Selenium inhibits apoptosis and GSH extrusion induced by DHA in human Paca-44 cell lines
L. Manzi, R. Molinari, L. Costantini, M.S. Gilardini Montani, N. Merendino

Department of Ecological and Biological Sciences (DEB), University of Tuscia, Largo dell’Università snc, 01100 Viterbo (Italy).

1Corresponding author: Nicolò Merendino, Laboratory of Cellular and Molecular Nutrition, Department of Ecological and Biological Sciences, University of Tuscia, Largo dell’Università, 01100 Viterbo, Italy; Tel.: +39-0761-357133; fax: +39-0761357751. E-mail address: merendin@unitus.it

Parole chiave: Selenio; Acido docosaesaenoico; Glutatione; Glutatione perossidasi.

Abstract
Aim of the Study. Considering that the possible interactions between selenium (Se) and docosahexaenoic
acid (DHA) in regulating various physiopathological responses in cancer cell lines have not been elucidated and defined yet, the aim of this work was to investigate the effects of the co-treatment of DHA with Se on cellular redox state, apoptosis induction and glutathione content in human pancreatic PaCa-44 cancer cells line.

Methods. The human PaCa-44 pancreatic adenocarcinoma cell line was treated with docosahexaenoic acid or arachidonic acid (ARA), alone or co-cultures with sodium selenite (Na2SeO3). After treatments cell viability was analyzed by Trypan blue dye exclusion assay and apoptosis was evaluated by Annexin V/FITC kit. Moreover cellular oxidative status was assessed measuring the levels of ROS by cell-permeable 2',7'-dichlorofluorescein diacetate (DCFH-DA), the glutathione peroxidase (GSH-Px) activity by spectrophotometric analysis and lipid peroxidation by TBA-method. The intracellular glutathione level was determined by HPLC.

Results. The results herein reported show that sodium selenite inhibits both the pro-oxidant and the pro-apoptotic effects induced by DHA.

Conclusion. This study suggests that sodium selenite, is able to inhibit the GSH reduction and this gives an important indication for the study of a new functional property of Se. Moreover our experimental results indicated that sodium selenite interferes with the pro-oxidant and pro-apoptotic actions of DHA, which is considered as a potential adjuvant in the treatment of cancer.

Key words: Selenium; Docosahexaenoic acid; Glutathione; Glutathione peroxidase.

Introduction

Epidemiological and experimental studies have showed that polyunsaturated fatty acids (PUFAs) and in particular the omega-3 docosahexaenoic acid (DHA) may have a chemopreventive and therapeutic role on tumors development (Diggle C.P., 2002; Siddiqui R.A., 2004; Merendino N., 2013). This activity seems to be due to their ability to induce oxidative products, cell cycle arrest and apoptosis in cancer cells and to inhibit neo-angiogenesis (Larsson S.C., 2004). However the mechanisms involved are not completely understood (Siddiqui R.A., 2008; Calviello G., 2006). In our previous studies we provide evidence that, treatment of human PaCa-44 pancreatic cancer cell line with different concentrations of DHA induced apoptosis via active intracellular reduced GSH extrusion (Merendino N., 2003; Merendino N., 2005). These results suggested that the depletion of GSH is a relatively early event in the commitment of apoptosis, in agreement with other studies (Ghibelli L., 1998; Franco R., 2007).

Reduced GSH plays a central role in a multitude of biochemical process, like cell cycle and gene expression regulation, membrane transport of both endogenous and exogenous molecules and protection against oxidative damage. GSH is the cofactor of numerous members of the GSH-peroxidase family. GSH-peroxidase family includes both soluble and membrane-bound members, with selenium-independent and selenium-dependent enzymes, where Se is incorporated as a selenocysteine residue.

Selenium is an essential micronutrient for mammals and it is involved in endocrine and immune functions and in the inflammatory responses (Beckett G.L., 2005; Arthur J.R., 2003). Selenium is essential for the activity of cellular glutathione peroxidases and in particular for an important selenoprotein well-known as the phospholipid hydroperoxide glutathione peroxidase, (PHG-Px), which removes esterified lipid hydroperoxides and indirectly down-regulates the 5-lipoxygenase activity by reducing cellular peroxide tone (Weitzel F., 1993). Moreover, Se has been demonstrated to bind and to inhibit the 5-lipoxygenase activity by active site modification (Hammarberg T.,
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2001). Other biological roles ascribed to Se include prevention of cancer (Combs G.F., 2006) and of cardiovascular disease (De Lorgeril M., 2006) and protection of apoptosis induced by \( \text{H}_2\text{O}_2 \), caspase-3 dependent (Demelash A., 2004).

The possible interactions between Se and DHA in regulating various physiopathological responses in cancer cell line have not been elucidated and defined. Moreover, taking into consideration that DHA and selenium affect the glutathione peroxidase activity, the aim of this work was to investigate the effects of the co-treatment of DHA with Se on cellular redox state and apoptosis in human pancreatic PaCa-44 cancer cells line.

Materials and methods

Chemicals

Pure 99% docosahexaenoic acid (DHA), arachidonic acid (ARA) 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (MDA), b-nicotinamide adenine dinucleotide phosphate (NADPH) reduced form, glutathione (GSH) reduced form, sodium selenite (Na$_2$SeO$_3$), H$_2$O$_2$, GSH-reductase (type IV) from Bakers yeast were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Growth media and additives for cultures were purchased from Euroclone (Paignton, U.K.).

Cell cultures and treatments

The human PaCa-44 pancreatic adenocarcinoma cell line was kindly provided by Prof. A. Scarpa, Department of Pathology, University of Verona, Italy. Cells were maintained in RPMI-1640 medium supplemented with 10% Fetal Calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin and 10 mg/ml streptomycin in a humidified atmosphere of 5% CO$_2$ at 37°C. For the experiments, cells were grown into 24-well cell culture plates and allowed to adhere for 24 h. Then, medium was replaced by fresh medium supplemented with different concentrations of DHA (50, 100, 150, 200 µM) or ARA at 200 µM concentration, dissolved in ethanol, alone or in co-cultures with sodium selenite at 1 mM concentration, dissolved in H$_2$O.

Measurement of Reactive oxygen species (ROS).

The level of ROS was determined in the control and fatty acids treated cells by labeling them with cell-permeable 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Invitrogen Carlsbad, Ca,USA). 2 x 10$^5$ cells were seeded at in 60-mm dishes, allowed to adhere for 24 h and then treated for 24 hours. The samples were incubated for 30 min at 37 °C with 10 mM of DCFH-DA/ml in Hanks’ balanced salt solution at dark before being detached with trypsin/EDTA, washed once in PBS and analyzed immediately by cytofluorimetry (FACScalibur Becton-Dickinson, San José, CA).

GSH-Px enzyme assay.

Measurements of GSH-Px activity were performed spectrophotometrically according to the method of Flohè and Gunzler (Flohè R., 1984). Briefly, 4x10$^6$ cells were seeded in 25 cm$^2$ tissue culture flask. After a 48 h period, medium was replaced by fresh medium supplemented with 1 µM sodium selenite 20 h before treatment with 200 µM DHA. Cells were further incubated for 24 hours, washed twice in cold PBS, harvested by scraping and added with lysis solution (Tris-HCl 10 mM pH 7.6, KCl 300 mM, Triton X-100 1%) then sonicated. The suspension was homogenate and the resulting homogenates were used for enzyme measurement. The method is based on monitoring the oxidation of NADPH at 340 nm (Varian Cary 4 spectrophotometer) in presence of exogenous GSH, hydroperoxide and...
glutathione reductase. The enzyme activity was calculated using a molar extinction coefficient of 6.22 x 10³ M⁻¹/cm⁻¹ for NADPH. One unit (U) was defined as the amount of enzyme catalysing the oxidation by hydroperoxide of 1 μmol reduced GSH to GSSG per minute at pH 7.0 at 37°C.

Assessment of cell viability and apoptosis
Cell viability was evaluated by Trypan blue dye exclusion assay. For assessment of apoptosis, cells were stained with Annexin V/FITC kit (Bender MedSystem, Vienna, Austria). Fluorescence intensity was analysed by a FACS calibur flow cytometer (Becton-Dickinson, San José, CA) and the CellQuest software (Becton-Dickinson) (Vermes I., 1995).

Glutathione determination
Cells were detached with trypsin, treated with 1 ml of 10 mM Tris-HCL solution (pH 6.0) containing 0.5 M diethylenetriamine-penta-acetic acid (DETAPAC) and syringed several times with an insulin syringe for their lysis. Cell protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories CA), and bovine serum albumin was used as a standard. For glutathione determination, 100 ml DL-Dithiothreitol (DTT) 25 mM and 150 ml of 0.1 M Tris-HCL (pH 8.5) were added to 50 ml of cell lysate. For GSSG determination, 50 ml of N-ethylmaleimide 2 mM, 50 ml of 0.1 M Tris-HCL (pH 8.5), and after 1 min 50 ml DTT 50 mM were added to the cell lysate (150 ml). After 30 min on ice, proteins were precipitated by the addition of 750 ml 2.5% (w/v) 5-sulfosalicylic acid (SSA), and centrifuged at 13,000 x g for 4 min at 4 °C. The clear supernatant was used to measure GSH and GSSG by high performance liquid chromatography separation and fluorimetric detection of the glutathione-orthophthalaldehyde adduct, as described by Neuschwander-Tetri B.A. and Roll F.J. (Neuschwander-Tetri B.A., 1989).

Measurement of lipid peroxidation (TBA-method)
Cell culture conditions were the same as described above. After incubation for 6, 18 and 24 hours, PaCa-44 cells were detached with trypsin, washed, resuspended in 500 ml PBS and assayed for the amount of thiobarbituric acid-reactive substances (TBARS) as conventional index for lipid peroxidation according to the HPLC method of Chirico (Chirico S., 1994) modified by Nardini (Nardini M., 1998).

Briefly, cells were disrupted by sonication with strokes of medium intensity. 200 µl of cellular suspension was added with 600 µl phosphoric acid 0.44 M, 200 µl TBA 0.6%, and heated in boiling water for 30 minutes at 90°C. After cooling at room temperature, samples were added with 2 ml n-butanol and vortexed. The butanolic phase was evaporated under a stream of nitrogen and the residues were redissolved in 500 µl of mobile phase. Finally 20 µl of sample was injected in the chromatographic column (Supelcosil LC-18, 15 cm x 4.6 mm, 5 µm, Supelco). Separation of TBA-MDA adduct was carried out with isocratic elution (mobile phase, 65% potassium phosphate monobasic 50 mM pH 7 and 35% methanol) at flow rate of 1 ml/min in HPLC system (PERKIN ELMER Series 200 lc PUMP). Peaks were detected by fluorescence measurement at 553 nm after excitation at 515 nm (Perkin Elmer Fluorescence Detector series 240), using the retention time of MDA standard. Fluorescence intensity of samples was converted to nmol of MDA equivalence based on a standard curve (0-160 pmol) generated with 1,1,3,3- tetramethoxypropane. All solutions were freshly prepared on the day of assaying. The results were expressed as malondialdehyde (MDA)/mg protein, by extrapolation from the standard curve. Cell protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories CA), and bovine serum albumin was used as a standard.
Statistical analysis
Mean and standard deviation (SD) of three replicates were calculated for all experimental groups. Statistical analysis was performed using one-way ANOVA. Fisher’s least significant differences test was used to describe statistical differences between means at $p < 0.05$ significance level.

Results
**Effects of DHA and ARA, with or without sodium selenite, on cellular oxidative stress.**
In order to study the effect of DHA on redox state, first we determined the intracellular level of oxidant species by DCF fluorescence. The results, showed in figure 1, indicated that treatment for 24 hours with 50, 100, 150 and 200 mM of DHA caused a significant production of intracellular oxidant species in a dose dependent manner, as compared with untreated PaCa-44 pancreatic cancer cell line. Subsequently cells were treated for 24 hours with 200 µM of DHA or ARA, alone or co-treated with sodium selenite at 1 µM concentration. As it can be observed in figure 2, the pro-oxidant effect of DHA was completely inhibited by co-treatment with sodium selenite. Moreover only a slight increase in the oxidation of 2’-dichlorodihydrofluorescein to 2’7’-dichlorofluorescein was found after treatment of ARA at 200mM concentration at the same period of time and there was not change after ARA + sodium selenite treatment with respect to untreated cells. Sodium selenite alone seemed to reduce intracellular oxidant species.

The possible involvement of DHA in lipid peroxides production was investigated by measuring intracellular malondialdehyde (MDA). The results clearly have shown that incubation with 200 µM DHA induced a significant and time-dependent increase of MDA already after 6 hours of treatment, and that the co-treatment with sodium selenite reverted such effect after 18 and 24 hours incubation. Furthermore, MDA production in cells treated with alone sodium selenite was significantly reduced after 18 hours of incubation (figure 3).

The inhibition of GSH-Px activity induced by DHA is restored by the addition of sodium selenite.
The analysis of GSH-Px activity on DHA treated pancreatic cancer cells was performed through spectrophotometrically analysis. As the panel A of Figure 4 shows, a decrease in the GSH-Px activity was observed in PaCa-44 cells treated for 24 hours with 200 µM of DHA compared to untreated cells. On the other hand treatment with the same concentrations of ARA did not affect significantly the GSH-Px activity (Figure 4 panel B). When the cells were pre-treated with sodium selenite, the co-treatment was able to retrieve GSH-Px activity at the same level of control cells.

The DHA-mediated effects on the viability and on apoptosis in the PaCa-44 cell line are reverted by sodium selenite.
The PaCa-44 pancreatic cancer cell line was treated with 200 µM of DHA and the cell viability (using trypan blue exclusion) and the apoptosis (using Annexin V staining analysis) were assessed at 24 and 48 hours. As shown in figure 5 and in table 1, the DHA treatment decreased the cell viability and induced apoptosis of the Paca-44 cell line in a time-dependent manner. Both growth inhibitory effects and apoptosis, mediated by DHA, are in part reverted by co-treatment with sodium selenite at 1 µM (figure 5 and table 1).

The intracellular reduction of GSH mediated by DHA is inhibited by sodium selenite.
The effects of DHA, DHA + sodium selenite and sodium selenite on levels of intracellular glutathione
(GSH and GSSG), were measured and reported in Table 2. The treatment with 200 μM DHA induced a significant time-dependent intracellular GSH reduction, whereas GSSG levels were not changed at all the treatment times. The co-treatment of DHA and sodium selenite significantly inhibits the GSH reduction. Moreover the treatment with the alone sodium selenite increases significantly the GSH concentration.

Discussion
The modifications in fatty acids composition of the cellular membrane due to PUFA supplementation, influence the unsaturation index of the lipid membrane and consequently the cells are more susceptible to oxidative stress (Palozza P., 1996). In our precedent paper we showed that DHA treatment of PaCa-44 cancer cells induce a rapid and specific efflux of GSH, resulting in induction of apoptosis (Merendino N., 2003). Several studies indicated that, in some instances most of the cell injury occurring after GSH depletion may actually depend on the onset of extensive, uncontrolled oxidative process such as lipid peroxidation (Wu G., 2004). Moreover a reduction of cellular GSH content could impaired the functionality of the GSH peroxidase family, that it is a critical defense mechanism against potentially toxic hydrogen peroxide and other peroxides, including lipid hydroperoxides (Ghosh J., 2004).

In this work we showed that DHA treatment on PaCa-44 pancreatic cancer cell line reduces the GSH intracellular content, increases ROS production and lipid peroxidation and decreases the activity of GSH peroxidase. DHA is also able to block cell cycle progression and to induce apoptosis as previously demonstrated (Merendino N., 2005). These results indicate that DHA is able to modify the cellular redox status and enhance lipid peroxidation being an essential step for cellular proliferation and apoptosis. Based on the known effects of selenium on cellular anti-oxidant/pro-oxidant function, we evaluated the effect of DHA after the pretreatment of the PaCa-44 cells with sodium selenite at non-toxic concentration (1μM). Our results show that the co-treatment of the cells with DHA and sodium selenite induces a reduction of peroxides production, enhances the GSH peroxidase activity and partially inhibits the anti-proliferative and pro-apoptotic effect induced by DHA alone. In this work we first demonstrate that sodium selenite inhibits the GSH efflux. This effect seems to be specific for sodium selenite because preliminary data showed that treatments with vitamin E instead of sodium selenite does not have significant effect on GSH intracellular (data not shown). This result is very important because sodium selenite could act not only as antioxidant, but it may have an active role in GSH metabolism.

These results may bring two considerations: one is that sodium selenite is able to inhibit the GSH reduction and this gives an important indication for the study of a new functional property of selenium. The second is that sodium selenite interferes with anti-proliferative and pro-apoptotic effects of DHA on pancreatic cancer.

References
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64: 297-304.


Figures and Tables

Figure 1. Effects of DHA on cellular oxidative stress.
Intracellular oxidant species were determined by FACS analysis using 2',7'-dichlorofluorescein diacetate (DCFH-DA). Control cells (CTR) were compared with cells supplemented with DHA at four different concentrations (50, 100, 150 and 200 mM) after 24 hours of treatment. The figure illustrates one of three separate experiments which gave similar results.
Figure 2. Effects of DHA, ARA and sodium selenite, on cellular oxidative stress. Intracellular oxidant species were determined by FACS analysis using 2',7'-dichlorofluorescein diacetate (DCFH-DA). Cells were treated for 24 hours with 200 µM of DHA or ARA alone and co-treated with sodium selenite at 1 µM concentration. The figure illustrates one of three separate experiments which gave similar results.
Figure 3. Effects of DHA and sodium selenite on lipids peroxidation.
Lipids peroxidation was measured by MDA productions. PaCa-44 pancreatic cells were treated with 200 mM of DHA alone or together with sodium selenite at 1 mM concentration at different times. The values represent the mean ± SE of three independent experiments. Means with different letters, within the same group (6 h, 18 h, and 24 h) are statistically significant. (p < 0.05)
Figure 4. Effects of DHA, ARA and sodium selenite, on GSH-Px activity.
GSH-Px activity was measured by spectrophotometric analysis. PaCa-44 pancreatic cells were treated with 200 mM of DHA (Panel A) or ARA (Panel B) alone or together with sodium selenite at 1 mM concentration for 24 hours. The values represent the mean ± SE of three independent experiments. Mean with different letters are statistically significant. (p < 0.05)
Figure 5. Effects of DHA and sodium selenite on cell growth.

Cells growth was measured by the trypan blue dye exclusion assay. PaCa 44 pancreatic cells were treated with 200 mM of DHA alone or together with sodium selenite at 1 mM concentration for 24 and 48 hours. The values represent the mean ± SE of three independent experiments. Means with different letters, within the same group (24 h, and 48 h) are statistically significant. (p < 0.05)
<table>
<thead>
<tr>
<th>% Apoptotic cells</th>
<th>24 hours</th>
<th>48 hours</th>
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<tbody>
<tr>
<td>CTR</td>
<td>8.2 ± 1.1(^a)</td>
<td>6.98 ± 0.9(^a)</td>
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<tr>
<td>DHA</td>
<td>25.12 ± 2.5(^b)</td>
<td>31.34 ± 1.5(^c)</td>
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<tr>
<td>DHA + Na-selenite</td>
<td>10.73 ± 3.1(^c)</td>
<td>15.57 ± 1.2(^b)</td>
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<tr>
<td>Na-selenite</td>
<td>6.65 ± 1.3(^a)</td>
<td>6.96 ± 0.5(^a)</td>
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Table 1. Effects of DHA and sodium selenite on apoptosis.
Apoptotic cells were measured by Annexin V staining analysis. PaCa-44 pancreatic cells were treated with 200 mM of DHA alone or together with sodium selenite at 1 mM concentration for 24 and 48 hours. The values represent the mean ± SE of three independent experiments. Means with different letters, within the same group (24 h and 48 h) are statistically significant. (p < 0.05)
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<table>
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<th></th>
<th>CTR</th>
<th>DHA</th>
<th>DHA + Na selenite</th>
<th>Na selenite</th>
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<tr>
<td><strong>6 HOURS</strong></td>
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<tr>
<td>GSH</td>
<td>80.7 ± 2.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>70.1 ± 3.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>83.1 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100.6 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>GSSG</td>
<td>0.76 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.80 ±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.79 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.80 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<td><strong>12 HOURS</strong></td>
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<tr>
<td>GSH</td>
<td>83.14 ± 3.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.2 ± 3.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>88.07 ± 4.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>126.22 ± 4.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>GSSG</td>
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<td>0.83 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.92 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td><strong>24 HOURS</strong></td>
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<tr>
<td>GSH</td>
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<td>42.04 ± 2.6&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>1.2 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
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Table 2. Effects of DHA and sodium selenite on Glutathione concentration.
Intracellular Glutathione (GSH and GSSG) levels were determined by high performance liquid chromatography. PaCa-44 pancreatic cells were treated with 200 μM of DHA alone or together with sodium selenite at 1 μM concentration at different times. The values represent the mean ± SE of three independent experiments. Means with different letters, within the same group (6 h, 12 h, and 24 h) are statistically significant. (p < 0.05)