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PEP-1-glutaredoxin 1 protects against hippocampal neuronal cell damage from oxidative stress via regulation of MAPK and apoptotic signaling pathways

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Abstract. Oxidative stress is known to be a primary risk factor for neuronal diseases. Glutaredoxin (GLRX)-1, a redox-regulator of the thioredoxin superfamily, is known to exhibit an important role in cell survival via various cellular functions. However, the precise roles of GLRX1 in brain ischemia are still not fully understood. The present study investigated whether transduced PEP-1-GLRX1 protein has protective effects against oxidative stress in cells and in an animal model. Transduced PEP-1-GLRX1 protein increased HT-22 cell viability under oxidative stress and this fusion protein significantly reduced intracellular reactive oxygen species and levels of DNA damage. In addition, PEP-1-GLRX1 protein regulated RAC-a serine/threonine-protein kinase and mitogen-activated protein kinase signaling, in addition to apoptotic signaling including B cell lymphoma (Bcl)-2, Bcl-2 associated X, apoptosis regulator, pro-caspase-9 and p53 expression levels. In an ischemic animal model, it was verified that PEP-1-GLRX1 transduced into the Cornu Ammonis 1 region of the animal brain, where it markedly protected against ischemic injury. These results indicate that PEP-1-GLRX1 attenuates neuronal cell death resulting from oxidative stress *in vitro* and *in vivo*. Therefore, PEP-1-GLRX1 may exhibit a beneficial role in the treatment of neuronal disorders, including ischemic injury.

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Introduction

Oxidative stress is known to be a result of excessive levels of reactive oxygen species (ROS) including hydroxyl radicals, superoxide anions, and hydrogen peroxide, which are produced by cellular respiration in mitochondria and other cellular process. ROS is known to be associated with the pathogenesis of various diseases (1-3). Oxidative stress is associated with cell signaling and gene regulation systems in a number of molecular biological processes. Excessive ROS levels have resulted in various diseases via the dysfunction of cellular macromolecules, leading to the promotion of lipid peroxidation, DNA fragmentation, and protein damage, eventually resulting in cell death (4,5). Several studies have suggested that the regulation of intracellular ROS levels is very important to neuronal cell survival in various diseases (6,7).

Human glutaredoxin (GLRX)-1 is a member of the thioredoxin family, additionally termed glutathione (GSH)-dependent thiol oxidoreductase, and is a small molecular weight (12 kDa) protein (8). GLRX1 is distributed in various human tissues including the brain, which are involved in sulfhydryl homeostasis and exhibit roles in the regulation of redox signaling, and control a variety of cellular processes (9-11). A previous study demonstrated that GLRX1 has an important role as a ROS scavenger and protects against protein oxidation resulting from oxidative stress (12). However, the exact function of GLRX1 in ischemic injury remains to be fully elucidated.

In general, it is difficult for exogenous macromolecules to transduce into cells. Protein transduction domains (PTDs) or cell penetrating peptides (CPPs) consist of a short chain of amino acids, and effectively transduce exogenous macromolecules into cells and tissues (13). Of the various PTDs, PEP-1 peptide has several advantages including rapid transduction of proteins, high stability in physiological buffers, and high efficiency of transduction (14,15). Although the precise transduction mechanism is not clear yet, it has been demonstrated that a variety of therapeutic PTD fused proteins were successfully delivered into various cells *in vitro* and in *in vivo* animal models, transduced proteins effectively prevented cell death (16-22). The present study investigated whether PEP-1-GLRX1 protein has protective effects against oxidative stress in HT-22 cells and against ischemic brain injury in an animal model. It was revealed that transduced PEP-1-GLRX1 protein has protective effects against oxidative stress in HT-22 cells and in an animal model of ischemia.

Materials and methods

Materials and cell culture. Ni^{2+}-nitrilotri-acetic acid Sepharose Superflow was purchased from Qiagen, Inc. (Valencia, CA, USA). Histidine antibody was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). B-cell lymphoma 2 (Bcl-2) antibody was obtained from Abcam (Cambridge, UK). Phosporylated (p)-protein kinase B (Akt), Akt, p-mitogen-activated protein kinase 8 (JNK), JNK, p-p44/42 mitogen-activated protein kinase (ERK), ERK, p-mitogen activated protein kinase 14 (p38), p38, p-cellular tumor antigen p53 (p53), p53, Bcl-2 associated protein X (Bax), caspase-9 and β-actin primary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). 2',7'-Dichlorofluorescein diacetate (DCF-DA) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). 8-hydroxy-2-deoxyguanosine (8-OHdG) antibodies were purchased from Santa Cruz Biotechnology, Inc. The polymerase chain reaction (PCR) technique was applied to isolate human GLRX1 cDNA. All remaining chemicals and reagents used in this experiment were of the highest available commercial grade.

Cell culture. HT-22 murine hippocampal neuronal cells were cultured in Dulbecco's modified Eagle's medium (Lonza Group, Ltd., Basel, Switzerland) supplemented with 10% fetal bovine serum (Lonza Group, Ltd.) and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37˚C, under humidified conditions of 95% air and 5% CO_2.

Expression and purification of PEP-1-GLRX1 proteins. A cell permeable PEP-1 expression vector was prepared in the present laboratory as previously described (19,20). The cDNA sequence for human GLRX1 was amplified by PCR using the following primer sequences: Forward, 5'-GGTCTC CTCGAGATGGCTCAAGAGTTTG-3' and reverse, 5'-GGA TCCTTACTGCAGAGCTCCAATCTG-3'. PCR products were excised, eluted (Expin Gel; GeneAll Biotechnology Co., Ltd., Seoul, Korea), and ligated into a TA cloning vector (pGEM®-T easy vector; Promega Corporation, Madison, WI, USA) according to the manufacture's protocol. The purified TA vector containing human GLRX1 cDNA was ligated into the PEP-1 expression vector to produce a PEP-1-GLRX1 fusion protein. In a similar fashion, a control GLRX1 was constructed that expressed the GLRX1 protein without PEP-1. To produce the PEP-1-GLRX1 and control GLRX1 proteins, the plasmid was transformed into *Escherichia coli* BL21 cells. The transformed bacterial cells were grown in 100 ml of lysogeny broth media at 37˚C to a D_{600} value of 0.5-1.0 and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside at 37˚C for 6 h. Harvested cells were lysed by sonication and purified using a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column (Qiagen, Inc.) and PD-10 column chromatography (GE Healthcare, Chicago, IL, USA). The purified protein concentrations were estimated using a Bradford assay (23).

Transduction of PEP-1-GLRX1 protein into HT-22 cells. To examine the transduction ability of PEP-1-GLRX1 protein, HT-22 cells were treated with various concentrations of PEP-1-GLRX1 protein (0.5-1.5 μM) for 1 h or with 1.5 μM for various time periods (10-60 min). Cells were then treated with trypsin-EDTA and washed with phosphate-buffered saline (PBS). The cells were harvested for the preparation of cell extracts to perform western blot analysis. Transduced PEP-1-GLRX1 protein was detected using an anti-histidine antibody (1:1,000; cat no. sc-804).

Western blot analysis. Following PEP-1-GLRX1 protein transduction, HT-22 cells were harvested and homogenized with NP-40 protein extraction solution (Elpis Biotech, Inc., Daejeon, South Korea) at 4˚C for 20 min. Protein concentration was determined by the Bradford assay. Equal amounts of protein (30 μg/lane) was resolved by 15% SDS-PAGE and the gels were subsequently transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk for 1 h at 37˚C in a Tris-buffered saline buffer with 0.1% Tween 20. The blocked membrane was incubated with histidine (1:1,000; cat no. sc-804), p-Akt (1:1,000; cat no. 9272S), Akt (1:1,000; cat no. 4058S), p-JNK (1:1,000; cat no. 9251S), JNK (1:1,000; cat no. 9258S), p-ERK (1:1,000; cat no. 4376S), ERK (1:1,000; cat no. 9102S), p-p38 (1:1,000; cat no. 4631S), p38 (1:1,000; cat no. 9212S), p-p53 (1:1,000; cat no. 9284S), p53 (1:1,000; cat no. 9282S), Bax (1:1,000; cat no. 2772S), Bcl-2 (1:1,000; cat no. ab59348), capase-9 (1:1,000; cat no. 9504S) and β-actin (1:1,000; cat no. 4967S) primary antibodies overnight at 4˚C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:10,000; cat no. 7074S) for 1 h at 37˚C. Bands were visualized with a Chemidoc imaging system (version 5.2; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and an enhanced chemiluminescence kit according to the manufacturer's protocol (GE Healthcare) (24). Bands were quantified with ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA).

Fluorescence confocal microscopy analysis. The distribution of transduced proteins was assessed using fluorescence microscopy as previously described (20,22). HT-22 cells were grown on coverslips and treated with PEP-1-GLRX1 protein (1.5 μM) for 1 h and then washed twice with PBS, fixed with

4% paraformaldehyde at 37˚C for 5 min, permeabilized with 0.1% Triton X-100 and blocked at 37˚C for 40 min with 3% bovine serum albumin (Sigma-Aldrich; Merck KGaA) in PBS (PBS-BT) and washed with PBS-BT. The cells were incubated with an anti-histidine antibody (1:2,000; Santa Cruz Biotechnology, Inc.) for 1 h at 37˚C, followed by incubation with Alexa Fluor 488-conjugated secondary antibody (1:15,000; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in the dark for 1 h at 37˚C. Nuclei were then stained for 3 min with 1 μg/ml DAPI diluted 1:3,000 (Roche Diagnostics GmbH, Mannheim, Germany) At each step, the cells were washed with PBS-BT three times. Coverslips were mounted onto glass slides with Dako fluorescent mounting solution (Agilent Technologies, Inc., Santa Clara, CA, USA) The cells were analyzed by confocal microscopy using a model FV-300 microscope (magnification, x630; Olympus Corporation, Tokyo, Japan).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, HT-22 cells were pretreated with PEP-1-GLRX1 protein (0.5-1.5 μM) for 1 h and then treated with hydrogen peroxide (H_2O_2, 1 mM) for 3 h. A cell viability assay was performed using MTT as previously described (20,22). The absorbance was read at a wavelength of 570 nm using an ELISA microplate reader (Labsystems Multiskan MCC/340) and cell viability was defined as the % of untreated control cells.

Measurement of oxidative stress-induced intracellular ROS levels. DCF-DA dye was used to measure intracellular ROS levels, which was converted into fluorescent 2'7'-dichlorofluorescein (DCF) by ROS (22). HT-22 cells were incubated in the absence or presence of PEP-1-GLRX1 (1.5 μM) for 1 h prior to treatment with H_2O_2 (1 mM) for 10 min. Those cells were washed twice with PBS and then incubated at 37˚C for 30 min using DCF-DA (10 μM). The image was produced at 485 nm excitation and 538 nm emission using a Fluoroskan ELISA plate reader (Labsystems Oy, Helsinki, Finland).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. To investigate DNA fragmentation, TUNEL staining was performed using a Cell Death Detection Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol (22). Briefly, HT-22 cells were incubated in the absence or presence of PEP-1-GLRX1 (1.5 μM) for 1 h, and then treated with H_2O_2 (1 mM) for 5 h. Next, nuclei were stained at 37˚C for 3 min with 1 μg/ml DAPI and washed with PBS. Coverslips were mounted onto glass slides using Dako fluorescent mounting solution (CA, USA). Images were taken using a fluorescence microscope (Nikon Eclipse 80i; Nikon Corporation, Tokyo, Japan). TUNEL-positive cells were counted by phase-contrast microscopy in at least 5 random fields at x200 magnification.

Measurement of apoptotic protein expression. HT-22 cells were incubated in the absence or presence of PEP-1-GLRX1 (1-1.5 μM) for 1 h, and then treated with H_2O_2 for various time periods. The expression levels of Bcl-2 (1 h), Bax (2 h), and pro-caspase-9 (4 h) in whole cell lysates were analyzed by western blotting using respective antibodies as described above. The bands were quantified by ImageJ software (version 1.4.8; National Institutes of Health).

Experimental animals and induction of cerebral forebrain ischemia. Male Mongolian gerbils (70-80 g; 6 months old; n=40) were obtained from the Experimental Animal Center of Soonchunhyang University (Cheonan, Korea). The animals were housed at an adequate temperature (23˚C) and humidity (60%) with a 12 h light/12 h dark cycle, and free access to food and water. All experimental procedures involving animals and their care conformed to the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research & Quarantine Service of Korea and were approved by the Institutional Animal Care and Use Committee of Soonchunhyang University (Cheonan-Si, Korea; SCH16-0024).

Cerebral forebrain ischemia damage was induced as previously described (25,26). Briefly, the animals were anesthetized with a mixture of 2.5% isoflurane (Baxtor Healthcare, Deerfield, IL, USA) in 33% oxygen and 67% nitrous oxide. Bilateral common carotid arteries were isolated and occluded using nontraumatic aneurysm clips. The complete interruption of blood flow was verified by observing the central retinal artery using an ophthalmoscope. Following 5 min of occlusion, the aneurysm clips were removed from the common carotid arteries. The body temperature under free-regulating or normothermic (37±0.5˚C) conditions was monitored with a rectal temperature probe (TR-100; Fine Science Tools, Foster City, CA, USA) and maintained using a thermometric blanket prior to, during, and following surgery until the animals completely recovered from anesthesia. Thereafter, the animals were kept on the thermal incubator (Mirae Medical Industry, Seoul, South Korea) to maintain body temperature until the animals were euthanized.

Treatment of ischemic animals with PEP-1-GLRX1 and immunohistochemistry. To explore the protective effects of PEP-1-GLRX1 protein against ischemic damage, the animals were divided into 4 groups (n=10 per group); control sham group (normal control), model group (ischemia control), control GLRX1-treated group, and PEP-1-GLRX1-treated group (each 2 mg/kg). The control GLRX1 and PEP-1-GLRX1 proteins were administered intraperitoneally 30 min following ischemia-reperfusion.

Immunohistochemistry was performed as described in previous studies (20,22). The brain tissue samples were obtained 7 days following ischemia-reperfusion. To examine the protective effects of PEP-1-GLRX1 protein against ischemic damage, the sections were stained overnight at 4˚C with histidine (1:500; cat no. sc-804), neuronal nuclei (NeuN; 1:100; cat no. MAB377; EMD Millipore, Billerica, MA, USA), 0.5% cresyl violet acetate (CV; cat no. 10510-54-0; Sigma-Aldrich; Merck KGaA), Fluoro-Jade B (FJB; 1:500; cat no. AG310-30MG; EMD Millipore), glial fibrillary acidic protein (GFAP; 1:500; cat no. AB5804; EMD Millipore) and ionized calcium-binding adapter molecule 1 (Iba-1; 1:500; cat no. 019-19741; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Subsequently, sections were incubated with biotinylated goat anti-mouse IgG antibody (1:200; cat no. BA-9200; Vector Laboratories, Inc. Burlingame, CA, USA) or biotinylated goat anti-rabbit IgG antibody (1:200: cat no. BA-1,000;

Vector Laboratories, Inc.) for 1 h at 25˚C. A cell count was conducted in the hippocampal area to quantify immunostaining as described in a previous study (20,22).

Statistical analysis. Data are expressed as the mean ± standard error of the mean of three experiments. Differences between groups were analyzed by one-way analysis of variance followed by a Bonferroni's post-hoc test using GraphPad Prism software (version 5.01; GraphPad Software Inc., San Diego, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Construction and purification of PEP-1-GLRX1 protein. The present study constructed a cell permeable PEP-1-GLRX1 protein expression vector. As presented in Fig. 1A, the vector contained a cDNA sequence with an amino-terminal tag containing human GLRX1, PEP-1 peptide and 6 histidine residues. A control GLRX1 expression vector without the PEP-1 peptide was also constructed. PEP-1-GLRX1 and control GLRX1 proteins were overexpressed in bacterial cells and purified by using Ni^{2+}-NTA and PD-10 column chromatography. The purified PEP-1-GLRX1 and control GLRX1 proteins were verified using SDS-PAGE and western blot analysis (Fig. 1B and C).

Transduction of PEP-1-GLRX1 protein into HT-22 cells. To examine of transduction ability of PEP-1-GLRX1 protein, HT-22 cells were treated with PEP-1-GLRX1 proteins at various concentrations (0.5-1.5 μM) for 1 h. Cells were also treated with the same concentration of PEP-1-GLRX1 protein (1.5 μM) for various times (10-60 min). The transduction of PEP-1-GLRX1 protein was verified by western blotting. As presented in Fig. 2A and B, PEP-1-GLRX1 protein concentration- and time-dependently transduced into HT-22 cells. However, control GLRX1 protein did not transduce into the cells. Furthermore, transduced PEP-1-GLRX1 protein was examined using DAPI and histidine immunostaining. Through immunostaining signals, it was verified that PEP-GLRX1 protein efficiently transduced into HT-22 cells (Fig. 2C). Since the stability of protein is a significant factor in the development of therapeutic agents, the stability of transduced PEP-1-GLRX1 protein was measured. As presented in Fig. 2D, the concentration of transduced PEP-1-GLRX1 protein in the cells decreased following 24 h, however remained present for up to a maximum of 60 h.

Effects of PEP-1-GLRX1 on cell survival from oxidative stress. In order to examine whether transduced PEP-1-GLRX1 protein has protective effects against H_2O_2-induced HT-22 cell death, the present study measured cell viability using an MTT assay. As presented in Fig. 3A, when cells were treated with H_2O_2, cell survival was ~50%. PEP-1-GLRX1 protein markedly increased cell viability to up to 73% dose-dependently. However, control GLRX1 protein did not demonstrate the protective effects in H_2O_2 treated cells. There was no protective effect on cell viability when the PEP-1 peptide alone was used for treatment under the same conditions (data not shown).

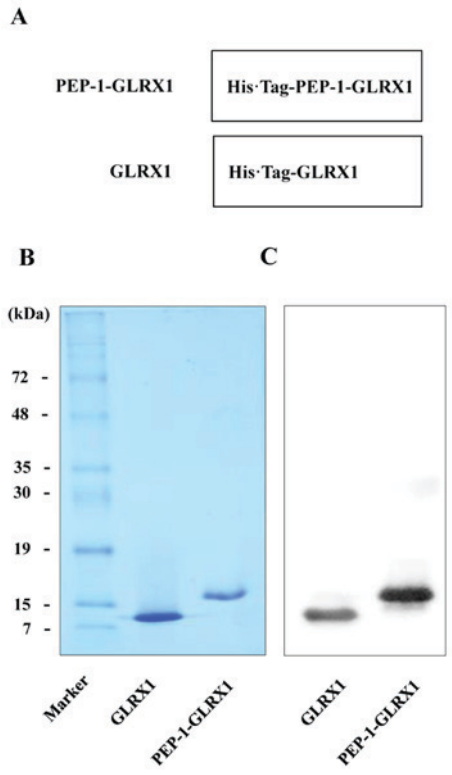

Figure 1. Construction and purification of PEP-1-GLRX1 proteins. (A) Constructed map of PEP-1-GLRX1 based on the pET15-b vector and diagrams of the expressed PEP-1-GLRX1 proteins. Purified recombinant PEP-1-GLRX1 and control GLRX1 proteins were identified by (B) 15% SDS-PAGE and were verified by (C) western blot analysis using an anti-histidine antibody. His, histidine; GLRX1, glutaredoxin 1.

In addition, intracellular ROS production levels were investigated. In H_2O_2 exposed cells, intracellular ROS levels were markedly increased, whereas ROS levels were significantly reduced by PEP-1-GLRX1 protein. However, control GLRX1 protein did not affect ROS levels (Fig. 3B). It was also investigated whether transduced PEP-1-GLRX1 protein inhibits DNA damage resulting from H_2O_2 using TUNEL and 8-OHdG staining (Fig. 3C and D). In the H_2O_2 exposed cells, TUNEL or 8-OHdG-positive cells were markedly increased compared with the non-treated control cells. However, TUNEL and 8-OHdG-positive cells were significantly decreased by PEP-1-GLRX1 protein. Conversely, there was no protective effect against H_2O_2-induced DNA damage in the control GLRX1 protein treated cells. These results indicated that transduced PEP-1-GLRX1 protein protected against H_2O_2-induced HT-22 cell death via suppression of intracellular ROS production and DNA damage.

PEP-1-GLRX1 prevents H_2O_2-induced RAC-a serine/threonine-protein kinase (Akt) and mitogen activated protein kinase (MAPK) activation. Excessive intracellular ROS production leads to the activation of MAPK pathways in various cell types. H_2O_2 is known as an oxidizing agent which induces the activation of MAPK pathways (5). Therefore, the present study investigated whether the role of PEP-1-GLRX1 protein is in response to the activation of Akt and MAPK [JUN N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK)1/2, and p38) in H_2O_2 exposed HT-22 cells (Fig. 4). When cells were

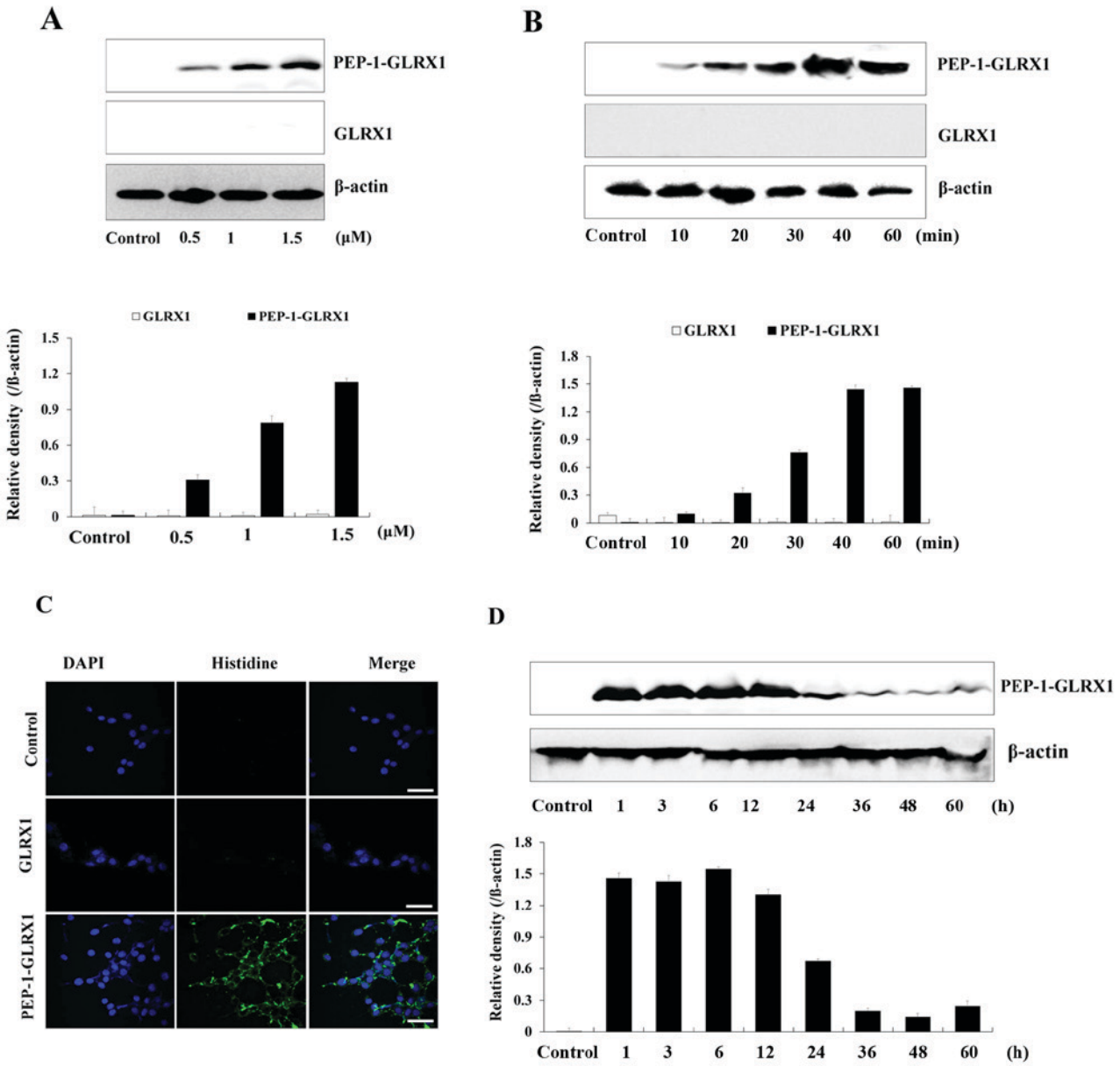

Figure 2. Transduction of PEP-1-GLRX1 proteins into HT-22 cells. (A) PEP-1-GLRX1 and control GLRX1 proteins (0.5-1.5 μM) were added to the HT-22 culture medium for 1 h. (B) PEP-1-GLRX1 and GLRX1 proteins (1.5 μM) were added to the HT-22 culture medium for 10-60 min and analyzed by western blotting using an anti-histidine antibody, and the band intensity was measured by densitometry. (C) Cellular localization of transduced PEP-1-GLRX1 proteins was verified by fluorescence microscopy. Scale bar=20 μm. (D) Stability of the transduced PEP-1-GLRX1 proteins (1.5 μM) in the HT-22 cells was analyzed by western blotting and the band intensity was measured by densitometry. β-actin was used as a loading control. GLRX1, glutaredoxin 1.

exposed H_2O_2, the activation levels of Akt and MAPK [phosphorylated (p)-Akt and p-MAPK] were markedly increased, however PEP-1-GLRX1 protein significantly inhibited p-Akt and p-MAPK levels in a concentration-dependent manner. However, levels of p-Akt and p-MAPK were not significantly altered in control GLRX1 protein and non-treated control cells. The results provided evidence that transduced PEP-1-GLRX1 inhibited the activation of Akt and MAPK in H_2O_2 exposed HT-22 cells.

PEP-1-GLRX1 protects HT-22 cells against H_2O_2-mediated apoptosis. It is well known that oxidative stress promotes activation of apoptotic signaling pathways (27,28). Therefore, the present study determined the ability of PEP-1-GLRX1 protein to inhibit H_2O_2-induced apoptotic pathways. Firstly, the expression levels of p-p53 were verified as oxidative stress increases expression levels of p-p53. As presented in Fig. 5A, the levels of p-p53 were significantly increased in the H_2O_2 exposed cells, whereas the levels were markedly inhibited in cells treated with PEP-1-GLRX1 protein in a concentration-dependent manner. However, control GLRX1 protein did not alter the expression level of p-p53 in H_2O_2 exposed cells.

The mechanism of the anti- or pro-apoptotic effects of PEP-1-GLRX1 protein were additionally investigated. As presented in Fig. 5B, increased Bax expression levels resulting from H_2O_2 were significantly reduced in the cells treated with PEP-1-GLRX1 protein. Conversely, Bax expression levels were not reduced in the control GLRX1 protein treated cells.

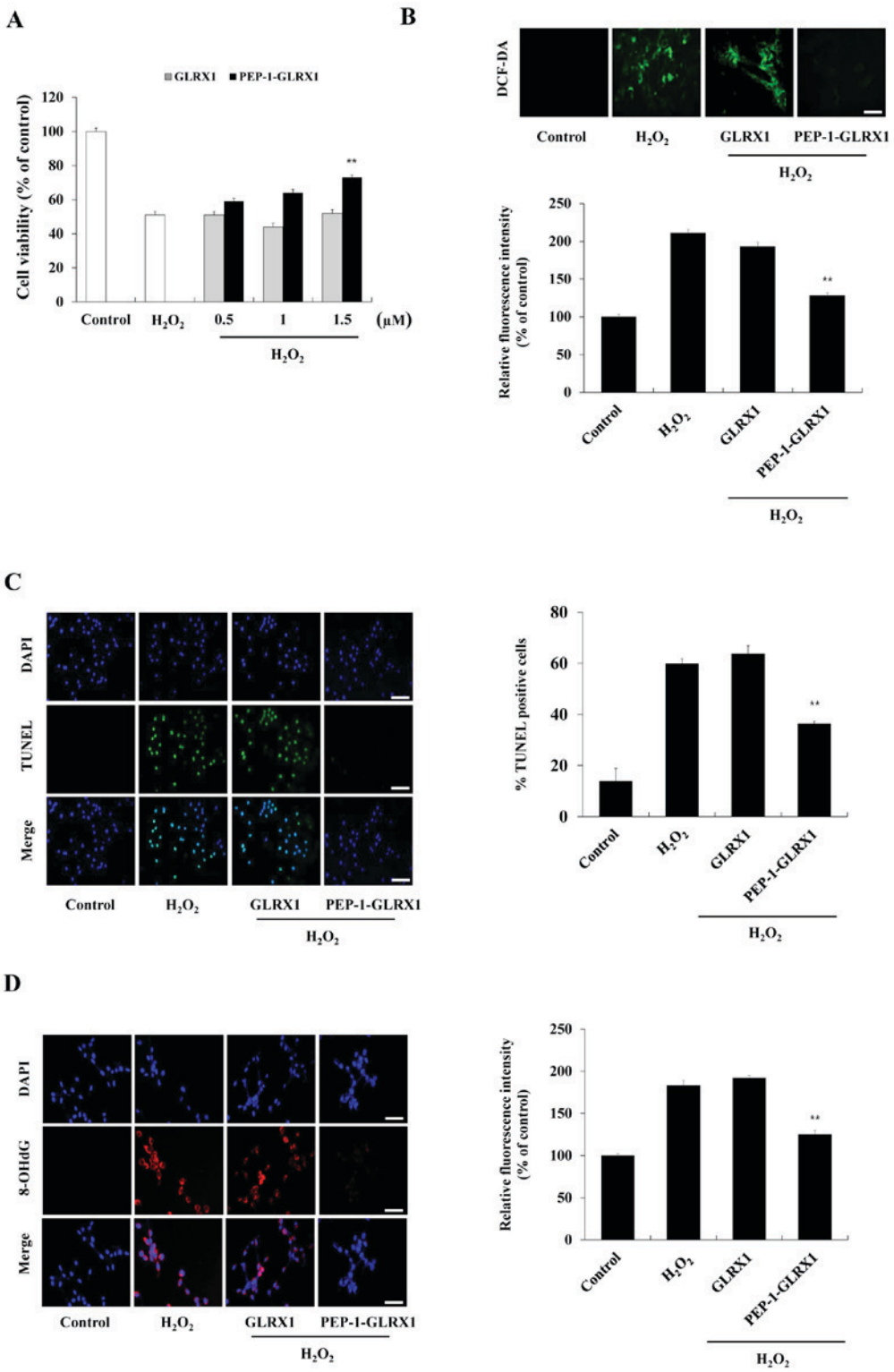

Figure 3. Effect of PEP-1-GLRX1 protein against oxidative stress-induced cell death. HT-22 cells were pretreated with PEP-1-GLRX1 and control GLRX1 (1.5 μM) proteins for 1 h prior to addition of hydrogen peroxide (1 mM) to the culture medium. (A) Cell viabilities were assessed by the MTT-based colorimetric assay. (B) Intracellular reactive oxygen species levels were measured by DCF-DA staining and the fluorescence intensity was measured using an ELISA plate reader. DNA damage was detected by (C) TUNEL and (D) 8-OHdG staining. Scale bar, 50 μm. Images were taken using a fluorescence microscope and the fluorescence intensity was measured using an ELISA plate reader. **P<0.01 vs. H_2O_2-treated cells. GLRX1, glutaredoxin 1; DCF-DA, 2',7'-Dichlorofluorescein diacetate; 8-OHdG, 8-hydroxy-2-deoxyguanosine; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; H_2O_2, hydrogen peroxide.

As presented in Fig. 6, Bcl-2 and pro-caspase-9 expression levels were significantly decreased in the H_2O_2 exposed cells compared with the non-treated control cells, whereas Bcl-2 and pro-caspase-9 expression levels were markedly recovered in a concentration-dependent manner in cells treated with PEP-1-GLRX1 protein. However, the alterations of Bcl-2 and pro-caspase-9 expression levels in control GLRX1 protein treated cells were

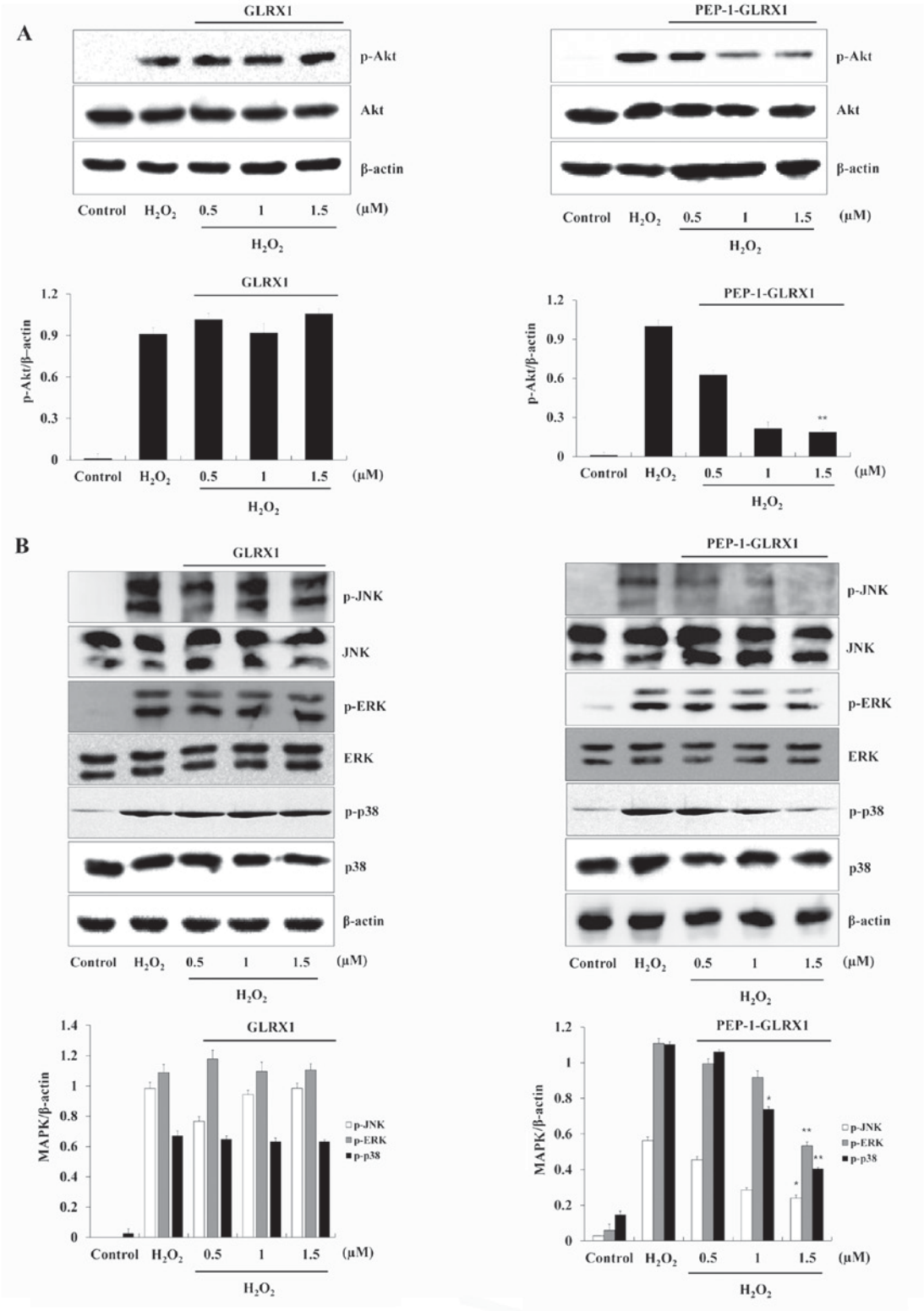

Figure 4. Effect of transduced PEP-1-GLRX1 protein against H_2O_2-induced Akt and MAPK activation. HT-22 cells were treated with PEP-1-GLRX1 and control GLRX1 (0.5-1.5 μM) for 1 h, and then exposed to H_2O_2 (1 mM). Then, the expression levels of (A) Akt and (B) MAPKs were measured by western blotting and the band intensity was measured by densitometry. *P<0.05; **P<0.01 vs. H_2O_2-treated cells. Akt, RAC-a serine/threonine-protein kinase; MAPK, mitogen activated protein kinase; GLRX1, glutaredoxin 1; H_2O_2, hydrogen peroxide; p, phosphorylated; JNK, JUN N-terminal kinase; ERK, extracellular signal-regulated kinase.

similar to the alterations induced by H_2O_2, indicating that PEP-1-GLRX1 efficiently inhibited H_2O_2-induced HT-22 cell death by preventing the activation of apoptotic signaling pathways.

PEP-1-GLRX1 protects against neuronal cell death in ischemic animal model. PTD fusion proteins are well known for their ability to transduce into the animal brain, crossing the blood-brain barrier (BBB) and improving neuronal

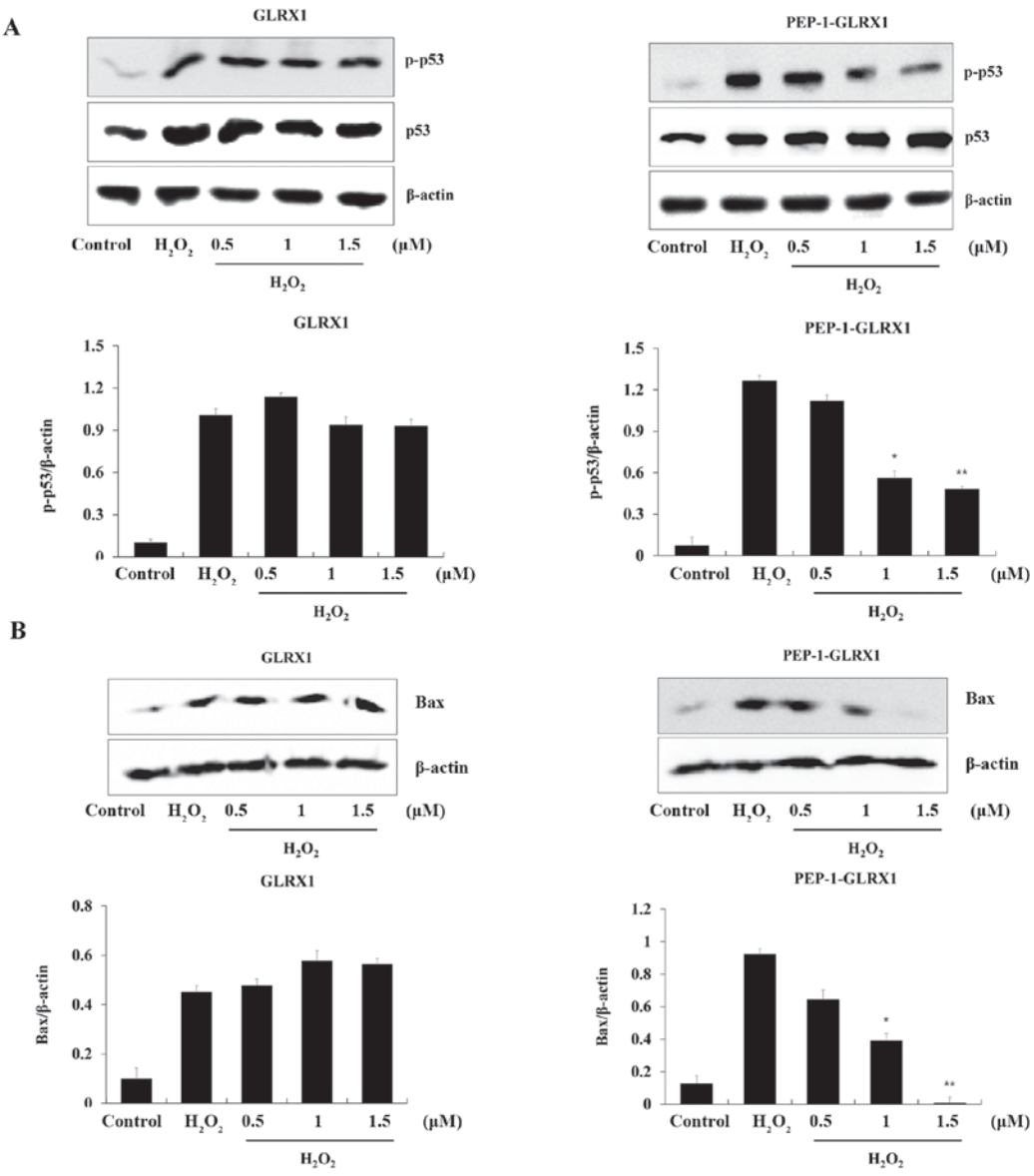

Figure 5. Effect of PEP-1-GLRX1 protein against H_2O_2-induced p53 and Bax expression. HT-22 cells were exposed to H_2O_2 (1 mM) with or without pretreatment with PEP-1-GLRX1 protein for 1 h. (A) p53 and (B) Bax expression levels were analyzed by western blotting and the band intensity was measured by densitometry. *P<0.05; **P<0.01 vs. H_2O_2-treated cells. GLRX1, glutaredoxin 1; H_2O_2, hydrogen peroxide; p, phosphorylated; Bcl-2, B cell lymphoma-2; Bax, Bcl-2 associated X, apoptosis regulator.

cell survival (13). Therefore, the present study determined whether PEP-1-GLRX1 protein transduced into the animal brain and the neuroprotective effects of PEP-1-GLRX1 in an animal model of ischemic injury. PEP-1-GLRX1 protein (2 mg/kg) was intraperitoneally injected into gerbils 30 min following ischemia-reperfusion. A total of 7 days following ischemic brain injury, the transduction of PEP-1-GLRX1 protein into the Cornu Ammonis (CA)1 region was verified, and cell viability determined. As presented in Fig. 7, in the sham control-, vehicle-, and control GLRX1 protein-treated groups, there were no differences in the Histidine antibody staining. However, in the PEP-1-GLRX1 protein treated groups, fluorescent stained signals were markedly increased compared with the sham control groups. In addition, through NeuN-immunostaining it was revealed that PEP-1-GLRX1 protein increased neuronal cell survival in the CA1 region.

The protective effect of PEP-1-GLRX1 proteins against ischemic brain injury were investigated by CV and FJB staining, which are known to be sensitive markers for the detection of neuronal injury (Fig. 8). In the PEP-1-GLRX1 protein treated groups, CV-positive neurons were significantly increased compared with the vehicle-treated groups. However, the number of CV-positive neurons were similar in the control GLRX1 protein-treated and vehicle-treated groups. In addition, FJB positive neuronal cells were not evident in the sham control group, whereas FJB positive neuronal cells were observed in the vehicle- and control GLRX1 protein treated group of the CA1 regions. FJB positive neuronal cells were significantly reduced in the PEP-1-GLRX1 protein-treated group compared with the vehicle-treated group.

Since the activation of astrocytes and microglia are known risk factors in ischemic injury (29), glial fibrillary acidic protein (GFAP) and Iba-1 immunostaining were performed to examine

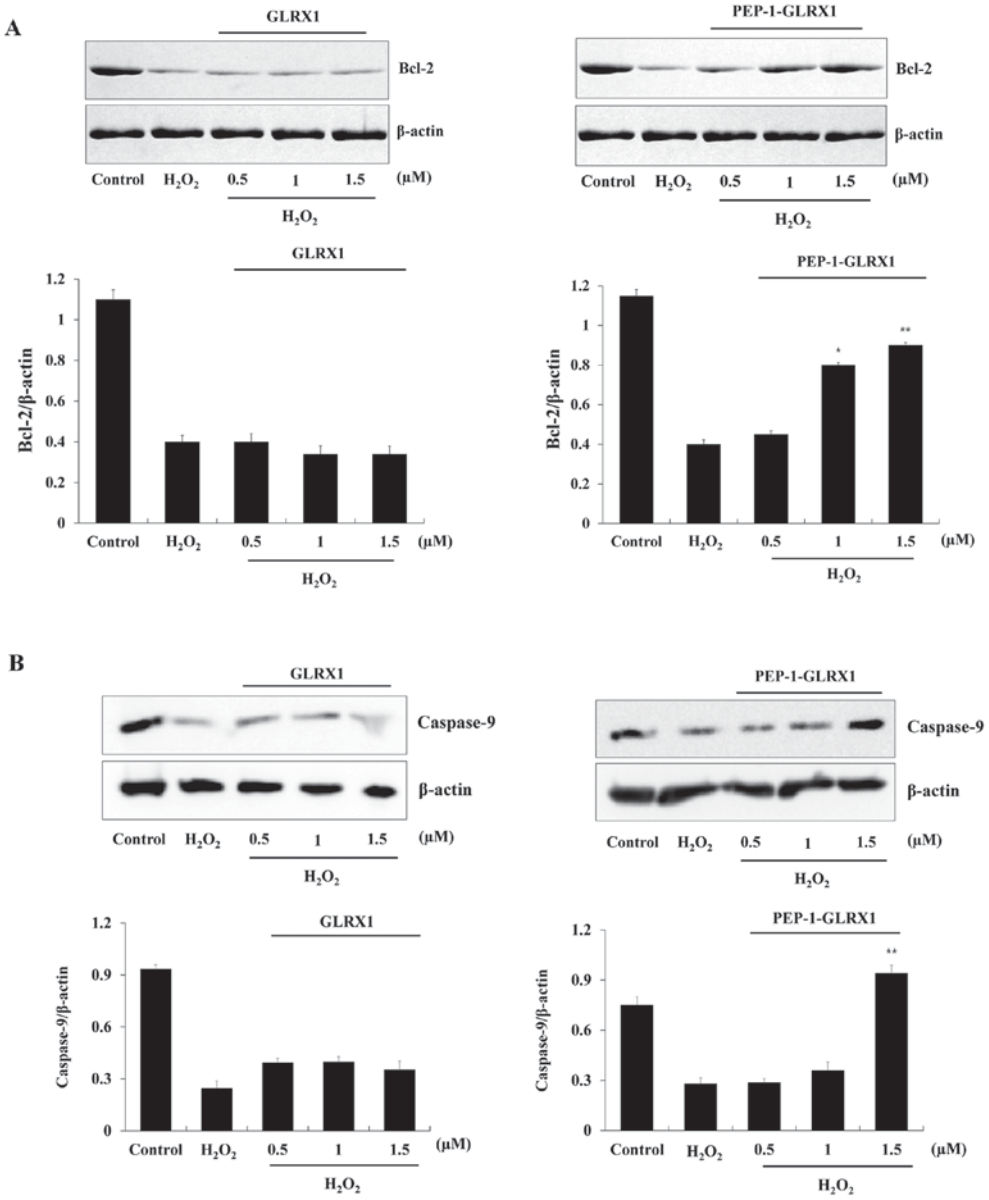

Figure 6. Effect of transduced PEP-1-GLRX1 on the expression levels of Bcl-2 and pro-caspase-9 in HT-22 cells. The cells were treated with PEP-1-GLRX1 (0.5-1.5 μM) for 1 h, and then exposed to H_2O_2 (1 mM). (A) Expression levels of Bcl-2 and (B) pro-caspase-9 were measured by western blotting and the band intensity was measured by densitometry. *P<0.05; **P<0.01 vs. H_2O_2-treated cells. GLRX1, glutaredoxin 1; H_2O_2, hydrogen peroxide; Bcl-2, B cell lymphoma-2.

whether transduced PEP-1-GLRX1 protein inhibited the activation of astrocytes and microglia in the CA1 region (Fig. 8). In the vehicle-treated group, GFAP-immunoreactive astrocytes were significantly increased in the CA1 regions, similar to those of control GLRX1 protein-treated groups. However, GFAP-immunoreactive astrocytes were markedly reduced in the PEP-1-GLRX1 protein-treated group compared with the vehicle-treated group. In addition, Iba-1-immunoreactive microglia in the CA1 region were significantly increased and aggregated in the vehicle-treated group, similar to that of the control GLRX1 protein-treated group. In the PEP-1-GLRX1 protein-treated groups, Iba-1-immunoreactive microglia aggregation recovered to a similar level as the sham control group. Overall, the results provided evidence that transduced PEP-1-GLRX1 protein markedly protected against neuronal cell death and may act as a therapeutic agent against ischemic brain injury.

Discussion

Human GLRX1, a member of GLRX family, is a GSH-dependent thiol oxidoreductase and is a small multifunctional protein. The two predominant forms (GLRX1 and GLRX2) exist in mammalian systems and GLRX1 is primarily located in the cytoplasm and GLRX2 in the mitochondria and nucleus (30). A previous study demonstrated that overexpressed GLRX1 protects Akt from H_2O_2-induced oxidation and protects cells from apoptosis via regulation of the redox state of Akt in cardiac H9c2 cells (31). Conversely, other studies have demonstrated that GLRX1 has an important role in defense against oxidative stress and cellular redox homeostasis (32). However, the roles of the GLRX1 protein against oxidative stress-induced neuronal cell damage are not yet clear.

Although beneficial proteins were considered as potential therapeutic agents, the application of these proteins has been

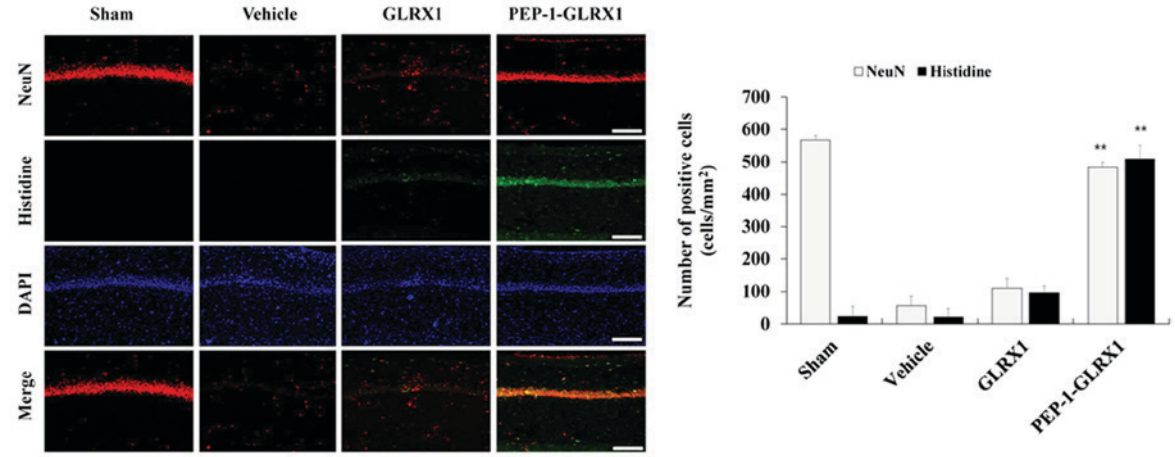

Figure 7. PEP-1-GLRX1 protein transduction into the animal brain. Gerbils were treated with single injections of PEP-1-GLRX1 (2 mg/kg). A total of 7 days following ischemic insults, transduced PEP-1-GLRX1 protein and its effects were verified using an anti-histidine, NeuN, and DAPI immunostaining. Scale bar=50 μm. **P<0.01 vs. vehicle group. GLRX1, glutaredoxin; NeuN, neuronal nuclei.

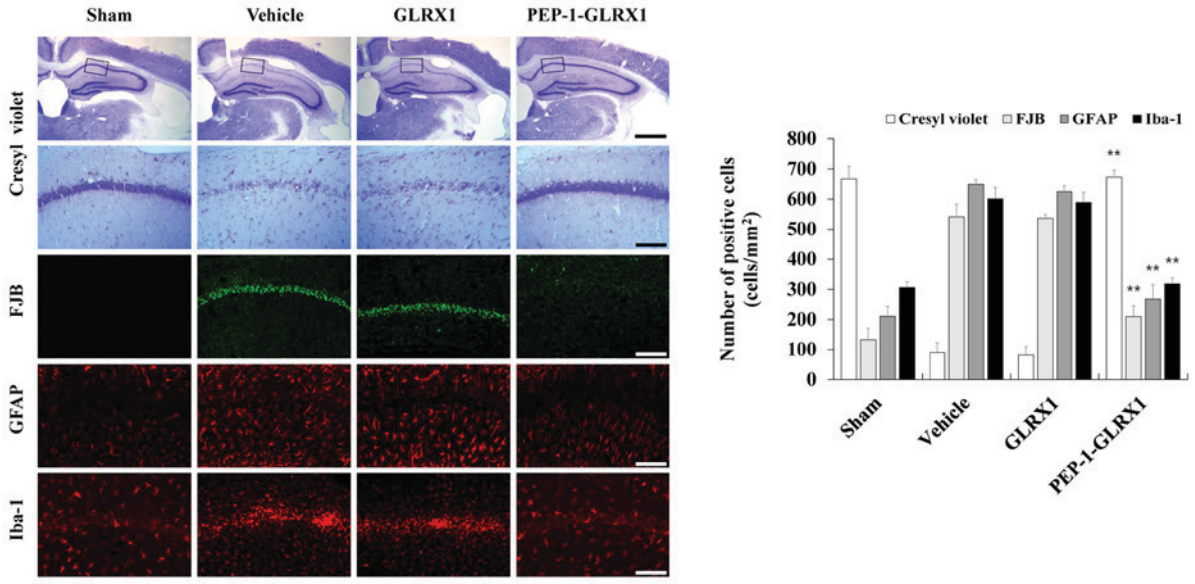

Figure 8. Effects of transduced PEP-1-GLRX1 protein against neuronal cell death in an animal model of ischemia. Neuroprotective effects of transduced PEP-1-GLRX1 protein were analyzed by CV, FJB, GFAP, and Iba-1 immunohistochemistry in the Cornu Ammonis 1 region of each group. Immunohistochemistry was performed in each of the control sham-, vehicle-, control GLRX1-, and PEP-1-GLRX1-treated groups 7 days following ischemia-reperfusion. Scale bars, 50 μm (FJB, GFAP, Iba-1 and bottom CV panel) and 400 μm (top CV panel). **P<0.01 vs. vehicle group. GLRX1, glutaredoxin; CV, cresyl violet; FJB, Fluoro-Jade B; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule 1.

restricted due to their inability to transduce into cells. PTDs, additionally termed CPPs, are small peptides consisting of 10 to 16 basic amino acids which transduce the plasma membrane either alone or combined with various macromolecules including proteins, without the aid of a special receptor (33). The present study used a PEP-1 peptide PTD, which has the ability to deliver a variety of proteins into cells and tissues including the brain (34,35). Even though numerous studies demonstrate that PEP-1 fused proteins transduce into cells and cross the BBB, the exact transduction mechanism is not yet fully understood.

ROS, including the superoxide anion ($\cdot O^{2-}$), hydroxyl radicals ($\cdot$OH) and H_2O_2 are generated by oxidative stress and lead to destruction of macromolecules including proteins, lipids, and DNA (36). ROS are associated with various diseases including ischemia, diabetes mellitus, Parkinson's and Alzheimer's disease (37). The present study examined whether PEP-1-GLRX1 suppressed the H_2O_2-induced cell damage and elevated cellular ROS levels. H_2O_2-induced cell death was significantly decreased by PEP-1-GLRX1. Additionally, PEP-1-GLRX1 significantly inhibited intracellular H_2O_2-induced ROS production levels, which indicated that PEP-1-GLRX1 protected against H_2O_2-induced cell death by inhibiting intracellular ROS generation. These results are consistent with other studies in which overexpressed GLRX1 protein protects H9c2 cells against H_2O_2 toxicity (38,39).

Previous studies have revealed that H_2O_2 is the primary oxidizing agent which induces neuronal cell death by activation of Akt and MAPK signaling pathways, including JNK, ERK and p38 (40,41). Conversely, other studies have indicated that activation of Akt and MAPKs by H_2O_2 appears to regulate

distinct cellular responses including cell survival in different cell types (42-44). The present study investigated whether transduced PEP-1-GLRX1 protein inhibited the activation of Akt and MAPK signaling induced by H_2O_2. Transduced PEP-1-GLRX1 protein markedly inhibited phosphorylation of Akt and MAPK signaling in the H_2O_2 treated cells. A previous study suggested that GLRX1 protein inhibits oxidative stress through the regulation of Akt and MAPK signaling pathways (45). Kwon *et al* (41) demonstrated that *Lonicera japonica* THUNB (LJ) markedly inhibits the activation of Akt and MAPKs, suggesting that LJ prevents H_2O_2-induced apoptosis in SH-SY5Y cells by inhibition of the activation of Akt and MAPKs (41). Studies have additionally demonstrated that Akt and MAPKs are important in neuronal apoptosis in response to environmental stresses and apoptotic agents (46,47). The results of the present study are consistent with reports that PEP-1-GLRX1 inhibits oxidative stress-induced HT-22 cell death by suppressing the activation of Akt and MPAK signaling.

Oxidative stress is highly associated with apoptotic signaling pathways and p53 is known to be a key regulator of apoptosis in neurons following ischemic injury (48-51). PEP-1-GLRX1 protein significantly reduced activation of p53 expression levels in a dose-dependent manner. Control GLRX1 did not affect the activation of p53 expression levels. Furthermore, the present study examined the effects of transduced PEP-1-GLRX1 protein against H_2O_2-induced alterations in Bcl-2, Bax and pro-caspase-9 expression levels in addition to H_2O_2-induced DNA damage, as these proteins and DNA damage are known to be primary markers of apoptotic pathways (52,53). Transduced PEP-1-GLRX1 protein significantly increased Bcl-2 and pro-caspase-9 expression levels compared with H_2O_2 treated cells. Conversely, Bax expression levels were markedly reduced. However, Bcl-2, Bax, and pro-caspase-9 expression levels did not alter in the control GLRX1 protein treated cells. In addition, TUNEL staining and 8-OHdG fluorescence staining were performed, which are well-known markers of DNA oxidation, in order to assess DNA damage (39). The results demonstrated that PEP-1-GLRX1 protected against H_2O_2-induced DNA damage in HT-22 cells, indicating that H_2O_2-induced neuronal cell death is markedly inhibited by transduced PEP-1-GLRX1 protein via regulation of apoptotic signaling pathways.

Although the exact mechanism of ischemic injury is not known, intracellular oxidative stress is considered to be one of the primary factors resulting in neurodegeneration including stroke and ischemia (28,54). To investigate the function of PEP-1-GLRX1 against ischemic injury, the present study used a gerbil ischemia-reperfusion model. First, immunohistochemical staining was performed on hippocampal neuronal cells in the CA1 region to examine whether the PEP-1-GLRX1 fusion protein transduced into the CA1 region across the BBB in an animal model of ischemia. Crossing the BBB is a necessary step for therapeutic proteins to treat neuronal diseases as the majority of proteins cannot easily cross the BBB. Using His and NeuN staining, it was demonstrated that PEP-1-GLRX1 protein transduced into the CA1 region by crossing the BBB, and markedly protected against neuronal cell death in an animal model of ischemia. To verify the neuronal damage, CV and F-JB staining were conducted, which are known to be sensitive markers for neuronal damage. As presented by CV and F-JB staining, PEP-1-GLRX1 protected against neuronal damage in the CA1 region, whereas the control GLRX1 protein did not affect the neuronal damage, which indicated that PEP-1-GLRX1 protected against ischemic injury in the animal model. These results are consistent with the author's previous reports, where it was demonstrated that cell permeable PTD proteins have a protective effect against neuronal cell death in the hippocampal CA1 region (20,22).

Microglial activation is a primary cause of degenerative brain diseases and astrocytes significantly affect the survival of neuronal cells during brain damage including ischemia. Therefore, the activation of microglial and astrocytes are used as markers for the detection of ischemic neuronal injury (55,56). Using Iba-1 and GFAP staining, which are microglia and astrocytes markers, the present study determined the effects of PEP-1-GLRX1 protein against microglial and astrocyte activation in an animal model of ischemia. PEP-1-GLRX1 protein significantly reduced the levels of Iba-1 and GFAP immunoreactive cells in the animal model. The control GLRX1 protein treated group did not demonstrate the same activation of microglia and astrocytes compared with the vehicle-treated group. Although the exact mechanisms need to be examined to develop therapeutic treatments for ischemic injury using this protein, it was demonstrated that PEP-1-GLRX1 protein has neuroprotective effects on neuronal cell death in an ischemic animal model and this fusion protein may be a potential therapeutic agent for various neuronal diseases associated with to oxidative stress.

In conclusion, cell permeable PEP-1-GLRX1 protein was constructed and it was demonstrated that PEP-1-GLRX1 protein efficiently transduced into HT-22 cells and inhibited intracellular ROS production, and inhibited activation of the Akt, MAPK, and apoptotic signaling pathways. In an ischemic animal model, PEP-1-GLRX1 protein transduced into the animal brain following crossing the BBB and revealed strong neuroprotective effects against ischemic injury, suggesting that PEP-1-GLRX1 may have potential applications for treating ischemic injury and neuronal diseases.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Author's contributions

EJR, DWK and MJS designed the research and experiments. HSJ, JHP, SBC, CHL, HJY, EJY and YJC contributed the

reagents and analytic tools and assisted with the analysis and interpretation of the data. DSK, EJS and OS performed the cell and animal experiments. KWL, SWC, YJC, KHH and JP analyzed and interpreted the data. WSE and SYC designed the study and wrote the manuscript.

Ethics approval and consent to participate

All experimental procedures involving animals and their care conformed to the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research & Quarantine Service of Korea and were approved by the Institutional Animal Care and Use Committee of Soonchunhyang University (Cheonan-Si, Korea; SCH16-0024).

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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