Published in final edited form as:
Gut. 2012 September ; 61(9): 1250–1260. doi:10.1136/gutjnl-2011-301078.

Glutathione Peroxidase 7 Protects Against Oxidative DNA Damage in Oesophageal Cells

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Abstract

Objective—Exposure of the oesophageal mucosa to gastric acid and bile acids leads to accumulation of reactive oxygen species (ROS), a known risk factor for Barrett's oesophagus (BO) and progression to oesophageal adenocarcinoma (OAC). In this study we investigated the functions of glutathione peroxidase 7 (GPX7), frequently silenced in OAC, and its capacity in regulating ROS and its associated oxidative DNA damage.

Design—Using in vitro cell models, we performed experiments that included GPX activity, Amplex UltraRed, CM-H_2DCFDA, Annexin-V, 8-oxoguanine, phospho-H2A.X, quantitative real-time PCR and Western blot assays.

Results—Enzymatic assays demonstrated a limited GPX activity of the recombinant GPX7 protein. However, GPX7 exhibited a strong capacity to neutralize H_2O_2 independent of glutathione. Reconstitution of GPX7 expression in immortalized BO cells, BAR-T and CP-A, led to resistance to H_2O_2-induced oxidative stress. Following exposure to acidic bile acids cocktail (pH 4), these GPX7-expressing cells demonstrated lower levels of H_2O_2, intracellular ROS, oxidative DNA damage and double strand breaks (DSB), as compared to control (*P<0.01*). In addition, these cells demonstrated lower levels of ROS signaling, indicated by reduced phospho-JNK (Thr183/Tyr185) and phospho-p38 (Thr180/Tyr182), and demonstrated lower levels of apoptosis following the exposure to acidic bile acids or H_2O_2-induced oxidative stress. The knockdown of endogenous GPX7 in immortalized oesophageal squamous epithelial cells (HET1A) confirmed the protective functions of GPX7 against pH4 bile acids by showing an

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Conflict of Interest: All authors indicated "no conflict of interest".

increase in the levels of H_2O_2, intracellular ROS, oxidative DNA damage, DSB, apoptosis and ROS-dependent signaling (*P<0.01*).

Conclusion—The dysfunction of GPX7 in oesophageal cells increases the levels of ROS and oxidative DNA damage which are common risk factors for BO and OAC.

Keywords

Glutathione peroxidase 7; Barrett's; Oesophagus; Cancer; DNA damage; Bile acids; ROS

Introduction

Gastro-oesophageal reflux disease (GORD) [1, 2] is a condition where gastric acid, usually mixed with bile acids, refluxes into the lower oesophagus. GORD- associated chronic mucosal injury and inflammation is a major risk factor for the development of Barrett's oesophagus (BO), a premalignant condition that is closely associated with the development of oesophageal adenocarcinoma (OAC). [3] BO is prevalent in the general population; it is present in 10–20% of patients with GORD. [4, 5] In the United States, based on data from the Surveillance, Epidemiology, and End Results (SEER) program, [6] the incidence rate for oesophageal adenocarcinoma has increased 4–10% per year among men since 1976, more rapidly than for any other type of cancer. [7, 8] Patients with BO can progress to low-grade dysplasia (LGD), high-grade dysplasia (HGD), and OAC at 30 to 60 times that of the general population. [5, 8] The natural and molecular history of progression of BO to OAC is poorly understood.

Clinical studies have identified glycocholic, taurocholic, glycodeoxycholic and glycochenodeoxycholic acid as the predominant bile acids appearing in the oesophagus of patients with GORD. [9, 10] Deoxycholic acid, a more toxic, unconjugated bile acid usually formed by the action of colonic bacteria, is also present in the refluxate of patients with Barrett's oesophagus. [1] Gastric acid and/or in combination with bile acids has been reported to induce a significant increase in intracellular reactive oxygen species (ROS) in oesophageal epithelial cells. The ROS levels are known to be higher in tissues with Barrett's oesophagus and OAC in both human [11, 12] and animal models. [13] Consistent with these observations, several recent studies have documented higher levels of oxidative DNA damage, as well as single and double strand breaks in human Barrett's oesophagus and OAC compared to normal oesophageal and duodenal epithelia. [12, 14, 15]

Normal cells have intact anti-oxidative properties that protect cells from ROS-induced DNA damage. [16, 17] Among these systems, the glutathione peroxidase (GPXs) family is a major antioxidative enzyme family that catalyzes the reduction of hydrogen peroxide, organic hydroperoxide, and lipid peroxides by reduced glutathione. [18] We have recently reported a frequent dysfunction of glutathione peroxidase 7 (GPX7) in OAC and its precancerous BO and dysplasia [19], suggesting that impairment of the anti-oxidative capacity may contribute to the development of OAC. GPX7 is one of the recently identified GPX family members, based on structural homology, with unknown biological functions. [20] In this study, we investigated the anti-oxidant functions of GPX7 and its capacity in protecting oesophageal epithelial cells from acid and bile acid-induced DNA damage.

Materials and Methods

Cell lines

Immortalized Barrett's oesophagus cell lines BAR-T [21] and CP-A (American Type Culture Collection, ATCC, Manassas, VA, USA) were cultured with Epithelial Cell

Medium-2 (ScienCell, Carlsbad, CA, USA), supplemented with 5% fetal bovine serum (FBS) and antibiotics on Primaria plates and flasks (BD Biosciences, Bedford, MA, USA). The immortalized human oesophageal epithelia cell line HET1A was obtained from ATCC. Dr. Hiroshi Nakagawa in University of Pennsylvania kindly provided the hTERT-immortalized human oesophageal epithelial cell line EPC2. EPC2 cells were grown in keratinocyte-SFM (KSFM) medium supplemented with 40 μg/ml bovine pituitary extract and 1.0 ng/ml epidermal growth factor (Invitrogen). The HEK293 AD cell line (Cell BioLabs Inc, San Diego, CA, USA) was used for propagating adenoviral particles. HET1A and HEK293 AD cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) media supplemented with 10% FBS and antibiotics (Invitrogen, Carlsbad, CA, USA). All cell lines were grown at 37°C in 5% CO_2.

Chemicals

A bile acid cocktail was prepared, consisting of an equimolar mixture of sodium salts of glycocholic acid, taurocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid and deoxycholic acid. In all experiments, we used 0.1 mM final concentration of the bile acids cocktail (20 μM of each of the above bile acids). This cocktail reflects the mixture of bile acid to which the distal oesophagus is ordinarily exposed during gastrooesophageal reflex disease, as previously reported. [1, 9]

Antibodies

Rabbit anti-human GPX7 polyclonal antibody was purchased from ProteinTech Group (Chicago, IL, USA). Rabbit anti-β-actin antibody, anti-phospho-histone H2A.X (ser139), anti-H2A.X, anti-SAPK/JNK, anti-phospho-p38 (Thr180/Tyr182), anti- caspase 3 and cleaved caspase 3 antibodies were purchased from Cell Signaling (Danvers, MA, USA). Mouse anti-8-oxoguanine monoclonal antibody (clone 483.15) was purchased from Millipore (Temecula, CA, USA), mouse anti-phospho-JNK (Thr183/Tyr185) was purchased from Santa Cruz (Santa Cruz, CA, USA).

RNA extraction and quantitative real-time RT-PCR (qRT-PCR)

RNA from 28 normal oesophageal squamous (NS), 6 Barrett's oesophagus (BO), 6 Barrett's dysplasia and 43 oesophageal adenocarcinoma (OAC) samples was available. All samples were obtained coded from the archives of the Pathology at Vanderbilt University and National Cancer Institute Cooperative Human Tissue Network (CHTN) in accordance with approved protocols at Vanderbilt University. All samples were histologically verified. The dysplasia samples were diagnosed as 1 LGD, 2 HGD, and 3 unclassified. Total RNAs were isolated using the RNeasy mini kit (Qiagen, Valencia, CA, USA). Single-stranded cDNA was subsequently synthesized using the iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA, USA). The sequence of GPX7 and HPRT primers was previously described [19]. The qRT-PCR was performed using an iCycler (Bio-Rad) with the threshold cycle number determined by use of iCycler software version 3.0. Reactions were performed in triplicate, the threshold numbers were averaged, and fold expression was calculated as previously reported. [19, 22]

Cloning and construction of GPX7 expression plasmid

A full length of GPX7 coding sequence with Flag tag was amplified from normal cDNA by PCR using Platinum PCR SuperMix High Fidelity (Invitrogen) and was cloned into the pACCMV.pLpA plasmid. [23] The pACCMV.pLpA-GPX7 plasmid was then co-transfected with pJM17 plasmid into 293 AD cells to generate and to propagate the full adenoviral-GPX7 particles as previously described. [23] The viruses were plaque purified, and the titer of the virus was determined using the Adeno-X qPCR Titration Kit (Clontech, CA, USA)

following the manufacturer's instructions. Two Barrett's cell lines, BAR-T and CP-A, in which GPX7 expression were significantly downregulated, were infected with 25 copies per cell of adenoviral-GPX7 particles (Ad-GPX7) and adenoviral empty particles (Ad-CTRL) in the culture medium. 48h after infection, the cells were harvested for GPX7 expression validation in both mRNA levels using qRT-PCR and in protein levels by Western blotting (Figure 2A).

Knockdown of GPX7 expression by lentiviral shRNA

To knockdown GPX7 expression in non-malignant cells, the oesophageal squamous cell line HET1A was transfected with the same number of copies of scrambled shRNA control (Sc shRNA) and GPX7 validated specific shRNA (GPX7 shRNA) lentiviral particles; the sequence of the GPX7 shRNA is 5'-agcctgacagcaacaagga-3' (GeneCopoeia, Rockville, MD, USA). GPX7 knockdown was confirmed using qRT-PCR and Western blotting (Figure 2B).

Cell viability assay

Cell viability was evaluated with the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA). BAR-T and CP-A cells were infected with control viral (Ad-CTRL) and GPX7 overexpressed particles (Ad-GPX7). 24h after infection, cells were split and seeded at 8000 cells/well density in 96-well plate and cultured for another 24h. Cells were then treated with different concentrations of H_2O_2 for 16h. For cell viability after acidic bile acids exposure, cells were treated with medium with acidic bile acids cocktail (pH 4, 0.1 mM) for 10, 30, and 60 min. After pH 4 bile acids treatment, cells were cultured in full medium for another 24h, and then CellTiter-Glo® Luminescent Cell Viability Assay was performed following the manufacturer's guidelines. HET1A cells with GPX7 shRNA and scrambled control shRNA (as described above) were also treated with H_2O_2 for 16h and pH 4 bile acids. The results were expressed as relative cell viability compared to the PBS-treated controls.

Confirmation of glutathione peroxidase activity of GPX7 protein

Because GPX7 is a new member of the GPX family where its activity has not been fully established, we carried out a Glutathione Peroxidase Activity Assay using the Glutathione Peroxidase Activity Kit from Assay designs (Enzo Life Science, Plymouth Meeting, PA) and recombinant GPX7 protein (Enzo Life Science) following the manufacturer's instructions. The absorbance of samples was measured at 340 nm for every 1 min over a 10 min period on a FLUO Star OPTIMA microplate reader (BMG Labtech, Cary, NC USA). The results are expressed as the change in absorbance per minute (ΔA340/min).

Detection of H_2O_2 level by Amplex UltraRed Assay

To determine if GPX7 can neutralize H_2O_2 directly, independent of glutathione, a direct peroxidase assay was carried out using Amplex UltraRed (Invitrogen). Briefly, an equal volume of 800 nM H_2O_2 diluted in 1× reaction buffer was added with different concentrations (from 0 ng/μl to 10 ng/μl) of recombinant GPX7 protein (Enzo Life Science). An equal volume of Amplex UltraRed reagent was then added to the samples and standards. After 30 min incubation, the plate was read on a FLUO Star OPTIMA microplate reader (BMG Labtech) at excitation/emission of 540 nm/590 nm. The final concentration of H_2O_2 was calculated from a standard curve. To determine if GPX7 can reduce H_2O_2 accumulation in oesophageal cells after acidic bile acid stimulation, BAR-T cells with reconstitution of Ad-GPX7 or Ad-CTRL, and HET1A cells with GPX7 shRNA knockdown or control Sc shRNA were stimulated with acidic bile acids (pH 4, 0.1 mM) for 10, 30, or 60 min. Immediately after acidic bile acids exposure, the media were collected and subjected to hydrogen peroxide assay using Amplex UltraRed (Invitrogen) as described above.

Detection of intracellular reactive oxygen species (ROS)

BAR-T cells with Ad-CTRL and Ad-GPX7, and HET1A cells with GPX7 shRNA and Sc shRNA were prepared as described above. 24h after infection, cells were split and seeded at 2×10^5 cells per well in a 12-well plate and cultured overnight. The next day cells were incubated with 5 μM of 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate and acetylester (CM-H_2DCFDA, Invitrogen) in phenol red free medium at 37°C for 20 min. Cells were washed with PBS and incubated at 37°C in phenol red free DMEM (Gibco, Carlsbad, CA, USA) containing 5% serum for 15 min to allow de-esterification of the dye. Cells were then incubated with acidic bile acids cocktail pH 4.0 (0.1 mM bile acid) for 30 min. Cells were trypsinized and resuspended in phenol red free DMEM with 1% serum and were immediately analyzed by flow cytometry at the channel for FITC. Experiments were performed in triplicate and 10,000 events were collected.

Detection of oxidative DNA damage

Oxidative DNA damage after cell exposure to acidic bile acid was evaluated using immunofluorescence assay of anti-8-oxoguanine and FITC-avidin. For immunofluorescence assay of anti-8-oxoguanine, cells were exposed to pH 4 bile acids cocktail (0.1 mM) for 60 min, fixed with freshly-made 4% paraformaldehyde for 45 min at room temperature, followed by permeabilization with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Cells were then incubated with 10% normal goat serum (Invitrogen) for 20 min at room temperature. Cells were incubated with primary antibody against 8-oxoguanine (mouse, 1:200, Millipore) overnight at 4°C followed by secondary goat anti-mouse antibody conjugated with TRITC (1:1000) at room temperature for 45 min. The slides were mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA) and viewed under a fluorescence microscope. To quantify the fluorescence intensity for 8-oxoguanine, ImageJ software (National Institutes of Health, USA) was used and at least 1000 cells were measured. The result is expressed as fluorescence intensity per cells as previously described. [24] The red fluorescence images were converted into pseudo green for better visualization. For FITC-avidin, we replaced the 8-oxo-guanine antibody with FITC-conjugated avidin (1:200, Santa Cruz) and incubated at 37°C for 1h in the dark. The slides were then mounted using Vectashield with DAPI (Vector Laboratories) and viewed under a fluorescence microscope.

Detection of double strand breaks

Because phospho-H2A.X (ser139) is a reliable marker for double strand breaks, [25] we used immunofluorescence to detect the phospho-H2A.X level in BAR-T cells and the HET1A cells. Cells were exposed to acidic medium (pH 4) with 0.1 mM bile acid cocktail for 20 min. After exposure, cells were washed with PBS and cultured with full medium for another 20 min as previously described. [15] Cells were fixed with 1:1 methanol/acetone for 20 min at −20°C followed by incubation in 10% normal goat serum (Invitrogen) for 20 min at room temperature. The primary antibody against phospho-H2A.X (ser139, 1:100, Cell Signaling) was incubated with cells overnight at 4°C. Cells were washed twice and incubated with secondary goat anti-rabbit antibody (1:1000) for 45 min at room temperature. The slides were mounted using Vectashield with DAPI (Vector Laboratories) and viewed under a fluorescence microscope. At least 1000 cells were counted using ImageJ software under the same threshold and the percentage of phospho-H2A.X positive cells was calculated.

Annexin V flow cytometry

To detect apoptosis after exposure to acidic bile acids, BAR-T cells and HET1A cells were exposed to pH 4 bile acids (0.1 mM) for 10 and 30 min. After exposure, cells were washed

with PBS and cultured in full medium for another 24h. Cells were then trypsinized and stained with annexin V-FITC or annexin V-PE (for HET1A to avoid GFP effect) for 5 min. FACS was done immediately after staining to determine the annexin V positive cells.

Western blotting

Cells with and without treatments were lysed in the presence of proteinase inhibitor cocktail and phosphatase inhibitor (Thermo Scientific, Rockford, IL, USA). Protein concentration was determined by a Bio-Rad Protein Assay using a FLUO Star OPTIMA microplate reader (BMG). An equal amount of proteins were loaded and separated by SDS-PAGE and transferred to nitrocellulose membranes and Western blotting analysis was performed using standard protocols. ImageJ software was used for quantitating the bands density of caspase 3, and was presented as the ratio of cleaved caspase 3/total caspase 3.

Statistical analysis

All experiments were performed in triplicate. Analysis of variance (ANOVA) was used to test for significant differences between means within a group. Student *t* test was used to test for significant differences between GPX7 transfected cells (Ad-GPX7) and adenoviral control (Ad-CTRL) cells, and between Sc shRNA control cells and GPX7 shRNA cells. Linear regression analysis was used to analyze the correlation between GPX7 concentration and the H_2O_2 level. All analyses were done using Prism 4 software. The error bars represent standard error from the three experiments. For all tests, $p<0.05$ was considered statistically significant. Statistically significant differences are marked in the Figures; * $p<0.05$ and ** $p<0.01$.

Results

GPX7 exhibits limited glutathione peroxidase activity and neutralizes H_2O_2 *in vitro* in glutathione-independent manner

GPX7 shares about 45% amino acid sequence with other GPX family members, such as GPX1 (42%) and GPX4 (44%).[20, 26] To explore if GPX7 has GPX activity, a glutathione peroxidase activity assay was performed using a commercial recombinant GPX7 protein. As shown in Figure 1A, compare to GPX control, GPX7 protein displayed some but limited glutathione peroxidase activity. However, the same recombinant GPX7 protein significantly reduced the H_2O_2 level with a significant inverse relationship between GPX7 concentration and the H_2O_2 level (linear regression, $r=0.9296$, $p<0.0001$) (Figure 1B) in a system without existence of glutathione. Of note, our results indicated that the levels of GPX7 are significantly downregulated in OAC and Barrett's dysplasia as compared to normal oesophagus (Figure 1C). This is consistent with our earlier report that demonstrated frequent DNA methylation of GPX7 promoter in OAC. [19] Overall, Barrett's esophagus (BO) samples did not show significant downregulation, due to small sample size. However, two of the six BO samples displayed significant downregulation; ≤0.5 expression fold as compared to normal NS.

GPX7 protects oesophageal epithelia from H_2O_2-induced oxidative stress

We next tested the protective functions of GPX7 against H_2O_2-induced oxidative stress. The reconstitution of GPX7 in BAR-T cells (Figure 2A) and CP-A cells (Supplementary Figure 2A) led to significantly higher cell viability, as compared to control cells, when exposed to H_2O_2 ($p<0.01$ for both 200 μM and 400 μM H_2O_2) (Figure 2C and supplementary Figure 2B). Conversely, the knockdown of endogenous GPX7 in HET1A cells (Figure 2B) led to a significantly lower cell viability following exposure to H_2O_2, as compared to controls ($p<0.01$ for 100 μM, 200 μM and 400 μM H_2O_2) (Figure 2D), suggesting that the

downregulation of endogenous GPX7 has sensitized the HET1A cells to H_2O_2-induced oxidative stress. Since H_2O_2-induced oxidative stress usually manifests as cell apoptosis, we performed Western blotting analysis for cleaved caspase 3. As shown in Figure 2E (BAR-T) and 2F (HET1A), cells with GPX7 expression (Ad-GPX7 in BAR-T and Sc shRNA in HET1A) displayed significantly weaker cleaved caspase 3 signal upon exposure to 200 μM H_2O_2 for 16 h.

GPX7 decreased acidic bile acid-induced H_2O_2 level and intracellular ROS level in oesophageal epithelial cells

We exposed oesophageal epithelial cells to a bile acids cocktail adjusted to pH 4 to mimic in vivo GORD conditions and performed the Amplex UltraRed Hydrogen Peroxide Assay to measure H_2O_2 levels. BAR-T cells with reconstitution of GPX7 showed significantly lower H_2O_2 levels as compared to control cells (Figure 3A). On the other hand, upon exposure to pH 4 bile acids, HET1A cells with knockdown of endogenous GPX7 displayed higher H_2O_2 levels than control cells transfected with scrambled shRNA (Figure 3B). Next, we performed analysis of CM-H_2DCFDA (Invitrogen), [15, 27] that detects predominantly H_2O_2 as well as other forms of ROS. The reconstitution of GPX7 in BAR-T cells (Figure 3C) and CP-A (Supplementary Figure 3) significantly decreased the intracellular ROS on exposure to acidic bile acids in comparison with control cells. In HET1A cells, knockdown of endogenous GPX7 significantly increased both baseline ROS level and the ROS level on exposure to pH 4 bile acids as compared to control (Figure 3D).

GPX7 protects oesophageal epithelial cells from acidic bile acid-induced oxidative DNA damage

We used immunofluorescence staining for 8-oxoguanine, one of the most identified oxidative DNA damage sites. On exposure of BAR-T cells to pH 4 bile acids, the reconstitution of GPX7 expression significantly decreased the oxidative DNA damage as compared to control cells ($p<0.05$) (Figure 4A). A similar result was obtained using avidin, another oxidative DNA damage marker [28] (Supplementary Figure 4) and in the CP-A cell line (Supplementary Figure 5). In contrast, knockdown of GPX7 in HET1A cells generated significantly more oxidative DNA damage than control cells on exposure to pH 4 bile acid ($p<0.05$, Figure 4B).

GPX7 protects oesophageal epithelial cells from acidic bile acid-induced DNA double strand breaks

Gastric acid, particularly in combination with bile acids, has been shown to induce double strand breaks in oesophageal epithelial cells. [14, 15] The immunocytochemical staining of phospho-H2A.X, a reliable marker for DNA double strand breaks, [25] demonstrated significantly lower levels of positive phospho-H2A.X sites following reconstitution of GPX7 in BAR-T cells ($p<0.05$, Figure 5A) and CP-A cells ($p<0.05$, Supplementary Figure 6) cells than in control cells, upon exposure to pH 4 bile acids. On the contrary, the knockdown of endogenous GPX7 in HET1A cells significantly increased the phospho-H2A.X sites, as compared to control ($p<0.05$, Figure 5B).

GPX7 suppresses ROS-dependent signaling in oesophageal epithelial cells

Following exposure to pH 4 bile acids, the BAR-T cells expressing GPX7 (Ad-GPX7) had lower levels of p-JNK and p-p38, as compared to control cells (Ad-CTRL). On the other hand, the knockdown of GPX7 in HET1A enhanced the p-JNK and p-p38 signaling on exposure to pH 4 bile acids (Figure 6).

GPX7 protects oesophageal epithelial cells from acidic bile acids-induced apoptosis

Because GPX7 expression protected cells from acidic bile acids-induced DNA damage, it may protect oesophageal epithelial cells from acidic bile acids-induced apoptosis. BAR-T cells and HET1A cells were treated with pH 4 bile acids for 10 and 30 min, followed by the replacement with a regular culture media for 24h. The ATP-Glo cell viability assay was then used to determine cell viability at this 24h time-point. As shown in Figure 7A, the short-term exposure (10 min) did not change the cell viability at 24h. However, the exposure for 30 min demonstrated a significant reduction in cell viability in BAR-T cells lacking GPX7 (Ad-CTRL, $p<0.01$). The flow cytometry analysis of Annexin-V positive cells, a widely accepted apoptotic cell marker, [29] confirmed these findings (Figure 7B, and representative flow cytometry histograms are shown in Supplementary Figure 7). Annexin-V positive cells were significantly lower in GPX7 expressing BAR-T cells as compared to control cells ($p<0.01$). A cleaved caspase 3 Western blotting analysis further demonstrated less apoptosis in GPX7 expressing cells ($p<0.01$, Figure 7C). Conversely, the knockdown of endogenous GPX7 in HET1A cells sensitized cells to the 10 and 30 min acidic bile acids treatments, as indicated by lower cell viability at the 24h time-point ($p<0.01$) (Figure 7D). Consistent with this finding, the GPX7 knockdown cells displayed significantly more apoptosis after acidic bile acids exposure as indicated by Annexin-V assay ($p<0.05$, Figure 7E) and Western blotting of cleaved caspase 3 ($p<0.01$, Figure 7F).

GPX7 expression is upregulated in normal squamous oesophageal epithelial cells after exposure to acidic bile acids

To investigate if GPX7 expression levels can be upregulated by exposure to acidic bile acids, as part of inherent protective capacity of normal squamous epithelial cells, we exposed HET1A cells and EPC2 cells to pH4 bile acids (0.1 mM) for 30 min. Following this exposure, cells were washed in PBS and cultured in regular medium for 30 min, 1h, 2h, 4h, 8h, and 16h time points. mRNA and protein levels were measured by real time RT-PCR and Western blotting respectively. In response to this treatment, we noticed significant up-regulation of the mRNA levels of GPX7 followed by increase in protein level at later time points (8h, 16h) in both cell lines (Figure 8). The concordance of the results between these two cell lines suggest that the presence of T antigen in HET1A did not play a significant role in our experimental in vitro system. On the contrary, similar induction of GPX7 was not observed in BAR-T cells. BAR-T-T cells have medium level of GPX7 expression at the base line as compared to normal oesophageal cells (Supplemental Figure 1). The failure to induce GPX7 in these cells could be due to the presence of partial epigenetic suppression of GPX7 expression. However, following reconstitution of GPX7 in BAR-T cells by an adenoviral system, GPX7 was significantly up-regulated after exposure to acidic bile acids (Supplementary Figure 8). This finding suggests that upregulation of GPX7 expression could be due to post transcriptional mechanisms.

Discussion

Our results indicate that the levels of GPX7 are significantly downregulated in both OAC and Barrett's dysplasia as compared to normal oesophagus and BO. This is consistent with our earlier report that demonstrated frequent DNA methylation of GPX7 promoter in OAC. [19] Glutathione peroxidase (GPX) is a widespread protein family that is evolutionarily conserved. Based on structural homology, GPX7 is a recently identified member of the glutathione peroxidase family, discovered so far in mammals. [20, 26] Unlike most of GPX family members, GPX7 incorporates cysteine instead of selenocysteine into its third conserved residue in the catalytic center. [20] While a recent study has shown lack of glutathione peroxidase activity of E.coli recombinant GPX7 [30], our results suggest that mammalian GPX7 conserves some of the glutathione activity. At the same time, GPX7 also

exhibited a potent capacity to directly neutralize H_2O_2. These results suggest the presence of anti-oxidant functions of GPX7 that could protect oesophageal epithelial cells from acid and/or bile acids-induced ROS and oxidative stress. Indeed, our results indicate that GPX7 can reduce ROS levels and inhibit H_2O_2 accumulation and release on exposure to acidic bile acids. These functions are retained in the immortalized oesophageal epithelial cells but partially lost in BO cells. This is of clinical importance since the reflux of gastric and bile acids in patients with gastrooesophageal reflux disease increases the burden of ROS on epithelial cells, elevating the risk for the development of BO and progression to OAC. [3, 31] The knockdown of endogenous GPX7 in HET1A cells led to a significant increase in the intracellular ROS, suggesting that endogenous GPX7 is necessary to maintain low ROS levels in oesophageal cells. One of the major outcomes of ROS is the induction of oxidative DNA damage which can manifest as oxidized purines and pyrimidines, single strand DNA breaks (SSBs) and double strand DNA breaks (DSB).[32, 33] Two of the most common endogenous DNA base modifications are 8-oxo-7,8-dihydroguanine(8-oxoG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy guanine). [32, 33] Acid and/or bile acids are potent inducers of oxidative DNA damage in oesophageal epithelia. [12, 34] The reconstitution of GPX7 into BAR-T cells significantly reduced the acid/bile acids – induced oxidative DNA damage, as measured by 8-oxoguanine and FITC-avidin staining. Moreover, using phosphorylation of H2A.X as a reliable measure of DSB [25, 35], our findings indicate that GPX7 can also protect against the accumulation of DSB in oesophageal cells. This protective capacity of oesophageal cells against acid/bile-acids –induced oxidative DNA damage was attenuated upon knockdown of endogenous GPX7 in HET1A cells. Although most of the oxidative DNA damage such as AP sites and 8-oxoG are not considered lethal to the cells, unless in high levels, they have a high mutagenic potential, if not repaired properly and promptly. The conversion of the unrepaired or mis-repaired DNA lesions-to-mutations seems to be the driving force directing a cell(s)-to- neoplastic transformation and carcinogenesis. [36] DSBs disrupt the continuity of the DNA template, essential for DNA replication and transcription. Omitted or imprecise repair of DSBs may therefore result in cell killing, small scale mutations, chromosome rearrangements, and carcinogenesis. [37] The observed frequent methylation of GPX7 [19] and its down-regulation in Barrett's dysplasia and OAC suggest that loss of GPX7 in oesophageal cells could be a turning point defining loss of the inherent protective antioxidant capacity of the oesophageal mucosa.

The fact that acidic bile acids induce oxidative DNA damage and double strand breaks may also lead to apoptosis, if the DNA damage is not repaired. Therefore, ROS may regulate cell survival, proliferation or apoptosis. [38, 39] In this context, we found that downregulation of GPX7 leads to more apoptosis following a short term 10 minute exposure to acid/bile acids in normal squamous epithelia than in Barrett's oesophagus cells. This may indicate that GPX7 functions are highly protective in normal squamous epithelia, but not in Barrett's oesophagus cells. Alternatively, this may also suggest that BO cells have acquired some pro-survival properties that are not present in normal squamous cells. Of note, the presence of GPX7 was sufficient to inhibit apoptosis in both BAR-T and HET1A cells. ROS, more importantly H_2O_2, can also function as second messengers leading to activation of a complex set of ROS signaling events. Several studies have shown that ROS activates JNK and p38 by promoting their phosphorylation at Thr183/Tyr185 and THr180/Tyr182, respectively. [40] Our findings that GPX7 can regulate the JNK and p38 are consistent with GPX7 activity against ROS and H_2O_2 accumulation. Although our findings are in agreement with the anti-ROS protective functions of GPX7, we can not exclude the presence of additional mechanisms such as those recently described suggesting that GPXs could be more than anti-oxidants and may be involved in the direct peroxide-dependent activation of transcription factors (for review[26]).

GPX7 expression was upregulated in normal oesophageal epithelial cells (HET1A and EPC2) after exposure to pH4 bile acids. A similar induction was not observed in BAR-T cells. This finding could be related to the presence of epigenetic suppression mechanism of GPX7 expression in BAR-T cells. Of note, BAR-T cells have lower levels of GPX7 expression as compared to normal oesophageal cells. Epigenetic downregulation of GPX7 has been shown in a subset of BO samples [19]. On the other hand, following full reconstitution of GPX7 using adenoviral system restored the ability of BAR-T cells to respond to acid bile acids and GPX7 levels were upregulated following treatment. This suggests that upregulation of GPX7 expression could be due to post transcriptional mechanisms in the form of increased RNA stability and/or protein stability; future studies are needed to verify the mechanism(s).

In summary, our data suggest that normal oesophageal epithelial cells have an inherent protective capacity to respond to acidic bile acids; this capacity could be defective during progression to OAC. We have demonstrated that GPX7 suppresses acidic bile acids- induced ROS and protects against oxidative DNA damage and double strand breaks in oesophageal epithelial cells. The progressive epigenetic downregulation of GPX7 during the multi-step Barrett's tumorigenesis is likely a risk factor for accumulating DNA damage events, a driving force for OAC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by grants from the National Institute of Health; R01CA106176 (WER) and R01CA138833 (AZ); Vanderbilt SPORE in Gastrointestinal Cancer (P50 CA95103), Vanderbilt Ingram Cancer Center (P30 CA68485) and the Vanderbilt Digestive Disease Research Center (DK058404). The contents of this work are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute or Vanderbilt University.

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What is already known about this subject

- Glutathione peroxidase 7 GPX is a novel protein,0020identified through sequence homology, with unknown functions
- Oesophageal epithelial cells express high levels of GPX7; GPX7 is frequently silenced by epigenetic mechanisms in the progression of Barrett's oesophagus and oesophageal adenocarcinomas.
- Acid and Bile acids induced reactive oxygen species and oxidative stress play a critical role in the development of Barrett's oesophagus and its progression.

What are the new findings

- This study demonstrates the potent protective anti-oxidant functions of glutathione peroxidase 7 in oesophageal cells.
- GPX7 regulates the levels of reactive oxygen species following acid/bile-acid treatments in glutathione dependent and independent manners. This function neutralizes the levels of ROS in oesophageal epithelial cells.
- Loss of GPX7 leads to accumulation of increase in the levels of H2O2, intracellular ROS, oxidative DNA damage and double stranded DNA breaks in the cells.
- GPX7 regulates ROS signaling by modulating p38 and JNK pathways in response to acid/bile-acids.

How might it impact on clinical practice in the foreseeable future?

- This study, taken together with our previous study of human tissues (Gut, 2009 Jan;58 (1):5–15), provide a direct evidence of the value of GPX7, suggesting that loss of GPX7 could be a turning point defining an altered response to acid/bile-acid reflux in patients with GORD. Therefore, it is possible that patients with hypermethylation of GPX7 could be at higher risk of accumulation of mutations, DNA damage lesions and progression of BO.

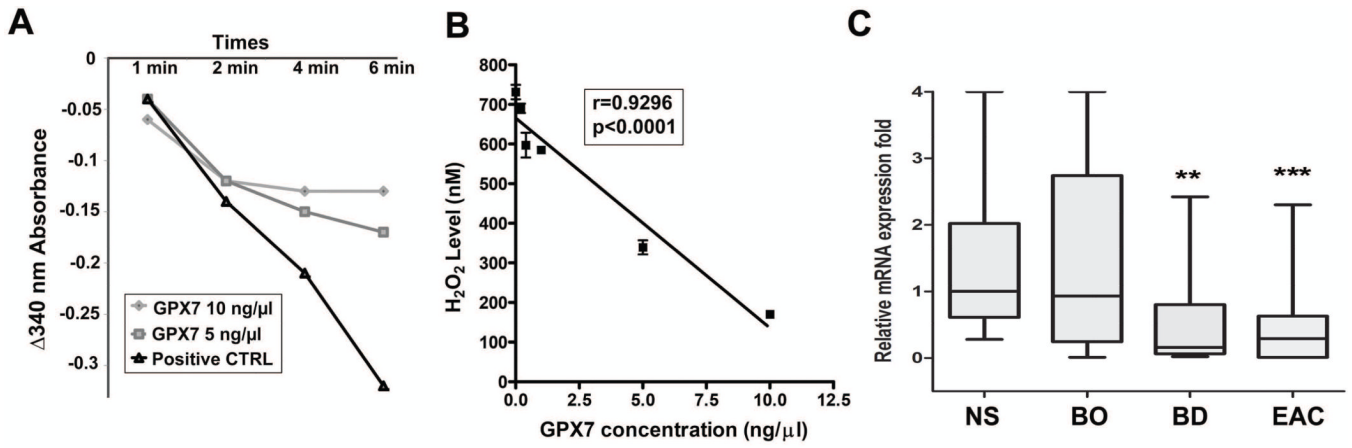

Figure 1. GPX7 neutralizes H_2O_2 independent of glutathione

A) Glutathione peroxidase activity assay was performed using a recombinant GPX7 protein. The change of the absorbance at 340 nm (Δ340 nm) was used to evaluate the relative glutathione peroxidase activity compared to a glutathione peroxidase positive control. **B)** Amplex UltraRed peroxidase assay was performed using recombinant GPX7 protein. An inverse relationship between the level of GPX7 and H_2O_2 was observed ($r=0.92$, $p<0.0001$). C) GPX7 expression was significantly downregulated in Barrett's dysplasia (BD) and adenocarcinomas (EAC), in comparison with normal oesophagus (NS).

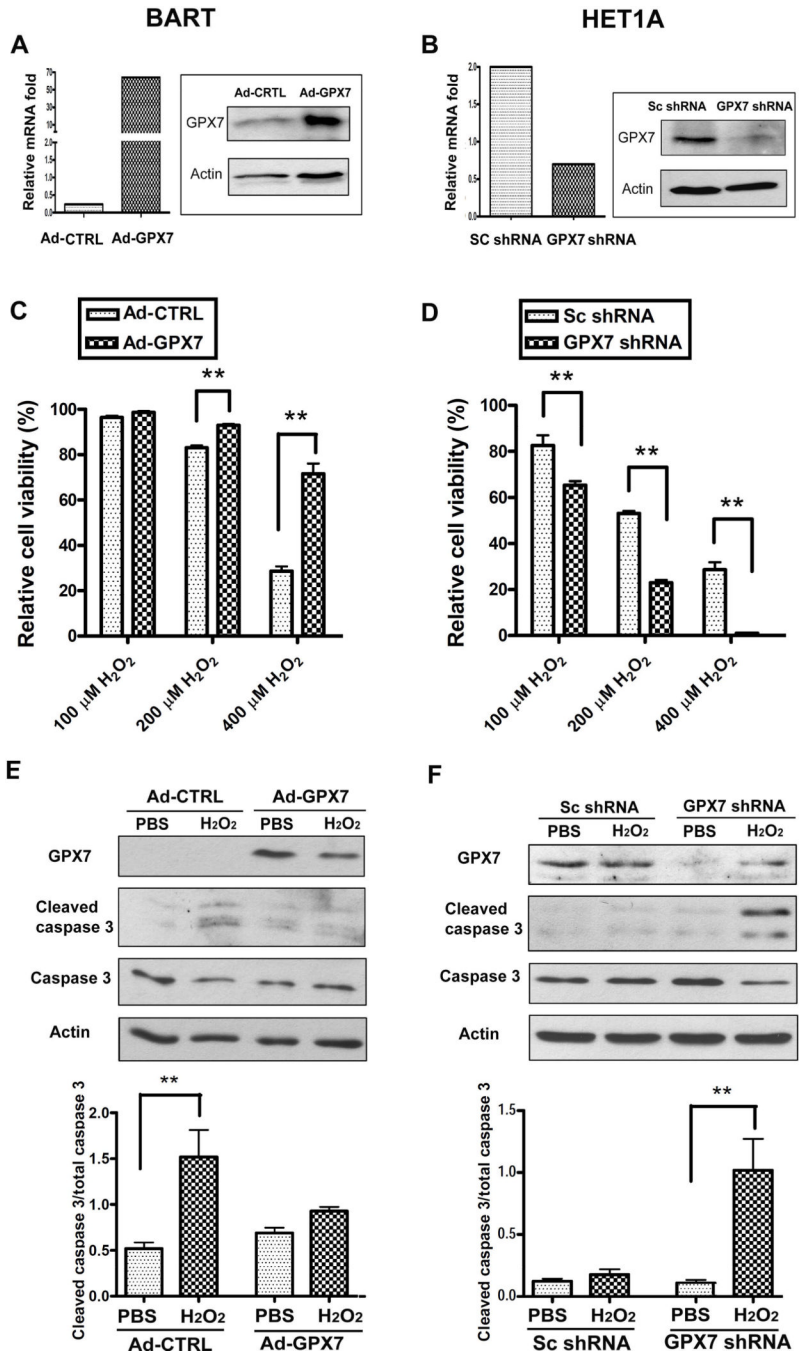

Figure 2. GPX7 protects oesophageal epithelial cells from H_2O_2 induced oxidative stress

A) BAR-T cells were reconstituted with a GPX7 expression adenoviral system. The levels of mRNA and protein in the control (Ad-CTRL) and GPX7 expressing cells (Ad-GPX7) are shown. **B)** HET1A cells were used for knockdown of GPX7 with a GPX7 shRNA lentiviral system. The levels of mRNA and protein in control (Sc shRNA) and GPX7 knockdown cells (GPX7 shRNA) are shown. BAR-T cells and HET1A cells were treated with different concentrations of H_2O_2 for 16h, and then cell viability was measured using ATPGlo assay. The results were normalized to the values in cells treated with PBS. The results demonstrate an increase in cell viability in GPX7 expressing BAR-T cells **(C)** and a decrease in cell

viability in GPX7 knockdown HET1A cells **(D). E–F)** Western blot analysis for cleaved caspase 3 in BAR-T (E) and HET1A cells (F). ImageJ software was used to determine the bands density of cleaved and total caspase 3. A histogram presenting the ratio of cleaved caspase 3 to total caspase 3 is displayed below the western blot images of BAR-T and HET1A respectively. **, $P<0.01$.

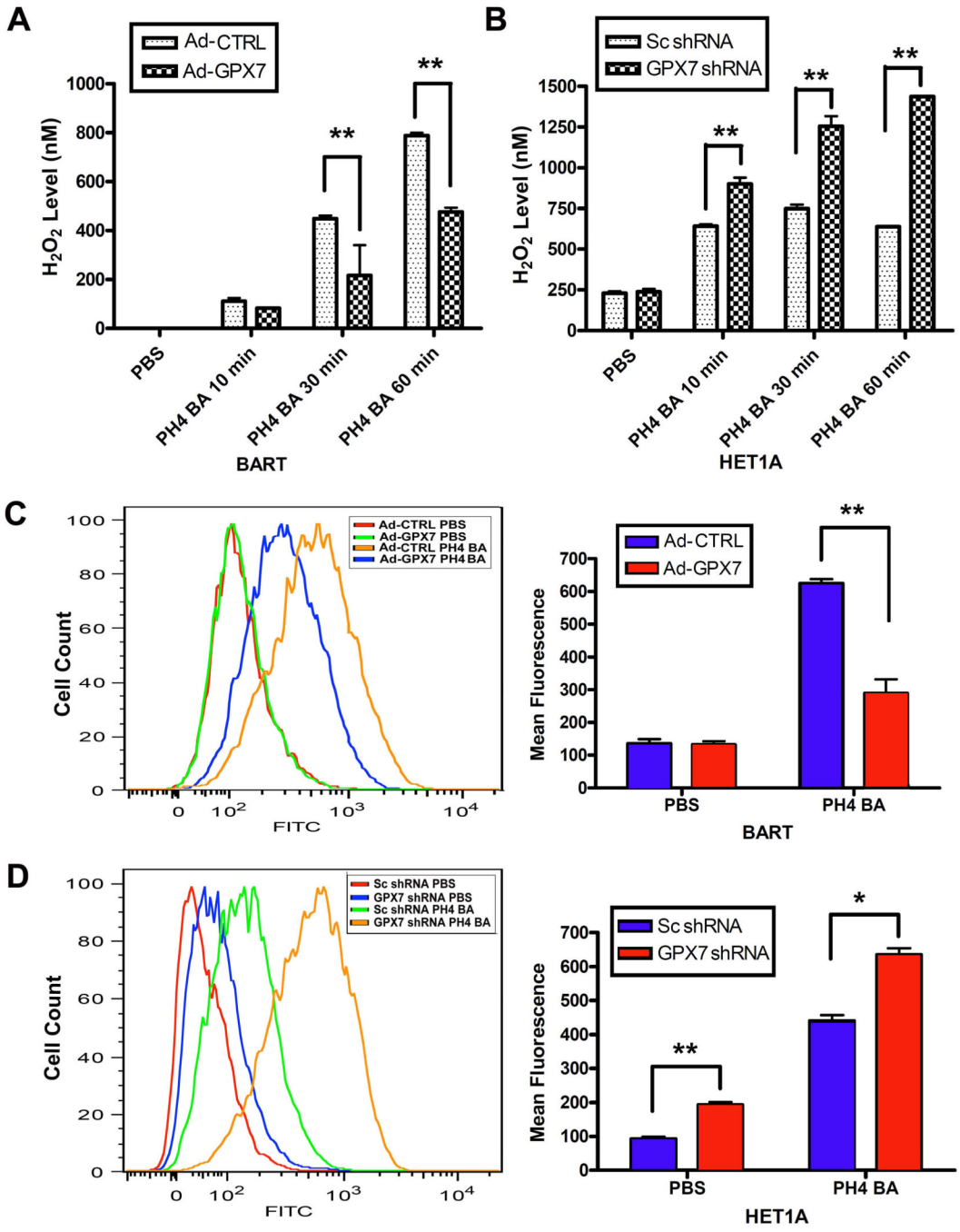

Figure 3. GPX7 decreases the H_2O_2 levels and intracellular ROS level in oesophageal epithelial cells upon pH 4 bile acids exposure

A–B) BAR-T cells and HET1A cells, as in Figure 2, were exposed to pH 4 bile acids (PH4 BA) for 10, 30, and 60 min. The media from BAR-T (**A**) and HET1A (**B**) cells were then subjected to Amplex UltraRed hydrogen peroxide assay to measure the H_2O_2 level. H_2O_2 concentrations were retrieved by comparing to standard curve. **C–D)** To measure intracellular ROS, BAR-T (**C**) cells and HET1A (**D**) cells were treated with pH 4 bile acids for 30 min, and then subjected to flow cytometry analysis of CM-H_2DCFDA. In (**C**) (BAR-T) and (**D**) (HET1A), left panels show representative flow cytometry histograms whereas the right panels depict the quantitative data from flow cytometry. * P<0.05, ** P<0.01.

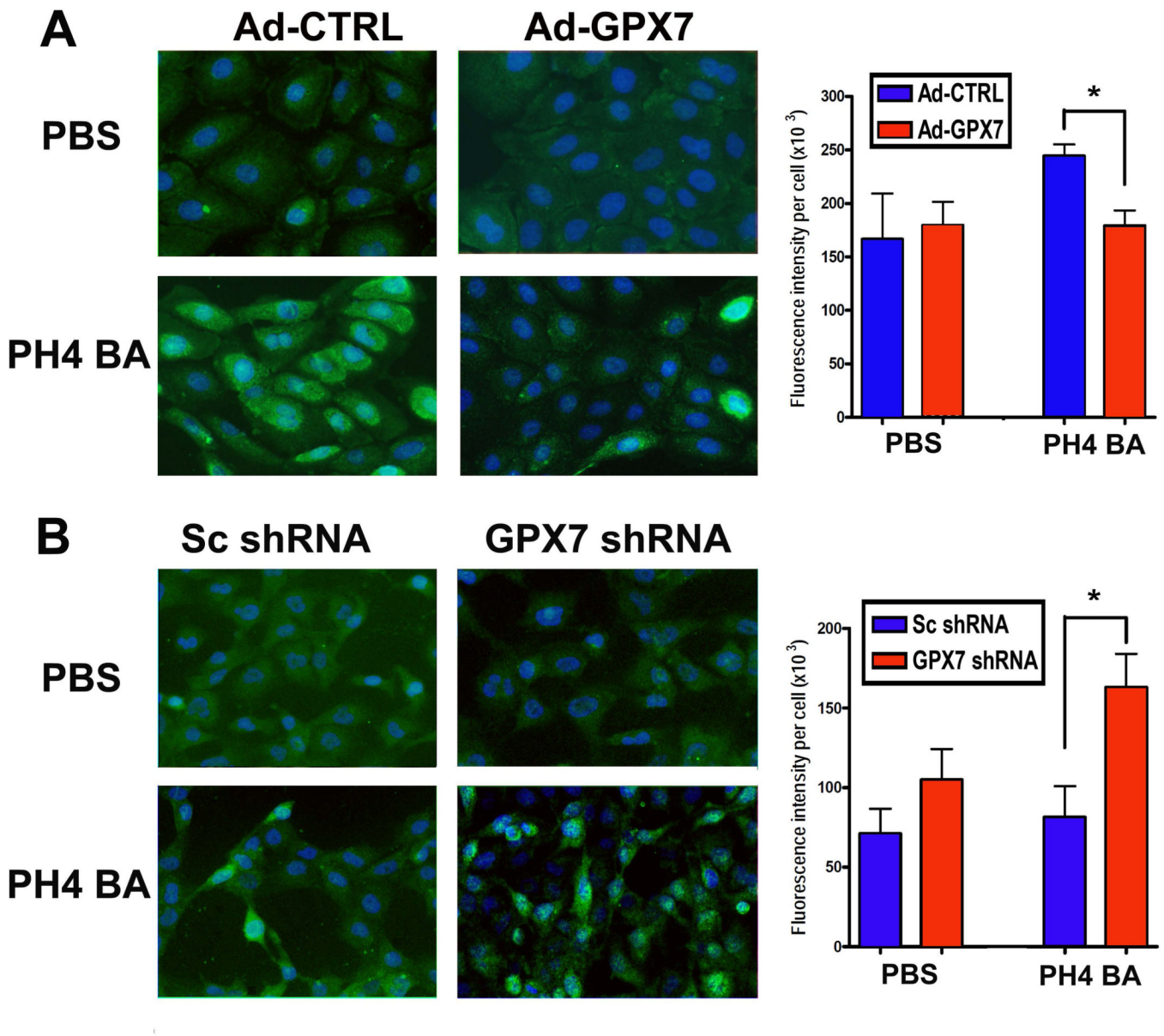

Figure 4. GPX7 decreased pH 4 bile acids-induced oxidative DNA damage

A–B) Immunofluorescence staining of 8-oxoguanine (green) in BAR-T cells (**A**) and HET1A cells (**B**) following the exposure to pH 4 bile acids (PH4 BA). The quantification of immunostaining results using ImageJ is shown in the right panels. * P<0.05. The reconstitution of GPX7 in BAR-T led to a significant reduction in fluorescence intensity per cell for 8-oxoguanine (**A**) whereas the knockdown of GPX7 in HET1A cells led to a significant increase in the fluorescence intensity per cell for 8-oxoguanine (**B**).

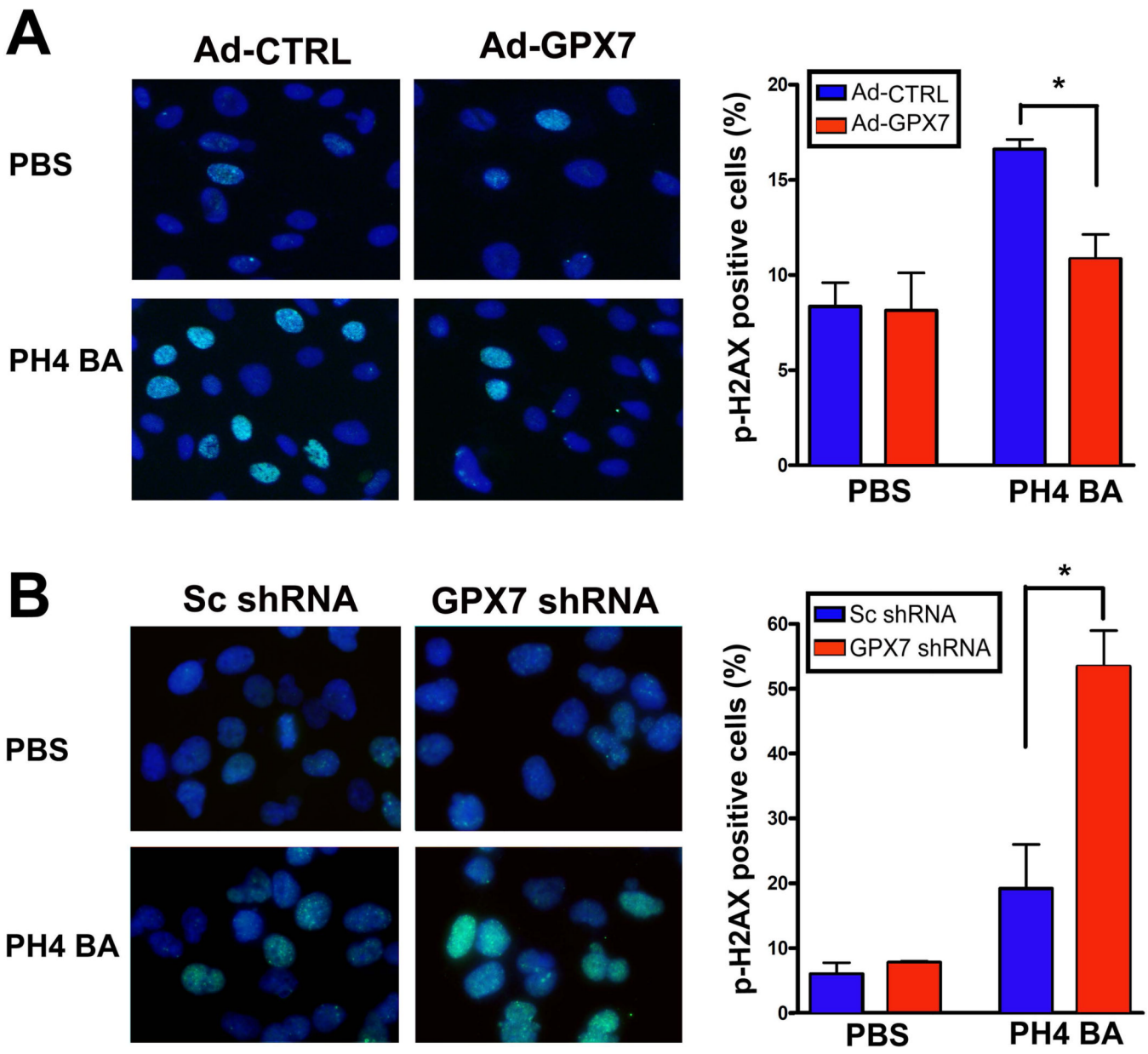

Figure 5. GPX7 reduces pH 4 bile acid-induced double strand breaks

A–B) Immunofluorescence staining of phospho-H2A.X (green) in BAR-T cells **(A)** and HET1A cells **(B)** following the exposure to pH 4 bile acids (PH4 BA). The quantification of immunostaining results from ImageJ is shown in the right panels. * P<0.05. The reconstitution of GPX7 in BAR-T led to a significant reduction in percentage of cells positive for phospho-H2A.X **(A)** whereas the knockdown of GPX7 in HET1A cells led to a significant increase in the percentage of cells positive for phospho-H2A.X **(B)**.

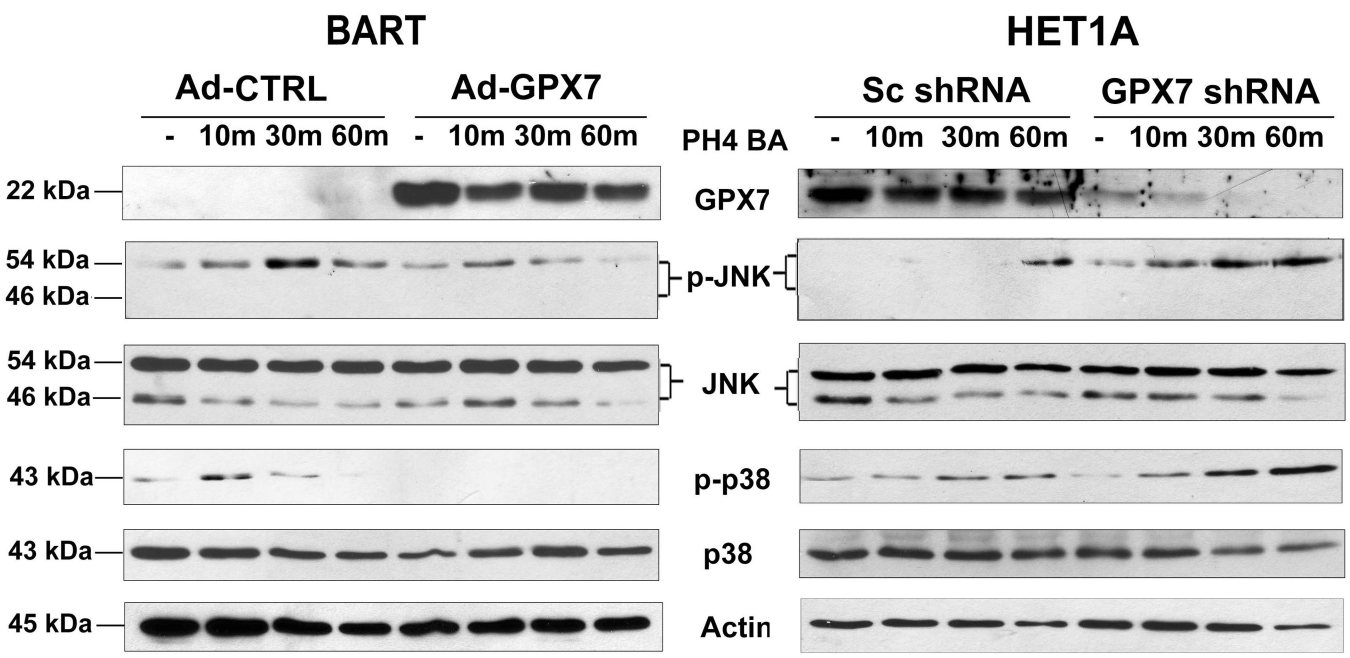

Figure 6. GPX7 suppresses ROS signaling on exposure to pH 4 bile acids

BAR-T cells and HET1A cells were exposed to pH 4 bile acids (PH4 BA) for 10, 30, and 60 minutes. Western blotting analysis demonstrated lower levels of phospho-JNK and phospho-p38 in BAR-T cells following reconstitution of GPX7 (Ad-GPX7) as compared to control (AD-CTRL) (left panel), whereas HET1A cells demonstrated higher levels of phospho-JNK and phospho-p38 following shRNA knockdown of GPX7 as compared to control sc shRNA (right panel).

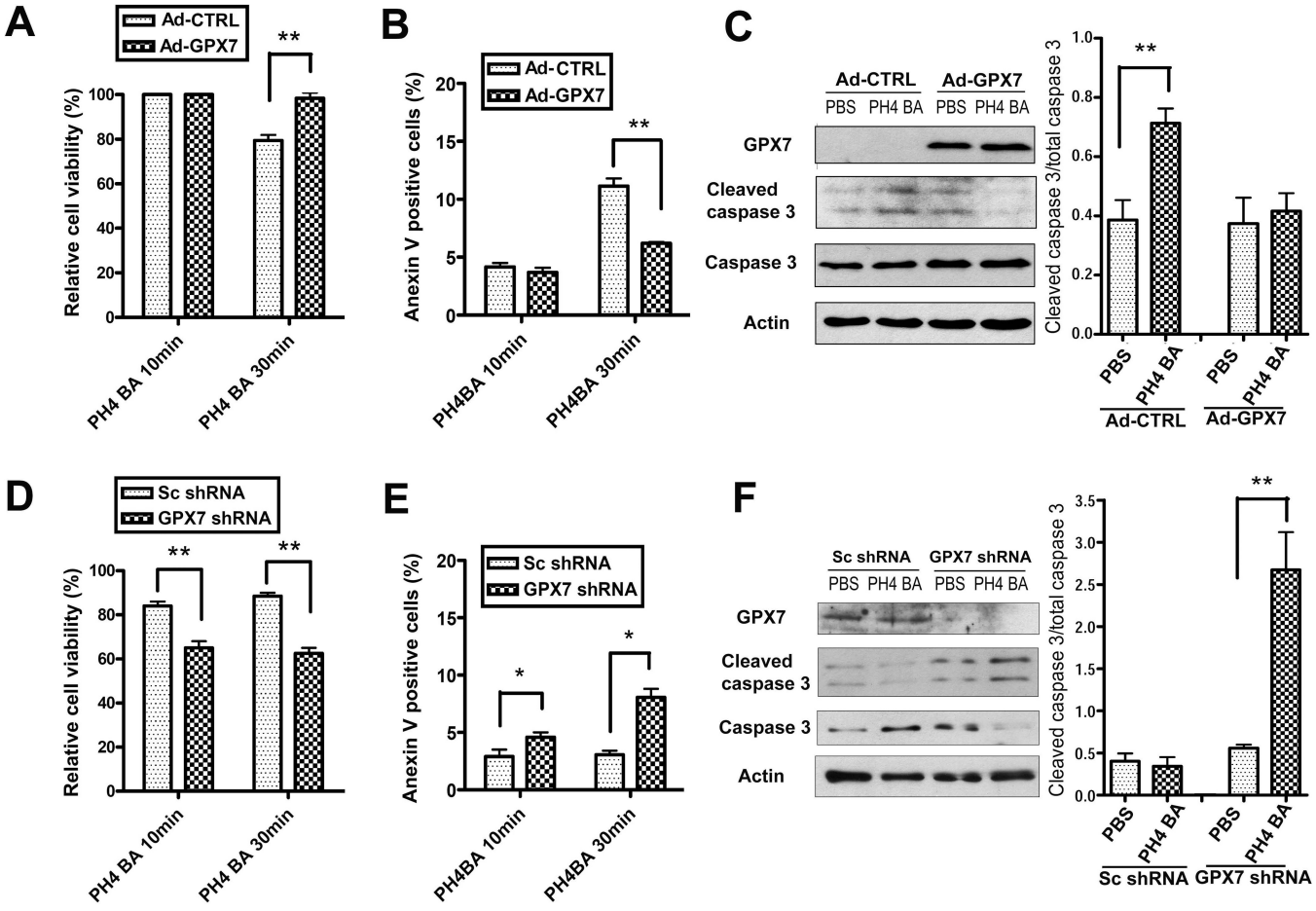

Figure 7. GPX7 protects oesophageal epithelial cells from acidic bile acids-induced apoptosis

BAR-T **(A–C)** and HET1A **(D–F)** cells were exposed to pH 4 bile acids (PH4 BA) for 10 and 30 minutes. **A)** ATPGlo cell viability assay demonstrated a significant increase in the percentage of viable cells following reconstitution of GPX7 (Ad-GPX7) as compared to control (Ad-CTRL) at the 30 min time point. **B**) Flow cytometry analysis of Annexin V apoptosis assay showed lower percentage of apoptotic cells following reconstitution of GPX7 (Ad-GPX7) as compared to control (Ad-CTRL) at the 30 min time point. **C)** Western blot analysis of cleaved caspase 3. The ratio of cleaved caspase 3 to total caspase 3 was quantified using ImageJ and is shown in the right of the western blotting images. **, $P<0.01$. The results demonstrate lower levels of cleaved caspase 3 in cells with reconstitution of GPX7, following exposure to PH4 BA. **D–F)** The knockdown of GPX7 in HET1A as compared to control sc shRNA cells, led to reduction in cell viability (**D**), increase in Annexin V positive cells (**E**) and an increase in the level of cleaved caspase 3 (**F**), following exposure to PH4 BA.

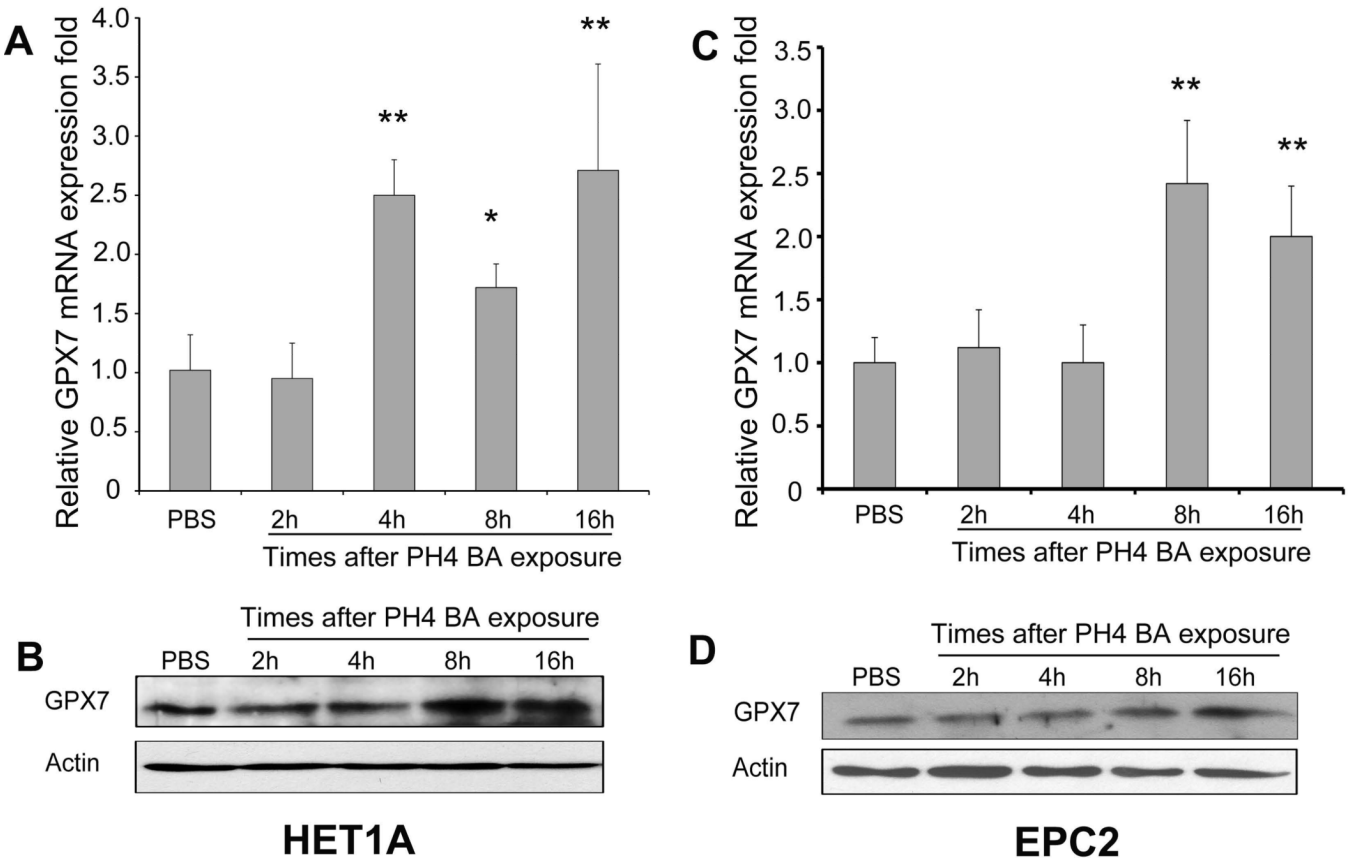

Figure 8. GPX7 expression was upregulated after acidic bile acids exposure in HET1A and EPC2 cells

HET1A and EPC2 cells were exposed to pH4 bile acids for 30 min, then cells were washed in PBS and cultured in regular medium for the indicated time points. Quantitative real time RT PCR and western blot analysis were used to determine mRNA (A & C) and protein levels (B & D) in HET1A and EPC2.