Fish Oil Derived Omega 3 Fatty Acids Suppress Adipose NLRP3 Inflammasome Signaling in Human Obesity

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Context: The NLRP3 inflammasome is a multiprotein danger-sensing complex that serves as a critical link between obesity-related adipose inflammation and insulin resistance and has been shown in animal models to be inhibited by fish oil-derived long chain omega-3 polyunsaturated fatty acids (n-3 PUFA).

Objective: We conducted a clinical trial and in vitro experiments to test our hypothesis that n-3 PUFA suppress NLRP3 inflammasome in human obesity through downregulation of inflammasome gene expression in adipocytes and macrophages.

Design: Placebo-controlled clinical trial and in vitro coculture experiments with primary human adipocytes (from biopsy specimens) and human THP-1 monocyte-derived macrophages treated with eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA) vs vehicle control.

Setting: General community, research laboratory.

Patients and Other Participants: Obese (body mass index $\geq 30$ kg/m$^2$), nondiabetic males and females age 18 to 50. N = 25.

Interventions: Clinical trial: Eight-week treatment with 4 g Lovaza (EPA and DHA) or placebo. Cells culture: EPA and/or DHA at 100 $\mu$g/mL or vehicle control in culture medium.

Main Outcome Measures: Adipose tissue or adipocyte/macrophage mRNA expression of IL-1$\beta$ and IL-18 and circulating IL-18 levels.

Results: Treatment of obese human subjects with fish oil supplements reduced expression of adipose inflammatory genes including inflammasome-associated IL-18 and IL-1$\beta$ and circulating IL-18 levels. Both EPA and DHA reduced inflammasome gene expression in obese human adipose and human adipocyte and macrophages.

Conclusions: N-3 PUFA reduce NLRP3 inflammasome in human adipose through downregulation of gene expression in adipocytes and monocytes/macrophages and has potential as nutritional therapeutic agent in prevention of obesity-related inflammation.

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Obesity is well-recognized as a low-grade inflammatory state; inflammation in metabolically active tissues, including adipose tissue, can contribute to obesity-related metabolic dysfunction and cardiometabolic disease. Factors including expansion of the adipose tissue depot and free fatty acid excess lead to activation of inflammatory pathways that recruit proinflammatory macrophages to adipose tissue. Together, adipocytes and activated macrophages interact to increase secretion of inflammatory cytokines (including TNFα and IL-1β) that directly and indirectly act upon insulin signaling pathways to promote insulin resistance [1–13].

A critical link between obesity-induced adipose inflammation and insulin resistance is the NLRP3 [nucleotide-binding and oligomerization domain-like receptor, leucine–rich repeat and pyrin domain-containing 3] inflammasome—a multiprotein danger-sensing complex composed of NLRP3, adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) and the cysteine protease caspase-1 [14]. The NLRP3 inflammasome is a sensor of metabolic stress “danger signals,” which include exogenous and endogenous signals, including free fatty acids and fatty acid derivatives [15], reactive oxygen species, and ATP. Activation of the inflammasome is a two-step process. Phase 1 (priming) occurs when toll-like receptor 4 is stimulated via saturated fatty acids, lipopolysaccharide, or inflammatory cytokines (e.g., TNFα and IL-6) and triggers the nuclear factor κB (NF-κB) pathway. This results in increased production of pro-IL-1β and pro-IL-18, as well as increased NLRP3 expression. The second phase is initiated by recognition of danger signals, upon which NLRP3 interacts with ASC, which then binds procaspase-1 forming the inflammasome complex [16]. This formation leads to cleavage of procaspase-1 to active caspase-1, which subsequently cleaves the prointerleukins to active IL-1β and IL-18 [17–19]. Inflammasome activation has been implicated in a multitude of inflammatory, metabolic, and neoplastic diseases [20].

IL-1β results in insulin resistance through activation of NF-κB and JNK signaling [21], which promotes serine phosphorylation of IRS1, targeting it from destruction instead of the tyrosine-phosphorylation required for insulin signaling [22]. Although it is less clear how IL-18 impacts insulin signaling, a human study showed that macrophages from subjects with type 2 diabetes had higher IL-18 secretion than healthy controls, and that treatment with the insulin-sensitizing agent metformin reduced IL-18 secretion [23].

Expression of NLRP3 components have been found to be increased in adipose tissue of obese humans and mice [24, 25] and weight loss in humans resulted in reduced expression in white adipose tissue [25]. Several animal models have demonstrated that absence of inflammasome components is associated with protection from obesity-related insulin resistance [25–27]; though there is inconsistency in this response as another group has shown that obesity-related inflammation was not associated with increased caspase-1 cleavage and Nlrp3 null mice were not protected from adipose tissue inflammation [28]. Lending further credence to a likely role of NLRP3 in obesity and metabolic dysregulation, genome wide association studies (thus far reported in Chinese Han populations) have shown association of an NLRP3 single nucleotide polymorphism with obesity [29] and with type 2 diabetes and insulin resistance [30, 31].

Several studies utilizing animal and in vitro models have demonstrated that saturated fatty acids are potent activators of the NLRP3 inflammasome [15, 16, 32–34]. In contrast, unsaturated fatty acids, particularly fish oil-derived long chain omega-3 polyunsaturated fatty acids (n-3 PUFA), may inhibit the inflammasome [35]. A general anti-inflammatory effect of fish oils in obesity-induced adipose inflammation has been shown repeatedly in animal and human models [36–39]. In 3T3-L1 adipocytes, coculture with macrophages from mice fed a high-fat diet (HFD) with fish oil had reduced IL-1β, IL-18, and caspase-1 expression, as well as reduced caspase-1 activity compared with HFD without fish oil [35]. To date, there have been no published studies on the impact of n-3 PUFA on the inflammasome in human or human cell culture models.

We conducted a clinical trial of n-3 PUFA supplementation on adipose tissue NLRP3 inflammasome activity in obesity as well as ex vivo adipose and coculture experiments with
human primary adipocytes and THP-1 monocyte-derived macrophages to demonstrate the effects of n-3 PUFA on the NLRP3 inflammasome.

1. Materials and Methods

A. Clinical Trial

All clinical studies described were performed with approval of the University of Pennsylvania (UPenn) Institutional Review Board after written informed consent was obtained from all research participants and was registered at clinicaltrials.gov (NCT02010359). We recruited healthy obese subjects age 18 to 50 with body mass index $\geq 30 \text{mg/m}^2$ from the general community for the Fish Oils and Adipose Inflammation Reduction study. A total of 25 subjects (13 Lovaza, 12 placebo) completed the study and are included in these analyses.

Exclusions included diagnosis of diabetes (glucose fasting $> 126 \text{mg/dL}$, or random $> 200 \text{mg/dL}$, or use of any antidiabetic agent), use of any lipid-lowering medications, inflammatory disease or use of anti-inflammatory agents (including over the counter analgesics, and inhaled/topical/nasal steroids), habitual fish intake of $>3$ servings/month and/or unwilling to eliminate all fish intake during the study, fish oil supplement intake within 6 months, conditions that would contraindicate use of Lovaza (liver dysfunction, anemia, arrhythmia, coagulopathy), pregnancy, and breastfeeding.

Participants who met initial criteria by telephone/E-mail interview underwent a screening visit with medical history, physical examinations, electrocardiogram, urine pregnancy test in females, and laboratories for fasting glucose, complete blood count, liver function panel, and lipids. If eligible, they returned for a randomization visit that included fasting blood draw and gluteal adipose biopsy, as previously reported [40, 41]. Briefly, subcutaneous adipose samples were collected by core needle aspiration through a 4-mm gluteal incision. Subjects were assigned a subject ID and randomized to Lovaza 4 g/d or placebo in a double-blind manner (managed via UPenn Investigational Drug Service). Subjects returned for study completion visit scheduled after 8 to 10 weeks of study drug, which again involved fasting blood draw and adipose biopsy from contralateral gluteal site. Adipose tissue and blood samples were aliquoted and stored at $-80^\circ\text{C}$ until further analysis.

B. Measurement of Circulating Metabolites and Inflammatory Markers

Fasting glucose insulin and lipid measurements were performed by the Translational Core Laboratory of the University of Pennsylvania using the Roche Cobas c311 (Roche, Basel, Switzerland). HOMA-IR was calculated using the following formula: fasting insulin level (\(\mu\text{IU/mL}\)) $\times$ fasting glucose level (in mM)/22.5. Inflammatory markers [IL-6, monocyte chemoattractant protein-1 (MCP-1), IL-18, and IL-1\(\beta\)] and high molecular weight (HMW) adiponectin were quantified by enzyme-linked immunosorbent assay run in duplicate according to manufacturer’s directions (R&D Systems, Minneapolis, MN) [42–45] and read on an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT). Coefficients of variance were $<15\%$ for all samples and assays run.

C. Human Adipose Ex Vivo Experiments

Human abdominal visceral and subcutaneous adipose tissue collected from bariatric surgery performed on obese subjects was supplied by the Penn Human Adipose Resource Center under a protocol approved by the UPenn Institutional Review Board. The tissue was dissected into 1-g samples and plated on a 12-well plate. The tissue was then incubated in adipocyte growth media containing 100 $\mu\text{M}$ eicosapentaenoic acid (EPA), 100 $\mu\text{M}$ docosahexaenoic acid (DHA), a combination of 50 $\mu\text{M}$ EPA and 50 $\mu\text{M}$ DHA, or vehicle [dimethyl sulfoxide (DMSO)] for 48 hours.

D. Primary Human Adipocyte Isolation

Primary adipocytes were isolated from fresh abdominal adipose tissue collected from bariatric surgery provided by the Penn Human Adipose Resource. The adipose tissue was digested
with collagenase (1 mg/mL) in serum free DMEM/F12 culture media using the protocol described with minor modifications [27]. The digested tissue was then filtered and resuspended in 10 mL adipocyte growth media (DMEM/F12 with Primocin (InvivoGen, San Diego, CA), biotin (4 mg/L), pantothenate (8 mg/L), epidermal growth factor (5 g/L), fibroblast growth factor (5 µg/L), and 10% fetal bovine serum (Sigma-Aldrich Corp., St. Louis, MO). The suspension was then centrifuged and the cell pellet was resuspended in OP media and plated at 40,000 cells/cm². The cells were grown to 80% confluence before being differentiated as previously described [41].

E. THP1 Macrophage Differentiation and Polarization

THP1 monocytes commercially purchased (Sigma-Aldrich Corp.) were maintained in culture at $1 \times 10^6$ cells/mL in THP1 culture media [RPMI medium supplemented with L-glutamine (2 mM), HEPES (10 mM), sodium pyruvate (1 mM), D-glucose (625 mg/L), betamercaptaethanol (100 µL/L), and 10% fetal bovine serum]. Differentiation was achieved by plating $3 \times 10^3$ cells/cm² in THP1 culture media with 200 µM phorbol 12-myristate 13-acetate (PMA) for 72 hours. At this point the PMA was removed and the cells were cultured in PMA-free THP1 culture media for 5 days before being polarized. Preliminary time course and dose response experiments determined these culture conditions to produce neutral, nonpolarized (M0) macrophages (data not shown). The M0 macrophages were then polarized to M1 classically activated, proinflammatory macrophages by culturing the cells in THP1 culture media with lipopolysaccharide (100 ng/mL) and INF-γ (20 ng/mL) for 24 hours.

F. Primary Adipocyte/Macrophage Co-Culture Experiments

Primary adipocytes were cultured with classically activated THP1 macrophages using the Costar Transwell coculture system (Corning Life Sciences, Corning, NY). Prior to coculture, primary adipocytes were plated in the lower compartment of a 12-well plate, at $1 \times 10^5$ cells/mL and differentiated. In a separate 12-well plate, without any cells in the lower compartment, $1 \times 10^5$ THP1 cells were plated, differentiated, and polarized in the trans well insert upper compartment. Once both primary adipocytes and THP1 macrophages were differentiated and polarized, the trans well inserts containing classically activated macrophages were placed inside of the lower compartment containing adipocytes.

Primary adipocyte and THP1 macrophage cocultures were treated with fish oil derived LC n-3 PUFA EPA and DHA dissolved in DMSO. Both cell types were treated with 100 µM EPA, 100 µM DHA, a combination of 50 µM EPA and 50 µM DHA, or vehicle (DMSO) for 48 hours. Treatment timing and concentration was determined based upon preliminary dose response and time course experiments (data not shown).

G. RNA Extraction, cDNA Synthesis, and Quantitative PCR

Whole adipose tissue was homogenized, or, for in vitro experiments adipocytes and monocytes were washed and lysed for RNA isolation using TRIzol reagent (Life Technologies, Foster City, CA). RNA concentration and quality were determined with an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc.), and cDNA was prepared using the High Capacity cDNA Reverse transcription kit (Life Technologies). Expression of genes was determined by quantitative real-time PCR using TaqMan Universal PCR MasterMix and primers and probes from Life Technologies and the QuantStudio6 quantitative PCR system. Gene expression levels were normalized to the housekeeping gene GAPDH, and relative expression was determined using the $2^{-∆∆Ct}$ method [46].

H. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 software. The statistical significance of difference between control and cocultured cells was determined by Student’s t test or Mann-Whitney U test depending on whether normality could be assumed. When
measuring the statistical difference between EPA and DHA treated cells, one-way ANOVA was used. A two-way ANOVA was used to determine the statistical significance between gene expression in visceral and subcutaneous tissue in ex vivo experiments.

2. Results

A. Fish Oil Supplements Reduced Adipose Tissue Expression of NLRP3 Inflammasome Genes and Circulating IL-18 in Human Obesity

Demographic information and measurements of circulating metabolic and inflammatory markers for all subjects at randomization and study completion is presented in Table 1. Both groups had similar age and sex. Although the placebo group had a greater number of subjects self-identified as black, and less as white, the proportions in these racial groups by treatment arm did not reach statistical significance between treatment groups. Body mass index and metabolic parameters (glucose, lipids, HMW adiponectin) were similar between groups and did not change significantly during study. Notably, while the classic inflammatory marker IL-6 and the inflammatory adipochemokine MCP-1 were not altered by Lovaza, there was a modest reduction in the NLRP3 inflammasome-associated markers IL-18 (36.2 ± 16.7 to 34.3 ± 16.9, P < 0.0001). IL-1β levels in plasma were below the lower limits of detection in our assay and thus could not be compared.

In contrast, comparison of adipose tissue pre- and posttreatment demonstrated a marked decrease in expression of IL-6, MCP-1 (CCL2), IL-7R, and CX3CL1 (adipose tissue cytokines and chemokines associated with obesity) in the Lovaza group (Fig. 1a). Lovaza also resulted in significant reduction in adipose tissue expression of the NLRP3 inflammasome genes IL-18, IL-1β, and CASP-1 though NLRP3 did not change (Fig. 1b).

B. Both EPA and DHA Reduce NLRP3 Gene Expression in Human Adipose From Obese Subjects

To determine whether inflammasome gene expression was suppressed by EPA or DHA, we designed ex vivo experiments treating obese human subcutaneous and visceral adipose tissue

Table 1. Demographic, Metabolic, and Inflammatory Parameters at Baseline and After 8 Weeks of Study Drug

|                        |Lovaza (n = 13) | Placebo (n = 12) |
|------------------------|---------------|-----------------|
|                        |Randomization | Completion      |Randomization | Completion      |
|Age (y)                |35.9 (9.41)   |35.9 (9.12)      |37.4 (8.36)   |35.7 (6.42)      |
|% Female                |62             |67              |
|% White                 |69             |23              |
|% African American      |23             |42              |
|Body mass index (kg/m²) |36.3 (5.61)   |35.9 (5.12)      |35.7 (6.42)   |36 (5.9)         |
|Glucose, mg/dL         |86.5 (11.6)   |79.3 (11.8)      |86.8 (10.2)   |84.3 (7.62)      |
|Insulin, uIU/mL         |17.6 (4.97)   |17.0 (6.82)      |16.2 (3.31)   |17.8 (4.91)      |
|HOMA-IR                |3.14 (0.9)    |3.53 (1.52)      |3.3 (1.17)    |3.45 (1.22)      |
|Triglycerides, mg/dL   |139 (108)     |106 (62.9)       |92.9 (35.1)   |85.4 (37)        |
|LDL, mg/dL             |111 (24.5)    |116 (21.6)       |119 (26.8)    |115 (37.4)       |
|HDL, mg/dL             |47.3 (20)     |49.2 (22)        |49.1 (9.21)   |50.2 (7)         |
|HMW adiponectin, µg/mL |1.79 (1.56)   |1.86 (1.71)      |2.44 (2.07)   |2.5 (2.21)       |
|IL-6, pg/mL            |1.72 (1.17)   |2.55 (2)         |2.03 (1.51)   |2.02 (1.91)      |
|MCP-1, pg/mL           |128 (28.2)    |133 (29.1)       |134 (52.2)    |123 (54.9)       |
|IL-18, pg/mL           |36.2 (16.7)   |34.3 (16.9)      |34 (9.83)     |38.5 (17.2)      |

Data are presented as mean (SD) for continuous variables or as a percentage for categorical variables.

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein.

\(aP < 0.05\) between groups.

\(bP < 0.05\) within group over time.
obtained during bariatric surgery with 100 μM DHA, 100 μM EPA, combination of 50 μM DHA + 50 μM EPA or DMSO vehicle control. Both EPA and DHA treatment of adipose tissue diminished gene expression of IL-18, IL-1β, NLRP3, and caspase 1 as shown in Fig. 2, though effects were overall more pronounced in subcutaneous adipose tissue. Expression changes were more likely with DHA as compared with EPA and were not additive, suggesting at least partial overlap of mechanistic pathways.

C. Pretreatment With EPA and DHA Reduces NLRP3 Inflammasome Gene Expression in Both Adipocytes and Macrophages in Coculture

To determine contribution of cell types found in adipose tissue to our observed effects on the inflammasome, we designed a coculture system with primary human adipocytes and human THP-1 monocyte-derived macrophages. Differentiated adipocytes and M1-activated macrophages were cultured together using the Transwell system for 48 hours and treated with 100 μM EPA, 100 μM DHA, 50 μM EPA + 50 μM DHA, and vehicle control and gene expression evaluated in both cell types. Coculture led to increased expression of the NLRP3 inflammasome genes IL-18, IL-1β, CASP-1, and NLRP3 in both cell types. In primary adipocytes, both EPA and DHA led to significant reduction in IL-18, CASP-1, and IL-1β expression, though NLRP3 expression was unchanged (Fig. 3). Similarly, M1 macrophage gene expression of inflammasome genes was reduced by EPA and DHA, though effects of DHA were larger in this cell type (Fig. 4). These results suggest involvement of adipocytes and adipose-tissue macrophages in the response to N-3 PUFA.

3. Discussion

These studies, which assess the role of n-3 PUFA on the NLRP3 inflammasome in human adipose, demonstrate inhibition of NLRP3-associated gene expression by EPA and DHA in adipocytes and macrophages. In a placebo-controlled clinical trial of obese participants, there was a decrease in adipose tissue NLRP3 gene expression and circulating levels of IL-18, which is a downstream product of NLRP3 signaling.

In recent years, n-3 PUFA fish oil supplements have been widely used and recommended for prevention and treatment of disease. In addition to the well-known clinical utility of n-3 PUFA on lowering triglyceride levels, there is also evidence, such as the PREDIMED study of the Mediterranean diet, demonstrating protection from cardiovascular mortality with increased intake of marine n-3 PUFA [47] and the American Heart Association supports
addition of fish to the diet for cardiovascular prevention in recent guidelines [48]. Yet, over several decades, overall data from clinical trials assessing effect of n-3 PUFA on primary and secondary prevention of cardiovascular disease is conflicting [49–56]. Thus, better understanding of the pleiotropic effects of n-3 PUFA to guide evidence-based recommendations on their use is critical.

Mechanisms by which n-3 PUFA exert their anti-inflammatory effects is also not fully clarified. Recent evidence has highlighted mechanisms involving EPA-derived resolvin E1 and DHA-derived protectin D1 [57]. In high-fat–fed mice, there are decreased levels of protectin in adipose and skeletal muscle, whereas transgenic restoration prevents the HFD–induced insulin resistance [58]. EPA and DHA also bind to G protein-coupled receptor (GPR) 120 and GPR40, and inhibit NF-κB and JNK through which effects on inflammasome signaling may also be mediated [59]. One study in monocyte cell culture models found that treatment with a GPR120 and GPR40 agonist decreased caspase-1 activation and secretion of IL-1β and IL-18, and short hairpin RNA targeting GPR40 and GPR120 mRNA partially abolished DHA and EPA effects on NLRP3 inflammasome activation. Further, deficiency of beta arrestin 2 (Arrb2), a downstream product of GPR120 signaling, using Arrb2 −/− mice only partially inhibited DHA activity suggesting other pathways may be involved [60]. There is minimal data on mechanisms by which n-3 PUFA interact with the NLRP3 inflammasome in adipose, though one study in murine 3T3-L1 adipocytes suggested that inhibition of IL-18, IL-1β, and caspase-1 by DHA and EPA were predominantly dependent upon adiponectin, as they were largely attenuated by use of an adiponectin-neutralizing antibody [35].
Our studies highlight the utilization of human adipocytes and macrophages and the experimental design of the coculture model, which better replicates the complex interactions in adipose tissue than isolated cell types. Further, we acquired adipose tissue from the natural state of human obesity, to increase applicability to real-world situations in which fish oil supplements would be clinically used. Finally, we incorporated a placebo controlled clinical trial with pre- and postexposure measurements to attempt to isolate the differences attributable to the EPA and DHA supplement.

We acknowledge that there are limitations to our studies, including the lack of mechanistic data. Further, our focus on adipocytes and macrophages specifically may have missed contribution from the myriad other cell types populating the adipose depot (vascular and neural cells, fibroblasts, stem cells, monocytes, T lymphocytes, and other immune cells) [61]. Conversely, as the stromal vascular fraction from adipose biopsy was used to create the primary adipocyte cell lines, there may be some contribution of immune cells to these models. The fish oil preparation in the clinical trial was a mixture of EPA and DHA that did not allow exploration of their individual effects, though the *in vitro* studies were designed to test specific effects of each agent. Finally, in this short-term 8-week study, determination of long-term effects and outcomes was not feasible.

Despite these limitations, our studies add to the emerging body of knowledge regarding the utilization of human adipocytes and macrophages and the experimental design of the coculture model, which better replicates the complex interactions in adipose tissue than isolated cell types. Further, we acquired adipose tissue from the natural state of human obesity, to increase applicability to real-world situations in which fish oil supplements would be clinically used. Finally, we incorporated a placebo controlled clinical trial with pre- and postexposure measurements to attempt to isolate the differences attributable to the EPA and DHA supplement.

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Despite these limitations, our studies add to the emerging body of knowledge regarding potential beneficial effects of fish oils and their derivatives. Further exploration of mechanism is needed, such as experimental models of fatty acid receptor deficiencies and polymorphisms and characterization of systemic and tissue-specific metabolites with fish oil supplementation to map involved pathways. Further studies into the effects of specific agents, inflammasome signaling in other metabolically active tissues (muscle, liver), and longer term studies looking at chronic effects are needed.
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