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Tat-aldose reductase prevents dopaminergic neuronal cell death by inhibiting oxidative stress and MAPK activation

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Abstract. Aldose reductase (AR) is known to detoxify aldehydes and prevent oxidative stress. Although AR exerts antioxidant effects, the role of AR in Parkinson's disease (PD) remains unclear. The objective of the present study was to investigate the protective effects of AR protein against 1-methyl-4-phenylpyridinium (MPP^+)-induced SH-SY5Y cell death and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD in a mouse model using the cell permeable Tat-AR fusion protein. The results revealed that when Tat-AR protein was transduced into SH-SY5Y cells, it markedly protected the cells against MPP^+-induced death and DNA fragmentation. It also reduced the activation of mitogen-activated protein kinase (MAPKs) and regulated the expression levels of Bcl-2, Bax and caspase-3. Immunohistochemical analysis revealed that when Tat-AR protein was transduced into the substantia nigra (SN) of mice with PD, it markedly inhibited dopaminergic neuronal cell death. Therefore, Tat-AR may be useful as a therapeutic protein for PD.

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Key words: Tat-aldose reductase, Parkinson's disease, dopaminergic cells, oxidative stress, MAPKs, protein therapy

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder. It is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta with Lewy bodies in the brain (1,2). Patients with PD are clinically characterized by tremors, rigidity, bradykinesia and postural instability (3,4). Several studies have indicated that oxidative stress and environmental factors are highly associated with PD (5-7). It has been reported that oxidative stress can induce a high level of toxic aldehydes, such as 3,4-Dihydroxyphenylacetaldehyde (DOPAL) in brains of PD patients. Such aldehydes can then trigger dopaminergic neuronal cell death (5,6,8). Neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydro pyridine (MPTP) can cause PD-like symptoms. It is metabolized to 1-methyl-4-phenylpyridinium (MPP^+) which can then lead to dopaminergic neuronal cell death (9,10).

Cytosolic enzyme aldose reductase (AR) is widely expressed in human tissues. It catalyzes the reduction of toxic aldehydes, including 4-hydroxy-2-nonenal (4HNE) produced by lipid peroxidation. 4HNE is known as a biomarker of oxidative stress (11-13). AR can also reduce toxic aldehyde DOPAL and induce dopaminergic neuronal cell death when this aldehyde is increased in the brain under conditions of oxidative stress (14,15). Several studies have demonstrated that AR can protect cells against oxidative stress by decreasing reactive oxygen species (ROS) levels in various cells, such as smooth muscle and lens epithelial cells (16-18).

It is known that macromolecule protein fused with protein transduction domains (PTDs) consisting of 10-16 amino acids can be transduced into cells (19). A number of studies have reported that PTD fused protein can be efficiently transduced into cells as a tool for protein therapy (20-26). In the presents study, a Tat-AR protein expression vector was constructed

based on the pET-15b vector. The Tat-AR expression vector contained a cDNA sequence encoding human AR, Tat PTD and an amino-terminal tag consisting of 6 histidine residues. Tat PTD was cloned into the *Nde*I and *Xho*I sites, and human AR cDNA was cloned into the *Xho*I and *BamH*I sites of pET-15b vector. Tat-AR protein or control AR expression vector consist of a His tag consisting of 6 histidine residues. A recent study demonstrated that a deficiency of AR can lead to dopaminergic neuronal loss (27). Thus, the objective of the present study was to investigate whether transduced Tat-AR protein can protect dopaminergic neuron cells against oxidative stress both *in vitro* and *in vivo*.

Materials and methods

Cell lines and reagents. Human neuroblastoma SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (Lonza/BioWhittaker) containing 15% FBS, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO_2 incubator.

Dichlorofluorescein diacetate (DCF-DA), methyl-4-phenyl-pyridinium (MPP^+) and 1-methyl-4-phenyl-1,2,3,6-tetrahydro pyridine (MPTP) were obtained from Sigma-Aldrich; Merck KGaA. Tat peptide was synthesized from Peptron, Inc. Enhanced chemiluminescence agent was purchased from Amersham; Cytiva. Antibodies were obtained from Cell Signaling Technology, Inc. Tyrosine Hydroxylase (TH) was purchased from Santa Cruz Biotechnology, Inc. All other chemicals and reagents, unless otherwise stated, were of the highest analytical grade available.

Purification and transduction of Tat-AR proteins into SH-SY5Y cells. Tat-AR protein was prepared as described in a previous study (28). To develop a therapeutic protein, a human AR gene fused with a Tat PTD to produce a cell permeable Tat-AR expression vector which contains 6X His, Tat PTD (RKKRRQRRR) and the AR gene. Bovine serum albumin was used as a standard and the purified Tat-AR protein concentration was measured by Bradford assay (29).

To examine the Tat-AR protein transduction efficiency, SH-SY5Y cells were exposed to various concentrations of Tat-AR and AR protein (0.5-3 μM) for 1 h. The SH-SY5Y cells were exposed of Tat-AR and AR protein (3 μM) for various periods of time (15-60 min). The cells were then washed with PBS and treated with trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.). The intracellular stability of Tat-AR protein was also determined. To confirm the stability of Tat-AR protein, the cells were further cultured (1-30 h) following transduction. The levels of transduced proteins were measured by western blot analysis using anti-histidine antibody.

Western blot analysis. Cell lysates were prepared with RIPA lysis buffer containing a cocktail of protease inhibitors (Elpis-Biotech, Inc.). The protein concentration was measured using the Bradford method. Equal amounts of each protein (30 μg) were loaded into 12% SDS-PAGE and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, Inc.). The membrane was blocked with TBS-T (25 mM Tris-HCl, 140 mM NaCl, 0.1% Tween-20, pH 7.5) buffer containing 5% non-fat dry milk for 1 h at room temperature. After washing with TBST, the membrane was incubated with the indicated primary antibodies overnight at 4˚C and followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at 37˚C. His (1:5,000; sc-804; Santa Cruz Biotechnology, Inc.), 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), cleaved caspase-3 (1:1,000; #9661), β-actin (1:5,000; #4967), and appropriate secondary antibodies (1:10,000; #7074). All of the above-mentioned antibodies were purchased from the Cell Signaling Technology, Inc. The membrane was then washed with TBST buffer 3 times and the protein bands were identified using chemiluminescent reagents as recommended by the manufacturer (Amersham; Cytiva). Bands were quantified using ImageJ software (version 1.48; National Institutes of Health) (25,30).

Confocal fluorescence microscopy analysis. To determine the intracellular distribution of transduced Tat-AR protein in SH-SY5Y cells, confocal fluorescence microscopy was performed, as previously described (24). SH-SY5Y cells were placed on coverslips and treated with 3 μM Tat-AR protein 1 h. The cells were washed with PBS twice and fixed with 4% paraformaldehyde for 5 min. The cells were treated in PBS containing 3% bovine serum albumin, 0.1% Triton X-100 (PBS-BT) at room temperature for 30 min and washed with PBS-BT. Histidine primary antibody (sc-804; Santa Cruz Biotechnology, Inc.) was diluted 1:1,500 and incubated at room temperature for 3 h. Alexa Fluor 488-conjugated secondary antibody (#32723; Invitrogen; Thermo Fisher Scientific, Inc.) was diluted 1:1,500 and incubated in the dark for 1 h at room temperature. Nuclei were stained with 1 μg/ml DAPI (Roche Applied Science, Mannheim, Germany) for 2 min at room temperature. The stained cells were analyzed by confocal fluorescence microscopy using a confocal laser-scanning system (Bio-Rad MRC-1024ES, Bio-Rad Laboratories, Inc.).

MTT assay. Cell viability was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described (24). The SH-SY5Y cells were pre-treated with Tat-AR (0.5-3 μM), AR (0.5-3 μM) and Tat peptide (0.5-3 μM) for 1 h and MPP^+ (5 mM) was then added to the culture medium for 13 h. The absorbance was measured at 570 nm using an ELISA microplate reader (Labsystems Multiskan MCC/340; Thermo Fisher Scientific, Inc.) and the cell viability was defined as the percentage of untreated control cells.

Terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick-end labelling (TUNEL) staining. To examine whether transduced Tat-AR proteins protect against MPP^+-induced DNA damage in cells, the SH-SY5Y cells were pre-treated with Tat-AR (3 μM), AR (3 μM) and Tat peptide (3 μM) for 1 h and MPP^+ (5 mM) was added to the culture medium for 16 h. TUNEL staining was performed using a Cell Death Detection kit (Roche Applied Science). Nuclei were stained with DAPI (1 μg/ml) for 2 min at room temperature. Fluorescent images were obtained using a fluorescence

microscope (Nikon eclipse 80i, Nikon Corporation) and cells exhibiting fluorescence were counted under a phase contrast microscope (x200 magnification; Nikon Corporation), as previously described (24,25).

Measurement of ROS production. Intracellular ROS levels were determined by 2',7'-dichlorofluorescein diacetate (DCF-DA) staining as previously described (28,31). To determine the effects of Tat-AR protein against MPP^+-induced intracellular ROS production in SH-SY5Y cells, the cells were placed on coverslips in 24-well plates, incubated for 12 h, and washed twice with PBS. After pretreated with Tat-AR, AR, and Tat peptide (3 μM) for 1 h, MPP^+ (5 mM) was added to the culture medium for 1 h. The cells were washed with PBS and incubated for 30 min with DCF-DA (10 μM). Protein was separately processed as mentioned above to obtain the fluorescent image and fluorescence intensity. One was used to obtain a fluorescent image. To obtain fluorescent images for each well, the cells were washed with PBS, mounted, and the cell images were obtained using a fluorescence microscope (Nikon eclipse 80i, Nikon Corporation).

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. The fluorescence intensity of the samples was measured using a Fluoroskan ELISA plate reader (Labsystems Diagnostics Oy) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Animal experiments and immunohistochemistry. Male C57BL/6 mice (total, n=35), 8 weeks old (weighing 16-20 g), were acquired from the Hallym University Experimental Animal Center. They were housed at 23˚C and a humidity of 60%. They were exposed to regulated 12 h cycles of light and dark and were provided with *ad libitum* access to food and water. The procedures for the care of animals conformed to the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research and Quarantine Service of Korea and approved by the Institutional Animal Care and Use Committee of Soonchunhyang University (SCH16-0051).

To determine whether transduced Tat-AR protein protects against PD, the mice were divided into 5 groups (n=7/each group) as follows: The normal control, MPTP-treated, Tat-AR-treated, control AR-treated and Tat peptide-treated groups. The mice received 4 injections of MPTP (20 mg/kg) at 2-h intervals. Mice were intraperitoneally (i.p.) injected with Tat-AR (2 mg/kg) 12 h prior to MPTP treatment. At 1 week after the final injection, the animals were deeply anesthetized with a mixture of 3% isoflurane (Baxter Healthcare Corporation) in 33% oxygen and 67% nitrous oxide. The brains of these animals were then harvested for immunohistochemistry.

Immunohistochemistry was performed as described in a previous study (31). The frozen and sectioned midbrains were prepared and fixed with 4% paraformaldehyde for 10 min. For removal of non-specific immunoreactivity, free-floating sections were first incubated with 0.3% Triton X-100 and 10% normal goat serum in PBS for 1 h at room temperature. They were then incubated with a rabbit anti-tyrosine hydroxylase (TH) monoclonal antibody (diluted 1:200; sc-14007; Santa Cruz Biotechnology, Inc.) for 48 h at 4˚C and sequentially incubated with a biotinylated goat anti-rabbit IgG (diluted 1:250; BA-1000; Vector Laboratories, Inc.) for 2 h at room temperature. The sections were then visualized with 3,3-diaminobenzidine (DAB) (40 mg DAB, 0.045% H_2O_2 in 100 ml PBS) mounted on gelatin-coated slides. To detect viable cells, cresyl violet (0.1%, Sigma-Aldrich; Merck KGaA) counterstaining for Nissl bodies was conducted for 20 min at room temperature following TH immunostaining. The sections were visualized with 3,3'-diaminobenzidine in 0.1 M Tris buffer and mounted on gelatin-coated slides. Images were captured and analyzed using an Olympus DP72 digital camera and a DP2-BSW microscope digital camera software. Figures were prepared using Adobe Photoshop 7.0. The manipulation of images was restricted to threshold and brightness adjustments applied to the entire image. The images shown are representatives from each group and the sections were processed and analyzed by a blinded observer. To establish the specificity of the immunostaining, a negative control test was carried out with pre-immune serum instead of the primary antibody. The negative control resulted in the absence of immunoreactivity in any structures.

For the quantification of TH immunostaining, a cell count was performed. TH immunostaining images (10 sections/mouse) were captured in the same region. Images were sampled from at least 5 different points within each SN section. Thereafter, the number of TH-positive cells was actually counted within the sampled images. All immunoreactive cells were counted regardless the intensity of labeling. Cell counts were performed by 2 different investigators who were blind to the classification of the tissues.

Statistical analysis. Data are expressed as the means ± SEM of 3 experiments. Differences between groups were analyzed by ANOVA followed by a Bonferroni's post-hoc test (using GraphPad Prism 8; GraphPad Software, Inc.). Statistical significance was considered at $P<0.05$.

Results

Transduction of Tat-AR protein into SH-SY5Y cells. To produce cell-permeable Tat-AR protein, the Tat-AR protein expression vector was constructed by subcloning the cDNA encoding human AR into a pET-15b plasmid containing a Tat PTD. Tat-AR protein expression vector contained a continuous cDNA sequence encoding human AR, a Tat PTD and 6 histidines. In addition, a control AR expression vector containing no Tat PTD was constructed (Fig. 1A). The purified Tat-AR and AR proteins are presented in Fig. 1B. To investigate the transduction efficiency of Tat-AR protein, SH-SY5Y neuroblastoma cells were treated with Tat-AR protein (0.5-3 μM) for 1 h or with Tat-AR protein at 3 μM for 15-60 min. Tat-AR protein was transduced into the cells in a concentration- and time-dependent manner (Fig. 2A and B). Tat-AR protein was also transduced into the cytosol and nucleus of the cells (Fig. 2C). Since stability is one of the major factors in protein therapy, the stability of the transduced Tat-AR protein was examined. Transduced Tat-AR protein persisted until 12 h in the cells (Fig. 2D). These results indicate that Tat-AR protein can be efficiently transduced into the SH-SY5Y cells and can exist for at least 12 h in the cells.

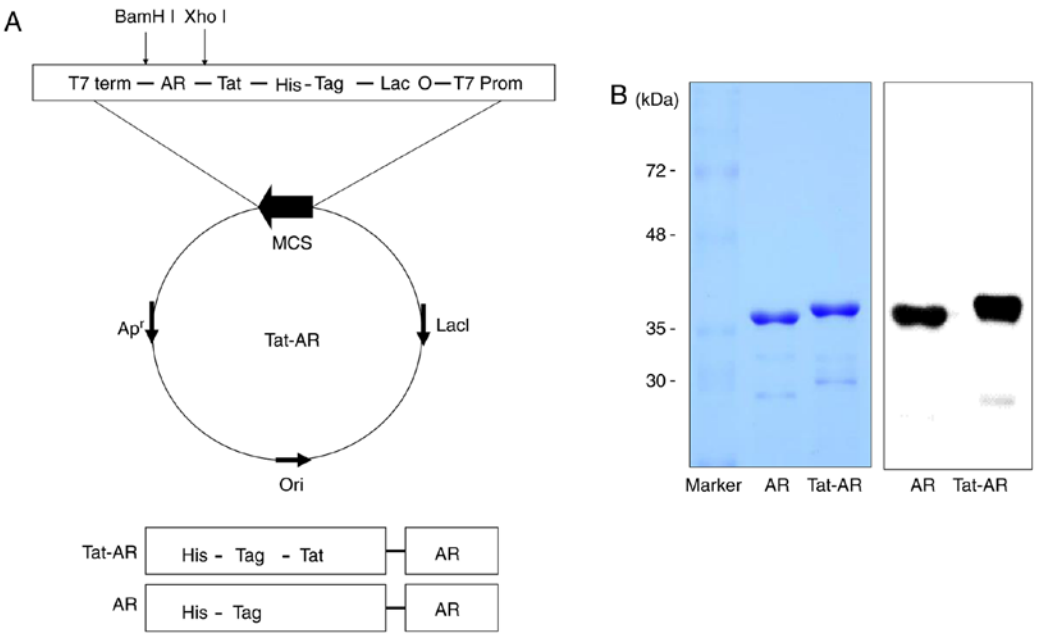

Figure 1. Purification of Tat-AR protein. Construction of the Tat-AR expression vector system was founded on the vector pET-15b. A synthetic Tat PTD was cloned into the *Nde*I and *Xho*I sites, and human AR cDNA was cloned into the *Xho*I and *BamH*I sites of pET-15b. (A) A diagram of the expressed Tat-AR and control AR proteins is depicted. Each consist of a His tag consisting of 6 histidine residues. (B) Purified Tat-AR and AR proteins were identified by 15% SDS-PAGE and were detected by western blot analysis using an anti-histidine antibody. Tat-AR, Tat-aldose reductase.

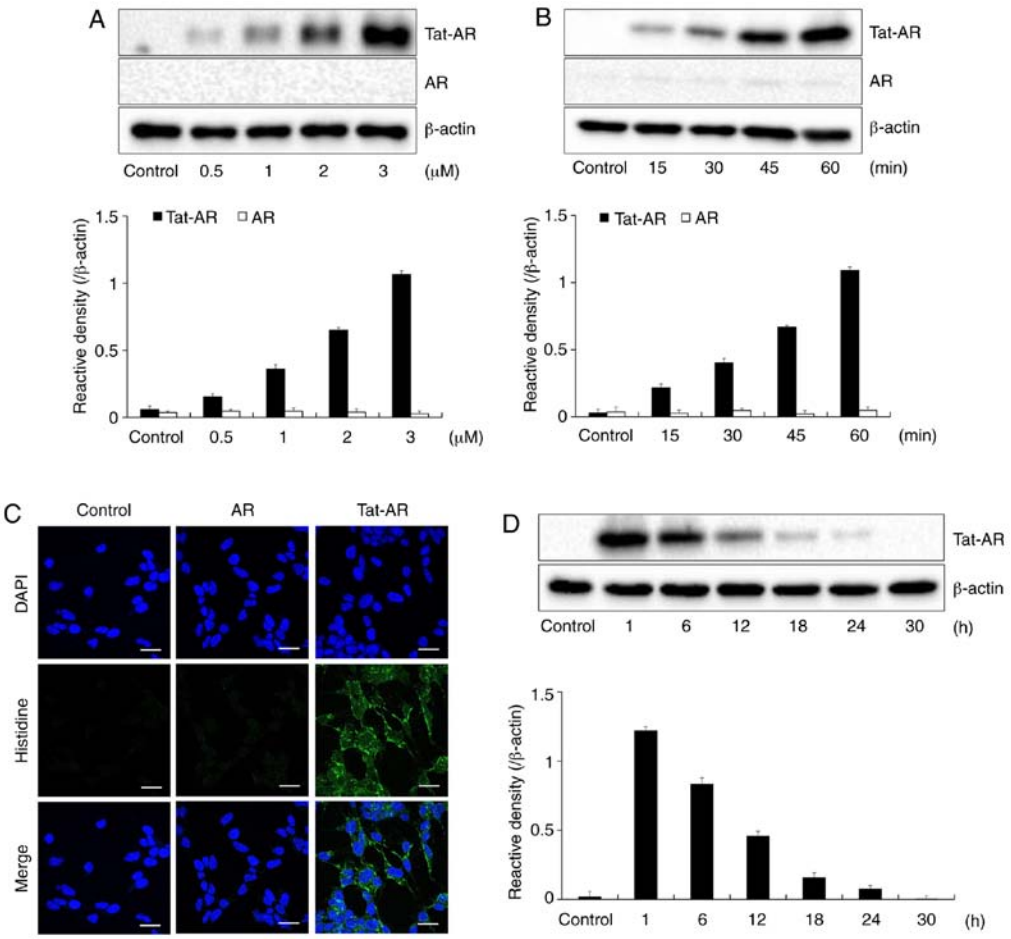

Figure 2. Transduction of Tat-AR proteins into SH-SY5Y cells. (A) The cell culture media were treated with Tat-AR protein at various concentrations (0.5-3 μM) and AR protein (0.5-3 μM) for 1 h. (B) The cell culture media were treated with Tat-AR protein (3 μM) and AR protein (3 μM) for different periods of time (15-60 min). (C) The localization of transduced Tat-AR protein was examined by confocal fluorescence microscopy. Scale bar, 20 μm. (D) Intracellular stability of transduced Tat-AR protein. SH-SY5Y cell culture media were incubated for 30 h following transduction of Tat-AR protein for 1 h. Transduction of Tat-AR protein was measured by western blot analysis and the intensity of the bands was measured by a densitometer. Tat-AR, Tat-aldose reductase.

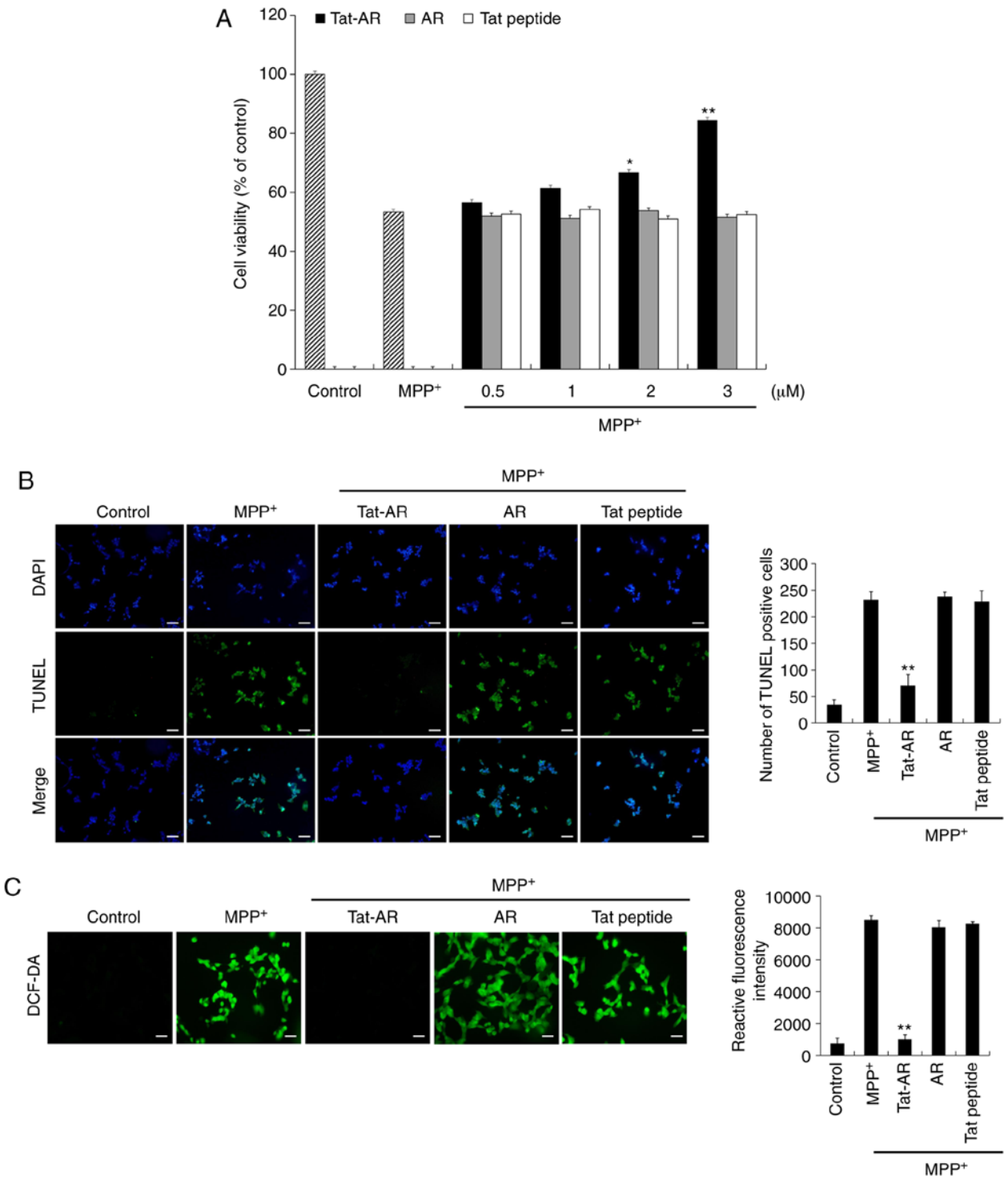

Figure 3. Effects of transduced Tat-AR protein against MPP+-induced SH-SY5Y cell death. The cells pretreated with Tat-AR protein (0.5-3 μM), AR protein (0.5-3 μM) and Tat peptide (0.5-3 μM) for 1 h and exposed to MPP+ (5 mM) for 13 h. (A) Cell viabilities were estimated using a colorimetric assay using MTT. (B and C) Effects of Tat-AR protein on MPP+-induced ROS production and DNA fragmentation. The cells were treated with Tat-AR (3 μM) for 1 h, and then exposed to MPP+ (5 mM). (B) DNA fragmentation was detected by TUNEL staining and the quantitative evaluation of TUNEL-positive cells was confirmed by cell counting under a phase-contrast microscopy (x200 magnification). (C) Intracellular ROS production levels were determined by DCF-DA staining. Fluorescence intensity was quantified using an ELISA plate reader. Scale bar, 20 μm. *P<0.05 and **P<0.01, compared with MPP+-exposed cells. Tat-AR, Tat-aldose reductase; MPP+, 1-methyl-4-phenylpyridinium.

Effects of Tat-AR protein against MPP+-induced SH-SY5Y cells. It is known that MPP+ can induce ROS production in dopaminergic neuronal cells, and can cause DNA damage and cell death (32). Thus, the protective effects of transduced Tat-AR protein against MPP+-induced cell death were examined. Cell viability was 53% in the cells exposed only to MPP+. However, cell viability was markedly increased up to 84% in the cells treated with Tat-AR protein following exposure to MPP+. By contrast, AR protein and Tat peptide failed to prevent cell death under the same experimental conditions (Fig. 3A).

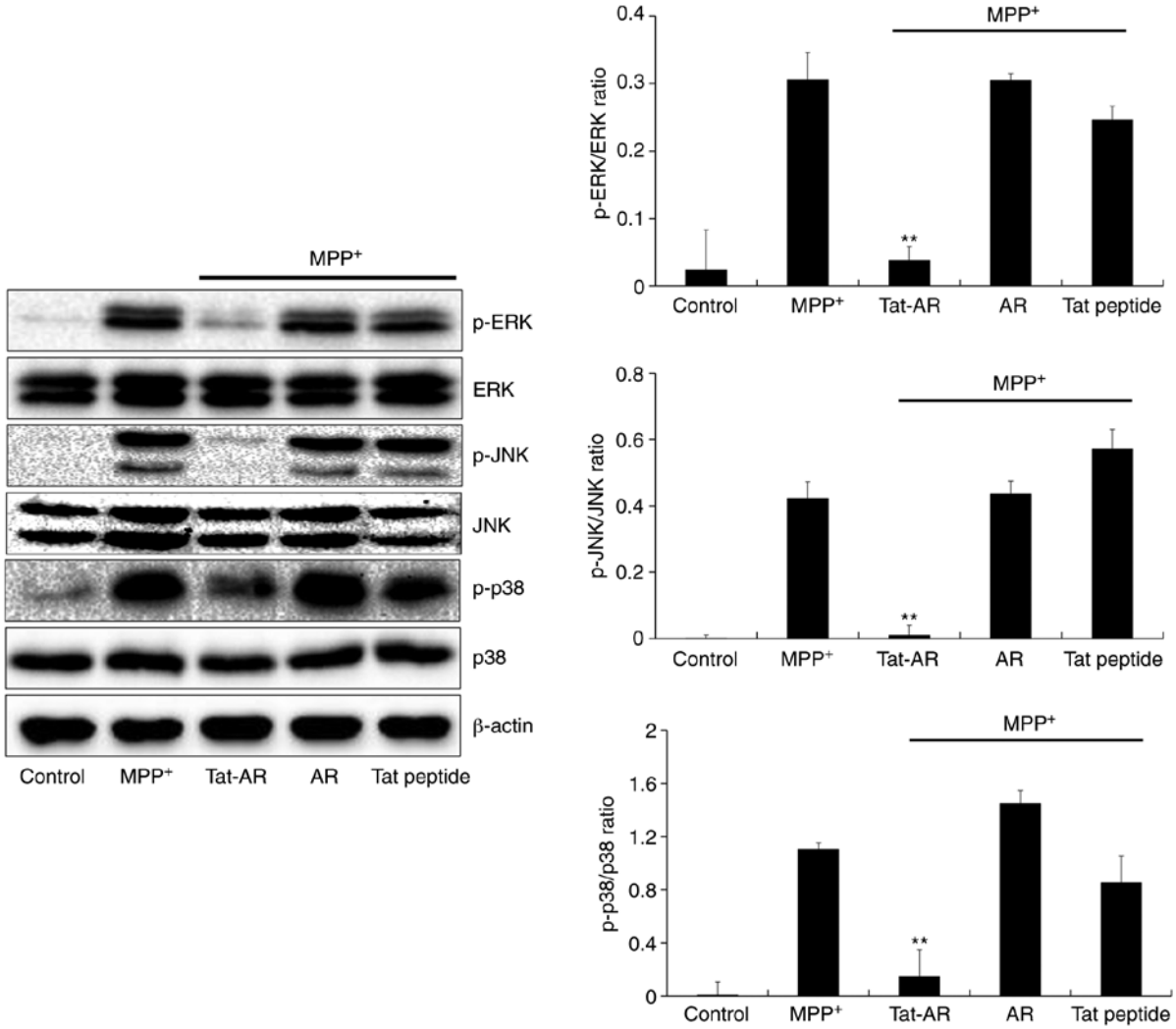

Figure 4. Effects of Tat-AR protein on MPP+-induced MAPK activation in SH-SY5Y cells. The cells were pre-treated with Tat-AR protein (3 μM), AR protein (3 μM) and Tat peptide (3 μM) for 1 h and stimulated with MPP+ (5 mM). The cells were then prepared and analyzed for the analysis of the phosphorylation of JNK, ERK, p38 levels by western blot analysis, and the band intensities were measured by a densitometer. **P<0.01, compared with MPP+-exposed cells. Tat-AR, Tat-aldose reductase; MPP+, 1-methyl-4-phenylpyridinium.

The protective effects of Tat-AR protein against MPP+-induced DNA damage and intracellular ROS production were also determined by TUNEL and DCF-DA staining, respectively (Fig. 3B and C). DNA damage and intracellular ROS production levels were increased in the cells exposed only to MPP+. However, DNA damage and intracellular ROS production levels were markedly inhibited in the Tat-AR protein-treated cells following MPP+ exposure. However, AR protein and Tat peptide failed to inhibit DNA damage and intracellular ROS production. These results indicate that Tat-AR protein can inhibit SH-SY5Y cell death by decreasing DNA damage and intracellular ROS production, functioning as an antioxidant in the cells.

Effects of Tat-AR protein on MPP+-induced signaling pathways. MPP+ can trigger mitogen-activated protein kinase (MAPK) signaling pathway activation (9,33,34). Thus, the effects of Tat-AR proteins on MPP+ induced MAPKs signaling pathways were determined in the present study. In the SH-SY5Y cells exposed to MPP+, the expression levels of phosphorylated MAPKs were higher than those in the control cells. By contrast, Tat-AR protein significantly decreased expression the levels of phosphorylated MAPKs in the cells exposed to MPP+. However, the expression levels of phosphorylated MAPKs in the cells treated with AR protein or Tat peptide were similar to those in the untreated cells exposed to MPP+ (Fig. 4).

It is well known that the neurotoxin, MPP+, can cause the overproduction of ROS in cells and activate apoptosis signaling pathways (32). Thus, effects of Tat-AR proteins on the MPP+-induced expression levels of Bax, Bcl-2 and caspase 3 were investigated. As shown in Fig. 5A, the Bcl-2 expression levels were decreased in the MPP+-exposed SH-SY5Y cells. By contrast, Tat-AR protein significantly increased the Bcl-2 expression levels in cells the exposed to MPP+. However, the expression levels of Bax exhibited opposite results from those of Bcl-2. Tat-AR protein also markedly increased the expression of caspase-3 in the MPP+-exposed cells. The cleaved caspase-3 expression levels were significantly decreased in the cells exposed only to MPP+. In addition, Tat-AR protein reduced the

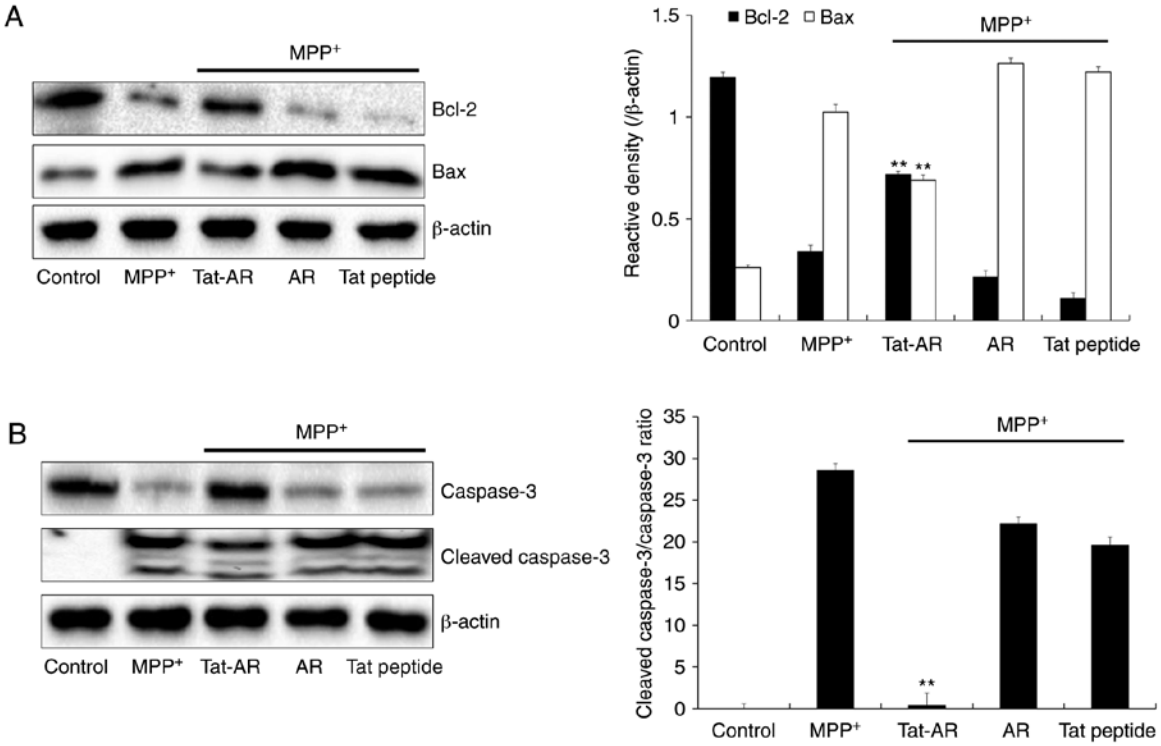

Figure 5. Effects of Tat-AR protein against MPP+-induced Bcl-2, Bax and caspase-3 expression in SH-SY5Y cells. The 1-h pre-treatment of SH-SY5Y cells with Tat-AR protein (3 μM), AR protein (3 μM) and Tat peptide (3 μM) was followed by exposure to MPP+ (5 mM). The expression levels of (A) Bcl-2 and Bax, and (B) caspase-3 and cleaved caspase-3 were determined by western blot analysis and the band intensity was measured by a densitometer. **P<0.01, compared with MPP+-exposed cells. Tat-AR, Tat-aldose reductase; MPP+, 1-methyl-4-phenylpyridinium.

cleaved Caspase-3/Caspase-3 ratio in MPP+ treated cells. AR protein and Tat peptide failed to affect the expression levels of caspase-3 and cleaved caspase-3 proteins induced by MPP+ (Fig. 5B). These results indicate that transduced Tat-AR protein can prevent SH-SY5Y cell death from MPP+ by regulating phosphorylation levels of MAPKs and apoptosis-related protein expression.

Protective effects of Tat-AR protein against MPTP-induced cell death in an animal model of PD. After Tat-AR protein (2 mg/kg) was i.p. injected into the mice, immunohistochemistry was performed using histidine antibody to determine whether Tat-AR protein was transduced into the mouse brains (Fig. 6A). Tat-AR protein was highly expressed in the SN region of the midbrain. However, AR protein was not detected in the SN region of the midbrain. These results indicate that Tat-AR protein can be transduced into mouse brains by crossing the blood-brain barrier (BBB).

Dopaminergic neuronal cell death is major indicator of PD. Thus, the protective effects of Tat-AR protein against MPTP-induced dopaminergic neuronal cell death were determined by immunohistochemistry using a TH antibody and cresyl violet staining. As shown in Fig. 6B and C, the TH-positive cell numbers were increased in the SN of the Tat-AR protein-treated group. In addition, neuronal cell survival was markedly increased in the Tat-AR protein-treated group. By contrast, the AR protein- and Tat peptide-treated groups did not exhibit such protective effects. These results indicate that Tat-AR protein can markedly prevent dopaminergic neuronal cell death.

Discussion

PD is a progressive neurodegenerative disorder, characterized by the loss of dopamine neurons in the SN pars compacta and the presence of Lewy bodies in the brain (1-3). Since oxidative stress is highly associated with the pathogenesis of various diseases, including PD, the regulation of oxidative stress is crucial to preventing these diseases (5-7,35-39). The definitive cause of dopamine neuron cell death in PD and precise etiological mechanism remain unclear.

AR can reduce levels of toxic aldehydes, including 4HNE. It can increase cell survival by decreasing oxidative stress and toxic aldehydes (11-15). Although several studies have suggested that AR plays a crucial role in cellular responses to oxidative stress by detoxifying ROS and decreasing reactive aldehydes in keratinocytes (40) and by neutralizing the toxicity of lipid peroxidation in arterial wall injuries (41), the precise protective role of AR in dopaminergic cells against oxidative stress remains poorly understood. Therefore, the present study investigated whether Tat-AR protein can protect SH-SY5Y cells against MPP+-induced dopaminergic neuronal cell death whether it can protect animals against MPTP-induced PD. The results revealed that purified Tat-AR protein could be transduced into SH-SY5Y cells in a time- and dose-dependent manner, and that such a transduced protein could significantly enhance cell survival and inhibit DNA damage in MPP+-exposed SH-SY5Y cells.

It is well known that neurotoxin MPP+ can induce oxidative stress and lead to dopaminergic neuronal death via mitochondrial damage (9,10,32). Other studies have demonstrated that

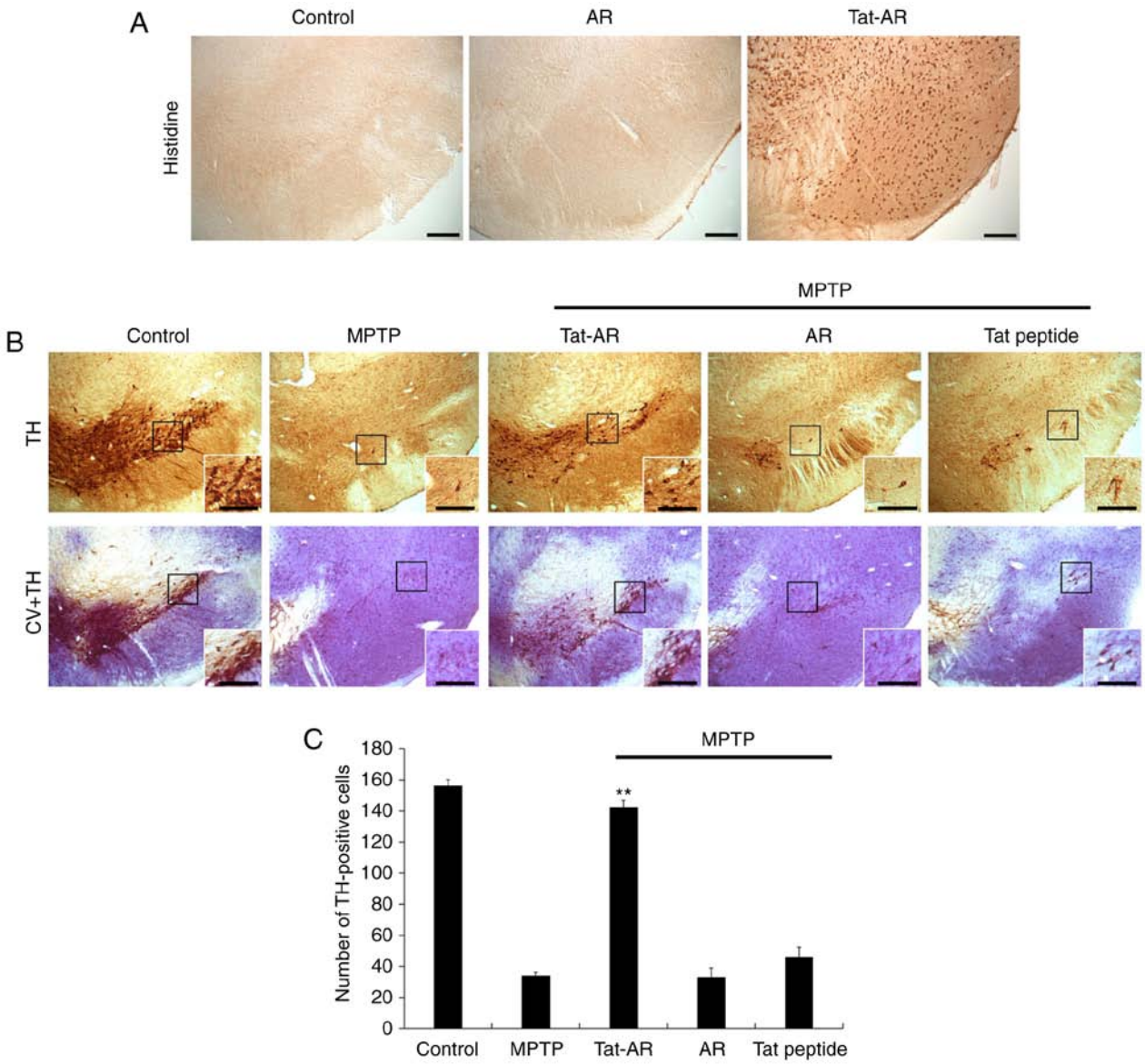

Figure 6. Transduced Tat-AR protein inhibits dopaminergic neuronal cell death in an animal model of PD. Transduction of Tat-AR protein into the SN. Tat-AR protein (2 mg/kg) was injected i.p. into mice, followed by collecting the brains 12 h later. (A) Brain tissues were immunostained with an anti-histidine antibody. Scale bars, 100 μm. (B) Protective effects of transduced Tat-AR protein in the animal model of PD. Tat-AR protein (2 mg/kg) was injected i.p. into mice, followed by collecting the brains 1 weeks. Brain sections showing TH immunoreactivity and double staining with cresyl violet (CV) and TH immunoreactivity. Scale bars, 100 and 50 μm. (C) Number of TH-positive neurons. Quantification of the number of positive dopaminergic neurons in 250x250 μm^2 is shown in the graph. $^{**}P<0.01$, statistically significant difference between MPTP and other groups. Tat-AR, Tat-aldose reductase; PD, Parkinson's disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TH, tyrosine hydroxylase.

the overexpression of AR protein can protect cells against methylglyoxal (MG)-induced aortic smooth muscle cell damage (42) and prevent aldehyde-induced human lens epithelial cell death (18). AR is also involved in cell survival as a detoxification enzyme (43). The results of the present study demonstrated the same protective pattern. Further studies are required to confirm such results.

MPP^+ can trigger cellular signaling pathways, including MAPKs and apoptosis signaling pathways (32). Thus, the effects of Tat-AR protein on MAPK signaling pathways, and the expression levels of Bax, Bcl-2 and caspase-3 were determined in the present study. In the MPP^+-exposed SH-SY5Y cells, Tat-AR protein markedly inhibited Bax and cleaved caspase-3 expression levels, but significantly increased the Bcl-2 expression levels. Other studies have also demonstrated that the overexpression of AR can increase the Bcl-2 level, but can decrease Bax expression levels and the phosphorylation level of JNK and p38 in aldehyde-induced human lens epithelial cells (18).

In addition, AR can reduce ultraviolet-B (UVB)-induced JNK and p38 phosphorylation in HaCaT cells (40). The phosphorylation levels of EKR1/2 have been shown to be significantly increased in MPTP-exposed $AR^{-/-}$ mouse brains (27). These reports suggest that dopaminergic neuronal loss is associated with the phosphorylation of EKR1/2 and that AR plays an important role in protecting dopaminergic neurons in PD (27,40). The results of the present study also demonstrated that Tat-AR protein reduced the activation of MAPKs in MPP^+-exposed SH-SY5Y cells, suggesting that Tat-AR protein plays a protective role against MPP^+-induced

dopaminergic neuronal cell death via the modulation of MAPKs and apoptosis signaling pathways.

The MPTP-induced mouse model of PD has been generally used to examine the pathological mechanisms of PD as this model exhibits a similar pathophysiology to human PD (44,45). In animal models of MPTP-induced PD, Tat-AR protein was shown to significantly protect dopaminergic neuronal cell death in the present study. Goldstein *et al* previously demonstrated that the overexpression of AR protein significantly reduced cell death in patients with PD (8). In addition, a number of studies have reported that the levels of toxic aldehyde DOPAL are markedly increased in patients with PD and that AR can decrease DOPAL levels (5,6,8), suggesting that AR plays a pivotal role in detoxifying DOPAL in PD (14,15,46). A recent study demonstrated that AR deficiency in MPTP-exposed mice led to increases in the characteristics of PD and that AR protected dopaminergic neurons against neurotoxic metabolites (27).

In conclusion, the presents study demonstrated that Tat-AR protein could be transduced into SH-SY5Y cells and SN in mouse brains, and that Tat-AR protein could significantly protect against MPP^+- and MPTP-induced dopaminergic neuronal cell death both *in vitro* and *in vivo*. Although further studies are required to elucidate the precise protective mechanisms, the present results suggest that Tat-AR protein may be useful as a therapeutic agent for PD.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SBC, WSE, MJS, DWK and SYC were involved in the conceptualization of the study. SBC, WSE, MJS, HJK and DSK were involved in the study methodology. HJY, EJY and YJC were involved in data validation. SWC, JP, KHH, KWL, JKP and SYC were involved in data curation. DWK and SYC were involved in the writing and editing of the manuscript and provided final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The procedures for the care of animals conformed to the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research and Quarantine Service of Korea and approved by the Institutional Animal Care and Use Committee of Soonchunhyang University (SCH16-0051).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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