

POLYUNSATURATED FATTY ACIDS ALTER EXPRESSION OF GENES ENCODING ANTIOXIDANT ENZYMES IN A549 CELLS EXPOSED TO DOXORUBICIN

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Reactive oxygen species (ROS) include a number of chemically reactive molecules derived from oxygen. ROS such as superoxide, hydrogen peroxide, and lipid hydroperoxides are known to modulate cell proliferation and apoptosis, and induce synthesis of growth factors that play an important role in tumor growth and invasion. Anticancer drugs such as doxorubicin (DX), cisplatin, paclitaxel, bleomycin, and irradiation up-regulate the level of ROS, which accumulation in tumor cells enhances cytostatic activity of these agents (1). While acute oxidative stress triggers cell apoptosis or necrosis, persistent oxidative stress induces genomic instability and has been implicated in tumor progression and drug resistance. Antioxidant enzymes (AOEs) and thiol proteins regulating cellular redox state constitute a major cellular protection against oxidants. Consequently, they are not only associated with carcinogenesis and tumor progression, but also with resistance of tumor cells to cytostatic drugs and radiation (2).

Polyunsaturated fatty acids (PUFAs) play an important role in both induction and prevention of carcinogenic process. It is well known that several types of neoplastic cells show decreased total PUFAs content, which contributes to their resistance to lipid peroxidation and chemotherapy. In this context, it is interesting to note that PUFAs, particularly eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), induce growth inhibition and/or apoptosis as well as inhibit and/or reverse drug resistance in various tumor cells

(3–5). Several mechanisms have been proposed to explain the anticancer activity of PUFAs including formation of ROS and cytostatic and cytotoxic compounds after peroxidation (6), alteration in transcription factors and gene expression, modification of signal transduction pathways (7, 8), modulation of AOEs (9–11), cellular differentiation, proliferation, and apoptosis (4, 12).

The present study was undertaken to investigate the effects of different concentrations of EPA and DHA on genes expression encoding antioxidant enzymes, such as superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), catalase (CAT), phospholipid hydroperoxide glutathione peroxidase (GPx-4), and glutathione S-transferase pi (GST-pi) in low PUFAs content human lung adenocarcinoma cells (A549) exposed to DX.

EXPERIMENTAL

Cell culture

A549 human lung adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC) and cultured (25000/cm²) in Modified Eagle's Medium (MEM) supplemented with 10% heat inactivated fetal bovine serum (FBS; PAA The Cell Culture Company), 10 mM buffer HEPES (Sigma), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

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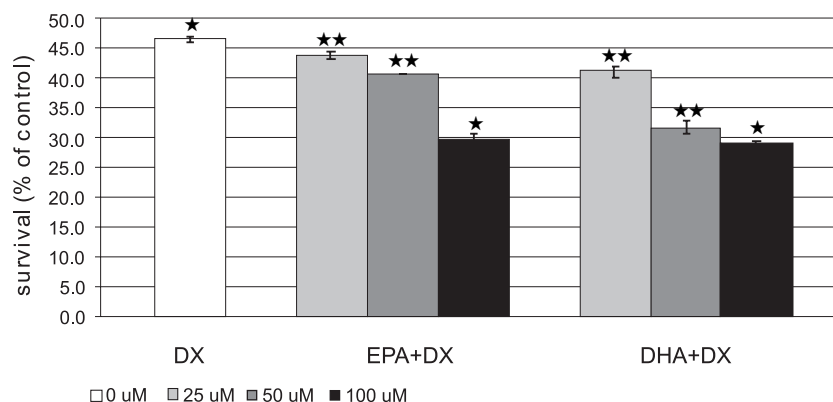


Figure 1. Survival (% of control) of A549 cells exposed to doxorubicin (DX; 0.078 $\mu\text{g}/\text{mL}$) in the presence or absence of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) at various concentrations (25–100 μM). Data were calculated as the means \pm SD from three independent experiments. Statistically significant differences ($p < 0.05$): ★ vs. control and DX; ★★ vs. control and DX, EPA+DX vs. DHA+DX

Cell exposure to DX and PUFAs

Doxorubicin, eicosapentaenoic acid (EPA, 20:5 n-3), and docosahexaenoic acid (DHA, 22:6 n-3) were purchased from Sigma. Stock solutions of doxorubicin were prepared in physiological saline solution, NaCl 0.9%. The fatty acids were dissolved in 99% ethanol and stored as stock solutions (100 mM) under nitrogen at -20°C . To achieve experimental conditions, PUFAs and DX were prepared freshly from stock solutions and diluted with the appropriate volumes of the growth medium.

Twenty-four hours after cell seeding, the medium was removed and replaced with the medium supplemented with EPA or DHA (25 μM , 50 μM or 100 μM). After following 24 h, the culture medium was replaced with the medium containing additionally DX at IC_{50} concentration (0.078 $\mu\text{g}/\text{mL}$) for 4 h. The DX concentration was chosen on the basis of previous studies performed in our laboratory and preliminary viability tests for this study. Control cells were cultured in the medium containing the same concentration of ethanol (0.1%, v/v) as the experimental cultures. Previous observation showed that ethanol at this concentration is not toxic to the cells.

Cytotoxicity assay

Survival of cells exposed to DX at IC_{50} (0.078 $\mu\text{g}/\text{mL}$) or PUFAs (25 μM , 50 μM or 100 μM) and DX at IC_{50} was assessed by the XTT method (In Vitro Toxicology Assay Kit XTT Based, TOX-2, Sigma) with a commercial kit according to the manufacturer's instruction. The method based on the ability of mitochondrial dehydrogenases of viable cells to cleave the tetrazolium ring of XTT (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxyanilide inner salt), yielding orange

formazan crystals, which are soluble in aqueous solutions. Absorbance of formazan was measured at 450 nm with plate reader (Triad LT Multimode Detector, Dynex Technologies). Cell viability was expressed as a percentage of absorbance measured in the treated wells relative to that in the untreated control wells.

mRNA extraction and analysis

Total RNA was extracted from cells using TRIZOL (Invitrogen) following the manufacturer's instruction and quantified by UV absorbance spectrophotometry using GeneQuant II (Pharmacia Biotech).

Quantitative RT-PCR was performed using QuantiTect Probe RT-PCR Kit (Qiagen) and the number of mRNA copies of SOD1, SOD2, CAT, GST-pi, GPx-4 genes was quantitated with ABI PRISM 7000 Sequence Detection System (Applied Biosystems), according to the manufacturer's protocols. TaqMan gene expression assays for analyzing the mRNA expression of SOD1 (assay ID Hs00166575_m1), SOD2 (assay ID Hs00167309_m1), CAT (assay ID Hs00156308_m1), GST-pi (assay ID Hs00168-310_m1) and GPx-4 (assay ID Hs00157812_m1) were obtained from Applied Biosystems.

mRNA expression levels of target genes were normalized to GAPDH mRNA (Applied Biosystems; assay ID Hs99999905_m1). The amount of target genes mRNA expression in each sample was expressed as a percentage of control.

Statistical analysis

The data obtained from three independent experiments were expressed as the mean values \pm standard deviations. Statistical significance analysis was based on analysis of variance (ANOVA) fol-

lowed by Tukey's HSD test. The p-value of less than 0.05 was considered significant. Statistical analysis was performed using Statistica 8 PL software for Windows (StatSoft, Poland).

RESULTS

Effect of PUFAs on DX cytotoxicity

Figure 1 shows the cell survival of A549 cells exposed to DX (IC₅₀) in the presence or absence of PUFAs. Both, DHA and EPA (25–100 μM) increased the antitumor activity of DX in these cells in a concentration dependent manner. Treatment with DX + EPA (100 μM) or DX + DHA (100 μM) caused nearly 70% reduction of cell viability of the A549 cells.

Effect of PUFAs on AOE's mRNA level

It was investigated whether DHA and EPA were able to modulate AOE's mRNA expression in A549 cells exposed to DX. The treatment of A549 cells with DX (IC₅₀) caused an increase of SOD1, SOD2, and GST-pi mRNA levels. The relative quantification of mRNA/GAPDH ratio of the SOD1, SOD2, and GST-pi showed marked decrease after DX treatment. The target/reference ratio for these genes, expressed as percentage of control, increased to 233.6, 273.55 and 199.9%, respectively. The observed changes in mRNA levels of CAT were not statistically significant. Compared to DX alone, EPA and DHA addition down-regulated SOD1, SOD2, GPx-4, and GST-pi genes expression in DX treated A549 cells in a concentration dependent manner (Tab. 1). After supplementation of A549 cells exposed to DX with EPA and DHA (100 μM), the value of target/reference ratio decreased below the control value.

DISCUSSION AND CONCLUSION

The cytotoxic action of DX has been mainly related to inhibition of topoisomerase II and to the production of ROS (11, 13). Therefore, the antioxidant status appears to be crucial determinant in the sensitivity of tumors to ROS generating anticancer therapy. Some tumor cells enhance total antioxidant capacity by up-regulation of the expression of AOE's, allowing them to survive damage from chemotherapy or radiotherapy (2). Major human AOE's include superoxide dismutases (SODs), CAT and enzymes associated with glutathione (GSH) metabolism such as glutathione peroxidases (GPx) and glutathione-S-transferases (GSTs). SODs reduce superoxide anion into hydrogen peroxide (14, 15). There are three SODs, all of which are expressed also in human lung. SOD2 (MnSOD) is mitochondrial, SOD1 (CuZnSOD) cytosolic and extracellular. CAT dismutates hydrogen peroxide. GPx, a selenoenzyme, detoxifies hydrogen and fatty acid peroxides by using GSH as a hydrogen donor. GSTs catalyze the conjugation of electrophilic metabolites or drugs to GSH to facilitate their excretion from the cells (2, 14, 15).

The redox-regulating proteins are highly expressed in lung tumors and are associated with lymph node status and prognosis in non-small cell lung cancer (NSCLC) (2, 14). A large study showed significantly elevated levels of SOD2 (the mRNA, protein and/or activity) in lung cancer (2). Mesothelioma cells with highest (10-fold) SOD2 activity also demonstrate elevated resistance to cytotoxic drugs such as epirubicin (anthracycline) (2). Conversely, the basal or induced level of SOD2 did not cause the development of drug resistance in A549 cells and human mesothelioma cells (M14K)

Table 1. PUFAs-induced alterations of antioxidant enzymes (AOE's) mRNA expression in A549 cells exposed to doxorubicin (DX, 0.078 μg/mL). QRT-PCR data are expressed as AOE's/GAPDH mRNA ratio (% of control). Data were calculated as the means ± SD from three independent experiments; * statistically significant differences vs. DX (p < 0.05).

DX + PUFAs concentration	Target/reference ratio (% of control)				
	SOD1	SOD2	CAT	GPx-4	GST-pi
DX	233.6 ± 21.8	273.6 ± 19.3	69.8 ± 2.8	32.7 ± 7.0	199.9 ± 1.2
DX + EPA (25 μM)	103.2 ± 5.8*	169.0 ± 25.3*	66.2 ± 10.9	29.8 ± 2.6	116.73 ± 4.8*
DX + EPA (50 μM)	68.3 ± 3.8*	161.2 ± 3.9*	81.9 ± 6.9	20.6 ± 4.4*	62.4 ± 3.5*
DX + EPA (100 μM)	21.4 ± 4.4*	79.1 ± 4.9*	79.0 ± 6.8	10.3 ± 2.5*	49.8 ± 1.2*
DX + DHA (25 μM)	177.5 ± 26.2*	185.6 ± 16.3*	81.9 ± 12.9	32.8 ± 1.0	86.1 ± 1.9*
DX + DHA (50 μM)	56.2 ± 12.7*	70.9 ± 12.7*	89.2 ± 14.5	23.4 ± 3.1*	53.5 ± 2.2*
DX + DHA (100 μM)	23.4 ± 2.8*	63.0 ± 3.3*	81.0 ± 5.9	21.6 ± 0.5*	43.9 ± 1.4*

during epirubicin treatment (15). Very little is known about CAT in malignant cells or about its role in drug resistance. It has been shown that CAT is not important in the drug resistance of human mesothelioma cells and A549 cells, and inhibition of CAT did not enhance epirubicin-related toxicity (15). A number of studies have shown that the amount of GST isoenzymes is even higher in tumors of the lung relative to the surrounding normal tissues (14). The shorter survival of the patients with elevated GST-pi could be due to lesser effectiveness of administered antineoplastic agents. The high GST-pi protein level contributes to this process either *via* its direct detoxifying effect towards some of the drugs, or *via* the inhibitory effect of GST-pi on MAP kinase signal pathways of apoptosis, triggered by antitumor drugs (16). The reported findings with human mesothelioma cells and adenocarcinoma A549 cells suggest that glutathione-associated mechanisms play an important role in the resistance of these cells to anthracyclines *in vitro* (15). It was found that nuclear GST-pi accumulated in A549 cells in response to DX and inhibition of the nuclear transport of GST-pi increased the sensitivity of the cancer cells to DX (16). GPx-4 is a unique antioxidant enzyme that can directly reduce phospholipid hydroperoxides in membranes and lipoproteins (9). The results described in this paper, obtained after exposition of A549 cells to DX, demonstrated up-regulation of the expression of SOD1, SOD2, and GST-pi. It suggests that these enzymes may protect A549 cells from DX-dependent cytotoxicity.

PUFAs supplementation has been recognized as one of the agents involved in the modulation of AOE activity and the response of cells to oxidative stress during anticancer treatment. It has been shown that DHA selectively down-regulates SOD1 expression in human lymphoma DHL-4 cells (10) and reduces the level of protein expression of GPx-4 in various human cancer lines (9). It is known that DHA and EPA, polyunsaturated fatty acids found in fish oil, exert selectively cytotoxic effects on cancer cells and are significantly less toxic toward normal cells (8). One major hypothesis explaining the anti-tumor effects of n-3 PUFAs is their susceptibility to lipid peroxidation which makes them capable of rapidly generating lipid peroxides, which may directly cause cytotoxicity or influence intracellular signaling pathways (6). The cytotoxicity of n-3 PUFAs on malignant cells can be accelerated under conditions of oxidative stress. Furthermore, the increased membrane unsaturation, resulting from a PUFAs supplementation, provides more abundant

targets for ROS formation generated by DX metabolism (5, 17). Numerous *in vivo* and *in vitro* studies have reported an increased efficacy of DX against human tumors after fish oil supplementation (11, 13, 17). However, DHA enhanced the sensitivity of breast cancer cells to DX, this chemosensitization was not correlated to anticancer drug intracellular concentration (13).

The present study showed that both, EPA and DHA, increased the antitumor activity of DX in A549 cells in a concentration dependent manner. The concentrations of DHA and EPA used can be found in human plasma under physiological conditions or during supplementation (18). EPA and DHA down-regulated SOD1, SOD2, GPx-4, and GST-pi genes expression in DX treated A549 cells. The observed changes in mRNA levels of CAT were not statistically significant. Similarly, exposition of breast cancer cells to DX by DHA observed by Vibet et al., was associated with a marked decrease in GPx-1 and tumor regression during chemotherapy and was correlated to low GPx-1 activity what suggests that the inhibition of GPx-1 activity by DHA could participate in tumor sensitization to anthracyclines (17). Exactly how DHA regulates the expression of AOE has not been established. Advances in the understanding of the biology of PUFAs indicate that they are active signaling molecules that may enter the nucleus *via* fatty acid binding protein and affect gene transcription (19). Alternatively, lipid peroxidation products may destabilize the SOD1 mRNA, leading to lower expression of the enzyme (10). In contrast, the decreased GPx-1 activity induced by DHA can be associated with a decreased protein level but not with a decreased mRNA, suggesting an effect of DHA at a post-transcriptional level (17). GPx can be damaged by lipid peroxidation products, which lead to a loss of GPx activity, probably by modification of the selenocysteine residue at the active site of the enzyme (20).

Taken together, the presented results support the idea that DHA and EPA incorporated to the tumor cells may serve as potential adjuvants to DX-based chemotherapy. The altered antioxidant enzymes expression can be associated with the sensitization of A549 cells to the cytotoxic effect of DX.

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