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Peroxiredoxin V Silencing Elevates Susceptibility to Doxorubicin-induced Cell Apoptosis *via* ROS-dependent Mitochondrial Dysfunction in AGS Gastric Cancer Cells

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Abstract. *Background/Aim: Peroxiredoxin V (Prx V) plays crucial roles in cellular apoptosis and proliferation in various cancer cells by regulating the cellular reactive oxygen species (ROS) levels. Materials and Methods: Here, we examined the possible regulatory effects of Prx V on doxorubicin (DOX)-induced cellular apoptosis and its mechanisms in the human gastric adenocarcinoma cell line (AGS cells). Results: Our findings suggest that Prx V knockdown may significantly increase the DOX-induced apoptosis by aggravating intracellular ROS accumulation. We also found that DOX-induced mitochondrial ROS levels and membrane permeability were significantly higher in short hairpin Prx V cells than in mock cells, and these phenomena were dramatically reversed by ROS scavenger treatment. Prx V knockdown also significantly upregulated the cleaved caspase 9, 3, and B-cell lymphoma 2 (Bcl2)-associated agonist of cell death/Bcl2 protein expression levels, suggesting that Prx V knockdown activates mitochondria-dependent apoptotic signaling pathways. Conclusion: Taken together, this study suggests that Prx V may be a strong molecular target for gastric cancer (GC) chemotherapy, and further elucidates the role of Prx V in oxidative stress-induced cell apoptosis.*

Gastric cancer (GC) is a major health burden and the fifth most common cancer worldwide (1). However, the lack of clear molecular markers can lead to late diagnosis, resulting in a loss of the optimal time for surgery. Currently, traditional chemotherapy is regarded as the main treatment for advanced GC, especially in developing countries (2, 3). Chemotherapy has always been considered the best treatment option for advanced and metastatic GC.

Doxorubicin hydrochloride (DOX) is an anthracycline cytotoxic antibiotic, which is a broad-spectrum antineoplastic drug (4). DOX can induce cell death through oxidative stress (5, 6), energetic stress (7), and DNA damage (8) *via* the accumulation of intracellular reactive oxygen species (ROS) in several cancer cell types (6, 9). The administration of DOX alone or in combination with cisplatin, fluorouracil, or paclitaxel (10) is common in a variety of malignancy treatments, including GC. However, the effective reduction of DOX resistance or improvement in the tumor sensitivity of DOX remains the most significant problem in the treatment of GC.

Peroxiredoxin V (Prx V) is the fifth member of the Prx family and also known as atypical 2-Cys-Prx (11, 12). It is the smallest member of the family and exists in the cytoplasm,

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mitochondria, and peroxisomes. According to our previous studies, Prx V plays crucial roles in oxidative stress-induced cell apoptosis *via* regulating the cellular ROS levels and mitochondrial signaling cascades in the colon, liver, lung, and GC cells (13-15). It is also well known that Prx V serves as a scavenging agent for ROS and reactive nitrogen species upon inflammatory and oxidative damages in both tissues and immune cells in vivo and in vitro (16, 17). Previous research showed that Prx V may be a key molecule that plays a regulatory role in the treatment of various cancers by regulating intracellular ROS levels. When Prx V is overexpressed, it can protect HT22 neuronal cells against the apoptosis induced by glutamate induced Ca^{2+} and ROS increase (16, 18, 19). However, silencing Prx V can increase the sensitivity of colon cancer cells to ROS stimulation and promote cellular apoptosis in SW480 cells (13). At the same time, we reported that Prx V may also play a regulatory role in the emodin-induced AGS cell apoptosis. The Prx V content in AGS cells directly affects the proportion of emodin-induced AGS cell death *via* the regulation of intracellular ROS levels (14). However, the possible molecular and intracellular signaling mechanisms of Prx V have not yet been understood in GC cell apoptosis upon anticancer drug therapy, such as DOX.

In the present study, we used AGS cells [the human gastric adenocarcinoma cell line CRL-1739 from the American Type Culture Collection (ATCC)] and constructed mock and short hairpin (sh)RNA Prx V stable cell lines in order to understand the role of Prx V in DOX-induced cell apoptosis. Cellular apoptosis, ROS levels, mitochondrial membrane potentials, and apoptosis-related protein expression levels were measured in these two modified AGS cells after treatment with DOX. Our results provide novel insights for understanding the function of Prx V in GC treatment.

Materials and Methods

Cell culture and media. AGS human gastric adenocarcinoma cells were obtained from the ATCC (Manassas, VA, USA). AGS cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Solarbio Life Science, Beijing, PR China), at 37˚C in a humidified incubator with 5% CO_2.

DOX and N-acetyl cysteine (NAC) treatment. DOX was purchased from Sigma-Aldrich (St. Louis, MO, USA) and applied to AGS cells at the indicated concentrations (0, 0.5, 1, 2 and 4 μM) for 24 h. In the NAC pre-treatment group, we added 5 mM of NAC into the culture medium before DOX treatment for 30 min, followed by co-treatment with DOX for 24 h.

Construction of stable Prx V knockdown AGS cells. ShRNA specific to Prx V (shPrx V LV3, H1-Puromycin) and control shRNA LV3 (H1-Puromycin) lentivirus vectors were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The targeted sequence of shPrx V was 5'-GGAATCGACGTCTCAAGAGGT-3', and the targeted sequence of the negative control was 5'-GTTCTCCGAAC GTGTCACGT-3'. We seeded 1×10^4 AGS cells/well in a 6-well cell culture plate (NEST Biotechnology, Wuxi, China) for 24 h (37˚C and 5% CO_2) prior to transfection. The culture medium was replaced with polybrene (0.5 μg/ml; Shanghai GenePharma Co., Ltd., Shanghai, PR China) containing lentivirus with a multiplicity of infection of 50 (MOI=50) for 48 h and subsequently changed into complete culture medium (DMEM with 10% FBS and antibiotics). Infected cells were selected *via* treatment with puromycin. Western blotting was used to analyze the expression levels of Prx I-VI proteins 3 days after selection.

Western blot analysis. Cell proteins (20 μg), lysed using lysis buffer, were separated on 12% sodium dodecyl sulfate polyacrylamide gels and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blotted with primary antibodies against Prx I (#bsm-52127R; Bioss Biology, Beijing, PR China), Prx II (#LF-MA0144; AbFrontier, Seoul, Republic of Korea), Prx III (#sc-59661; Santa Cruz Biotechnology, Dallas, TX, USA), Prx IV (#sc-376668; Santa Cruz Biotechnology), Prx V (#sc-133073; Santa Cruz Biotechnology), Prx VI (#sc-134478; Santa Cruz Biotechnology), Caspase 9 (#9505; Cell Signaling Technology, Beverly, MA, USA), B-cell lymphoma 2 (Bcl2; #sc-7382; Santa Cruz Biotechnology), Bcl2-associated agonist of cell death (Bad; #bs-0892R; Bioss Biology), Caspase 3 (#9661; Cell Signaling Technology), and β-actin (#ab72911; Abcam, Cambridge, UK) (dilutions of 1:2,000) at 4˚C for 6 h after being blocked with skimmed milk for 1 h. The membranes were then washed five times with 10 mM Tris-HCl (pH 7.5), containing 150 mM NaCl (tris-buffered saline [TBS]) and 0.2% Tween 20, and subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sangon Biotech, Shanghai, PR China) or anti-mouse IgG (Sangon Biotech) for 1 h at room temperature. After the removal of excess antibodies by washing with TBS, specific binding was detected using a chemiluminescence detection system (Amersham, Berkshire, UK) according to the manufacturer's instructions. The data were analyzed in ImageJ and a histogram was generated using the same software.

Cell viability assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to examine cell viability. The cells were inoculated at a density of 1×10^4 cells/well in 96-well plates (NEST Biotechnology) and treated with DOX at different doses for 24 h (37˚C and 5% CO_2). The control cells were treated with normal medium alone. The accumulation of formazan (dissolved with dimethyl sulfoxide) was determined following the addition of MTT reagent (0.5 mg/ml), and the absorbance was measured at a wavelength of 490 nm. A UVmax kinetic microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) was used to detect the absorbance.

Cell apoptosis assay. The cells were recycled using trypsin, re-suspended in apoptosis stain buffer, and then stained with annexin V-phycoerythrin (PE) according to the manufacturer's protocol for the apoptosis detection kit (Solarbio Life Science). The cells were washed once with phosphate-buffered saline. Fluorescence microscopy and flow cytometry analysis were used to check for annexin V-PE-positive cells. The flow cytometry results were analyzed with the WinMDI (version 2.9; BD Biosciences, San Jose, CA, USA) software.

Intracellular and mitochondrial ROS analysis. Dihydroethidium (DHE; Beyotime Biotechnology, Shanghai, PR China) and MitoSOX Red (Thermo Fisher Scientific, Waltham, MA, USA) were used as fluorescent probes to indicate changes in intracellular and mitochondrial ROS levels. The DOX-treated AGS and control cells were collected, washed, and stained with DHE and MitoSOX. Fluorescence microscopy and flow cytometry analysis were used to measure the intracellular and mitochondrial ROS levels.

Mitochondrial membrane potential ability assay. The cell mitochondrial membrane potential was detected using JC-1 dye (Solarbio Life Science). DOX-treated and control AGS cells were collected and stained with JC-1 dye. Fluorescence microscopy was used to examine the JC-1-positive cells.

Statistical analysis. Data from at least three independent experiments are presented as mean±standard errors of the mean. A two-way analysis of variance was used to analyze changes over time and differences between groups in each experiment. For most experiments, Tukey's *post hoc* test (α=0.05) was used to determine the statistical significance between two groups. All statistical analyses were conducted using the SPSS Statistics software (version 25; IBM SPSS, Armonk, NY, USA). Differences were considered statistically significant if $p<0.05$ ($*p<0.05$, $**p<0.01$, and $***p<0.001$).

Results

Knockdown of Prx V increases DOX-induced cell death in AGS cells. In order to explore the regulatory mechanism of Prx V in the process of DOX-induced AGS cell death, we constructed Prx V gene-silenced (shRNA Prx V) and mock empty-vector AGS cell lines with lentiviral vectors. Western blot analysis showed that the lentiviral vector significantly reduced Prx V protein expression level while having no effect on the expression levels of other Prx family members in AGS cells (Figure 1A and B). To determine the effect of Prx V on DOX-induced cell death, the mock and shPrx V cells were treated with various DOX concentrations (0, 0.5, 1, 2, 4 μM) for 24 h. As shown in Figure 1C, knockdown of Prx V significantly decreased the DOX-induced viability of AGS cells.

Knockdown of Prx V increases DOX-induced apoptosis and ROS accumulation in AGS cells. To analyze the effect of Prx V on DOX-induced apoptosis, we performed apoptosis detection analysis *via* staining with annexin V-PE, a marker of apoptosis, and conducted flow cytometry and fluorescence microscopy analyses of the mock and shPrx V AGS cell lines. The results showed that DOX treatment significantly increased cellular apoptosis in a dose- and time-dependent manner both in mock and shPrx V AGS cells, and shPrx V AGS cells showed more susceptibility to DOX stimulation than mock cells (Figure 2A-D). Considering that the main function of Prx V is to target ROS, we measured the ROS levels in AGS cells after DOX treatment. As shown in Figure 2E and F, with increased DOX treatment concentrations, the intracellular ROS accumulation was also significantly elevated both in mock and shPrx V cells; however, shPrx V cells were more strongly affected than the mock cells.

Knockdown of Prx V increases DOX-induced apoptosis via mitochondria-dependent pathways. To further analyze the relationship between Prx V and cell apoptosis, we detected the ROS levels localized in mitochondria as well as the changes in mitochondrial membrane potentials, with MitoSox (a mitochondrial ROS detection dye) and JC-1 (a mitochondrial membrane potential detection dye) staining using flow cytometry and fluorescence microscopy analyses. Statistical analysis of the flow cytometry results showed that knockdown of Prx V markedly increased the mitochondrial ROS accumulation upon DOX stimulation in AGS cells (Figure 3A). Statistical analysis of the mitochondrial membrane potential results showed that Prx V silencing exacerbated the DOX-induced loss of mitochondrial membrane potential in AGS cells (Figure 3B). Analysis of proteins related to the classical intrinsic apoptosis pathway revealed that DOX treatment upregulated the pro-apoptotic proteins, such as cleaved caspase 3 and 9, and the Bad/Bcl2 protein expression ratio after DOX stimulation both in the mock and shPrx V AGS cells; however, these changes were more significant in shPrx V cells than in mock cells (Figure 3C-F).

Anti-apoptotic function of Prx V relies on the cellular ROS levels in AGS cells. To further confirm that DOX induces AGS cell apoptosis by increasing the intracellular ROS levels, we analyzed the ROS levels and mitochondrial membrane potentials in AGS cells after pre-treatment with ROS scavenger NAC and in combination with DOX treatments. The results showed that the increased ROS levels, which were induced by DOX treatment, declined after NAC pre-treatment in both mock and shPrx V AGS cells (Figure 4A). Simultaneously, the loss of mitochondrial membrane potentials and cellular apoptosis were also inhibited by NAC pre-treatment (Figure 4B-D), as observed using fluorescence microscopy and flow cytometry analyses. Furthermore, we also examined the effect of NAC treatment on the DOX-induced apoptotic protein expression levels in both mock and shPrx V AGS cells. As shown in Figure 5A-D, DOX induced the upregulation of pro-apoptotic proteins, such as cleaved caspase 3 and 9, and the Bad/Bcl2 protein expression ratio, all of which were significantly reversed by NAC treatment in both mock and shPrx V AGS cells.

Discussion

In recent years, the use of ROS to induce cell apoptosis has become a promising method for the treatment of malignant tumors (20). ROS, as a kind of ion and free radical with strong activity, is also a by-product of the normal metabolism of organisms and plays an indispensable role in

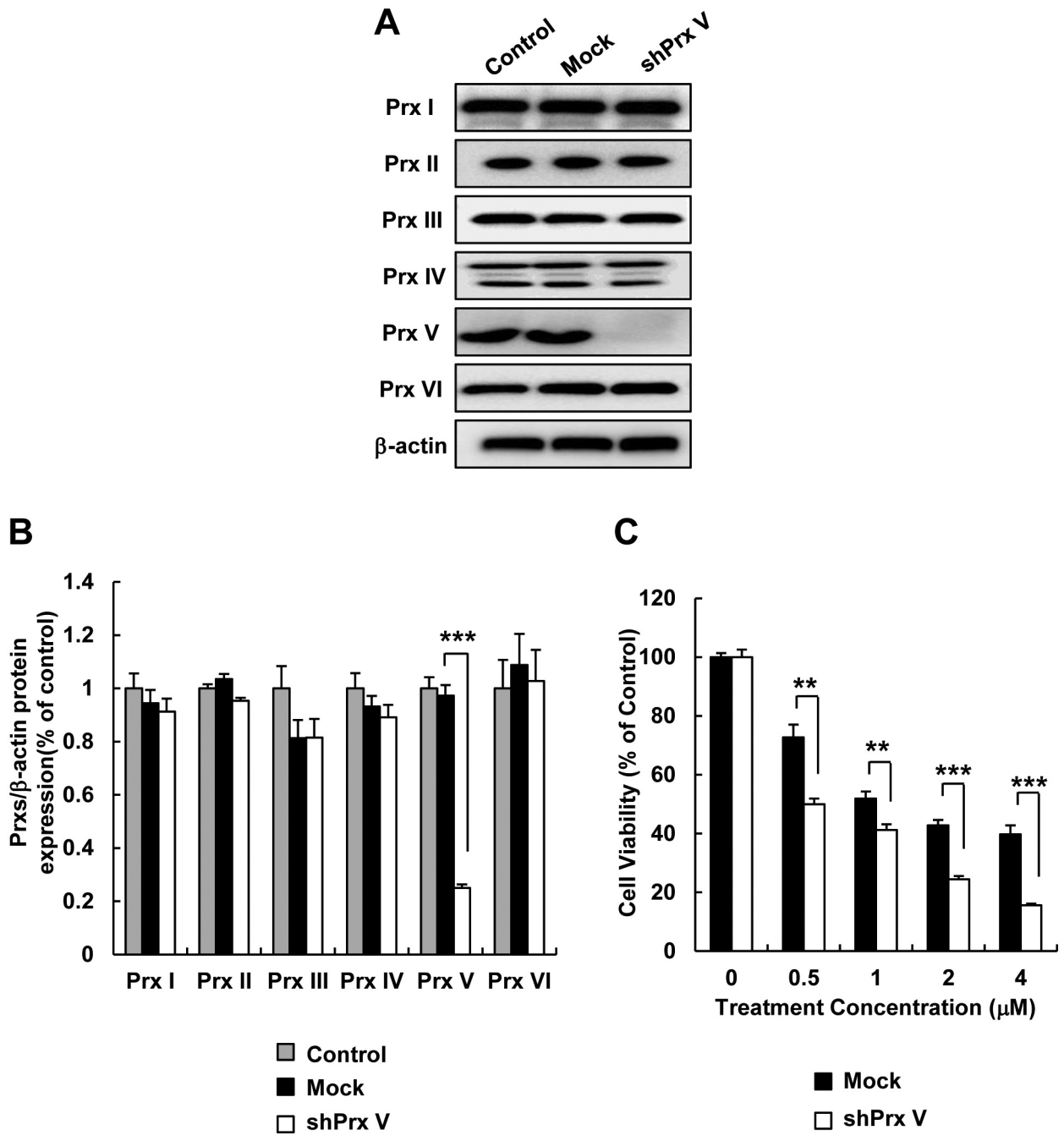

Figure 1. *Effect of DOX on the viability of AGS cells. (A) DOX construction. (B) Protein expression levels of Prx I-VI in control, mock, and shPrx V AGS cells. (C) The viabilities of mock and shPrx V AGS cells after treatment with DOX. *p<0.05, **p<0.01, and ***p<0.001 versus control. Data are presented as means; error bars indicate standard error of the mean of three different samples.*

many physiological processes (21). A low level of ROS can be used as a second messenger to regulate the migration, adhesion, and homeostasis of normal cells in vivo (22), whereas a high level of ROS exhibits cytotoxicity through lipid peroxidation (23), unsaturated fatty acids in protein amino acids, and other biological molecules (24), leading to irreversible DNA damage (25) that ultimately results in cell apoptosis or necrosis. It is widely known that redox states between cancer cells and normal cells differ, and that malignant tumor cells are more susceptible to exogenous

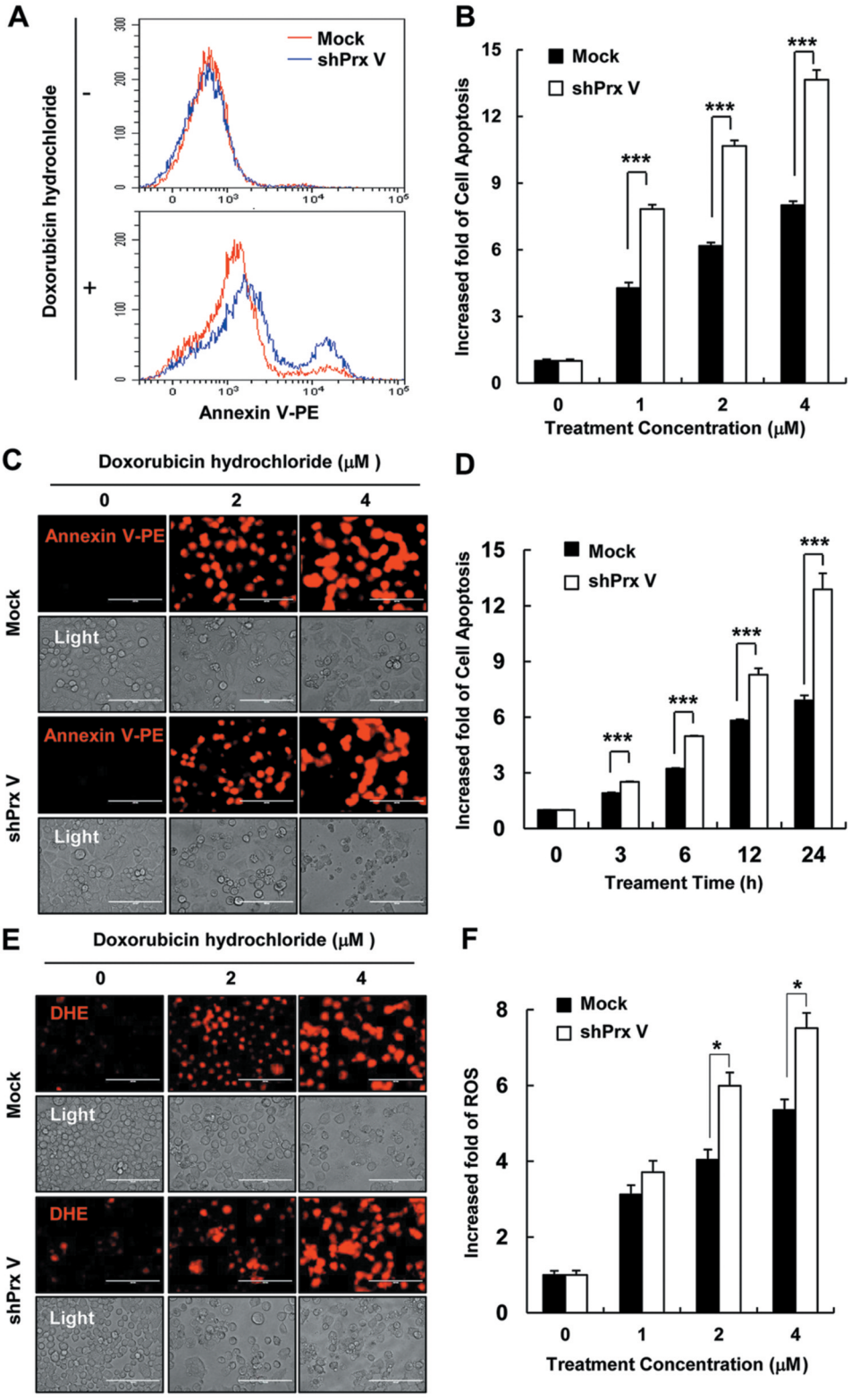

Figure 2. *Effects of DOX on the intracellular ROS levels and apoptosis. (A) Flow cytometry analysis. (B) Quantitative flow cytometry data. (C) Annexin V-PE staining to observe the effects of DOX-induced apoptosis in a dose-dependent manner (scale bar=100 μm). (D) Quantitative data from the flow cytometry analysis of DOX-induced apoptosis in a time-dependent manner. (E) DHE staining to observe the ROS levels (scale bar=100 μm). (F) Quantitative flow cytometry data. Data are presented as means; error bars indicate standard error of the mean of three different samples.*

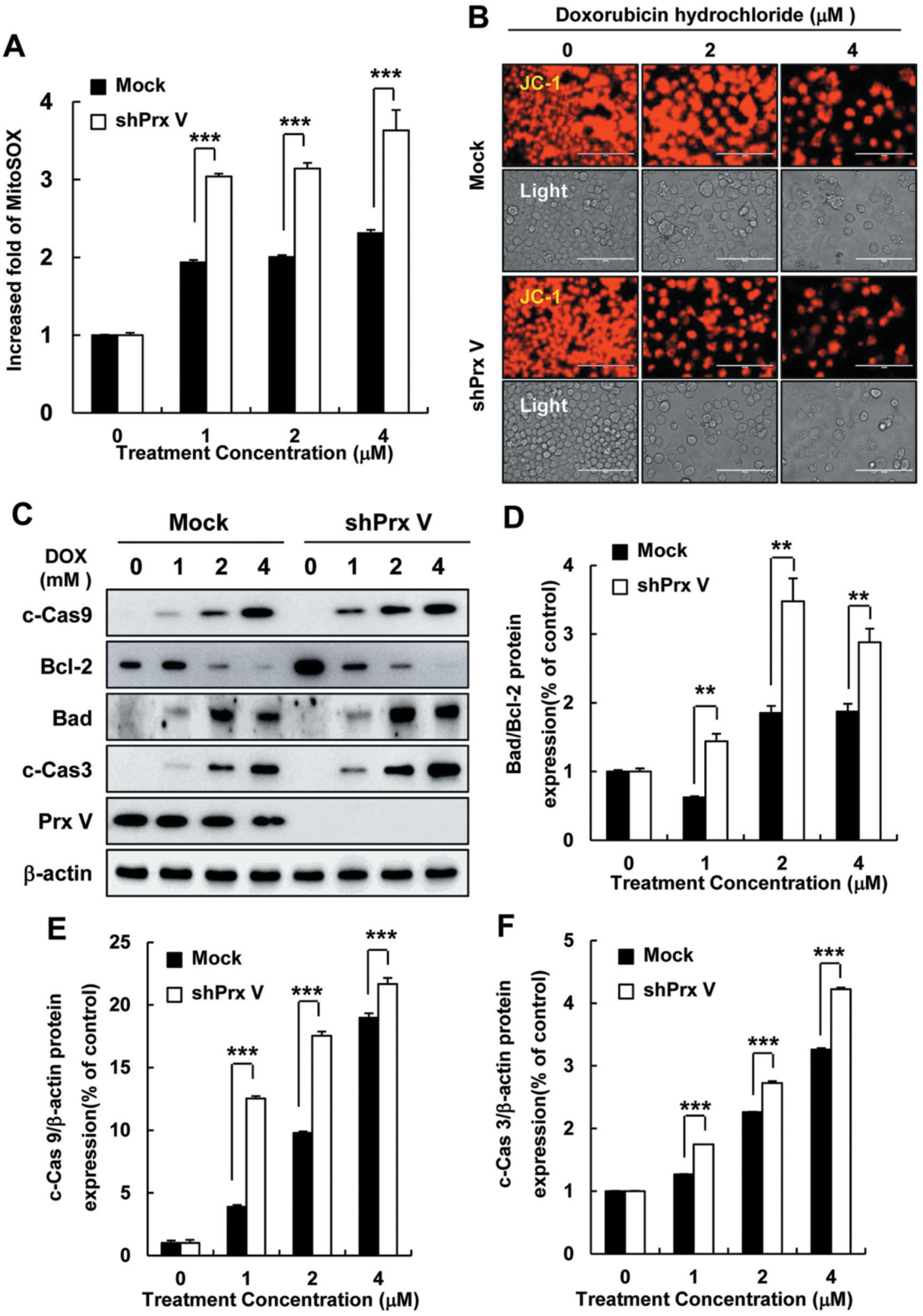

Figure 3. *DOX-induced AGS cell apoptosis via increased mitochondrial ROS levels. (A) Quantitative flow cytometry data. (B) MitoSOX staining to observe the mitochondrial ROS level. (B) JC-1 staining to observe the mitochondrial membrane potential (scale bar=100 μm). (C) Western blot analysis of the protein expression levels of the Bad/Bcl2 ratio as well as cleaved caspase 3 and 9 in mock and shPrx V AGS cells. (D-F) Quantitative western blot analysis data. Data are presented as means; error bars indicate standard error of the mean of three different samples.*

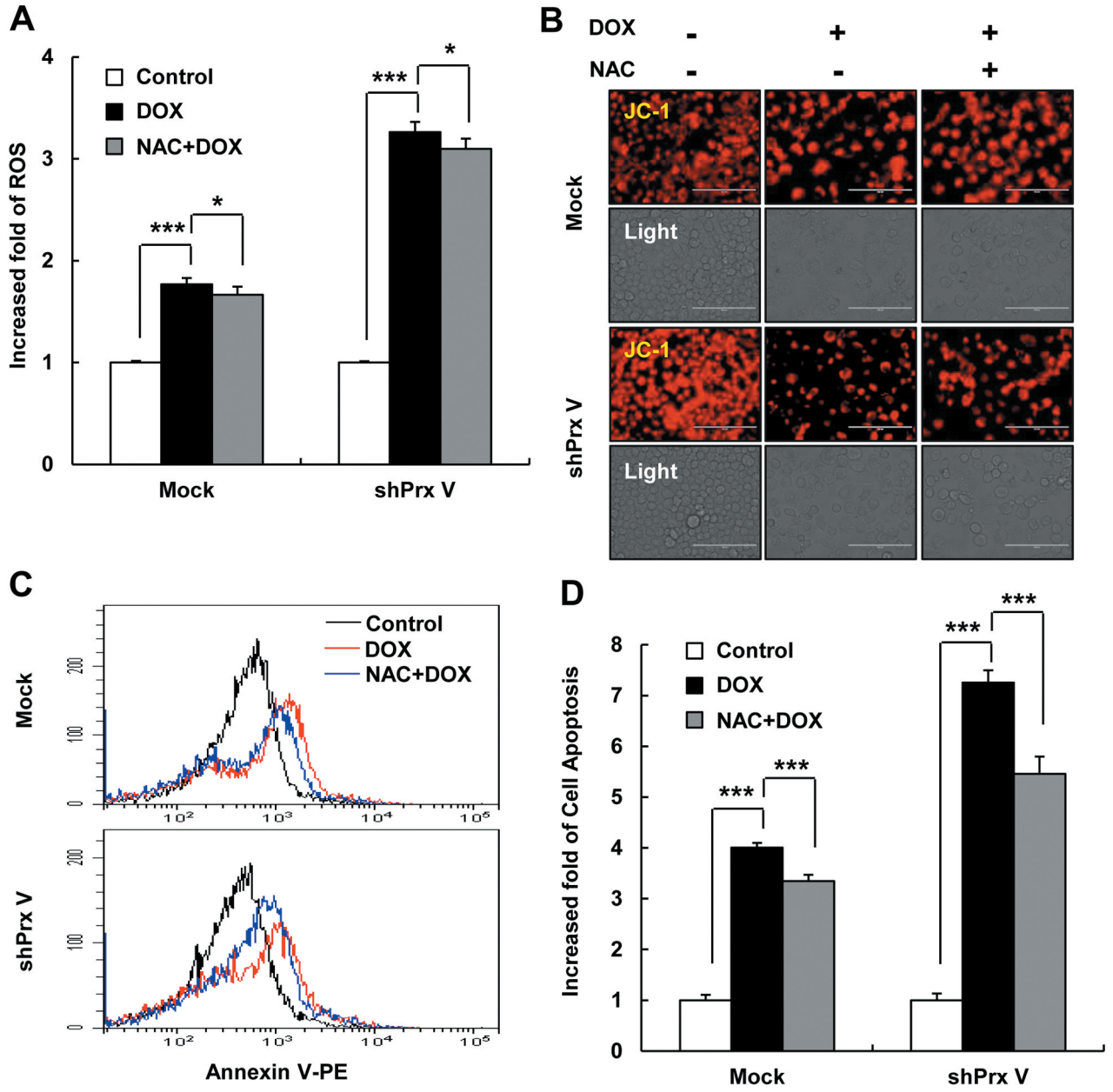

Figure 4. *Effects of NAC pre-treatment on ROS levels and the mitochondrial membrane potential. (A) Quantitative flow cytometry data. (B) DHE staining to observe the intracellular ROS levels (scale bar=100 μm). (C) MitoSOX staining to observe the mitochondrial ROS level. (D) JC-1 staining to observe the mitochondrial membrane potentials in the mock and shPrx V AGS cells treated with both NAC and DOX for 24 h. Data are presented as means; error bars indicate standard error of the mean of three different samples.*

redox than normal cells (26). Therefore, interrupting the intracellular ROS level balance is an effective way to induce cell death in cancer cells.

Anthracyclines have been used in clinical therapy for more than half a century (27, 28), and an increasing number of chemotherapy regimens administer anthracycline alone or in combination with non-anthracycline drugs to exert the greatest anti-tumor effect, which is one of the significant achievements in current tumor therapeutics (29, 30). While some chemotherapy drugs have been gradually replaced by targeted therapy in recent years, anthracyclines remain difficult to replace in the status of tumor chemotherapy. However, anthracyclines have obvious cardiotoxicity (31) and present several toxic side effects that may be associated

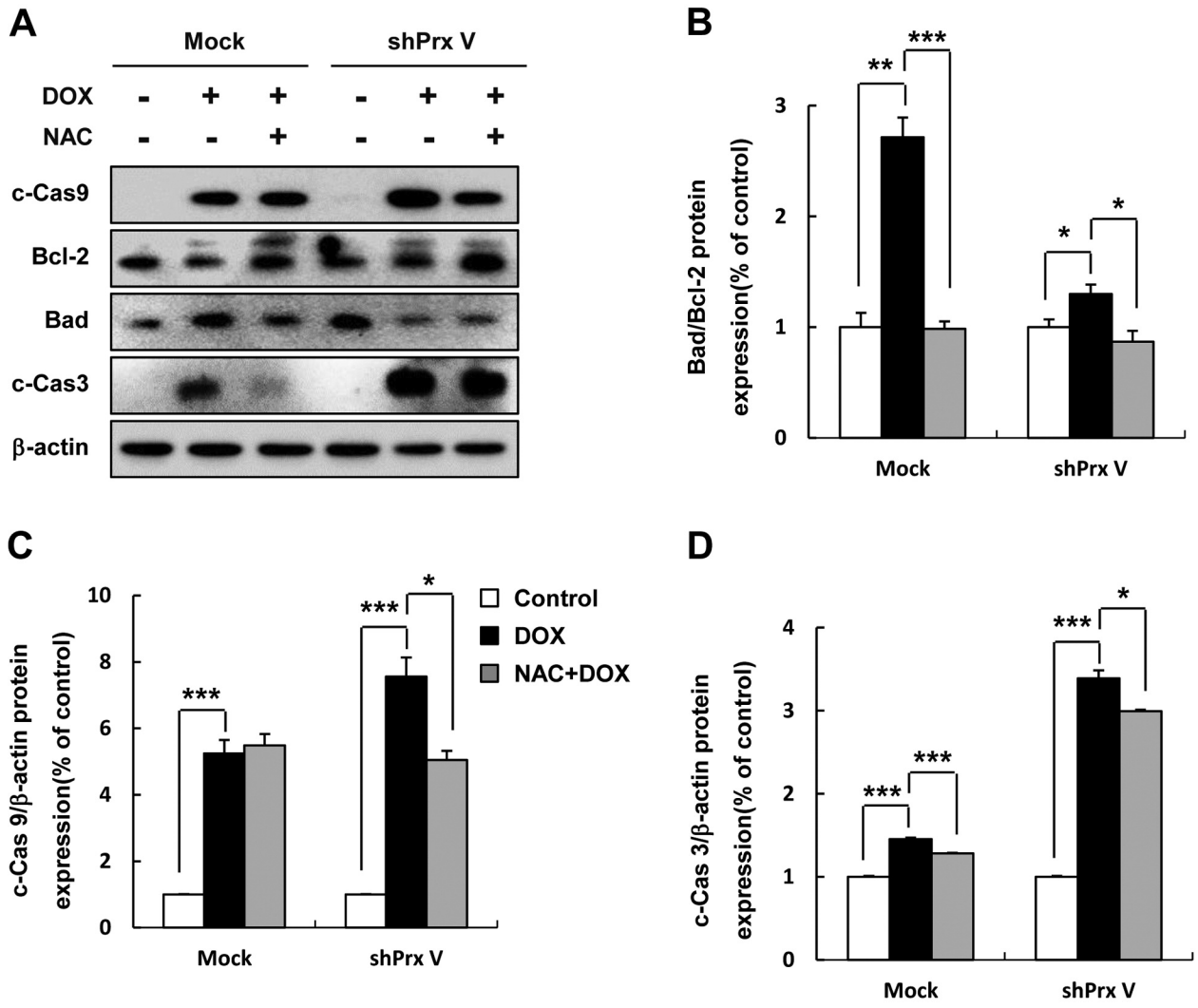

Figure 5. *Effect of NAC pre-treatment on cell apoptosis. (A) Western blot analysis of the protein expression levels of the Bad/Bcl2 ratio as well as cleaved caspase 3 and 9 in mock and shPrx V AGS cells. (B-D) Quantitative analysis of Bad/Bcl2 ratio as well as cleaved caspase 9 and 3 in mock and shPrx V AGS cells. Data are presented as means; error bars indicate standard error of the mean of three different samples.*

with exposure level, which limits the acceptable dosage. DOX, as an early-discovered anthracycline compound, was isolated from a Streptomyces species (32). The cell death induced by DOX may be due to a variety of factors, such as proinflammatory cytokines, DNA damage, and bursts of cellular ROS levels; oxidative stress is one of the mainstream theories that explain its mechanism (33). When a variety of harmful substances stimulate the internal environment of the body, the body generates excess ROS, which causes cells and tissues to undergo a series of physiological and pathological reactions (34, 35). Such reactions can destroy cell DNA, induce genetic mutations, and lead to protein denaturation, thereby triggering cell apoptosis (36, 37).

In the present study, we examined the possible regulatory function of Prx V in DOX-induced cell apoptosis in AGS GC cells, by constructing Prx V knockdown cell lines with lentivirus. We found that Prx V knockdown significantly increased the DOX-induced cell death and cellular ROS levels (Figures 1 and 2). These results are similar to our previous study, in which the deletion of Prx V elevated the susceptibility to emodin stimulation in AGS cells by increasing the cellular ROS accumulation (14). These findings strongly suggest that Prx V plays a protective role in GC cell apoptosis by attenuating the intracellular ROS accumulation. Furthermore, upon scavenging the ROS with NAC, both mock and shPrx V cellular ROS levels and apoptosis were significantly inhibited (Figure 4A, 4C, and

4D), suggesting the key role of ROS in the DOX-induced AGS cell apoptosis regulated by Prx V.

It is also widely known that mitochondrial dysfunction is the primary mechanism for cellular apoptosis. Elevated ROS can induce such dysfunction, thereby promoting cell death in cancer cells. In our previous study, we observed the regulatory role of Prx V in AGS cells *via* emodin stimulation; however, we did not assess the effect of Prx V on mitochondrial dysfunction (14). Therefore, in the present study, we evaluated the effect of Prx V on DOX-induced mitochondrial dysfunction in AGS cells. Our results showed that knockdown of Prx V significantly increased the DOX-induced mitochondrial membrane permeability, which was dramatically reversed by ROS scavenging (Figures 3A, 3B and 4B). These results indicate that knockdown of Prx V enhances DOX-induced mitochondrial ROS accumulation, which in turn increases the mitochondrial membrane permeability. We also examined mitochondria-dependent apoptosis-related protein expression levels after DOX treatment in both mock and shPrx V AGS cells. The results showed that knockdown of Prx V significantly enhanced the mitochondria-dependent cellular apoptosis upon DOX treatment, and these changes were reversed by ROS scavenging (Figures 3 and 5). These results strongly suggest that silencing of Prx V may increase GC cell apoptosis *via* mitochondria-dependent signaling pathways.

Therefore, according to our research results, we demonstrated that the effect of Prx V may depend on the mitochondria-dependent pathways that are involved in signal transduction by ROS, but the possible molecular mechanisms should be further examined. This is consistent with our previous study results of AGS treatment with emodin, which also showed that reducing Prx V could enhance the sensitivity of AGS cells to chemotherapeutics. Taken together, our findings illustrate that Prx V knockdown increases the accumulation of intracellular and mitochondrial ROS while it upregulates GC cell sensitivity by promoting mitochondria-dependent cellular apoptosis in DOX-treated AGS cells.

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

Authors' Contributions

Conceptualization: YZJ, YXG, TK, HNS. Methodology: TK, HNS. Software: TK, HNS. Validation: TK, HNS. Formal Analysis: YZJ, YXG, YL, DPX, CXR, SJL, HNS. Investigation: YZJ, YXG, YL, TK, HNS. Resources: TK, HNS. Data Curation: TK, HNS. Writing – Original Draft: YZJ, YXG, TK, HNS. Writing – Review & Editing: TK, HNS. Visualization: TK, HNS. Supervision: HNS, TK, DYX. Project Administration: TK, DYX. Funding Acquisition: TK, DYX. All Authors read and approved the final article.

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