2012 International Conference on Life Science and Engineering
IPCBEE vol.45 (2012) © (2012) IACSIT Press, Singapore
DOI: 10.7763/IPCBEE. 2012. V45. 20

Protective Effect of PEP-1-MT- III on Neuronal Cell Death to Oxidative Stress

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Abstract. Metallothioneins (MT)-Ⅲ can protect neuronal cells from the cytotoxicity of oxidative stress. However, biological function of MT-Ⅲ proteins is unclear in ischemia. In this study, we examined the protective effects of MT-Ⅲ proteins on oxidative stress-induced neuronal cell death and brain ischemic insult. A human MT-Ⅲ gene was fused with a protein transduction domain, PEP-1 peptide, to construct a cell permeable PEP-1-MT-Ⅲ protein. Purified PEP-1-MT-Ⅲ proteins transduced into astrocytes in a time- and dose-dependent manner and protected against oxidative stress-induced cell death. Also, transduced PEP-1-MT-Ⅲ proteins efficiently protected cells against DNA fragmentation. Furthermore, immunohistochemical analysis revealed that PEP-1-MT-Ⅲ prevented neuronal cell death in the CA1 region of the hippocampus induced by transient forebrain ischemia. Since the transduced PEP-1-MT-Ⅲ protein has neuroprotective roles as an antioxidant in vitro and in vivo, PEP-1-MT-Ⅲ protein may be used as a potential therapeutic agent against various human brain diseases such as stroke, Alzheimer's disease, and Parkinson's disease.

Keywords: Antioxidant, PEP-1-MT-Ⅲ, Protein transduction, Cell viability, Ischemia, ROS

1. Introduction

Metallothionein (MTs) are low molecular weight, cystein-rich, and metal-binding proteins [1-3]. MT-Ⅲ is known to be a growth inhibitory factor (GIF), which is a brain-specific member of the MT family [4]. Overexpression of MT-Ⅲ prevents neuronal cell death in an animal model of brain damage [5,6]. Reactive oxygen species (ROS) are natural and inevitable by-products of various cellular processes involving interactions with oxygen. The effects of ROS on the brain can be explored through the interruption and reperfusion of blood flow to the brain which induces an enormous increase of ROS in the hippocampal CA1 region and finally results in neuronal cell death [7,8]. Protein transduction technology has been successfully used to deliver a range proteins into mammal cells. In previous studies we have shown in vitro and in vivo that that various transduced proteins efficiently protected against cell death [9-11]. In the present study, we investigated the protective effects of cell permeable PEP-1-MT-Ⅲ protein on oxidative stress-induced neuronal cell death and transient forebrain ischemia.

2. Materials and Methods

2.1. Cells and Materials

Rat astrocytoma cell line C6, astrocyte cells, were obtained from the Korean Cell Line Research Foundation, in Seoul, Korea. The cells were cultured in DMEM supplemented with 10% fetal bovine serum **(**FBS) and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37 ℃ under humidified conditions of 95% air and 5% CO_2. Plasmid pET-15b and *Escherichia coli* strain BL21 (DE3) were obtained from Novagen. The FBS and antibiotics were purchased from Gibco BRL. Ni^{2+}-nitrilotri-acetic acid sepharose superflow was purchased from Qiagen. Synthetic PEP-1 peptides were purchased from PEPTRON (Daejeon, Korea). All other chemicals and reagents were of the highest commercial grade available.

2.2. Expression and Purification of PEP-1-MT-Ⅲ Proteins

A PEP-1 expression vector was prepared in our laboratory as described previously. The cDNA sequence for human MT-Ⅲ was amplified by PCR using the sense primer 5'-CTCGAGATGGACCCTGAGACCTGCCCCTGC-3' and the antisense primer, 5'-GGATCCTCACTGGCAGCAGCTGCACTTCTC-3'. The resulting PCR product was sub-cloned in TA cloning vector and ligated into the expression vector, PEP-1, to produce a genetic in-frame PEP-1-MT-Ⅲ protein. In a similar fashion, a control MT-Ⅲ was constructed that expressed the MT-Ⅲ protein without the PEP-1.

The recombinant PEP-1-MT-Ⅲ plasmid was transformed into *E. coli* BL21 cells and induced with 0.5 mM isopropyl-β-D-thio-galactoside (Duchefa, Haarlem, Netherlands) at 37℃ for 3-4 h. Harvested cells were lysed by sonication and the recombinant PEP-1-MT-Ⅲ was purified using a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column and PD-10 column chromatography (Amersham, Brauncschweig, Germany) according to the manufacturer's instructions. The protein concentration was estimated by the Bradford procedure using bovine serum albumin as a standard.

2.3. Transduction of PEP-1-MT-Ⅲ Protein into Astrocytes

For the transduction of PEP-1-MT-Ⅲ, the cells were treated with various concentrations of PEP-1-MT-Ⅲ for 1 h. Then the cells were 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.

2.4. Western Blot Analysis

The proteins in cell lysates were resolved by 15% 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 PBS. The membranes were immunoblotted with the indicated primary antibodies as recommended by the manufacturer. Proteins were detected by chemilluminescence according to the manufacturer's instructions (Amersham, Franklin Lakes, NJ, USA).

2.5. Fluorescence Microscopy Analysis

Astrocytes were grown on coverslips and treated with 3 μM of PEP-1-MT-Ⅲ proteins. Following incubation for 1 h at 37℃, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 5 min at room temperature. The cells were 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. The primary antibody (His-probe, Santa Cruz Biotechnology) was diluted 1:2000, and incubated for 3 h at room temperature. The secondary antibody (Alexa fluor 488, Invitrogen) was diluted 1:15000, and incubated for 1 h at room temperature in the dark. Nuclei were stained for 2 min with 1 μg/ml DAPI (Roche). The distributions of fluorescence were analyzed by fluorescence microscopy (Nikon eclipse 80i, Japan).

2.6. 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium Bromide (MTT) Assay

A MTT assay was used to determine the viability of astrocytes treated with hydrogen peroxide (H_2O_2). The cells were pretreated with PEP-1-MT-Ⅲ (0.5-3 μM) for 1 h, after which H_2O_2 (0.5 mM) was added to the culture medium for 16 h. The absorbance was measured at 540 nm using an ELISA microplate reader (Labsystems Multiskan MCC/340) and the cell viability was defined as the % of untreated control cells.

2.7. Measurement of Intracellular ROS Levels

Intracellular ROS levels were determined using DCF-DA, which is converted by ROS into fluorescent DCF. Astrocytes were incubated in the absence or presence of PEP-1- MT-Ⅲ for 1 h and then treated with hydrogen peroxide (100 μM) for 30 min. The cells were washed twice with PBS and incubated with DCF-DA (10 μM) for 30 min. The cellular fluorescence intensity quantification was measured at 485 nm excitation and 538 nm emission using a Fluoroskan ELISA plate reader (Labsystems Oy, Helsinki, Finland).

2.8. TUNEL Assay

Astrocytes were incubated in the absence or presence of PEP-1-MT-Ⅲ (3 μM) for 1 h, and then treated with H_2O_2 (80 μM) for 12 h. Terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated dUTP nick end labeling (TUNEL) staining was performed using the Cell Death Detection kit (Roche Applied Science)

according to the manufacturer's instructions. Images were taken using a fluorescence microscope (Nikon eclipse 80i, Japan).

2.9. Measurement of Mitochondrial Membrane Potential

Astrocytes were incubated in the absence or presence of PEP-1-MT-III (3 μM) for 1 h, and then exposed to H_2O_2 (1 mM) for 2 h. The mitochondrial membrane potential was measured using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazoly-carbocyanine iodine (JC-1) assay kit (Cayman, MI, USA) according to the manufacturer's instructions. Images were taken using a fluorescence microscope (Nikon eclipse 80i, Japan).

2.10. Experimental Animals and Induction of Cerebral Forebrain Ischemia

Mongolian gerbils (*Meriones unguiculatus*) were obtained from the Experimental Animal Center, at Hallym University. The animals were housed at a constant temperature (23℃) 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 (Permit number: Hallym 2009-116).

The cerebral forebrain ischemia models were performed according to a method previously described. To determine whether transduced PEP-1-MT-III protects against ischemic damage, gerbils (each $n = 5$) were i.p. injected with PEP-1-MT-III protein (3 mg/kg) 30 min before the occlusion of common carotid arteries. The 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 central artery in the eyeball using an ophthalmoscope. After 5 min occlusion, the aneurysm clips were removed. The restoration of blood flow (reperfusion) was observed directly under the ophthalmoscope.

Brain tissue samples were obtained at 4 days after ischemia-reperfusion, perfused transcardially with PBS (pH 7.4), followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4) in the sham-operated group, vehicle-treated, and PEP-1 peptide-treated group, control MT-III-treated group, and PEP-1-MT-III-treated group (each 3 mg/kg). The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. The tissues were then frozen and sectioned with a cryostat at 50 μm and consecutive sections were collected in six-well plates containing PBS. Cresyl violet staining was performed as previously described.

2.11. Measurement of Lipid Perxoidation in Hippocampus

Lipid peroxidation was measured according to the method described by Zhang et al.. An aliquot (100 μl) of brain supernatant was added to a reaction mixture containing 100 μl of 8.1% sodium dedecyl sulphate (SDS), 750 μl of 20% acetic acid (pH 3.5), 750 μl of 0.8% thiobarbituric acid and 300 μl distilled water. Samples were then boiled for 1 h at 95℃ and centrifuged at 4000 x *g* for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 532 nm.

2.12. Quantitative Analysis

Comparison between the groups was made by ANOVA followed by Dunnett test. A value of $P < 0.05$ was considered statistically significant. The neuronal number and intensity of immunoreactivity were calculated using an image analyzing system. The staining intensity of the immunoreactive structures was evaluated as the relative optical density (ROD): the relative % of the control level is shown in the graph.

3. Discussion

MT-III highly protected cells against the H_2O_2-induced production of ROS and DNA damage in human fibroblasts leading them to suggest that MT-III protein protects cells against oxidative stress and acts as antioxidant in mammalian cells [12,13]. Transduced PEP-1-MT-III protein protected against neuronal cell death in the hippocampal CA1 and CA3 regions after ischemia and attenuates neuronal damage after ischemic injury. We observed a significant increase in the levels of MDA in the brain, a marker of lipid peroxidation, 3 h after ischemic insult. However, increased MDA levels were markedly reduced by transduced PEP-1-MT-III protein. In conclusion, we demonstrated that human MT-III fused with PEP-1

peptide (PEP-1-MT-Ⅲ) can be efficiently transduced *in vitro* and *in vivo* and protects against oxidative stress-induced cell death and ischemic insults.

4. Acknowledgements

This work was supported by a research grant from the Brain Research Center of the 21st Century Frontier Research Program (2010K000808) and by the Priority Research Centers Program grant (2009-0093812) through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology.

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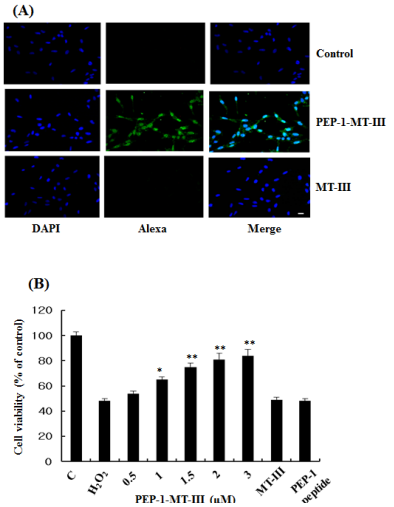

Fig. 1: Transduction of PEP-1-MT-III proteins into astrocytes and cell viability.

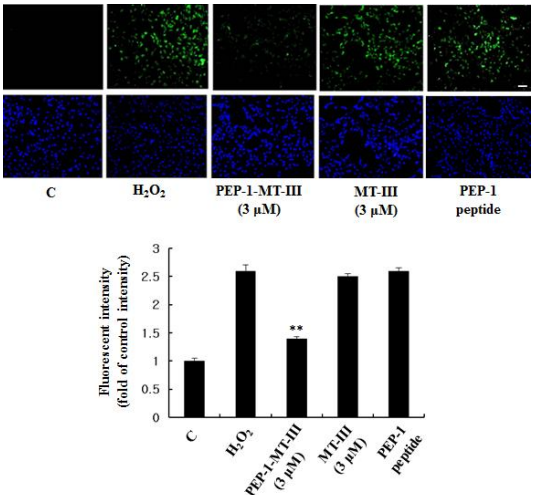

Fig. 2: Effects of transduced PEP-1-MT-III on ROS generation in cells exposed to hydrogen peroxide.

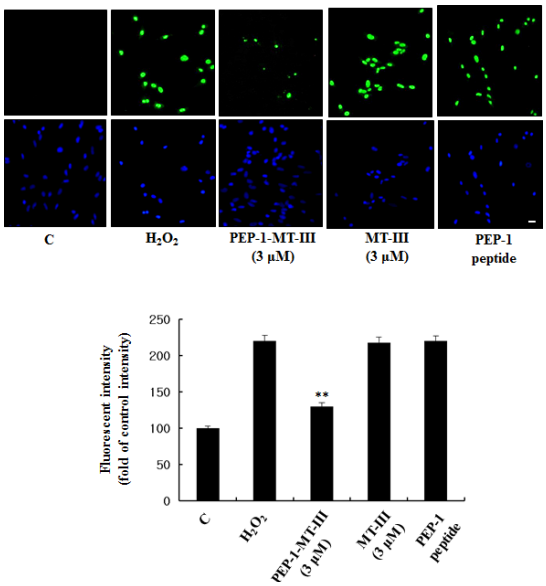

Fig. 3: PEP-1-MT-III protein protects against H_2O_2-induced DNA fragmentation.

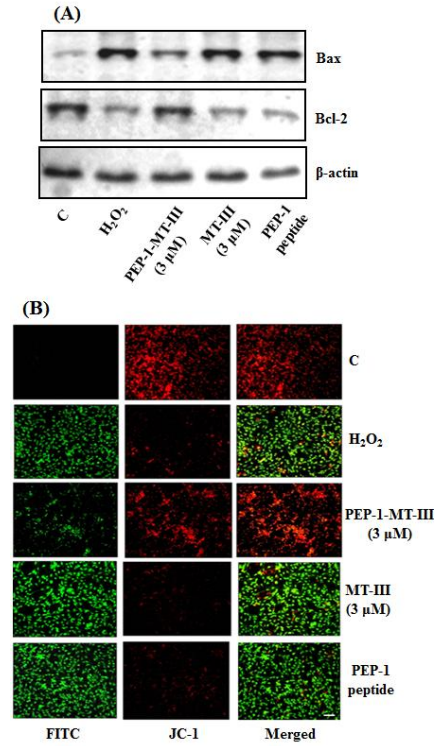

Fig. 4: Effect of PEP-1-MT-III protein on the Bax to Bcl-2 ratio and mitochondrial membrane potential.

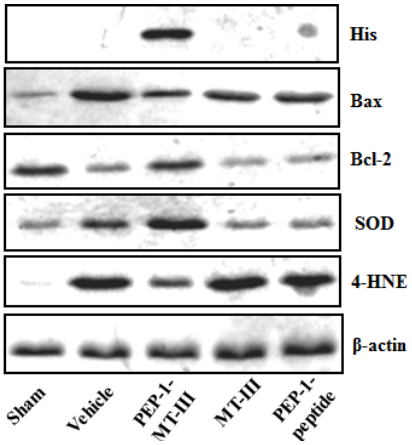

Fig. 5: Protective effect of transduced PEP-1-MT-III protein in the mice brain analyzed by Western blotting using histidine, Bax, Bcl-2, SOD and 4-HNE antibodies.

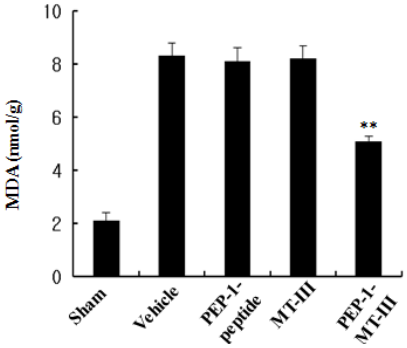

Fig. 6: Effects of transduced PEP-1-MT-III on brain malondiadehyde (MDA) level.