Identification of a Novel Putative Non-selenocysteine Containing Phospholipid Hydroperoxide Glutathione Peroxidase (NPGPx) Essential for Allaying Oxidative Stress Generated from Polyunsaturated Fatty Acids in Breast Cancer Cells*

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A dramatic reduction in the expression of a novel phospholipid hydroperoxide glutathione peroxidase (PHGPx), which incorporates cysteine instead of selenocysteine in the conserved catalytic motif was observed in a microarray analysis using cDNAs amplified from mRNA of Brca1-null mouse embryonic fibroblasts. This non-selenocysteine PHGPx named NPGPx is a cytoplasmic protein with molecular mass of ~22 kDa and has little detectable glutathione peroxidase activity in vitro. Ectopic expression of NPGPx in Brca1-null cells that were sensitive to oxidative stress induced by hydrogen peroxide conferred a similar resistance level to that of the wild-type cells, suggesting the importance of this protein in reducing oxidative stress. Expression of NPGPx was found in many tissues, including developing mammary gland. However, the majority of breast cancer cell lines studied (11 of 12) expressed very low or undetectable levels of NPGPx irrespective of BRCA1 status. Re-expression of NPGPx in breast cancer lines, MCF-7 and IICC1937, which have very little or no endogenous NPGPx, induced resistance to eicosapentaenoic acid (an omega-3 type of polyunsaturated fatty acid)-mediated cell death. Conversely, inhibition of the expression of NPGPx by the specific small interfering RNA in HCC1937 breast cancer cells that originally express substantial NPGPx by the specific small interfering RNA in HS578T breast cancer cells that originally express substantial NPGPx by the specific small interfering RNA in HS578T cell death. Conversely, inhibition of the expression of NPGPx by the specific small interfering RNA in HS578T breast cancer cells that originally express substantial NPGPx by the specific small interfering RNA in HS578T cell death. Conversely, inhibition of the expression of NPGPx by the specific small interfering RNA in HS578T breast cancer cells that originally express substantial NPGPx by the specific small interfering RNA in HS578T.

The complex interplay among genetic susceptibility, hormones, environmental carcinogens, and dietary consumption contributes to the complexity of breast cancer development, treatment, and prevention (1, 2). Hormone metabolism and environmental genotoxic agents generate reactive oxygen species (ROS) that may introduce genetic mutation and aberrant signal transduction that can lead to uncontrolled cell proliferation (3–5). Interestingly, many tumor susceptibility genes that have been linked to breast carcinogenesis such as p53, ATM, PTEN, BRCA1, and BRCA2 have either direct or indirect roles in cellular defense against oxidative stress as the result of excessive ROS production without concomitant up-regulation of antioxidants (6–10).

It has been reported that BRCA1-deficient embryonic stem cells are deficient in transcriptional-coupled oxidative damage repair (11). How BRCA1 exerts its role in this repair pathway remains unclear. BRCA1 serves as a transcriptional modulator to regulate the expression of the DNA damage response genes such as GADD45 (12, 13) and physically interacts with repair machinery proteins such as the Rad50-MRE11-NBS1 complex upon ionizing irradiation (14). It is therefore likely that ROS generated by either oxidative stress or IR may lead to a similar DNA damage response mediated by BRCA1 (15). However, it is not known whether BRCA1 has any direct role in mediating the removal of ROS generated by oxidative stress.

Extensive studies on animal cancer models have demonstrated that different forms of polyunsaturated fatty acids (PUFA) yield pronounced effects on growth of breast cancer cells (16). Omega-3 forms of PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid, which are highly enriched in fish oil, are known to inhibit proliferation of many different types of cancers (17). Because EPA has been demonstrated to increase peroxisomal β-oxidation, which generates hydrogen peroxides (18–20), the inhibitory effect of omega-3 PUFA has been attributed to the production of oxygen radicals and initiation of intracellular lipid peroxidation (21).

Lipid peroxidation involves the introduction of the polar hydroperoxy group into the hydrophobic moiety of unsaturated fatty acid (22). The resulting hydroperoxy lipids are sources for further generation of ROS and substrates for reduction by GPx or phospholipid hydroperoxide glutathione peroxidase

* This work was supported by National Institutes of Health Grants CA94170 and CA81020 (to A. U. and X. J.), Department of Defense pre-doctoral training Grant DAMD 17-99-1-9402 (to A. U. and X. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡‡ The abbreviations used are: ROS, reactive oxygen species; GPx, glutathione peroxidase; PHGPx, phospholipid hydroperoxide glutathione peroxidase; BRCA1, breast cancer susceptibility gene 1; MEF, mouse embryonic fibroblast; EPA, eicosapentaenoic acid methyl ester; PUFa, polyunsaturated fatty acid; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium, inner salt; siRNA, small interfering RNA; PCS, fetal calf serum; RT, reverse transcriptase; EGFP, enhanced green fluorescent protein; NPGPx, non-selenocysteine containing phospholipid hydroperoxide glutathione peroxidase.

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(PHGPx), a distinct member of the GPx family (22, 23). Three other members of the GPx family, cytoplasmic GPx (GPx1), gastrointestinal GPx (GPx2), and extracellular GPx (GPx3), generally prefer soluble alkyl hydroperoxides as substrates instead of hydroperoxides of complex lipid and phospholipid (24). It remains unclear how each individual GPx contributes to the elimination of oxidative stress generated from endogenous cellular respiration and metabolism of exogenous mutagens.

We have identified a novel GPx that shares a similar structural domain to GPx4. The presence of cysteine instead of selenocysteine, a conserved residue of the catalytic center, prompted the name non-selenocysteine phospholipid hydroperoxide glutathione peroxidase or NPGPx. Absence of NPGPx expression was found in the majority of breast cancer cell lines tested, and reconstitution of NPGPx expression in MCF-7 and HCC1937 breast cancer cells conferred resistance to EPA-mediated cell death. Conversely, inhibition of the normal expression of NPGPx in HS578T breast cancer cells by the specific siRNA increased their sensitivity to eicosapentaenoic acid-mediated cell death. These findings suggest a potential role for NPGPx in alleviating oxidative stress induced by dietary consumption of fatty acids in breast cancer cells.

**EXPERIMENTAL PROCEDURES**

**Chemicals and cDNAs—** Hydrogen peroxide and EPA were obtained from Sigma. EPA was dissolved in 100% ethanol to yield a stock concentration of 1 g/mL. MTS assay reagents were obtained from Promega (Madison, WI) and used according to the supplier’s instructions. Both mouse and human full-length NPGPx cDNA clones were obtained from ATCC with GenBank™ accession numbers BC003228 and BC032788, respectively, and sequenced to verify the authenticity.

**Cell Culture—** Breast cancer cell lines including MCF7, MDAMB231, MDAMB468, MDAMB157-7, MDAMB661, T47D, ZR75, MDAMB435, HS8578-T, HBL100, and SKBR3 were obtained from ATCC and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS), except for MCF-7 and HCC1937, which were grown in RPMI medium supplemented with 10% FCS. Mouse embryonic fibroblasts (MEF) (25) were grown in low glucose Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, except for MCF-7 and HCC1937, which were treated, in quadruplets, with different concentrations of EPA in changed into serum-free medium for an additional 24 h. The cells were then treated, in quadruplets, with different concentrations of EPA in 0.2% ethanol and 1% FCS. Cell viability was determined 4 days later by adding MTS reagent (Promega) as per the manufacturer’s instructions.

**Colonies Formation Assay—** About 3–5 × 10⁴ cells of mouse embryonic fibroblasts were seeded into 100-mm dishes and treated with freshly diluted H₂O₂ in 10% FCS. The medium was changed every 2 days for 2 weeks. Colonies were counted and the ratio of colony numbers between treated and untreated cells is expressed as percentage of survivability.

**Preparation of RNA for RT-PCR—** Total RNA was isolated from either tissues or cells as instructed by the manufacturer (TRizol, Carlsbad, CA). RT-PCR was performed as described (12). The sequence of oligonucleotide primers used for amplifying specific gene products is listed in Table I.

**Antibody Preparation—** Human NPGPx cDNA was fused to a glutathione S-transferase vector in-frame from amino acid 32 to 186. The fusion protein was expressed and purified from bacteria for serving as antigens to generate mouse polyclonal antibodies. In addition, the purified His-tagged NPGPx protein was used as antigen to generate monoclonal antibodies following the established procedures (30, 31).

**Generation of the High Titer Retrovirus for NPGPx Expression—** High titer RNA virus was achieved by placing cytomegalovirus promoter in front of an upstream 5′-terminal repeat (R5) (33). The vector contains p53 + /Brca1 − retrovirus driven by the phosphoglycerate kinase (PGK) promoter as a marker for infection. NPGPx cDNA was inserted at XhoI and HindIII sites under control of R5. The expression plasmid was co-transfected with vesicle stomatitis virus glycoprotein expressing plasmid into HEK 293-GP2 cells, which stably expressed viral packaging proteins (gag and pol) as described (34).

**TABLE I**

| Name of oligonucleotide | Sequence | Size of amplified products |
|-------------------------|----------|---------------------------|
| S16 (forward)           | 5′-tcgaagggccgctgctcatc-3′ | 100 (mouse and human) |
| S16 (reverse)           | 5′-cttaggcgggaggggggcc-3′ | 470 (mouse and human) |
| GPx1 (forward)          | 5′-tagggcggccagtcctg-3′   | 369 (mouse) |
| GPx1 (reverse)          | 5′-tagaaggcccggccagtcctg-3′ | 490 (mouse and human) |
| NPGPx mouse (forward)   | 5′-aagttcgctgatctcggaggag-3′ | 412 |
| NPGPx mouse (reverse)   | 5′-agtgtgtagtactgcagggc-3′ | 315 |
| NPGPx human (forward)   | 5′-cacggagaggctttcagggc-3′ | 500 |
| NPGPx human (reverse)   | 5′-cggctgctttcagggc-3′ | 350 |

**Oligonucleotide sequences for RT-PCR**

- S16 (forward): 5′-tcgaagggccgctgctcatc-3′
- S16 (reverse): 5′-cttaggcgggaggggggcc-3′
- GPx1 (forward): 5′-tagggcggccagtcctg-3′
- GPx1 (reverse): 5′-tagaaggcccggccagtcctg-3′
- NPGPx mouse (forward): 5′-aagttcgctgatctcggaggag-3′
- NPGPx mouse (reverse): 5′-agtgtgtagtactgcagggc-3′
- NPGPx human (forward): 5′-cacggagaggctttcagggc-3′
- NPGPx human (reverse): 5′-cggctgctttcagggc-3′

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human breast cancer cells were seeded at $2 \times 10^5$ into each 60-mm plate for 24 h and transfected with 0.4 nmol of NPGPx siRNA or luciferase siRNA using OligofectAMINE (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. For Western analysis, cells were harvested at the designated time intervals, and whole cell lysates (20 μg of protein) were subjected to 12% SDS-PAGE followed by immunoblotting using mouse monoclonal NPGPx antibody (GenTex, San Antonio, TX). These siRNA-treated cells were re-plated at $3 \times 10^5$ into each well of the 96-well plates after 24 h of transfection in the presence of different concentrations of EPA for the viability assay as described above.

RESULTS

Identification of NPGPx—To search for genes regulated by BRCA1, we initiated a microarray analysis using a microchip containing about 8680 genes with cDNAs amplified from the mRNA of wild type, p53$^{-/-}$, and p53$^{-/-}$-Brca1$^{-/-}$ (or Brca1-null) MEFs. The results indicated that the expression of several genes encoding lipoxygenase (37), follistatin-like (38), Src-suppressed C kinase substrate/SSECKS (39), and glutathione peroxidase-like (GenBank accession number BC032788 or AF320068) were significantly reduced in Brca1-null cells. Because loss of both wild-type alleles of Brca1 has been correlated with sensitivity to oxidative stress (11, 40), GPx-like was further characterized.

A comparison of amino acid sequences of this GPx-like with other GPx family members revealed 42% identity to mammalian cytoplasmic GPx1 and 44% to GPx4 (Fig. 1A). Three signature motifs of glutathione peroxidase are conserved in GPx-like, including the conservation of glutamine and tryptophan residues serving as the catalytic center of the enzyme (Fig. 1A). However, GPx-like incorporates cysteine instead of selenocysteine (encoded by an in-frame opal codon UGA), the third conserved residue in the catalytic center of most mammalian GPx enzymes (encoded by an in-frame opal codon UGA), the third conserved residue in the catalytic center of most mammalian GPx family members (23). Moreover, amino acid sequence alignment between mammalian GPx family members revealed the presence of gaps that are conserved among PHGPx from many species (Fig. 1A) (41, 42). Therefore, GPx-like is the first reported mammalian PHGPx that uses cysteine instead of selenocysteine in the presumed catalytic site and henceforth is referred to as NPGPx (non-selenocysteine containing phospholipid hydroperoxide glutathione peroxidase). Query to GenBank to determine the chromosomal localization of NPGPx revealed that the NPGPx sequence is identical to Gpx7 and is located on chromosome 1p32 (www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l = 27234). NPGPx is evolutionarily conserved between human and mouse, reaching up to 89% amino acid similarity, and shares ~37% amino acid similarity with yeast PHGPx1 through PHGPx3. Unlike GPx4, NPGPx has neither...
mitochondrial nor nuclear targeting sequences but can contain an N-terminal signal peptide.

**Identification of NPGPx Protein and Its Cellular Localization**—Using the NPGPx cDNA as template for in vitro transcription and translation, a doublet band with 26 kDa was detected (Fig. 1B). To confirm that the protein is authentic from the cDNA, we generated specific antibodies using antigens derived from either N-terminal glutathione S-transferase-tagged or His-tagged NPGPx proteins expressed in bacteria for identifying NPGPx. The in vitro translated NPGPx protein can be immunoprecipitated by the specific polyclonal antibodies against human NPGPx, but not by preimmune sera (Fig. 1B). The same anti-NPGPx polyclonal antibody, but not preimmune, recognized endogenous cellular NPGPx in immunoprecipitation followed by Western blot analysis (Fig. 1C, lane 2). Furthermore, a similar size of the protein was detected by direct Western blot analysis probed by αNPGPx polyclonal antibody (αNPGPx Mab-1) (Fig. 1C, lane 3). Intriguingly, the size of the endogenous NPGPx is about 22 kDa, which approximates the predicted molecular mass of NPGPx (186 amino acids), different from the in vitro synthesized protein. The mobility discrepancy in SDS-PAGE between the in vitro translated and the endogenous NPGPx suggests that NPGPx may be processed in vivo.

Because NPGPx is the first mammalian PHGPx that uses cysteine instead of selenocysteine in the presumed catalytic site (Fig. 1A), it remains to be seen whether the protein has glutathione peroxidase activity. We then purified hexahistidine-tagged NPGPx to near homogeneity by affinity chromatography using a nickel-agarose resin and performed glutathione peroxidase activity assay. Little or no peroxidase activity was found under this assay condition. Although this result is consistent with many seleno-independent GPxs that have been investigated (43), an alternative possibility exists and requires further exploration.

Next, the cellular localization of NPGPx in normal human diploid fibroblasts (HS27) was determined by immunostaining with either preimmune or anti-NPGPx polyclonal antibody (Fig. 1D). Specific staining was seen in the cytoplasm, with little or undetectable signal in the nucleus, suggesting that NPGPx is a cytoplasmic protein.

**Expression of GPx Family Members in Brca1-null MEF**—To validate the results obtained from the microarray analysis and to assess the expression of other GPx family members, we performed semiquantitative RT-PCR on total RNA collected from wild-type, p53/−, and p53/−;Brca1/− (Brca1-null) MEFs. As shown in Fig. 2A, there was a modest up-regulation of NPGPx mRNA expression in p53/− MEF in comparison to wild-type MEF. However, NPGPx mRNA was significantly down-regulated in Brca1-null cells. In contrast, the expressions of other members of the GPx family such as GPx1 and GPx4 were not changed. Consistent with the RT-PCR results, the NPGPx protein expression was readily detected in both wild-type and p53/− MEFs, but not in Brca1-null cells (Fig. 2B). Despite the similarity in the amino acid sequences among GPx family members, anti-NPGPx antibody was highly specific and recognized neither GPx1 nor GPx4, which were expressed in MEFs. Although there was a positive correlation between Brca1 and NPGPx expressions in these MEFs, we found no evidence to support a direct role for Brca1 in regulating the expression of NPGPx (data not shown and see below). Therefore, the reduction of NPGPx expression in the Brca1-null MEF may not be a direct consequence of Brca1 inactivation.

**Ectopic Expression of NPGPx Reduces Oxidative Stress of Brca1-null MEF**—Brca1-null fibroblasts remain a useful system to test the biological function of NPGPx because they contain very little or no endogenous NPGPx. Moreover, Brca1-deficient fibroblasts have been described to be sensitive to exogenous H2O2 (40). It is likely that reconstitution of NPGPx into Brca1-null MEFs will rescue H2O2-induced lethality. To express NPGPx ectopically in these MEFs, we used two types of high titer retroviruses carrying either EGFP alone or EGFP along with NPGPx expression units. As shown in Fig. 2B, Brca1-null MEF infected with NPGPx retrovirus expressed the protein at a significantly higher level when compared with that of wild-type or p53-null MEF. Parental Brca1-null cells infected with EGFP alone did not express detectable NPGPx protein. The ectopically expressed NPGPx mimicked the endogenous NPGPx in cytoplasmic localization as described above (Figs. 3C, right panel, and 1D). Consistently, the Brca1-null cells with little NPGPx expression did not show specific NPGPx staining.

Colonial formation assays were then performed on these cells to test the effect of exogenous H2O2 on cell viability. About 60–70% of p53/− and wild-type MEFs treated with 100 μM H2O2 formed colonies after 2 weeks (Fig. 3D), whereas only about 10% of parental Brca1-null cells or infected with EGFP virus alone survived. On the other hand, significant resistance to H2O2 was observed in Brca1-null cells infected with NPGPx virus (Fig. 3D), suggesting that NPGPx is crucial for reducing the toxic effect of H2O2. However, it was noticed that the resistance to H2O2 toxicity of NPGPx virus-infected Brca1-null cells is not better than that of the wild-type or p53-null MEF although the former contained much higher level of NPGPx.

**NPGPx Is Expressed Ubiquitously and in Developing Mammary Gland**—At least four members of the mammalian glutathione peroxidases have been characterized and their expressions have a distinct pattern of tissue distribution (23). To assess the expression pattern of NPGPx, we performed RT-PCR using specific primers directed against GPx1, GPx3, and GPx4 (Fig 4A). As reported, kidney and testis are the primary sites that express high levels of GPx3 and GPx4, respectively (Fig. 2B) (23). On the other hand, the expression of the NPGPx mRNA was detected in testis, lung, kidney, adipose tissue, and mammary gland (Fig. 3A). Similarly, NPGPx protein was expressed in all tissues tested, including the mammary gland. We then examined the expression of NPGPx in virgin, pregnant, and lactating mammary glands. Interestingly, reduction of NPGPx mRNA and protein expression (Fig. 4D) was seen in both pregnant and lactating mammary glands in comparison to...
peroxide. MEFs including wild-type, expression confers resistance to the toxicity of the exogenous hydrogen cytoplasm of the infected cells but not in non-infected cells.

anti-NPGPx antibodies as well as with 4,6-diamidino-2-phenylindole as the 5

rus construct. The EGFP high titer retrovirus was constructed based on p53 and (Brca1 cytoplasm.

an internal loading control. Nuclear matrix protein p84 was used anti-NPGPx antibodies. A specific band of 22 kDa was detected only in corresponding cell lysates were analyzed by Western blotting with p53, and GPx1, and GPx4. Ribosomal S16 mRNA was included in the analysis as an internal control. B, NPGPx protein expression from different tissues. Total proteins extracted from the same array of tissues as described in A were analyzed by Western blotted and probed with anti-NPGPx antibodies. A nuclear matrix protein, p84, was used as an internal loading control. C, NPGPx mRNA expression during mammary gland development. Total RNA was extracted from mammary glands of virgin (V), pregnant (P), or lactating (L) female mice for RT-PCR analysis with specific primers for NPGPx. D, Western blot analysis of NPGPx proteins from different stages of mammary glands. Total protein extracts were prepared from mammary glands of virgin (V), pregnant (P), or lactating (L) mice and analyzed by Western blotting with anti-NPGPx antibodies. A nuclear matrix protein, p84, was used as an internal loading control.

virgin glands. However, the level of GPx1 mRNA did not vary appreciably during mammary gland development, whereas the level of GPx1 mRNA was increased in lactating gland (Fig. 4C). These results suggest that NPGPx is ubiquitously expressed and its expression level may vary during mammary gland development.

Lack of NPGPx Expression in Breast Cancer Cells—It is well documented that an intimate connection between oxidative stress and breast carcinogenesis exists (1). Breast cancers have been described to display a deregulated glutathione peroxidation system (44, 45). Therefore, we examined a panel of breast cancer cell lines for the expression of NPGPx. As shown in Fig. 5B, 11 of the 12 breast cancer cell lines examined, regardless of their BRCA1 status, expressed little or no NPGPx protein except HS578T. NPGPx was detected in normal human mammary epithelial cells immortalized by ectopically expressed
expression of NPGPx in both HCC 1937 and MCF-7 cells conferred increased resistance to the toxic effects of eicosapentaenoic acid than that of control cells without NPGPx. This result supports the possibility that NPGPx is essential for reducing the oxidative stress generated by specific polyunsaturated fatty acids.

To further consolidate this observation, we treated HS578T breast cancer cells that expressed detectable amounts of endogenous NPGPx with NPGPx siRNA. Expression of NPGPx protein was monitored by straight Western blot analysis using anti-NPGPx antibodies. As shown in Fig. 7A, the prepared siRNA effectively inhibited the expression of NPGPx protein 24 h after transfection, whereas control siRNA for the luciferase gene had little or no effect. The transfected cells were then treated with EPA. Inhibition of the normal expression of NPGPx in HS578T breast cancer cells by the specific NPGPx siRNA significantly increased its sensitivity to eicosapentaenoic acid-induced cell death, whereas the luciferase siRNA had no effect.

**DISCUSSION**

We described here a novel type of mammalian PHGPx, henceforth named NPGPx, which was discovered serendipitously during microarray studies comparing gene expressions in Brca1 proficient and null mouse embryonic fibroblasts. NPGPx is the prototype of mammalian non-selenocysteine PHGPx and is ubiquitously expressed. Interestingly, a majority of breast cancer cells do not express NPGPx. Expression of NPGPx suppresses toxic effects of soluble hydroperoxide inducing reagents (H₂O₂), as well as adverse effects of omega-3 type PUFA.

We initially hypothesized that NPGPx may be a potential target gene of Brca1 because Brca1 is a coactivator/corepressor of gene transcription and loss of Brca1 induces hypersensitivity
to $\text{H}_2\text{O}_2$ (46–48). However, ectopic expression of wild-type Brca1 in Brca1-null MEF by retroviral infection did not result in the re-expression of NPGPx mRNA and protein. Similarly, Brca1-null MEF stably expressing either mouse or human Brca1 also did not show detectable expression of NPGPx. Furthermore, expression of Brca1 did not affect the activity of the NPGPx regulatory sequence (which comprised of a 4-kb promoter and 2 kb of intron 1) linked to a reporter gene in a transient transfection assay (data not shown). Therefore, the lack of detectable NPGPx expression in Brca1-null cells may be attributed to the indirect effect of Brca1 via genomic instability pathway.

PHGPx is a specific class of GPx enzyme, which is able to reduce complex membrane-bound lipid peroxides in addition to soluble hydroperoxide substrates (23). The first mammalian PHGPx described was GPx4 (49–51). To date, all members of mammalian GPx, including GPx4, incorporate the non-standard amino acid selenocysteine at their catalytic site (24). A non-selenocysteine GPx (GPx5) was recently found in mammalian testis but has structural similarity to cytosolic GPx1 instead of GPx4 (52). NPGPx, on the other hand, is unique because it incorporates regular cysteine at the catalytic center and shares structural similarity to the PHGPx from many different species (41, 42). There is one other non-selenocysteine GPx (NSGPx) that has been described (12); however, in contrast to NPGPx, NSGPx (also known as 1-Cys peroxiredoxin) bears very little resemblance to the conserved motifs of GPx family members (53).

Despite sharing structural similarity with mammalian GPx4 and other PHGPx, NPGPx has several distinct features. Our anti-NPGPx antibody that was prepared by using the entire protein as an antigen, specifically recognizes NPGPx but not other GPx, suggesting a significant difference in their structure. It is noted that the in vitro synthesized NPGPx migrates slower than the endogenous NPGPx, suggesting a post-translational modification event, possibly by proteolytic processing. In support of this notion, transient expression of NPGPx cDNA in human 293 cells yields two distinct bands (data not shown), 26 and 22 kDa. The upper band (26 kDa) comigrated with the in vitro translated NPGPx, whereas the lower band (22 kDa) comigrated with endogenous NPGPx protein from different cell types (data not shown). Although the precise nature of the in vivo processing of NPGPx is presently not clear, it is likely that the potential signal peptide at the N terminus may be cleaved after synthesis in vivo.

Immunostaining of NPGPx indicated a cytoplasmic location that differs from GPx4, which is distributed in mitochondria (54). Although the biological significance of NPGPx localization remains to be explored, we speculate that NPGPx may serve as an additional layer of defense to reduce lipid peroxidation products in the cytoplasm before they reach critical organelles such as the mitochondria and nucleus.

NPGPx appears to be functionally important in mediating oxidative stress. As shown in Brca1-null fibroblasts, which do not express endogenous NPGPx, re-expression of NPGPx significantly reduces the toxic effect of soluble hydroperoxides ($\text{H}_2\text{O}_2$) despite the expression of other GPx members in these cells. How NPGPx is critical to cells in reducing oxidative stress either from $\text{H}_2\text{O}_2$ or from PUFA remains to be explored. So far, low or no glutathione peroxidase activities were found in all seleno-independent GPx that have been investigated (43). Consistently, we cannot detect any significant enzymatic activity of NPGPx under the standard GPx assay. Thus, we referred to NPGPx herein as glutathione peroxidase solely based on its sequence similarity to known GPx. However, a cysteine containing thioredoxin peroxidase encoded by a glutathione peroxidase gene of Plasmodium falciparum is active with $\text{H}_2\text{O}_2$ and organic hydroperoxide but not with phosphatidylcholine hydroperoxide despite structural similarity to PHGPx (55). Similarly, two plants of GPx-like proteins with cysteine as the active site also have the peroxidase activity (56). Because different GPx may utilize distinct substrates and cofactors, investigating whether NPGPx has any enzymatic activity toward specific substrates is actively undergoing. Alternatively, NPGPx may cooperate with other GPx in a novel manner to reduce cellular oxidative stress.

Glutathione-based detoxification of reactive oxygen species has been reported to be deregulated in a number of different types of cancer (57–59). GPx3 is highly up-regulated in clear cell carcinoma, a highly aggressive subtype of epithelial ovarian carcinoma (60). Furthermore, the glutathione peroxidation system has been proposed to contribute to the mechanism of drug resistance pathways (61, 62). For instance, overexpression of GPx1 in the T47D breast cancer cell line leads to partial resistance to doxorubicin (63). In addition, overexpression of GPx4 in MCF-7 breast cancer cells confers resistance to photodynamic therapy, which is mediated by the delivery of singlet oxygen (64).

Interestingly, NPGPx mRNA and protein were consistently down-regulated in whole mammary glands of pregnant and lactating mice (where an outburst proliferation of epithelial cells took place), whereas the expression of GPx4 remained unchanged. Similarly, NPGPx was not expressed or expressed at a very low level in the majority of breast cancer cell lines, whereas GPx1 and GPx4 expression were detected in many breast cancer cell lines (65). Therefore, there seems to be an inverse relationship between NPGPx expression and cellular proliferation; in other words, it appears that, in the absence of NPGPx, transformed breast epithelial cells may acquire a selective advantage for uncontrolled proliferation. Intriguingly, the chromosome region of 1p32, where NPGPx is located, is among the most common sites of loss of heterozygosity in breast cancer (66, 67). Therefore, it is likely that loss of NPGPx expression may be a consequence of mutational events that occurred in these breast cancer cells. Further experiments are needed to substantiate this possibility.

Many lipid peroxidation metabolites generated by a variety of lipoxygenases, cyclooxygenases, and desaturases may influence the growth of preneoplastic and/or transformed mammary epithelial cells (28, 68, 69). PUFAs are noted for their different effects on breast cancer proliferation in experimental animal models (29). Our result that breast cancer cells lacking NPGPx expression become sensitive to omega-3 fatty acid may provide a plausible explanation of why EPA exerts anti-tumor activity of breast cancer (17).

Although in principle GPx4 may reduce and consequently remove the effect of PUFA, our results suggest that NPGPx is a critical enzyme that may affect the final outcome of polyunsaturated fatty acid metabolism on breast cancer growth and development. First, the expression of GPx4 is generally unchanged in the course of normal mammary gland development or tumorigenesis, whereas NPGPx expression is lost in the majority of breast cancer cell lines. Second, the effects of omega-3 fatty acid on breast cancer cells have been demonstrated using cell lines that are known to express GPx4 but lack NPGPx expression. Third, NPGPx expression, thus far has been correlated with non-transformed cells, whereas GPx4 expression has been found in both non-transformed and transformed cells. However, it remains to be determined whether the loss of NPGPx is a critical step in cellular transformation and that re-expression of NPGPx in breast cancer cells affects their tumorigenicity.
Acknowledgments—We thank Paula Garza and Diane Jones for the preparation of antibody, and Nicholas Ting for critically reading the manuscript.

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