Protective Role of Prx (Peroxiredoxin) I and II against H_2O_2-Induced Apoptosis of MCF7 Cell Lines

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Purpose: Apoptosis is known to be induced either by direct oxidative damage from oxygen free radicals or hydrogen peroxide, or from their generation in cells by injurious agents. Peroxiredoxin plays an important role in eliminating peroxides generated during metabolism. The aim of this study is to elucidate the role of Prx (peroxiredoxin) enzymes during the cellular response to oxidative stress.

Methods: The presence of Prx isoforms was demonstrated by immunoblot analysis using Prx isoforms-specific antibodies, and RT-PCR using Prx isozyme coding sequences. Annexin V-FITO apoptosis detection method was used to measure the cell death following exposure to H_2O_2.

Results: Treatment of MCF7 cell lines with H2O2 resulted in the dose-dependent expression of Prx I and II. Observed decreases in the mRNA expressions of Prx I and II, analyzed by RT-PCR, correlated well with the results of immunoblot analysis. The treatment of normal breast cell line, MCF10A, with H2O2 resulted in rapid cell death, while the breast cancer cell line, MCF7, was resistant. In addition, we confirmed that Prx I and II transfected MCF10A cells were more prone to cell death than MCF10A transfected with vector alone, after H2O2 treatment.

Conclusion: These findings suggest that Prx I and II have an important function as inhibitors of cell death during the cellular response to oxidative stress. **(Journal of Korean Breast Cancer Society 2003;6:68-74)**

Key Words: Prx (peroxiredoxin), H_2O_2, Breast cancer, Apoptosis, Oxidative stress

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INTRODUCTION

Oxidative stress has been implicated in the normal aging processes and numerous disorders including cancer, muscular atrophy, neurodegenerative diseases and atherosclerosis.(*1,2*)

Mammalian cells have two metal catalyzed oxidative mechanisms, namely the ascorbate oxidation system and the thiol oxidation system. These can both be inhibited by catalase or glutathione synthetase, which protects them from ROS.(*3*)

Peroxiredoxin, previously called thioredoxin peroxidase, is a 25 kDa protein that acts in the thiol oxidation system. It was first discovered in yeast in 1994(*4*) and since more than 100 isoforms, with some sequence homology, have been identified from protozoa(*5,6*) to mammalian species including the rat, mouse and human. Prx is thought to be of biological importance, because these molecules are highly conserved in eukaryotes and prokaryotes.(*7*) The gene locus for these proteins is suspected to be in chromosome 13q12.(*8*) Six Pix isoforms have been reported in humans, namely, NKEF-A, NKEF-B, MER 5, AOE372, TAS and ORF6.(*9*) These enzymes may have functions other than reductase activity, but their functional roles not yet been established.(*10,11*) Some investigators have reported that Prx has an anti-apoptotic effect in vitro.(*12*) A more well-known fact is that catalase and superoxide dismutase can protect cells form different initiators of apoptosis.(*13,14*) Moreover, H_2O_2 is thought to be a second messenger in the cell-signaling pathway and therefore, the reductase species may play an important role in cellar phenomena such as cell proliferation, differentiation and immune response. This proliferative action of Prx has only been reported in in vitro studies.(*12*)

In this study, we used Western immunoblot analysis using Prx isozyme antibodies and RT-PCR using the Prx isozyme coding sequences to determine the expressional pattern of Prx isozymes in the H_2O_2 treated MCF7 cell line. Also, we measured cell death by flow-cytometry in a H_2O_2 treated MCF7 cell line.

METHODS

1) Materials

PMSF, leupeptin, aprotinin, Triton X-100, DTT, HEPS and diethylpyrocarbonate (DEPC) were obtained from Sigma, Trizol reagent from Gibco BRL, the First strand cDNA synthesis kit from Boehringer Mannheim, *Taq* DNA polymerase from TaKaRa Shuzo Co, peroxidase conjugated goat anti-rabbit IgG antibody from Jackson ImmunoResearch Laboratories, Inc, and the Enhanced Chemiluminescence Detection (ECL) system from Amersham Corp. Protein concentrations were determined using a protein assay kit from Pierce. The Annexin V-FITO apoptosis detection kit was obtained from BD PharMingen, and rabbit antibodies raised against Prx isozymes were a generous gift from Dr. Ho-Zoon Chae.

2) Cell and cell culture

The human mammary carcinoma cell line MCF7 was obtained from the Korean Cell Line Bank and maintained in RPMI1640 with 10 % fetal bovine serum (FBS), containing 100 units of penicillin and 100mg of streptomycin/ml in a 37°C incubator under 5% CO_2.

3) RNA isolation and RT-PCR

Total RNA was isolated from H_2O_2 treated or control MCF7 cells using TRIzol reagent. The RNA pellet obtained was dissolved in diethylpyrocarbonate (DEPC) treated H_2O. at concentrations of 0.5μg/l to 1.0μg/μl and the stored at -70°C. The quantity and quality of the RNA preparations were determined by measuring absorbance at 260 nm and 280 nm. One microgram of total RNA sample was reverse transcribed using the first strand cDNA synthesis kit with random primer p $(dN)_6$, according to the manufacturer's instructions. RT-PCR analysis was performed as previously described. Primers used for amplification were synthesized as follows. The forward primers, which included a Hind III site and the initiation codon, were

5'GGGGCCAAGCTTATGTCTTCAGGAAATGCTAA3' for PrxI and 5'TTCAAAAAGCTTATGGCCTCCGGTAACGCGCG3' for Prx II.

The reverse primers, which included a XbaI site and the stop codon, were

5'CGGGCCTCTAGATCACTTCTGCTTGGAGAAAT3' for Prx I and 5'GGCCCCTCTAGACTAATTGTGTTTGGAGAAA3' for Prx II.

The amplification conditions used for Prx I were 35 cycles of 94°C for 30 sec, 65°C for 1 min, and 72°C for 1 min. The amplification conditions used for Prx II were 35 cycles of 94°C for 30 sec, 58°C for 1 min, and 72°C for 1 min. The PCR products were subcloned into pcDNA6B basic vector (Invitrogen) to yield pcDNA6B-prxI, and pcDNA6B-prxII. Clones containing the coding sequences in the correct orientation were selected and used for transfection.

4) Transfection of eukaryotic cells

MCF10A cells were plated at a density of 3×10^5 cells/ml/60 mm dish and allowed to recover for 24 hr. They were then incubated with 4 g of appropriate DNA and 20μl of LipofectAMINE™ in 3 ml of opti-MEM (Life Technologies, Inc) for 6h, after which 3 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum was added to the transfection mixture and the cells were then incubated for an additional 18 h. The efficacy and reproducibility of transfection were confirmed by immunoblot analysis of the Prx I and Prx II protein in each batch of cells.

5) Immunoblot analysis

H_2O_2 treated or control MCF7 cells were homogenized in 20 mM HEPES (pH 7.2), 150 Mm NaCl, 1% Triton X-100, 2% cholic acid, 1 mM EDTA, 1 mM EGTA, 0.1 mM DTT, 10μg/ml leupeptin, 10μg/ml aprotinine, and 10 mM phenylmethylsulfonyl fluoride (PMSF). Protein content was determined by the bicinchoninic acid (BCA) protein assay using bovine serum albumin as a standard. Cell lysates were released by heating samples at 95°C for 10 minutes with Laemmli cooking buffer and then separated by SDS-polyacrylamide gel electrophoresis. Samples were transferred onto nitrocellulose filters and incubated separately with their respective polyclonal antibodies for 2 hours. Immunoreactive bands were visualized using peroxidase conjugated goat anti-rabbit IgG antibody. Detection was performed with the ECL system.

6) Annexin V-FITO apoptosis detection

H_2O_2 treated or control MCF10A and MCF7 cells were washed twice with cold PBS and the cells resuspended in 1x

binding buffer at a concentration of 1×10^{o} cells/ml. 100μl of this mixture was then transferred to a 5 ml culture tube. 5 μl of Annexin V-FITC and 5μl of PI were then added. The cells were gently vortexed and incubated for 15 min at room temperature in the dark, and 400μl of 1x binding buffer was added to each tube. Flow cytometry was then performed within one hour.

7) Quantification of autoradiographs

A Biomedlab computing densitometer was used to digitize images. The optical density of bands was analyzed with background subtraction using ImageQuant software

RESULTS

H_2O_2 acts in a concentration-dependent manner to induce cell death in a variety of cell types. To examine the effect of H_2O_2 treatment on Prx expression, MCF7 cells were exposed to various doses of the H_2O_2 for 24 hrs. Using five isoform-specific antibodies, we investigated the presence of Prx I, II, III, IV and V by immunoblot analysis and RT-PCR in the H_2O_2-dose dependent treated MCF7 cell lines. As shown in Fig. 1, H_2O_2 treatment induced Prx expression in a dose-dependent manner. Significant decreases in Prx I and II were detected at H_2O_2 concentrations above 600μM. Western blotting was conducted for Prx III, IV and V, which were not dose-dependently decreased in MCF7 cell lines.

Using designated primers based on the conserved amino acid regions, we determined the presence of Prx I and II mRNAs by RT-PCR versus decreasing H_2O_2-dose in MCF7 the cell line. For normalization purposes, β-actin was amplified simultaneously. As shown in Fig. 1, H_2O_2 treatment resulted in the expression of Prx in a dose-dependent manner. Significant decreases in Prx I and II were detected at H_2O_2 concentration above 600μM. These results showed that decreases in Prx I and II mRNA expressions were well correlated with the results of immunoblot analysis.

Tryphan blue exclusion was employed to assess time-dependent cell viability after the H_2O_2 treatments. As shown in Fig. 2, treatment with H_2O_2 also resulted in a cell death in time-dependent manner.

We also examined expressions of Prx I and II in normal human breast tissues, cancer tissues, and in MCF10A and MCF7, and Prx I and II were detected in normal tissues, cancer tissues, MCF10A and MCF7 by RT-PCR and immunoblot analysis. Fig. 3 shows that Prx I and II were

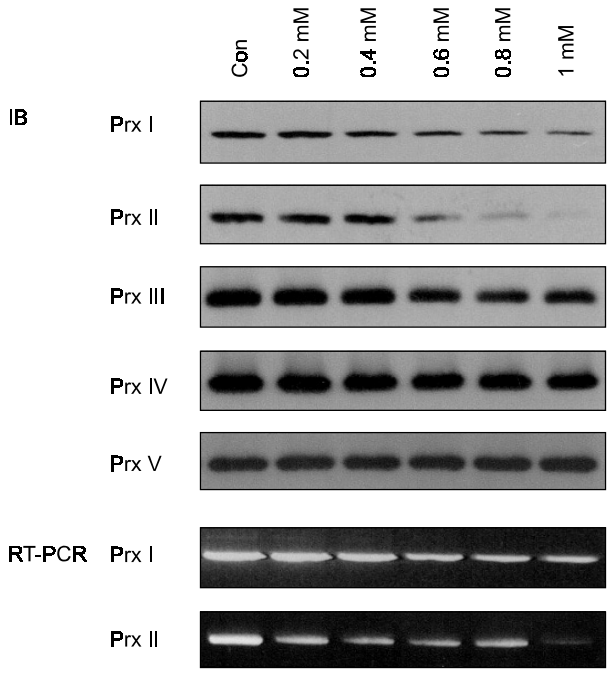

Fig. 1. Detection of Prx isozymes protein in H_2O_2 treated MCF7 cell by immunoblot analysis and RT-PCR. Expression of Prx by H_2O_2 was found to be concentration dependent. MCF7 were exposed to the indicated concentrations of H_2O_2 for 24 hrs. Western blotting and RT-PCR analysis was performed as described in Materials and Methods. Western blotting was employed to assess the levels of the Prx isozymes (I, II, III, IV, and V) using Prx isozyme-specific antibodies. The same membrane was stripped and re-blotted with Prx isozymes-specific antibodies to demonstrate the equal loading of protein in each lane.

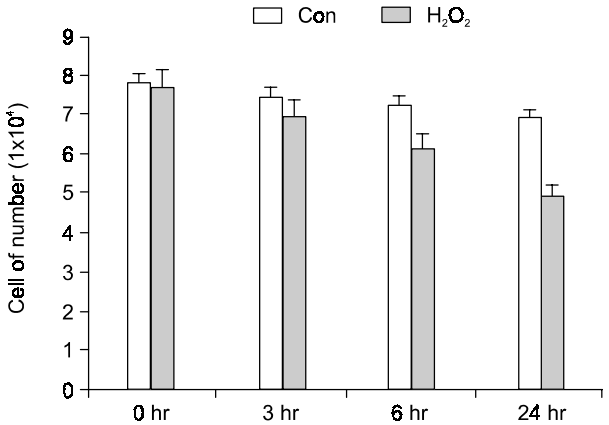

Fig. 2. Cell viability of H_2O_2 time dependent treated MCF7 cells. MCF7 were exposed to 800μM of H_2O_2 for 3, 6 and 24 hrs and surviving cells (those able to exclude trypan blue) were counted. Data are reported as percent survival relative to control (untreated) cells. Data are the means of at least 3 independent experiments and error bars correspond to standard errors of the mean.

overexpressed in human breast cancer tissues and MCF7 versus normal human breast tissues and MCF10A.

H_2O_2 was found to induce cell death in both MCF10A and MCF7 by annexin V assay. As shown in Fig. 4, we found that H_2O_2 800μM treatment in MCF10A cells increased cell death by 39.2%. H_2O_2 800μM treatment in MCF7 cells increased cell death by 11.4%.

We also investigated whether Prx I and II are able to protect MCF10A cells from the cell death induced by H_2O_2 by transiently expressing Prx I and II. The overexpression of these two proteins in MCF10A transfected cells was confirmed by immunoblot analysis.

As shown in Fig. 5, in response to stimulation by H_2O_2 800μM, the survival cells of MCF10A transfected with vector alone increased by 29% and increased by 3.6% in Prx I and II transfected cells.

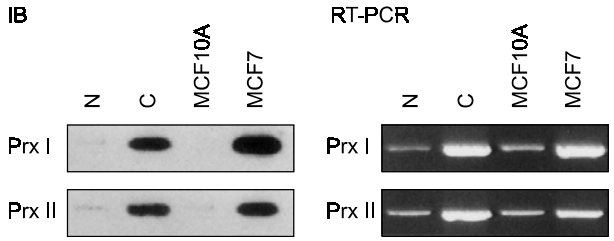

Fig. 3. Expression of Prx I, and II in normal human breast and breast cancer tissues, and in the MCF10A and MCF7 cell lines by immunoblot analysis and RT-PCR.

DISCUSSION

In mammalian cells, a variety of extracellular stimuli, including tumor necrosis factor-α,(*15*) PDGF,(*16,17*) EGF, (*18-21*) and FGF(*15*) induce a transient increase in the intracellular concentration of H_2O_2. The transient nature of receptor-mediated increase in intracellular H_2O_2 suggests that, in addition to its production, the rapid removal of this molecule is important for receptor signaling. Together with glutathione peroxidase and catalase, peroxiredoxin (which reduces H_2O_2 to H_2O) enzymes play an important role in eliminating peroxides generated during metabolism. Oxidative stress may be broadly defined as an imbalance between oxidant production and the antioxidant capacity of the cell to

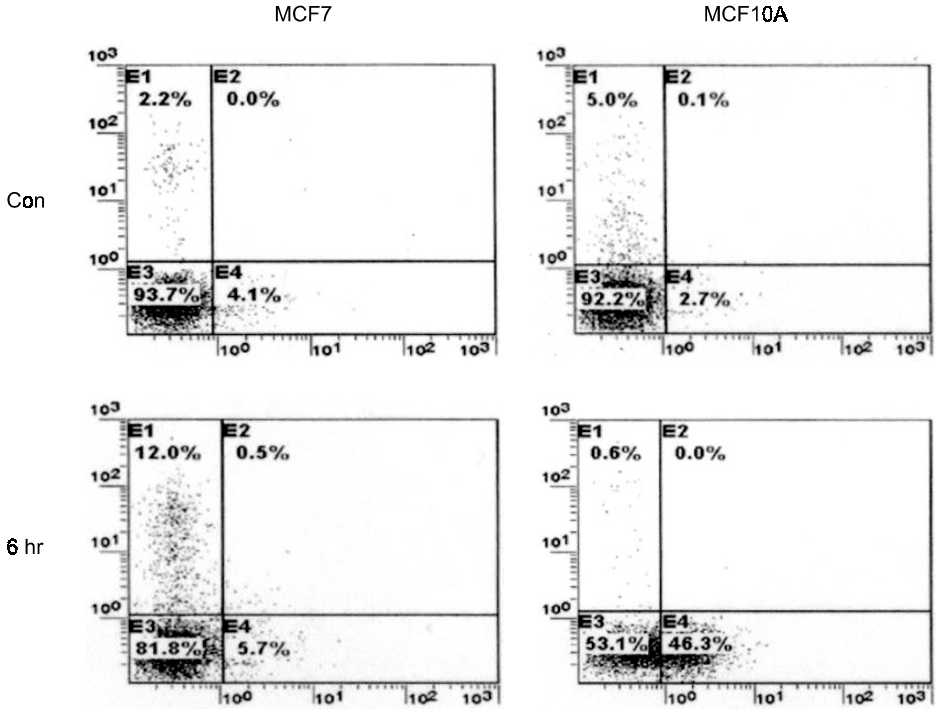

Fig. 4. H_2O_2 induced cell death in MCF10A and MCF7 cell lines MCF10A and MCF7 cells were left untreated or treated for 6 hr with 800μM H_2O_2. Cells were stained with fluorescein isothiocyanate-conjugated annexin V in a buffer containing propidium iodide and analyzed by flow cytometry. For each group of cells, the percentage of early apoptotic cells is shown in the lower right quadrant. Cells in the late stages of apoptosis had compromised membranes and stained for both annexin V and propidium iodide, and are shown in the top left quadrant. Dead cells only incorporate propidium iodide (top right). The lower left quadrant contains the vital population.

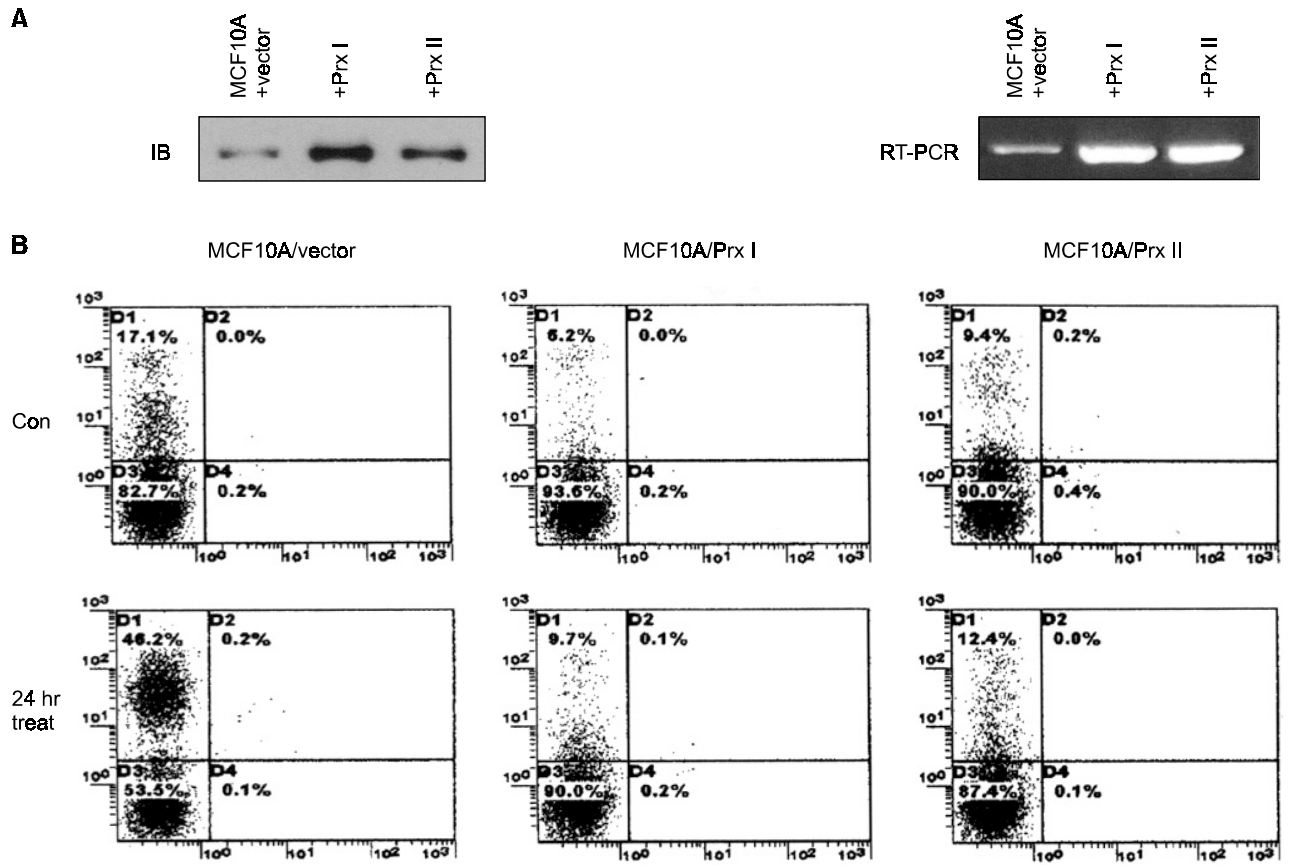

Fig. 5. H_2O_2 induced cell death in Prx I transfected MCF10A cells. (A) Expression of Prx I, and II in vector transfected MCF10A and Prx I transfected MCF10A cells by immunoblot analysis and RT-PCR. (B) Vector transfected MCF10A and Prx I transfected MCF10A cells were left untreated or treated for 24 hr with 800μM H_2O_2. The cells were stained with fluorescein isothiocyanate-conjugated annexin V in a buffer containing propidium iodide and analyzed by flow cytometry.

prevent oxidative injury. Thus, Oxidative stress has been implicated in a large number of human diseases including atherosclerosis, pulmonary fibrosis, cancer, neurodegenerative diseases, and aging.(*22,23*) Yet the relationship between oxidative stress and the pathobiology of these diseases is not clear, largely due to a lack of understanding of the mechanisms by which ROS functions in both normal physiological and disease states. Results from our laboratory demonstrate the overexpression of Prx in human breast cancer tissues.(*24*) However, we could not find any significant association between Prx overexpression and clinical parameters in these groups.

Apoptosis can be triggered by a variety of signals and pathophysiologic conditions, which include oxidative stress. Moreover, the Inhibition of apoptosis can cause abnormal cellular proliferation and may be connected to cancer development.

Endogenously produced ROI and H_2O_2 are known to act as intermediates in apoptotic signaling, based on the finding that ROI generation can be detected in cells undergoing apoptosis. Exposure of cells to low concentrations of exogenous ROI, i.e., H_2O_2, also induces apoptotic cell death, whereas higher amounts can cause necrosis.(*25*)

In the current study H_2O_2 was added to cells in the presence of serum at concentrations ranging from 200 to 1,000 μM H_2O_2 in the MCF7 breast cancer cell line. As shown in Figs. 2 and 3, H_2O_2 treatment resulted in Prx expression in a dose-dependent manner, and significant decreases in Prx I and II were detected at H_2O_2 concentrations above 600μM. Moreover, these results, which were obtained by RT-PCR correlated well with those obtained by immunoblot analysis. Furthermore, these results are in accord with a report upon the overexpressions of Prx I, II and III in human breast cancer tissues.(*24*)

Prx I and II, which are abundant in the cytosol, are likely to play an important role in eliminating H_2O_2 generated as a

by-product of metabolism in the cytosol.(*26*) Zhang et al. reported results of a cell transfection study, which linked Prx II and anti-apoptotic activity.(*12*) Prx was suggested to be an anti-apoptotic factor that inhibits(*27*) of PARP (poly ADP-ribosyl polymerase) cleavage triggered by diverse stimuli, such as serum deprivation, and etoposide and ceramide treatment, which induce apoptosis.(*12,27*) Kang et al. reported that Prx I and II might participate in the signaling cascades of growth factors and tumor necrosis factor-a by regulating the intracellular concentration of H_2O_2, and found that Prx II is able to inhibit the release of cytochrome C from mitochondria to the cytosol, thus preventing cellular hydrogen peroxide accumulation.(*28*)

The viabilities of MCF10A and MCF7 were severely compromised over the H_2O_2 concentration range, which strongly supports the biologic relevance of Prx I and II expressions and the role of Prx in protecting cells against H_2O_2-induced cell death (Fig. 4). To more accurately quantitative the fraction of the cells undergoing apoptosis we used the annexin V assay, which provides a way of determining the proportion of cells in the early stages of apoptosis, and the proportion of cells in the later stage of apoptosis, as well as the numbers undergoing necrosis. When MCF10A and MCF7 cells were treated for 6 hr with 800μM H_2O_2, cell death was increased about 2.6-fold more in the MCF10A cell-lines as compared to the MCF7 cell line (fig. 4). Furthermore, we investigated whether Prx I and II are able to protect MCF10 cell from cell death in response to H_2O_2 treatment by transiently expressing Prx I and II in these cells. We found that Prx I and II transfected MCF10A cells resisted H_2O_2 induced cell death compared with MCF10A cells transfected with vector alone (Fig. 5).

These findings suggest that Prx I and II have important functions as inhibitors of cell death during the cellular response to oxidative stress.

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