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L-PhGPx expression can be suppressed by antisense oligodeoxynucleotides

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Abstract

Phospholipid hydroperoxide glutathione peroxidase (PhGPx) directly reduces hydroperoxides of phospholipid and cholesterol to their corresponding alcohols. There are two forms of PhGPx: L-PhGPx localizes in mitochondria and S-PhGPx in cytosol. Antisense oligodeoxynucleotides can inhibit specific protein expression. We tested the hypothesis that antisense oligodeoxynucleotides could be designed to inhibit PhGPx expression and thereby sensitize cells to lipid peroxidation induced by singlet oxygen. We chose P4 cells, a cell line established from L-PhGPx cDNA transfected MCF-7 cells, as our cell model. Lipid peroxidation was induced by singlet oxygen generated by Photofrin and visible light. We found that the antisense oligodeoxynucleotide (5′GCCGAGGCT CATCGCGGCGG 3′) was effective in suppressing L-PhGPx mRNA, PhGPx protein, and activity. This antisense oligodeoxynucleotide did not interfere with S-PhGPx. When cells were exposed to singlet oxygen, lipid hydroperoxides were produced in the cells. L-PhGPx was able to remove these hydroperoxides; this removal was inhibited by antisense treatment. The inhibition of L-PhGPx by the antisense oligodeoxynucleotides also resulted in increased membrane damage as measured by trypan blue dye exclusion. These data demonstrate that PhGPx expression can be manipulated by antisense techniques.

Keywords: Free radicals; Lipid peroxidation; Lipid hydroperoxides; Photosensitization; Antisense oligodeoxynucleotides

Cells have evolved antioxidant systems to detoxify LOOH.[1] Among the antioxidant enzymes, PhGPx plays an important role in LOOH repair. Unlike cytosolic GPx-1, which cannot react with membrane-associated fatty acid hydroperoxides until the fatty acid ester groups are cleaved by phospholipase A_2, PhGPx (GPx-4) acts directly on phospholipid hydroperoxides in membranes [1]. It has been estimated that the rate of reduction of lipid hydroperoxide by PhGPx is 10^4-fold greater than those of PLA_2 and GPx-1 [2].

There are two forms of PhGPx in cells: the long form PhGPx (L-PhGPx) that is located in mitochondria and the short form PhGPx (S-PhGPx) that is located in the cytosol [3]. A clone (P4) that was derived from MCF-7 human breast cancer cells stably transfected with sense L-PhGPx cDNA has been established in our laboratory [4]. The increased L-PhGPx activity in these cells can lower the level of hydroperoxides and protect against the loss of plasma membrane

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[1] *Abbreviations used:* Anti, antisense; ATCC, American Type Culture Collection; dNTP, mixture of dATP, dGTP, dCTP, and dTTP; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetate; FBS, fetal bovine serum; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; LOOH, lipid hydroperoxide; MCF-7, human breast carcinoma cells; MEM, minimum essential medium; MMLV, Moloney-murine leukemia virus; $NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Oligo, oligodeoxynucleotide; PB, phosphate buffer; PBS, phosphate-buffered saline; PCOOH, phosphatidylcholine hydroperoxide; PCR, polymerase chain reaction; PDA, photodynamic action; PhGPx, phospholipid hydroperoxide glutathione peroxidase; PUFA, polyunsaturated fatty acid; RT, reverse transcriptase; SDS, sodium dodecyl sulfate; WT, wild-type.

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permeability when subjected to an oxidative stress that targets membranes [4].

Photodynamic action with Photofrin and visible light results in singlet oxygen formation [5]. The photosensitizer, Photofrin, selectively accumulates in cell membranes [5]. The singlet oxygen produced then reacts with polyunsaturated fatty acids to form LOOHs [6–8]. Upon reaction with ferrous iron, these LOOHs will form alkoxyl and peroxyl radicals that can initiate damaging free radical chain reactions that alter the structure and function of membranes.

To understand further the role of PhGPx in oxidative stress, we proposed to develop tools that can decrease PhGPx expression. Since there are no known specific chemical inhibitors of PhGPx that can effectively block the activity of this enzyme, antisense oligos seemed to be a useful approach. Antisense oligos are short sequences of DNA designed to inhibit the gene expression of a specific protein [9]. They hybridize to target RNA to form a DNA–RNA duplex and prevent the translation of the specific protein. They can also enter the nucleus and prevent transcription or transport from the nucleus. Antisense oligos offer the specificity of the genetic code and versatility of targeting any number of proteins.

The purpose of this research was to develop antisense oligos specific for PhGPx and test the effects of inhibition of PhGPx on the response of cells to an oxidative stress that targets membranes. We chose P4 cells, a cell line derived from MCF-7 cells with stable transfection of L-PhGPx sense cDNA. We hypothesized that PhGPx antisense oligos would block PhGPx production and sensitize cells to PDA-induced singlet oxygen toxicity. Our results indicate that our antisense oligo decreased L-PhGPx protein and activity levels to near WT levels; cells with PhGPx inhibition had more membrane damage and a higher level of lipid hydroperoxides when exposed to $^1O_2$. Thus, antisense techniques can be used to decrease the production of PhGPx and thereby sensitize cells to PDA. Antisense PhGPx could be an important avenue for treatment of disease, such as cancer.

Materials and methods

Reagents

Trypan blue was obtained from Fisher Scientific (Pittsburgh, PA). The primary polyclonal antibody against rat PhGPx was a gift from Dr. Qitao Ran (UT Health Science Center, San Antonio, TX). Horseradish peroxidase conjugated to goat anti-rabbit IgG and blocking reagents were purchased from Boehringer Mannheim (Indianapolis, IN). L-α-phosphatidyl-choline type III/S, lipoxidase type IV, sodium deoxycholate, glutathione (GSH), glutathione reductase (GR), NADPH, Triton-X-100 (peroxide-free), phosphatidylcholine, ethylenediaminetetraacetate (EDTA), sodium azide, sodium selenite, and ethidium bromide were purchased from Sigma (St. Louis, MO). Lipofectin, Eagle's minimum essential medium (MEM), fetal bovine serum (FBS), Hanks' buffer, Geneticin (G418), and Tris–HCl were purchased from Gibco (Grand Island, NY). Photofrin (porfimer sodium) was a gift from QLT Phototherapeutics (Vancouver, British Columbia, Canada). A stock solution was made in 5% dextrose (pH 7.4), sterile filtered (0.22 μm), and stored at −20 °C. When needed, metal-free buffers were prepared as outlined in [10].

Cell culture

MCF-7 cells (WT) were purchased from American Type Culture Collection (ATCC). The human-PhGPx overexpressing MCF-7 cells (P4) were established by Wang et al. [4]. Vector control cells (Neo), with the same PhGPx level as MCF-7 WT, were established by transfecting vector control plasmids into MCF-7 WT, which served as transfected controls. All cells were cultured in Eagle's minimum essential medium (MEM) with 1% non-essential amino acids, 10% fetal bovine serum (FBS), and 30 nM sodium selenite. P4 cells were grown in the above medium with antibiotic G418 (350 μg/mg). The antibiotic was removed one passage before an experiment. All cells were routinely incubated at 37 °C with 95% air and 5% CO_2 in a humidified incubator. In all cases, media were generally changed every 3–4 days. Cells were changed to fresh media 24 h before harvesting for the experiments.

Antisense oligos for L-PhGPx and controls

We chose to build 20-base long antisense oligos that included the transcriptional start codon [9]. The 37-base sequence that has the human-L-PhGPx start codon is [12]:

5′ CTCCCAGCCCCGCCGCG**ATG**AGCCTCGGC CGCCTTTG 3′

Three sequences were considered as L-PhGPx antisense oligos. They were:

$Anti_1$	5′ GCCGAGGCT<u>CAT</u>CGCGGCGG 3′
$Anti_2$	5′ CAAAGGCGGCCGAGGCT<u>CAT</u> 3′
$Anti_3$	5′ <u>CAT</u>CGCGGCGGGGCTGGGAG 3′

The marked **CAT** is a reflection of the transcription start site. Because there are four Gs in a row in $Anti_3$ and the G residue itself may target hybridization to mRNA, for economy, we only tested $Anti_1$ and $Anti_2$.

Mismatch, scrambled, and sense sequences were also designed for controls. Comparing these to $Anti_2$, the oligo sequences were:

$Anti_2$	5′ CAAAGGCGGCCGAGG CTCAT3′
Mismatch control	5′ CGACAACAACCAATG CTTAT3′
Scrambled control	5′ ACGTGCTACCGAGCA CGGAG3′
Sense control	5′ ATGAGCCTCGGCCGC CTTTG3′

Antisense oligo transfection

Cells were seeded in 100 mm dishes and allowed to reach 50–60% confluence. Twenty-four hours later, the cells were washed twice with serum-free MEM. Then, the Lipofectin plus antisense oligo mixture was added to the cells and incubated for 6 h. For 1 μM oligo, 8 μM Lipofectin was added; and for 10 μM oligo, 16 μM Lipofectin was added. Serum-free MEM was used in all procedures. After a 6 h incubation, the media were changed back to complete media. Forty-eight hours later, cells were harvested on ice using a rubber scraper or exposed to Photofrin.

Cell homogenization

The cell homogenization process was performed on ice to prevent denaturation of the antioxidant enzymes. Eighty percent confluent cells in 100-mm culture dishes were washed twice with phosphate-buffered saline (PBS, pH 7.8). Cells were harvested using a scraper. Cells were then centrifuged at 1000 rpm for 5 min at 4 °C and the supernatant was removed. The cell pellet was resuspended in 0.05 M phosphate buffer (PB, pH 7.8) and sonicated with four bursts of 30 s, using a Vibra Cell Sonicator (Sonics, Materials, Danbury, CN) with a cup horn at full power. The concentration of total cellular protein was determined with the Bio-Rad Protein assay kit (Bio-Rad Laboratories, Hercules, CA) using IgG as standard. Total cellular protein was used for activity assays and Western blotting analysis.

Western blot for PhGPx

Total denatured protein (200 μg) was separated on a 12.5% SDS–PAGE and electrotransferred onto a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). The membrane was blocked in 10% non-fat milk for 1 h at room temperature, followed by incubation with rabbit anti-rat PhGPx polyclonal antibody (1:200) overnight at 4 °C. After washing three times with TTBS (0.02 M Tris–HCl buffer, pH 7.5, 0.137 M NaCl, and 0.1% Tween 20), the membrane was incubated with horseradish peroxidase conjugated to goat anti-rabbit IgG (1:10,000). Detection by the chemiluminescence reaction was carried out using the enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech, Piscataway, NJ), followed by exposure to Kodak X-ray film.

Activity assay for PhGPx

The substrate, phosphatidylcholine hydroperoxides (PCOOHs) of PhGPx needed for the activity assay, was prepared by enzymatic hydroperoxidation of phosphatidylcholine using soybean lipoxidase type IV [4,13,14]. The activity was measured by a coupled enzymatic assay using GSH, glutathione reductase (GR), PCOOH, and NADPH with some modifications [4,13].

Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction on total RNA was accomplished exactly as detailed by Wang et al. [4].

Photofrin photosensitization

Forty-eighty hours after PhGPx antisense oligo transfection, 2×10^5 cells were seeded into 6-well plates and exposed to 6 μg/mL Photofrin in Hanks' buffer for 45 min. Cells were then irradiated with visible light (2.2 mW cm^{-2}) for 0, 5, 10, and 15 min. After Photofrin and light treatment, cells were allowed to recover for 6 h in full medium at 37 °C, 5% CO_2, and 95% air in the dark.

Trypan blue dye exclusion assay

After Photofrin and light exposure, cells were incubated for 6 h in the dark and then cells were trysinized and incubated with 0.2% trypan blue for 2 min at room temperature. Cells excluding dye and cells stained with trypan blue were counted with a hematocytometer. Cell membrane permeability was expressed as the percentage of cells excluding the dye.

Lipid hydroperoxide assay

The steady-state level of cellular LOOHs was determined using the Lipid Hydroperoxide Assay Kit (Cayman Chemical, Ann Arbor, MI). After transfection with PhGPx antisense oligo (1 μM, 48 h), cells were exposed to Photofrin and light for 10 min and then incubated for 6 h in full medium in the dark at 37 °C. Then, cells were washed with metal-free PBS (pH 7.8) and harvested and

sonicated in metal-free phosphate buffer (pH 7.8). Cellular lipids were extracted with 1.5 mL deoxygenated (N_2 for 45 min) chloroform:methanol (2:1, v/v) mixture by centrifuging at 5000 rpm for 5 min at 4 °C. Ferrous iron was added to the chloroform layer and the formation of ferric thiocyanate was measured at 500 nm on a Beckman DU-70 spectrophotometer. 13-Hydroperoxyloctadecadienoic acid was used as the standard. The amount of LOOHs was normalized to total cellular protein.

Results and discussion

Effective L-PhGPx antisense oligo sequence, concentration, and time

Antisense oligos are short sequences of DNA designed to inhibit the gene expression of a specific protein [9]. We tested two L-PhGPx antisense oligos to find the most effective one. We used these two antisense oligo sequences at concentrations of 0.1, 0.5, 1, and 10 μM. PhGPx activity was measured at 24, 48, and 72 h after antisense transfection. The PhGPx activity had no significant change after $Anti_1$ delivery, at all concentrations and at all time points measured (data not shown). $Anti_2$ decreased the PhGPx activity to about 25% of the initial activity of P4 cells, Fig. 1. Concentrations of $Anti_2$ at 1 and 10 μM were effective; these concentrations of $Anti_2$ suppressed PhGPx activity maximally at 48 h. There was no suppression at 24 h; at 72 h there was a partial return of activity to normal levels. Thus, we chose to use $Anti_2$ at a concentration of 1 μM and 48 h after antisense treatment to examine endpoints in the rest of the experiments.

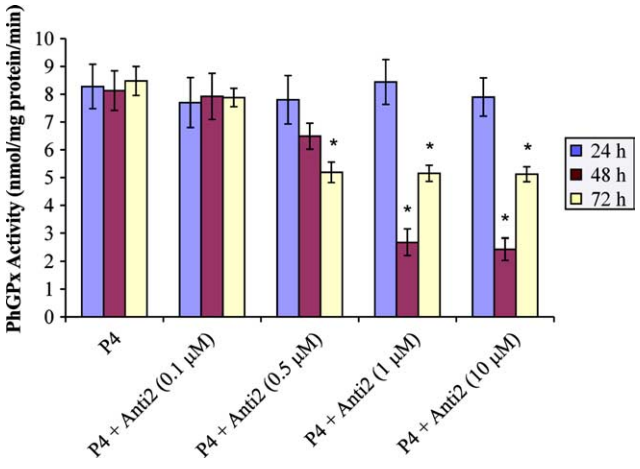

Fig. 1. $Anti_2$ Oligo inhibits L-PhGPx activity. PhGPx-overexpressing MCF-7 cells (P4) were transfected with 0.1, 0.5, 1 or 10 μM antisense oligo. At 24, 48 or 72 h post-transfection, cells were harvested and the total cellular protein was assayed for PhGPx activity. Results are means ± SE, $n = 3$, $^*p < 0.05$ compared to P4 cells at the same time. The ANOVA–Tukey test was used by Microsoft Excel program (Excel 97 SR-1) to compare the difference between two groups.

$Anti_2$ lowers L-PhGPx levels

Antisense oligos can disrupt gene expression and/or mRNA processing. Thus, to test if L-PhGPx antisense oligo inhibits the gene expression of L-PhGPx in P4 cells, we measured mRNA levels by RT-PCR, protein by Western blotting analysis, and PhGPx activity by enzymatic assay. As shown in Figs. 2A and B, PhGPx protein levels and activity were each decreased to about 25% of that of P4 cells, reverting these levels to near those of wild-type MCF-7 cells. After $Anti_2$ transfection, the mRNA level for L-PhGPx also decreased from the untreated levels, Fig. 2C. Note that mRNA levels for L-PhGPx in WT and Neo cells were below the limit of detection. These results demonstrate that $Anti_2$ acts by disrupting gene expression, but because the reduction in mRNA for L-PhGPx is to the same extent as the enzymatic activity as well as the reduction in PhGPx- protein, $Anti_2$ must also disrupt some other aspect of L-PhGPx, such as mRNA processing.

Inhibition of L-PhGPx increases $^1O_2$-induced membrane permeability in P4 cells

We next asked the question: will $Anti_2$ actually alter a biological response to oxidative stress? Because PhGPx removes LOOH in cells, we tested whether cells that had PhGPx inhibited by $Anti_2$ would have more membrane damage upon exposure to $^1O_2$. We treated P4 cells with $Anti_2$ and examined membrane permeability by trypan blue dye exclusion after treatment with photosensitizer and light. As shown in Fig. 3, cell membrane permeability was increased in P4 cells treated with $Anti_2$; in fact, the susceptibility to membrane damage was reversed to that of wild-type, MCF-7 cells. These data clearly show that the experimental results fall into two categories. All cells that have L-PhGPx overexpression are protected (upper group). All cells that do not have L-PhPGx, which includes P4 cells treated with $Anti_2$, show greater sensitivity to singlet oxygen exposure (lower group). Thus, these experiments suggest that $anti_2$ oligo treatment sensitized P4 cells to singlet oxygen and LOOH formation.

Suppression of L-PhGPx expression decreased repair of $^1O_2$-induced lipid hydroperoxides in MCF-7 cells

LOOH produced during lipid peroxidation can accumulate in cell membranes [8]. We hypothesized that cells with decreased L-PhGPx levels would more slowly remove these LOOHs. To test this hypothesis, cells with antisense treatment were incubated with Photofrin and then illuminated with visible light to produce LOOHs in

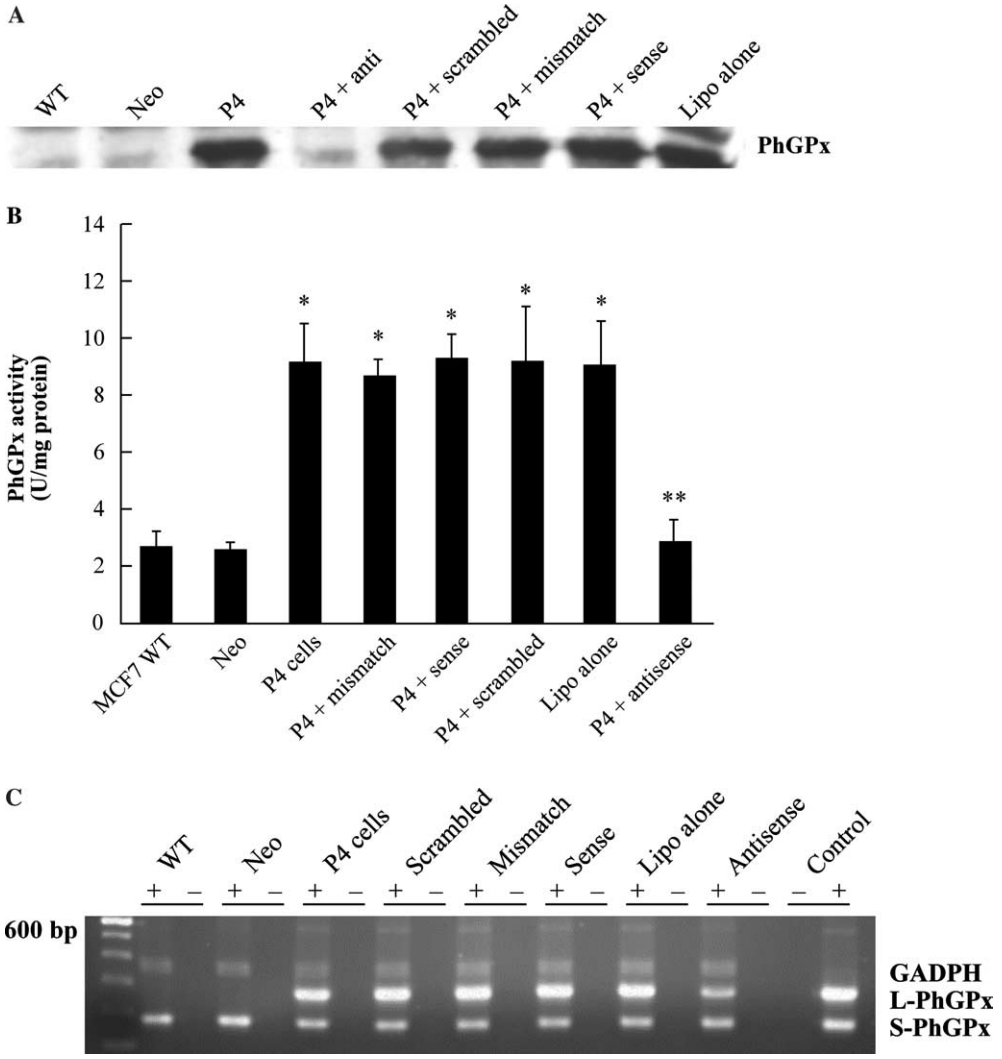

Fig. 2. $Anti_2$ Oligo inhibits the production of L-PhGPx at the protein, activity, and mRNA levels. Cells were transfected with 1 μM $Anti_2$. After 48 h, cells were harvested for PhGPx Western blot, activity assay, and RT-PCR. (A) Western blot for PhGPx. Protein (200 μg in each line) was separated on a 12.5% SDS–polyacrylamide gel. Decreased expression of PhGPx protein is observed in the ~20 kDa band in P4 cells treated with $Anti_2$. The blot was repeated with similar results. (B) Activity assay for PhGPx. Total cellular protein was assayed for PhGPx activity. $Anti_2$ reduces PhGPx activity in P4 cells to those of WT and Neo, whereas the control oligos had no effect on activity. Results are means ± SE, $n = 3$, $^*p < 0.05$ compared to MCF-7 WT and Neo, $^{**}p < 0.05$ compared to P4 cells. (C) RT-PCR for PhGPx. The upper bands are the GAPDH mRNA as RT-PCR loading control. The middle bands correspond to L-PhGPx mRNA. The lower bands indicate S-PhGPx mRNA. The gel experiment was repeated with similar results. "+" means cDNA template with primers. "−" means no addition of reverse transcriptase as RT controls for individual cells. Control (+): pure PhGPx cDNA plasmid was amplified in the PCR mixture. Control (−): the PCR mixture was amplified without template cDNA.

cell membranes. Fig. 4 shows that after Photofrin and light exposure, the antisense-treated P4 cells had a significantly higher level of LOOH than those without treatment. Because the predominant form of PhGPx in MCF7 WT is S-PhGPx and $Anti_2$ does not influence S-PhGPx (Fig. 2), we would expect $Anti_2$ to have no effect on Neo or WT cells. Also, we would expect P4 cells treated with $Anti_2$ to behave much like WT cells. Indeed the levels of LOOH observed with $Anti_2$ in P4 cells are identical to those seen in WT and Neo cells with or without $Anti_2$. Thus, $Anti_2$ is effective in blunting the activity of L-PhGPx as monitored by removal of LOOH.

Lipid peroxidation causes damage to cell membranes. PhGPx prevents this damage by reducing phospholipid hydroperoxides in cellular membranes to their corresponding alcohols [11]. We tested the hypothesis that inhibition of PhGPx activity by antisense oligos can sensitize cells to LOOH. We and others have shown that singlet oxygen production by Photofrin and light results in LOOH formation in cell membranes [4,6–8]. Thus, we used singlet oxygen as a tool to introduce LOOH into cell membranes by a non-free radical mechanism. Antisense oligos are short sequences of DNA that can enter cells and cause inhibition of synthesis of a specific protein by action at several possible sites, including

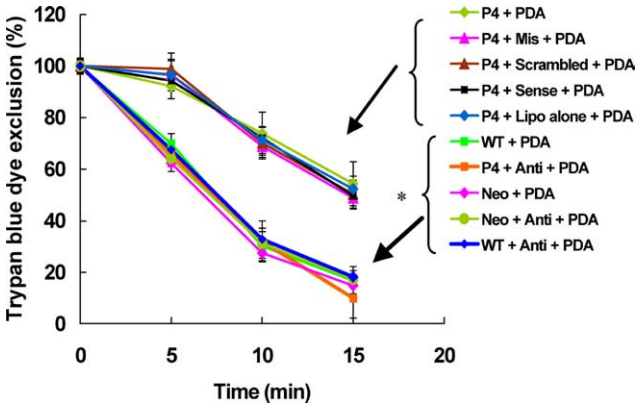

Fig. 3. Inhibition of L-PhGPx increases $^1O_2$-induced membrane permeability. Membrane permeability was assayed by trypan blue dye exclusion. After PhGPx transfection by $Anti_2$, cells were exposed to Photofrin in Hanks' buffer (45 min) and then irradiated with visible light (10 min). After light exposure, cells were incubated for 6 h in full medium in the dark at 37 °C to allow for damage repair. Cells were then trysinized and assayed for membrane permeability. The cell membrane permeability is expressed as the percentage of cells that excluded the dye. Results are means ± SE, $n = 3$, the results of each experiment in the upper group were significantly different from each experiment in the lower group, $^*p < 0.05$. After treatment with $Anti_2$, P4 cells showed the same survival as WT.

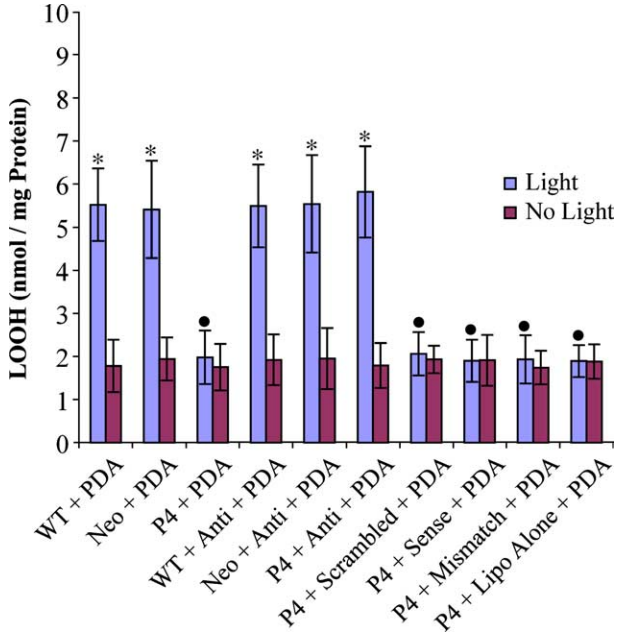

Fig. 4. Suppression of L-PhGPx decreased $^1O_2$-induced repair of lipid hydroperoxides in P4 cells. After PhGPx antisense oligo (1 μM) transfection for 48 h, cells were exposed to 6 μg/mL Photofrin in Hanks' buffer for 45 min and then irradiated with visible light (10 min). After light exposure, cells were allowed for another 6 h in full medium in the dark at 37 °C. Then, cellular lipids were extracted with chloroform:methanol (2:1, v/v). The chloroform layer was used for LOOH determination using the Cayman Lipid Hydroperoxide Assay Kit. Total LOOHs were normalized to total cellular protein. Results are expressed as means ± SE, $n = 3$, $^*p < 0.05$ compared the group with and without light, $^\bullet p < 0.05$ compared among the groups with light.

transcription and post-transcriptional levels [9]. In this study, we used PhGPx-overexpressing MCF-7 cells to test the efficacy of antisense oligos for PhGPx and to study the effect of L-PhGPx. These PhGPx-stably transfected MCF-7 cells (P4) have been characterized by Wang et al. [4]. P4 cells have a much higher level (4-fold) of PhGPx expression than WT MCF-7 cells. We developed an effective L-PhGPx antisense oligo sequence and treatment protocol to inhibit L-PhGPx production in P4 cells. RT-PCR also showed that L-PhGPx inhibition by $Anti_2$ does not interfere with S-PhGPx expression. By examining trypan blue exclusion, we demonstrated that $Anti_2$ caused P4 cells to revert to the susceptibility to LOOH of wild-type cells.

In summary, this work clearly demonstrates that:

1. By targeting the first transcription start site of PhGPx we can effectively block expression of L-PhGPx mRNA and PhGPx protein with appropriate antisense oligos.
2. As seen by PCR, our most effective antisense oligos for L-PhGPx did not interfere with S-PhGPx expression.
3. The levels of suppression of PhGPx, as seen by examining protein or measuring activity, are consistent. (Note that because L-PhGPx protein is converted to the same protein as S-PhPGx by cleavage of the mitochondrial target sequence once it enters the mitochondria, it is not possible to distinguish these proteins by Western blotting or activity assays.)
4. Our most effective antisense oligos are able to suppress PhGPx protein and activity levels to those of WT cells; this in conjunction with the PCR results implies that our antisense oligos are nearly 100% effective.
5. PhGPx protects cells against the toxicity of lipid hydroperoxides.
6. Using PCR, MCF-7 cells (cells of somatic tissue) only express S-PhGPx. (Choosing these cells to over-express L-PhGPx allowed us to clearly see the effect of L-PhGPx expression/suppression without interference from endogenous L-PhGPx.)
7. Among the possible mechanisms by which antisense oligos can work, we demonstrated that our most effective antisense oligos block at both the mRNA and protein levels.

We hope this approach will be useful as there are no specific chemical inhibitors of PhGPx. We conclude that antisense L-PhGPx oligos can effectively block the production of cellular PhGPx enzyme and sensitize cells to oxidative stress, such as those induced by singlet oxygen produced by photodynamic action.

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