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Stabilization of Rat Serum Proteins Following Oral Administration of Fish Oil

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The mechanism of action of fish oil (FO), currently used in different chronic inflammatory conditions such as rheumatoid arthritis (RA), is not completely understood, although it is thought that it could alter the metabolism of endogenous autacoids. In addition, we hypothesized that the known capability of fatty acids (FA) of stabilizing serum albumin and perhaps other proteins, may be of pharmacological relevance considering that it is shared by other anti-rheumatic agents (e.g. nonsteroidal antiinflammatory drugs). Thus, we studied the effect of oral administration of FO and corn oil (CO), a vegetable oil with a different composition, on the stability of rat serum proteins, evaluated by a classical *in vitro* method based on heat-induced protein denaturation. FO, and, to a lower extent, CO inhibited heat-induced denaturation of rat serum (RS): based on the inhibitory activity (EC_{50}) of the major fatty acids against heat-induced denaturation of RS *in vitro*, it was possible to speculate that *in vivo* effects of palmitic acid (C16:0) and eicosapentaenoic acid (EPA, C20:5, n-3) may be more relevant than that of linolenic acid (C18:2). To better investigate this phenomenon, we extracted albumin from the serum of animals treated or not with FO with a one-step affinity chromatography technique, obtaining high purity rat serum albumin preparations (RSA-CTRL and RSA-FO), as judged by SDS-PAGE with Coomassie blue staining. When these RSA preparations were heated at 70°C for 30 min, it was noted that RSA-FO was much more stable than RSA-CTRL, presumably due to higher number of long chain fatty acids (FA) such as palmitic acid or EPA.
In conclusion, we provided evidences that oral administration of FO in the rat stabilizes serum albumin, due to an increase in the number of protein bound long chain fatty acids (e.g. palmitic acid and EPA). We speculate that the stabilization of serum albumin and perhaps other proteins could prevent changes of antigenicity due to protein denaturation and glycosylation, which may trigger pathological autoimmune responses, suggesting that this action may be involved in the mode of action of FO in RA and other chronic inflammatory diseases.

Key words: Fish oil, Fatty acids, Antidenaturant agents, Rheumatic diseases.

INTRODUCTION

Fish oil (FO) is currently used in different chronic degenerative conditions including inflammatory diseases such as rheumatoid arthritis (RA) (Kremer, 1996). The mechanism by which FO could exert beneficial effects in these conditions is not completely understood but it is thought that supplementation of n-3 long chain polyunsaturated fatty acids (n-3-PUFA) may alter the metabolism of eicosanoids with formation of autacoids (PG_3 and LT_5 instead of PG_2 and LT_4) with lower pro-inflammatory activities (Kremer, 1996).

On the other end, it has been known for a long time that fatty acids (FA) stabilize serum albumin (Ballou *et al.*, 1944; Boyer *et al.*, 1946; Peters, 1985; Peters, 1996; Saso *et al.*, 1999) and perhaps other proteins. This property may be of pharmacological relevance considering that nonsteroidal anti-inflammatory drugs (NSAID) have both antidenaturant (Mizushima, 1964; Saso *et al.*, 1998) and antirheumatic activity and that other anti-denaturant, such as bendazac (Silvestrini, 1987) and bindarit (Cioli *et*

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al., 1992), possess beneficial activity in chronic-degenerative processes such as adjuvant arthritis in the rat (Cioli *et al.*, 1992; Saso *et al.*, 1992). On this basis, it is possible to speculate that fish oil activity in RA may be partly due to protein stabilization as a consequence of increased levels of selected FA.

The aim of this work was to study the effect of oral administration of FO and corn oil (CO), a vegetable oil with a completely different composition (Kinsella, 1990; White, 1992), on the stability of rat serum proteins, evaluated by a classical *in vitro* method based on heat-induced protein denaturation (Saso *et al.*, 1999; Mizushima, 1964; Saso *et al.*, 1998).

MATERIALS AND METHODS

Reagents

Human serum albumin (HSA, 96% pure, Cohns fraction V, cat. N°A-1653), sodium chloride, sodium citrate, heparin, fish oil (FO, cat. N° F-8020), corn oil (CO, cat. N° C-8267), fatty acids, Tris (cat. N° T-1503), sodium thiocyanate (cat. N° S-7757), and acrylamide (40% solution, cat. N° A-4058) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS, cat. N° 161-0301), bromophenol blue (cat. N° 161-0404), a mixture of molecular weight (Mr) markers [cat. N° 161-0309, containing prestained myosin (apparent Mr=205,000), β-galactosidase (apparent Mr= 116,500), bovine serum albumin (apparent Mr=77,000) and ovalbumin (apparent Mr=116,500)] and other reagents for electrophoresis were from Bio-Rad (Richmond, CA, USA). Coomassie blue R-250 and G-250 were from United States Biochemical Co. (Cleveland, Ohio, USA).

Heat-induced denaturation of rat plasma and serum

Wistar rats of about 250 g were anesthetized and blood was withdrawn by cardiac puncture and collected in tubes with or without heparin (0.2 mg/ml). After 2 hr at room temperature, the tubes were centrifuged at 2000 × g for 20 min and the samples were diluted with 0.066 mol/l sodium citrate pH 5.3 (at 22°C). Then, aliquots of 100 µl were pipetted in triplicate onto a 96-well microplate (Falcon 3911, Becton & Dickinson, Oxnard, CA, USA), the plate was covered with aluminum foil and incubated in oven at 70°C ± 0.1°C for 30 min. The turbidity of the samples was determined as absorbance at 595 nm, using an automatic microplate reader (model 3550 obtained from Bio-Rad, Hercules CA, USA).

Effect of fish oil and corn oil administration on heat-induced denaturation of rat serum

12 Wistar rats of about 250 g were divided in 3 groups and treated p.o. with saline (control group), FO (1 g/Kg body weight) and CO (1 g/Kg body weight). After 2 hrs the animals were anesthetized and blood was withdrawn by cardiac puncture and allowed to clot for 2 hrs at room temperature and heat-induced denaturation of sera was examined as described above.

Effect of major fatty acids of fish oil and corn oil on heat-induced denaturation of rat serum

The assay was performed according to the classical Hizushimo's assay (Mizushima, 1964) modified as previously described (Saso *et al*, 1998). Briefly, serum obtained from Wistar rats of about 250 g was diluted 1:4 with 0.066 mol/l sodium citrate (pH 5.3 at 22 C°), and the major fatty acids of FO and CO (according to reference 12) were added at different concentrations up to their solubility. Then, heat-induced denaturation of 100 µl aliquots was studied as described above. The antidenaturant activity of each substance at different concentrations was calculated using the following formula:

$$a_x=[1-(A_{x,70^oC}-A_{0,22^oC})/(A_{0,70^oC}-A_{0,22^oC})] \bullet 100$$

where $A_{X,\ 70^oC}$ is the absorbance at 595 nm of the protein solution heated in the presence of the substance X, and $A_{0,\ 22^oC}$ and $A_{0,\ 70^oC}$ are the absorbances of protein solutions before and after the heating, respectively. Each activity vs. concentration curve was analyzed statistically according to Tallarida and Murray (1986), to determine the concentration corresponding to a stabilizing activity of 50% (EC_{50}).

Rat serum albumin (RSA) purification by Affi-gel blue affinity chromatography

Affi-gel blue affinity chromatography was performed essentially as described in Cheng *et al.* (1988). Two 10 × 500 mm columns (Econo-columns®, Bio-Rad cat. N° 737-1052) were packed with 40 ml of Affi-gel blue (50-100 mesh, Bio-Rad cat. N° 153-7301) and equilibrated with buffer A (0.020 mol/l Tris pH 7.4 at 22°C) at the flow rate of 6 ml/hr. Then, two distinct pools of rat serum (10 ml), prepared from animals treated (RS-FO) or not with FO (RS-CTRL), were extensively dialyzed against buffer A, using a dialysis membrane with cut-off 6000-8000 daltons (Spectra/Por®, cat. N° 132650, Spectrum, Houston, Texas, USA), and loaded onto the columns at the flow rate indicated above. Afterwards, the columns were washed thoroughly with buffer A until no proteins were present in the eluate as judged by UV detection at 214 nm. Next, albumin was removed from the stationary phase by washing with 5 bed volumes (200 ml) of buffer B (0.05 mol/l Tris, pH 8.0 at 22°C, containing 0.2 mol/l sodium thiocyanate). Finally, the eluates were dialyzed against buffer A using an 8050 Amicon (Danvers, MA, USA) ultrafiltration unit, equipped with YM-10 membranes with cut-off 10000 daltons and concentrated down to 10 ml. The protein content of the two samples (RSA-

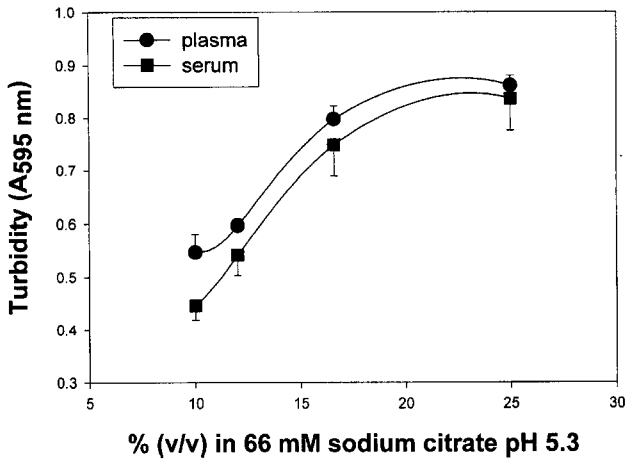

Fig. 1. Heat-induced denaturation of rat plasma and serum. 100 µl aliquots of normal rat plasma or serum were diluted in citrate buffer and pipetted in triplicate onto a 96-well microplate and incubated at 70°C for 30 min. Then, the turbidity was measured as absorbance at 595 nm. Data are means ± S.D.: no significant differences were observed between the two curves.

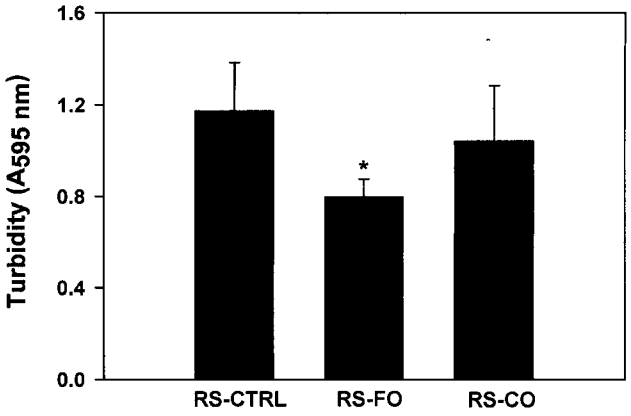

Fig. 2. Effect of fish oil and corn oil administration on heat-induced denaturation of rat serum. 100 µl aliquots of rat serum prepared from blood of control animals (RS-CTRL), and animals previously (2 hrs) treated p.o. with fish oil (RS-FO) or corn oil (RS-CO) were pipetted in triplicate onto a 96-well microplate and incubated at 70°C for 30 min. Then, the turbidity was measured as absorbance at 595 nm. The statistical analysis was performed by the Kruskal-Wallis ANOVA on ranks test and the Dunn's test, using the software Sigma-Stat®, version 2.0 for Windows 95™. *$p<0.05$ vs. RS-CTRL.

CTRL and RSA-FO) was estimated to be about 40 mg/ml by a Coomassie blue binding assay performed according to (Bradford, 1976) as modified by (Macart and Gerbaut, 1982).

Analysis of purified albumins by SDS-PAGE

Aliquots of about 20 µg of RS-CTRL, RS-FO, HSA, RSA-CTRL and RSA-FO were fractionated by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol on a 7.5% *T* gel, according to (Laemmli, 1970). Following electrophoresis, the gel was stained with Coomassie blue R-250.

Heat-induced denaturation of albumin purified from serum of rats treated with fish oil

Purified albumins (RSA-CTRL and RSA-FO) were dissolved at different concentrations in the range 0-1% (w/v) in 0.066 mol/l sodium phosphate (pH 5.3 at 22°C) and heat induced denaturation of 100 µl aliquots was examined in triplicate as described above.

Statistical analysis

Statistical analysis was performed using the Kruskal-Wallis ANOVA on ranks test combined with the Dunns test, performed with the software Sigma-Stat® version 2.0 for Windows 95™ (SPSS, Chicago IL, USA).

RESULTS

Heat-induced denaturation of rat plasma and serum

When rat plasma or serum, diluted in 0.066 mol/l sodium citrate pH 5.3, were heated at 70°C for 30 min, the turbidity vs. concentration curves reported in Fig. 1 were obtained: no significant differences between the behavior of the two fluids were observed.

Effect of fish oil and corn oil administration on heat-induced denaturation of rat plasma and serum

Oral administration of fish oil (FO, 1 g/Kg), but not corn oil (CO, 1 g/Kg), significantly inhibited heat-induced denaturation of serum prepared from blood withdrawn 2 hrs after the treatment (Fig. 2).

Effect of major fatty acids of fish oil and corn oil heat-induced denaturation of rat serum

When heat-induced denaturation of normal rat serum (NRS), spiked with the major fatty acids of FO and CO, was studied, sigmoidal turbidity vs. concentration curves were obtained and effective concentration 50 (EC_{50}) of each compound was calculated and plotted in Fig. 3 with variable symbol size according to the composition of the two oils (Kinsella, 1990; White, 1992). The most potent and abundant compounds were palmitic (C16:0) and eicosapentaenoic acid (C20:5, n-3) in the case of FO; and palmitic (C16:0) and linoleic acid (C18:2) in the case of CO (Fig. 3).

Purification of rat serum albumin by Affi-gel blue affinity chromatography and analysis by SDS-PAGE

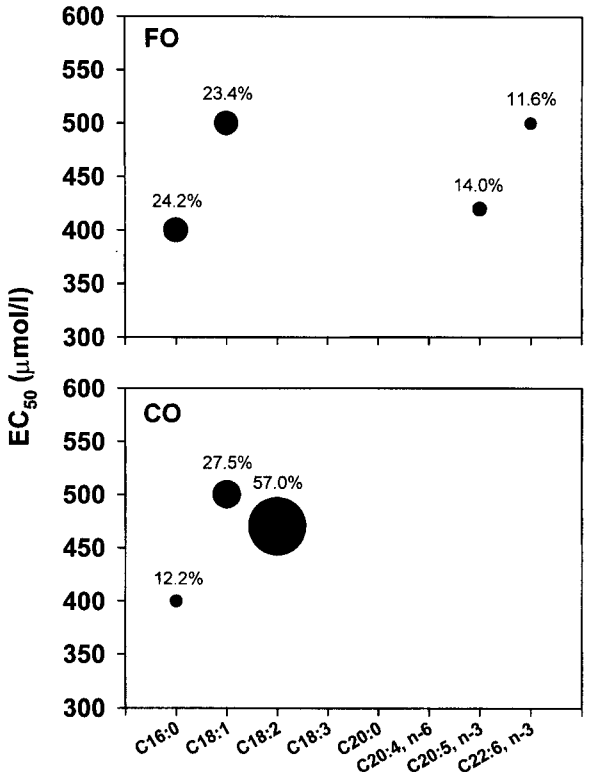

Fig. 3. Effect of major fatty acids of fish oil and corn oil on heat-induced denaturation of rat serum. The major fatty acids of fish oil (FO) and corn oil (CO) were added to normal rat serum (NRS) at increasing concentrations up to their solubility and their heat induced denaturation was studied: from the turbidity vs. concentration curves, the effective concentrations 50 (EC_{50}) were calculated and plotted using variable symbol sizes according to the known composition of the oils.

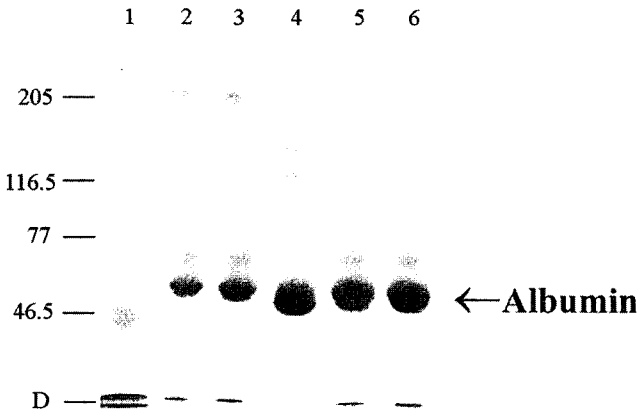

Fig. 4. Analysis of purified albumins by SDS-PAGE. Polyacrylamide gel electrophoresis (PAGE) was performed in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol on a 7.5% *T* gel, which was stained with Coomassie blue: lane 1, molecular weight (Mr) prestained markers (Bio-Rad, cat. N° 161-0309, 10 µl), i.e. myosin (205,000), β-galactosidase (116,500), bovine serum albumin (77,000) and ovalbumin (116,500); lane 2, normal rat serum (RS-CTRL, 0.3 µl); lane 3, rat serum from animals treated with fish oil (RS-FO, 0.3 µl); lane 4, 96% pure human serum albumin (HSA, 20 µg); lane 5, albumin purified from RS-CTRL (RSA-CTRL, about 20 µg); lane 6, albumin purified from RS-FO (RSA-FO, about 20 µg).

About 400 mg of rat serum albumin (RSA) were extracted from serum samples (10 ml) prepared from animals treated (RS-FO) or not (RS-CTRL) with FO, by affinity chromatography using the commercially available phase Affi-gel blue.

The purity of the protein preparations was checked by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol on a 7.5% *T* gel stained with Coomassie blue (Fig. 4): RS-CTRL (0.3 µl, lane 2), RS-FO (0.3 µl, lane 3), commercial human serum albumin (HSA, 20 µg, lane 4), RSA-CTRL (about 20 µg, lane 5) and RSA-FO (about 20 µg , lane 6) were analyzed; albumin was visualized as a strong band with apparent Mr of 68, 000, as compared with the Mr protein marker (lane 1). By comparison with commercial HSA (lane 4), the purity of our preparations (RSA-CTRL and RSA-FO) was estimated to higher than 96%.

Heat-induced denaturation of albumin purified from serum of rats treated with fish oil

When heat-induced denaturation of purified albumin (RSA-CTRL and RSA-FO), dissolved at different concentrations in the range 0-1% (w/v) in 0.066 mol/l phosphate (pH 5.3 at 22°C), was examined, it was noted that RSA-FO yielded much lower turbidities than RSA-FO at the same concentrations (Fig. 5).

DISCUSSION

Oral administration of fish oil (FO), and, to a lower extent, of corn oil (CO), inhibited heat-induced denaturation of rat serum (RS) (Fig. 2), probably due to increased serum concentration of free fatty acids (FA), which are well known stabilizers of serum albumin (Ballou *et al.*, 1944; Boyer *et al.*, 1946; Saso *et al.*, 1999; Brandt, 1976; Gumpen *et al.*, 1979; Ahmad *et al.*, 1995).

Based on both the potency (EC_{50}), and the relative abundance (reported in Kinsella, 1990) of the fatty acids of FO and CO (Fig. 3), we speculated that *in vivo* effects of palmitic acid (C16:0) and eicosapentaenoic acid (EPA, C20:5, n-3) were more relevant than those of linoleic acid (C18:2) (Fig. 3).

To better investigate this effect of FO, we extracted albumin from the serum of animals treated or not with this product, using a one-step affinity chromatography technique: the purity of the rat serum albumin preparations (RSA-CTRL and RSA-FO), as judged by SDS-PAGE using Coomassie blue staining (Fig. 4), was comparable,

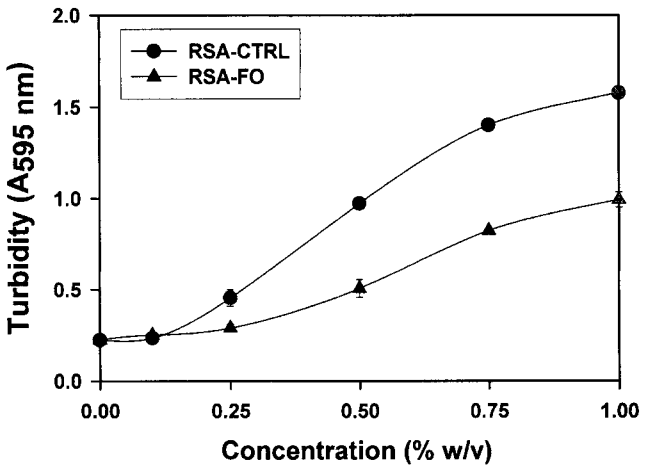

Fig. 5. Heat-induced denaturation of albumin purified from serum of rats treated with fish oil. Albumins purified from normal rat serum (RSA-CTRL) or serum of animals treated with fish oil (RSA-FO) were diluted in 0.066 mol/l sodium phosphate pH 5.3 at 22°C, and subject to heat-induced denaturation. Turbidity was evaluated as absorbance at 595 nm. Data are means ± S.D.

if not higher, than that of HSA marketed as 96% pure. When these RSA preparations, diluted in sodium phosphate buffer with pH close to the isoelectric point of the protein (Peters, 1985), were heated at 70°C for 30 min, it was noted that RSA-FO was much more stable than RSA-CTRL, presumably due to higher number of long chain fatty FA such as palmitic acid or EPA. Since the association constants of the binding of the latter compounds to albumin are very high ($>10^7$ M^{-1} according to *Richieri et al.*, 1993), they were not likely to dissociate from the protein molecule during the purification procedure.

The dose of FO administered to rats in this work (1 g/Kg) is higher than those evaluated in clinical studies (McCarthy *et al.*, 1992; Gerster, 1995), but we speculate that chronic therapies with a few grams a day for several weeks could have protein stabilizing effects comparable to those of single high doses. In fact, HSA molecule has 6 distinct binding sites for long chain FA (Peters, 1985; Peters, 1996), but carries only an average of 1-2 molecules of FA in physiological conditions (Peters, 1985; Peters, 1996). Thus, an increase in FA serum levels, which can be significant during FO supplementation [about 4-fold for EPA following administration of about 2 g/day for 12 weeks (Tempel *et al.*, 1990)], could further protect albumin and perhaps other proteins against denaturation (Opie, 1962; Silvestrini *et al.*, 1969) and abnormal glycosylation (Saso *et al.*, 1993), which are known to affect protein antigenicity and possibly trigger pathological autoimmune responses in certain rheumatic conditions.

In conclusion, we provided evidences that oral administration of FO stabilizes serum albumin and perhaps other proteins, due to an increase in the number of protein bound long chain fatty acids (e.g. palmitic acid and EPA), suggesting that this action may be involved in its mode of action in RA and other chronic inflammatory diseases.

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