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Transduced Tat-Frataxin Protects Dopaminergic Neuronal Cells Against MPP+/MPTP-induced Cytotoxicity

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Abstract. Parkinson's disease (PD) is caused by various factors such as reactive oxygen species (ROS), dysfunction of mitochondria, and aggregation of misfolded proteins, thereby leading to loss of dopaminergic (DA) neurons in the substantia nigra (SN) of the brain. Frataxin (FXN) is associated with iron homeostasis and biogenesis of iron-sulfur clusters in the electron transport chain complex. In this study, we investigated the potential of Tat-FXN to cross the blood brain barrier (BBB) and protect DA neurons against oxidative stress in a mouse model of PD. Therefore, these results suggest that Tat-FXN may provide neuroprotective therapy for ROS related diseases including PD.

Keywords: Frataxin, MPTP, Parkinson's disease, Protein transduction domain, Reactive oxygen species

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic (DA) neurons in the substantia nigra (SN) and symptomized by tremors, rigidity, and akinesia [1,2]. A number of studies have been reported that PD is associated with various factors such as oxidative stress, overproduction of reactive oxygen species (ROS), increased misfolded proteins, and damaged mitochondrial (mt) function. Frataxin (FXN) encoded by the FXN gene in the nuclear genome is a ubiquitously expressed mt protein [5]. Although the biological functions of FXN are still not clearly understood, FXN plays an important role in iron homeostasis and biogenesis of iron-sulfur clusters (ISC) in active sites of mt complexes I, II and aconitases [3,5,6]. In this study, we examined whether FXN fused with Tat (Tat-FXN) could be transduced into SH-SY5Y cells and brain tissues, and subsequently protect brain tissues in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced animal model of PD.

2. Materials and Methods

2.1. Materials and Cell Culture

A Tat peptide was synthesized from Peptron (Dajeon, Korea). The SH-SY5Y human neuroblastoma cell line was maintained in Eagle's Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA) and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin; Gibco BRL) at 37℃ in a humidified atmosphere of 95% air and 5% CO_2. All other chemicals and reagents, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were of the highest analytical grade available.

2.2. Transduction of Tat-FXN Fusion Protein into SH-SY5Y Cells

Expression vectors for FXN and Tat-FXN fusion proteins were constructed and the fusion proteins were prepared as previously described [7]. The protein concentration was determined by the Bradford procedure using bovine serum albumin as a standard [8]. To assess the dose-dependency of the transduction of FXN and Tat-FXN fusion proteins into SH-SY5Y cells, cells were grown to confluence in a 60 mm-diameter dish and incubated with various concentrations (0.1–2 μM) of FXN and Tat-FXN proteins for 1 h. To assess the

time-dependency of the transduction of each protein, cells were treated with either FXN or Tat-FXN protein (2 μM) for various times (10–180 min). To remove Tat-FXN or FXN attached to the outer cellular membrane, the cells were treated with trypsin for 10 min and washed with phosphate buffered saline (PBS) three times. The cells were harvested and cell extracts were prepared for Western blot analysis. Also, to assess the intracellular stability of Tat-FXN in SH-SY5Y cells, cells were treated with Tat-FXN (2 μM) for 1 h and harvested after various incubation times (1–60 h).

2.3. Western Blot Analysis

Equal amounts of β-actin normalized proteins from each cell lysate were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were electrotransferred to a nitrocellulose membrane, which was then blocked with 5% non-fat dry milk in TBS-T buffer (25 mM Tris-HCl, 140 mM NaCl, 0.1% Tween 20, pH 7.5). The membrane was probed with a rabbit anti-histidine, FXN or other antibodies (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by detection with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (1:10,000; Sigma-Aldrich). The bound antibody complexes were visualized with enhanced chemiluminescent reagents according to the manufacturer's instructions (Immobilon Western Chemiluminescent AP Substrate; Millipore, Billerica, MA, USA). Band intensity was measured using a densitometer and analyzed using Image J software (NIH, Bethesda, MD, USA).

2.4. Confocal Fluorescence Microscopy

Cells were seeded on glass coverslips and incubated with FXN and Tat-FXN proteins (2 μM) at 37ºC for 1 h. After washing twice with PBS, the cells were incubated with a growth medium containing a Mitochondria selective probe (MitoTrackerTM Red 580, Molecular probes) for 20 min, and fixed with 4% paraformaldehyde at room temperature for 5 min. The anti-histidine primary antibody was diluted at 1:2,000, and incubated with each sample for 2 h at room temperature. A secondary antibody conjugated with Alexa fluor 488 (Invitrogen, Carlsbad, CA, USA) was diluted at 1:15,000 and incubated for 45 min at room temperature in the dark. Nuclei were counterstained for 5 min with 1 μg/ml 4'6-diamidino-2-phenylindole (DAPI) (Roche Applied Science, Basel, Switzerland). Also, to investigate the cellular localization of Tat-FXN in fixed or non-fixed cells, Tat-FXN was labeled using an EZ-Label fluorescein isothiocyanate (FITC) protein labeling kit (Pierce, Rockford, IL, USA). Fluorescence images were obtained using a model FV-300 confocal fluorescence microscope (Olympus, Tokyo, Japan).

2.5. Viability Assay

An established assay [9] based on 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) and a trypan blue exclusion assay were used to assess the viability of SH-SY5Y cells. Cells were seeded into wells of a 96-well plate and grown to 70% confluence. The cells were pretreated with various concentrations of each protein for 1 h. After washing with Dulbecco's phosphate buffered saline (DPBS), the cells were exposed to 1-methyl-4-phenylpyridinium (MPP+) (4 mM) for 18 h. For the MTT assay, MTT was added to the culture medium for 4 h. Precipitated formazan crystal was dissolved in dimethyl sulfoxide and absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader. Cellular viability was expressed as a percentage of a MPP+ untreated control. For the trypan blue exclusion assay, cells were stained in trypan blue (0.4%) and the numbers of viable and dead cells were counted. Quantitative data represent three independent experiments.

2.6. Measurement of ROS Level

Intracellular ROS levels were determined using 2',7'-dichlorofluorescein diacetate (DCF-DA), which is oxidized by ROS into highly fluorescent 2',7'-dichlorofluorescein (DCF) [10]. SH-SY5Y cells were incubated with Tat, FXN, and Tat-FXN (2 μM) for 1 h, followed by treatment with MPP+ (4 mM) for 1 h. After the cells were washed twice with DPBS, DCF-DA was added to a final concentration of 20 μM for 30 min and the cells were then washed twice with DPBS. The level of DCF fluorescence in each sample was monitored at 485 nm (excitation) and 538 nm (emission) using an ELISA plate reader (Labsystems Oy, Helsinki, Finland).

2.7. Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-end-labeling (TUNEL) Assay

SH-SY5Y cells were seeded on glass coverslips and incubated with Tat, FXN, and Tat-FXN (2 μM) at 37℃ for 1 h. After the medium was removed, cells were washed twice with DPBS and treated with MPP+ (4 mM) for an additional 12 h. TUNEL staining was performed using the Cell Death Detection kit (Roche Applied Science, Basel, Switzerland) according to the manufacturer's instructions. Fluorescence micrographs were acquired using an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan).

2.8. Animal Model Study

Male, 8-week-old, C57BL/6 mice were purchased from the Hallym University Experimental Animal Center. The animals were housed at a constant temperature (23℃) and relative humidity (60%) with alternating 12 h cycles of light and dark. They were provided with food and water ad libitum. 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 and Quarantine Service of Korea, and were approved by the Hallym Medical Center Institutional Animal Care and Use Committee.

To examine the in vivo transduction of FXN and Tat-FXN into mice tissues, each protein was intraperitoneally (i.p.) injected into mice (5 mice / group) at a dose of 2 mg/kg. In order to investigate the distribution of FXN and Tat-FXN in each tissue, several different tissue samples, including brain, liver, kidney, heart, and lung, were obtained at the desired times (0-48 h). The levels of FXN and Tat-FXN in the samples were assessed by Western blot analysis using a FXN antibody. Brain tissues were removed 12 h later for histological analysis. Samples were immunostained using a rabbit anti-histidine polyclonal antibody (1:400) and biotinylated goat anti-rabbit secondary antibody (1:200).

To examine the effect of Tat-FXN on oxidative stress in the brains of mice, Tat, FXN, and Tat-FXN were i.p. injected at a dose of 2 mg/kg, followed by four injections of MPTP. The brains from each group (5 mice / group) were harvested at 1, 6, 12, 24, and 48 h after treatment with MPTP. The levels of 4-hydroxynonenal (4-HNE) and SOD were measured by Western blot analysis using a 4-HNE and SOD antibody. Also, to identify whether Tat, FXN, or Tat-FXN can protect DA neurons in the SN against PD, the mice were sacrificed one week after MPTP treatment and surviving DA neurons were identified via a histological analysis.

2.9. Tissue Processing and Immunohistochemistry for Tyrosine Hydroxylase (TH) Expression in the SN

Tissues processing and immunostaining were performed in a similar fashion to previous studies [11,12]. Briefly, animals were anesthestized by i.p. injection of sodium pentobarbital (100 mg/kg body weight) and fixed transcardially with 4% paraformaldehyde. The brains were removed, post-fixed in the same fixative for 4 h, frozen and sectioned with a cryostat at a thickness of 30 μm. The sections were blocked with 3% bovine serum albumin in PBS for 30 min at room temperature and then incubated overnight at room temperature with a rabbit anti-TH IgG primary antibody (1:500, Santa Cruz Biotechnology) in PBS containing 0.3% Triton X-100. After being washed three times with PBS, sections were incubated with the biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA, USA). Cresyl violet counterstaining for Nissl bodies was conducted. Then, the sections were visualized with 3,3'-diaminobenzidine (DAB) in 0.1 M Tris buffer and mounted on gelatin-coated slides. Images were captured and analyzed using an Olympus DP72 digital camera and DP2-BSW microscope digital camera software. Figures were prepared using Adobe Photoshop 7.0 (San Jose, CA, USA). Manipulation of images was restricted to threshold and brightness adjustments applied to the entire images. Histological data are representative images from each group and sections were processed and analyzed by a blinded observer.

2.10. Statistical Analysis

Data are expressed as the means ± SD. Comparison between groups was performed by Student's *t* test. Values of $P < 0.01$ and $P < 0.05$ were considered to be statistically significant.

3. Discussion

The present study suggests that Tat-FXN possesses the capability to traverse the BBB, remove endogenous ROS, and protect DA neurons in the SN. These results suggest that Tat-FXN may have potential as a therapeutic agent for PD. In addition, previous reports have suggested that anti-oxidant enzymes and endogenous or exogenous anti-oxidants can inhibit the progressive development of PD [4,13,14]. In this study, we produced clear evidence that Tat-FXN can be efficiently transduced into various tissues, suggesting that PTD is one useful delivery tool for peptide or protein based drugs. Our results demonstrated that Tat-FXN in the brain relieves oxidative stress induced by MPTP through up-regulating the expression of SOD and consequently inhibiting the generation of 4-HNE, thereby protecting DA neurons.

4. Acknowledgements

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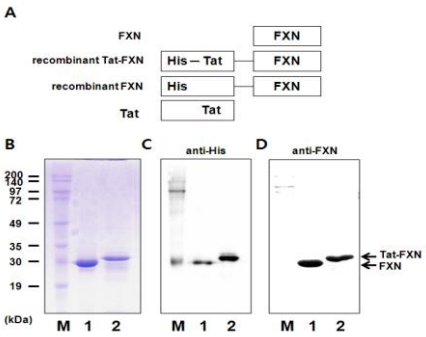

Fig. 1: Purification of Tat-FXN and FXN fusion proteins.

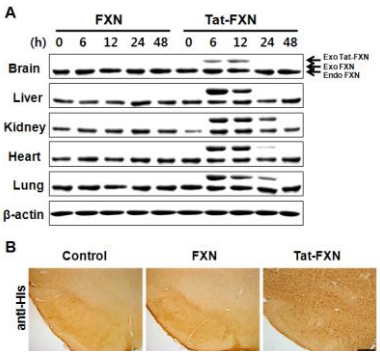

Fig. 4: Tat-FXN is delivered to mice brain following i.p. administration.

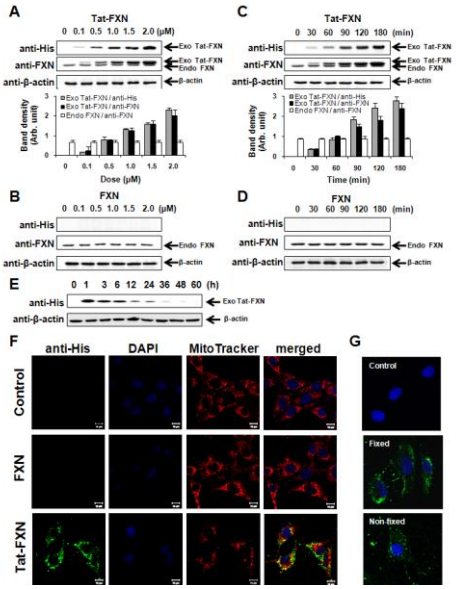

Fig. 2: Purification of Tat-FXN and FXN fusion proteins.

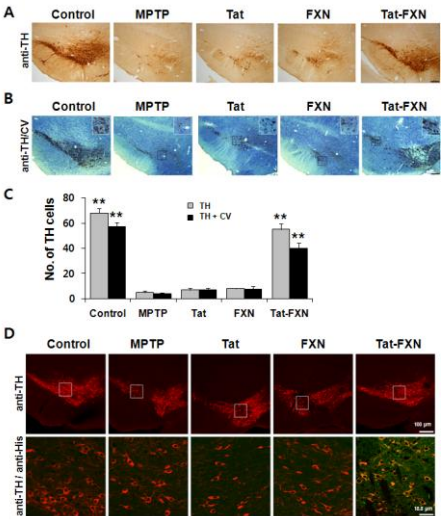

Fig. 5: Transduced Tat-FXN protects DA neuronal cells from MPTP-induced oxidative stress in a mouse model of PD.

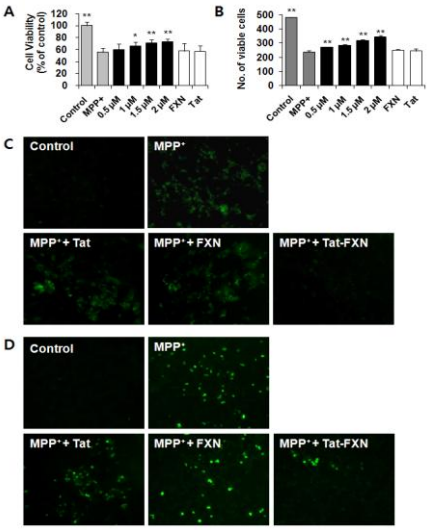

Fig. 3: Tat-FXN protects SH-SY5Y cells against MPP+-induced oxidative stress.

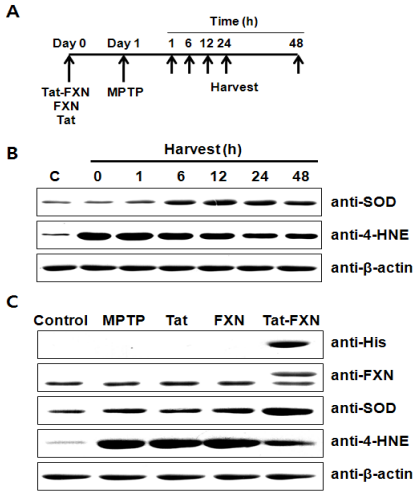

Fig. 6: Tat-FXN affects cellular level of SOD and 4-HNE in the brain.