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A high-fish-oil diet prevents adiposity and modulates white adipose tissue inflammation pathways in mice☆

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Abstract

Fish oil improves obesity and its comorbidities, but its mechanisms of action remain unknown. We evaluate the effects of a diet rich in fish oil in white adipose tissue (WAT) inflammation pathways, renin–angiotensin system (RAS) and mitogen-activated protein kinases (MAPKs). To achieve our aims, four groups of male C57BL/6 mice were fed different diets: standard chow diet (SC; 10% energy from fat), SC+fish oil diet (SC-FO; 10% energy from fat), high-fat lard diet (HF-L; 50% energy from lard) and HF fish oil diet (HF-FO; 50% energy from fish oil). We evaluated body mass, epididymal fat pad mass, food intake and glucose tolerance. In WAT, we assessed adipocyte hypertrophy, monocyte chemotactic protein-1 immunofluorescence, and gene and protein expression of insulin signaling, inflammation, MAPKs, RAS, peroxisome proliferator-activated receptors (PPARs) and AMP-activated protein kinase (AMPK). In relation to the results, the HF-L group, as expected, showed elevated body mass and adiposity, glucose intolerance and hypertrophied adipocytes. In WAT, we found a defect in insulin signaling, infiltration of macrophages and inflammatory markers with the associated activation of MAPKs and local RAS. On the contrary, the HF-FO group did not present increased body mass, adiposity or glucose intolerance. In this group, insulin signaling, macrophage infiltration and inflammation were reduced in WAT in comparison with the HF-L group. We also observed decreases of MAPKs and local RAS and elevation of PPAR and AMPK. In summary, fish oil activates PPAR (the three isoforms) and AMPK, decreases WAT insulin resistance and inflammation, and inhibits MAPK and RAS pathways activation.

Keywords: Fish oil; n-3 PUFA; White adipose tissue; Inflammation; PPAR; Local RAS

1. Introduction

Obesity is considered a proinflammatory state in which excess white adipose tissue (WAT) releases a range of inflammatory cytokines [e.g., interleukin (IL)-6 and tumor necrosis factor (TNF)-α] that can lead to the development and/or worsening of obesity-related comorbidities such as insulin resistance (IR) and nonalcoholic fatty liver disease [1]. The presence of these cytokines is closely related to the activation of nuclear factor kappa B (NFκB), which in turn stimulates the expression of these inflammatory mediators [2]. Moreover, inflammation in WAT is also associated with activation, through phosphorylation, of the mitogen-activated protein kinases (MAPKs), including extracellular-signal-regulated kinase (ERK)1/2 and c-Jun N-terminal kinase (JNK), which induce proinflammatory cytokines production [3].

More recently, elevation of the local renin–angiotensin system (RAS) in WAT has been associated with obesity, and local RAS plays important roles in WAT inflammation [4]. Angiotensin II type 1 receptor (AT1r) blockage decreases NFκB activation [5], while its stimulation activates ERK and JNK [6,7], showing a cross talk among these pathways towards inflammation. Interestingly, peroxisome proliferator-activated receptor (PPAR)γ appears to suppress the expression of AT1r [8], together with NFκB and ERK inhibition [5,7], thus abrogating its proinflammatory effects.

Lifestyle modifications continue to be important factors in the treatment of obese people [9]. In this context, fish oil [rich in n-3 polyunsaturated fatty acids (PUFAs)] has been studied as an adjuvant therapy for obesity and its comorbidities. The most important components of fish oil are the eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids [10]. Fish oil improves IR [11], hepatic steatosis [12,13] and some inflammatory diseases such as asthma [14] and Crohn's disease [15]. The ability of these n-3 PUFAs to be agonists of the PPARs, especially PPARγ, is recognized as one of the mechanisms of their beneficial effects [10]. Besides that, phosphorylation and the consequent

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activation of AMP-activated protein kinase (AMPK) may be another pathway integrating the benefits of fish oil on adipose tissue [16].

In the context of inflammation, fish oil (n-3 PUFAs) supplementation has positive effects on the inflammatory process in WAT [13,17,18]. Nevertheless, the mechanisms linking fish-oil-activated pathways like PPAR and AMPK and the inflammatory pathways RAS and MAPK in WAT are not entirely understood. Therefore, the aim of the present study was to evaluate the effects of a diet rich in fish oil on WAT inflammation pathways RAS and MAPKs.

2. Material and methods

2.1. Animals and diet

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication number 85-23, revised 1996). The protocol was approved by the Ethics Committee for Animal Experimentation of the State University of Rio de Janeiro (Protocol Number CEUA/028/2012). All efforts were made to minimize suffering. The animals were maintained under controlled conditions (20°C±2°C and 12-h/12-h dark/light cycle) with free access to food and water, and their feed intake was measured daily and body mass (BM), weekly. Three-month-old C57BL/6 male mice were randomly assigned to four groups (n=10 each group):

1. Standard-chow group (SC; 40 g soybean oil/kg diet, 10% of the total energy content originating from lipids),
2. Standard-chow fish oil group (SC-FO; 4 g soybean oil and 36 g fish oil/kg diet, 10% of the total energy content originating from lipids),
3. High-fat lard group (HF-L; 40 g soybean oil and 238 g lard/kg diet, 50% of the total energy content originating from lipids) and
4. High-fat fish oil group (HF-FO; 40 g soybean oil and 238 g fish oil/kg diet, 50% of total energy content originating from lipids).

The diets were made with purified nutrients (PragSolucoes, Jau, SP, Brazil) in accordance with the American Institute of Nutrition's recommendations (AIN 93M) and were administered over an 8-week period [19]. The detailed composition of the diets was shown in Table 1.

2.2. Oral glucose tolerance test (OGTT)

The OGTT was performed at the end of the experiment. Mice were fasted for 6 h before glucose administration. Glucose (1 g/kg) was administrated orally at time 0; tail blood was collected at fasting (i.e., baseline) and then at 15, 30, 60 and 120 min after glucose loading for glucose determination (Glucometer Accu-Chek, Roche, SP, Brazil).

2.3. Euthanasia and tissue extraction

The animals were anesthetized with sodium pentobarbital (intraperitoneal, 150 mg/kg). Epididymal fat pad (the abdominal fat in the lower part of the abdomen and connected to the epididymis) was carefully dissected, weighed and prepared for analyzes. Samples were rapidly frozen and stored at −80°C for molecular studies; alternatively, the samples were kept in freshly made fixative solution (4% formaldehyde w/v, 0.1 M phosphate buffer, pH 7.2).

Table 1
Composition and energy content of the diets (AIN-93M-based diets).

Ingredients (g/kg)	SC	SC-FO	HF-L	HF-FO
Casein (≥85% of protein)	140.0	140.0	175.0	175.0
Cornstarch	620.692	620.692	347.692	347.692
Sucrose	100.0	100.0	100.0	100.0
Soybean oil	40.0	4.0	40.0	40.0
Lard	–	–	238.0	–
Fish oil	–	36.0	–	238.0
Fiber	50.0	50.0	50.0	50.0
Vitamin mix[a]	10.0	10.0	10.0	10.0
Mineral mix[a]	35.0	35.0	35.0	35.0
L-Cystin	1.8	1.8	1.8	1.8
Choline	2.5	2.5	2.5	2.5
Antioxidant	0.008	0.008	0.060	0.060
Total mass	1,000.0	1,000.0	1,000.0	1,000.0
Proteins (% energy)	14	14	14	14
Carbohydrates (% energy)	76	76	36	36
Lipids (% energy)	10	10	50	50
Energy content (kcal/kg)	3811	3811	5000	5000

Abbreviations: standard-chow (SC), standard-chow fish oil (SC-FO), high-fat lard (HF-L), and high-fat fish oil (HF-FO) diets.
[a] Mineral and vitamin mixtures are in accordance with AIN 93M.

2.4. Epididymal WAT

2.4.1. Adipocyte microscopy

Fixed epididymal adipose tissue samples were embedded in Paraplast Plus (Sigma-Aldrich, St. Louis, MO, USA), sectioned at a nominal thickness of 5 μm and stained with hematoxylin and eosin. Ten nonconsecutive random microscopic fields were analyzed per animal in a blinded manner with a light microscope (Leica Microsystems GmbH, Wetzlar, Germany) and an Infinity 1-5c camera (Lumenera Co., Ottawa, ON, Canada). The mean diameter of at least 50 adipocytes per animal was measured using Image Pro Plus software v. 7.01 (Media Cybernetics, Silver Spring, MD, USA).

2.4.2. Adipocyte immunofluorescence

For monocyte chemotactic protein-1 (MCP-1) immunofluorescence, tissue sections were submitted to citrate buffer, pH 6.0, at 60°C for 20 min for antigen retrieval, glycine 2% and blocking buffer [phosphate-buffered saline (PBS)/5% bovine serum albumin (BSA)]. The WAT sections were incubated overnight at 4°C with anti-MCP-1 antibody (SC-1785; Santa Cruz Biotechnology), diluted 1:50 in PBS/1% BSA, followed by incubation for 1 h at room temperature with fluorochrome-conjugated secondary antibody anti-goat IgG-Alexa 488 (Invitrogen, Molecular Probes, Carlsbad, CA, USA), diluted 1:50 in PBS/1% BSA. After rinsing in PBS, the slides were mounted with SlowFade Antifade (Invitrogen, Molecular Probes, Carlsbad, CA, USA). Digital immunofluorescence images were captured using confocal microscopy (Nikon Confocal Laser Scanning Microscopy, Model C2; Nikon Instruments, Inc., Melville, NY, USA).

2.4.3. Western blotting

Total proteins were extracted in homogenization buffer containing protease inhibitors. Equal quantities of total protein were resuspended in sodium dodecyl sulfate (SDS)-containing sample buffer, heated for 5 min at 100°C, and separated by SDS polyacrylamide gel electrophoresis. After electrophoresis, the proteins were electroblotted onto polyvinyl difluoride transfer membranes (Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked with nonfat dry milk. Homogenates were incubated with the following primary antibodies: AKT (60 kDa; 44-609G; Invitrogen), p-AKT^{ser473} (65 kDa; 44-621G; Invitrogen), IL-6 (23 kDa; AB1423, Millipore), TNF-α (26 kDa; SC-1350, Santa Cruz Biotechnology), adiponectin (30 kDa; A6354, Sigma-Aldrich), NFκB (65 kDa; SC-109, Santa Cruz Biotechnology), ERK1/2 (42-44 kDa; SC-135900; Santa Cruz Biotechnology), p-ERK1/2$^{thr202/tyr204}$ (42–44 kDa; SC-81492; Santa Cruz Biotechnology), JNK (46–54 kDa; SC-7345; Santa Cruz Biotechnology), p-JNK$^{thr183/tyr185}$ (54–46 kDa; SC-12882; Santa Cruz Biotechnology), renin (38 kDa; SC-137252; Santa Cruz Biotechnology), angiotensin-converting enzyme (ACE; 150 kDa; ab11734; Abcam), AT1r (43 kDa; SC-579; Santa Cruz Biotechnology), PPARα (55 kDa; SC-9000; Santa Cruz Biotechnology), PPARγ (67 kDa; SC-7273; Santa Cruz Biotechnology), p-PPARγ^{ser112} (57 kDa; SC-28001; Santa Cruz Biotechnology), AMPKα 1/2 (63 kDa; SC-25792; Santa Cruz Biotechnology) and p-AMPKα 1/2^{thr172} (SC-33524; Santa Cruz Biotechnology). Beta-actin (SC81178; Santa Cruz Biotechnology) served as a loading control. All protein expression was detected using an ECL detection system and the Bio-Rad Molecular Imaging ChemiDoc XRS Systems (Bio-Rad, Hercules, CA, USA). The chemiluminescence intensity of the bands was quantified using the ImageJ software, version 1.48 (NIH, imagej.nih.gov/ij, USA). The integral absorbance values were measured, and the results are shown as percentages of the SC group.

2.4.4. Reverse transcriptase quantitative polymerase chain reaction (PCR)

Total RNA was extracted from approximately 50 mg of adipose tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The quantity of RNA was determined using Nanovue spectroscopy (GE Life Sciences), and 1 μg RNA was treated with DNAse I (Invitrogen). First-strand cDNA was synthesized using Oligo (dT) primers for mRNA and Superscript III reverse transcriptase (both Invitrogen). Quantitative real-time PCR was performed using a BioRad CFX96 cycler and SYBR Green mix (Invitrogen). The following primers are described in Table 2: IL-6, TNF-α, renin, ACE, AT1r, PPARα, PPARβ/δ and PPARγ2. The endogenous expression of β-actin was used to normalize the expression of the selected genes. After a predenaturation and polymerase-activation program (4 min at 95°C), 44 cycles of 95°C for 10 s and 60°C for 15 s were followed by a melting curve program (60°C to 95°C with a heating rate of 0.1°C/s). Negative controls consisted of wells in which the cDNA was substituted for deionized water. The relative expression ratio of the mRNA was calculated using the equation $2^{-\Delta\Delta Ct}$, in which −ΔCT represents the difference between the number of cycles (CT) of the target genes and the endogenous control.

2.5. Data analysis

Values are shown as the mean and its respective standard error. In cases where we could confirm homoscedasticity of variances, comparisons among groups were made by one-way analysis of variance (ANOVA) followed by a Holm–Sidak *post hoc* test. A P value <.05 was considered statistically significant (GraphPad Prism version 6.05 for Windows).