Knockdown of peroxiredoxin V increases glutamate-induced apoptosis in HT22 hippocampal neuron cells

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Abstract. High concentrations of glutamate may mediate neuronal cell apoptosis by increasing intracellular reactive oxygen species (ROS) levels. Peroxiredoxin V (Prx V), a member of the Prx family, serves crucial roles in protecting cells from oxidative stress. The present study investigated the regulatory effect of Prx V on glutamate-induced effects on viability and apoptosis in HT22 cells. Western blotting was used for protein expression analysis and Annexin V/PI staining and flow cytometry for determination of apoptosis. The results demonstrated that glutamate may ROS-dependently increase HT22 cell apoptosis and upregulate Prx V protein levels. Furthermore, knockdown of Prx V protein expression with a lentivirus significantly enhanced HT22 cell apoptosis mediated by glutamate, which was reversed by inhibition of ROS with N-acetyl-L-cysteine. Inhibiting the extracellular signal-regulated kinase (ERK) signaling pathway with PD98059, a specific inhibitor for ERK phosphorylation, markedly decreased glutamate-induced HT22 cell apoptosis in Prx V knockdown cells, indicating the potential involvement of ERK signaling in glutamate-induced HT22 cell apoptosis. In addition, an increase in nuclear apoptosis-inducing factor was observed in Prx V knockdown HT22 cells following glutamate treatment, compared with mock cells, whereas no differences in B-cell lymphoma-2 and cleaved-caspase-3 protein expression levels were observed between mock and Prx V knockdown cells. The results of the present study indicated that Prx V may have potential as a therapeutic molecular target for glutamate-induced neuronal cell death and provide novel insight into the role of Prx V in oxidative-stress induced neuronal cell death.

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Introduction

Alzheimer's disease (AD) is a late-onset neurodegenerative disorder that is characterized by the deposition of extracellular amyloid-β protein and construction of intracellular neurofibrillary tangles, and results in a decline in memory and cognition as well as alterations in behavior and personality (1-3). Plaques and tangles present primarily in the hippocampus, entorhinal cortex, amygdala and basal forebrain, and induce neuronal cell death, which is associated with AD progression. Thus, protecting neuronal cells against cell death induced by various types of stress is essential in delaying the progression of neurodegenerative diseases.

The HT22 mouse hippocampal cell line has been widely used as an *in vitro* model for investigating oxidative stress-induced cell death. It is established that the HT22 cells lack an ionotropic glutamate receptor; thus, when the cells are treated with a high concentration of glutamate, intracellular glutathione is depleted and reactive oxygen species (ROS) levels increase, and this process is independent of the ionotropic glutamate receptor (4-6). In addition, it has been reported that oxidative stress-induced neuronal cytotoxicity is mediated by the mitogen-activated protein kinase (MAPK) signaling pathway and apoptosis-inducing factor (AIF) (7-11), and the activities of these pathways are widely associated with cellular ROS levels. Conversely, antioxidant enzymes such as superoxide dismutase 2 effectively protect against oxidative stress-induced cell death in HT22 cells (12), indicating the involvement of antioxidant enzymes in oxidative stress-induced neuronal cell death.

Peroxiredoxins (Prxs) are a ubiquitous family of antioxidant enzymes that catalyze the reduction of hydrogen peroxide or alkyl peroxides. Previously, the function of Prxs has interested researchers due to their ability to reduce ROS and peroxynitrite, and their modulation of cytokines and growth factor signaling cascades associated with cell apoptosis and proliferation (13-15). Prxs have been reported to be present in several cellular compartments, including the mitochondria, nucleus, cytosol and peroxisomes. Prx V, also termed atypical 2-Cys-Prx, is the smallest member of the family and has only 10% sequence identity with typical 2-Cys-Prxs, and is widely expressed in cellular compartments (16-19). According a previous study, Prx V regulated the microglial activation process by modulating the generation of ROS/nitrogen oxide, which was regulated by the activation of thec-Jun N-terminal kinase (JNK) signaling cascade activation (20). Reports have demonstrated that recombinant Prx V attenuated ibotenate-mediated mouse brain neurotoxicity and inhibited p53-induced HeLa cell apoptosis (21,22). In addition, peroxisomal overexpression of human Prx V protected against glial cell death induced by ROS, while knockdown of Prx V inhibited osteoarthritic chondrocyte growth via wnt/β-catenin signaling (23,24). Furthermore, mitochondrial Prx V may regulate the1-methyl-4-phenylpyridinium $(MPP)^+$mitochondrial pathway of apoptosis via crosstalk between the endoplasmic reticulum and mitochondria (25). It was also reported that Prx V is widely expressed in cellular and subcellular structures in mammalian cells, which indicated the important roles of Prx V in normal peripheral nerves (2,25). These reports indicate that Prx V may be involved in apoptosis-associated neuronal damage. However, the role of Prx V in glutamate-induced HT22 cell apoptosis is not well understood at present.

The present study investigated the role of Prx V in the glutamate-mediated apoptosis of HT22 cells. The results revealed that Prx V protein expression levels were upregulated partially in a time- and dose-dependent manner by glutamate stimulation. Furthermore, knockdown of Prx V increased apoptosis in HT22 cells, potentially via extracellular signal-regulated kinase (ERK) and AIF signaling pathways, which were mediated by glutamate. The findings of the current study may provide a novel therapeutic target for glutamate-induced hippocampal cell apoptosis.

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). ERK, p38 MAPK and JNK inhibitors (PD98059, SB203580 and SP600125, respectively) were all purchased from EMD Millipore (Billerica, MA, USA).

Cell culture. HT22 cells, which were immortalized from murine hippocampal neuroncells was kindly provide by Professor Dong-Seok Lee, 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 HT22 cells. Short hairpin RNA (shRNA) specific to Prx V (shPrx V LV3, H1/GFP&Puro) and control shRNA LV3 (H1/GFP&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 the negative control was 5'-GTTCTCCGAACG TGTCACGT-3'. To construct the Prx V knockdown cells, $2x10^5$/well of the HT22 cells 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 the packed lentivirus with a multiplicity of infection of 20 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 (26). The expression of Prx V protein levels were examined by western blotting 3 days after infection. The proficiency of shRNA lentiviruses in selectively suppressing Prx V expression, with no effects on Prx II expression, was determined in virus-infected cells by western blotting.

Cell treatments. HT22 cells at a density of ~70% were treated with various doses (0, 2, 4, 6, 8 and 10 mM) glutamate (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 12 h at 37˚C and 5% CO_2 to analyze cell viability and apoptosis. The selection of doses for glutamate in the current study was based on other reports that treated HT22 cells with glutamate to analyze the toxicity of glutamate on neuronal cell death (9-12). For NAC treatment, HT22 cells in were pretreated with NAC (1 or 5 mM) for 30 min at 37˚C then flowed by glutamate (4 mM) treatment for 12 h. For MAPK inhibitor treatment, approximately at 70% density of the mock and short hairpin (sh)RNA-transfected HT22 cells were pretreated with SB203580 (5 μM, for p38 MAPK inhibition), PD98059 (5 μM, for ERK MAPK inhibition) and SP600125 (5 μM, for JNK MAPK inhibition) for 30 min (37˚C and 5% CO_2), followed by glutamate (4 mM) treatments (12 h, 37˚C and 5% CO_2).

Cell viability assay. Cell viability was examined via an MTT assay. HT22 cells were seeded at a density of 5,000 cells per well in 96-well plates and treated with glutamate at 0-10 mM for 24 h (37˚C and 5% CO_2) and the control cells were treated with media alone without glutamate. 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 Kineticmicroplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Cell apoptosis detection. To determine cell apoptosis, $1x10^6$ glutamate-treated HT22 cells were harvested using trypsin and resuspended with PBS and stained with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI), according to the manufacturer's protocol of the apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA). The Annexin V-FITC/PI positive cells were analyzed by flow cytometry on a BD FACSCalibur (BD Biosciences). The results were analyses with Win MDI (version 2.9; BD Biosciences) software.

Cellular ROS detection. To determine the cellular ROS levels, $1x10^6$ of the HT22 cells were treated with glutamate (0, 2, 4, 6, 8 and 10 mM) for 6 h. Subsequently, the cells were fixed with 4% formaldehyde (room temperature, 15 min) and treated with 2.5% Triton X-100 for 5 min at room temperature, followed by staining with 5 μM dihydroethidium (Beyotime Institute of Biotechnology, Haimen, China) and 10 μg/ml DAPI (BIOSS, Beijing, China) for 15 min at room temperature. Subsequently, the cellular ROS levels were detected with a fluorescence microscope (5 field of view, magnification, x200) and the density of the fluorescence was determined with ImageJ (K 1.45) software (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis. Cells were lysed using lysis buffer containing 20 mM HEPES-OH (pH 7.0), 50 mM NaCl, 10% glycerol and 0.5% triton X-100 and proteinase inhibitors 0.5 μg/ml of leupeptin; 0.7 μg/ml of pepstatin A; 0.1 mM of AEBSF [4-(2-aminonethly)-benzenesulfony fluoride]; 2 μg/ml of aprotinin. Nuclear and cytosol proteins were obtained using nuclear extraction kit (NXTRACT, Sigma-Aldrich, Merck KGaA). The protein concentrations were analyzed with Bradford assay. A total of 30 μg protein lysateswere separated on 12% SDS gels and transferred onto nitrocellulose membranes (EMD Millipore). Membranes were subsequently blocked with 5% skim milk (Sigma-Aldrich, Merck KGaA) for 30 min at room tmeperature and incubated with antibodies against Prx V (cat. no. SC-130337, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Prx II (cat. no. SC-515428), B-cell lymphoma-2 (Bcl-2; cat. no. SC-783), cleaved-caspase-3 (cat. no. SC-136219), AIF (cat. no. AC-5586), laminB (all Santa Cruz Biotechnology, Inc.) and β-actin (Sigma-Aldrich; Merck KGaA) at a dilution of 1:2,000 at 4˚C overnight. Membranes were then probed with horseradish peroxidase-conjugated goat anti-mouse IgG (cat. no. SAB3701105, Sigma-Aldrich; Merck KGaA) or anti-rabbit IgG (cat. no. SAB3700878, Sigma-Aldrich; Merck KGaA) at a dilution of 1:5,000 for 1 h at room temperature. Following the removal of excess antibody by washing with TBS, specific binding was detected using a chemiluminescence detection system with chemiluminescence detection kit (cat. no. RPN2135; GE Healthcare, Chicago, IL, USA) according to the manufacturer's protocol, and the densitometric analysis was performed by ImageJ (K 1.45) software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Statistical significance between experimental groups was determined with mean ± standard deviation by a one-way analysis of variance and a Tukey test. The data were analyzed by SPSS (19.0) software (IBM Corp., Armonk, NY, USA). All experiments were done at least 3 times independent. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Induction of neuronal cell death by glutamate in HT22 cells. HT22 cells were treated with glutamate for 12 h, and HT22 cells treated with glutamate for 12 h appeared to be less viable compared with control cells (Fig. 1A). Furthermore, the present study also determined the effect of glutamate on cell viability (Fig. 1B) by an MTT assay and cellular apoptosis by fluorescence-activated cell sorting analysis (Fig. 1C and D) in HT22 cells. The results demonstrated that treatment with glutamate significantly decreased the cell viability and increased cell apoptosis in a concentration-dependent manner in HT22 cells.

ROS is implicated in glutamate-induced apoptosis in HT22 cells. It was previously reported that glutamate treatment may increase the cellular ROS levels in HT22 cells (4,5,27). To confirm whether ROS was associated with glutamate-induced HT22 cell apoptosis, the cells were treated with the indicated concentrations (0, 2, 4, 6, 8 and 10 mM) of glutamate for 6 h and the cellular ROS levels were determined by dihydroethidium staining. Stained cells were observed with a fluorescence microscope. The results demonstrated that the cellular ROS levels were dose-dependently increased by glutamate treatment (Fig. 2A and B). Furthermore, inhibition of cellular ROS accumulation with NAC, an ROS scavenger, significantly reduced glutamate-induced HT22 cell apoptosis (Fig. 2C and D), as observed by flow cytometric analysis. These findings indicateda potential involvement of ROS in glutamate-stimulated HT22 cell apoptosis.

Glutamate treatment induces upregulation of Prx V protein expression in HT22 cells. To investigate whether Prx V protein expression levels are altered upon glutamate stimulation in HT22 cells, the cells were stimulated for the indicated durations and concentrations with glutamate and subjected to western blotting to analyze Prx V protein expression. The protein expression of Prx V by glutamate treatment was increased up to 4 h, and then decreases at 8 and 12 h and up to 4 mM glutamate, then decreased at 6, 8 and 10 mM. (Fig. 3A-D), whereas Prx II expression was not altered across any of the treatments. To verify whether glutamate-induced Prx V protein upregulation was associated with intercellular ROS accumulation, HT22 cells were pretreated with NAC for 30 min following glutamate stimulation for 12 h. The results demonstrated that treatment with NAC successfully reduced Prx V protein expression levels in HT22 cells that also received glutamate treatment (Fig. 3E and F). These results indicated that Prx V may be involved in the glutamate-induced HT22 cell apoptosis process that was associated with intercellular ROS accumulation.

Knockdown of Prx V increases glutamate-induced apoptosis in HT22 cells. As demonstrated in Fig. 4A, the Prx V protein expression levels were selectively knocked down in the Prx V-shRNA lentivirus-infected cells (by ~80%), while the protein expression of Prx II was not affected. To investigate the effect of Prx V shRNA on cell viability, mock and Prx V shRNA-transfected HT22 cells were treated with glutamate and cell viability was detected via an MTT assay. As demonstrated in Fig. 4B, decreased cell viability was observed in Prx V shRNA-transfected cells at 4 and 6 mM compared with mock shRNA-transfected cells. Furthermore, the apoptotic ratio between mock and Prx V shRNA-transfected HT22 cells following glutamate treatment was analyzed by flow cytometry. The results demonstrated that cellular apoptosis was significantly increased in Prx V shRNA-transfected cells compared with mock cells in HT22 cells treated with 4 mM

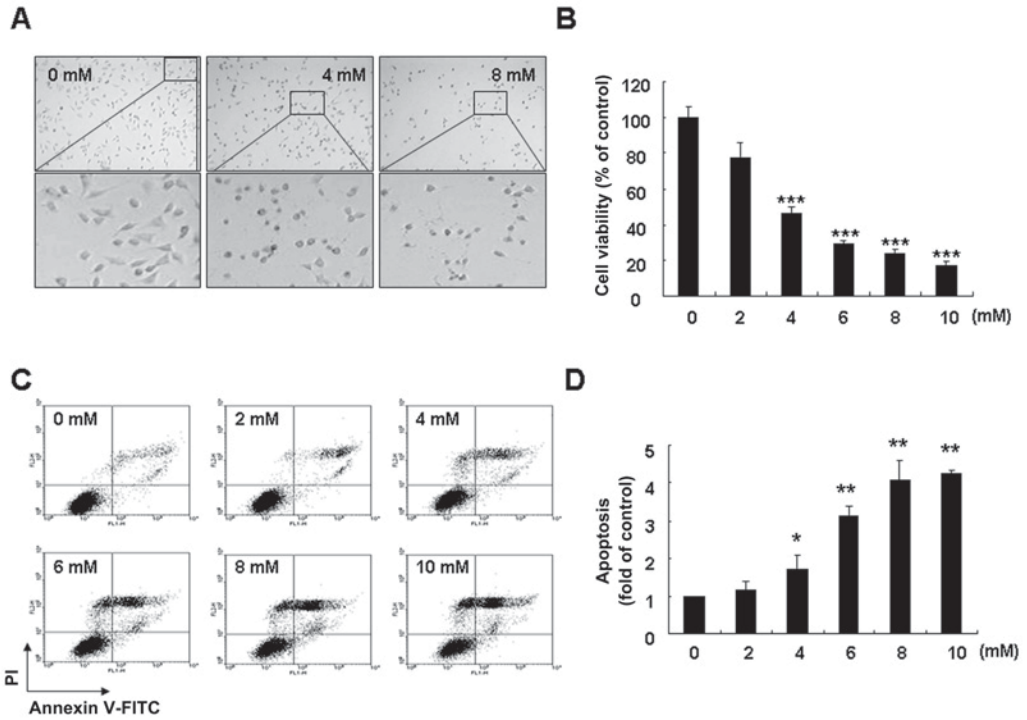

Figure 1. Induction of neuronal cell death by glutamate in HT22 cells. (A) HT22 cells were incubated with glutamate at the indicated concentrations for 12 h. Cell morphologies were observed and images were captured with a phase-contrast microscope (magnification, x200). (B) HT22 cells were treated with glutamate for 24 h and the cell viability was determined with an MTT assay. HT22 cells were stimulated with glutamate at the indicated concentrations for 12 h and stained with Annexin V-FITC/PI for apoptosis analysis. (C) Representative flow cytometry plots following Annexin V-FITC/PI staining in each group. (D) Flow cytometry results (upper right) were quantified and statistical analysis performed among groups. Data are presented as the mean ± standard deviation (n=3). *P<0.01, **P<0.01 and ***P<0.001 vs. 0 mM glutamate. FITC, fluorescein isothiocyanate; PI, propidium iodide; PE, phycoerythrin.

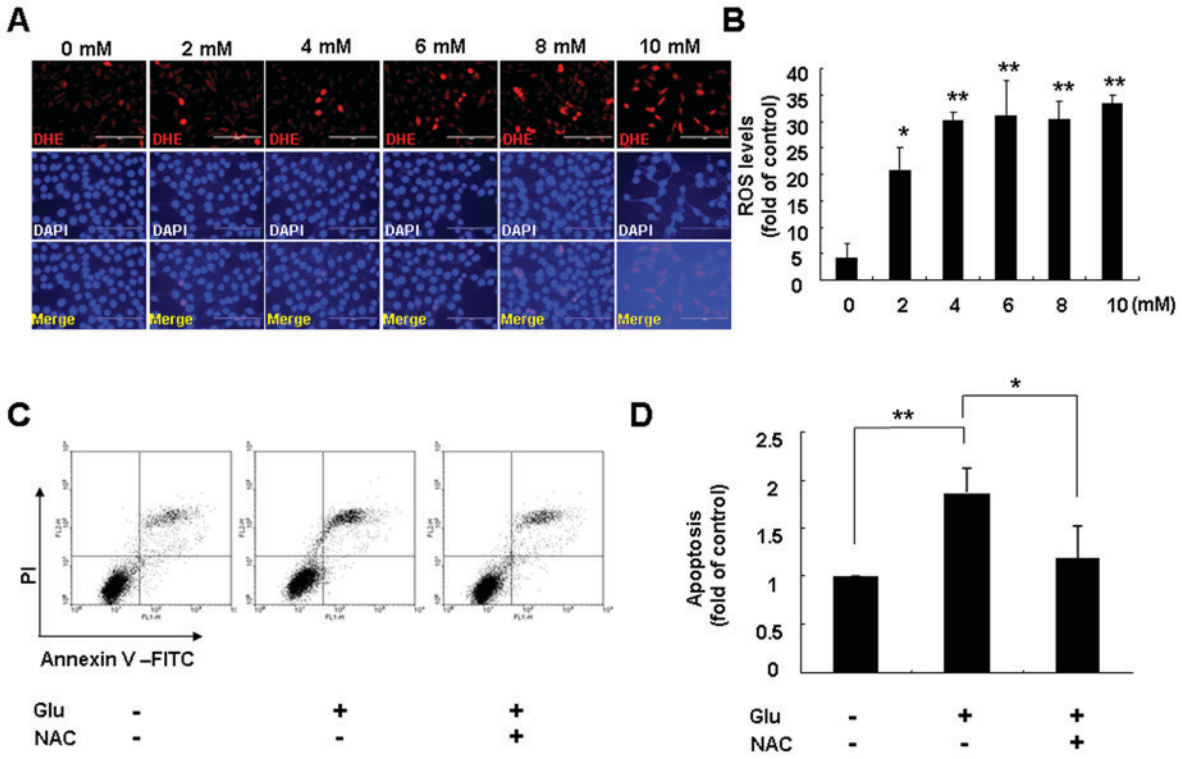

Figure 2. Glutamateinduced oxidative cytotoxicity in HT22 cells. HT22 cells were treated with glutamate at the indicated concentrations for 6 h. (A) Intracellular ROS accumulation was analyzed using a fluorescence microscope following DHE staining (scale bar, 100 μm). (B) Percentage of DHE-positive cells were counted as ROS accumulating cells and n=5 for each treatment group. HT22 cells were treated with 4 mM glutamate for 12 h with or without pretreatment with of NAC (5 mM) and stained with Annexin V-FITC/PI. *P<0.05 and **P<0.01 vs. 0 mM glutamate. (C) Representative flow cytometry plots following Annexin V-FITC/PI staining in each group (upper right hand side cells were the apoptotic cells). (D) Flow cytometry results were quantified and statistical analysis performed among groups. *P<0.05 and **P<0.01, as indicated. Data are presented as the mean ± standard deviation (n=5). ROS, reactive oxygen species; DHE, dihydroethidium; NAC, N-acetyl-L-cysteine; FITC, fluorescein isothiocyanate; PI, propidium iodide; PE, phycoerythrin; Glu, glutamate.

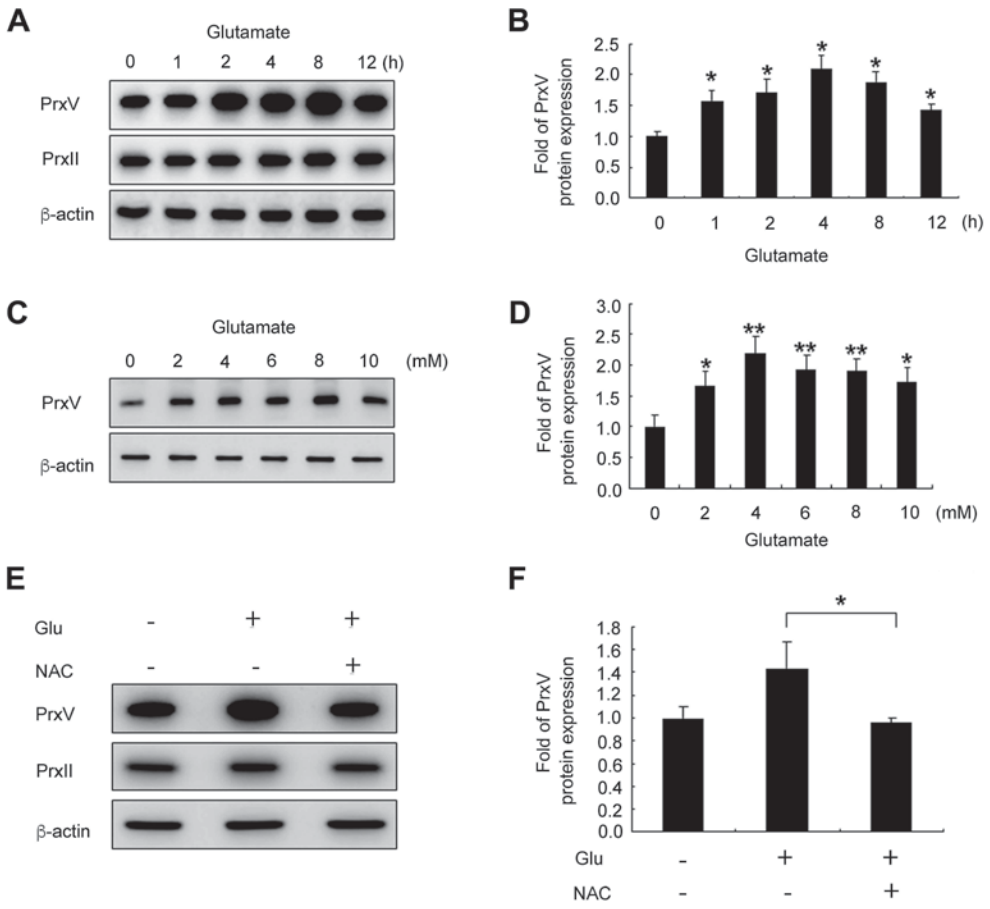

Figure 3. Prx V is upregulated by glutamate in HT22 cells. (A) Protein expression levels of Prx V and Prx II were determined by western blotting in HT22 cells treated with 4 mM glutamate for the indicated durations. (B) Western blotting results were quantified by densitometric analysis. (C) HT22 cells were stimulated with the indicated concentrations of glutamate for 4 h and then Prx V protein expression was analyzed by western blotting. (D) Densitometry was subsequently performed to quantify the protein expression levels of Prx V. (E) HT22 cells were treated with 4 mM glutamate for 12 h with or without pretreatment with NAC (5 mM) and harvested for western blot analysis using Prx V, Prx II and β-actin antibodies. (F) Densitometric analysis was performed to quantify the protein expression of Prx V following glutamate treatment with or without NAC. Data are presented asthe mean ± standard deviation (n=3). For parts B and D, *P<0.05 and **P<0.01 vs. 0 mM glutamate; for part F, *P<0.05, as indicated. Prx, peroxiredoxin; NAC, N-acetyl-L-cysteine.

glutamate, and increases in apoptosis and decreases in cell viability were reversed by NAC treatment (Fig. 4C-E).

Prx V regulates HT22 cell apoptosis via ERK, MAPK and AIF signaling pathways. To understand the underlying molecular mechanism of Prx V in glutamate-induced cell apoptosis of HT22 cells, three types of MAPK inhibitors, including SB203580 (for p38 MAPK), PD98059 (for ERK MAPK) and SP600125 (for JNK MAPK) were used in combination with glutamate treatment. The increased apoptotic ratios were analyzed between mock shRNA and Prx V shRNA-transfected HT22 cells by flow cytometry. As demonstrated in Fig. 5A, increased apoptosis following glutamate treatment was suppressed by PD98059 treatment, which selectively inhibits the ERK signaling pathway, both in mock shRNA and Prx V shRNA-transfected cells, while the other compounds exhibited no effect. Furthermore, western blotting results demonstrated that the protein expression levels of the proapoptotic protein cleaved-caspase-3 and the antiapoptotic protein Bcl-2 were not significantly different between mock shRNA and Prx V shRNA-transfected cells (Fig. 5B). In addition, the current study also investigated the translocalization of AIF between the mock shRNA and Prx V shRNA-transfected HT22 cells. As demonstrated in Fig. 5C and D, increased nuclear translocalization of AIF was observed in Prx V shRNA-transfected cells compared with mock shRNA-transfected cells following treatment with glutamate.

Discussion

Previously, protective role of antioxidants in glutamate-induced neuronal cell death has been investigated extensively. Phosphoproteomic analysis demonstrated that 17 types of proteins were up- or down-regulated in glutamate-treated HT22 cells, and overexpression of Prx II and SOD1 efficiently protected neuron cell death induced by ischemia and oxidative insult, but not glutamate excitotoxicity (28,29). In addition, delivery of Prx VI into cells attenuated tumor necrosis factor-α- and glutamate-induced retinal ganglion cell death (30). These reports suggested an association between Prxs and glutamate-induced neuronal cell death; however, little is known regarding Prx V expression and function in HT22 mouse hippocampal cells. We previously reported high expression levels of Prx V in neuron cells (31) and microglia Prx V in mouse brains and primary cell could be up-regulated by lipopolysaccharide (LPS) stimulation (20). The results of the current study demonstrated that glutamate treatment

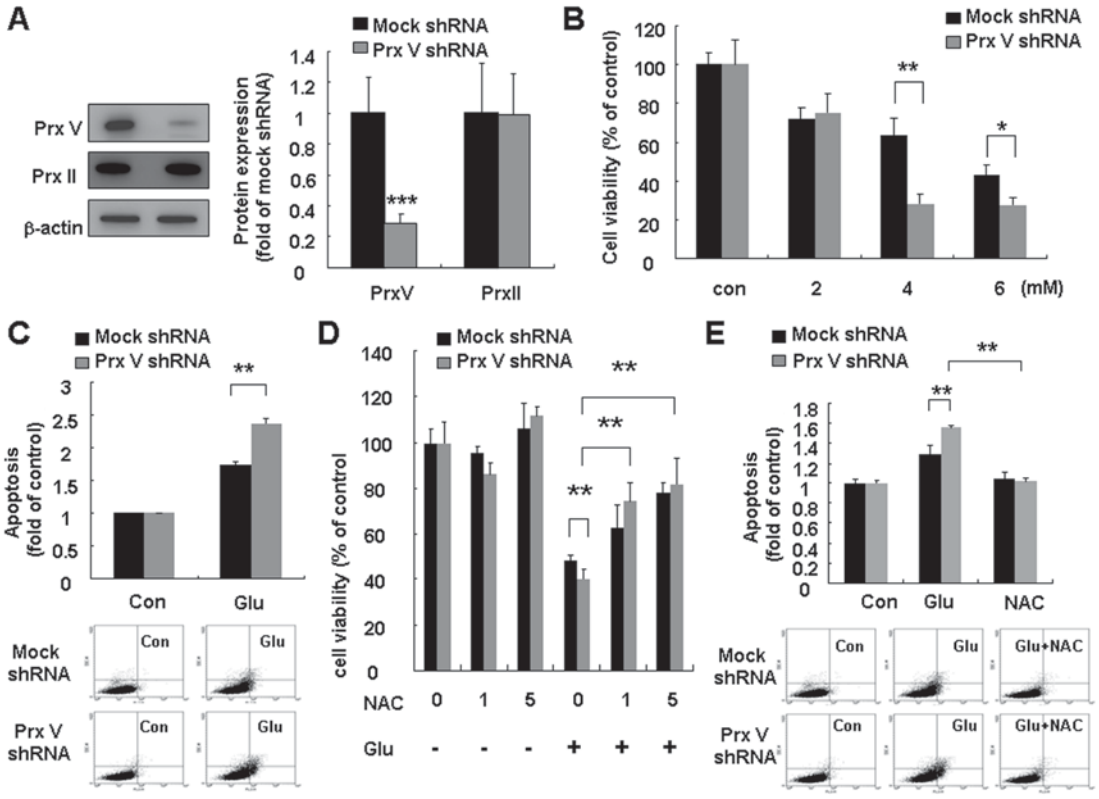

Figure 4. Effect of Prx V on glutamate-induced apoptosis in HT22 cells. (A) HT22 cells were stably transfected with the Prx V shRNA or with a scrambled non-targeting shRNA lentivirus vector, and western blot analysis was performed to determine Prx V and Prx II protein expression levels. The results were quantified by densitometric analysis. (B) Mock shRNA and Prx V shRNA HT22 cells were stimulated with glutamate at the indicated dosages and cell viability was determined via an MTT assay. (C) Mock shRNA and Prx V shRNA HT22 cells were treated with 4 mM glutamate and stained with Annexin V-FITC/PI. Cell apoptosis was assessed by flow cytometry and the results were quantified and statistically analyzed. (D) Mock shRNA and Prx V shRNA HT22 cells were pretreated with NAC (1 and 5 mM) for 30 min followed by glutamate treatment (4 mM), and cell viability was analyzed by an MTT assay. (E) Mock shRNA and Prx V shRNA HT22 cells were pretreated with NAC following treatment with 4 mM glutamate and stained with Annexin V-FITC/PI. Cell apoptosis was assessed by flow cytometry, and the results were quantified and statistically analyzed. Data are presented as themean ± standard deviation (n=3). For part A, ***P<0.001 vs. mock shRNA; for parts B-E, *P<0.05 and **P<0.01, as indicated. Prx, peroxiredoxin; shRNA, short hairpin RNA; FITC, fluorescein isothiocyanate; PI, propidium iodide; PE, phycoerythrin; NAC, N-acetyl-L-cysteine; Con, control; Glu, glutamate.

significantly reduced cell viability and increased apoptosis in a concentration-dependent manner, which was associated with increased ROS levels stimulated by glutamate.

In addition, in the current study, Prx V expression levels were upregulated during glutamate-stimulated apoptosis in HT22 cells, indicating a potential regulatory effect of Prx V in HT22 cell apoptosis. Numerous reports have demonstrated that antioxidant expression depends on oxidative conditions. For example, Prx I and Prx VI may be effectively induced in response to oxidative insults, such as tert-butyl hydroquinone and LPS, via binding of nuclear factor erythroid 2-related factor 2 to antioxidant response elements on their promoter regions (32-34). The present study also challenged HT22 cells with the chemical antioxidant NAC prior to stimulation with glutamate. The results demonstrated that the Prx V protein expression levels were downregulated compared with those treated with only glutamate, which was accompanied by reduced cell apoptosis. These findings indicated that ROS signaling pathways may be implicated in glutamate-stimulated Prx V induction in HT22 cells.

According to the results of the present study, Prx V protein expression was upregulated during glutamate-induced apoptosis in HT22 cells. Knockdown of Prx V expression in HT22 cells enhanced glutamate-induced decreases in cell viability and increased cellular apoptosis, which may be associated with increases in the cellular ROS levels. The MAPK signaling pathway is widely involved in cell proliferation, migration, apoptosis and differentiation, and has been reported to be closely associated with cellular ROS levels (35-37). However, to the best of our knowledge, no previous evidence has demonstrated an association between Prx V and MAPK in neuronal cell apoptosis. It has been reported that Prx V may prevent amyloid-β oligomer-induced neuronal cell death through inhibiting the ERK signaling pathway in HT22 cells, indicating an association between Prx V and MAPK signaling pathways in cellular apoptosis (38). In the current study, experiments that employed various MAPK inhibitors demonstrated that increased cell apoptosis in Prx V knockdown cells was reversed by ERK inhibitor treatment, indicating that the regulatory effects of Prx V on HT22 cell apoptosis may be regulated via ERK MAPK signaling pathways. Additionally, it has previously been reported that glutamate-stimulated apoptosis may be mediated via the translocation of AIF from the cytosol to nuclei in HT22 cells (10). Thus, the present study also assessed the translocation of AIF from the cytosol to nuclei and apoptosis-associated protein expression levels between mock and Prx V shRNA-transfected cells following glutamate treatment. The results demonstrated that increased translocation

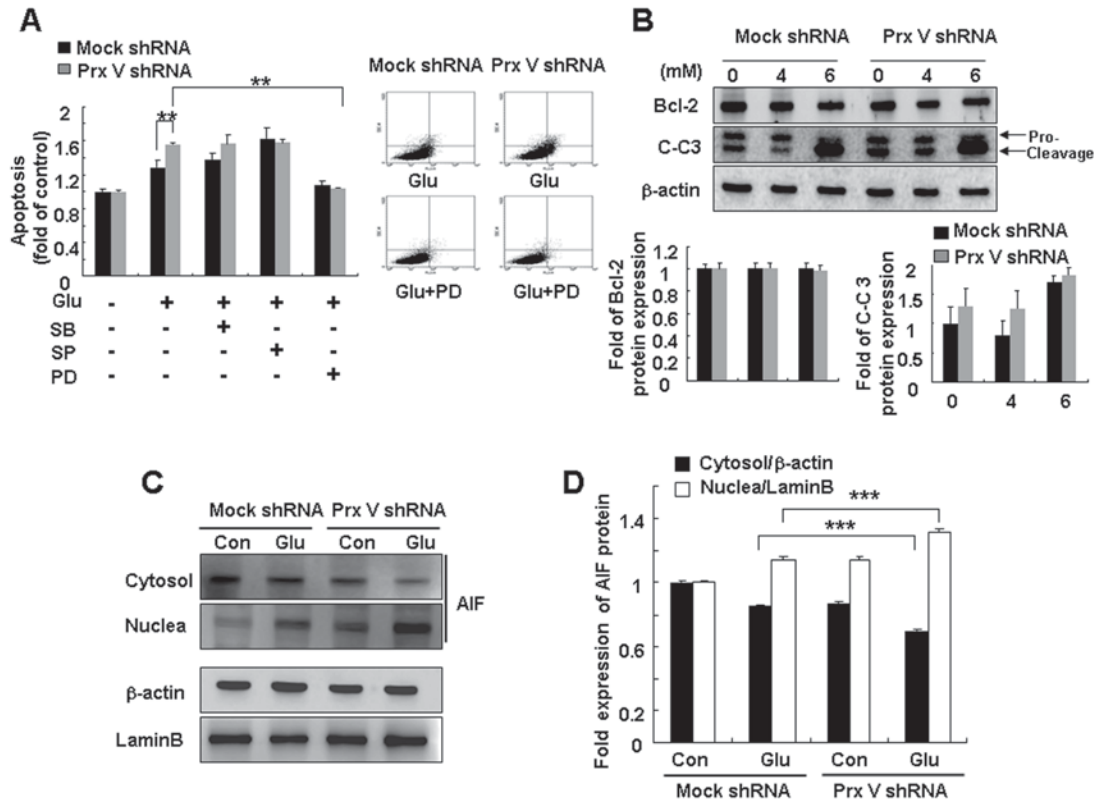

Figure 5. Prx V regulates HT22 apoptosis via ERK and AIF signaling pathways. (A) Mock shRNA and Prx V shRNA HT22 cells were pretreated with SB203580, PD98059 and SP600125, which are inhibitors of p38, ERK and JNK mitogen-activated protein kinases, respectively, for 30 min, followed by treatment with 4 mM glutamate (12 h) and staining with Annexin V-FITC/PI. Cell apoptosis was assessed by flow cytometry and the results were quantified and statistically analyzed. (B) Western blot analysis using Bcl-2 and cleaved-caspase-3 antibodies was performed in mock shRNA and Prx V shRNA HT22 cells treated with 4 and 6 mM glutamate (12 h). (C) Cytosolic and nuclear AIF protein levels were determined by western blot analysis following glutamate treatment (4 mM, 12 h) in mock shRNA and Prx V shRNA HT22 cells. (D) Cytosolic and nuclear AIF protein levels were quantified by densitometric analysis. Data are presented asthemean ± standard deviation (n=3). **P<0.01 and ***P<0.001, as indicated. Prx, peroxiredoxin; ERK, extracellular signal-regulated kinase; AIF, apoptosis-inducing factor; shRNA, short hairpin RNA; JNK, c-Jun N-terminal kinase; FITC, fluorescein isothiocyanate; PI, propidium iodide; PE, pychoerythrin; Bcl-2, B-cell lymphoma-2; Glu, glutamate; SB, SB203580; SP, SP600125; PD, PD98059; c-c3, cleaved-caspase-3; Con, control.

of AIF from the cytosol to nuclei was observed in Prx V shRNA cells, whereas there was no difference in the protein expression of Bcl-2 and cleaved-caspase-3 in both transfection groups, indicating that AIF translocation maybe a key process for Prx V in regulating HT22 cell apoptosis. Although the findings of the current study may provide insight for understanding the role of Prx V in neuronal cell death, certain limitations exist. For example, the results in the current study do not provide direct evidence of the mechanism by which Prx V regulates AIF translocation and the ERK signaling pathway and signaling pathways involved in glutamate-mediated Prx V induction in HT22 cells remain unclear. However, according to the results of the present study, it may be hypothesized that Prx V may serve a protective role via the ROS/ERK/AIF signaling cascade, which was induced by glutamate in HT22 cells. However, this mechanism requires further investigation.

In conclusion, the results of the present study indicated that glutamate-stimulated activation of ROS may provoke the induction of Prx V, which has an antioxidative function in glutamate-stimulated HT22 cell apoptosis, and PrxV may serve a role in neural apoptosis. Identification of the mechanisms underlying this may provide novel therapeutic targets for treatment of oxidative stress induced neuronal cell death.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GNS and LL performed the cell culture and wrote the manuscript. LF, YJ and MHJ performed the data analysis. YHH, CHJ and YZJ construct the shRNA cell lines and preformed image analysis. DSL, THK and YDC were involved in drafting the manuscript and data analysis. HNS was a major contributor

in writing the manuscript and made substantial contributions to conception and design.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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