n-3) fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is beneficial against a number of diseases including cardiovascular disease. Several clinical studies suggest that a higher consumption of fish and (n-3) fatty acids is associated with reduced coronary heart disease morbidity and mortality (2–6). Furthermore, fish oil is reported to reduce atherosclerosis in different animal models as well as in human subjects (7–9). Importantly, fish oil has been shown to be protective against dyslipidemia and atherosclerosis in LDL receptor-deficient (LDLR<sup>−/−</sup>) mice, a widely used animal model to study atherosclerosis (8). However, the exact mechanism involved in the hypolipidemic and antiatherosclerotic effects of fish oil is not known. Although reduced hepatic triglyceride synthesis and increased β-oxidation are known to contribute to the hypolipidemic effect of fish oil (10), the possible involvement of white adipose tissue (WAT) in mediating the hypolipidemic and the atheroprotective effects of fish oil remains unstudied.

Abbreviations used: CE, cholesterol ester; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LDLR<sup>−/−</sup>, LDL-receptor deficient; MAPK, mitogen activated protein kinase; NEFA, nonesterified fatty acid; PL, phospholipids; SAA, serum amyloid A; TC, total cholesterol; TG, triglycerides; TNF, tumor necrosis factor; WAT, white adipose tissue.

1 Supported by NIH grants GM15431, DK48831; and ES13125. A. H. Hasty was also supported by a Junior Faculty Award from the American Diabetes Association (1-04-JF-20), a Pilot and Feasibility Award from the Vanderbilt Digestive Diseases Research Center (VDDRC, DK088404), and a Scientist Development Grant from the AHA (0330011N). A. Chait is supported by NIH grants DK002456 and HL030086.

2 Author disclosures: V. Saraswathi, L. Gao, J. D. Morrow, A. Chait, K. D. Niswender, and A. H. Hasty, no conflicts of interest.

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WAT plays a crucial role in the regulation of systemic lipid homeostasis by storing excess energy in the form of triglycerides (TG). Several lines of evidence suggest that inflammation in WAT can modulate its storage and secretory functions (1,11,12). WAT-specific inflammation can be mediated by at least 2 mechanisms. First, adipocytes themselves can secrete certain inflammatory mediators such as IL-6 and plasminogen activator inhibitor (13). Second, the accumulation of macrophages in WAT in the obese state contributes to local inflammation (11,12). It is becoming clear that local inflammation in adipose tissue can modulate WAT insulin signaling, which in turn could modulate the lipid storage function of adipose tissue (1,14). Fish oil, a potent antiinflammatory agent, may therefore influence not only inflammation in WAT, but also downstream storage and/or secretory functions. Therefore, we hypothesized that fish oil may mediate its antiatherosclerotic effect in part by reducing WAT-specific inflammation, thereby modulating WAT storage and/or secretory functions.

Materials and Methods

**Animals and diets.** LDLR−/− mice used in the present study were originally purchased from Jackson Laboratory and were on the C57BL/6 background. Because LDLR−/− mice develop atherosclerosis only when fed a high fat diet, 2-3 mo old female mice were fed a high fat diet [AIN-93G diet (15,16); 39% of energy from fat, 0.5% cholesterol, Dyets supplemented with 6% olive oil or menhaden oil (fish oil containing 140 mg EPA and 95 mg DHA/g of oil) for 12 wk (Table 1). The fat mix used (209 g/kg) contained 45% coconut oil, 30% olive oil, 15% corn oil, and 10% soybean oil. Thus, both diets contained a base level of (209 g/kg) contained 45% coconut oil, 30% olive oil, 15% corn oil, and 10% soybean oil. Thus, both diets contained a base level of (209 g/kg), whereas the “fish oil” diet was supplemented with 60 g/kg 10% soybean oil. Therefore, we hypothesized that fish oil may modulate its antiatherosclerotic effect in part by reducing WAT-specific inflammation, thereby modulating WAT storage and/or secretory functions.

<table>
<thead>
<tr>
<th>Ingredient</th>
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<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
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<td>200</td>
</tr>
<tr>
<td>t-Cystine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Carbohydrates</td>
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<td></td>
</tr>
<tr>
<td>Sucrose</td>
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<td>100</td>
</tr>
<tr>
<td>Cornstarch</td>
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</tr>
<tr>
<td>Dextrose</td>
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<td>125</td>
</tr>
<tr>
<td>Fat</td>
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<td></td>
</tr>
<tr>
<td>Fat mixture</td>
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<td>209</td>
</tr>
<tr>
<td>Olive oil</td>
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<td>0</td>
</tr>
<tr>
<td>Menhaden oil</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Choline bitartrate</td>
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<td>2.5</td>
</tr>
<tr>
<td>Vitamins and minerals</td>
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<td></td>
</tr>
<tr>
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<td>10</td>
</tr>
<tr>
<td>Mineral mix AIN 83 #210025</td>
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<td>35</td>
</tr>
<tr>
<td>Antioxidant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-Butylhydroquinone</td>
<td>0.054</td>
<td>0.054</td>
</tr>
</tbody>
</table>

1 Fat mixture contained (g/kg): coconut oil, 450; olive oil, 300; corn oil, 150; soybean oil, 100.
2 (15).
3 (16).

**Plasma and tissue lipid analyses.** Lipids were extracted from plasma and tissue samples using the method of Folch-Lees (18). Individual lipid fractions were analyzed by the Lipid Core Laboratory of the Vanderbilt Mouse Metabolic Phenotyping Center, using GC, as previously described (19).

**Perigonadal WAT cholesterol measurements.** WAT samples were homogenized in a 3:1 ethanol:ether mixture as described by Johnston et al. (20). The lipid samples were solubilized with 3 mL of 2-propanol and then assayed using Cholesterol Reagent from Raichem.
**Oil Red O staining of liver sections for neutral lipids.** Frozen liver sections were cut and stained with Oil Red O for 4 h. Sections were counterstained with hematoxylin for 3 min.

**Atherosclerotic lesion area quantification.** Cross-sections through the aortic root were obtained from frozen hearts according to the method of Paigen et al. (26). Sections were stained with Oil Red O to detect neutral lipids. Lesion area was quantified using Kinetic Histomex 6 imaging and analysis software.

**Statistical analysis.** Student's t test was used to compare the olive oil and fish oil–fed groups. Differences were considered significant at P < 0.05.

**Results**

**Body weight and plasma components in LDLR−/− mice.** The body weights of the olive oil and fish oil–fed mice before the start of the diet were 19.1 ± 1.3 g and 18.5 ± 1.2 g, respectively. After feeding the diets for 12 wk, the groups gained similar amounts of weight (Table 2). Plasma insulin and glucose concentrations did not differ between the olive oil and the fish oil–fed mice. In mice fed the fish oil diet, plasma NEFA concentrations did not differ between the olive oil and the fish oil diets, whereas TG, the major lipid fraction stored in the adipose tissue, was greater in fish oil–fed mice than in olive oil–fed mice (Table 2). In addition, WAT lipid storage and adiponectin secretion.

**WAT lipid storage and adiponectin secretion.** Whereas body weight was similar in the 2 groups, the total fat mass was greater in fish oil–fed mice than in olive oil–fed mice (Table 2). In addition, the perigonadal WAT mass was greater in fish oil–fed mice (P < 0.05). Analysis of specific lipids stored in perigonadal WAT showed that concentrations of PL, FFA, and CE were lower, per gram of tissue mass, in the fish oil group (P < 0.05), whereas TG, the major lipid fraction stored in the adipose tissue, did not differ between the groups (Table 3). Surprisingly, the level of TC in WAT of fish oil–fed mice was 5-fold that of the olive oil–fed group (P < 0.001). The increased perigonadal WAT mass in fish oil–fed mice was coincident with a greater mean adipocyte size (90.4 ± 4.8 µm²) than in olive oil–fed mice (68.0 ± 1.6 µm², P < 0.05, Fig. 1A–C), suggesting that fish oil effects on WAT mass are mediated in part by its ability to induce adipocyte hypertrophy, possibly due to increased cholesterol storage. The levels of adiponectin protein in WAT were increased to 90% higher in fish oil compared with olive oil–fed mice (Fig. 1D). This was accompanied by elevated plasma adiponectin for all molecular weight forms (Fig. 1E and F).

**Adipose tissue macrophage accumulation, inflammation, and insulin resistance.** Despite the significant increase in perigonadal WAT mass in the fish oil–fed mice, expression in WAT of macrophage markers such as MAC-1 (52%, P < 0.05) and CD68 (76%, P < 0.01) were lower than in mice fed olive oil. In addition, fish oil–fed mice showed downregulation of inflammatory markers such as TNFa (65%, P < 0.05), MMP3 (80%, P < 0.01), and SAA3 (58%, P < 0.01) in WAT (Fig. 2A). Because TNFa is an important inflammatory marker in adipose tissue, we further determined protein concentrations of this cytokine in WAT samples. The TNFa protein concentration was 29% lower in fish oil–fed mice than in olive oil–fed mice. Furthermore, WAT from mice supplemented with fish oil had less phosphorylation of MAPK pathway members such as ERK1/2 (61%) and p38 MAPK (88%) (P < 0.05). Phosphorylated JNK levels were not different between the groups (Fig. 2B). In addition to reduced local inflammatory markers in WAT, circulating concentrations of SAA were lower in the fish oil–fed mice (7.5 ± 0.7 mg/mL) compared with olive oil–fed mice (15.6 ± 1.8 mg/mL) (P < 0.001), suggesting that not only WAT-specific inflammation but also systemic inflammatory responses can be improved by fish oil supplementation. Finally, fish oil feeding improved insulin signaling in WAT as evidenced by the degree of insulin receptor phosphorylation in 5-h food-deprived fish oil–fed mice (5.22 ± 0.43 units/mg protein) compared with olive oil–fed mice (3.81 ± 0.35 units/mg protein) (n = 6; P < 0.05).

**Hepatic lipid accumulation and atherosclerotic lesion formation.** Increased WAT lipid storage was also associated with reduced hepatic lipid content as visualized by Oil Red O staining (Fig. 3A and B). Analysis of the liver lipid profile by GC confirmed lower concentrations of CE (43%, P < 0.01) and TG (20%, P < 0.05) in mice fed fish oil than in those fed olive oil (Table 3). However, fish oil–fed mice had a liver PL concentration 24% greater than that of olive oil–fed controls (P < 0.01). Analysis of atherosclerotic lesion area in aortic root sections revealed that the degree of lesion formation was significantly increased in fish oil–fed mice (46% (P < 0.001), 46% (P < 0.05), respectively, in the plasma of fish oil–fed mice. 2 To convert from mg/g to mg/L, multiply by 173; to convert from mg/dL (glucose) to mmol/L, divide by 18 and to convert from mg/dL to mmol/L, multiply by: 0.0113 for TG, 0.02586 for TC, CE, and UC, and 0.01416 for PL.

**TABLE 2** Body weight, fat mass, and plasma lipid concentrations in LDLR−/− mice fed fish oil or olive oil for 12 wk.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Olive oil</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>29 ± 1</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>Total fat mass, g</td>
<td>2.48 ± 0.28</td>
<td>4.97 ± 0.91*</td>
</tr>
<tr>
<td>Perigonadal fat mass, g</td>
<td>1.05 ± 0.13</td>
<td>1.44 ± 0.10*</td>
</tr>
</tbody>
</table>

**TABLE 3** Liver and WAT lipid concentrations in LDLR−/− mice fed fish oil or olive oil for 12 wk.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Olive oil</th>
<th>Fish oil</th>
<th>Olive oil</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL</td>
<td>15.8 ± 0.8</td>
<td>19.6 ± 0.9</td>
<td>1.4 ± 0.2</td>
<td>0.9 ± 0.1*</td>
</tr>
<tr>
<td>FFA</td>
<td>4.7 ± 0.1</td>
<td>4.7 ± 0.3</td>
<td>1.6 ± 0.4</td>
<td>0.7 ± 0.02*</td>
</tr>
<tr>
<td>TG</td>
<td>74 ± 5</td>
<td>59 ± 4*</td>
<td>409 ± 24</td>
<td>355 ± 31</td>
</tr>
<tr>
<td>CE</td>
<td>32.8 ± 2.1</td>
<td>18.7 ± 2.5</td>
<td>1.2 ± 0.3</td>
<td>0.2 ± 0.06*</td>
</tr>
<tr>
<td>TG</td>
<td>36.0 ± 2.3</td>
<td>22.1 ± 2.5</td>
<td>9.4 ± 1.4</td>
<td>47.0 ± 4.5*</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM; n = 4–6/group. Different from olive oil group, *P < 0.05, **P < 0.01 or ***P < 0.001.

2 To convert from mg/g to mg/L, multiply by 173; to convert from mg/dL (glucose) to mmol/L, divide by 18 and to convert from mg/dL to mmol/L, multiply by: 0.0113 for TG, 0.02586 for TC, CE, and UC, and 0.01416 for PL.

3 For liver TC, the values of CE and unesterfied cholesterol were added, whereas for WAT, TC was measured as described in text.

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lower in the fish oil–fed mice (63,021 ± 8343 μm²/section) than in olive oil–fed mice (100,287 ± 7041 μm²/section) (n = 13, P < 0.05, Fig. 3C and D).

**Formation of F2-, F3- and F4-isoprostanes.** Fish oil feeding resulted in a lowering of the concentrations of F2-isoprostanes by 54% (P < 0.001) in liver, 35% (P < 0.05) in heart, and 59% (P < 0.01) in WAT (Fig. 4A). In fish oil–fed mice, formation of F3 isoprostanes (Fig. 4B) was increased to 11.7-fold (P < 0.001) in liver, 2.1-fold (P < 0.05) in heart, and 6.3-fold (P < 0.001) in WAT compared with those of olive oil–fed mice. Similarly, F4-isoprostanes (Fig. 4C) were increased to 5.2-fold (P < 0.001) in liver, 2.2-fold (P < 0.001) in heart, and 4.0-fold (P < 0.001) in WAT.

**Discussion**

In the current study, we have demonstrated that supplementation of a high fat–olive oil–containing diet with fish oil invokes an antiinflammatory effect as evidenced by reduced macrophage infiltration and inflammatory gene expression in WAT, with a concomitant improvement in WAT insulin signaling. This is associated with increased lipid storage, in particular, cholesterol storage within WAT, associated with reduced liver and plasma lipids, as well as ameliorated atherosclerotic lesion formation.

An interesting finding of the present study is that total adiposity and perigonadal WAT mass were significantly elevated upon fish oil feeding. Fish oil has been shown to have differential effects with regard to adiposity in different models. For example, fish oil supplementation has been shown to be negatively associated with adiposity in wild type C57BL/6J mice and Wistar rats (27,28). In contrast, EPA, one of the (n-3) fatty acids present in fish oil, preserves WAT mass in cancer cachexia (29) and has been shown to increase adiposity in ICR mice (30). The increased adiposity in the ICR mice was associated with improved insulin sensitivity and decreased hepatic steatosis. Consistent with this report, our current study demonstrates that although fish oil feeding increased WAT mass in LDLR−/− mice, it remarkably reduced hepatic steatosis and atherosclerosis. Thus, although fish oil can increase or decrease adipose tissue mass depending upon the model and dietary regimen used, it appears to universally improve metabolic outcomes.

It is well-established that adipose tissue contains large amounts of cholesterol and performs a buffer function for circulating cholesterol (31–33). One of the most striking findings of our study was the observation that the total cholesterol in perigonadal WAT was significantly increased in fish oil–fed mice. These data suggest that improved cholesterol storage in WAT can be associated with reduced total and free cholesterol in plasma, and cholesterol esters in liver, and that this may be one mechanism by which fish oil exerts its hypolipidemic and antiatherosclerotic effects in LDLR−/− mice. Despite the fact that WAT is the major storage site of unesterfied cholesterol in the body (34), little attention has been paid to the role of WAT in cholesterol homeostasis. Vassiliou and McPherson (35) reported that cholesterol ester transfer protein (CETP) expressed by adipocytes is involved in selective extraction of cholesterol ester from HDL; however, mice do not express CETP. It has also been
reported that endocytosis of VLDL via VLDLR occurs in differentiated 3T3-L1 preadipocytes (36) although the role of VLDLR in cholesterol uptake by adipocytes in vivo is not known. Both improved insulin signaling and dietary (n-3) fatty acids have been shown to increase hepatic uptake of HDL-cholesterol via enhanced expression of scavenger receptor class B, type 1 (SR-B1) (37,38). Insulin can also induce translocation of SR-B1 from intracellular sites to the plasma membrane of adipocytes (39). In this study, we detected improved insulin signaling in WAT from fish oil–fed mice. Thus, it is possible that by improving insulin signaling, fish oil may also increase SR-B1 mediated cholesterol uptake in WAT. Although it is tempting to speculate that fish oil has a direct effect on adipose tissue cholesterol storage, it is also possible that the primary effect of fish oil on cholesterol metabolism occurs at the level of the liver. Future studies are needed to determine how fish oil mediates cholesterol distribution between the liver and adipose tissue.

It was previously shown that increased adiposity is associated with increased macrophage infiltration into WAT, and that these macrophages are a major contributing factor to local inflammatory cytokine production (11,12). In our study, fish oil feeding decreased macrophage and inflammatory markers in fat despite increased adiposity. Thus, factors other than adipose tissue mass may contribute to macrophage recruitment to WAT. Todoric et al. (40) also reported that fish oil reduces macrophage infiltration and inflammation in WAT in obese diabetic mice, although they did not report differences in WAT mass in their model. The mechanisms by which (n-3) fatty acids decrease macrophage infiltration into WAT are not known. Our previous studies indicated that plasma lipids do not impact macrophage recruitment to WAT (41); thus, the hypolipidemic effects of fish oil are likely not responsible for this effect. More studies are needed to determine how dietary fatty acids can directly impact adipose tissue and lead to altered macrophage recruitment and inflammation.

Emerging evidence suggests that fish oil exerts its beneficial effects via adipose tissue by increasing the secretion of adiponectin, an antiinflammatory and antiatherogenic adipokine (28,42). Our current data support this observation (Fig. 1D–F). It is also thought that adiponectin secreted from adipose tissue may provide a direct link between adipose tissue function and protection against cardiovascular diseases (43). Thus, fish oil may mediate its antiatherosclerotic effects not only by improving WAT lipid storage but also by promoting appropriate adipokine secretion.

To determine the potential metabolites involved in mediating the effects of fish oil, isoprostanes, the prostaglandin-like non-enzymatic oxidation products, were measured in different tissues. Our data demonstrate that the formation of F2-isoprostanes from arachidonic acid is significantly reduced in fish oil–fed animals (Fig. 4A). These metabolites have been used as indicators of oxidative stress in various clinical conditions including atherosclerosis (44,45). Interestingly, our data also provide evidence for the increased formation of both F3- and F4-isoprostanes derived from EPA and DHA, respectively, upon fish oil feeding (Fig. 4B and C). Evidence suggests that F3-isoprostanes are biologically
In conclusion, our data suggest that fish oil can mediate its hypolipidemic and antiatherosclerotic effects in part via its anti-inflammatory properties, with a concomitant increase in WAT adipose tissue mass. Although obesity is an independent risk factor for the development of metabolic syndrome, our findings suggest that preservation of adipose tissue mass may be beneficial as a protection against dyslipidemia, hepatic steatosis and atherosclerosis.

**FIGURE 4** Isoprostane formation in LDLR−/− mice fed fish oil or olive oil for 12 wk. Isoprostane levels were assessed in liver, heart, and WAT. F₂-isoprostanes (A), F₃-isoprostanes (B), and F₄-isoprostanes (C). Values are means ± SEM (n = 6). Different from olive oil group, *P < 0.05, #P < 0.01, or ^P < 0.001.

**Literature Cited**


