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PEP-1-p18 prevents neuronal cell death by inhibiting oxidative stress and Bax expression

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P18, a member of the INK4 family of cyclin-dependent kinase inhibitors, is a tumor suppressor protein and plays a key cell survival role in a variety of human cancers. Under pathophysiological conditions, the INK4 group proteins participate in novel biological functions associated with neuronal diseases and oxidative stress. Parkinson's disease (PD) is characterized by loss of dopaminergic neurons, and oxidative stress is important in its pathogenesis. Therefore, we examined the effects of PEP-1-p18 on oxidative stress-induced SH-SY5Y cells and in a PD mouse model. The transduced PEP-1-p18 markedly inhibited 1-methyl-4-phenyl pyridinium-induced SH-SY5Y cell death by inhibiting Bax expression levels and DNA fragmentation. Additionally, PEP-1-p18 prevented dopaminergic neuronal cell death in the substantia nigra of a 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine-induced PD mouse model. These results indicate that PEP-1-p18 may be a useful therapeutic agent against various diseases and is a potential tool for treating PD. [BMB Reports 2012; 45(9): 532-537]

INTRODUCTION

Parkinson's disease (PD) is a chronic and progressive neuronal disorder that is characterized by the selective loss of dopaminergic neurons in the substantia nigra (SN) leading to dopamine depletion in the striatum. Typical symptoms of PD are muscle rigidity, bradykinesia, postural instability, and resting tremors (1-3). Although the exact mechanism of PD is yet not understood, several risk factors such as aging, environmental exposure, and genetic factors are involved in PD pathogenesis (4, 5). Many studies have made extensive use of 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP) and 1-methyl-4-phenyl pyridinium (MPP^+) PD models to understand the PD mechanism. However, no effective PD therapeutic agent has been identified to date. Thus, new strategies for PD therapy are needed to mitigate progressive dopaminergic neuronal damage (5-7).

The cell cycle is divided into four phases, and progression through each phase of the cycle is regulated by cyclin-dependent kinases (CKDs), whereas cyclin-dependent kinase inhibitors (CDKIs) arrest cell cycle progression by inhibiting the activities of CDKs (8). The CDKIs are divided into two families. The INK4 family includes p16, p15, p18, and p19, which specifically arrest the cell cycle in the early G_1 phase. The CIP/KIP family includes p21, p27, and p57, which extensively inhibit the cell cycle (9, 10). CDKI expression may play an important novel biological role related with neurodegenerative diseases under pathophysiological conditions (11).

To allow for the delivery of exogenous protein into cells, small regions of proteins, called protein transduction domains (PTDs) or cell penetrating peptides, have been developed. PTDs have demonstrated their ability to deliver various proteins into living cells (12, 13). Although the transduction mechanism remains unclear, PTD fusion proteins have been used to deliver therapeutic proteins *in vitro* and *in vivo*. We have shown previously that various PTD fusion proteins transduced into neuronal cells cross the blood-brain barrier and efficiently protect against cell death (14-20). In this study, we investigated the protective effects of PEP-1-p18 against MPP^+-induced dopaminergic neuronal cell death and MPTP-induced PD mouse models.

RESULT AND DISCUSSION

Purification and transduction of PEP-1-p18 in SH-SY5Y cells

Studies have shown that INK4 group proteins play novel biological functions associated with DNA repair and neuronal diseases (11). However, the biological function and molecular mechanism of p18 in PD are still unclear.

We constructed a PEP-1-p18 expression vector to develop an expression and purification system for cell-permeable p18, which contained human p18 cDNA, the PEP-1 peptide, and six histidine residues. We also constructed a control p18 expression

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vector containing human p18 cDNA and six histidine residues (data not shown). PEP-1-p18 was purified using a Ni^{2+}-nitrilotriacetic acid/Sepharose affinity column and PD-10 column chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses of the purified PEP-1-p18 were performed. The purification results are shown in Fig. 1A. The proteins were confirmed by Western blot analysis using an antirabbit polyhistidine antibody.

We analyzed PEP-1-p18 transduction by adding it to SH-SY5Y cell culture medium at various concentrations (0.5-3 μM) for 60 min, and the levels of transduced proteins were measured by Western blotting (Fig. 1B). We also analyzed the time-dependency of PEP-1-p18 transduction by adding 3 μM PEP-1-p18 for various periods of time (10-60 min), and the levels of transduced proteins were measured by Western blotting (Fig. 1C). The results revealed that PEP-1-p18 was efficiently transduced into the cells in a time- and dose-dependent manner. However, control p18 was not transduced into cells. As shown in Fig. 1D, we examined the intracellular stability of transduced PEP-1-p18 in SH-SY5Y cells. Significant levels of transduced PEP-1-p18 persisted in the cells for 48 hr. To further clarify cellular localization of the transduced proteins in the cells, transduced cells were double stained with the nucleus-specific marker DAPI. As shown in Fig. 2A, PEP-1-p18 was detected in the cytoplasm and nucleus of transduced cells. These results indicate that PEP-1-p18 was efficiently transduced into the cells.

Efficient delivery of therapeutic proteins into cells is very important to gain the desired therapeutic effect. However, delivering protein into cells is limited due to their size and biochemical properties. Therefore, it has been problematic to utilize proteins as therapeutic agents (21). This problem was overcome by protein transduction domains, which are capable of delivering protein into cells (22).

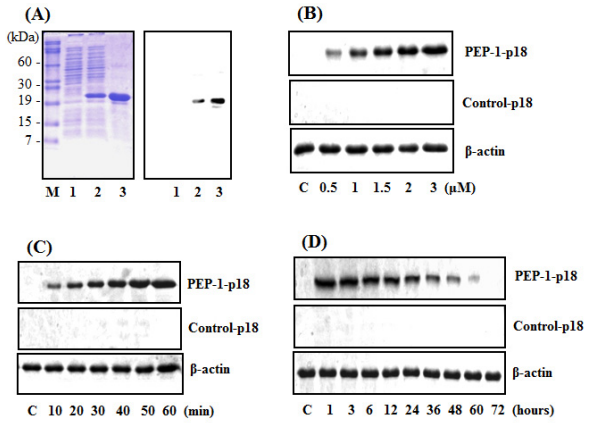

Fig. 1. Purification and transduction of the PEP-1-p18 protein. Protein extracts of cells and purified protein were analyzed by 15% SDS-PAGE and subjected to Western blot analysis with an antirabbit polyhistidine antibody (A). Lane 1, noninduced PEP-1-p18; lane 2, induced PEP-1-p18; lane 3, purified PEP-1-p18. Transduction of PEP-1-p18 protein into SH-SY5Y cells. PEP-1-p18 protein (0.5-3 μM) was added to the culture medium for 60 min (B). PEP-1-p18 (3 μM) was added to the culture media for 10-60 min (C). Cells pretreated with 3 μM PEP-1-p18 were incubated for various times prior to Western blotting (D).

Transduced PEP-1-p18 protects neuronal cells from MPP^+-induced cytotoxicity

MPP^+ is a well-known neurotoxin that can induce neuronal cell death by generating reactive oxygen species (ROS) and DNA damage (11, 23, 24). To determine whether the transduced PEP-1-p18 has a functional role in neuronal cell death, we examined the effect of MPP^+ on cell viability. The viability of cells treated with MPP^+ increased in a dose-dependent manner when pretreated with PEP-1-p18. Only 51% of the cells were viable

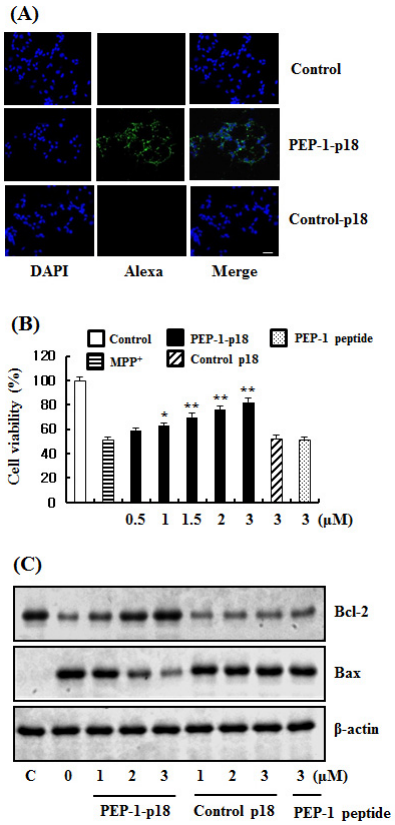

Fig. 2. Localization and inhibitory effect of transduced PEP-1-p18 protein. The distribution of cells transduced with PEP-1-p18 was observed by fluorescence microscopy (A). Scale bar = 50 μm. MPP^+ (5 mM) was added for 18 hr to SH-SY5Y cells that had been pretreated for 1 hr with PEP-1-p18 protein (0.5-3 μM). Cell viability was assessed by the MTT-based colorimetric assay (B). *P < 0.05 and **P < 0.01, compared with MPP^+-treated cells. Cells were pretreated with PEP-1-p18 (3 μM) for 1 hr and then exposed to MPP^+ (5 mM) for 8 hr. Equal amounts of whole cell extracts were separated by SDS-PAGE, and Bax and Bcl-2 were detected by Western blot analysis. β-actin was used as the control (C).

when they were exposed to 5 mM MPP^+ for 18 hr. However, the viability of cells pretreated with PEP-1-p18 increased in a dose-dependent manner, reaching > 82% at the maximum concentration used (Fig. 2B). However, the control p18 and PEP-1 peptide did not show protective effects under the same conditions, indicating that transduced PEP-1-p18 plays a defensive role against cell death induced by MPP^+ in cells without a cytotoxic effect.

We further examined whether PEP-1-p18 inhibits anti and pro-apoptotic protein (Bcl-2 and Bax) expression and DNA fragmentation. Thus, we performed Western blotting using the Bcl-2 and Bax antibodies. As shown in Fig. 2C, Bax expression levels increased markedly following MPP^+ treatment, whereas the level of Bax expression decreased significantly in PEP-1-p18 transduced cells. In contrast, Bcl-2 expression increased significantly in cells transduced with PEP-1-p18. Control p18 and PEP-1 peptide did not result in changes in Bcl-2 and Bax expression levels under the same conditions. These results indicate that PEP-1-p18 plays a defensive role against MPP^+-induced cell death.

The protective effect of transduced PEP-1-p18 against DNA fragmentation was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. As shown in Fig. 3, the number of stained cells among MPP^+ treated cells increased markedly compared with that in the control group, whereas the number of cells treated with PEP-1-p18 that were stained decreased significantly. However, control p18 and PEP-1 peptide treated cells were similarly stained as MPP^+-treated cells. Apoptosis can be triggered by various factors such as UV radiation, oxidative stress, and DNA damage. The pro-apoptotic protein Bax is active during apoptosis, whereas the antiapoptotic protein Bcl-2 is inactive during apoptosis (25-27). Deng *et al.* (2003) demonstrated that Bcl-2 retards cell cycle progression by regulating intracellular ROS levels (28).

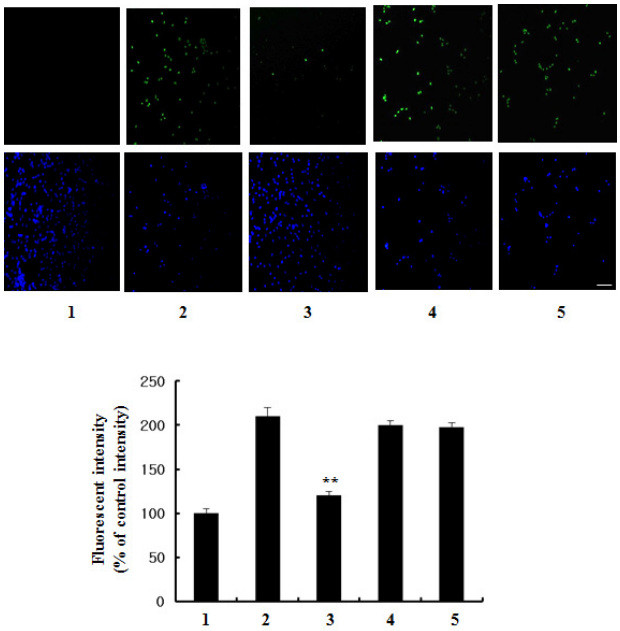

Fig. 3. The PEP-1-p18 protein protects against MPP^+-induced DNA fragmentation. Cells were treated with PEP-1-p18 (3 μM) for 1 hr, and then exposed to MPP^+ (4 mM) for 2 hr. DNA fragmentation was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Upper panels are TUNEL positive cells (green) and lower panels are DAPI stained images (blue). Panels are as follows: 1, control cells; 2, MPP^+ treated cells; 3, PEP-1-p18 (3 μM) treated cells; 4, control p18 (3 μM) treated cells; 5, PEP-1 peptide (3 μM) treated cell. Scale bar = 50 μm.

Neuroprotective effect of PEP-1-p18 in a PD mouse model

To determine whether PEP-1-p18 crosses the blood-brain barrier, we performed immunohistochemistry on the SN in brain sec-

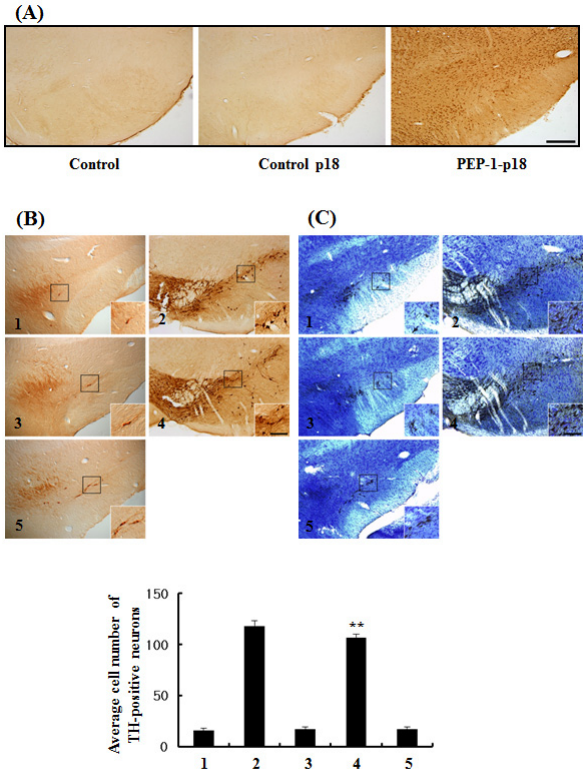

Fig. 4. Effects of transduced PEP-1-p18 on dopaminergic neuronal cell viability. Transduction of the PEP-1-p18 protein across the blood-brain barrier (A). Transduction of the PEP-1-p18 protein in mouse brain was analyzed by immunohistochemistry using anti-histidine antibody. Animals were treated with a single injection of PEP-1-p18 and killed after 8 hr. Scale bar = 100 μm. Representative photomicrographs of the tyrosine hydroxylase (TH) (B) and cresyl-violet (C) immunostained substantia nigra (SN) of PEP-1-p18 (2 mg/kg) treated mice. The number of TH immunoreactive neurons was counted in the SN of mice. Panels in (B and C) are as follows: 1, MPTP treated; 2, control; 3, control p18 treated; 4, PEP-1-p18 treated; 5, PEP-1 peptide treated. Scale bar = 50 μm. **P < 0.01 compared with MPTP-treated mice.

tions from PEP-1-p18 treated mice. As shown in Fig. 4A, transduced PEP-1-p18 levels increased significantly throughout the SN of PEP-1-p18 treated animals. However, control p18 was not transduced into the SN.

PD is the second most common neurodegenerative disorder and is associated with progressive degeneration of dopaminergic neurons in the SN pars compacta. It is well known that exposure of C57BL/6 mice to the neurotoxin MPTP is one of the most valuable approaches to analyze critical aspects of PD in animal models. MPTP is a selective neurotoxicant and is known to deplete striatal dopamine initiated by MPP^+ (29). We examined the protective effect of PEP-1-p18 against dopaminergic neuronal cell death in a MPTP-induced mouse model of PD. PEP-1-p18 (2 mg/kg) was injected intraperitoneally (i.p.) one time, and dopaminergic neuronal cell levels were assessed by tyrosine hydroxylase (TH) and cresyl violet histochemistry. As shown in Fig. 4B and 4C, the PEP-1-p18 protein-treated groups were efficiently protected against dopaminergic neuronal injury caused by MPTP. Under the same experimental conditions, the PEP-1 peptide and control p18-treated groups demonstrated no protective effect. These results indicate that PEP-1-p18 was efficiently transduced into the SN beyond the blood-brain barrier and protected against MPTP-induced dopaminergic neuronal cell death. Thus, the PEP-1-p18 protein may be a therapeutic agent for treating PD.

In summary, we demonstrated that PEP-1-p18 was efficiently transduced *in vitro* and *in vivo* and protected against MPP^+-induced and MPTP-induced neuronal cell death. Although further study will be needed to ascertain the mechanisms of neuronal cell protection, our success using PEP-1-p18 transduction indicates the therapeutic potential of this protein in the treatment of PD.

MATERIALS AND METHODS

Materials

Antibodies against histidine, Bcl-2, Bax, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human neuroblastoma SH-SY5Y cells were obtained from the Korean Cell Line Research Foundation (Seoul, Korea). Human p18 cDNA was isolated using the polymerase chain reaction (PCR) technique. The synthetic PEP-1 peptides were purchased from Peptron (Daejeon, Korea). All other chemicals and reagents were of the highest analytical grade available.

Expression and purification of PEP-1-p18 proteins

A PEP-1 expression vector was prepared in our laboratory as described previously (14). The human p18 cDNA sequence was amplified by PCR using the sense primer 5'-CTCGAGGCCG GCCTTG-3' and the antisense primer 5'-GGATCCTTGAAGATTT GTGGCTC-3'. The PCR product was sub-cloned in a TA-cloning vector, ligated into the PEP-1 vector, and cloned in *Escherichia coli* DH5α.

The plasmid was transformed into *E. coli* BL21 cells to produce PEP-1-p18. The transformed bacterial cells were grown in 100 ml of LB media at 37°C to an optical density 600 value of 0.5-1.0 and induced with 0.5 mM of isopropyl-beta-D-thiogalactopyranoside at 37°C for 3-4 hr. Harvested cells were disrupted by sonication in a binding buffer. After centrifugation, the supernatant was immediately loaded onto a Ni^{2+}-nitrilotriacetic acid/Sepharose affinity column. Protein containing the PEP-1-p18 fraction was combined, and the salts were removed using PD-10 column chromatography. The protein concentration was estimated using bovine serum albumin (BSA) as the standard (30).

Transduction of the PEP-1- p18 protein into SH-SY5Y cells

SH-SY5Y cells were cultured in MEM Eagle medium with Eagle's balanced salt solution and L-glutamine containing 10% heat-inactivated fetal bovine serum and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37°C under humidified conditions of 95% air and 5% CO_2. The cells were grown to confluence in six-well plates for PEP-1-p18 transduction. The culture medium was replaced with 1 ml of fresh solution. After the cells were treated with various concentrations of PEP-1-p18 for 1 hr, they were treated with trypsin-EDTA and washed with phosphate-buffered saline (PBS). The cells were harvested to prepare cell extracts for Western blot analysis.

Western blot analysis

Proteins in cell lysates were resolved by 15% SDS-PAGE. The proteins were electrotransferred to a nitrocellulose membrane, which was blocked with 5% nonfat dry milk in PBS. The membrane was probed with the indicated antibodies, and the immunoreactive bands were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Buckinghamshire, UK).

Fluorescence microscopic analysis

SH-SY5Y cells were grown on coverslips treated with 3 μM PEP-1-p18. Following a 1 hr incubation at 37°C, 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 30 min with 3% BSA, 0.1% Triton X-100 in PBS (PBS-BT), and washed with PBS-BT. The cells were then exposed to the primary antibody (His-probe, 1 : 2,000; Santa Cruz Biotechnology) for 1 hr at room temperature. The secondary antibody (Alexa Fluor 488, 1 : 15,000; Invitrogen, Carlsbad, CA, USA) was applied for 1 hr at room temperature in the dark. Nuclei were stained for 5 min with 0.1 μg/ml 4'6-diamidino-2-phenylindole (DAPI; Roche, Basel, Switzerland). The distribution of fluorescence was analyzed by confocal microscopy using a model FV-300 microscope (Olympus, Tokyo, Japan).

Cell viability assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) assay was used to determine viability of SH-SY5Y cells treated with MPP^+. The cells were pretreated with PEP-1-p18 (0.5-3 μM) for 1 hr before MPP^+ (5 mM) was added to the culture medium for 18 hr. Cell viability was estimated by MTT colorimetric assay. The absorbance was measured at 540 nm using

an enzyme-linked immunosorbent assay microplate reader (Labsystems Multiskan MCC/340), and cell viability was defined as the percentage of untreated control cells.

TUNEL assay

SH-SY5Y cells were incubated in the absence or presence of PEP-1-p18 (3 μM) for 1 hr, and then treated with MPP^+ (4 mM) for 2 hr. TUNEL staining was performed using a Cell Death Detection kit (Roche Applied Science, Piscataway, NJ, USA) according to the manufacturer's instructions. Images were taken using an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan).

Experimental animals and immunohistochemistry

Male C57BL/6 mice (6-weeks-old, 22-25 g) were used. The mice were obtained from the Experiment Animal Center at Hallym University. The animals were housed at a constant temperature (23°C) and relative humidity (60%) with a fixed 12 hr light/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 and Quarantine Service of Korea and were approved by the Hallym Medical Center Institutional Animal Care and Use Committee. The mice received four injections of MPTP (20 mg/kg) at 2 hr intervals. To determine whether transduced PEP-1-p18 protects against PD, the mice were i.p. injected with PEP-1-p18 or control p18 and PEP-1 peptide (2 mg/kg) 12 hr before the MPTP treatment (n = 5 mice/each group). The mice were killed 1 week after the last injection. The frozen and sectioned midbrains were prepared and fixed with 4% paraformaldehyde for 10 min. Free-floating sections were first incubated with 0.3% Triton X-100 and 10% normal goat serum in PBS for 1 hr to remove nonspecific immunoreactivity. The sections were then incubated with a rabbit antiTH monoclonal antibody (Vector Laboratories, Burlingame, CA, USA; 1 : 200), visualized with 3,3-diaminobenzidine (DAB) (40 mg DAB, 0.045% H_2O_2 in 100 ml PBS), and mounted on gelatin-coated slides. Cell counts were performed on SN TH-immunoreactive neurons. Neurons were counted using the optical fractionator method, an unbiased quantitative technique that is independent of neuronal size and shape or any conformational changes in the tissue.

Statistical analysis

Data are expressed as mean ± standard deviation from three independent experiments. Differences among means were analyzed using one-way analysis of variance (ANOVA). Newman-Keuls post hoc analysis was employed when differences by ANOVA testing were observed ($P < 0.05$).

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