

Docosahexaenoic Acid Induces Apoptosis in the Human PaCa-44 Pancreatic Cancer Cell Line by Active Reduced Glutathione Extrusion and Lipid Peroxidation

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Abstract: We investigated the ability of fatty acids to induce growth inhibition and apoptosis in the human PaCa-44 pancreatic cancer cell line and the mechanism(s) underlying apoptosis. Butyric acid, α -linoleic acid, and docosahexaenoic acid (DHA) were supplemented at 200 μ M concentration in the medium of cell cultures. Our results showed that all fatty acids inhibited cell growth, whereas only DHA induced cell apoptosis. An oxidative process was implicated in apoptosis induced by DHA because butylated hydroxytoluene and vitamin E prevented lipid peroxidation and reversed apoptosis. Intracellular and extracellular glutathione [reduced glutathione (GSH) and oxidized glutathione (GSSG)] concentrations were measured following DHA treatment in the presence or in the absence of GSH extrusion inhibitors such as cystathionine or methionine. DHA induced intracellular GSH depletion without affecting intracellular GSSG concentration and increased extracellular GSH and GSSG levels. Intracellular GSH depletion and extracellular GSH increase were both reversed by cystathionine. Inhibition of active GSH extrusion from the cell by cystathionine or methionine completely reversed lipid peroxidation and apoptosis. These data document the antiproliferative and apoptotic activities of DHA. The data provide evidence that intracellular GSH depletion represents an active extrusion process rather than a consequence of an oxidative stress, suggesting a causative role of GSH depletion in DHA-induced apoptosis.

Introduction

There is evidence that n-3 and n-6 dietary polyunsaturated fatty acids (PUFAs) induce growth inhibition and/or apoptosis as well as inhibit and/or reverse drug resistance in a variety of tumor cells (1–4). These results have led to propos-

ing a number of PUFAs as possible anticancer therapeutic agents or potential adjuvants to radiotherapy or chemotherapy (5,6). The mechanism underlying apoptosis induced by PUFAs is unclear. Although a role for oxidative stress has been indicated, whether it represents a cause or a consequence of their apoptotic activity is controversial (6–8). The most abundant antioxidant compound in the cell is glutathione, and depletion of intracellular reduced glutathione (GSH) has been described to occur following different apoptotic stimuli (9,10). However, whether it reflects an intracellular oxidation or a specific GSH extrusion from the cell is debated (11,12). Among the different n-3 and n-6 PUFAs, docosahexaenoic acid (DHA; 22:6 n-3) has been shown to be the most potent inducer of apoptosis in human colon cancer cells, and lipid peroxidation has been indicated to be involved in the apoptotic process (13). However, whether glutathione is implicated in lipid peroxidation induced by DHA has not been elucidated.

Pancreatic cancer represents the fifth leading cause of cancer death in western countries (14). It is associated with severe cachexia (15), and it is almost incurable, displaying a high degree of resistance to conventional radiotherapy and chemotherapy (14). In particular, elevated GSH levels in pancreatic carcinoma have been reported to be associated with resistance to chemotherapy (16). In this study, we documented the antiproliferative and apoptotic activities of DHA and α -linoleic acid (LA; 18:2 n-6) in the human PaCa-44 pancreatic cancer cell line. We chose PaCa-44 cells because this cell line has been extensively characterized at both the molecular (17) and the functional (18) levels, and it seems to represent an in vitro model comparable to human pancreatic ductal carcinoma. Our results indicate a role for glutathione depletion via active intracellular GSH extrusion from pancreatic cancer cells induced to apoptosis by DHA.

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Materials and Methods

Cell Cultures and Treatments

The human PaCa-44 pancreatic adenocarcinoma cell line was kindly provided by Professor A. Scarpa, Department of Pathology, University of Verona, Italy. Cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 10 mg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. For the experiments, cells were seeded onto 24-well cell culture plates and allowed to adhere for 24 h. Then the medium was replaced with fresh medium supplemented with 200 µM DHA, LA, or as controls with butyric acid (BA; 4:0; Sigma Chemical Co. Milano, Italy) dissolved in ethanol or ethanol alone. At 24, 48, and 72 h, cells were detached with trypsin and analyzed for their proliferation or apoptosis. In some experiments, cells were pretreated with 10 µM butylated hydroxytoluene (BHT) or 40 µM vitamin E 1 h before DHA treatment. For the experiments of inhibition of GSH extrusion, cells were pretreated with 1 mM cystathionine or 1 mM methionine (Sigma Chemical Co.) (19) 1 h before DHA treatment.

Assessment of Cell Growth and Apoptosis

Cell growth was assessed (in triplicate) by counting the number of cells in a Neubauer cell chamber (Brand, Werhem, West Germany). During cell count, cell viability was controlled by the tripan blue dye exclusion assay. For assessment of apoptosis, permeabilized cells were stained with propidium iodide (PI) and analyzed by flow cytometry (20). Cells were incubated with 0.75 ml hypotonic fluorochrome solution containing 50 µg/ml PI, 0.1% sodium citrate, and 0.1% Triton X-100 for 1 h at 4°C. The percentage of apoptotic cells was determined on the basis of the number of subdiploid DNA peaks corresponding to nucleosomes in the DNA fluorescence histogram. We acquired 5,000 events per sample. In some experiments, apoptosis was assessed by the annexin V assay (21) using the Annexin V/FITC Kit (Bender MedSystems, Vienna, Austria). Fluorescence intensity was analysed by a FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA) and a CellQuest software (Becton-Dickinson).

Cell Cycle Analysis

Cell cycle was analyzed by PI staining and flow cytometric analysis. Briefly, cells were fixed in 70% ethanol, washed with PBS, incubated with 1 µg/ml RNAase A for 30 min at 37°C, and stained with 5 µg/ml PI. Fluorescence intensity was analyzed by a FACScalibur flow cytometer (Becton-Dickinson).

Glutathione Determination

Cells were detached with trypsin, treated with 1 ml of 10 mM Tris-HCL solution (pH 6.0) containing 0.5 M

diethylenetriaminepentaacetic acid, 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, Hercules, CA), and bovine serum albumin was used as a standard. For total glutathione determination, 100 µl DL-Dithiothreitol (DTT) 25 mM and 150 µl of 0.1 M Tris-HCL (pH 8.5) were added to 50 µl of cell lysate. For oxidized glutathione (GSSG) determination, 50 µl of N-ethylmaleimide 2 mM, 50 µl of 0.1 M Tris-HCL (pH 8.5), and after 1 min, 50 µl DTT 50 mM were added to the cell lysate (150 µl). After 30 min on ice, proteins were precipitated by the addition of 750 µl 2.5% (wt/vol) 5-sulfosalicylic acid, and centrifuged at 13,000 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 and Roll (22).

Measurement of Lipid Peroxidation (TBA-RS Method)

Cells were detached with trypsin, washed, and resuspended in 500 ml PBS. Protein concentration was measured as described previously. A measurement of 1 ml of thiobarbituric acid (TBA) reagent (0.375 % 2-TBA, 15% TBA, 0.25 N HCl) was added to the cell suspension. The samples were heated at 95°C for 20 min and then chilled to room temperature and centrifuged at 1,500 g for 10 min. TBA-reactive substances (RS) produced by lipid peroxidation was measured in the supernatant at 535 nm according to the TBA method (23). The results were expressed as malondialdehyde (MDA) ng/mg protein.

Statistical Analysis

Statistical analysis was performed using one factor analysis of variance and two-tailed *t*-test.

Results

DHA Inhibits Growth and Induces Apoptosis in Human Pancreatic Cancer Cells

The PaCa-44 pancreatic cancer cell line was treated with 200 µM of DHA, LA, or BA, and cell growth and apoptosis were assessed at 24, 48, and 72 h using cell count and PI staining analysis, respectively. All the fatty acids tested inhibited growth of PaCa-44 cells (Fig. 1), whereas only DHA induced apoptosis in the pancreatic cancer cell line (Fig. 2A). Indeed, DHA induced more than 30% of apoptotic cells at 48 h, and apoptosis increased reaching more than 50% of apoptotic cells at 72 h (Fig. 2A). Cell death due to nonapoptotic toxic events could be excluded

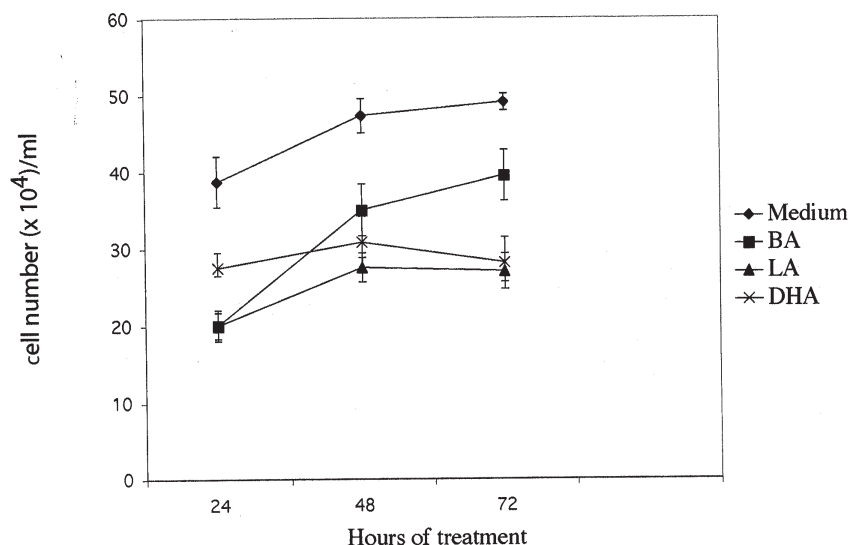


Figure 1. Proliferation of the human PaCa-44 pancreatic cancer cell line treated with fatty acids. Cells were treated with 200 μM docosahexaenoic acid (DHA), α -linoleic acid (LA), butyric acid (BA), or medium alone (control) for different time periods, and cell counts were performed. Results are reported as means of three different experiments \pm SD. Significant values ($P < 0.001$) were obtained in treated cells as compared to controls.

by the tripan blue dye exclusion assay. To further ascertain whether PI intracellular staining reflected specifically an apoptotic event, DHA treated pancreatic tumor cells were stained with annexin V. This molecule binds phosphatidylserine, which, in the apoptotic cell, is redistributed from the inside to the outside of the cell membrane, representing an early specific event in the apoptotic process (24). As shown in Fig. 2B, in contrast to the untreated cells, DHA treated PaCa-44 cells were stained with annexin V at 6, 18, 24, and 48 h, confirming that DHA induced apoptosis in pancreatic cancer cells. Moreover, treatment of PaCa-44 cells with different doses of DHA ranging from 50 to 200 μM was analyzed. Our results show that 150 μM of DHA was the minimal concentration required to induce apoptosis at 24, 48, and 72 h (Fig. 3).

DHA Inhibits Cell Cycle Progression in Human Pancreatic Cancer Cells

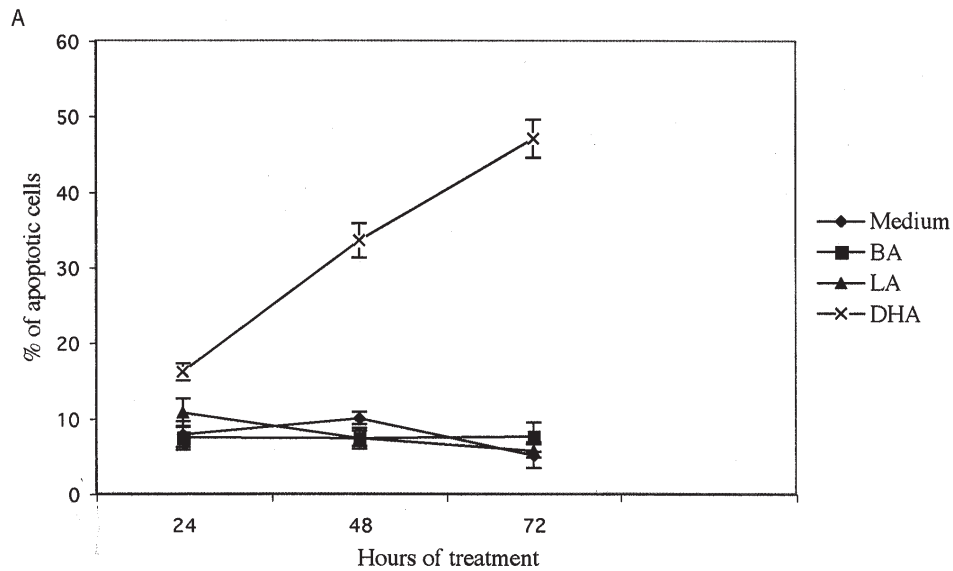
The analysis of cell cycle in DHA treated pancreatic cancer cells was performed using PI staining and flowcytometric analysis. Comparing to the untreated cells, a decrease in the S + G2-M phase and an increase in the cell cycle G₀-G₁ fractions were observed in PaCa-44 cells treated with 200 μM of DHA for 12 h (Fig. 4 and Table 1). These results show that DHA induced cell cycle arrest in pancreatic cancer cells. Moreover, the addition to DHA treatment of 40 μM α -tocopherol, used as an antioxidant molecule, reverted cell cycle arrest induced by DHA, indicating that oxidation was involved in this process.

Active GSH Extrusion Is Involved in Lipid Peroxidation and Apoptosis Induced by DHA

We analyzed the implication of oxidative processes in the induction of apoptosis by DHA. We observed that pretreatment of PaCa-44 cells with the antioxidants BHT (10 μM) or vitamin E (40 μM) reversed the apoptosis induced by DHA (200 μM ; Fig. 5). We also observed that treatment of PaCa-44 cells with DHA induced an increase in MDA concentration that was strongly inhibited by pretreatment of cells with BHT or vitamin E (Fig. 6).

To investigate a possible role of glutathione in the induction of apoptosis by DHA, PaCa-44 cells were treated with DHA, and both intracellular and extracellular GSH and GSSG concentrations were measured in the presence or in the absence of an inhibitor of GSH extrusion such as cystathionine (19). As illustrated in Table 2 and Table 3, intracellular GSH was dramatically reduced (more than 60%) following 6 h of DHA treatment, whereas GSSG levels were not changed at 6 and 18 h. Extracellular GSH and GSSG levels were increased approximately 40% and 30%, respectively, following DHA treatment (Table 3). Moreover, intracellular GSH depletion and extracellular GSH increase were both reversed by cystathionine (Table 2 and Table 3).

To verify whether an active mechanism of GSH extrusion from the cell could be involved in apoptosis and lipid peroxidation, experiments were performed in the presence of two specific inhibitors of carrier-mediated GSH extrusion such as cystathionine or methionine (19). Our results showed that pretreatment of PaCa-44 cells with cystathionine (1 mM)



B

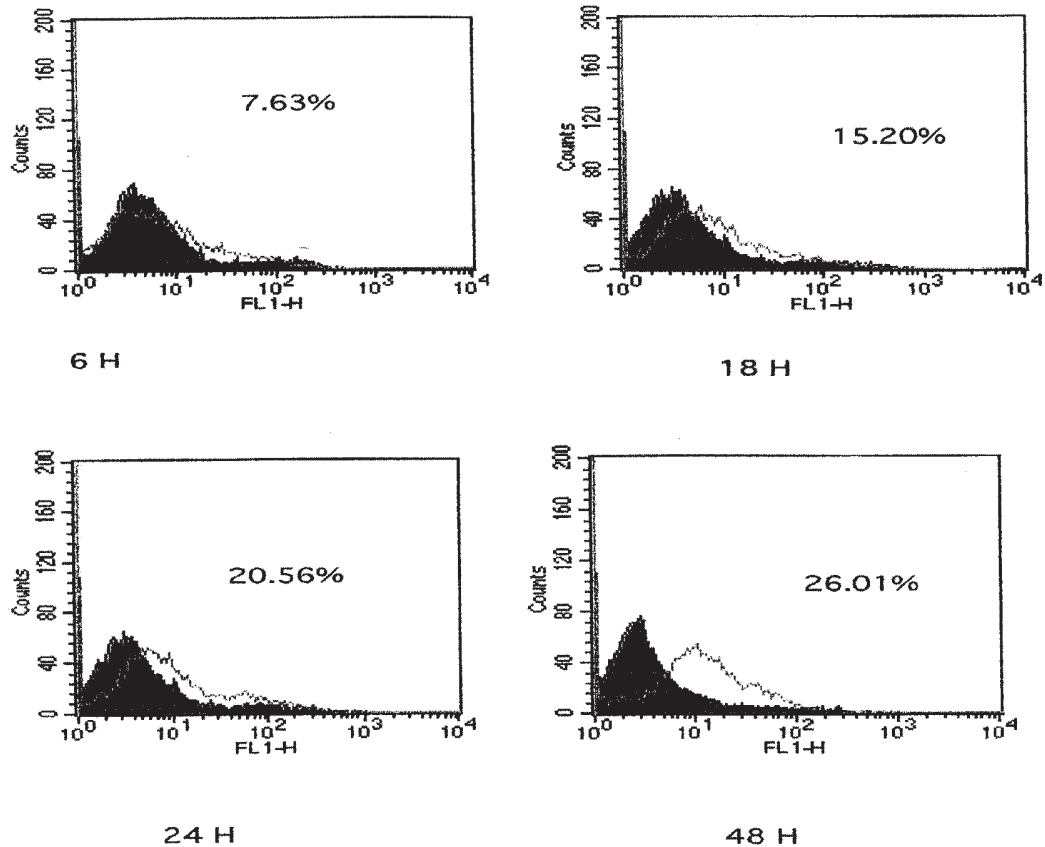


Figure 2. Apoptosis of the human PaCa-44 pancreatic cancer cell line treated with fatty acids. Cells were treated with 200 μ M docosahexaenoic acid (DHA), α -linoleic acid (LA), butyric acid (BA), or medium alone for different time periods, and apoptosis was analyzed. A: Apoptosis was assessed by propidium iodide intracellular staining and flow cytometric analysis. Results are reported as means of three different experiments \pm SD; significant values ($P < 0.05$ at 24 h; $P < 0.001$ at 48 and 72 h) were obtained in DHA-treated cells. B: Apoptosis was also assessed by the Annexin V assay; the data are representative of three different experiments that gave similar results. FL 1-H, measure of fluorescence intensity.

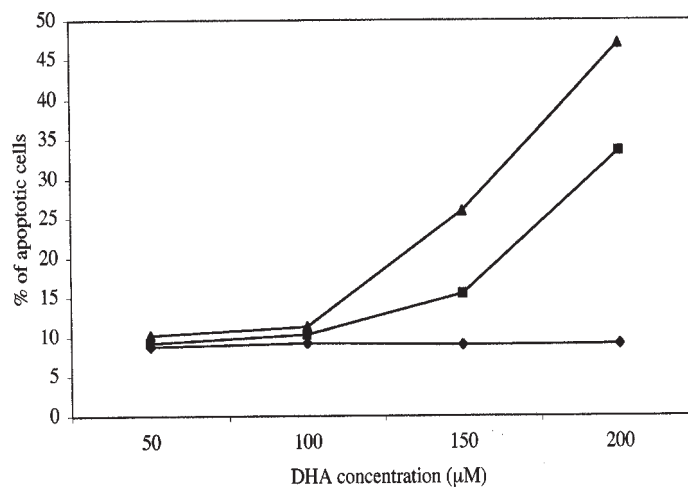


Figure 3. Apoptosis of PaCa-44 cells treated with different concentrations of docosahexaenoic acid (DHA). Cells were treated with 50, 100, 150, and 200 μM of DHA for 24 (○), 48 (□) or 72 (▲) h, and apoptosis was assessed by propidium iodide staining and flow cytometric analysis. The data are representative of three different experiments that gave similar results.

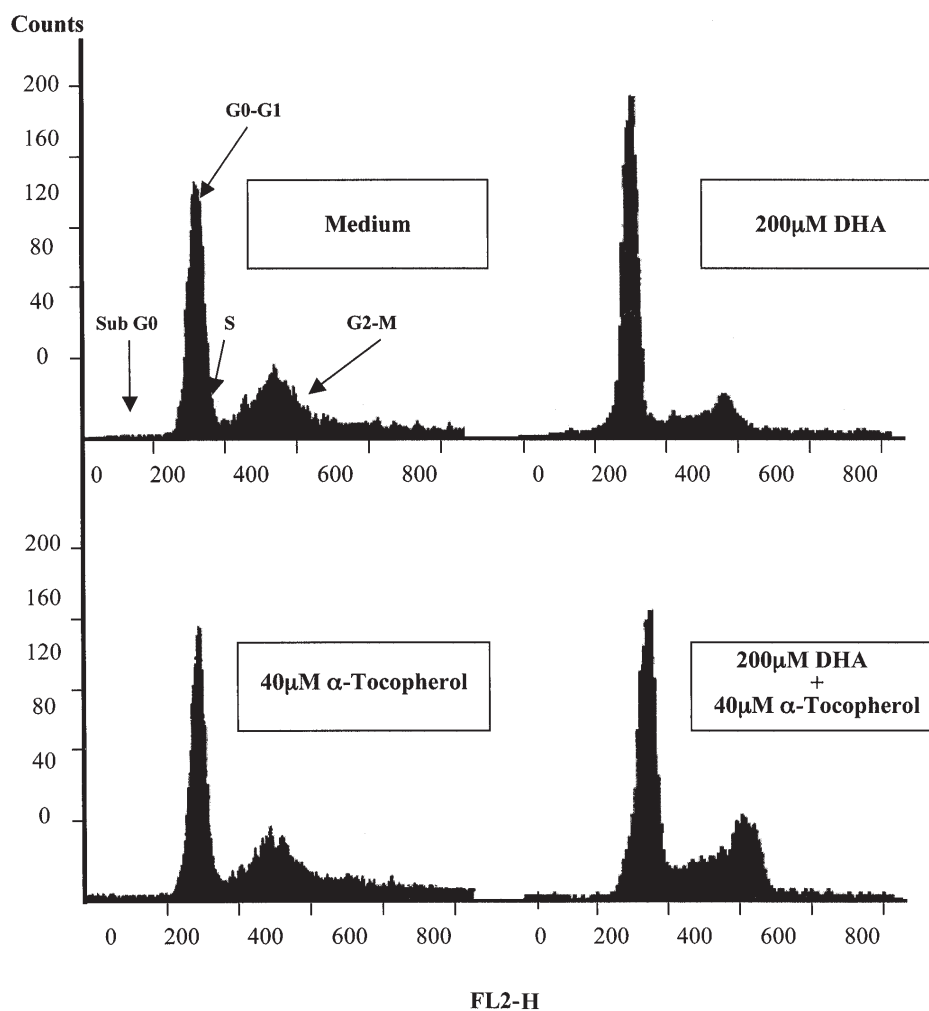


Figure 4. Cell cycle analysis in PaCa-44 cells treated with docosahexaenoic acid (DHA) in the presence or in the absence of an antioxidant molecule. Cells were pretreated with α -tocopherol or medium alone 1 h before DHA treatment, and cell cycle was analyzed at 12 h using propidium iodide staining and flow cytometric analysis. The data are representative of three different experiments that gave similar results.

Table 1. Cell Cycle Analysis in PaCa-44 Cells Treated With docosahexaenoic Acid (DHA) in the Presence or in the Absence of an Antioxidant Molecule^a

Treatment (12 h)	% Sub G0	% G0-G1	% S + G2-M
Medium	2.51 ± 0.55	50.76 ± 1.22	46.73 ± 1.38
DHA (200 μM)	3.42 ± 0.61	85.21 ± 2.11	11.37 ± 1.96
α-Tocopherol (40 μM)	2.91 ± 0.81	52.13 ± 1.82	44.96 ± 2.12
α-Tocopherol + DHA	2.22 ± 0.56	59.11 ± 1.21	38.67 ± 1.71

^a: Results are reported as means of three different experiments ± SD.

or methionine (1 mM) 1 h before DHA treatment inhibited both apoptosis (Fig. 7) and MDA production (Fig. 8).

Overall, these data indicated that intracellular GSH depletion induced by DHA was the result of a specific GSH extrusion from pancreatic cancer cells and that it played an active role in the control of lipid peroxidation.

Discussion

Our results show that DHA, used at a concentration achievable in vivo (24), induced growth inhibition, cell cycle arrest, and apoptosis in the human PaCa-44 pancreatic cancer cell line. LA was less effective in cell growth inhibition than DHA and in contrast to DHA, did not induce apoptosis in pancreatic cancer cells. These results are consistent with the data found in the literature, indicating that the antiproliferative and the apoptotic activities of fatty acids are generally influenced by the carbon chain length and the number of double bonds, being more effective those with longer carbon chain and more unsaturation degree (1,6,25–27).

We investigated the mechanism(s) underlying apoptosis induced by DHA, and we analyzed a possible active role of glutathione in this process. Our results, which show an early strong decrease of intracellular GSH together with an increase of extracellular GSH levels following DHA-induced apoptosis and inhibition of intracellular GSH depletion by specific inhibitors of carrier-mediated GSH extrusion, indicate that intracellular GSH depletion observed DHA-induced was the result of an active extrusion process of glutathione from the cell. Moreover, the GSH extrusion inhibitors completely prevented lipid peroxidation and reversed apoptosis. Overall, our results are consistent with the data reported in the literature indicating a role for glutathione depletion via active GSH extrusion in cells induced to apoptosis by a number of agents that did not imply a direct oxidative stress (11,12,19) and suggesting the GSH extrusion as the cause of oxidative stress.

In conclusion, our data provide evidence that depletion of intracellular GSH, observed following DHA-induced apoptosis in the human PaCa-44 pancreatic cancer cell line, reflected an active GSH extrusion process rather than a consequence of oxidative stress. Considering that GSH levels

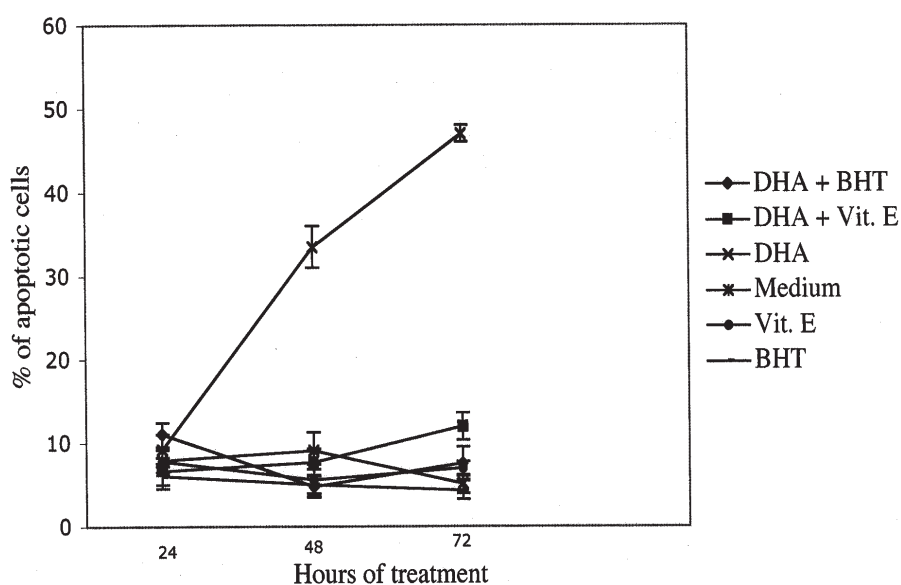


Figure 5. Effect of antioxidant molecules on apoptosis of PaCa-44 cells treated with docosahexaenoic acid (DHA). Cells were pretreated with 10 μM of butylated hydroxytoluene (BHT), 40 μM of Vitamin E (Vit. E), or medium alone 1 h before DHA (200 μM) treatment, and apoptosis was assessed by propidium iodide staining and flow cytometric analysis. Results are reported as means of three different experiments ± SD; significant values ($P < 0.001$) were obtained in DHA and DHA + antioxidant treated cells as compared with DHA + antioxidants and antioxidants alone treated cells.

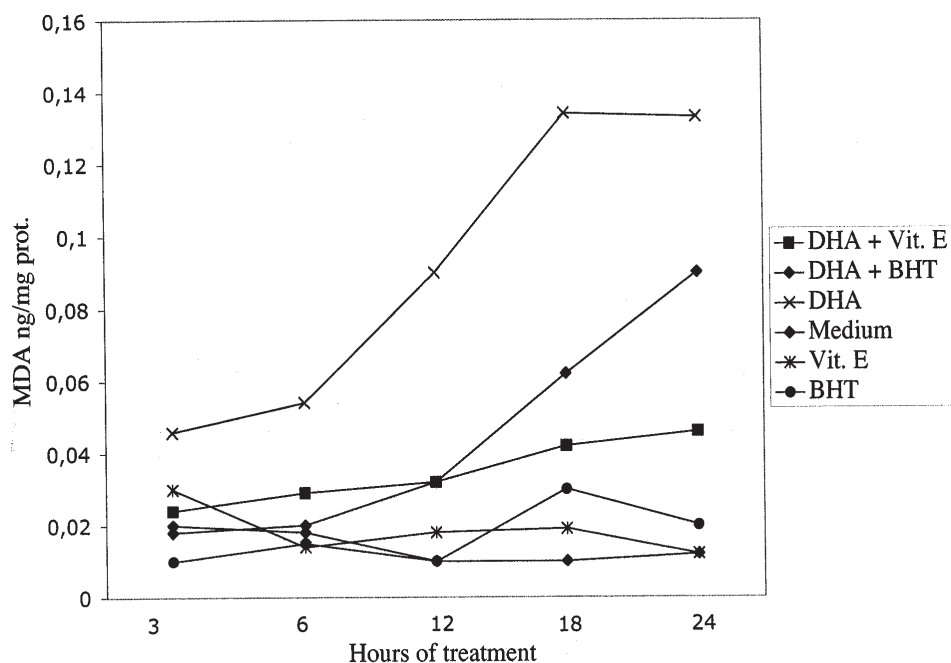


Figure 6. Lipid peroxidation in PaCa-44 cells treated with docosahexaenoic acid (DHA) in the presence or in the absence of antioxidants. Cells were pretreated with 10 μ M of butylated hydroxytoluene (BHT), 40 μ M of Vitamin E (Vit. E), or medium alone 1 h before DHA (200 μ M) treatment; malondialdehyde (MDA) concentrations were assessed by the TBA method. The data are representative of three different experiments that gave similar results. Prot., protein.

Table 2. Intracellular Glutathione (GSH and GSSG) Concentrations Following Treatment of PaCa-44 Cells With docosahexaenoic Acid (DHA) in the Presence or in the Absence of an Inhibitor of GSH Extrusion^a

Hours	Medium		Treated DHA		DHA + Cystathionine	
	GSH	GSSG	GSH	GSSG	GSH	GSSG
0	69.6 \pm 2.2	0.50 \pm 0.10	69.6 \pm 1.9	0.50 \pm 0.18	65.6 \pm 1.6	0.50 \pm 0.10
6	66.6 \pm 2.6	0.58 \pm 0.10	20.28* \pm 3.1	0.28* \pm 0.09	58.4* \pm 2.3	0.35 \pm 0.08
18	60.4 \pm 3.2	1.15 \pm 0.30	15.67* \pm 3.8	0.28* \pm 0.10	56.9* \pm 4.8	0.35 \pm 0.13

^a: Results are reported as means of three different experiments \pm SD. Abbreviations are as follows: GSH, reduced glutathione; GSSG, oxidized glutathione. GSH and GSSG are measured in nmol/mg protein. * $P < 0.001$.

Table 3. Intracellular and Extracellular GSH and GSSG Levels Following Treatment of PaCa-44 Cells With docosahexaenoic Acid (DHA) in the Presence or in the Absence of an Inhibitor of GSH Extrusion^a

	GSH		GSSG	
	Intracellular	Extracellular	Intracellular	Extracellular
RPMI 1640 Medium	46.5 \pm 2.3	14.7 \pm 1.9	0.4 \pm 0.07	15.0 \pm 1.9
DHA	24.3* \pm 2.5	32.8* \pm 2.1	0.3 \pm 0.08	22.3* \pm 1.7
DHA + cystathionine	40.1 \pm 3.2	19.0 \pm 2.8	0.5 \pm 0.11	22.3 \pm 2.4

^a: Results are reported as means of three different experiments \pm SD. Abbreviations are as follows: GSH, reduced glutathione; GSSG, oxidized glutathione. GSH and GSSG are measured in nmoles. * $P < 0.001$.

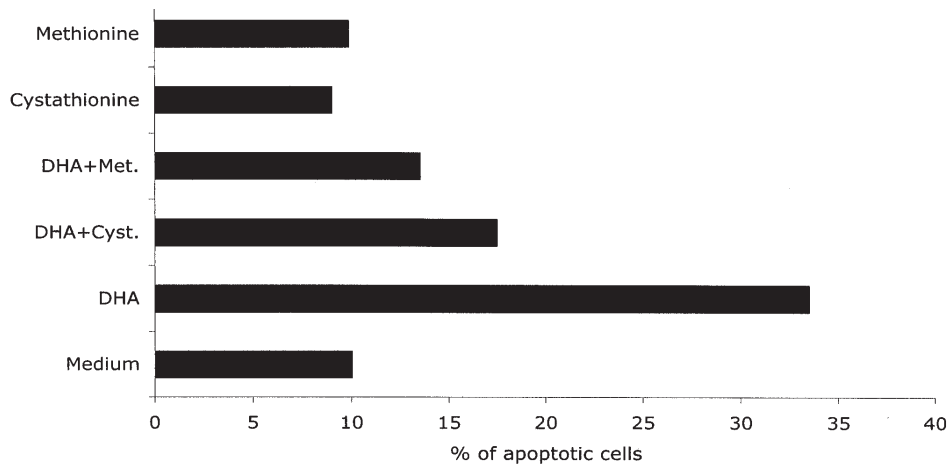


Figure 7. Effect of reduced glutathione (GSH)-carrier inhibitors on apoptosis of PaCa-44 cells treated with docosahexaenoic acid (DHA). Cells were pretreated with 1 mM of cystathionine, 1 mM of methionine, or medium alone 1 h before DHA (200 μ M) treatment, and apoptosis was assessed at 48 h. The data are representative of at least three different experiments that gave similar results.

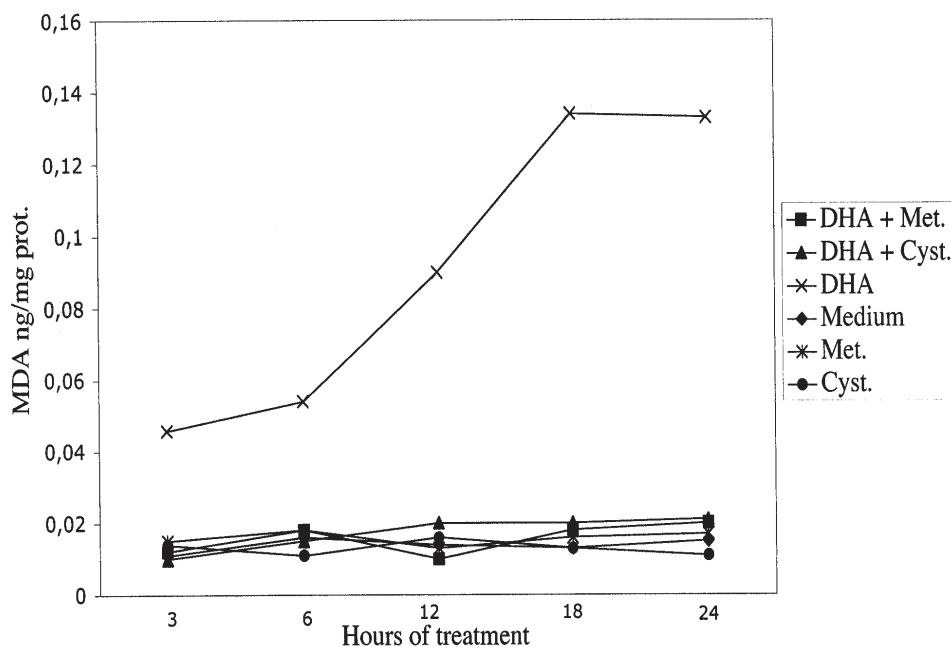


Figure 8. Effect of reduced glutathione (GSH)-carrier inhibitors on lipid peroxidation induced by docosahexaenoic acid (DHA) treatment. PaCa-44 cells were pretreated with 1 mM of cystathionine (Cyst.), 1 mM of methionine (Met.), or medium alone 1 h before DHA (200 μ M) treatment, and malondialdehyde (MDA) concentrations were measured. The data are representative of at least three different experiments that gave similar results. Prot., protein.

have been found elevated in pancreatic carcinomas compared to normal tissues, and that GSH depletion resulted in growth inhibition and enhanced apoptosis in pancreatic cancer cells (16), a possible role of DHA as adjuvant in radiotherapy or chemotherapy should be investigated in future studies.

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