Increased susceptibility of cellular membranes to the induction of oxidative stress after ingestion of high doses of fish oil: effect of aging and protective action of dl-α tocopherol supplementation

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Feeding young and aged rats (2 and 18 months old, respectively) with sardine oil (10 g/kg body weight) for 14 days increases the content of eicosapentaenoic acid and docosahexaenoic acid in erythrocyte membranes. These changes are associated with an increased membrane susceptibility to the induction of oxidative stress. Supplementation of the dietary oil with dl-α tocopherol (1 g/kg oil) protects the membranes from young rats against this increased susceptibility, while membranes from aged animals appear equally susceptible to oxidation when compared with membranes obtained from rats fed nonsupplemented oil. Following fish oil ingestion, plasma and membrane dl-α tocopherol levels are reduced to undetectable levels. Supplementation of the oil with the antioxidant, restores dl-α tocopherol pool but to a different level in young and aged animals. The differential response of membranes from young and aged rats to the induction of oxidative stress can be ascribed to a different membrane availability of dl-α tocopherol and therefore to a different free radical scavenging capacity.

Keywords: fish oil; membrane lipid peroxidation; plasma dl-α tocopherol levels; polyunsaturated fatty acids

Introduction

Fish oil consumption has been associated with a reduced risk of cardiovascular diseases, atherosclerosis, and some inflammatory diseases.[1,2] The preventive effect of fish oil ingestion has been ascribed to its relatively high content of n-3 polyunsaturated fatty acids, such as eicosapentaenoic acid (C20:5, n-3. EPA) and docosahexaenoic acid (C22:6, n-3. DHA).[3] Fish oil of different sources and/or composition and some edible mixtures containing fish oil or n-3 fatty acid concentrates are expended in many countries as medicinal or nutritional supplements.[4] These products are sometimes exaggeratedly consumed by individuals who expect increased health benefits.

Recently it has been reported that some metabolic risks may be associated with the consumption of high doses of marine oils rich in n-3 fatty acids.[5] While feeding animals a diet rich in polyunsaturated fatty acids increases their content in cell membrane phospholipids,[6] an increased susceptibility to the induction of oxidative stress, expressed mainly as lipid peroxidation, has been observed in animals fed fish oil or fish oil products contained in diets insufficiently supplemented with antioxidants.[7] The present study was undertaken to determine whether an increased susceptibility to the induction of oxidative stress can be prevented in erythrocyte membranes from young and aged

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rats when animals are fed high doses of fish oil supplemented with dl-α tocopherol.

Materials and methods

Sixty-day-old male Wistar rats (120–150 g body weight), referred to as young rats, and 18-month-old male Wistar rats (550–580 g), referred to as aged rats, were fed a standard diet and water ad libitum. One-third of each group of animals (young and aged) received by intragastric cannula 10 g of sardine oil/kg body weight daily (9 a.m.) for 14 days. Another third received the fish oil in the same conditions as above but supplemented with dl-α tocopherol (1 g/kg oil). Control animals were maintained in the same conditions as experimental animals but without receiving fish oil. Sardine oil (31–34%, EPA plus DHA) was obtained from a local fish meal factory (Corpesca, S.A., Mejillones, Chile), deodorized by high vacuum distillation as previously described[8] and kept under N_2 atmosphere until use. Some fractions of distilled oil were preserved with dl-α tocopherol (1 g/kg oil) as antioxidant. The peroxide value of the distilled oil was always below 1 meq/kg.

At the end of the 14-day treatment, the animals were exsanguinated by cardiac puncture under light anesthesia. Blood was centrifuged at 300*g* for 10 min at 4° C, and after removal of the buffy coat, erythrocytes were washed three times with PBS buffer (150 mmol/L NaCl and 5 mmol/L sodium phosphate, pH 8). Membrane ghosts were obtained according to Burton et al.[9]

Erythrocyte membrane lipid peroxidation was induced by $Fe2+$ ascorbate (50 μmol/L $Fe2+$, 400 μmol/L ascorbate).[5] Lipid peroxidation was assessed as thiobarbituric acid reactive substances (TBARS) as described.[10] Proteins were measured according to Albro.[11] Fatty acid methyl esters were prepared by interesterification with a boron trifluoride-methanol mixture[12] of the lipid extracts obtained from erythrocyte membranes. Fatty acids determination was carried out by gas chromatography using a Carlo Erba GC-6000 Vega-2 (Milano, Italy) equipped with an SP 2330, 30 mts column (Supel Co., USA) (o.d. 0.75 mm) and a Spectra-Physics SPA 4270 integrator (San Jose, CA USA) for peak area measurement.

dl-α tocopherol extraction from plasma and from erythrocyte membranes was performed according to Burton et al.[13] dl-α tocopherol was assessed by high pressure liquid chromatography as described by Lehmann et al.[14] dl-α tocopherol was a gift of Productos Roche (Santiago, Chile). All other chemicals (reagent grade) were obtained from Sigma Chemical Co. (St. Louis, MO USA). Statistical analysis of results was by a two-way ANOVA followed by the Duncan's test for multiple comparisons.

Results

As it has been previously reported,[5] feeding either young or aged rats with sardine oil for 14 days produces several changes in the fatty acid content of erythrocyte membranes. *Figure 1* shows the profile of some unsaturated fatty acids obtained from erythrocyte membranes from young *(Figure 1-A)* and aged *(Figure 1-B)* rats who received either sardine oil or sardine oil supplemented with dl-α tocopherol. From both profiles it is evident a decrease in the content of oleic acid (18:1, n-9) and arachidonic acid (20:4, n-6), and a significant increase in the contents of EPA and DHA. On the other hand, the content of linoleic acid (18:2, n-6) decreases in membranes obtained from aged rats only. Interestingly, the EPA and DHA content of membranes obtained from rats fed dl-α tocopherol-supplemented sardine oil is significantly higher than that seen in membranes obtained from animals fed nonsupplemented oil.

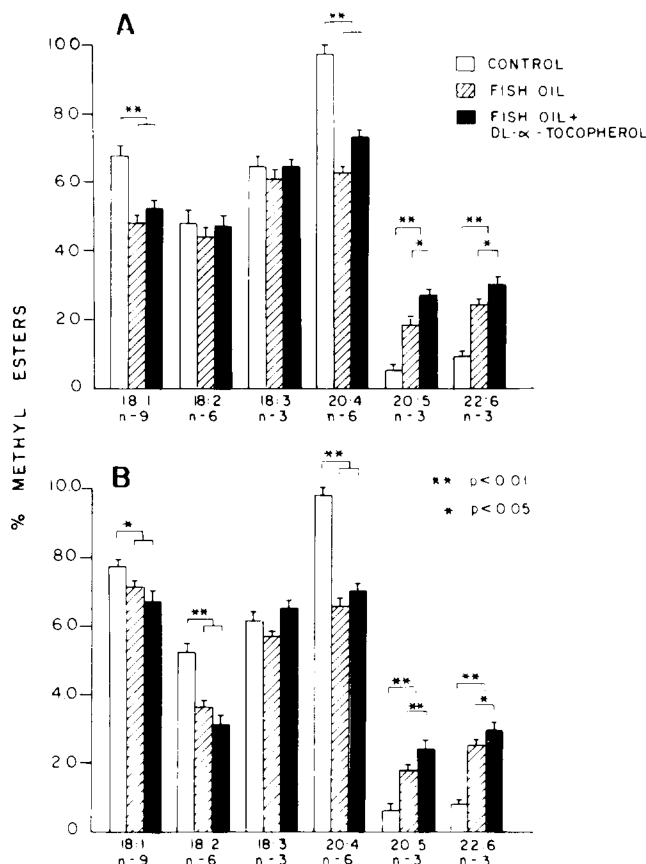

Figure 1 Changes (%) in the composition of some unsaturated fatty acids from erythrocyte membranes obtained from young (A) and aged rats (B) after feeding fish oil and fish oil supplemented with dl-α tocopherol for 14 days. Results represent the mean ± S.E.M. of six experiments, each one constituted by three animals.

In vitro exposure of membranes from young or aged rats to the prooxidant effect of $Fe2+$ ascorbate, leads to a progressively increased formation of TBARS *(Figure 2 A and B)*. When data are analyzed after 60 min of incubation, membranes obtained from rats fed sardine oil show a higher TBARS production compared with controls. However, the response of membranes obtained from rats fed sardine oil added to dl-α tocopherol is quite different. While membranes obtained from young rats appear resistant to the induction of oxidative stress, those from aged rats develop incubation TBARS levels higher than membranes obtained from animals who received fish oil without the antioxidant after 60 min.

Figure 3 (A and B) shows dl-α tocopherol levels in plasma and in erythrocyte membranes. Young and aged rats show similar dl-α tocopherol levels in plasma *(Figure 3-A)*. In erythrocyte membranes, however, young rats show dl-α tocopherol levels 50% higher

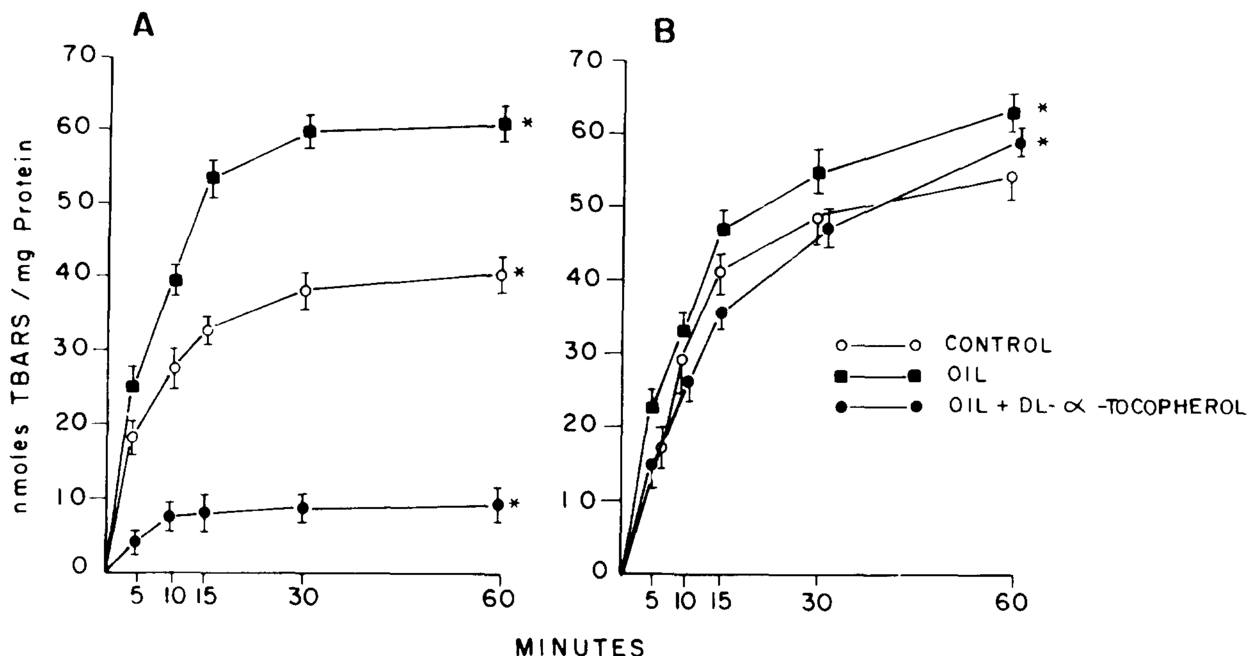

Figure 2 Effect of feeding fish oil and fish oil supplemented with dl-α tocopherol (1 g/kg oil) on the susceptibility of erythrocyte membranes obtained from young rats (A) and aged rats (B) to the induction of lipid peroxidation. Results are expressed as the mean ± S.E.M. of five experiments, each one constituted by three animals. Statistical significance of data was performed for 60 min incubation. (* $P < 0.001$)

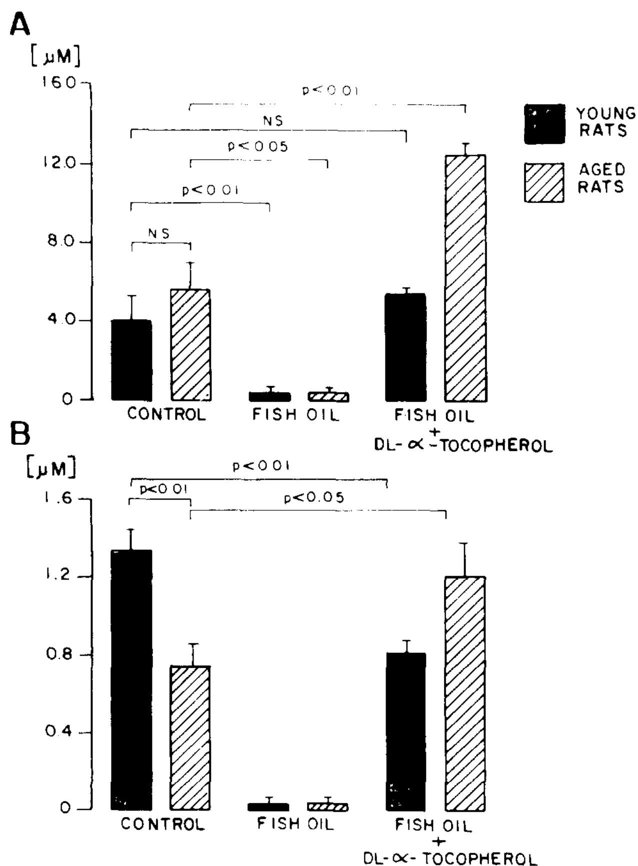

Figure 3 Changes in plasma (A) and erythrocyte membrane (B) dl-α tocopherol content after feeding young and aged rats with fish oil and fish oil supplemented with dl-α tocopherol. Results represent the mean ± S.E.M. of five experiments, each one constituted by three animals.

(Figure 3-B). Feeding fish oil drastically decreases dl-α tocopherol levels in plasma and in membranes *(Figure 3 A and B)*, both in young and in aged rats. Supplementation of the oil with dl-α tocopherol restores, although to a different level, the plasma and the membrane concentration of the antioxidant. While young rats show dl-α tocopherol levels equivalent to their respective controls, the concentration of dl-α tocopherol in plasma from aged rats increases by over 100%. Oil supplementation with dl-α tocopherol does not restore the levels of the antioxidant in erythrocyte membranes from young rats, as compared with their respective controls. However, aged rats show a higher dl-α tocopherol concentration than their respective controls.

Discussion

The increased susceptibility of erythrocyte membranes to the induction of oxidative stress associated with the ingestion of high doses of fish oil may be a consequence of an increase in the content of n-3 polyunsaturated fatty acids of such membranes. As previously described by us,[5] EPA and DHA content in erythrocyte membranes and hepatic microsomes is increased following the ingestion of fish oil. Similar results have been found by Leibovitz et al.,[15] who reported increased TBARS values after incubation of liver and kidney homogenates obtained from rats fed with a diet supplemented with menhaden oil.

Polyunsaturated fatty acid profiles obtained from young rats are not substantially different from those obtained from aged rats, linoleic acid being an exception. This latter fatty acid appears significantly reduced

in aged animals. The reason for the increase in the EPA and DHA content seen in membranes from rats fed sardine oil supplemented with dl-α tocopherol remains to be elucidated. However, it may be hypothesized that the antioxidant either enhances the incorporation of these fatty acids into the membrane structure or it protects them from the deleterious effect of in situ lipid peroxidation. dl-α tocopherol would preserve the content of EPA and DHA and other polyunsaturated fatty acids in the erythrocyte membrane.

Feeding young rats sardine oil supplemented with dl-α tocopherol decreases considerably the susceptibility of erythrocyte membranes to the induction of oxidative stress. This protective effect is, however, not observed in membranes obtained from aged animals. Such a differential response may relate to differences in the bioavailability of the antioxidant in young and aged animals. Plasma levels of dl-α tocopherol in aged rats rise over two fold when animals received fish oil supplemented with the antioxidant. Similarly, the levels of dl-α tocopherol assessed in the erythrocyte membranes of these animals show a significant increase. These changes suggest a good absorption of the antioxidant in the digestive tract and its adequate transfer from plasma to erythrocyte membranes in aged rats. In contrast, in young rats dl-α tocopherol plasma levels show only a discrete increase after feeding animals fish oil supplemented with the antioxidant. In these latter animals, erythrocyte membranes show a drastic decrease in their dl-α tocopherol content. This apparent paradoxical behavior may be explained as follows: an increased susceptibility to the induction of lipid peroxidation in membranes of aged animals may, in spite of their high dl-α tocopherol content, occur due to an actual decreased availability of the antioxidant needed to act as a scavenger for those free radicals involved in lipid peroxidation. Therefore, although the antioxidant is found to be present in high concentration, it may be unable to trap pro-oxidant free radicals. The decreased dl-α tocopherol concentration observed in membranes obtained from young animals may, in turn, result from the antioxidant activity of the tocopherol as a free radical scavenger, avoiding lipid peroxidation in these structures by transforming into α-tocopheryl quinone,[16] a product of the antioxidant activity of dl-α tocopherol. The protective effect of the antioxidant is demonstrated when these membranes are subjected to the $Fe2+$ ascorbate peroxidative assay. In addition, Kitabchi and Wimalasena[17] described the existence of specific binding sites for α tocopherol in erythrocytes, where it has been shown that the antioxidant has a wide variety of roles other than its free radical scavenger properties.[18] Although changes in these receptors have not been described in aged animals, it can be hypothetized that membranes of erythrocytes from aged rats may lack these specific receptors, dl-α tocopherol being nonspecifically bound to the membrane matrix may be unable to act as antioxidant.

Ingestion of fish oil without antioxidant reduces the plasma and erythrocyte membrane dl-α tocopherol concentration to undetectable levels, and increases considerably the susceptibility of these membranes to the induction of lipid peroxidation both in young and aged rats. These observations indicate that fish oil ingestion in high doses may lead to a depletion of plasma and erythrocyte membrane dl-α tocopherol. This effect has also been observed by us in other cellular fractions such as hepatic microsomes and brain homogenates (unpublished results).

Our results suggest that ingestion of high doses of fish oil are accompanied by an increased susceptibility to the induction of oxidative stress in erythrocyte membranes of both young and aged rats. Supplementation of the oil with dl-α tocopherol to a concentration of 1 g/kg oil may prevent this oxidation but only in membranes obtained from young rats. The absence of protective effect in aged rats may be attributed not to a lack of tissue antioxidant, but to a decrease in the ability of dl-α tocopherol to act as a free radical scavenger. The activity of protective enzymes such as superoxide dismutase, catalase, and glutathione peroxidase has not been studied yet in experimental animals given high doses of fish oil. The possibility of adding higher concentrations of dl-α tocopherol or other antioxidant to the oil to balance the cellular antioxidant capacity may thus be considered. Further experiments to evaluate this possibility and to assess the effect of fish oil ingestion in other tissues are currently being conducted in our laboratory.

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