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Fish oil supplementation for two generations increases insulin sensitivity in rats

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Abstract

We investigated the effect of fish oil supplementation for two consecutive generations on insulin sensitivity in rats. After the nursing period (21 days), female rats from the same prole were divided into two groups: (a) control group and (b) fish oil group. Female rats were supplemented with water (control) or fish oil at 1 g/kg body weight as a single bolus for 3 months. After this period, female rats were mated with male Wistar rats fed on a balanced chow diet (not supplemented). Female rats continued to receive supplementation throughout gestation and lactation periods. The same treatment was performed for the next two generations (G1 and G2). At 75 days of age, male offspring from G1 and G2 generations from both groups were used in the experiments. G1 rats did not present any difference with control rats. However, G2 rats presented reduction in glycemia and lipidemia and improvement in *in vivo* insulin sensitivity (model assessment of insulin resistance, insulin tolerance test) as well as *in vitro* insulin sensitivity in soleus muscle (glucose uptake and metabolism). This effect was associated with increased insulin-stimulated p38 MAP kinase phosphorylation and lower n-6/n-3 fatty acid ratio, but not with activation of proteins from insulin signaling (IR, IRS-1 and Akt). Global DNA methylation was decreased in liver but not in soleus muscle. These results suggest that long-term fish oil supplementation improves insulin sensitivity in association with increased insulin-stimulated p38 activation and decreased n-6:n-3 ratio in skeletal muscle and decreased global DNA methylation in liver.
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1. Introduction

Evidence suggests that omega-3 (n-3) polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, have positive impact on health in humans and animals models. These fatty acids have been suggested to provide beneficial effects on several disorders such as dyslipidemias, cardiovascular diseases, hypertension, insulin resistance, cancer, type 2 *diabetes mellitus* (DM2) and metabolic syndrome [1–5]. Lack of n-3 PUFA in the diet has been related to increased risk for DM2 and cardiovascular diseases [6,7], while dietary enrichment with n-3 PUFA has been associated with improved insulin sensitivity and reduced risk factors for several diseases [8,9].

Diet enrichment with fish oil prevents insulin resistance and obesity induced by a high-fat diet [10–16], a high-sucrose diet [17–23], both [24–26] and tumor necrosis factor (TNF) infusion [27]. In addition, dietary enrichment with n-3 PUFA prevents or reduces insulin resistance and/or cardiovascular risks in animal models of obesity [28,29], metabolic syndrome [30,31] and diabetes [32], as well as in humans with overweight [33–35], obesity [36], diabetes [37,38] and aging [39,40]. However, in some studies, n-3 PUFA had no effect or even impaired insulin sensitivity and/or cardiovascular risk factors in obese Zucker rats [41], diet-induced obese humans [42] and diabetic patients [37,43,44]. Differences are probably due to variations in the experimental model or design, treatment time and n-3 PUFA dose, for example.

Supplementation with n-3 PUFA has several effects on diet-induced obese animals and obese humans, including the following: (a) reduction in body weight, adiposity, total body fat and lipolysis [16,23,30]; (b) reduction in glucose plasma concentration, insulin, lipids (triacylglycerol, total cholesterol and nonesterified fatty acids) and in some inflammatory markers (tumor necrosis factor-α [TNF-α], interleukin-6 [IL-6], C-reactive protein [CRP], and plasminogen activator inhibitor-1 [PAI-1]) [26,34,37,45,46]; (c) improvement in the glucose tolerance test, insulin sensitivity [insulin tolerance test (ITT), homeostasis model assessment of insulin resistance (HOMA-IR)] and insulin signaling (insulin binging, Akt, ERK) [25,29,47,48]; (d) decrease in hepatic steatosis and lipid accumulation and their derivatives in liver, skeletal muscle and pancreas [20,28,49,50]; (e) improvement in plasmatic levels of adipokines (leptin, adiponectin

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and visfatin) [2,51,52]; (f) increase in the n-3 fatty acid derivatives in the phospholipids (hepatocytes, adipocytes and myocytes) [4,11,13,24,36,47,53,54]; (g) modulation of gene expression towards glucose metabolism (glucose transporter-4 [GLUT-4]), lipid oxidation (carnatine palmitoyltransferase-1 [CPT-1], acyl-CoA oxidaxe [ACO], and uncoupling protein-3 [UCP-3]) and hepatic glucose production (glucose-6-phosphatase [G6Pase], malic enzyme, glucose-6-phosphate dehydrogenase [G6PDH]], and phosphoenolypyruvate carboxykinase [PECK]), rather lipid accumulation (decreased acetyl-CoA carboxylase [ACC], fatty acid synthase [FAS], sterol regulatory element-binding protein-1 [SREBP-1], and nuclear factor Y [NFY]) [5,14,22,31,32,55–57]; (h) reduction in other cardiovascular risks (reduced blood pressure and platelet aggregation, improved endothelial function) [9,33,39] and (i) increase in resolvins, protectins and maresins and decrease in proinflammatory eicosanoids [58–61].

Studies in healthy animals or humans, however, have failed to show beneficial effects of fish oil supplementation on insulin sensitivity [1,42,62]. In addition, the mechanisms involved are not completely known yet. Thus, the aim of this work was to evaluate the long-term effect of fish oil supplementation for two generations on insulin sensitivity in rats by measuring the following parameters: (a) glucose uptake and metabolism in incubated soleus muscle; (b) plasma lipid [free fatty acids, triacylglycerol, total cholesterol, low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol] levels; (c) ITT; (d) insulin signaling pathways (insulin receptor, IRS-1, Akt and p38 MAP kinase); (e) translocation of GLUT-4 from cytoplasm to plasmatic membrane and (f) fatty acid profile in skeletal muscle. We hypothesized that n-3 PUFA supplementation has effect on healthy animals only after a long period of supplementation.

2. Materials and methods

2.1. Materials

Enzymes and reagents for all assays were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Regular insulin was obtained from Eli Lilly and Company (Indianapolis, IN, USA). D-[U-^{14}C]-glucose, 2-deoxy-D-[2,6-^{3}H]-glucose and ECL Western Blotting System Kit were obtained from Amersham International Plc. (Bucks, UK). L-[1-^{14}C]-glucose was obtained from NEN Life Sciences Products, Inc. (Boston, MA, USA). Anti-phosphoserine (473)-Akt, anti-phosphothreonine (308)-Akt and anti-phosphotyrosine (182)-p38 MAP kinase antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-phosphotyrosine, anti-insulin receptor-β and anti-IRS-1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-GLUT-4 antibody was from Abcam (Cambrigde, UK). Anti-IgG polyclonal antibody conjugated to horseradish peroxidase was obtained from ICN Biomedicals, Inc. (Irvine, CA, USA). Fish oil (obtained from cold sea fishes) was generously donated by Naturalis Nutrition and Pharma Ltd. (São Paulo, Brazil). Standard chow diet containing 4% fat was obtained from Nuvital Nutrients Ltd. (Curitiba, Brazil). Total fatty acid composition of the standard chow diet and fish oil was determined by high-performance liquid chromatography as previously described (Shimadzu model LC-10A, Shimadzu, Kyoto, Japan). Fish oil used in this study was composed by lauric (0.5%), myristic (11.4%), palmitic (14.2%), palmitoleic (0.9%), stearic (3.1%), oleic (8.7%), linoleic (0.5), γ-linolenic (1.7%), arachidonic (12.1%), EPA (26.0%) and DHA (20.2%). Fatty acids from standard chow diet were composed by myristic (0.2%), palmitic (15.6%), stearic (4.2%), oleic (27.2%), linoleic (49.0%) and linolenic acid (2.9%).

2.2. Animals

Wistar rats were obtained from the Department of Physiology, Biological Sciences Building, Federal University of Paraná, Curitiba, Brazil. The animals were maintained at 12:12-h light–dark cycle, 23°C±1°C, and received a standard diet for rats containing 52% carbohydrates, 22% proteins and 4% lipids (CR-1, Nuvilab-Nuvital Nutrients Ltd., Parana, Brazil) and water *ad libitum*. The chow was removed 6 h before the beginning of the experiments, which was always carried out at 02:00 p.m. The Brazilian Animal Ethics Committee approved this study.

2.3. Study design

After the nursing period (21 days), female rats from the same prole were divided into two groups: (a) control group supplemented with water and (b) experimental group supplemented with fish oil. Both supplementations were daily and orally given at 1 g/kg body weight as a single bolus. Female rats were supplemented for 3 months [generation 0 (G0)]. After this period, female rats were mated with male Wistar rats fed on a normal chow diet (not supplemented). The female rats continued to receive supplementation throughout gestation and lactation periods. The same treatment was performed for the next two generations (G1 and G2).

At 75 days of age, male offspring from G1 and G2 generations from both groups (control and fish oil) were used in the experiments. Body weight was determined every 2 days for adjustment of the fish oil supplementation and water administration. Daily food intake was measured in the last week of the experimental period.

2.4. Glucose uptake and metabolism in isolated soleus muscle

Soleus muscles were isolated and incubated as previously described [63,64]. The chow was removed 6 h before the beginning of the experiment, which was always carried out at 2:00 p.m. The rats were killed by cervical dislocation, and the muscles were rapidly and carefully isolated, split longitudinally in portions weighing 25–35 mg and preincubated, at 35°C, in Krebs–Ringer bicarbonate buffer containing 5.6 mM glucose, pH 7.4, pregassed for 30 min with 95% O_2/5% CO_2, with agitation at 100 oscillations/min for 30 min. Subsequently, the muscles were transferred to the same buffer with 0.3 μCi/ml D-[U-^{14}C]glucose and 0.2 μCi/ml 2-deoxy-[2,6-^{3}H]D-glucose and incubated under similar conditions for 1 h in the absence or presence of 0, 100, 1000 or 10,000 μU/ml insulin. Phenylethylamine, diluted 1:1 v/v in methanol, was added into a separate compartment for $^{14}CO_2$ adsorption. After the incubation period, the muscles were briefly washed in saline at 4°C and frozen in liquid N_2. [^{14}C]-glycogen synthesis (as estimated by D-[^{14}C]-glucose incorporation into glycogen) was determined as described by Leighton and Cooper [65] and in our previous studies [66–68]. Lactate production was determined as described by Engel and Jones [69]. The decarboxylation of D-[^{14}C]-glucose and the uptake of 2-deoxy-D-[2,6-^{3}H]-glucose were measured as previously described [70–72]. For determination of the extracellular space, some muscles were incubated in the presence of 0.1 μCi/ml L-[1-^{14}C]-glucose [72,73].

2.5. Determination of blood metabolites

Serum glucose concentration was measured by a glucose-oxidase-based assay kit as described by Trinder [74] at 505 nm. Serum cholesterol, high density lipoprotein (HDL) cholesterol and triacylglycerol (TAG) concentrations were determined as previously described [75,76] at 500 nm (cholesterol) and 540 nm (TAG). Serum lactate was assayed as described by Engle and Jones [69]. Insulin was measured by radioimmunoassay as previously described [77].

2.6. Insulin tolerance test

Insulin tolerance test was performed as previously described [78]. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneal injection). Two international units of insulin per kilogram of body weight was intraperitoneally injected. Blood was collected from tail vein at 0 (basal), 5, 10, 15, 20, 25 and 30 min thereafter. Plasma was then separated, and serial glucose concentration of each animal was determined. Glucose disappearance rate (K_{ITT}) was calculated using the formula $0.693/t_{1/2}$. The glucose $t_{1/2}$ was calculated from the slope of the least square analysis of blood glucose concentration during the linear phase of decline [79].

2.7. Liver and muscle glycogen contents

Glycogen content of the liver, soleus and gastrocnemius muscles was measured by digesting 50–75 mg in 0.5 ml KOH solution (1 M) for 20 min at 70°C. After that, triethanolamine buffer and amyloglucosidase were added to the samples and then incubated for 2 h at 25°C. Samples were centrifuged at 800 *g* for 5 min, and the supernatant was added to glucose assay buffer [80]. Glycogen content was quantified by measuring the absorbance at 340 nm.

2.8. Fatty acid composition

Total lipid was extracted from gastrocnemius muscles according to Folch et al. [81] using chloroform–methanol (2:1), and fatty acid profile was determined by high-performance liquid chromatographer [82]. The fatty acids were saponified using 2 ml of an alkaline methanol solution (1 mol/L NaOH in 90% methanol) at 37°C for 2 h in a shaking water bath. Afterwards, samples were acidified to pH 3 with HCl solution (1 mol/L). Fatty acids were then extracted 3 times with hexane and derivatized with 4-bromomethyl-7-coumarin, and the analysis was performed in a Shimadzu LC-10 high-performance liquid chromatographer using a C8 column (2.5 cm×4.6 mm i.d.; particle size of 5 μm) with a C8 pre-column (25 cm×4.6 mm i.d.; particle size 5 μm). Fatty acids were resolved using a mobile phase of acetonitrile–water (gradient from 77:23, vol/vol) at 1-ml/min flow rate and detected by fluorescence (325 nm, excitation; 395 nm, emission) [83]. The following fatty acids (Sigma-Aldrich, St. Louis, MO, USA) were used as standards: lauric (C12:0), myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1 n6), stearic (C18:0), oleic (C18:1 n9), linoleic (C18:2 n6), linolenic (C18:3 n6), arachidonic (C20:4), eicosapentaenoic (C20:5 n3), docosahexaenoic (C22:6 n3) and margaric (C17:0) acids. A calibration curve was established for each fatty acid standard in order to obtain correlation and regression coefficients. Limit for detection ranged from 1 to 10 ng of the fatty acids.

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