n–3 PUFA dietary supplementation inhibits proliferation and store-operated calcium influx in thymoma cells growing in Balb/c mice

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Abstract  The antitumor effect of daily individual administration of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (2 g/kg body weight) in Balb/c mice bearing a transplantable thymoma was investigated. Mice received oleic acid (control group), EPA and DHA ethyl esters starting 10 days before tumor inoculation. Analysis of phospholipid composition of neoplastic cell revealed that EPA and DHA levels were significantly increased (33 and 22% increase) after EPA and DHA treatments, respectively. Conversely, decreased levels of arachidonic acid were found in both cases (19 and 24% decrease in EPA and DHA groups, respectively). EPA and DHA delayed the appearance of macroscopic ascites (100% of animal, from 7 to 28 days), prolonged animal survival (100% of animal, from 22 to 33 and 33 days, respectively) and reduced the percentage of proliferating tumor cells detected by immunostaining of proliferating cell nuclear antigen (PCNA) (80 and 85% decrease) after EPA and DHA treatments, respectively. This effect was related to the inhibition of cell proliferation in thymoma cells growing in Balb/c mice.

Supplementary key words  DHA • EPA • fatty acids • tumor growth • phospholipids • signaling

There is increasing evidence for a protective effect of n–3 polysaturated fatty acids (PUFAs) against the development and progression of several types of cancer (1, 2). We have previously reported the antiproliferative effect of a dietary supplementation with a mixture of EPA and DHA in the colonic mucosa of human subjects at high risk for colon cancer (3). Recently, we found that EPA dietary supplementation is able to inhibit the development of focal hepatic neoplastic lesions during chemical carcinogenesis in rats (4). Moreover, we observed that individual administration of purified EPA and DHA inhibited the growth of Morris hepatocarcinoma 3924A transplanted in rats (5). In this study we found, in agreement with other authors (6–8), that the antitumoral action of EPA and DHA was related to the inhibition of cell proliferation and apoptosis.

The mechanism(s) underlying the antitumor effect of n–3 PUFAs have not been completely elucidated. The reduction in the synthesis of eicosanoids derived from arachidonic acid is considered to be involved in the growth inhibitory effect of n–3 PUFAs (9, 10). It has also been suggested that cancer cells are killed by lipid peroxidation products generated in larger amount in cells enriched with n–3 PUFAs (11). We recently suggested that the enhancement of β-oxidation activity by n–3 PUFAs and the consequent overproduction of H2O2 may originate a mild oxidative stress able to inhibit neoplastic growth (4). Finally, different studies have reported that n–3 PUFAs can modulate the activity of components of intracellular signaling (12–14). It is well established that Ca2+ is among the major intracellular factors involved in the signaling transduction pathways evoking cell growth and proliferation, and other key processes such as gene expression, contraction, secretion, and cellular metabolism (15, 16). Many of these processes require a sustained

Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PCNA, proliferating cell nuclear antigen; PUFAs, polysaturated fatty acids; SOCI, store-operated Ca2+ influx; Tg, thapsigargin.

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increase in the concentration of intracellular free Ca\(^{2+}\) ([Ca\(^{2+}\)\_]) that is mainly elicited by the influx of the cation into the cell from the extracellular compartment (16). Recently, it has been established that n-3 PUFAs suppress voltage-gated L-type Ca\(^{2+}\) currents in rat cardiac myocytes (17). In non-excitable cells, the major pathway of Ca\(^{2+}\) entry is the so-called store-operated Ca\(^{2+}\) influx (SOCI), triggered by agonists (such as inositol 1,4,5-trisphosphate) that act by emptying the high affinity intracellular Ca\(^{2+}\) stores (18). In particular, SOCI has been involved in the cell signaling occurring in non-excitable cells to evoke different cell processes including gene regulation and cell growth (19). Recently it has been observed that the addition of PUFAs to different strains of cells cultured in vitro modify the extent of SOCI (20). However, at the moment, the effect of a physiological membrane incorporation of PUFAs on this pathway of Ca\(^{2+}\) entry, obtained in vivo after a dietary treatment, has never been studied. In particular, the effect of n-3 PUFAs is almost completely unknown.

The purpose of the present study was to evaluate the effects of an oral administration of EPA and DHA on the growth of a highly malignant thymoma transplanted in the peritoneal cavity of Balb/c mice. We intended also to study the regulatory effect of these dietary fatty acids incorporated in cell phospholipids on the store-dependent pathway of Ca\(^{2+}\) influx.

**MATERIALS AND METHODS**

**Animals and treatments**

Male inbred Balb/c mice, aged 6 weeks (average body weight: 23.4 ± 1.7 g), were housed in cages and fed a non-purified commercial diet (Altromin-Rieper, Bolzano, Italy). The composition of the diet was (% w/w) crude protein, 23; fat, 5.5; fiber, 5; mineral, 8; carbohydrates, 58.5; water, 12. The vitamin mixture was supplied by Hoffmann-La Roche, Basel, Switzerland) was verified by analysis. The animals received OA, EPA, or DHA for 10 days before inoculation of a highly malignant thymoma in ascites form and until the end of the experiment. Ten days represents the time required to obtain the maximal incorporation of EPA and DHA (data not shown). All the animals of this experiment were used to study the appearance of tumor and survival.

In the second experiment the animals were divided into three groups (44 mice/group) and treated as reported above for the first experiment. Eight animals from each group were killed 4 days after tumor transplantation to evaluate tumor cell proliferation by immunostaining of proliferating cell nuclear antigen (PCNA). After 3 additional days, 20 animals from each group were killed and tumor thymocytes were harvested for cell counting and evaluation of fatty acid composition and Ca\(^{2+}\) influx. The remaining mice were used to study the appearance of tumor and survival. The animal-use protocol was approved by the Ministry of Health, Veterinary Service, Rome, Italy.

**Tumor cells**

A highly malignant thymoma growing in ascitic form in the peritoneum of Balb/c mice was used. The neoplastic cell line derived from a strain of malignant spontaneous thymoma of Balb/c mice (originally propagated in the Institute of Pathology of the University of Perugia, Italy) (22). The ascites thymoma cells were maintained by weekly intraperitoneal transplantation in syngeneic Balb/c mice.

**Tumor cell inoculation and count**

Tumor cells were aseptically inoculated (1 × 10\(^7\) in 0.3 ml 0.9% NaCl) in the peritoneal cavity of Balb/c mice. Tumor cell count was performed after harvesting the cells from the peritoneum of mice 7 days after transplantation, because these cells grow exponentially for 5 days, reaching a plateau at 6-7 days. To ensure the complete collection of cells, the peritoneum was washed twice with 5 ml 0.9% NaCl. Cell suspension in ascitic fluid was diluted in Ringer-HEPES 50 mM, pH 7.4 (130 mM NaCl, 5 mM KCl, 1 mM MgSO\(_4\), 1.3 mM CaCl\(_2\), and 50 mM HEPES) to avoid agglutination, and tumor thymocytes were immediately counted using a Trypan Blue dye exclusion method using a Burker chamber.

**Cell proliferation**

The percentage of proliferating cells was determined by immunostaining of proliferating cell nuclear antigen (PCNA), a well-known cell cycle-related antigen detected by immunofluorescence in S-phase nuclei (23). Cells were harvested from peritoneum at the 4th day after transplantation, during the exponential phase of duplication. They were cyt centrifuged, fixed with acetone, and incubated for 1 h with the anti-PCNA antibody (PC10, Boehringer Mannheim, Indianapolis, IN) at a dilution of 1:1000. The cells were counterstained with hematoxylin after visualization of the reaction product with diaminobenzidine (DAB) and routine streptavidin–biotin–peroxidase treatment. The labeling index (LI%), defined as the percentage of positively staining nuclei, was determined from a count of 1000 cells at high power magnification (400×).

**Phospholipid fatty acid composition of tumor cells**

Tumor cells were harvested from the peritoneum of mice at the 7th day after transplantation. The extraction of lipids from tumor cells was performed according to the method of Bligh and Dyer (24). Total phospholipids were separated from neutral lipids by thin-layer chromatography using toluene–diethyl ether–ethanol 35:3:5.1 (v/v/v) as the solvent system (25). After transmethylation and extraction with n-hexane, fatty acid methyl esters were separated by gas–liquid chromatography using a Carlo Erba HRGC 5300 Mega-series chromatograph (5). Individual fatty acid methyl esters were identified by comparing their retention times to known standards (Sigma, St. Louis, MO). The areas under the peaks of each fatty acid were measured with a Carlo Erba Mega-
series integrator and expressed as percentages of the total area of all major fatty acid peaks.

**Intracellular free Ca\(^{2+}\) measurements**

\([\text{Ca}^{2+}]\) was evaluated by measuring the fluorescence of the Ca\(^{2+}\)-indicating dye, Fura-2 (26). The cells were loaded with 3 \(\mu\)M Fura-2/AM (Fura-2/acetoxymethylester), an esterified derivative of Fura-2, as previously described (22). After loading, Fura-2/AM was hydrolyzed by intracellular esterases to Fura-2 that become entrapped in the cytosol and bind to free Ca\(^{2+}\). Briefly, cells (2.5 \(\times\) 10\(^6\) ml) harvested at the 7th day from inoculum were washed twice and suspended in Ringer-HEPES 50 mm, pH 7.4. They were incubated in the dark at 34\(^\circ\)C for 10 min and then supplemented with Fura-2/AM (Sigma, St. Louis, MO). After further 15 min at 34\(^\circ\)C, to allow the incorporation of Fura-2/AM into the cells, the cell suspension was diluted 1:5 in the same buffer and incubated for an additional 10 min to allow complete hydrolysis of the Fura-2/AM. Cells were then washed and resuspended at the same initial cell concentration and kept at room temperature until used. Just before use, 1 ml of the stock suspension was rapidly centrifuged and resuspended at a final concentration of 1.25 \(\times\) 10\(^6\) cells/ml in Ca\(^{2+}\)-free Ringer-HEPES 50 mm, pH 7.4, in which CaCl\(_2\) was omitted and 0.2 mm EGTA was added. The measurements were performed in a Perkin-Elmer LS3B fluorometer (excitation 340 nm, emission 510 nm) at 30\(^\circ\)C, to minimize dye leakage from the cells. Calibration was performed according to the protocol described by Arslan et al. (27). A value of 184 nm was used as the \(K_0\) for the Ca\(^{2+}\)-Fura-2 complex at 30\(^\circ\)C.

SOCl\(_2\) was investigated using thapsigargin (Tg), an endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor that, leading to the depletion of intracellular Ca\(^{2+}\) stores, directly triggers this pathway of Ca\(^{2+}\) influx. SOCl\(_2\) was measured after cell pretreatment with 500 nm Tg in a Ca\(^{2+}\)-free medium and subsequent readmission of 1.5 mm CaCl\(_2\) (20).

\(\text{Mn}^{2+}\) quenching of Fura-2 fluorescence was evaluated as surrogate of Ca\(^{2+}\) to trace Ca\(^{2+}\) influx mechanism (20). The measurements were performed at 360 nm, the excitation wavelength at which the indicator fluorescence is not influenced by changes in Ca\(^{2+}\) concentration (isosbestic point).

**Statistical analysis**

The results were expressed as the means \(\pm\) SE. Comparisons among groups were made by one-way analysis of variance (ANOVA) using Minitab software (Minitab, State College, PA). When significant differences were found, post hoc comparisons of means were made using Fisher’s test. Differences were considered significant at \(P < 0.05\).

**RESULTS**

Dietary supplementations with n-3 PUFAs at the dose used in this study did not modify daily food intake of mice. Weight gain, measured prior to tumor inoculation, was not significantly different (ANOVA, \(P < 0.001\)) among the groups of animals (data not shown).

**Tumor growth**

Figure 1 shows macroscopical ascites appearance and animal survival after transplantation. All the animals of the OA group exhibited maximal ascites growth within 7 days (panel A) and died within 21 days after tumor inoculation (panel B). Treatment with both EPA and DHA delayed ascites appearance and prolonged animal survival.

**Phospholipid fatty acids of neoplastic thymocytes**

The content of the principal PUFAs in total phospholipids (PL) from OA-, EPA- and DHA-treated mice is shown in Fig. 2. The content of the PUFAs was significantly affected by the treatment with the two n-3 fatty acids with respect to the control group. EPA treatment significantly increased EPA (20:5n–3) and its metabolite docosapentaenoic acid (DPA, 22:5n–3) without modifying the level of DHA (22:6n–3). Conversely, DHA supplementation increased the amount of DHA itself (22:6n–3). Levels of linoleic acid were unchanged in both groups of animals and the content of arachidonic acid (20:4n–6) was significantly decreased in both groups.

**Fig. 1.** Development of tumor (A) and animal survival (B) after tumor cell transplantation in OA-, EPA-, and DHA-treated mice. Values are means of two experiments (16 mice for each experiment). Statistical comparisons were performed at the 7th (panel A) and 22nd day (panel B) after tumor transplantation, when 100% OA-treated animals had tumors or were dead, respectively. * Significantly different from OA group (\(P \leq 0.001\); one-way ANOVA, followed by Fisher’s test).
Cell count and proliferation

Figure 3 shows the effects of EPA or DHA on the number (A) and proliferation (B) of tumor cells growing in the peritoneal cavity of control and treated mice. Both n−3 PUFA treatments significantly reduced the number of neoplastic thymocytes (A) harvested 7 days after tumor inoculation. Cell proliferation (B) was evaluated by PCNA immunostaining at the 4th day after inoculum. PCNA-LI% of thymoma cells isolated from EPA and DHA was decreased 5- and 7-fold, respectively, as compared to cells from OA-treated mice.

Store-dependent Ca\(^{2+}\) influx of neoplastic thymocytes

Figure 4 shows the modification in SOCl\(_{i}\) of tumor thymocytes enriched in vivo with EPA and DHA and determined by the Ca\(^{2+}\)-free/ Ca\(^{2+}\)-reintroduction protocol after pretreatment with 500 nm Tg. Tg was used in supramaximal concentration to ensure the complete release of Ca\(^{2+}\) stored in the endoplasmic reticulum. The [Ca\(^{2+}\)]\(_{i}\) increase, measured in the absence of extracellular Ca\(^{2+}\) and triggered by the Tg-induced depletion of intracellular stores, was small and not different among control and EPA- or DHA- enriched cells. After 6 min of Tg treatment, calcium readmission, at the concentration of 1.5 mm, resulted in a rapid [Ca\(^{2+}\)]\(_{i}\) rise, which is known to be dependent on activation of store-operated Ca\(^{2+}\) influx. The rise ended in a plateau phase and, as better shown by Fig. 5, both EPA and DHA significantly reduced Ca\(^{2+}\) increase at the same degree.
To exclude that the reduced increase in cytosolic Ca\(^{2+}\) concentration after Tg/Ca\(^{2+}\) addition was due to the enhancement of Ca\(^{2+}\) extrusion through the plasma membrane, the influx of Mn\(^{2+}\) was also analyzed (Fig. 6). This cation enters the cytosol through the same store-dependent pathway as Ca\(^{2+}\) and its flux is almost completely unidirectional towards the interior of the cell being poorly extruded from the cytosol (28). The figure shows the effect of the dietary supplementations with EPA or DHA on Mn\(^{2+}\) influx in Tg-pretreated neoplastic cells. Mn\(^{2+}\) influx has been measured as percentage of Mn\(^{2+}\) quenching of Fura-2 signal. As shown by the figure, both PUFAs markedly inhibited Mn\(^{2+}\) influx in Tg-pretreated neoplastic thymocytes.

**DISCUSSION**

Different epidemiological and experimental studies demonstrate the antitumor action of n–3 PUFAs and support the hypothesis that they may have an anticancer effect also in humans (1–2, 29).

The present study investigated the effect of separate dietary administration of EPA and DHA on the growth of a highly malignant thymoma transplanted in mice. This tumor grows in the peritoneum as a cell suspension and is particularly suitable to study the effects of dietary manipulation on different cellular parameters.

Both EPA and DHA inhibited tumoral development and growth to the same extent, delaying macroscopical tumor appearance, and animal death. The antitumoral effect of EPA and DHA treatment was further proven by...
the reduced number of cells growing in the peritoneum and by the marked inhibition of thymoma cell proliferation, as demonstrated by PCNA immunostaining. We cannot exclude that EPA and DHA may act also by modulating the apoptotic process, as shown previously by Finstad et al. (8) in cell lines or by us in animal models (5, 30).

However, in the present work, the number of apoptotic cells, evaluated in the peritoneum of Balb/c mice by the in situ nick end labeling method, was very low and it was not possible to appreciate the differences among the three treatment groups (data not shown). Presumably, the physiological phagocytosis occurring in the peritoneum in vivo did not allow the correct evaluation of the process.

Analysis of phospholipid fatty acid composition revealed increased levels of EPA and its metabolite docosapentaenoic acid (DPA, 22:5n-3) during EPA treatment and increased levels of DHA during DHA treatment. Conversely, the percentage of arachidonic acid was diminished in phospholipids from both groups. Such modifications confirmed those largely reported in vitro and in vivo models and in normal and tumor tissue and cells (5, 25, 31).

Among the mechanisms invoked to explain the n-3 PUFA inhibitory effect on cellular growth is their ability to modulate the activity of component of intracellular signaling (i.e., protein kinases, eicosanoids, reactive oxygen species, etc.) (4, 12-14). It is well known that Ca2+ is an essential component in the signaling pathways evoking key cellular processes including cell proliferation and growth (15, 16). For this reason we investigated Ca2+ metabolism in thymoma cells, whose proliferation was markedly inhibited by dietary treatment with EPA and DHA. In particular, we studied Ca2+ influx, which in non-excitable cells regulates different processes as exocytosis, contraction, enzyme control, gene regulation, cell proliferation, and apoptosis (19). The predominant Ca2+ entry pathway in non-excitable cells is SOCI, in which Ca2+ entry is regulated by the filling state of the agonist-sensitive intracellular Ca2+ stores (18). When the stores are full, they prevent extracellular Ca2+ entry, but after emptying, Ca2+ influx is promoted. Thymoma cells derived from both EPA- and DHA-treated mice showed a lower level of SOCI with respect to control cells. We studied SOCI after Tg-induced store depletion and subsequent Ca2+ addition. Moreover, the finding that n-3 PUFA s also inhibited Mn2+ influx in Tg-pretreated neoplastic cells strongly suggested that EPA and DHA treatments specifically inhibited the SOCI pathway. Our results are in good agreement with those recently obtained by Gamberucci, Fulcieri, and Benedetti (20). They demonstrated the inhibition of the SOCI pathway by unsaturated fatty acids added in vitro to different strains of cells treated with Tg. This inhibition was related to the concentration and the degree of unsaturation of the fatty acids used. However, in the present work, thymoma cell phospholipids, in addition to an enrichment in n-3 PUFA s, showed also a decreased content of arachidonic acid after n-3 PUFA treatment. Concerning arachidonic acid, there is wide debate in the literature. Khorodova and Astashkin (32) observed that arachidonic acid added to lymphocytes inhibited or induced store-dependent Ca2+ entry depending on the concentration of the fatty acid. The activation of SOCI by arachidonic acid administration was also reported by Rzigalinski, Blackmore, and Rosenthal (33). Finally, it has also been recently shown (33, 34) that during the rise of Ca2+ associated to SOCI, phospholipase A2 (PLA2) is activated and arachidonic acid is released. These authors hypothesized that arachidonic acid may function as a second messenger involved in the coupling between store depletion and Ca2+ entry in SOCI. In agreement with these results, the decreased levels of arachidonate acid in thymoma phospholipids after treatments with EPA or DHA could explain the lower activity of SOCI, even though further work is needed to prove the existence of a PLA2/arachidonic acid coupling signal between store emptying and Ca2+ influx also in thymoma cells.

Moreover, our results are in agreement with those obtained in muscle cells cultured in vitro by Hirofuji et al. (35). They observed that treatment with DHA for 48 h inhibited Ca2+ influx induced by angiotensin II. These authors hypothesized that the incorporation of DHA in membrane phospholipids is a necessary prerequisite for its inhibitory effect on angiotensin II-induced Ca2+ influx. Conversely, previous studies (36) had shown that EPA and DHA, added as free fatty acids in Jurkat cells cultured in vivo, induced a sustained increase of [Ca2+], within a minute. However, this increase was related only to Ca2+ released from intracellular stores and not to Ca2+ influx through the plasma membrane.

The present data demonstrate for the first time that n-3 PUFA s incorporated into membrane phospholipids after a dietary treatment inhibited the store-dependent pathway of Ca2+ influx. This pathway in addition to its more obvious role as a mechanism to refill empty stores, has been involved in cellular signalling (19), particularly in the regulation of cell proliferation (37, 38).

The results obtained suggest that the antitumoral effect of EPA and DHA is related to their inhibition of cell proliferation. This inhibition, in turn, could be explained, at least in part, by their ability to decrease the store-dependent Ca2+ entry. In agreement with our hypothesis, Kokoska, Smith and Miller (39) have recently found that SOCI is inhibited by indomethacin and other inhibitors of prostanoid synthesis. They hypothesize that the known anti-proliferative role of these nonsteroidal antiinflammatory drugs on gastric mucosa cells could be explained by their ability to inhibit the SOCI pathway in these cells.

In conclusion, the data herein presented demonstrate that dietary treatment with EPA or DHA inhibits the growth of highly malignant ascites thymoma and delays the animal’s death. They suggest a possible use of these fatty acids as chemopreventive agents. The antitumoral effect of n-3 PUFA s can be explained by the inhibition of cell proliferation and the decrease in the activity of SOCI, a Ca2+ influx pathway that appears to be involved in the signaling that evokes many key cell processes.

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REFERENCES


