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Reduced protein oxidation in Wistar rats supplemented with marine ω3 PUFAs

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ABSTRACT

The potential effects of various dietary eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6) ratios (1:1, 2:1, and 1:2, respectively) on protein redox states from plasma, kidney, skeletal muscle, and liver were investigated in Wistar rats. Dietary fish oil groups were compared with animals fed soybean and linseed oils, vegetable oils enriched in ω6 linoleic acid (LA; 18:2) and ω3 α-linolenic acid (ALA; 18:3), respectively. Fish oil treatments were effective at reducing the level of total fatty acids in plasma and enriching the plasmatic free fatty acid fraction and erythrocyte membranes in EPA and DHA. A proteomic approach consisting of fluorescein 5-thiosemicarbazide (FTSC) labeling of protein carbonyls, FTSC intensity visualization on 1-DE or 2-DE gels, and protein identification by MS/MS was used for the protein oxidation assessment. Albumin was found to be the most carbonylated protein in plasma for all dietary groups, and its oxidation level was significantly modulated by dietary interventions. Supplementation with an equal EPA:DHA ratio (1:1) showed the lowest oxidation score for plasma albumin, followed in increasing order of carbonylation by 1:2 < 2:1 ≈ linseed < soybean. Oxidation patterns of myofibrillar skeletal muscle proteins and cytosolic proteins from kidney and liver also indicated a protective effect on proteins for the fish oil treatments, the 1:1 ratio exhibiting the lowest protein oxidation scores. The effect of fish oil treatments at reducing carbonylation on specific proteins from plasma (albumin), skeletal muscle (actin), and liver (albumin, argininosuccinate synthetase, 3-α-hydroxysteroid dehydrogenase) was remarkable. This investigation highlights the efficiency of dietary fish oil at reducing in vivo oxidative damage of proteins compared to oils enriched in the 18-carbon polyunsaturated fatty acids ω3 ALA and ω6 LA, and such antioxidant activity may differ among different fish oil sources because of variations in EPA/DHA content.

Reactive oxygen species (ROS)[1] are formed from the electron-transfer reactions involved in aerobic metabolism, but they are also generated from exposure to various toxicants. ROS are essential in vital biochemical processes such as intracellular messaging, cellular differentiation, growth arrestment, apoptosis, and immunity against microorganisms [1]. However, impairment in the normal reduction–oxidation (redox) balance provokes ROS overproduction, a condition known as oxidative stress that triggers oxidative damage of cellular biomolecules (i.e., lipids, proteins, and DNA) and has been linked to the development of a wide range of metabolic diseases, including atherosclerosis, type 2 diabetes, and obesity, and even to cellular aging [2]. Several oxidized products of lipids (e.g., F2-isoprostane and malondialdehyde), DNA (typically 8-oxo-7,8-dihydro-2′-deoxyguanosine), and proteins (e.g., protein carbonyls) are generally monitored to evaluate oxidative stress in vivo [3].

Regular consumption of ω3 PUFAs of marine origin, principally EPA (20:5 *n*-3) and DHA (22:6 *n*-3), has been implicated in prevention of autoimmune disorders, diabetes, and coronary heart disease [4–6]. EPA and DHA (a) alter membrane fluidity; (b) interact with transcription factors such as peroxisome proliferator-activated receptor (PPAR), nuclear factor κB, and sterol regulatory element

Abbreviations: 1/2-DE, one/two-dimensional gel electrophoresis; AKR, 3-α-hydroxysteroid dehydrogenase; ALA, α-linolenic acid; ARA, arachidonic acid; ASS, argininosuccinate synthetase; AST, aspartate aminotransferase; DHA, docosahexaenoic acid; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPA, eicosapentaenoic acid; FTSC, fluorescein 5-thiosemicarbazide; GC/FID, gas chromatography/flame ionization detector; LA, linoleic acid; LC–ESI–IT–MS/MS, high-performance liquid chromatography coupled with electrospray ionization ion trap tandem mass spectrometry; MDA, malondialdehyde; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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binding proteins that engage genes that influence cellular differentiation and growth and metabolism of lipid, protein, and carbohydrates; and (c) are substrates for enzymes including cyclooxygenase, lipoxygenase, and cytochrome P450 [7,8]. As a result, the healthy outcome of marine *n*-3 PUFA consumption has been mainly attributed to their effects in reducing elevated plasma triglyceride levels, increasing high-density lipoprotein cholesterol, reducing platelet aggregation, improving endothelial function, and preventing inflammatory pathways [9,10].

Several investigations have also indicated a potential modulation of marine lipids on in vivo redox balance, although both in vitro and in vivo experiments have yielded contradictory results. In vitro supplementation of human platelets showed a biphasic effect of DHA with antioxidant and pro-oxidant effects at low and high concentrations, respectively [11]. This pro-oxidant action was ascribed to the high DHA accumulation in membranes and the theoretical direct relation between PUFA oxidation and unsaturation degree. A similar tendency was found in a nutritional intervention study in healthy men [12]. Intake of 200 mg of DHA/day provided antioxidative protection by increasing the endogenous antioxidant α-tocopherol and reducing urinary F2-isoprostanes, whereas supplementation of 1600 mg of DHA exerted a pro-oxidant effect by increasing isoprostane levels. Conversely, elevated supplementations of fish oil or purified EPA and DHA, ranging from 3.6 to 4.4 g per day, were effective at reducing urinary F2-isoprostanes in non-insulin-dependent type 2 diabetic patients [13,14], but had no effect in reducing oxidative stress in exercise-trained men, considering plasma malondialdehyde (MDA), antioxidant capacity, and protein carbonylation [15]. Another nutritional intervention showed an increment of plasma MDA after supplementation with fish oil containing 2.5 g EPA and 1.8 g DHA, but it did not modify plasma protein carbonylation [16]. Recent investigations have suggested that both DHA and fish oil, but not EPA, have beneficial effects on lipoprotein metabolism and oxidative stress [17]. A differential redox modulation by EPA and DHA, together with methodological limitations, can explain in part the above-mentioned contradictory results. Numerous studies evaluate exclusively free MDA; however, it is fundamentally bound to proteins because of the affinity of MDA and other reactive carbonyls to form protein adducts. Protein carbonylation is a good alternative to estimate jointly the formation of reactive carbonyl compounds and free radical processes that also lead to protein carbonyls. Moreover, carbonylation alters biochemical properties of proteins such as enzymatic activity, DNA-binding activity of transcription factors, and susceptibility to proteolytic degradation, and the oxidation of low-density lipoprotein (LDL) is suggested to induce important events triggering atherosclerosis pathogenesis [18]. At present, the spectrophotometric measurement of the reaction of protein carbonyls with 2,4-dinitrophenylhydrazine (DNPH) is routinely used for monitoring protein carbonylation despite its reduced sensitivity and selectivity [19]. Recent advances in mass spectrometry and proteomic tools make possible a massive identification and quantification of posttranslational modifications such as protein carbonylation, although these methodologies have been scarcely implemented to evaluate diet influence on protein oxidation.

This investigation was aimed to delve deeper into the effect of dietary interventions with various EPA:DHA ratios on the individual redox state of proteins from plasma, kidney, skeletal muscle, and liver. To achieve such purpose, healthy Wistar rats were employed as an animal model. Rats were fed standard diets enriched with fish oil containing three different EPA:DHA ratios (1:1, 2:1, and 1:2) and were compared with diets containing soybean oil and linseed oil. Soybean oil is a rich source of the ω6 LA (18:2), whereas linseed oil has an elevated content of ω3 ALA (18:3), the dietary precursor of long-chain ω3 PUFA biosynthesis. The influence of fatty acid supplementation on redox regulation of proteins was evaluated by using a proteomic procedure based on labeling protein carbonyls with fluorescein 5-thiosemicarbazide (FTSC), protein separation on 1-DE or 2-DE gels, and protein identification by tandem mass spectrometry.

Materials and methods

Materials and reagents

Oils differing in EPA:DHA ratio were obtained by mixing appropriate quantities of the commercial fish oils AFAMPES 121 EPA (AFAMSA, Vigo, Spain), EnerZona Omega 3 RX (Milan, Italy), and Oligen liquid DHA 80% (IFIGEN-EQUIP 98, S.L., Barcelona, Spain). Soybean oil, obtained from unrefined organic soy oil (first cold pressing) was from Clearspring Ltd. (London, UK), and linseed oil, obtained from unrefined organic flax oil (first cold pressing), was from Biolasi S.L. (Ordizia, Guipuzcoa, Spain). Nonadecanoic acid was purchased from Larodan Fine Chemicals (Malmö, Sweden). Diethyl ether, formic acid, and methanol were purchased from Merck (Darmstadt, Germany). FTSC was purchased from Invitrogen (Carlsbad, CA, USA) and porcine sequencing-grade modified trypsin was from Promega (Madison, WI, USA). Ketamine chlorhydrate (Imalgene 1000) was purchased from Merial Laboratorios S.A. (Barcelona, Spain) and xylazine (Rompun 2%) was from Quimica Farmaceutica S.A. (Barcelona, Spain). ProteoBlock protease inhibitor cocktail was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Dichloromethane, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), iodoacetamide, ethylenediaminetetraacetic acid (EDTA), trichloroacetic acid (TCA), Tris–HCl, 3,3-cholaminopropyldimethylammonio-1-propanesulfonate (Chaps), sodium phosphate, magnesium chloride anhydrous, and bicinchoninic acid (BCA) were purchased from Sigma (St. Louis, MO, USA). Urea, thiourea, sodium dodecyl sulfate (SDS), glycine, glycerol and Serdolit MB-1 were obtained from USB (Cleveland, OH, USA). Immobiline DryStrip gels (pH 3–10, 11 cm), IPG buffer, pharmalyte 3–10, ammonium persulfate, bromophenol blue, and 1,2-bis(dimethylamino)ethane were purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Acrylamide, bis-*N,N′*-methylene-bis-acrylamide, and Bio-Rad protein assay were obtained from Bio-Rad (Hercules, CA, USA). All other chemicals and reagents used were of analytical reagent grade and water was purified using a Milli-Q system (Millipore, Billerica, MA, USA).

Animals and diets

Briefly, experiments were performed with 35 female Wistar rats (Janvier, St. Berthevin, France) with an initial age of 13 weeks. Animals were kept in an isolated room with a constantly regulated temperature (22 ± 2 °C) and controlled (50 ± 10%) humidity on a 12-h artificial light cycle. They were randomized into five groups: soybean group ($n = 7$), linseed group ($n = 7$), EPA:DHA 1:1 group ($n = 7$), EPA:DHA 2:1 group ($n = 7$), and EPA:DHA 1:2 group ($n = 7$). All groups were fed the standard pelleted chow diet A04 from Harlan Iberica (Barcelona, Spain), which contained, on a wet basis, 16.0% protein, 60.0% carbohydrate, 4.0% fiber, 3.0% fat, and 5.0% mineral and provided an energy density of 2.9 kcal per gram. The levels of the micronutrients per kilogram of standard chow diet were 8400 mg for Ca, 5700 mg for P, 2500 mg for Na, 6400 mg for K, 70 mg for Mn, 6600 UI for vitamin A, 900 UI for vitamin D3, and 30 mg for vitamin E. The animals had ad libitum access to water and food. Each specific group was given a weekly oral dose of 0.8 ml/kg of the corresponding oil as listed in Table 1. The incorporation of PUFAs from the diet was