1-cys peroxiredixin (1-cysPrx), a member of the peroxiredxin superfamily, reduces phospholipid hydroperoxides as well as organic peroxides and \( \text{H}_2\text{O}_2 \). To determine the physiological function(s) of 1-cysPrx, we have used an antisense strategy to suppress endogenous 1-cysPrx in L2 cells, a rat lung epithelial cell line. A 25-base antisense morpholino oligonucleotide was designed to bind a complementary sequence overlapping the translational start site (+18 to +7) in the rat 1-cysPrx mRNA, blocking protein synthesis. Treatment with an antisense oligonucleotide for 48 h resulted in approximately 60% suppression of the 1-cysPrx protein content as measured by immunoblot analysis and an approximately 44% decrease of glutathione peroxidase activity as compared with random oligonucleotide treated cells. Accumulation of phosphatidylethanolamine hydroperoxide in plasma membranes was demonstrated by high pressure liquid chromatography assay for conjugated dienes (260 pmol/10^6 cells for antisense versus 70 pmol/10^6 cells for random oligonucleotide and control cells) and by fluorescence of diphenyl-1-pyrenylphosphine, a probe for lipid peroxidation. The percentage of cells showing positive staining for annexin V and propidium iodide after antisense treatment was 40% at 28 h and 80% at 48 h. TdT-mediated dUTP nick end labeling assay at 48 h indicated DNA fragmentation in antisense-treated cells that was blocked by prior infection with adenovirus encoding a fluorescein isothiocyanate (FITC) labeled fluorescein protein; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

The peroxiredoxins (Prx)\(^{1}\) constitute a recently described and widely distributed superfamily of nonselenium glutathione peroxidases. 1-cysPrx, also named peroxiredoxin VI, is a member of this superfamily that can reduce phospholipid hydroperoxides (PLOOH) as well as organic peroxides and \( \text{H}_2\text{O}_2 \). To determine the physiological function(s) of 1-cysPrx, we have used an antisense strategy to suppress endogenous 1-cysPrx in L2 cells, a rat lung epithelial cell line. A 25-base antisense morpholino oligonucleotide was designed to bind a complementary sequence overlapping the translational start site (+18 to +7) in the rat 1-cysPrx mRNA, blocking protein synthesis. Treatment with an antisense oligonucleotide for 48 h resulted in approximately 60% suppression of the 1-cysPrx protein content as measured by immunoblot analysis and an approximately 44% decrease of glutathione peroxidase activity as compared with random oligonucleotide treated cells. Accumulation of phosphatidylethanolamine hydroperoxide in plasma membranes was demonstrated by high pressure liquid chromatography assay for conjugated dienes (260 pmol/10^6 cells for antisense versus 70 pmol/10^6 cells for random oligonucleotide and control cells) and by fluorescence of diphenyl-1-pyrenylphosphine, a probe for lipid peroxidation. The percentage of cells showing positive staining for annexin V and propidium iodide after antisense treatment was 40% at 28 h and 80% at 48 h. TdT-mediated dUTP nick end labeling assay at 48 h indicated DNA fragmentation in antisense-treated cells that was blocked by prior infection with adenovirus encoding a fluorescein isothiocyanate (FITC) labeled fluorescein protein; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

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An Antisense Oligonucleotide to 1-cys Peroxiredoxin Causes Lipid Peroxidation and Apoptosis in Lung Epithelial Cells*
EXPERIMENTAL PROCEDURES

**Materials**—Glutathione, glutathione reductase, NADPH, tert-butyl hydroperoxide, and H$_2$O$_2$ were purchased from Sigma. All cell culture medium components were from Invitrogen. Trolox, a water-soluble form of vitamin E, and acrylamide glycerylphosphocholine (DPPP) was purchased from Difco Laboratories. The plasmids containing the insert of 1-cysPrx were verified by sequencing, as described previously (2).

**Construction of a Recombinant Adenovirus Encoding Rat 1-cysPrx**—The assembly and production of recombinant adenovirus was carried out by GeneTools, LLC. The plasmid DNA from transformed colonies was isolated for the presence of the insert DNA and its orientation by restriction enzyme digestion. The plasmids containing the insert of 1-cysPrx were verified by sequencing, as described previously (2).

**Oligonucleotide Treatment**—A 25-base morpholinoo oligonucleotide (5'-CCGGCATGCGGCGGATGATCAGGG-3') complementary to 18 nucleotides was introduced into the upstream primer (5'-CCTCTTACCTCAGTTA-GAGGGCTGCTT-3') for the production of a 675-bp PCR product. Purified 675-bp PCR products were double digested with KpnI and I-CeuI and ligated to the similarly digested pShuttle mammalian expression vector, and transformed into competent Escherichia coli. The plasmids containing the insert of 1-cysPrx were verified by sequencing, as described previously (2).

**Northern Blot Analysis**—Total RNA was isolated from adenovirus-infected cells using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA (5 μg) was separated by electrophoresis on a 1% agarose gel containing formaldehyde. The size-fractionated RNA was then transferred onto a nylon membrane (Schleicher & Schuell) by capillary action and hybridized to 32P-labeled rat 1-cysPrx cDNA probe generated by random priming (Amersham Biosciences). After a high stringency wash, the membrane was exposed to Kodak x-ray film with an intensifying screen at −70 °C for 14 h and quantitated as described above for immunoblots. To normalize for loading, the membrane was stripped of the 1-cysPrx probe, rehybridized with 32P-labeled glyceraldehyde-3-phosphate dehydrogenase cDNA probe and re-exposed to x-ray film.

**Enzyme Assay**—GPx activity was determined by coupled NADPH/glutathione reductase assay in the presence of GSH with PCOHO or tert-butyl hydroperoxide, and lysozyme as a substrate, as described previously (3). The enzyme reaction was measured at pH 4 with a liposome-based assay using radiochemical detection as described previously (3).

**Analysis of Phospholipids by HPLC**—L2 cells from the three experimental conditions (control, antisense and random oligonucleotide-treated) that were adherent to 60-mm culture dishes were extracted by the Bligh and Dyer procedure (25). The extract was dried under a stream of N$_2$, and the pellet of phospholipids was redissolved in 50 μl of methanol for analysis by HPLC (Waters, Milford, MA). The samples were injected onto a reverse phase high resolution C$_{18}$ column (3.9 × 150 mm) (NovaPack), eluted isocratically with methanol, 50 mM ammonium acetate (60:40 v/v, pH 5.0) at a flow rate of 1 ml/min using an Alliance 2690 separation unit, and detected with PDA 996 UV and RI 442 detectors. The data were processed using Millenium$^2$ software. Phosphatidylcholine hydroperoxide (PCOHO) was detected by absorbance of conjugated dienes at 234 nm. A PCOHO standard was prepared using 15-lipoxygenase (Cayman Chemical Company Inc., Ann Arbor, MI) and 1-palmitoyl-2-linolenoyl-sn-glycero-3-phosphocholine (Sigma) as reported previously (2).

**Phospholipid Hydroperoxide Detection in Real Time**—DPPP was used as a membrane-localized fluorescent probe that reacts specifically with hydroperoxides and becomes highly fluorescent when oxidized (26). Control, antisense and random oligonucleotide-treated L2 cells adherent to 12 × 25-mm plastic slides (Aclar; Allied Signal, Morris Plains, NJ) for 24 h in culture dishes of two identical sets of dishes (i.e. control, antisense and random oligonucleotide treatment) were then extracted and quantitated using the FluorS multi-imager (Bio-Rad). Supernatant containing 30 μg of protein was mixed with 2× sample buffer, boiled for 3 min, and applied to a 12% SDS-PAGE gel. After electrophoresis, the proteins were transferred onto nitrocellulose membrane. The blot was probed with 1:3000 diluted rabbit anti-1-cysPrx (27) and then incubated with 1:5000 diluted horseradish-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratory, West Grove, PA) with detection by ECL and quantitated by densitometric scanning of x-ray film using the FluorS multi-imager (Bio-Rad). The membrane then was washed with stripping solution (0.25 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol, 2% SDS) at 55 °C and reprobed with 1:500 diluted rabbit anti-actin polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to normalize for protein loading.

**Detection of Cell Damage**—To detect the early stages of injury, translocation of phosphatidylserine in the cell membrane was examined by staining with annexin V according to the manufacturer's instructions (ClonTech, Palo Alto, CA). At 20, or 28, h after antisense or random morpholino oligonucleotide treatment, the cells were labeled in situ with annexin V-FITC and propidium iodide (PI), and six randomly selected fields were counted for fluorescent cells using an Nikon Diaphot.
mixture containing the terminal deoxynucleotidyl transferase. 4
midino-2-phenylindole dihydrochloride (DAPI, 300 nM) was used to
higher fluorescence in the L2 cells
1-cysPrx antisense oligonucleotide, which showed a 5-fold
effect on 1-cysPrx protein expression in the primarily isolated
cells. The reason for the difference
immunoreactive
in the primary isolated cells.
Enzymatic activities were evaluated in crude cell extracts
tube-treated L2 cells showed no change in immunoreactive
proteins. The values for L2 cells are indicated as percentages of the
corresponding value in type II cells (100%).

DNA cleavage, a characteristic of apoptosis, was detected by the
TdT-mediated nick end labeling (TUNEL) assay as described by
the manufacturer (Roche Molecular Biochemicals). At 5, 28, or 48 h
after antisense or random oligonucleotide treatment, L2 cells cultured
on two chamber slides were washed twice with PBS and fixed with 4%
paraformaldehyde in PBS at room temperature for 20 min. The fixed
cells were permeabilized with 0.1% Triton X-100, 0.1% sodium citrate at
4°C for 2 min and incubated for 1 h at 37°C with TUNEL reaction
mixture containing the terminal deoxynucleotidyl transferase. 4′-Diamino-
di-2-phenylindole dihydrochloride (DAPI, 300 nm) was used to
counterstain double-stranded DNA in nuclei after TUNEL staining.

The cells were air-dried, and coverslips were applied with 3 µl of Mowiol
(Calbiochem, San Diego, CA). Images of nuclear fluorescence typical of
apoptotic cells were collected by fluorescence microscopy as described
above.

Data Analysis—The data are expressed as the means ± S.E. Statisti-
cal significance was evaluated by Mann Whitney t test or one-way
analysis of variance, followed by comparison of the treated versus
untreated by the Bonferroni procedure using SigmaStat software (Jandel,
San Rafael, CA). The level of significance was taken as p < 0.05.

RESULTS

Cell Growth—L2 cells treated with antisense oligonucleotide
grew poorly compared with control and random oligonucleo-
tide-treated cells and exhibited a round cell shape by micros-
copy with a high rate of detachment from the dishes (data not
shown). Cellular morphology and cell attachment in antisense
oligonucleotide-treated cells was improved significantly by the
addition of Trolox to the culture medium.

Effect of Antisense Oligonucleotide on 1-cysPrx Expression—
Control L2 cells expressed 1-cysPrx at ~35% of the level ob-
erved in primarily isolated rat type II alveolar epithelial cells
after 24 h in culture as determined by Northern and immuno-
bLOTS (Fig. 1). Control (vehicle only) and random oligonucleo-
tide-treated L2 cells showed no change in immunoreactive
1-cysPrx during 48 h of culture (Fig. 2). By contrast, treatment
of L2 cells with the antisense oligonucleotide for 48 h decreased
the immunodetectable 1-cysPrx protein level by ~60% (Fig.
2A). Antisense oligonucleotide treatment had no significant
effect on 1-cysPrx protein expression in the primarily isolated
rat type II cells (data not shown). The reason for the difference
in efficacy was evaluated by the use of fluorescein-labeled
1-cysPrx antisense oligonucleotide, which showed a 5-fold
higher fluorescence in the L2 cells versus type II alveolar
epithelial cells (data not shown), indicating resistance to transfe-
cion in the primarily isolated cells.

Enzymatic activities were evaluated in crude cell extracts
from vehicle only (control) and antisense and random oligonu-
clide-treated L2 cells at 48 h of culture. Antisense oligonu-
clide-treated cells demonstrated decreased activity by 44% for GPx
and 48% for PLA2 relative to control, whereas random oligonu-
clide had no effect on activities (Fig. 2B).

Detection of Cellular Phospholipid Peroxidation—Analysis of
lipid extracts of antisense and random oligonucleotide-treated
L2 cells by HPLC showed similar levels of PCOOH at the start
of incubation (Fig. 3A). After 48 h of culture, antisense oligonu-
clide-treated cells showed a marked increase in the phospholipid
hydroperoxide peak on the HPLC tracing, whereas the cor-
responding peak in random oligonucleotide-treated cells was
unchanged (Fig. 3A). The increase in PCOOH at 48 h of
culture was ~3.5-fold (p < 0.05) in antisense oligonucleotide
compared with random oligonucleotide-treated L2 cells (Fig.
3B). Accumulation of PCOOH in antisense oligonucleotide-
treated cells was significantly reduced by the addition of Trolox
to the culture medium (Fig. 3B). Lipid peroxidation was ana-
yzed further by use of the specific fluorescent probe, DPPP.
Control (vehicle only) L2 cells cultured for 48 h, then labeled
with DPPP, and analyzed in a fluorometer showed low level
fluorescence emission with a peak at 380 nm (Fig. 4A). Random
oligonucleotide-treated cells showed a similar level of DPPP
fluorescence. There was approximately four times more oxidi-
dized DPPP fluorescence in antisense oligonucleotide-treated
L2 cells after 48 h of incubation as compared with random
oligonucleotide-treated and control cells (Fig. 4A). These re-
results indicate lipid peroxidation of antisense oligonucleotide-
treated cells during growth in normal culture medium.
The Cu²⁺-Asc chemical reaction that results in the generation of OH (27) and DPPP were used to evaluate lipid peroxidation in antisense oligonucleotide-treated cells in response to oxidative stress. DPPP fluorescence of cells was normalized to background fluorescence before the Cu²⁺-Asc treatment. Cu²⁺-Asc treatment of L2 cells showed an instantaneous increase in DPPP fluorescence with a rate of fluorescence change that was ~60% greater in antisense oligonucleotide-treated cells as compared with that of random oligonucleotide-treated or vehicle only (control) cells (Fig. 4B). This suggests that antisense oligonucleotide-treated cells with decreased 1-cysPrx have a diminished capacity to reduce plasma membrane phospholipid.

Fig. 3. HPLC detection of PCOOH accumulation in antisense- and random-treated L2 cells. A, HPLC traces of lipid extracts of antisense and random oligonucleotide-treated L2 cells before and 48 h after treatment. Absorbance values for corresponding peaks were recalculated into concentration using Beer’s law (ε = 23,000 M⁻¹ cm⁻¹ at 234 nm). B, cellular PCOOH content for antisense and random oligonucleotide-treated cells with or without addition of Trolox. The values are the means ± S.E. for at least three independent experiments. *, p < 0.05 compared with zero time; #, p < 0.05 for Trolox compared with the corresponding condition without Trolox.
hydroperoxides generated by oxidant stress.

**Detection of Cell Injury**—To determine whether antisense oligonucleotide treatment resulted in translocation of phosphatidylserine to the outer leaf of the plasma membrane, we examined cellular binding of annexin V as an index of perturbation of the membrane bilayer. PI staining was used as a marker of increased plasma membrane permeability. Approximately 10% of cells showed annexin V and PI fluorescence at zero time (prior to treatment with oligonucleotide) (Fig. 5A). No change in annexin V binding or PI uptake was seen at 5 h of incubation, but a significant increase in annexin V- and PI-labeled cells (40%) was detected at 28 h after antisense treatment with a further increase to 80% at 48 h (Fig. 5). In addition, there was a marked decrease in the number of cells that remained attached to the plate as shown in the phase contrast image (Fig. 5B). By contrast, control (vehicle only; not shown) or random oligonucleotide-treated cells did not show any increase in annexin V- or PI-labeled cells during the 48-h incubation, and the number of attached cells did not appear to be affected significantly (Fig. 5).

The development of apoptosis was assessed by DNA fragmentation through TUNEL assay. The appearance of fluorescence in the nuclei of antisense-treated cells shows evidence of apoptosis at 48 h, whereas only minimal DNA fragmentation was observed in cells treated with random oligonucleotide (Fig. 6) or in control (vehicle only) cells (not shown). There is a marked decrease in the number of antisense oligonucleotide-treated cells on the culture dish compared with the random oligonucleotide-treated cells as shown by phase contrast and DAPI staining (Fig. 6). Pretreatment with Trolox prevented the development of apoptosis and cell loss in antisense oligonucleotide-treated cells (Fig. 6).

**Ad.1-cysPrx-infected L2 Cells**—To further investigate whether the decrease in 1-cysPrx expression was linked to apoptosis, 1-cysPrx was overexpressed in cells by adenovirus-mediated transfection. The efficiency of L2 cell infection with adenovirus was examined with Ad.GFP. At a multiplicity of infection of 2, 5, or 10 plaque forming units/cell, transfection efficiency as determined by fluorescence microscopy was 50, 80, and 98% at 24 h post-infection, respectively (data not shown). The cells were then treated with adenovirus harboring the coding region of 1-cysPrx cDNA. Northern blot analysis of total RNA using a full-length 1-cysPrx cDNA probe revealed the presence of a more rapidly migrating 1-cysPrx message only in Ad.1-cysPrx-infected L2 cells and not in Ad.GFP-infected or noninfected (control) L2 cells (Fig. 7A). This truncated message (about 0.7 kb) is consistent with the vector design of Ad.1-cysPrx, which does not contain the 3’-untranslated region. Immunoblot analysis showed increased expression of...
1-cysPrx protein in cells infected with Ad.1-cysPrx, confirming that the 1-cysPrx mRNA detected by Northern hybridization was correctly translated in the cells (Fig. 7B). The 1-cysPrx protein content was not increased by infection of cells with Ad.GFP.

To study whether 1-cysPrx overexpression could “rescue” L2 cells from the effect of antisense treatment, cells were incubated with Ad.1-cysPrx at an multiplicity of infection of 8 plaque forming units/cell for 2 h prior to exposure to antisense or random oligonucleotide. For comparison, L2 cells were infected with the same multiplicity of infection of Ad.GFP or were left untreated (control). Immunoblot analysis of antisense oligonucleotide-treated L2 cells following infection with adenovirus encoding 1-cysPrx (original magnification, ×200).

1-cysPrx protein in cells infected with Ad.1-cysPrx, confirming that the 1-cysPrx mRNA detected by Northern hybridization was correctly translated in the cells (Fig. 7B). The 1-cysPrx protein content was not increased by infection of cells with Ad.GFP.

To study whether 1-cysPrx overexpression could “rescue” L2 cells from the effect of antisense treatment, cells were incubated with Ad.1-cysPrx at an multiplicity of infection of 8 plaque forming units/cell for 2 h prior to exposure to antisense or random oligonucleotides. For comparison, L2 cells were infected with the same multiplicity of infection of Ad.GFP or were left untreated (control). Immunoblot analysis of antisense oligonucleotide-treated L2 cells showed a significant increase in expression of 1-cysPrx at 48 h after infection with Ad.1-cysPrx to levels that were comparable with vehicle only and random oligonucleotide-treated cells that were not infected with adenovirus (Fig. 7C). Increases in 1-cysPrx expression after Ad.1-cysPrx infection also were observed in vehicle only and random oligonucleotide-treated cells (Fig. 7C). There was no change in 1-cysPrx expression compared with control in cells infected with Ad.GFP (Fig. 7C).

The effect of Ad.1-cysPrx infection on lipid peroxidation was determined by DPPP fluorescence. Control (noninfected) cells treated with antisense oligonucleotide showed a marked increase in DPPP fluorescence of the cell extracts compared with vehicle only and random oligonucleotide-treated cells (Figs. 4A and 8). Antisense oligonucleotide-treated L2 cells infected with Ad.1-cysPrx demonstrated levels of DPPP fluorescence that were similar to random oligonucleotide-treated cells (Fig. 8).

Both DNA fragmentation as measured by the TUNEL assay and loss of cells from the plate as shown by phase contrast and DAPI staining were attenuated in the antisense-treated cells in response to Ad.1-cysPrx infection (Fig. 6). Ad.1-cysPrx infection had no effect on the results of TUNEL assay in random oligonucleotide-treated cells (not shown). These results suggest that a certain level of 1-cysPrx is essential to protect L2 cells from apoptosis mediated by ROS generated during normal cellular metabolism.

**DISCUSSION**

Prx have been classified into two major groups, one containing a single conserved cysteine in the N-terminal region (1-cysPrx) and the other (2-cysPrx) containing an additional conserved cysteine near the C-terminal region, which is separated from the first cysteine by roughly 120 amino acids (28, 29). The presence or absence of the second cysteine is correlated with the consensus sequence neighboring the first cysteine: Pro-Val-Cys-Thr for 1-cysPrx and Phe-Val-Cys-Thr for 2-cysPrx. The
with control or random oligonucleotides, led to a significant reduction of 1-cysPtx expression and a proportional decrease in both peroxidase and PLA2 activities in L2 cells at 48 h post treatment. The decrease in the 1-cysPtx protein level was associated with diminished cell viability and increased cell membrane hydroperoxide content as indicated by HPLC analysis and DPPP fluorescence. The increased hydroperoxide content was most likely the result of decreased reduction of PLOOH formed in cellular membranes because of endogenous oxidants. To show that the presence of increased PLOOH perturbed the lipid bilayer, we used the annexin V-FITC test for phosphatidyl serine translocation to the outer leaf of the bilayer (37). Annexin V-FITC-associated fluorescence was detected after 28 h of incubation with antisense oligonucleotide, and nearly all cells were positive at 48 h. These cells also showed increased plasma membrane permeability by the PI test, indicating damage to cell membranes. A positive TUNEL assay indicating an apoptotic death was detected at 48 h of incubation but not at the earlier time point. These data suggest that peroxidative injury to the plasma membrane is followed by the development of apoptosis in antisense oligonucleotide-treated L2 cells incubated for 28−48 h in cell culture medium. Pretreatment of cells with Trolox, a soluble form of vitamin E, prevented both lipid peroxidation and apoptosis, providing evidence for their linkage. Ad.1-cysPtx infection restored 1-cysPtx expression to control levels and also prevented cellular apoptosis, confirming that the effects of antisense treatment were due to the decreased cellular expression of 1-cysPtx. Thus, 1-cysPtx appears to function as an important cellular antioxidant enzyme in this lung epithelial cell line.

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REFERENCES

1. Kang, S. W., Baines, I. C., and Rhee, S. G. (1998) J. Biol. Chem. 273, 6303−6311
2. Fisher, A. B., Dodia, C., Manevich, Y., Chen, J. W., and Feinstein, S. I. (1999) J. Biol. Chem. 274, 21326−21334
3. Chen, J. W., Dodia, C., Feinstein, S. I., Jain, M. K., and Fisher, A. B. (2000) J. Biol. Chem. 275, 28421−28427
4. Shiichi, H. A., and Demar, J. C. (1990) Exp. Eye Res. 50, 513−520
5. Singh, A. K., and Shiichi, H. A. (1998) J. Biol. Chem. 273, 26171−26178
6. Peshenko, I. V., Novoselov, V. I., Evdokimov, V. A., Nikolaev, Y. V., Shuravova, T. M., Lipkin, V. M., and Fesenko, E. E. (1996) FEBS Lett. 381, 12−14
7. Peshenko, I. V., Novoselov, V. I., Evdokimov, V. A., Nikolaev, Y. V., Kamalov, S. S., Shuravova, T. M., Lipkin, V. M., and Fesenko, E. E. (1998) Free Radic. Biol. Med. 25, 100−107
8. Kim, T. S., Dodia, C., Chen, X., Hennigan, B. B., Jain, M. K., Feinstein, S. I., and Fisher, A. B. (1998) Am. J. Physiol. 274, L750−L761
9. Akiba, S., Dodia, C., Chen, X., and Fisher, A. B. (1998) Comp. Biochem. Physiol. 120, 393−404
10. Seo, M. S., Kang, S. W., Kim, K., Baines, I. C., and Rhee, S. G. (2000) J. Biol. Chem. 275, 20346−20354
11. Peshenko, I. V., and Shiichi, H. (2001) Free Radical Biol. Med. 31, 292−303
12. Peshenko, I. V., Singh, A. K., and Shiichi, H. (2001) J. Ocucal Pharmacol. Therap. 17, 83−89
13. Marnett, L. J. (2000) Carcinogenesis 21, 361−370
14. Henegly, K., and Floyd, R. A. (2002) Arch. Biochem. Biophys. 397, 377−383
15. Jacobson, M. D. (1996) Trends Biochem. Sci. 21, 83−86
16. Droge, W. (2002) Physiol. Rev. 82, 47−95
17. Parassassi, T., Battillon-Go, B., Brunilli, R., Cazzolato, G., Krasnowska, E. K., Mei, G., Sevanian, A., and Ursini, F. (2001) Free Radical Biol. Med. 31, 63−89
18. van Knijn, F. J. G. M., Sevanian, A., Handelman, G. J., and Dratz, E. A. (1987) Trends Biochem. Sci. 12, 31−34
19. Roveri, A., Maiorino, M., and Ursini, F. (1994) Methods Enzymol. 233, 202−212
20. Wang, H. P., Qian, S. S., Schader, F. Q., Deman, F. C., Oberley, L. W., and Buettner, G. R. (2001) Free Radical Biol. Med. 30, 825−835
21. Hurst, R., Yongping, B., Jeneth, P., Mannervick, B., and Williamson, G. (1998) Biochem. J. 332, 97−109
22. Yang, Y., Cheng, J. Z., Singhal, S. S., Saini, M., Pandya, U., Awasthi, S., and Awasthi, Y. C. (2001) J. Biol. Chem. 276, 19220−19230
23. Manevich, Y., Sweitzer, T. D., Pak, J. H., Feinstein, S. I., Muzikantov, V. R., and Fisher, A. B. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 11599−11604
24. Kim, H. S., Pak, J. H., Gonzalez, L. W., Feinstein, S. I., and Fisher, A. B. (2002) Am. J. Respir. Cell Mol. Biol. 27, 227−233

Antisense Oligonucleotide to 1-cysPtx

FIG. 8. Measurement of lipid peroxidation in Ad. 1-cysPtx infected (+Ad.Ptx) and noninfected cells (Control) that were then treated with antisense or random oligonucleotide or vehicle only as described in the legend to Fig. 7. Lipid peroxidation was measured by incubation of cells with DPPP followed by extraction and assay of fluorescence. Fluorescence was normalized for total protein concentration in the cell extracts. Each data set represents the mean ± S.E. of three independent experiments. * p < 0.05 for corresponding value of random oligonucleotide-treatment. Adenovirus infection of control cells was not evaluated. # p < 0.05 for uninfected compared with the corresponding value for adenovirus-infected cells.

Prx are distinct from other peroxidases in that they do not utilize cofactors such as metals or prosthetic groups. Members of both groups of Prx exist as homodimers with two monomers oriented in a head-to-tail manner (30, 31). The 2-cysPrx, also referred to as thioredoxin peroxidases, reduce peroxides with electrons provided by thioredoxin (28, 29). 1-cysPrx, also named Prx VI, is capable of reducing peroxides in the presence of small thiols such as dithiobreitol (1, 2, 7) or glutathione (2, 3, 4, 6). We have recently demonstrated that depletion of glutathione diminishes activity of the enzyme in intact cells, indicating that it can serve as a physiological electron donor (23).

Recombinant human 1-cysPtx has peroxidase activity against H2O2, small organic hydroperoxides, fatty acid hydroperoxides, and PLOOH in vitro (2). The present study provides evidence that 1-cysPtx functions as an essential antioxidant enzyme to detoxify PLOOH as a result of ROS generated in intact cells. This result complements our previous demonstration that overexpression of 1-cysPtx in NCI-H441 cells protects against ‘OH-mediated phospholipid peroxidation (23). Additional evidence for an antioxidant role for 1-cysPtx is its induction in human umbilical vein endothelial cells by hydroperoxides (32), in rat lungs immediately after birth, (24, 33) and in kidney following intraperitoneal injection of chloroform (33). Thus, 1-cysPtx is induced by oxidative stress, and its expression appears to protect against oxidative-mediated cell injury.

We chose an antisense strategy to decrease 1-cysPtx expression in L2 cells, an epithelial cell line that was derived originally through clonal isolation from rat lung alveolar epithelial cells in primary culture (34). A morpholino antisense oligonucleotide, 25 bases in length, was designed to target the region overlapping the translation start site, thus inhibiting 1-cysPtx synthesis. This mechanism is different from the more commonly used phosphorothioate oligomers in which the RNA-DNA duplex that is formed becomes accessible to RNase H, leading to mRNA degradation. Advantages of morpholino-substituted oligonucleotides include complete resistance to nucleases, good targeting predictability, excellent sequence specificity, minimal nonspecific activity, and less toxicity (35, 36). A relatively minor disadvantage is that an effect of the antisense oligomer cannot be detected by Northern blot analysis.

Treatment with 1-cysPtx antisense oligonucleotides, but not
Antisense Oligonucleotide to 1-cysPrx

25. Bligh E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917
26. Takahashi, M., Shibata, M., and Niki, E. (2001) Free Radical Biol. Med. 31, 164–174
27. Manevich, Y., Held, K. D., and Biaglow, J. E. (1997) Radical Res. 148, 580–591
28. Rhee, S. G., Kang, S. W., Netto, L. E., Seo, M. S., and Stadtman, E. R. (1999) Biofactors 10, 207–209
29. Rhee, S. G., Kang, S. W., Chang, T. S., Jeong, W., and Kim, K. (2001) IUBMB Life 52, 35–41
30. Choi, H. J., Kang, S. W., Yang, C. H., Rhee, S. G., and Ryu, S. E. (1998) Nat. Struct. Biol. 5, 400–406
31. Hidatsu, S., Abe, Y., Okada, K., Nagahara, N., Hori, H., Nishino, T., and Hakoshima, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12333–12338
32. Mitsumoto, A., Takezawa, Y., Okawa, K., Iwamatsu, A., and Nakagawa, Y. (2001) Free Radical Biol. Med. 30, 625–635
33. Fujii, T., Fujii, J., and Taniguchi, N. (2001) Eur. J. Biochem. 268, 218–225
34. Douglas, W. H., and Kaighn, M. E. (1974) In Vitro 10, 230–237
35. Summerton, J., and Weller, D. (1997) Antisense Nucleic Drug Dev. 7, 187–195
36. Summerton, J. (1999) Biochim. Biophys. Acta. 1489, 141–156
37. van Engeland, M., Nieland, L. J. W., Ramaekers, F. C. S., Schutte, B., and Reutelingsperger, C. P. M. (1998) Cytometry 31, 1–9