Supplementary Information

Tat-Thioredoxin-like protein 1 attenuates ischemic brain injury by regulation of MAPKs and apoptosis signaling

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Running title: Tat-TXNL1 ameliorates hippocampal neuronal cell damage

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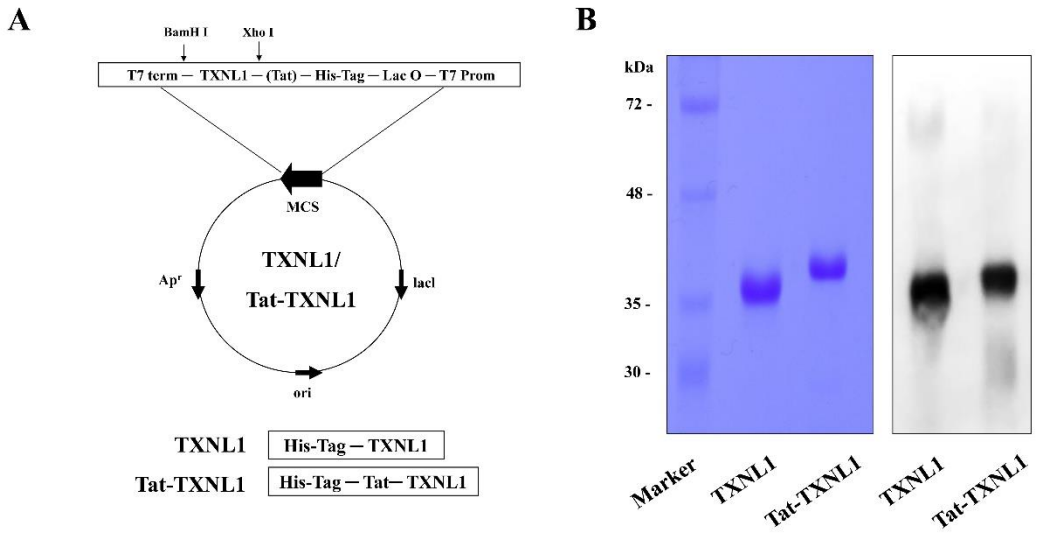
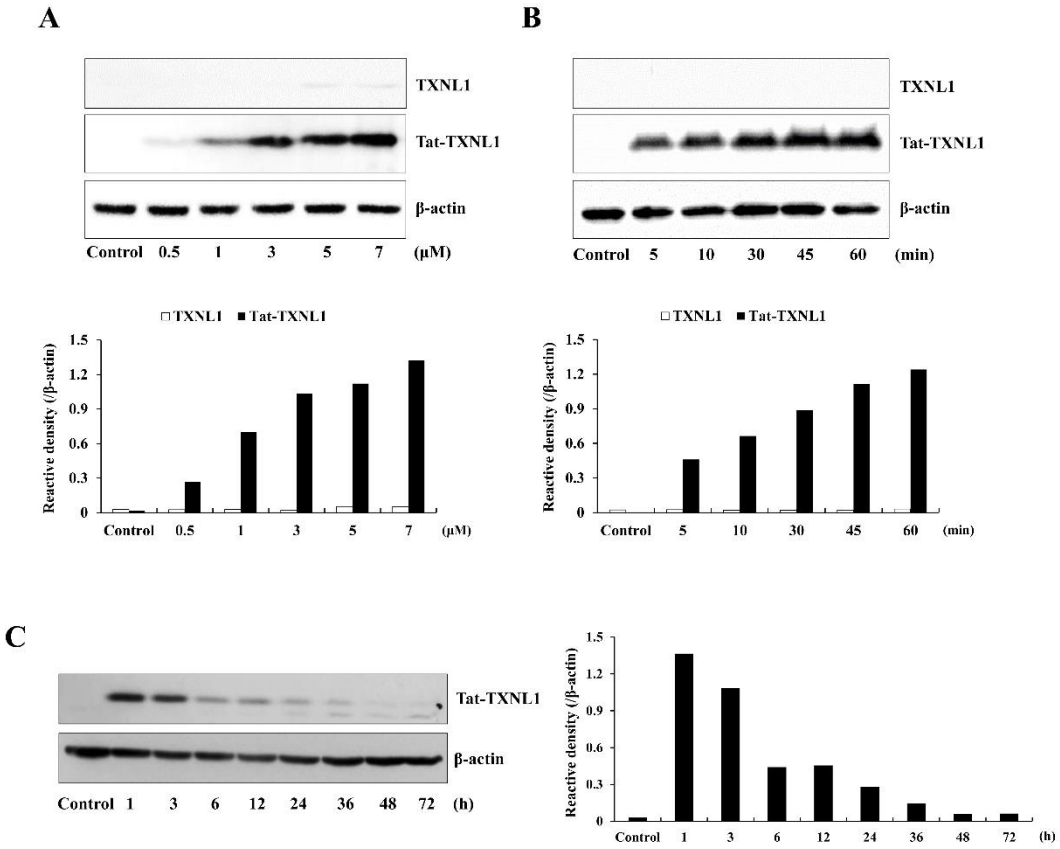
FIGURES



Supplementary Fig. S1

Figure S1. Purification of Tat-TXNL1 protein. (A) Diagram of Tat-TXNL1 and TXNL1 expression vector. (B) Purified Tat-TXNL1 and control TXNL1 were identified by 12% SDS-PAGE and detected by Western blotting using rabbit anti-polyhistidine antibody.



Supplementary Fig. S2

Figure S2. Transduction of Tat-TXNL1 proteins into HT-22 cells. (A) The cells were treated with Tat-TXNL1 at different doses (0.5-7 μM) or TXNL1 for 1 h and transduced levels of Tat-TXNL1 was measured by Western blotting using rabbit anti-polyhistidine antibody. Purified TXNL1 protein was used for positive control to detection of transduced TXNL1. (B) The cells were treated with Tat-TXNL1 (7 μM) or TXNL1 for different time periods (5-60 min) and transduced levels of Tat-TXNL1 was measured by Western blotting using rabbit anti-polyhistidine antibody. Purified TXNL1 protein was used for positive control to detection of transduced TXNL1. (C) Intracellular stability of transduced Tat-TXNL1. The cells were more incubated for 72 h after transduction of Tat-TXNL1 for 1 h. Then, transduction of Tat-TXNL1 was measured by Western blotting using rabbit anti-polyhistidine antibody. The intensity of the bands was measured by a densitometry. (D) The localization of transduced Tat-TXNL1 was examined by confocal fluorescence microscopy. Scale bar = 20 μm.

MATERIALS AND METHODS

Materials and cell culture

Used antibodies were acquired from the following companies: Histidine antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and other antibodies from Cell Signaling Technology (Beverly, MA, USA). Fetal bovine serum (FBS) and antibiotics (streptomycin and penicillin) were obtained from Gibco BRL (Grand Island, NY, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Lonza/ BioWhittaker (Walkersville, MD, USA). All the chemical and reagents used in this study were of analytical grade.

HT-22 cells were maintained in an environment at of 95% oxygen and 5% CO_2 at 37˚C, and cultured in DMEM consisting of 5 mM $NaHCO_3$, 20 mM HEPES/NaOH (pH 7.4), 10% FBS and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin). To examine time and concentration dependent transduction ability of Tat-TXNL1, HT-22 cells were exposed to different concentration (0.5-7 μM) of Tat-TXNL1 and TXNL1 for 1 h and to 7 μM for various time periods (5-60 min.). Cells were then washed with PBS and treated with trypsin-EDTA (Gibco BRL). The amounts of transduced proteins were measured by Western blotting as described previously (1).

Purification of Tat-TXNL1 protein

After human TXNL1 gene was amplified by PCR using the sense primer 5'-CTCGAGGTGGGGGTGAAGC-3' and the antisense primer 5'-GGATCCTCATTAGT GGCTTTCTCCTTT-3', the PCR products were ligated into a TA vector and Tat expression vector to generate Tat-TXNL1 protein as described previously (1,2). TXNL1 protein was generated without the Tat peptide as a control. Both proteins were cultured in *Escherichia coli* BL21 (DE3) and induced by adding 0.5 mM isopropyl-β-D-thio-galactoside (IPTG; Duchefa, Haarlem, the Netherlands) at 18˚C for over 24 h. Subsequently, Tat-TXNL1 and TXNL1 proteins were purified by a Ni^{2+}-nitrilotri-acetic acid Sepharose affinity column (Qiagen, Valencia, CA, USA) and PD-10 column chromatography (Amersham, Braunschweig, Germany) according to the manufacturer's instructions. After the proteins were purified and protein concentration was determined, the proteins were analyzed by SDS-PAGE and Western blotting as described a previous study (1-4).

Western blot analysis

After transduction of Tat-TXNL1 protein, protein extraction was performed using cell lysi buffer (RIPA; ELPIS BIOTECH, Daejeon, Korea) according to the manufacturer's instruction. Then, equal amount of proteins were loaded into 12% sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) and electrotransferred to a polyvinylidene difluoride (PVDF) membrane, and subsequently incubated with primary antibodies: His (1:5,000; sc-804; Santa Cruz Biotechnology), Akt (1:2,000; #9273), p-Akt (1:2,000; #4058), JNK (1:1,000; #9258), p-JNK (1:1,000; #9251), ERK (1:2,000; #9102), p-ERK (1:2,000; #4376), p38 (1:2,000; #9212), p-p38 (1:2,000; #4631), Bcl-2 (1:1,000; #2876), Bax (1:1,000; #2772), Caspase-3 (1:1,000; #9662), Caspase-9 (1:1,000; #9504S), β-actin (1:5,000; #4967), and appropriate secondary antibodies (1:10,000; #7074). All of the above antibodies were purchased from the Cell Signaling Technology (Beverly, MA, USACity, Country), except for the His antibody. Then the membranes were washed with TBS-T (25 mM Tris-HCl, 140 mM NaCl, 0.1% Tween 20, pH 7.5) buffer three times and the protein bands were identified using chemiluminescent reagents as recommended by the manufacturer (Amersham, Franklin Lakes, NJ, USA).

Immunofluorescence staining

For immunofluorescence staining, HT-22 (5×10^4 cells/well) cells were cultured on coverslips and treated with 7 μM of Tat-TXNL1 or TXNL for 1 h. The cells were washed with phosphate buffered saline (PBS) twice and fixed with 4% paraformaldehyde for 5 min. The cells were treated with in PBS containing 3% bovine serum albumin and 0.1% Triton X-100 (PBS-BT) at room temperature for 1 h and washed with PBS-PT. The histidine primary antibody was diluted 1:2000 and incubated at room temperature for 4 h. The Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) was diluted 1:1500 and incubated in the dark for 1 h. Nuclei were stained with 1 μg/mL 4'6-diamidino-2-phenylindole (DAPI; Roche Applied Science, Mannheim, Germany) for 2 min. Then stained cells were analyzed by confocal fluorescence microscopy using a confocal laser-scanning system (MRC1024, Bio-Rad, CA, USA) (1,2,5).

Cytotoxicity assay

To determine the cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay performed (2,6).

HT-22 cells were divided into 4 groups: normal control, hydrogen peroxide (H_2O_2) treated group, Tat-TXNL1 + H_2O_2 treated group, and TXNL1 + H_2O_2 treated group. The cells were seeded into 96-well plates and treated with Tat-TXNL1 (0.5-7 μM) and TXNL1 for 1 h. Then, the cells were incubated with 1 mM H_2O_2 for 90 min. The absorbance was measured at 450 nm using an ELISA microplate reader (Infinite 200 nanoquant, TECAN, Switzerland) and cell viability was defined as the % of untreated control cells.

Intracellular ROS levels were determined used 2′,7′-dichlorofluorescein diacetate (DCF-DA) staining as described previously (1,2,7). To determine the effect of Tat-TXNL1 against H_2O_2-induced intracellular ROS production in HT-22 cells, the cells (5×10^4 cells/well) were placed on coverslips in 24-well plates, incubated for 12 h, and washed twice with PBS. After pretreated with Tat-TXNL1, TXNL1, and Tat peptide (7 μM) for 1 h, H_2O_2 (1 mM) was added to the culture medium for 20 min. The cells were washed with PBS and incubated for 30 minutes with DCF-DA (10 μM). We separately processed the protein as mentioned above to obtain the fluorescent image and fluorescence intensity. One was used to obtain a fluorescent image. To take fluorescent images for each well, the cells were washed with PBS, mounted, and the cell images were obtained by fluorescence microscopy (Nikon eclipse 80i, Japan).

The other was used to obtain the fluorescence intensity. To detect the fluorescence intensity for each well, the cells were collected and washed with PBS. After added 300 μl of PBS buffer and resuspended the cells, the cells (100 μl) were transferred into 96-well plate reader. Then, the fluorescence intensity of samples was measured using a Fluoroskan ELISA plate reader (Labsystems Oy, Helsinki, Finland) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

DNA fragmentation was determined using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining (1,2,8). HT-22 cells were treated with Tat-TXNL1 (7 μM) and TXNL1 protein for 1 h and exposed to H_2O_2 (1 mM) for 4 h. Then, fluorescent images were obtained by fluorescence microscopy (Nikon eclipse 80i, Japan). Cytotoxicity was normalized to that of the control groups.

Experimental animals and treatment

Male gerbils (65-75 g; 6 months old) obtained from the Experimental Animal Center, at Hallym University (Chuncheon, Korea) were housed at a temperature of 23±C, with humidity of 60%, and exposed to 12 hour periods of light and dark with 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, Korea) [SCH 15-0002-3].

Cerebral forebrain ischemia was induced as previously described (1,2). 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.

To explore the protective effects of Tat-TXNL1 against ischemic injury, the animals were divided into four groups (n = 10 per group): the sham group, vehicle-treated group, TXNL1 (2 mg/kg)-treated group, and Tat-TXNL1 (2 mg/kg)-treated group. The TXNL1 and Tat-TXNL1 proteins were administered intraperitoneally 30 min before ischemia-reperfusion. All animals were euthanized utilizing general inhalants methods in the euthanasia chamber for fill rate of 30-70% of the chamber volume per minute with Carbon dioxide (CO_2) according AVMA Guidelines for the Euthanasia of Animals.

The brain tissue samples were obtained 7 days following ischemia-reperfusion. To examine the protective effects of Tat-TXNL1 against ischemic injury, the sections were stained at 37 ˚C with Cresyl violet (CV; 0.5% CV solution; Junsei Chemical Co. Ltd., Saitama, Japan), Fluoro-Jade B (F-JB; 1:500; EMD Millipore, Billerica, MA, USA), ionized calcium-binding adapter molecule 1 (Iba-1; 1:500;

Wako, Osaka, Japan) and glial fibrillary acidic protein (GFAP; 1:500; EMD Millipore). Subsequently, sections were incubated with biotinylated goat anti-mouse IgG antibody (1:200; Vector Laboratories, Inc. Burlingame, CA, USA) or biotinylated goat anti-rabbit IgG antibody (1:200; Vector Laboratories, Inc.).

The positive neuronal cell number and intensity of immunoreactivity were calculated using an image analyzing system equipped with a computer based CCD camera (software: Optimas 6.5, CyberMetrics, USA). The staining intensity of the immunoreactive structures was evaluated as the relative optical density (ROD). A ratio of the ROD was calibrated as % as described in a previous study (1,2).

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.

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