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Peroxiredoxin V Inhibits Emodin-induced Gastric Cancer Cell Apoptosis *via* the ROS/Bcl2 Pathway

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Abstract. *Background/Aim: Peroxiredoxin (Prx) protein family is aberrantly expressed in various cancers including gastric cancer. Among the six family members, Prx V has been known as an antioxidant enzyme which scavenges intracellular reactive oxygen species (ROS) and modulates cellular apoptosis. This study aimed at investigating the role of Prx V in apoptosis of gastric cancer cells. Materials and Methods: Stably constructed Prx V knockdown, over-expression and mock AGS cells (a human gastric adenocarcinoma cell line) were used to study the effect of Prx V on emodin-induced apoptosis by western blotting, cell viability, apoptosis and ROS detection assays. Results: Overexpression of Prx V significantly decreased emodin-induced cellular apoptosis and ROS levels compared to Mock and Prx V knockdown AGS cells. Also, overexpression of Prx V down-regulated the expression of pro-apoptotic proteins, Bad and cleaved PARP, and increased the expression of anti-apoptotic protein, Bcl2. Conclusion: Prx V suppresses AGS cell apoptosis via scavenging intracellular ROS and modulating apoptosis-related markers.*

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Gastric cancer (GC) is one of the most common malignancies worldwide; unfortunately, the majority of GC patients is diagnosed at an advanced stage and die within 24 months after surgery because of recurrence and metastasis (1). The cure rate of gastric cancer is extremely low and the risk of treatment is large.

It is well known that reactive oxygen species ROS levels are high in cancer cells compared to normal cells. ROS such as hydrogen peroxide, superoxide anions *etc*. are produced during mitochondria metabolism (2). Low levels of ROS can act as second messengers by participating in a variety of cellular physiological activities such as signal transduction, apoptosis, aging, proliferation and migration of cells. On the contrary, high levels of ROS have been recognized as driving factors in numerous diseases including cancer, aging, neurodegenerative disease, diabetes, cardiovascular disease, stroke, and asthma (3, 4), by inducing lipid, protein and DNA oxidation (5) thereby resulting in increased cellular apoptosis. On the other hand, there are cellular antioxidant mechanisms that clear excessive ROS (6, 7), such as superoxide dismutase (SOD), catalase (Cat), glutathione peroxidase (Gpx) and peroxiredoxin (Prxs).

Prx V is a member of the Prxs family, which plays crucial roles in protecting cells from oxidative stress. Prxs are

classified into three types, referred to as typical 2-Cys (Prx I-IV), atypical 2-Cys (Prx V) and 1-Cys (Prx VI) Prxs (8). Prx V is also known as ROS/RNS scavenger and is widely distributed in cytoplasm and mitochondria. It has been reported that IFN-γ increases Prx V gene expression *via* a MyD88- and TNF-dependent pathways and the MAPKs have been shown to be downstream of IFN-γ and LPS signaling pathways, leading to Prx V induction (9). Furthermore, our previous study has shown that the expression of Prx V can significantly vary due to LPS stimulation in microglia. Induced Prx V expression was associated with the cooperative action of ROS, RNS and the JNK signaling cascade. Interestingly, knockdown of Prx V increased microglial activation by augmenting ROS generation and JNK-dependent NO production, suggesting that Prx V might indeed induce a ROS-dependent signaling cascade (10). Furthermore, over-expression of Prx V enhanced carcinogenicity by increasing invasion and proliferation of gastric cancer cells *via* up-regulation of Snail (11), and was also associated with poor prognosis of breast cancer (12). It has also been reported that over-expression of Prx V can enhance proliferation, migration and invasion of colon cancer cells by promoting epithelial-mesenchymal transition (13).

Emodin (1,3,8-Trihydroxy-6-methylanthraquinone) is an anthraquinone natural extract of the rhizome of *Polygonum cuspidatum* and has a variety of pharmacological activities, including anticancer, anti-inflammation, antibacterial, diuretic and laxative *etc*. Recent studies have shown that emodin has inhibitory effects on various cancers such as colorectal cancer, liver cancer, prostate cancer, pancreatic cancer, cervical cancer and breast cancer by inducing the cellular apoptosis (14-17).

In the present study, the effect of emodin on AGS cells [a human gastric adenocarcinoma cell line (ATCC® CRL-1739™)] apoptosis and cellular ROS levels was examined. Furthermore, to understand the regulatory function of Prx V on emodin-induced AGS cell apoptosis, Prx V shRNA, mock and Prx V cDNA infected (O/V) AGS cells were generated with Lentiviral vectors. Cellular apoptosis, ROS levels and apoptosis-related protein expression were measured in those three modified AGS cells after treatment with emodin.

Materials and Methods

Chemicals. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamic acid and N-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Cell culture. The AGS human gastric cancer cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in DMEM supplemented with 10% (v/v) FBS and penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively), and incubated at 37˚C and 5% CO_2. Cells were sub-cultured once every two days.

Construction of stable Prx V knockdown and over-expression AGS cells. Short hairpin RNA (shRNA) specific to Prx V (shPrx V LV3, H1&Puro), his-tag Prx V LV3 (H1&Puro) and control shRNA LV3 (H1&Puro) 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 negative control was 5'-GTTCTCCGA ACGTG TCACGT-3'. AGS cells at a density of 2×10^5/well were seeded in a 6-well tissue culture plate for 24 h (37˚C and 5% CO_2) prior to infection. The culture medium was replaced by polybrene (5 μg/ml; Shanghai GenePharma Co., Ltd.) and packed lentivirus with a multiplicity of infection 20 were added for 12 h, and subsequently replaced with complete culture medium (DMEM with 10% FBS and antibiotics). Infected cells were selected by treatment with puromycin and sub-cultured every 5-7 days. The expression of Prx V protein levels was examined by western blotting 3 days after infection.

Western blotting analysis. Cell protein lysates 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, II and V, his-tag, cleaved-PARP, Bcl-2, Bad (Santa Cruz, CA, USA), and β-actin (Sigma-Aldrich) at 4 ˚C, overnight. The membranes were 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 (Sigma-Aldrich) or anti-mouse IgG (Sigma-Aldrich) 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.

Cell viability assay. Cell viability was examined *via* the MTT assay. AGS cells were seeded at a density of 5,000 cells per well in 96-well plates and treated with 0-10 mM of Emodin for 24 h (37˚C and 5% CO_2), and the control cells were treated with media alone without Emodin. The accumulation of formazan (the dimethylsulfoxide was used as solvent) was determined following the addition of MTT reagent (5 mg/ml) and the absorbance was measured at a wavelength of 560 nm. Absorbance was detected by a UV max Kinetic microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Cell apoptosis and ROS detection. To determine cellular apoptosis and ROS levels, emodin-treated AGS cells were harvested using trypsin, resuspended in PBS and stained with annexinV-fluorescein isothiocyanate (FITC)/propidium iodide (PI) (for apoptosis) and DCF-DA (for ROS), according to the manufacturer's protocols of the apoptosis/ROS detection kit (BD Biosciences, Franklin Lakes, NJ, USA). The annexin V-FITC/PI and DCF-DA positive cells were analyzed by fluorescence microscope and flow cytometry on a BD FACSCalibur (BD Biosciences). The results were analyzed with WinMDI (version 2.9; BD Biosciences) software.

Statistical analysis. The data are depicted as the means±SEM. Student's *t*-tests were performed using the GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA), and $p<0.05$ was considered to indicate a significant difference.

Results

Emodin induces AGS gastric cancer cell apoptosis and diminishes the expression level of Prx V. To investigate whether emodin induces cellular apoptosis, Annexin V-FITC/PI staining was conducted for AGS gastric cancer cells treated with 30 μM of emodin in a time-dependent manner for 0, 3, 6, 12, and 24 h. As shown in Figure 1A, the PI and Annexin V-FITC positive AGS cell population was increased with increasing treatment time of emodin. Flow cytometry analysis was then conducted to confirm the above observed cellular apoptosis results. As shown in Figure 1B, cellular apoptosis was increased in a time-dependent manner following treatment with emodin, confirming that emodin induces cellular apoptosis of AGS gastric cancer cells.

It has been reported that overexpression of Prx V enhances carcinogenicity of gastric cancers by increasing cell proliferation and invasiveness and decreasing cellular apoptosis (11). Similarly, Peroxiredoxin I (Prx I) and Peroxiredoxin II (Prx II) have also been reported to be involved in the apoptotic pathway of various cancer cells (18, 19). Therefore, western blot analysis was conducted to check whether Prx I, Prx II and Prx V contribute to emodin-induced AGS cell apoptosis. As shown in Figure 1C and D, treatment with emodin resulted in a time-dependent decrease in the expression of Prx V. Expression of Prx I and Prx II was not affected. Altogether, these data showed that emodin induces AGS cell apoptosis and reduces the expression of Prx V in a time-dependent manner.

Emodin-induced apoptosis of AGS cells is associated with intracellular ROS. ROS derived from cellular metabolism, cause oxidative stress, which can then lead to cellular apoptosis of various cancer cells including gastric cancer cells (20). Therefore, we further investigated whether ROS is related with the emodin-induced cellular apoptosis of AGS cells. First, AGS cells were pre-treated with 5 mM of ROS scavenger (NAC) for 15 min. Then, cells were treated with 30 μM of emodin for 12 h, as a 12 h treatment was shown to cause a significant effect on AGS cell apoptosis. As shown in Figure 2A, flow cytometry showed that the population of Annexin V/PI positive cells increases with emodin treatment. However, pre-treatment with NAC decreased the Annexin-V/PI positive cell population. These results demonstrated that the emodin-induced AGS cell apoptosis can be reversed by pre-treating with a ROS scavenger, indicating the association of ROS with emodin-induced apoptosis.

Our results showed that treatment with emodin results in a time-dependent decrease in the levels of Prx V (Figure 1A-D). Similarly, emodin treatment resulted in a time-dependent increase in the levels of ROS (Figure 1E). Therefore, we conducted western blotting analysis to examine whether emodin induced intracellular ROS is related with the reduced expression levels of Prx V, as reported previously in non-small cell lung cancer cells (21). Extracts of AGS cells incubated with or without emodin were analyzed by western blot to examine the effect of emodin on the expression levels of Prx I, Prx V, Prx II and PRX (Figure 2B-E). Treatment with emodin did not affect the levels of Prx I and II but it significantly decreased the levels of Prx V. Interestingly, the emodin-induced reduction of Prx V expression was reversed following pre-treatment with NAC, indicating that ROS mediates the effect of emodin on the reduction of Prx V. Therefore, emodin-induced apoptosis and reduction in Prx V expression are associated with intracellular ROS.

Overexpression of Prx V reduces emodin-induced apoptosis of AGS cells. To examine the possible regulatory role of Prx V in emodin-induced intracellular ROS production and apoptosis, AGS cells were infected with lentiviral vectors to silence Prx V (shPrx V) or overexpress Prx V (Prx V-his). As a control AGS cells were mock infected with empty lentiviral vector. Prx V knockdown or overexpression was confirmed by western blotting (Figure 3A). Further experiments were then conducted using shPrx V, Prx V-his and Mock AGS cells.

To examine the effects of Prx V on emodin-induced apoptosis and intracellular ROS levels, Annexin-V/PI and DCF-DA positive cells were measured separately by flow cytometry. First, the three types of infected AGS cells, shPrx V, Prx V-his and Mock, were treated with different concentrations of emodin (0, 10, 20 and 30 μM) for 12 h and with 30 μM of emodin for the indicated time periods (0, 3, 6, 12 and 24 h). Then, flow cytometry was used to detect Annexin-V/PI and DCF-DA positive cell populations. As shown in Figure 3B and C, apoptosis and intracellular ROS levels were significantly reduced in emodin treated Prx V-his cells but not in shPrx V compared to Mock cells. As shown in Figure 3D and E, treatment with 30 μM emodin resulted in time-dependent reduction in the intracellular levels of ROS and apoptosis in Prx V-his AGS cells while shPrx V cells did not show any significant difference compared to Mock cells. These results showed that Prx V overexpression reduces the emodin-induced intracellular ROS level and apoptosis of AGS gastric cancer cells.

Overexpression of Prx V significantly modulates the expression of apoptosis-related proteins in emodin-treated AGS cells. To further understand the mechanisms of the effect of Prx V on emodin-induced apoptosis of AGS cells, the expression levels of Bad, cleaved-PARP and Bcl2 in shPrx V, Prx V-his and Mock cells were examined following treatment with 30 μM of emodin for the indicated times (0, 12 and 24 h) by western blotting. As shown in Figure 4A, B and D, treatment of AGS Mock cells with 30 μM emodin resulted in a time-dependent increase in the levels of pro-apoptotic proteins Bad and cleaved-PARP. In contrast, the

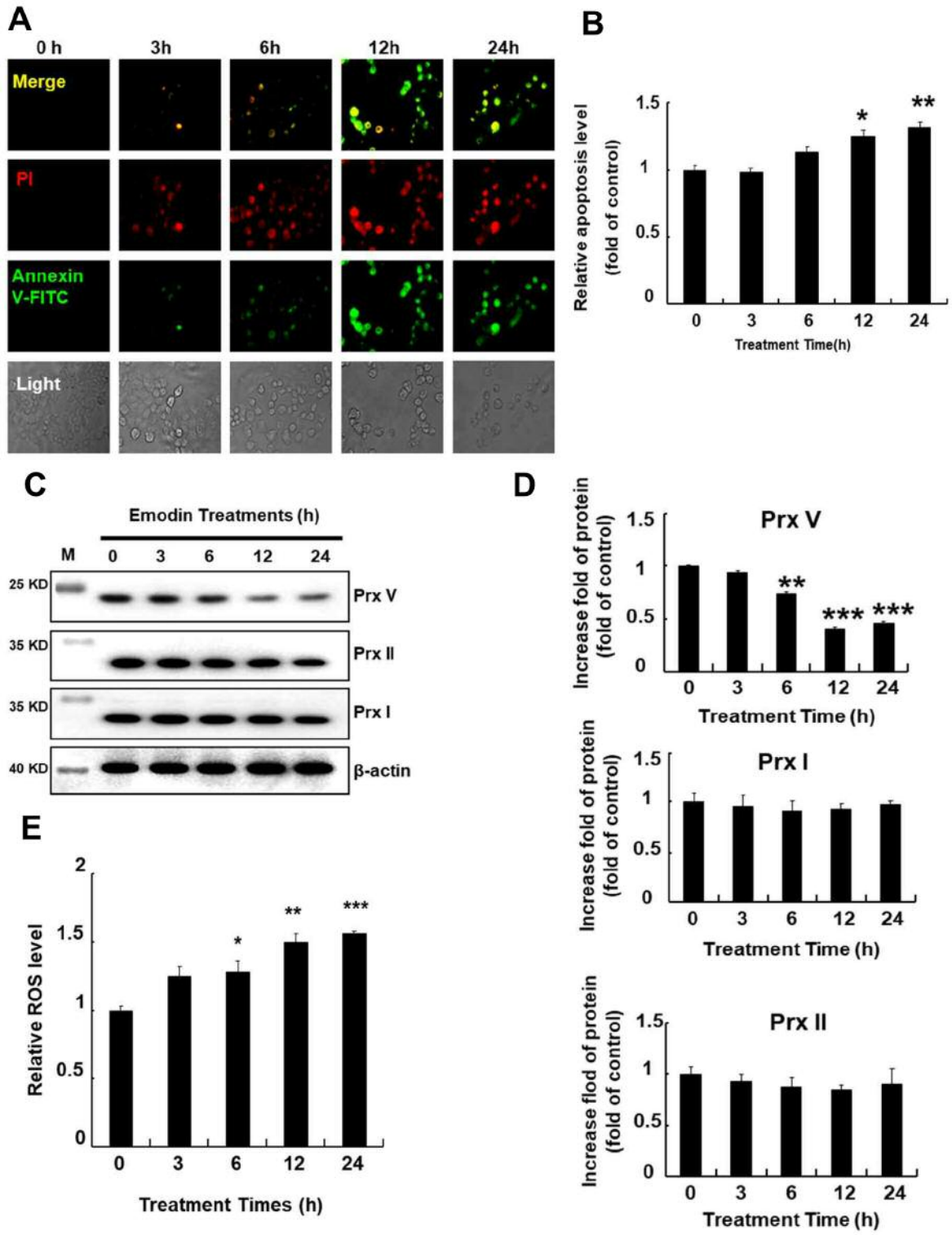

Figure 1. *Emodin induces ROS and apoptosis of AGS cells and a decrease in the Prx V protein expression levels. (A) AGS gastric cancer cells were treated with emodin (30 μM) for 0, 3, 6, 12, and 24 h, and cellular apoptosis was analyzed by fluorescence microscope and (B) flow cytometry (fold increase in apoptosis was analysed by WinMDI software). (C, D) The expression levels of Prx V, Prx I, Prx II were analyzed by western blot. (E) AGS cells were treated with emodin (30 μM) for 0, 3, 6, 12 and 24 h. Cellular ROS levels were determined with flow cytometry by detecting the DCF-DA-positive cells, and the fold increase of ROS levels were represented at mean means±standard deviation. Protein expression is presented as mean±standard deviation, *p<0.05, **p<0.01, ***p<0.01. Three independent replicates were performed for all the experiments.*

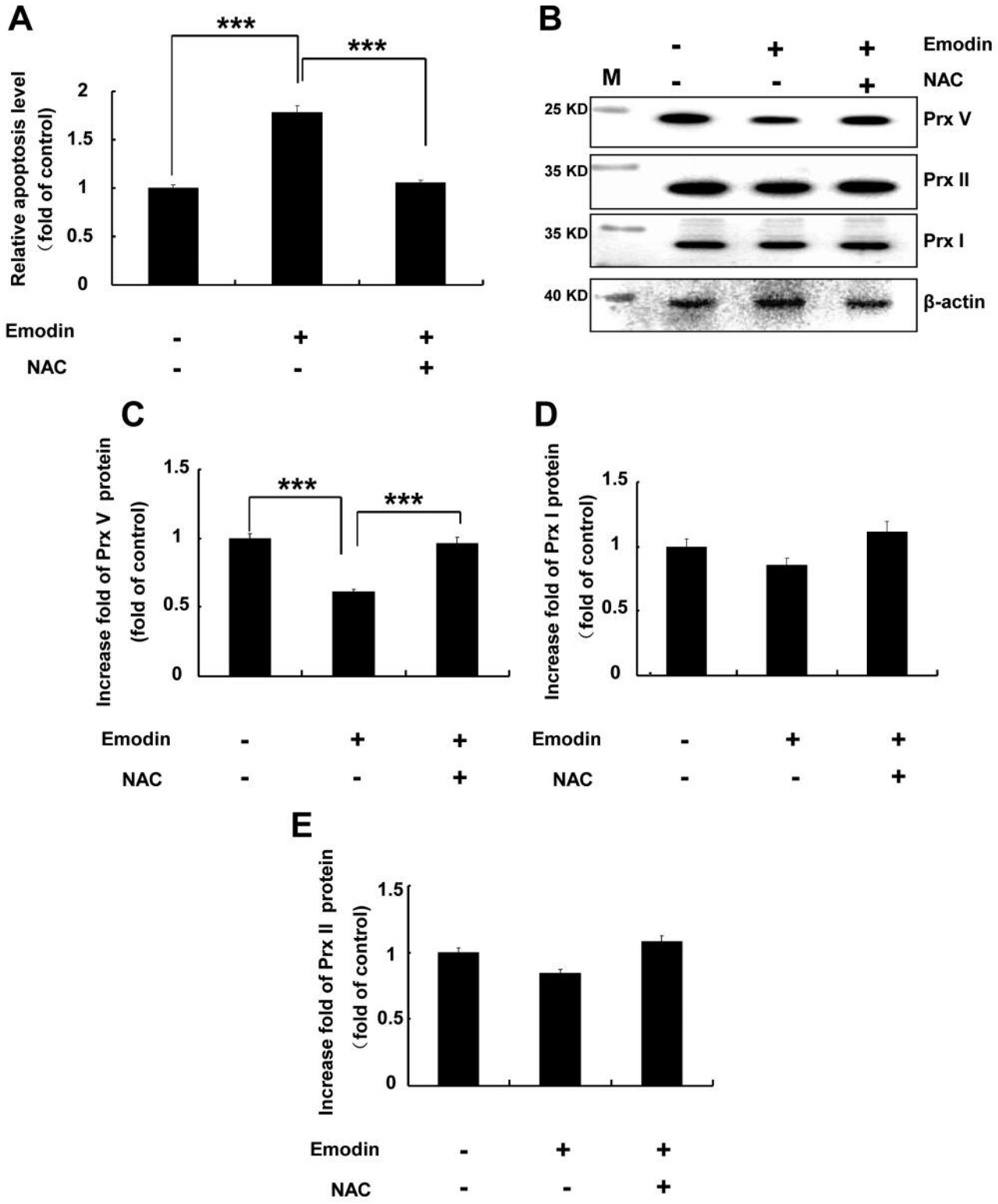

Figure 2. *Emodin induced-apoptosis in AGS cells is associated with intracellular ROS. (A) AGS gastric cancer cells were incubated in the absence or presence of emodin (30 μM) or with emodin after being pretreated with NAC, and cellular apoptosis was analyzed with flow cytometry (fold increase in apoptosis was analyzed by WinMDI software). (B) The protein expression levels of (C) Prx V, (D) Prx I, (E) Prx II were analyzed by western blot. Protein expression is presented as the mean ±standard deviation, *p<0.05, **p<0.01, ***p<0.01. Three independent replicates were performed for all the experiments.*

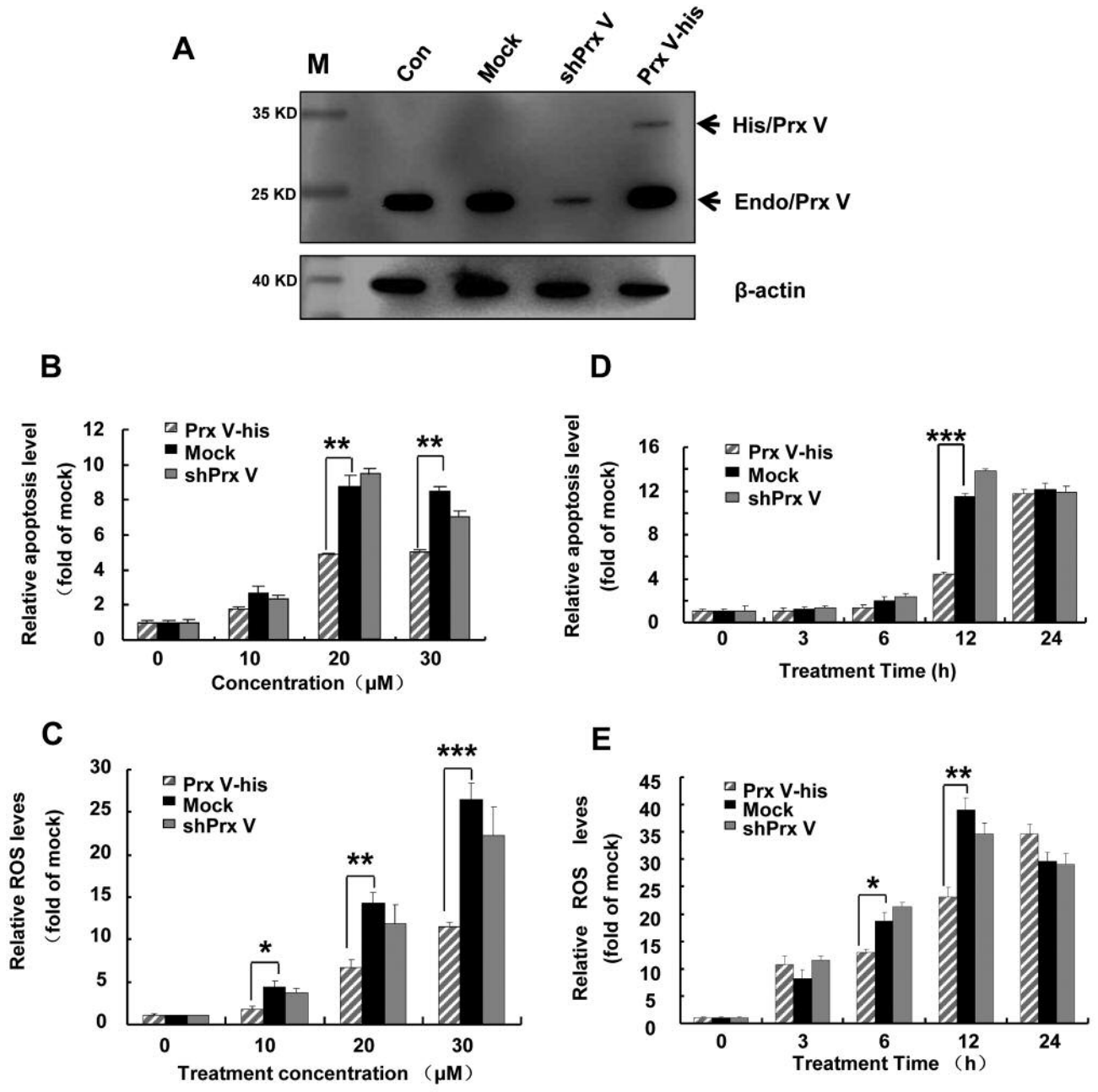

Figure 3. *Over-expression of Prx V can reduce emodin-induced AGS apoptosis. (A) AGS gastric cancer cells were infected with lentivirus. The expression levels of Prx V His-tag were analyzed by western blot. (B, D) The infected AGS gastric cancer cells were treated with emodin (0, 10, 20 and 30 μM) for 24 h and with emodin (30 μM) for 0, 3, 6, 12, and 24 h. Apoptosis was analyzed by flow cytometry (fold increase in apoptosis was analyzed by WinMDI software), and (C, E) the intracellular ROS levels were also analyzed by flow cytometry (fold increase in ROS was analyzed by WinMDI software). Fold increase in apoptosis and ROS is presented as the mean±standard deviation, *p<0.05, **p<0.01, ***p<0.01. Three independent replicates were performed for all the experiments.*

expression of Bad and cleaved-PARP was decreased in Prx V-his AGS cells compared with Mock cells. However, there was a trend of increased expression in shPrx V AGS cells compared with Mock cells. Parallel, expression of the anti-apoptotic protein Bcl2 expression was reduced in emodin-treated AGS Mock cells in a time-dependent manner (Figure 4A and B). In contrast, the expression of Bcl2 was increased in emodin-treated Prx V-his AGS cells compared with AGS

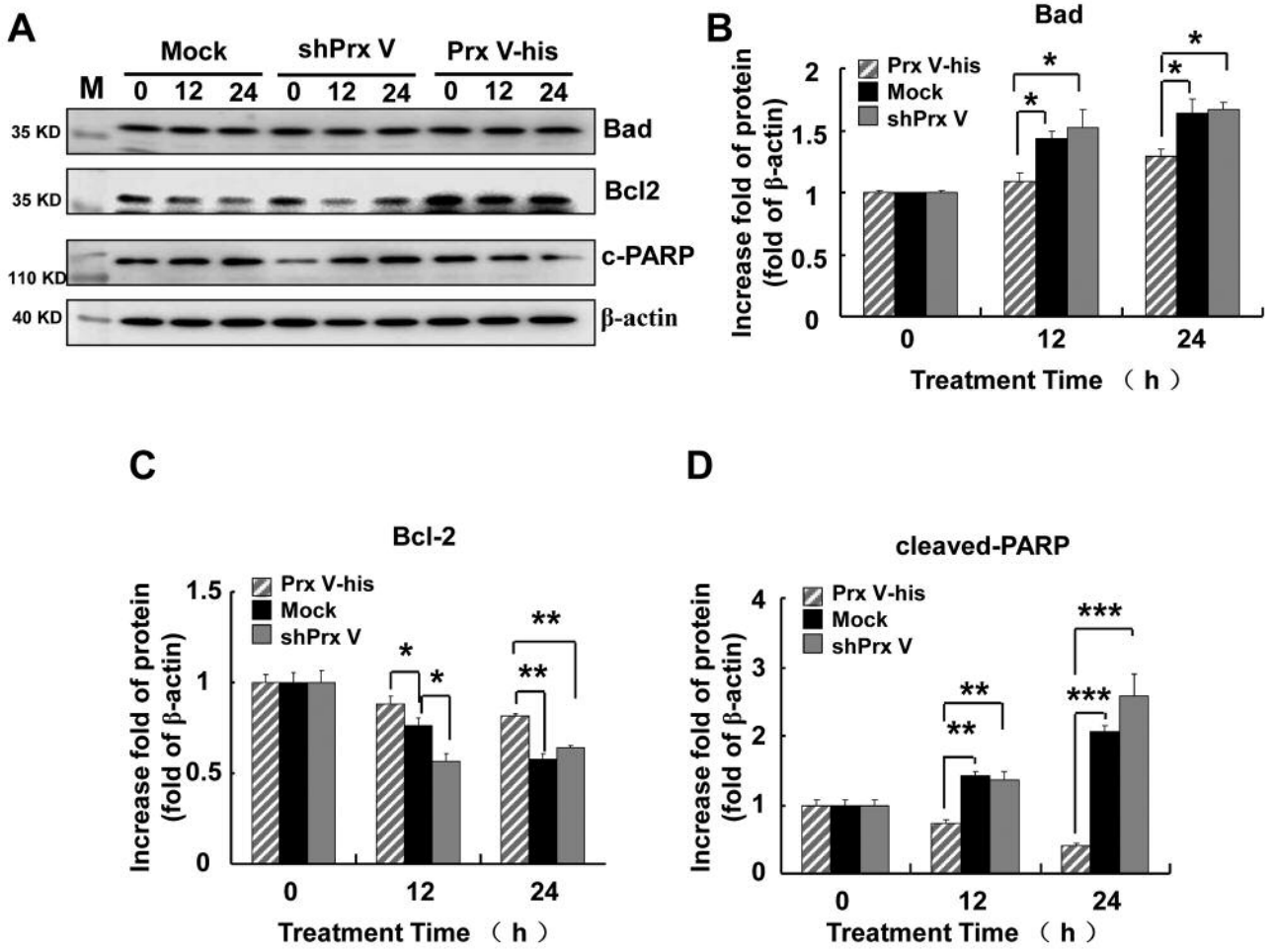

Figure 4. *Over-expression of Prx V significantly inhibited emodin-induced apoptosis-related protein expression. (A) Infected AGS gastric cancer cells were treated with emodin (30 μM) for 0, 12, and 24 h. The expression levels of (B) Bad, (C) Bcl2, (D) cleaved-PARP were analyzed by western blot. Protein expression is presented as the mean±standard deviation, *p<0.05, **p<0.01, ***p<0.01. Three independent replicates were performed for all the experiments.*

Mock and shPrx V AGS cells. There was no statistically significant difference in Bcl2 expression between emodin-treated Mock and shPrx V AGS cells. Altogether, these data suggested that overexpression of Prx V suppresses the emodin-induced apoptosis in AGS gastric cancer cells.

Discussion

Gastric cancer (GC) is the second most common cause of cancer-related deaths worldwide (22). The 5-year survival rate of gastric cancer is around 10-20% (22). Also, the rate of early diagnosis is low. Therefore, most of gastric cancer patients have advanced-stage disease at the time of diagnosis and miss the best surgical window (23). Thus, new therapeutic strategies to diagnose and treat GCs are yet to be developed.

Recently, the intake of antioxidants has been considered as a possible strategy to improve overall health (24, 25). Antioxidants play important roles in redox signaling and redox status of cells and thereby the maintenance of cellular integrity and homeostasis (26). Also, several clinical trials have suggested that antioxidants benefit cancer therapy (27). Among these, Prx protein family (Prx I-VI) has been reported to be aberrantly expressed in various tumors, implying an important role of Prxs in carcinogenesis (28). Prx V is an atypical 2-cys-Prx which is widely expressed in cellular compartments. It has been reported that Prx V is involved in cellular apoptosis induced by oxidative stress in various cells such as Hela cervical cancer cells and HT22 hippocampus cells through several pathways (29-31). The role of Prx V in the apoptosis of GC cells has not yet been reported. However, it has been reported that overexpression

of Prx V could enhance tumorigenicity and epithelial-mesenchymal transition (EMT) in gastric cancers both in patients and cancer cell lines. Prx V is also associated with the 5-year survival rate of patients (11). These findings implied that Prx V plays and pivotal role in GCs.

In this study, for the first time, the role of Prx V in GC cell apoptosis was examined using emodin-treated AGS gastric cancer cells. According to our results, emodin induced an increase in intracellular ROS levels and apoptosis and a decrease in the expression of Prx V in AGS gastric cancer cells. Induction of apoptosis and reduction in Prx V expression were associated with increased levels of ROS in emodin-treated AGS GC cells as was observed by the pre-treatment with NAC, a ROS scavenger. Previous study has demonstrated that ROS are generated during the progression of cancer and provide several useful markers for cancer diagnosis and prognosis (32). The intracellular antioxidant enzymes, including Cat, SOD, Trx, Gpx and Prxs control ROS levels to maintain redox status and cellular homeostasis; their levels play important role in apoptosis (33). Prx V has also been identified as a ROS and RNS scavenger among Prx family members and is involved in oxidative stress-induced apoptosis (34, 35). Therefore, we hypothesized that down-regulation of Prx V in emodin-treated AGS cells is associated with induction in the intracellular ROS levels.

To study the role of Prx V in gastric cancer cell apoptosis and intracellular ROS induced by emodin treatment, we constructed three stable AGS cell lines including shPrx V (Prx V silenced), Prx V-his (Prx V overexpressed) and control (Mock). With the use of these cell lines, we showed that overexpression of Prx V in GC cells significantly suppresses the emodin-induced apoptosis and ROS levels compared with Mock AGS cells. However, there was no observable difference between apoptosis and ROS levels in shPrx V and Mock cells. Similarly, another study has already shown that overexpression of Prx V significantly prevented ferric ammonium citrate-induced cell apoptosis in HT22 cells. But, knockdown of Prx V had no significant effect compared to Mock cells (36). These findings suggested that regulation of oxidative stress and apoptosis is more effective in Prx V overexpressing cells, but the mechanisms involved should be further studied.

Apoptosis is critical for normal development and maintenance of homeostasis of multicellular organisms (37). Various stimuli can cause apoptosis, including reduction in growth factors, application of chemotherapeutic drugs, and cross linking of death signal transmitting receptors (37, 38). In the process of cell apoptosis, inherent mitochondria dependent signaling pathways play an important role. Furthermore, Bcl2 (B-cell CLL/lymphoma 2) family is probably the best example of apoptosis regulators, by either inducing (pro-apoptotic) or inhibiting (anti-apoptotic) cell death (39, 40). With different kinds of stimulations, Bcl2 can inhibit the release of cytochrome C to prevent the cell apoptosis (41, 42). Therefore, we used few of apoptosis related markers, Bcl2, Bad and cleaved PARP, to further evaluate the role of Prx V on AGS cell apoptosis. Our results again showed that overexpression of Prx V suppresses GC cell apoptosis, as observed through the down-regulation of Bcl2 levels and up-regulation of Bad and cleaved-PARP levels in Prx V-his AGS cells compared with shPrx V and Mock AGS cells. Therefore, we hypothesized that emodin-induced apoptosis of AGS cells is associated with these pro-apoptotic and anti-apoptotic proteins.

Furthermore, based on our results, it is suggested that the effect of Prx V may depend on mitochondria-dependent pathways which were also involved in ROS-induced signaling, but the possible molecular mechanisms should be further studied. Although there is no direct evidence verifying the protective role of Prx V on emodin-induced cancer cell apoptosis, our results provide a novel view of the possible regulatory function of Prx V in AGS gastric cell apoptosis, which was stimulated by emodin. Taken together, our findings showed that overexpression of Prx V reduces the accumulation of intracellular ROS, enhances cell survival and inhibits mitochondria dependent cellular apoptosis in emodin-treated AGS gastric cancer cells.

Conflicts of Interest

The Authors declare that there are no conflicts of interest regarding this study.

Authors' Contributions

Y.Z.J., H.N.S., Y.L., T.K., D.Y.X., and Y.J., designed and wrote the whole manuscript; Y.Z.J., H.N.S., Y.L., T.K., performed the experiments; D.H.L., J.S.K., S.U.K., B.Y.J., Y.H.H., M.H.J., G.N.S., D.S.L. contributed to the revision of the manuscript. All Authors read and approved the final manuscript.

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