Phospholipid hydroperoxide glutathione peroxidase plays a role in protecting cancer cells from docosahexaenoic acid–induced cytotoxicity

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Abstract

Docosahexaenoic acid (DHA; 22:6, n-3) is known to exert cytotoxic effects against various types of tumors via lipid peroxidation. Whereas several enzymes influence the response of cells to oxidative stress, only one enzyme, phospholipid hydroperoxide glutathione peroxidase (GPx-4), directly reduces lipid hydroperoxides in mammalian cells. The present study was designed to examine the involvement of GPx-4 in determining the effects of DHA addition to various human cancer cell lines. Although baseline levels of GPx-4 did not correlate with the relative sensitivity of human cancer cell lines to DHA, DHA reduced the level of protein expression of GPx-4 by at least 50% in all six lines. Knockdown of GPx-4 by small interfering RNA technique in a human ovarian cancer cell line significantly enhanced the cytotoxic effect of DHA in a time- and concentration-dependent manner. This cytotoxic effect of DHA was reversed by pretreatment with vitamin E, suggesting that the enhanced toxicity of GPx-4 knockdown is due to changes in the ability of the cells to handle oxidative stress. Neither baseline superoxide dismutase-1 nor catalase expression correlated with the relative sensitivity of the cells to DHA treatment. These results illustrate that susceptibility to the oxidative stress imposed by DHA, and possibly other therapeutic agents, is due to complex interactions among multiple antioxidant systems. The modulation of GPx-4 levels by DHA administration is of potential importance and may influence the cellular response to other oxidant stresses. [Mol Cancer Ther 2007;6(4):1467 – 74]

Introduction

n-3 polyunsaturated fatty acids (n-3 PUFAs) have been shown to exert selective cytotoxicity against various types of cancer cells (1). Multiple cellular mechanisms have been proposed to explain the anticancer effects of n-3 PUFAs, including inhibition of arachidonic acid–derived eicosanoid biosynthesis, influences on transcription factors and gene expression, modification of signal transduction pathways, induction of differentiation of cancer cells, and/or enhancement of lipid peroxidation (2, 3). Of these, a large amount of work indicates a central role of lipid peroxide generation in explaining the cytotoxicity of n-3 PUFAs (4–6).

Multiple enzymes, such as catalase, the superoxide dismutases (SOD), and those involved in both the glutathione redox cycle and the thioredoxin cycle, are involved in cellular defenses against oxidant stresses. Each of the antioxidant enzymes has selective substrates and distinct distributions in various cellular compartments (7). Phospholipid hydroperoxide glutathione peroxidase (GPx-4) was the fourth selenium-containing glutathione peroxidase discovered and has been purified (8), cloned (9–11), and characterized (12). Among the primary cellular enzymes that reduce hydrogen peroxides, GPx-4 is known to have a broad range of cellular substrates (13) but it is the only glutathione peroxidase to directly act on phospholipid hydroperoxides (13, 14). This implies that it has a unique involvement in the protection of cells against damage induced by lipid peroxidation. GPx-4 is widely expressed in normal tissues and is likely to play a role in protecting a variety of normal and neoplastic cells from lipid peroxidation (7).

Docosahexaenoic acid (DHA) is one of the most potent cytotoxic n-3 PUFAs, likely due to its possessing a greater number of double bonds than other naturally occurring PUFAs, which increases its susceptibility to peroxidation (15, 16). Although it is logical to suppose that GPx-4 might determine how a cell responds to DHA exposure, the multiplicity of enzymes that can influence how a cell responds to oxidant stress requires that the role of each be examined separately. The role of GPx-4 in determining the effect of DHA on cancer cell viability has not previously been investigated. The present study provides the first evidence indicating that GPx-4 is involved in antagonizing DHA cytotoxic effects in human cancer cell lines, although not due simply to its baseline expression. These findings provide new insights into our understanding of the anticancer properties of n-3 PUFAs and further substantiate the role of lipid peroxidation in DHA-induced cytotoxicity.

Materials and Methods

Materials

CellTiter 96 Aqueous ONE Solution Cell Proliferation Assay [3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS assay)]
reagent was from Promega (Madison, WI). Antibodies for GPx-4, SOD-1, and β-actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). SuperScript II reverse transcription kit and Taq polymerase were from Invitrogen (Carlsbad, CA). DHA and anti-catalase antibody were from Sigma (St. Louis, MO). All other chemicals and reagents were of analytic grade.

**Cell Culture and Cell Viability Assay**

The A2780 human ovarian cancer cell line and A2780/CP70 (a cisplatin-resistant sub-line) were kindly provided by Dr. Stephen B. Howell (University of California, San Diego, CA). The MM1.S and MM1.R lines were obtained from Dr. Steven Rosen (Northwestern University, Chicago, IL) and the C8161 line from Dr. Mary Hendricks (Northwestern University). The other cell lines were obtained from American Type Culture Collection (Manassas, VA). Cells were routinely grown in 75-mm flasks, in an environment containing 5% CO₂, and passaged every 3 days. HL-60, Raji, CEM, MCF-7, MM1.S, MM1.R, A2780, and A2780/CP70 cells were cultivated in RPMI 1640 supplemented with 2 mmol/L l-glutamine and 10% FCS. HT29 and Panc-1 cells were cultivated in DMEM supplemented with l-glutamine and 10% FCS. C8161 cells were maintained in Ham’s F10 medium supplemented with 15% FCS.

Cell viability was analyzed by MTS assay (Promega). Cells (3,000–10,000) were plated in each well of a 96-well tissue culture plate with 100 μL of fresh medium containing DHA or other reagents, and the cells were incubated at 37°C for 48 h. After centrifugation, the RNA pellet was washed once with 70% ethanol and air-dried. Reverse transcription was done at 42°C for 50 min in 20 μL of mixture containing 5 μg of RNA, 200 units of SuperScript II, 40 units of RNase inhibitor, 0.5 mmol/L of each deoxynucleotide triphosphate, and 100 ng of oligo-dT primers. The reaction was stopped by incubation at 70°C for 15 min. Primers for PCR amplification of GPx-4 were developed using the National Center for Biotechnology Information Entrez nucleotide database (accession no. NM_002085): sense primer (100/116), 5′-TGGGGAAGGTGAAGGTCGG-3′; antisense primer (701/684), 3′-CTAGAAATAGTGGGGCAG. The PCR reaction contained 1 μL of cDNA, 0.2 μmol/L sense and antisense primers, 0.4 mmol/L deoxynucleotide triphosphate mix, 1.5 mmol/L Mg²⁺, and 2.5 units of Taq polymerase in a 25-μL volume.

**Small Interfering RNA Technique of GPx-4 Expression**

Sequence information about mature human GPx-4 mRNA was obtained from the National Center for Biotechnology Information Entrez nucleotide database. Two target sites within the gene were chosen from the mRNA sequence of GPx-4 (GenBank accession no. NM_002085). Each target site was searched with National Center for Biotechnology Information BlastN to confirm its specificity. Two different small interfering RNAs (siRNA) were designated GPx-4-siRNA-1 and GPx-4-siRNA-2, which target nucleotides 316 to 334 and 515 to 533 of the human GPx-4 mRNA sequence, respectively. The 64-mer sense and antisense oligonucleotide templates (19 × 2 nucleotides specific to the targeted genes and 26 nucleotides for restriction enzyme sites and hairpin structure) were synthesized by Integrated DNA Technologies (18). The DNA oligonucleotides were annealed, phosphorylated, and ligated to the Pub/Bsd/H1 vector at the BamHI/HindIII sites. This vector was provided by Dr. Leonidas Tsikas (University of Oklahoma Health Sciences Center, Oklahoma City, OK) and contains the blasticidin resistance gene (19). Successful ligation was confirmed by enzyme digestion and by direct sequencing. The constructs (4 μg of the mixture of GPx-4-siRNA-1 and GPx-4-siRNA-2) were transfected into A2780 cells using Lipofectamine reagent (Invitrogen). Three days after transfection, the cells were selected by the addition of blasticidin (10 μg/mL, final concentration) to the medium for 6 days. The selected cells were immediately subjected to reverse transcription-PCR or viability assay experiments.

**Reverse Transcription-PCR**

A2780 cells were grown and treated in six-well plates (300,000 per well). Total RNA was isolated using TRIzol reagent (Invitrogen). Cells were denatured with 500 μL of TRIzol Reagent at room temperature for 5 min. RNA was extracted by addition of 100 μL of chloroform and centrifuged for 15 min at 10,000 × g, 4°C. The aqueous phase was collected and the RNA precipitated with 2 volumes of isopropanol at room temperature for 10 min. After centrifugation, the RNA pellet was washed once with 70% ethanol and air-dried. Reverse transcription was done at 42°C for 50 min in 20 μL of mixture containing 5 μg of RNA, 200 units of SuperScript II, 40 units of RNase inhibitor, 0.5 mmol/L of each deoxynucleotide triphosphate, and 100 ng of oligo-dT primers. The reaction was stopped by incubation at 70°C for 15 min. Primers for PCR amplification of GPx-4 were developed using the National Center for Biotechnology Information Entrez nucleotide database (accession no. NM_002085): sense primer (100/116), 5′-TGGGGAAGGTGAAGGTCGG-3′; antisense primer (701/684), 3′-CTAGAAATAGTGGGGCAG. The PCR reaction contained 1 μL of cDNA, 0.2 μmol/L sense and antisense primers, 0.4 mmol/L deoxynucleotide triphosphate mix, 1.5 mmol/L Mg²⁺, and 2.5 units of Taq polymerase in a 25-μL volume. The cDNA was first denatured at 95°C for 3 min and thermal cycles included 94°C for 1 min, 51°C for 2 min, and 72°C for 2 min, for a total of 30 rounds. Elongation was done at 72°C for 12 min. The PCR reaction products were separated in a 1% agarose gel containing ethidium bromide and visualized under UV light. The expression level of GPx-4 was analyzed by densitometry and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers used for GAPDH amplification were sense, 5′-TGCGGAAAGTGTAAGTCCGG-3′, and antisense, 5′-GGGATCTCCGCTCCGAAG-3′. SOD-1 mRNA level was analyzed using primers as previously reported.

**Thiobarbituric Acid Reactive Substance Assay**

Thiobarbituric acid reactive substance assay was done as previously described (20). In brief, cells were harvested by suspending them in cold PBS, sonicated, and subjected to reactions containing butylated hydroxytoluene, chloroacetic acid, HCl, thiobarbituric acid, and SDS. The color was
developed at 95 °C for 60 min and the reaction was cooled down on ice. The samples were centrifuged and the supernatant from each tube was measured at 540 nm with reference to a reagent blank. Thiobarbituric acid reactive substances were normalized to the protein concentration of each sample and expressed as arbitrary units relative to the level in control cells.

**Statistical Analysis**

One-way ANOVA analysis, IC₅₀ calculations, and non-linear regression were done with Prism 4 (GraphPad Software, Inc., San Diego, CA).

**Results**

**Effects of DHA on the Expression of GPx-4 in Human Cancer Cells**

n-3 PUFAs have been shown to alter activity of antioxidant enzymes in mammalian systems (21–27). To understand the role of GPx-4 in DHA-induced cytotoxicity, we first examined whether DHA could affect the expression of this enzyme in six human cancer cell lines. These include A2780 (ovarian carcinoma), Raji (B-cell lymphoma), CEM (T-cell leukemia), HL-60 (myeloid leukemia), MM1.R (myeloma), and MM1.S (myeloma). Analysis of expression of SOD-1 and catalase was also included in the experiments. As shown in Fig. 1, using protein specific antibodies, expression of GPx-4 was detected as a size of ~20 kDa, SOD-1 at 23 kDa, and catalase at 60 kDa. Treatment of the cells with 100 μmol/L DHA for 16 h reduced the protein level of GPx-4 by at least 50% in all six cell lines tested. SOD-1 expression was also down-regulated by DHA in these cell lines, supporting our previous findings (20). On the other hand, catalase protein level was somewhat increased in HL-60, Raji, and MM1.R cellsthat had been treated with DHA (Fig. 1). We have previously shown that in human lymphoma DHL-4 cells, DHA reduces both SOD-1 mRNA and protein expression levels (20). The effects of DHA on GPx-4 expression in cancer cells have never been reported. These results indicate that both GPx-4 and SOD-1 enzymes are targeted by DHA, which may contribute to DHA-induced lipid peroxidation and killing of cancer cells.

**Effects of DHA on the Viability of A2780 Cells**

Because the human ovarian cancer cell line A2780 is known to be sensitive to oxidative stress (28) and exhibits reduced GPx-4 expression on treatment with DHA, we chose it for further study. We first examined the effects of DHA on the viability of A2780 and A2780/CP70 cells (a subline resistant to cisplatin). As shown in Fig. 2A, treatment with increasing concentrations of DHA for 72 h caused a reduction of cell viability in both cell lines. The IC₅₀ of DHA for A2780 cells was 277 μmol/L and for A2780/CP70 cells, 300 μmol/L, indicating a similar sensitivity of these two cell lines to the cytotoxic effect of DHA. The effects of cisplatin on these two cell lines were also investigated (Fig. 2B). In line with previous reports, A2780 cells were shown to be more sensitive to cisplatin than the A2780/CP70 subline (29). The IC₅₀ of cisplatin was 3 μmol/L for A2780 and 12 μmol/L for A2780/CP70 cells. These results verify the phenotype of A2780 cells and suggest that DHA induces cytotoxicity through cellular mechanisms that are different from those used by cisplatin.

**Knockdown of GPx-4 Enhances DHA Cytotoxicity in A2780 Cells**

To determine whether GPx-4 plays a role in protecting cells against the cytotoxic effects of DHA, a siRNA approach was taken to knock down the expression of GPx-4 in A2780 cells. The cells were transfected with vectors containing short DNA sequences that can be transcribed to generate a double-stranded hairpin RNA structure (18). Transfected cells were selected by incubation with blasticidin (10 μg/mL) and knockdown of GPx-4
expression was confirmed by reverse transcription-PCR and Western blot analysis (Fig. 3A). A clear and prominent band was detected in A2780 cells, corresponding to the size of GPx-4 mRNA seen in other cells (30). This mRNA species was significantly lower in GPx-4-knockdown cells whereas the mRNA levels of SOD-1 were the same in both cell lines (data not shown). Densitometric analysis indicated an 80% reduction of GPx-4 mRNA expression in A2780 cells after knockdown of the gene. The protein level of GPx-4 was also reduced to the same extent. A previous study reported that knockout of GPx-4 in mice results in growth impairment of the animal. GPx-4(−/−) embryos die in utero by midgestation and exhibit a lack of normal structural compartmentalization. GPx-4(+/-) mice display reduced levels of GPx-4 mRNA and protein in various tissues, and cell lines derived from GPx-4(+/-) mice are markedly sensitive to inducers of oxidative stress (31). However, in our hands, knockdown of this enzyme in A2780 cells did not affect cell proliferation (Fig. 3B). This suggests that low-level expression of GPx-4 is sufficient for maintaining cell growth of A2780 cells. However, knockdown of GPx-4 enhanced DHA-mediated cytotoxicity toward A2780 cells. This enhancement was detected in a concentration-dependent manner (Fig. 4A) as early as 24 h after DHA treatment (Fig. 4B). Detailed analysis revealed that the IC50 of DHA toward GPx-4-knockdown cells was 66 μmol/L whereas in mock-transfected cells, it was 166 μmol/L. The cytotoxic effects of the PUFA eicosapentaenoic acid (20:5, n-3) was also enhanced in GPx-4-knockdown cells, but the saturated long-chain fatty acid docosanoic acid (22:0) at the same concentration did not affect cell viability of either control A2780 cells or GPx-4-knockdown cells (Fig. 4C). This clearly shows that knockdown of GPx-4 sensitizes A2780 cells to cytotoxicity caused by long-chain n-3 PUFAs. No enhancement of cisplatin-induced cytotoxicity was detected in GPx-4-knockdown cells (Fig. 4D).
To ensure that the enhanced cytotoxicity of DHA in GPx-4-knockdown cells was due to a change in their susceptibility to oxidative stress, we pretreated the cells with 100 μmol/L vitamin E for 15 min before addition of varied concentrations of DHA for 72 h. As shown in Fig. 5, vitamin E completely reversed the cytotoxic effects of 100 μmol/L DHA both in control and GPx-4-knockdown cells (Fig. 5B). However, vitamin E was more effective in counteracting DHA cytotoxicity in control cells than in GPx-4-knockdown cells when 300 μmol/L DHA was used, suggesting that it is the enhanced oxidative stress that contributes to the enhanced cytotoxicity after the knockdown of GPx-4 in these cells. Indeed, knockdown of GPx-4 enhanced DHA-induced lipid peroxide levels in A2780 cells as analyzed by the thiorbarbituric acid reactive substance assay (Fig. 5A), an effect that was reversed by vitamin E (data not shown).

To further confirm that GPx-4 modulates DHA cytotoxic effects, A2780 cells were transiently transfected with a cDNA construct encoding for GPx-4 (pcDNA3-GPx-4; kindly provided by Dr. Larry Oberley, University of Iowa, Iowa City, IA). Enhanced expression of GPx-4 was observed by Western blot analysis 48 h after transfection (Fig. 5C) and DHA-induced cytotoxicity was attenuated in cells that overexpress GPx-4 (Fig. 5D).

**No Correlation Was Evident between the IC₅₀ Values of DHA and the Baseline Expression Levels of GPx-4, SOD-1, or Catalase in 10 Human Cancer Cell Lines**

Because DHA reduces GPx-4 expression and knockdown of GPx-4 enhances DHA toxicity, we speculated that the level of expression of GPx-4 in each cancer cell line may determine their sensitivity to DHA. To test this hypothesis, IC₅₀ of DHA for each cell line was determined. The protein expression levels of GPx-4, SOD-1, and catalase were determined by Western blotting. The detected protein bands were quantified by densitometry and expressed as percentages of the highest expression level detected for each protein. The relationship between the IC₅₀ value for DHA and the protein expression of the antioxidant enzymes in 10 cell lines (Table 1) was analyzed by nonlinear regression. As shown in Fig. 6, there was no significant correlation between the basal expression levels of any of the enzymes studied and the IC₅₀ values for DHA (GPx-4, \( r^2 = 0.07218 \); SOD-1, \( r^2 = 0.04977 \); catalase, \( r^2 = 0.2640 \)).

**Discussion**

Lipid peroxidation has been recognized as one of the key mechanisms involved in the selective anticancer activity of n-3 PUFAs, and defense systems that modulate the response of cells to oxidant stresses (32) are likely candidates to explain the differential effects of these compounds. Among those defenses is the family of glutathione peroxidases. Of the family members identified to date, GPx-4 is the only one that is able to directly detoxify lipid hydroperoxides (7), and it therefore seemed a likely candidate to explain the variable effects of DHA on tumor cell viability. The experiments reported here show that the baseline level of key antioxidant enzymes, including GPx-4, does not correlate with the sensitivity of 10 different cancer cell lines to DHA treatment. Although, at first glance, these results might be taken to indicate that GPx-4 is not an important modulator of DHA effects, the studies reported here suggest otherwise.

It should first be noted that the baseline expression of two other well-recognized antioxidant enzymes, catalase and SOD-1, did not correlate with the cellular sensitivity to DHA. By examining the expression of these enzymes...
following exposure to DHA, we were able to show that there is a dynamic element to cellular antioxidant defenses, which seems to be important in determining cell fate with this oxidant stress. We found reduced expression of both SOD-1 and GPx-4 in a number of cell lines. On the other hand, catalase expression was actually increased by DHA under the same experimental condition. Whereas the mechanisms of differential effects of DHA on the expression of these antioxidant enzymes remain to be elucidated, these findings indicate a complex response to DHA by antioxidant enzymes in a given cell model system. Previous studies have reported that cells with reduced GPx-4 level are vulnerable to oxidative stress (31). To determine whether the change in GPx-4 expression itself could determine how a cell responds to DHA exposure, we used the siRNA technique to knock down GPx-4 expression

Table 1. IC50 values of DHA and relative protein expression levels of GPx-4, catalase, and SOD-1 in human cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cancer type</th>
<th>IC50, μmol/L</th>
<th>GPx-4 relative level, %</th>
<th>Catalase relative level, %</th>
<th>SOD-1 relative level, %</th>
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<tbody>
<tr>
<td>HL-60</td>
<td>Myeloid</td>
<td>78</td>
<td>40</td>
<td>100</td>
<td>60</td>
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<tr>
<td>C8161</td>
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<td>5</td>
<td>10</td>
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<td>156</td>
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<td>8</td>
<td>90</td>
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<tr>
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<td>Ovarian</td>
<td>277</td>
<td>40</td>
<td>20</td>
<td>65</td>
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</table>
without altering SOD-1 expression and showed that GPx-4 plays a role in protecting cells from DHA-induced oxidative injury. Thus, down-regulation of this important antioxidant enzyme seems to be an important part in understanding DHA mechanism of action.

One might hypothesize that n-3 PUFA exposure would increase expression of antioxidant enzymes so that cells could maximize their potential to attenuate oxidative stress. The literature indicates, however, both increases and decreases in antioxidant enzyme activity in animals fed PUFA-enriched diets (21–27). We have previously reported that DHA targets SOD-1 gene expression in human lymphoma DHL-4 cells, which may contribute to the particular sensitivity of that cell line to DHA (20). The present study indicates that SOD-1 down-regulation is not unique to that cell line and that a second antioxidant enzyme, GPx-4, is similarly affected. Exactly how DHA regulates expression of these enzymes 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 (33), affecting gene transcription. Thus, rather than simply altering enzyme expression by imposing an oxidant stress on the cell, it seems likely that DHA regulates expression of antioxidant enzymes at the transcriptional level. This may be of importance in considering some of the putative health benefits of some n-3 PUFAs in preventing or treating cancer, as well as treating or preventing other types of disease.

In conclusion, the results from the present study show that GPx-4 plays a role in modulating the effects of DHA exposure on human cancer cells. Given that GPx-4 is the only major antioxidant enzyme known to directly reduce phospholipid hydroperoxides within membranes and lipoproteins (7), these findings suggest that the anticancer effects of n-3 PUFAs may be augmented if safe interventions can be identified that decrease intracellular GPx-4 expression and/or function.

References


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