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Effects of low doses of Tat-PIM2 protein against hippocampal neuronal cell survival

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ABSTRACT

Oxidative stress is considered a major factor in various neuronal diseases including ischemia-reperfusion injury. Proviral Integration Moloney 2 (PIM2) proteins, one of the families of PIM kinases, play crucial roles in cell survival. However, the functions of PIM2 protein against ischemia are not understood. Therefore, the protective effects of PIM2 against oxidative stress-induced hippocampal HT22 cell death and brain ischemic injury were evaluated using Tat-PIM2, a cell permeable fusion protein. Tat-PIM2 protein transduced into hippocampal HT22 cells. Low doses of transduced Tat-PIM2 protein protected against oxidative stress-induced cell death including DNA damage and markedly inhibited the activation of mitogen activated protein kinase (MAPKs), NF-κB and the expression levels of Bax protein. Furthermore, Tat-PIM2 protein transduced into the CA1 region of the hippocampus and significantly prevented neuronal cell death in an ischemic insult animal model. These results indicated that low doses of Tat-PIM2 protein protects against oxidative stress-induced neuronal cell death, suggesting low doses of Tat-PIM2 protein provides a potential therapeutic agent against oxidative stress-induced neuronal diseases including ischemia.

1. Introduction

Proviral Integration Moloney (PIM) proteins are a family of serine/threonine kinases involved in cell survival and apoptosis composed of three members, PIM1, PIM2, and PIM3. The PIM proteins are highly conserved in multicellular homologous organisms [1–3]. PIM2 was first discovered in lymphomas in mice and found to have anti-apoptotic effects as well as several other functions including cell cycle regulation, cell proliferation, and transcriptional activity [2,4–8]. Several studies have demonstrated that PIM2 plays an important role in cell survival and apoptosis in tumors [9–12].

Cerebral ischemia occurs when blood flow to the brain is reduced as a result of transient or permanent occlusion of the cerebral artery. Cerebral ischemia potentially triggers neuroapoptosis, which leads to irreversible brain damage [13]. Reactive oxygen species (ROS) induce cell damage by mediating signal transduction pathways. ROS-induced direct or indirect damage to cells is seen as a trigger of a variety of neurological disorders such as stroke. Especially superoxide anion ($O_2^{\bullet-}$) is known to induce neuroapoptosis, playing a crucial role in the pathophysiology of brain ischemia [14,15]. Recent studies have shown that oxidative stress is associated with ischemic injury and suggest that regulation or inhibition of oxidative stress is beneficial against ischemic injury [16,17].

Proteins are difficult to deliver into cells because of their high molecular weights. Thus, we used protein transduction domains (PTDs) to deliver the protein into cells. PTDs have the ability to traverse the plasma membrane and enter cells on their own without the aid of a specific receptor [18,19]. Tat PTD has been extensively studied and demonstrated the ability to transduce into various cells and in various animal models [20–23]. We also demonstrated the protective effects of PTDs fusion protein against cell death *in vitro* and *in vivo* [24–28].

In the present study, we investigated the neuroprotective effects of transduced Tat-PIM2 protein against oxidative stress-induced neuronal cell death and in an ischemic insult animal model. We demonstrated that low doses of transduced Tat-PIM2 protein significantly protected against oxidative stress-induced neuronal cell death *in vitro* and *in vivo* suggesting that low doses of Tat-PIM2 protein may potentially

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represent a therapeutic agent against oxidative stress-induced diseases including brain ischemia.

2. Materials and methods

2.1. Materials, animals and HT22 cell cultures

The indicated primary and β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tat peptides were purchased from PEPTRON (Daejeon, Korea). Unless otherwise stated all other agents were of the highest grade available.

Mongolian gerbils (*Meriones unguiculatus*) were obtained from the Experimental Animal Center, at Hallym University. The animals were housed at a constant temperature (23 °C) and relative humidity (60%) with a fixed 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 Hallym Medical Center Institutional Animal Care and Use Committee.

Mouse hippocampal HT22 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 20 mM Hepes/NaOH (pH 7.4), 5 mM $NaHCO_3$, 10% fetal bovine serum (FBS) and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37 °C in a humidity chamber with 5% CO_2 and 95% air.

2.2. Expression and purification of Tat-PIM2 proteins

A Tat expression vector was prepared in our laboratory as described previously [29]. The cDNA sequence for human PIM2 was amplified by PCR using the sense primer 5′-CTCGAGATGTTGACCAAGCCTCTAC-3′ and the antisense primer, 5′-GGATCCTTAGGGTAGCAAGGACCA-3′. The resulting PCR products were eluted (Kanto Chemical, Tokyo, Japan), subcloned in a TA cloning vector and ligated into the Tat or control expression vector. Then, the Tat-PIM2 plasmid was transformed into *Escherichia coli* BL21(DE3) cells and induced with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG; Duchefa, Haarlem, Netherlands) at 37 °C for 6 h. Harvested cells were lysed by sonication and the Tat-PIM2 protein was purified using a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column (Qiagen, Valencia, CA, USA) and PD-10 column chromatography (Amersham, Braunschweig, Germany). The purified protein concentrations were estimated using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA) [30].

2.3. Transduction of Tat-PIM2 protein into HT22 cells

To examine the transduction ability of Tat-PIM2 protein, the cells were treated with various concentrations of Tat-PIM2 protein (0.5–3 μM) for 1 h or with 3 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 Tat-PIM2 protein was detected using a rabbit anti-polyhistidine antibody (Santa Cruz, CA, USA).

2.4. Western blot analysis

Equal amounts of proteins in cell lysates were separated by 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked with a TBS-T (25 mM Tris–HCl, 140 mM NaCl, 0.1% Tween 20, pH 7.5) buffer containing 5% non-fat dry milk. After the membrane was incubated with primary antibodies and appropriate secondary antibodies, the protein bands were detected using chemiluminescent reagents as recommended by the manufacturer (Amersham, Franklin Lakes, NJ, USA) [27,31].

2.5. Fluorescence microscopy analysis

The distribution of transduced protein was analyzed using a fluorescence microscopy assay as described previously [27,32]. HT22 cells were grown on coverslips and treated with Tat-PIM2 protein (3 μM) for 1 h, the cells were washed twice with PBS, fixed with 4% paraformaldehyde, permeabilized and blocked for 40 min with 3% bovine serum albumin, 0.1% Triton X-100 in PBS (PBS-BT) and washed with PBS-BT. Then the cells were incubated with a primary antibody (His-probe, 1:2000) and a secondary antibody (Alexa Fluor 488, 1:15,000) in the dark. After nuclei stained for 2 min with 1 μg/ml DAPI (Roche, Mannheim, Germany), the cells were analyzed by confocal microscopy using a Model FV-300 microscope (Olympus, Tokyo, Japan).

2.6. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability was analyzed by a colorimetric assay using MTT as described in previous studies [27,33]. Briefly, HT22 cells were pretreated with Tat-PIM2 protein (0.2–0.8 μM) for 1 h, after which H_2O_2 (1 mM) was added to the culture medium for 1 h 30 min. Cell viability was measured at 570 nm using an ELISA microplate reader (Labsystems Multiskan MCC/340) and cell viability was defined as the percentage of untreated control cells.

2.7. Measurement of intracellular ROS levels

Intracellular ROS levels were measured using dichlorofluorescein diacetate (DCF-DA) as described previously [27,34]. HT22 cells were incubated with Tat-PIM2 protein (0.8 μM) for 1 h and then treated with hydrogen peroxide (H_2O_2, 1 mM) for 30 min. The cells were washed twice with PBS and incubated with DCF-DA (20 μM) for 30 min. Then, the fluorescence intensity was measured at 485 nm excitation and 538 nm emission using a Fluoroskan ELISA plate reader (Labsystems Oy, Helsinki, Finland).

2.8. TUNEL assay

DNA fragmentation was determined by Terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick-end labeling (TUNEL) staining as described previously [27]. HT22 cells were incubated in the absence or presence of Tat-PIM2 protein (0.8 μM) for 1 h and then treated with H_2O_2 (1 mM) for 18 h. After TUNEL staining, images were taken using a fluorescence microscope (Nikon eclipse 80i, Japan) and fluorescence intensity was measured using a Fluoroskan ELISA plate reader (Labsystems Oy, Helsinki, Finland) at 485 nm excitation and 538 nm emission.

2.9. Experimental animals and treatment

The cerebral forebrain ischemia model was performed as described previously [27]. Briefly, the animals were anesthetized, common carotid arteries were isolated, freed of nerve fibers, and occluded with non-traumatic aneurysm clips. Complete interruption of blood flow was confirmed by observing the retinal artery using an ophthalmoscope. After 5 min occlusion, the aneurysm clips were removed. The restoration of blood flow (reperfusion) was observed directly under the ophthalmoscope.

To explore the protective effects of Tat-PIM2 protein against ischemic damage, the animals were divided into 5 groups (each $n = 10$); control sham group, vehicle-treated group, Tat peptide-treated group, control PIM2-treated group, and Tat-PIM2-treated groups (each 2 mg/kg)