

Transduced Tat-aldose Reductase Protects Hippocampal Neuronal Cells against Oxidative Stress-induced Damage

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Aldose reductase (AR) protein, a member of the NADPH-dependent aldo-keto reductase family, reduces a wide range of aldehydes and enhances cell survival by inhibition of oxidative stress. Oxidative stress is known as one of the major pathological factor in ischemia. Since the precise function of AR protein in ischemic injury is fully unclear, we examined the function of AR protein in hippocampal neuronal (HT-22) cells and in an animal model of ischemia in this study. Cell permeable Tat-AR protein was produced by fusion of protein transduction domain in Tat for delivery into the cells. Tat-AR protein transduced into HT-22 cells and significantly inhibited cell death and regulated the mitogen-activate protein kinases (MAPKs), Bcl-2, Bax, and Caspase-3 under oxidative stress condition. In an ischemic animal model, Tat-AR protein transduced into the brain tissues through the blood-brain barrier (BBB) and drastically decreased neuronal cell death in hippocampal CA1 region. These results indicate that transduced Tat-AR protein has protective effects against oxidative stress-induced neuronal cell death *in vitro* and *in vivo*, suggesting that Tat-AR protein could be used as potential therapeutic agent in ischemic injury.

Key words: Tat-AR, Oxidative stress, Ischemia, MAPKs, Cytotoxicity, Protein therapy

INTRODUCTION

Cerebral ischemia is a neuronal disease that occurs when the blockage of blood flow to the brain causes reperfusion injury and insufficient amount of oxygen induced by oxidative stress leads to neuronal cell death [1, 2]. It is well known that the high levels

of oxidative stress critically increase neuronal cell death, whereas inhibition of oxidative stress by overexpression of antioxidant proteins reduced neuronal cell death [3]. Many studies have shown that high levels of oxidative stress destroy intracellular macromolecules including DNA and proteins and finally leads to cell death [4, 5]. Conversely, natural products and proteins such as curcumin and caveolin-1/-3, which have antioxidant functions, have been shown to inhibit cell death including ischemic insults via the inhibition of oxidative stress [6, 7].

Aldose reductase (AR; EC 1.1.1.21), a member of the NADPH-dependent aldo-keto reductase family, reduces aldehydes detoxification during lipid peroxidation and several studies have demonstrated that AR plays protective roles as an antioxidant protein against reactive oxygen species (ROS) [8-10]. Thus this protein

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detoxifies ROS induced lipid aldehyde materials and protects against cell death via the inhibition of oxidative stress in a variety of cells such as SH-SY5Y cells [10]. Other studies also showed that AR protein enhanced cell survival by inhibiting cell toxicities in smooth muscle and lens epithelial cells [11].

Protein transduction domains (PTDs) carries not only proteins and peptides but also antisense, plasmids, microbeads, liposomes, and other molecules into cells without any special receptor [12]. Since PTD-fused proteins can pass through the membrane and enter into the inner part of cell, cell permeable PTD like Tat-peptide can be useful tools in protein therapy when it fused with target protein [13, 14]. We have demonstrated that various PTD fusion proteins showed protective effect against cell damage *in vitro* and *in vivo* [15-22].

In this study, we fused AR with Tat PTD to transduce into cells and examined whether this Tat-AR fusion protein protects against oxidative stress-induced hippocampal HT-22 cell death and in an ischemic animal models.

MATERIALS AND METHODS

HT-22 cell culture and materials

Mouse hippocampal HT-22 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics (100 µg/ml streptomycin, 100 µg/ml penicillin) at 37°C in a humidity chamber with 5% CO₂ and 95% air.

Ni²⁺-nitrilotriacetic acid Sepharose Superflow was purchased from Qiagen (Valencia, CA, USA). PD-10 columns were purchased from Amersham (Braunschweig, Germany). The indicated primary and β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). An enzyme-linked immunosorbent assay (ELISA) kit for hexa histidine was obtained from Cloud-Clone Corp. (Houston, TX, USA). Unless otherwise stated, all other agents were of the highest grade available.

Purification and transduction of Tat-AR protein into HT-22 cells

Preparation of the Tat expression vector has been described in a previous study [15]. Human AR was amplified by PCR with two primers. The sense primer 5'-CTCGAGGCAAGCCGTCCT-3' contained an *Xho*I restriction site.

The antisense primer 5'-GGATCCTCAAACTCTTCAT-GGAAGG-3' contained a *Bam*HI restriction site. The resulting PCR products were ligated into a TA vector and digested with *Xho*I and *Bam*HI restriction enzyme. Fragments were then ligated into the Tat expression vector to generate Tat-AR. Also, AR was

prepared without the Tat peptide as a control. Recombinant Tat-AR plasmid was transformed into *Escherichia coli* (Rosetta) and cultured in 0.5 mM isopropyl-β-D-thiogalactoside (IPTG; Duchefa, Haarlem, the Netherlands) at 18°C for 24 h. Harvested cells were lysed by sonication and Tat-AR protein was purified using a Ni²⁺-nitrilotriacetic acid Sepharose affinity column and PD-10 column chromatography. Bovine serum albumin was used as a standard and purified Tat-AR protein concentration was measured by Bradford assay [23].

To examine whether Tat-AR protein transduced efficiently in a time and concentration dependent, HT-22 cells were exposed to different concentrations (0.5~5 µM) of Tat-AR and AR protein for 1 h. HT-22 cells were exposed 5 µM of Tat-AR and AR protein for various time periods (10~60 min). Cells were then washed with trypsin-EDTA and washed twice with PBS. The amounts of transduced proteins were measured by Western blotting. We also determined the intracellular stability of Tat-AR protein by culturing the cells (1~36 h) after transduction. Then transduced levels were measured by Western blotting using an anti-histidine antibody.

Western blot analysis

Equal amounts of proteins were loaded into 12% SDS-PAGE and electrotransferred to a polyvinylidene difluoride (PVDF) membrane. 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. After being washed with TBST, the membrane was incubated with the indicated primary and appropriate secondary antibodies recommended by the manufacturer. Then the membranes were washed with TBST buffer three times and the protein bands were identified using chemiluminescent reagents as recommended by the manufacturer (Amersham, Franklin Lakes, NJ, USA) [16].

Measurement of transduced Tat-AR protein levels

HT-22 cells (1×10⁶) were pretreated with Tat-AR proteins and AR (0.5~5 µM for 1 h or 5 µM for 10~60 min). Cells were then washed with PBS and treated with trypsin-EDTA. Transduced Tat-AR protein levels were analyzed using an ELISA kit for hexa histidine (Cloud-Clone Corp.) according to the manufacturer's instruction.

Confocal fluorescence microscopy analysis

To determine the intracellular distribution of transduced Tat-AR protein in HT-22 cells, we performed confocal fluorescence microscopy as described previously [16, 17]. HT-22 cells were placed on coverslips and treated with 5 µM of Tat-AR protein for 1 h. The cells were washed with PBS twice and fixed with 4% para-

formaldehyde for 5 min. The cells were treated in PBS containing 3% bovine serum albumin and 0.1% Triton X-100 (PBS-BT) at room temperature for 30 min and washed with PBS-BT. The histidine primary antibody was diluted 1:1500 and incubated at room temperature for 3 h. The Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) was diluted 1:1500 and incubated in the dark for 1 h. Nuclei were stained with 1 $\mu\text{g}/\text{ml}$ DAPI (Roche Applied Science, Mannheim, Germany) for 2 min. Then stained cells were analyzed by confocal fluorescence microscopy using a confocal laser-scanning system (Bio-Rad MRC-1024ES, 4BIOROD, CA, USA).

Cell viability assay

Cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [19, 24]. HT-22 cells were seeded on a 96-well plate and treated with Tat-AR or AR protein for 1 h. Then the cells were incubated with 1 mM hydrogen peroxide (H_2O_2) for 8 h. The absorbance was determined at 540 nm using a microplate reader (Infinite 200 nanoquant, TECAN, Switzerland) and cell viability was defined as the percentage of untreated control cells.

DNA fragmentation staining

To examine whether transduced Tat-AR proteins protect against H_2O_2 -induced DNA damage in cells, HT-22 cells were pretreated with 5 μM Tat-AR protein for 1 h and exposed to 1 mM H_2O_2 for 6 h. Terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling (TUNEL) staining was performed using a Cell Death Detection kit (Roche Applied Science). Each fluorescent image was obtained using a fluorescence microscope (Nikon eclipse 80i, Tokyo, Japan). Fluorescence positive cells were counted

under a phase-contrast microscopy ($\times 200$ magnification) [16, 17].

Experimental animals and treatment

Male gerbils (65~75 g, 6 months) obtained from the Hallym University Experimental Animal Center, were housed at a temperature of 23°C, with humidity of 60%, and exposed to 12 hour periods of light and dark with 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 Institutional Animal Care and Use Committee of Soonchunhyang University [SCH16-0009].

The transient forebrain ischemia model was performed as described previously [16, 17]. 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-AR protein against ischemic damage, the animals were divided into 4 groups (each $n=10$): control sham group, vehicle-treated group, AR-treated group, and Tat-AR-treated groups. The AR and Tat-AR proteins (2 mg/kg) were administered intraperitoneally 30 min before ischemia-reperfusion.

Immunohistochemistry

Immunohistochemistry was performed as described in previous studies [16-18]. Brain tissue samples were obtained at 7 days after ischemia-reperfusion. To examine the protective effects of trans-

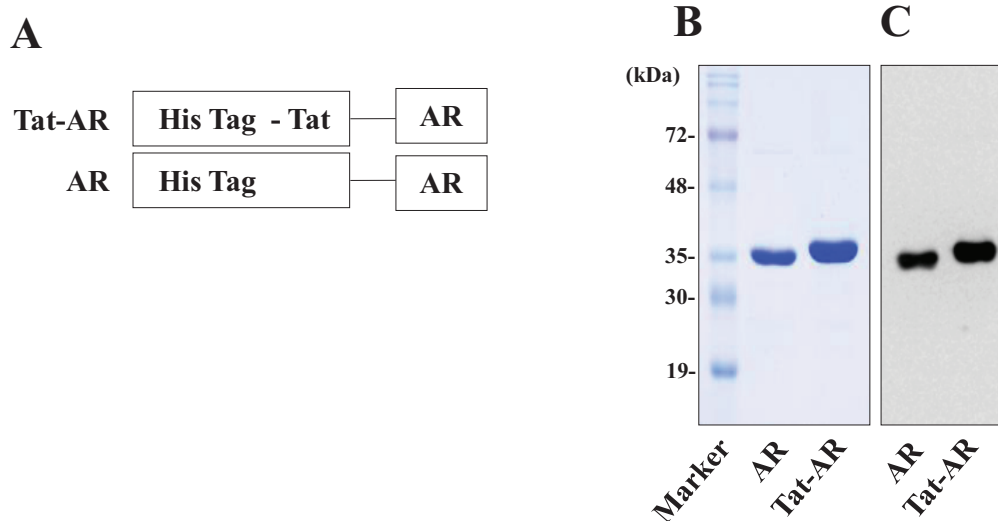


Fig. 1. Construction and purification of Tat-AR protein. Constructed map of Tat-AR based on the pET15b vector and diagrams of the expressed Tat-AR proteins (A). Purified recombinant Tat-AR and AR proteins were identified by 15% SDS-PAGE (B) and detected by Western blot analysis using an anti-histidine antibody (C).

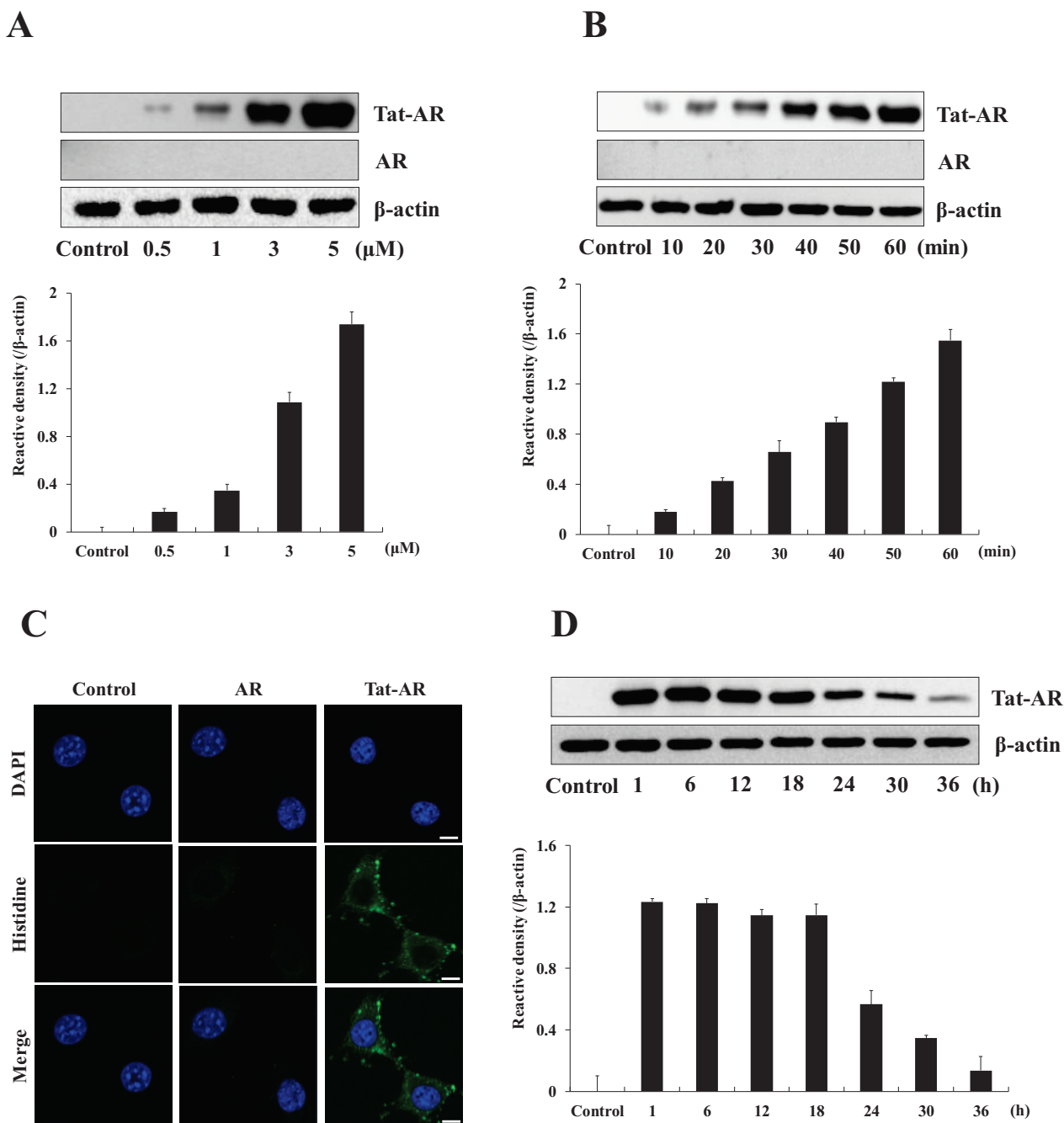


Fig. 2. Transduction of Tat-AR proteins into HT-22 cells. HT-22 cell culture media were treated with Tat-AR protein at different doses (0.5~5 μM) or the AR protein for 1 h (A). The cell culture media were treated with Tat-AR protein (5 μM) or AR protein for different time periods (10~60 min) (B). Then, transduction of Tat-AR protein was measured by Western blotting and the intensity of the bands was measured by a densitometer. The localization of transduced Tat-AR protein was examined by confocal fluorescence microscopy (C). Scale bar=5 μm. Intracellular stability of transduced Tat-AR protein. HT-22 cell culture media were incubated for 36 h after transduction of Tat-AR protein for 1 h (D). Transduction of Tat-AR protein was measured by Western blotting and the intensity of the bands was measured by a densitometer. Quantitative analysis of transduced Tat-AR protein level in HT-22 cells. After HT-22 cells were treated with Tat-AR protein, transduced Tat-AR protein level determined using an ELISA kit (E). Data are repressed as mean±SEM (n=3).

duced Tat-AR protein against ischemic damage, the sections were incubated in 10% normal goat serum in PBS for 30 min and the sections were stained with a histidine antibody, Cresyl violet (CV),

Fluoro-Jade B (FJB), ionized calcium-binding adapter molecule 1 (Iba-1) and neuronal nuclei (NeuN). The positive neuronal cell number and intensity of immunore-

E

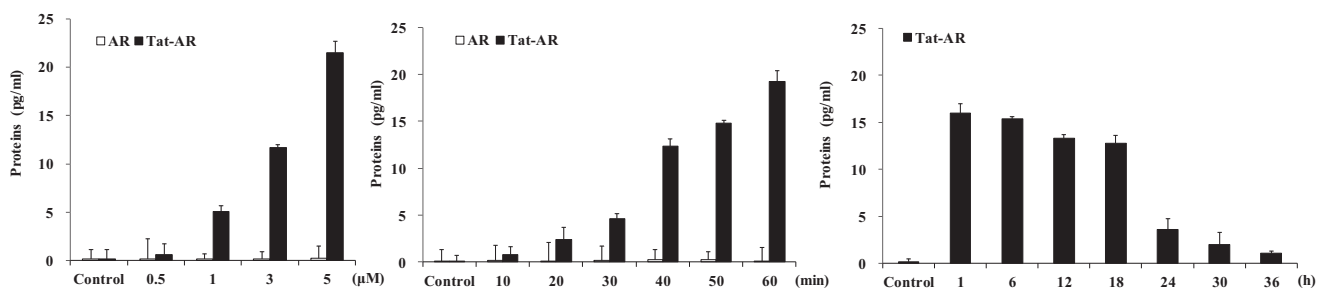
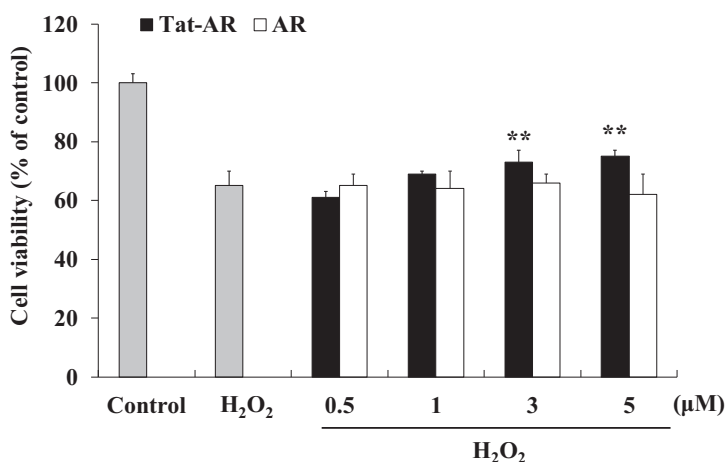


Fig. 2. Continued.

A



B

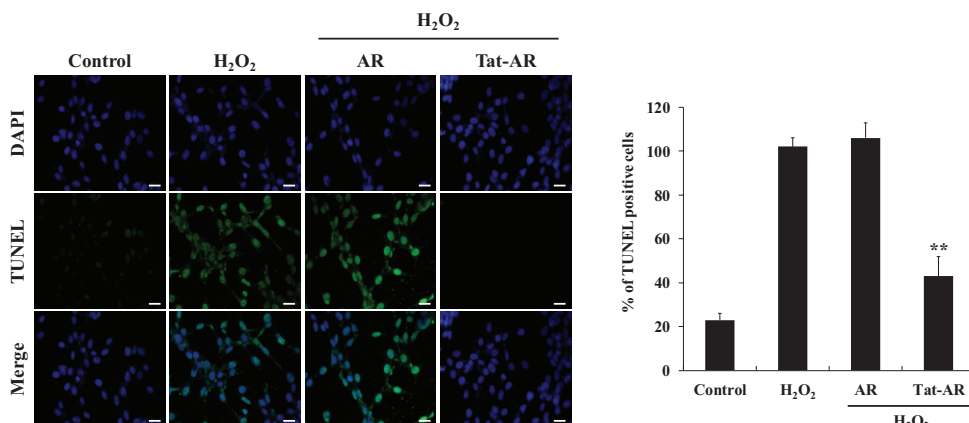


Fig. 3. Effects of transduced Tat-AR protein on cell viability in response to oxidative stress. Effect of transduced Tat-AR protein on cell viability. HT-22 cells were pretreated with Tat-AR protein (0.5~5 μM) for 1 h and exposed to H₂O₂ (1 mM) for 8 h. Cell viabilities were estimated using a colorimetric assay using MTT (A). Effect of transduced Tat-AR protein on DNA damage. The cells were treated with Tat-AR (5 μM) for 1 h, and then exposed to H₂O₂ (1 mM) for 3 h. Then, DNA fragmentation was detected by TUNEL staining and quantitative evaluation of TUNEL positive cells confirmed by cell counting under a phase-contrast microscopy (×200 magnification) (B). Scale bar=20 μm. **p<0.01 compared with H₂O₂-treated cells. Data are repressed as mean±SEM (n=3).

activity were calculated using an image analyzing system equipped with a computer based CCD camera (software: Optimas 6.5, CyberMetrics, USA). The staining intensity of the immunoreactive

structures was evaluated as the relative optical density (ROD). A ratio of the ROD was calibrated as % [16, 17].

Statistical analysis

Data are expressed as the mean±SEM of three experiments. Differences between groups were analyzed by ANOVA followed by a Bonferroni's post-hoc test. Statistical significance was considered at p<0.05.

RESULTS

Purification and transduction of Tat-AR protein into HT-22 cells

A human AR gene was fused with a Tat PTD to produce cell-permeable Tat-AR protein. Also, we constructed an AR protein expression vector as a control (Fig. 1A). As shown in Fig. 1B and 1C, Tat-AR and AR proteins were purified and confirmed by SDS-PAGE and Western blotting. Purified Tat-AR and AR proteins showed to the expected molecular weights of approximately 37 and 36 kDa, respectively.

To investigate whether Tat-AR and AR protein possesses the capacity to transduce into HT-22 cells, the cells were treated with various concentrations of Tat-AR proteins (0.5~5 μM) protein for 1 h or various time periods (10~60 min) of Tat-AR proteins (5 μM). Then, cells were washed with PBS and cell lysates were

analyzed by Western blotting. Tat-AR proteins were detected in the cell lysates from transduced HT-22 cells both a concentration- and time-dependently (Fig. 2A and 2B). Also, we determined the distribution of transduced proteins in HT-22 cells using Alexa Fluor 488 and DAPI immunostaining. As expected, the transduced Tat-AR proteins were detected in the cytoplasm and nucleus of the cells. In contrast, AR protein was not transduced under the same conditions (Fig. 2C). Furthermore, we assessed the stability of transduced Tat-AR protein. HT-22 cells were incubated with different time periods (1~36 h) after transduction of Tat-AR protein to persist in the cells. Tat-AR protein was observed in the cells up to 36 h (Fig. 2D). We also examined the transduced Tat-AR protein levels using an ELISA kit. As shown in Fig. 2E, transduced Tat-AR protein levels were increased concentration- and time-dependently.

Effects of Tat-AR protein against H₂O₂-induced HT-22 cell damages

The protective effects of Tat-AR protein against H₂O₂-induced HT-22 cell damages were determined, as shown in Fig. 3. To examine the effect of Tat-AR protein on cell viability, we performed an MTT assay. Cell viability was about 60% in the cells treated with

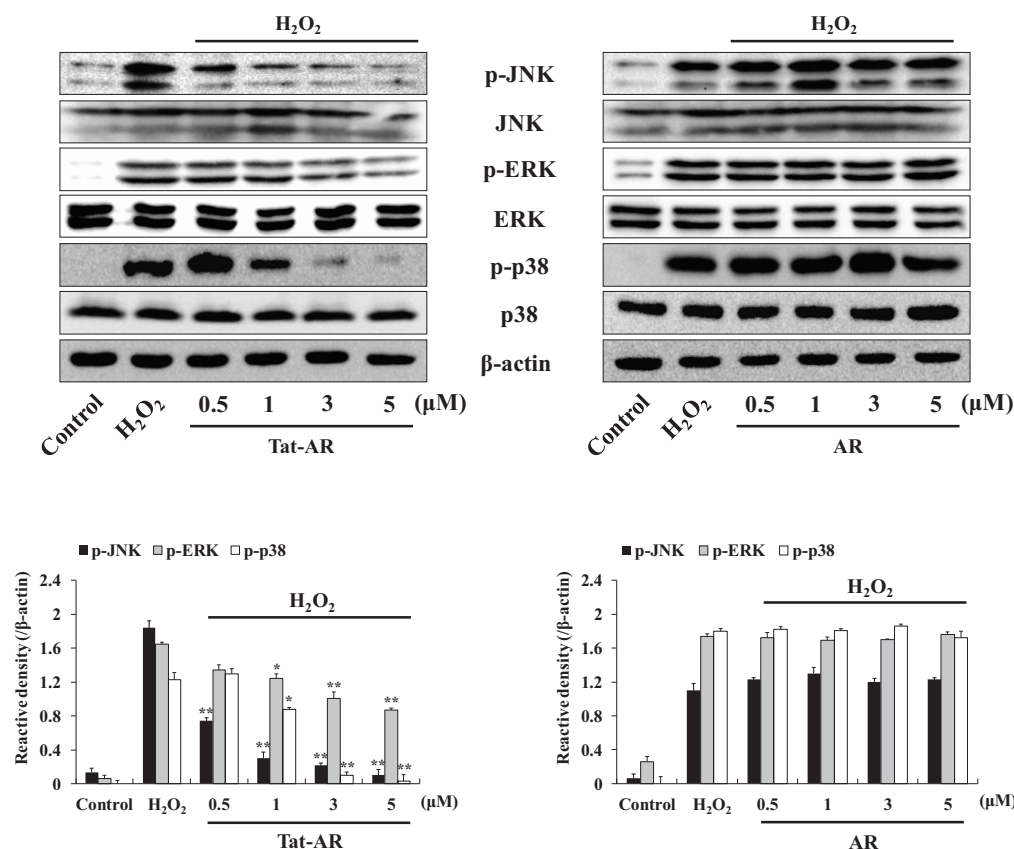


Fig. 4. Effects of Tat-AR protein on H₂O₂-induced MAPK activation in HT-22 cells. The cells were stimulated with H₂O₂ (1 mM) for 30 min with or without being pretreated with Tat-AR protein (0.5~5 μM) for 1 h. Then, the cells were prepared and analyzed for phosphorylation of JNK, ERK, p38 levels by Western blotting and the band intensities were measured by densitometer. *p<0.05 and **p<0.01 compared with H₂O₂-treated cells. Data are repressed as mean±SEM (n=3).

only H₂O₂ (1 mM, 8 h), whereas Tat-AR protein increased cell viability in an accordance with Tat-AR concentration up to 75% (Fig. 3A).

To determine whether Tat-AR protein inhibits DNA fragmentation, TUNEL staining was performed. DNA fragmentation significantly increased in the cells treated with only H₂O₂ (1 mM, 3 h)

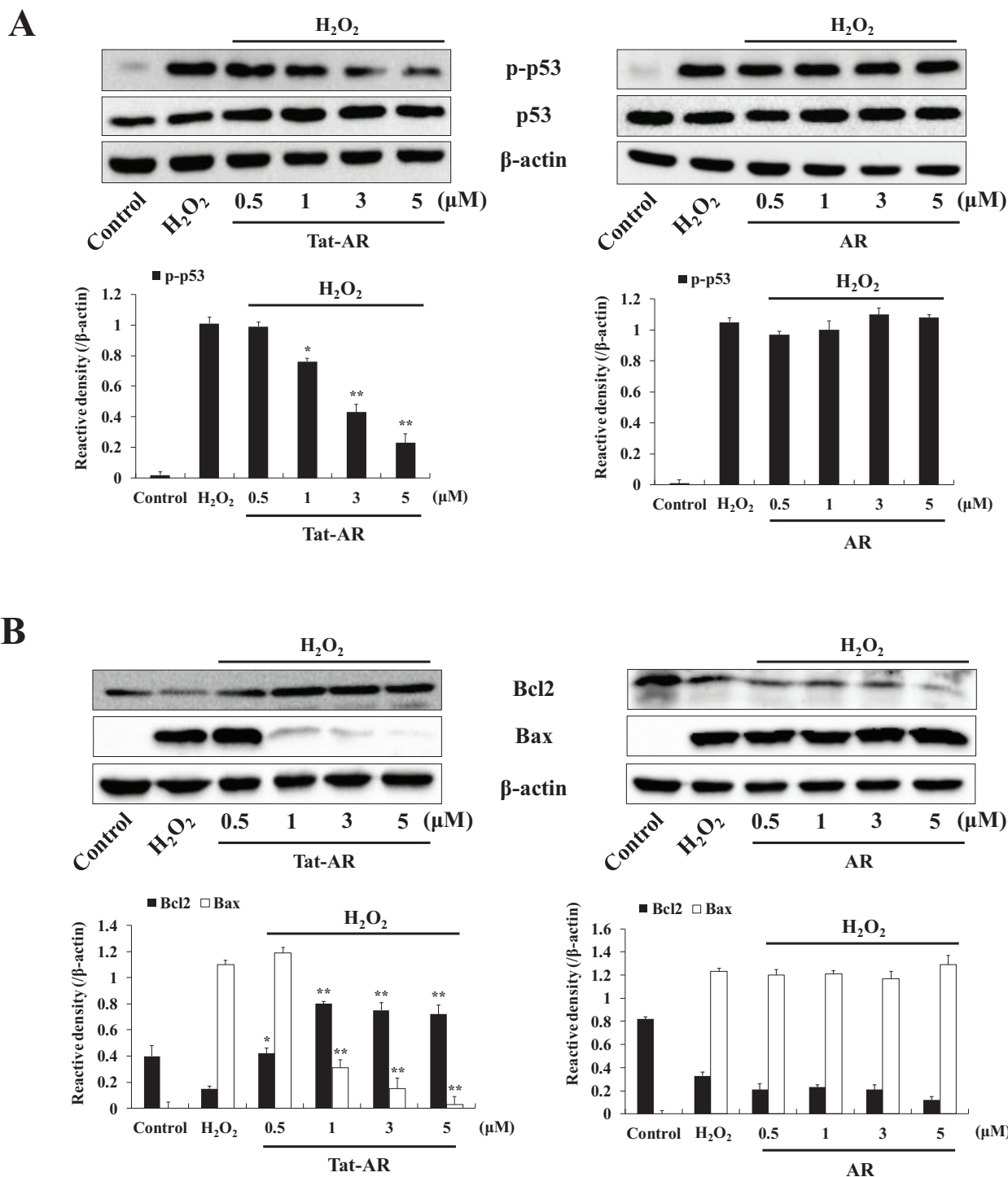


Fig. 5. Effect of Tat-AR protein against H₂O₂-induced p53, Bcl-2, Bax, and Caspase-3 expression in HT-22 cells. One-hour pretreatment of HT-22 cells with Tat-AR protein (0.5~5 μM) or AR protein was followed with treatment with H₂O₂ (1 mM) for 20 min (p53), 90 min (Bcl-2), 120 min (Bax), and 6 h (Caspase-3), respectively. The expression levels of p53 (A), Bcl-2 and Bax (B), and Caspase-3 (C) were determined by Western blot analysis and the band intensity was measured by densitometer. *p < 0.05 and **p < 0.01 compared with H₂O₂-treated cells. Data are repressed as mean ± SEM (n=3).

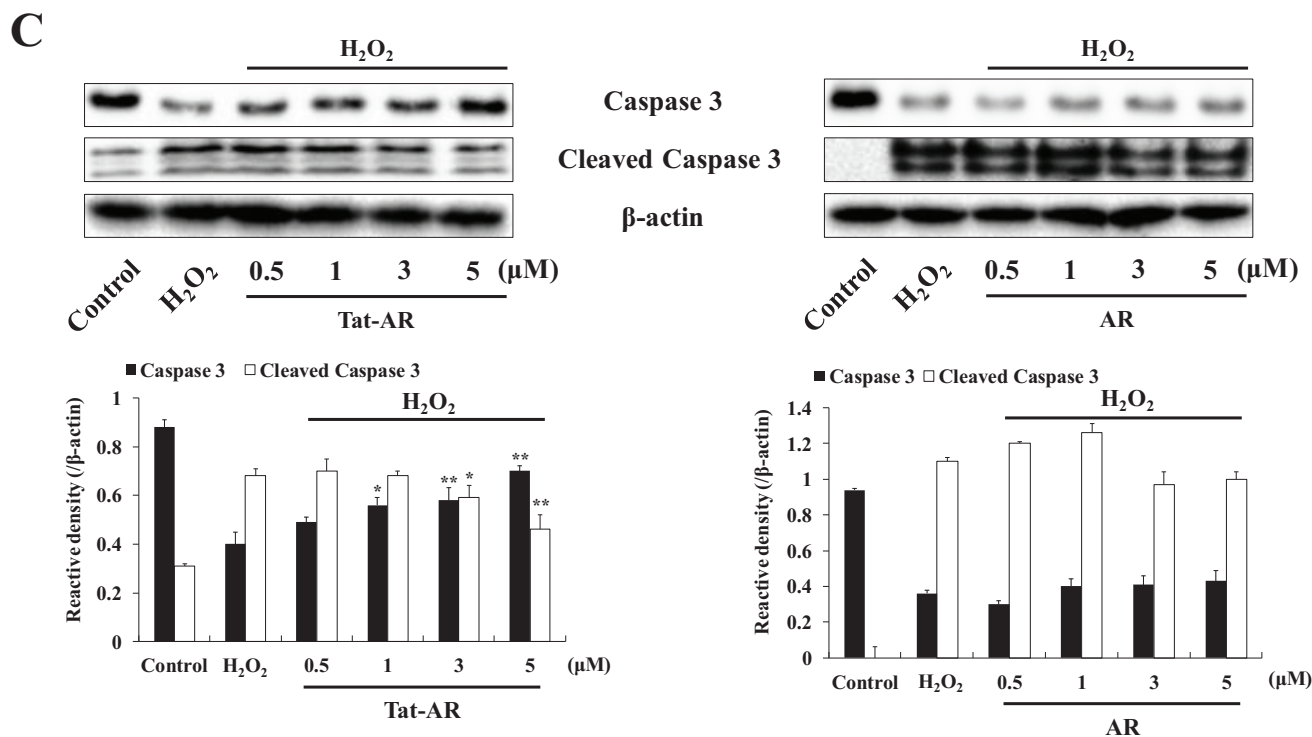


Fig. 5. Continued.

treatment cells. Tat-AR protein markedly inhibited DNA fragmentation (Fig. 3B).

Effects of Tat-AR proteins on H₂O₂-induced signaling pathways in HT-22 cells

The mitogen-activated protein kinases (MAPKs) signaling pathways, such as extracellular signal regulating kinase 1/2 (ERK1/2 or p44/42), c-Jun N-terminal kinase (JNK), and p38, are highly associated with ROS and finally lead to cell death [25]. We found that Tat-AR protein markedly and concentration-dependently reduced the expression of MAPKs phosphorylation in cells treated with H₂O₂ (1 mM). However, AR protein displayed no change in expression levels compared to the cells treated with H₂O₂ alone (Fig. 4).

Next, we examined whether Tat-AR protein inhibits H₂O₂-induced apoptosis because H₂O₂ is known to induce apoptosis. Phosphorylation p53 expression levels cause apoptosis [26, 27]. As shown in Fig. 5A, Tat-AR protein markedly inhibited the phosphorylated p53 expression concentration-dependently compared to the only H₂O₂ treated cells. Bax, Bcl-2, and Caspase-3 protein expression are associated with the oxidative stress induced apoptotic processes [28-30]. We showed that Tat-AR protein concentration-dependently elevated the Bcl-2 expression in the H₂O₂ treated cells, whereas the expression of Bax showed the op-

posite effect compared to the Bcl-2 (Fig. 5B). Further, we showed that Caspase-3 expression reduced in the H₂O₂ only treated cells. However, Tat-AR protein drastically increased the Caspase-3 expression and cleaved Caspase-3 expression showed the opposite effect compared to Caspase-3 (Fig. 5C). However, AR protein did not show the same changes of expression of apoptotic related proteins under the same experimental conditions.

Protective effect of Tat-AR protein on ischemic injury

To investigate the protective effects of transduced Tat-AR protein on ischemic injury in an animal model, we performed immunohistochemistry using a histidine antibody and NeuN staining. NeuN is known as a marker for neurons generally used to detect neurons. As shown in Fig. 6A, we observed that Tat-AR protein transduced into the hippocampal CA1 region, crossing the blood-brain barrier (BBB), where it markedly protected against neuronal cell death compared to the vehicle- and AR protein treated groups. We also showed that transduced Tat-AR protein significantly increased hippocampal neuronal cell survival. However, the AR protein treated group showed a similar pattern compared with vehicle treated group. To investigate the endogenous levels of AR protein in gerbil brain, gerbils treated with Tat-AR protein were sacrificed and obtained brain tissue. Then, endogenous AR protein levels were confirmed by Western blotting with AR antibody. As shown

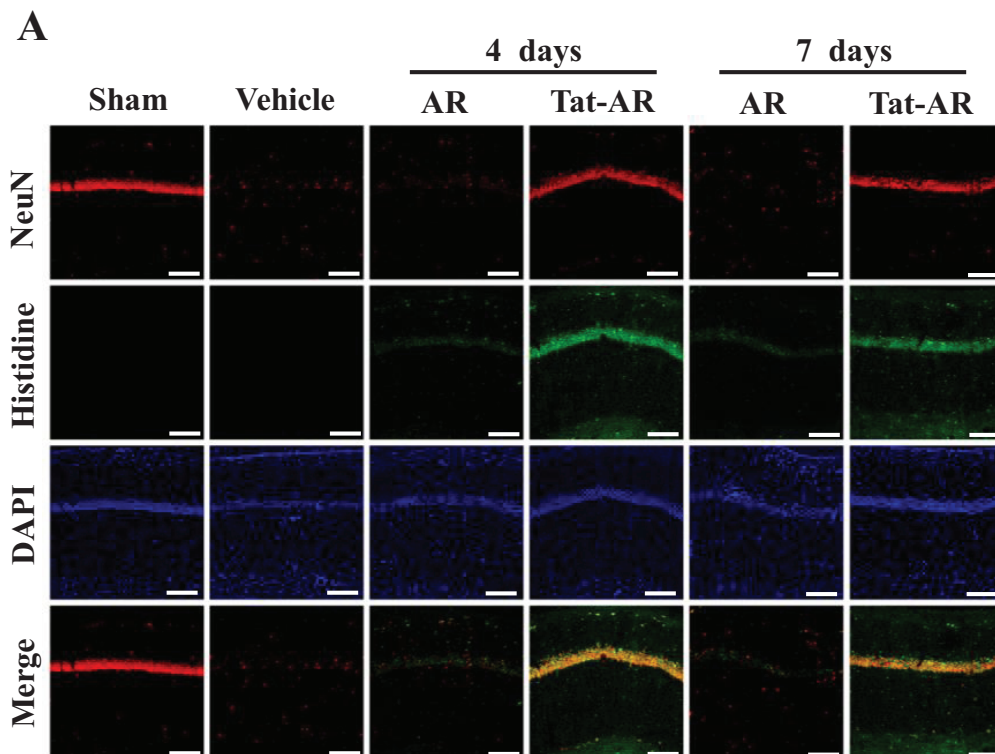
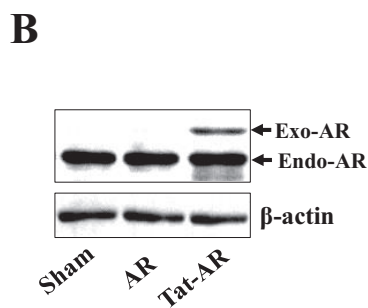


Fig. 6. Transduction of Tat-AR protein into gerbil brain and its protective effects. Gerbils were treated with single injections Tat-AR (2 mg/kg) proteins and killed after 4 and 7 days (n=10 per groups). Then the transduction of Tat-AR protein was analyzed by immunohistochemistry using a Histidine antibody and DAPI. Ischemic neuronal damage was analyzed by NeuN-immunohistochemistry (A). Endogenous and transduced AR protein levels in gerbil brain were analyzed by Western blot using an AR protein antibody (B). Scale bar=50 μ m.



in Fig. 6B, transduced Tat-AR protein doesn't affect the level of endogenous AR protein significantly.

Furthermore, we examined whether transduced Tat-AR protein inhibits neuronal cell injury and activation of microglia and astrocytes using FJB, Iba-1, and GFAP staining (Fig. 7). In the vehicle- and AR protein-treated groups, FJB, Iba-1, and GFAP fluorescence signals were intensively detected in the hippocampal CA1 region. In contrast, intensively fluorescence signals were markedly reduced in the Tat-AR treated group. These results indicate that Tat-AR protein transduced into hippocampal CA1 region, traversing the BBB, and protected against neuronal cell damage resulting from ischemic injury by decreasing microglia and astrocyte activation.

DISCUSSION

The generation of ROS induced by oxidative stress is involved in the pathogenesis of neurodegenerative disorders, cancers, and inflammatory diseases. Since excessive oxidative stress induces serious cell damage and finally leads to cell death, the inhibition of excessive oxidative stress may prevent various disorders [31-33]. The role of AR protein in a variety of disorders has been widely investigated. Several studies have shown that expression of AR protein protects against ROS formation and plays an important role as an antioxidant in neuronal cell [9-11]. On the other hand, some studies have demonstrated that inhibition of AR dramatically prevents production of LPS-induced cytokines, and inflammatory mediator proteins in Raw 264.7 cells, suggesting that inhibitors

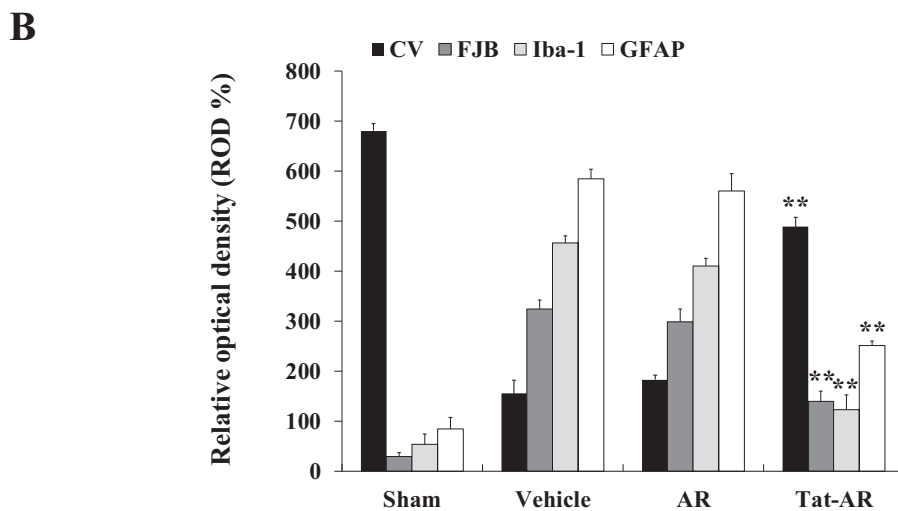
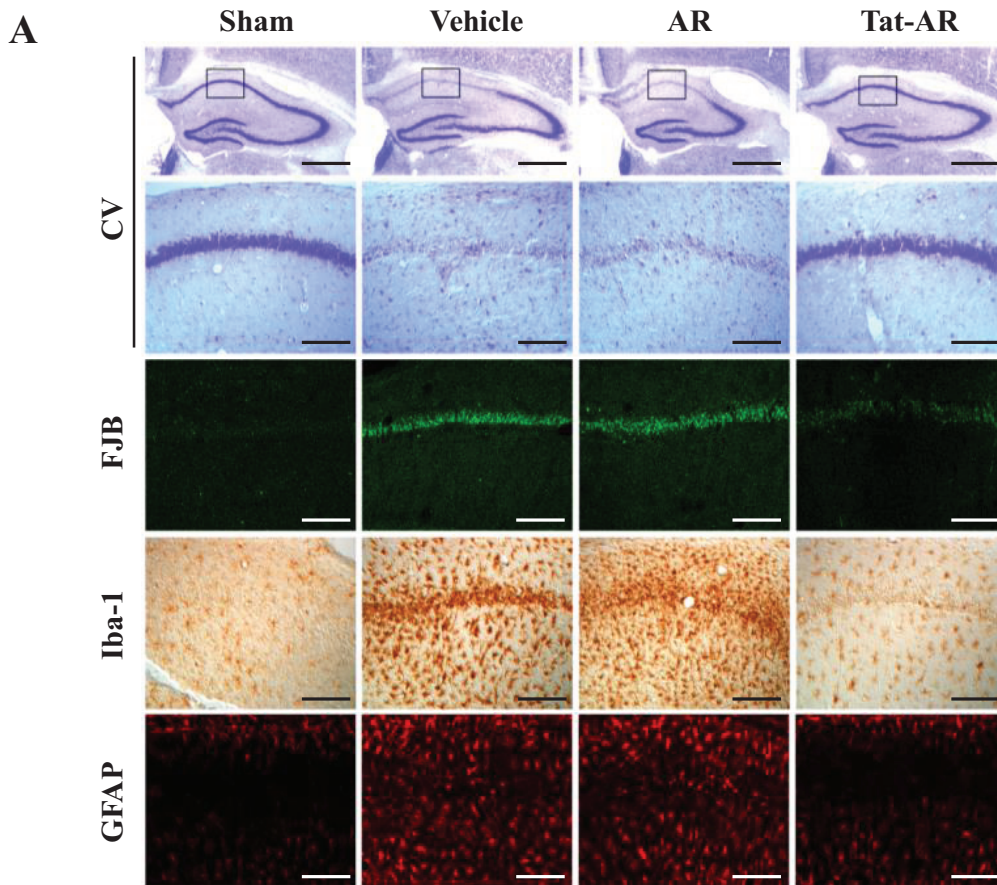


Fig. 7. Effects of transduced Tat-AR protein on neuronal cell viability after ischemic insults using Cresyl violet, FJB, Iba-1 and GFAP immunostaining (n=10 per groups). The hippocampus was stained with CV, FJB, Iba-1 and GFAP in sham-, vehicle-, AR and Tat-AR treated animals 7 days after I/R (A). Relative numeric analysis of CV-, FJB-, Iba-1- and GFAP-positive neurons in CA1 region (B). Scale bar=400 and 50 μ m. **p<0.01, significantly different from the vehicle group.

of AR could be used for therapeutic agent in inflammation [34]. Thus, the AR protein shows contradictory effects depending on cell type or disease. Therefore, we examined whether AR protein has a protective effect against oxidative stress-induced HT-22 cell death and in an ischemic injury animal model.

Many studies have demonstrated that PTD fusion proteins transduce into cells and tissues suggesting that PTD fusion proteins can be attractive therapeutic tools for various diseases [12-14, 35]. Tat, human immunodeficiency virus transactivator of transcript, is identified as the 11 amino acid sequence YGRKKRRQRRR

and Tat fusion protein has ability to deliver a variety of proteins crossing the cell membranes and the BBB. Thus, transduction of Tat PTD fusion protein can represent a novel strategy for treating a majority of neuronal disorders [36, 37]. In previous studies, we reported that transduced Tat fused proteins have protective effects against oxidative stress induced cell deaths and in animal models of various diseases [15-22].

It is well known that excessive cellular ROS generation leads to cell death and macromolecules damage including DNA [4, 5]. Therefore we investigated the cell viability and TUNEL staining assay to show the protective effect of Tat-AR against ROS. The data showed that Tat-AR protein inhibits cell death and DNA fragmentation induced by oxidative stress. Several studies have shown that AR protein inhibits ROS toxicity. Overexpression of AR protein increased human lens epithelial cell survival in aldehyde-induced toxic condition and inhibited UVB-induced cell death and intracellular ROS generation in HaCaT cell. Thus, they suggested that AR proteins inhibit the oxidative stress by sequestering ROS [11, 38]. Our results showed same patterns as those reports suggesting Tat-AR protein protected H₂O₂-induced cell death and ROS generation in HT-22 cells.

Next, we investigated the effect of Tat-AR protein against oxidative stress-induced MAPKs signaling pathways. We showed that transduced Tat-AR protein inhibited the activation of MAPKs in the HT-22 cells. Increased ROS generation is known highly associated with cell signaling pathways by stimulation of redox-sensitive transcription factors and MAPKs (JNK, ERK1/2 and p38), which are known as a superfamily of serine/threonine kinases [25]. In smooth muscle cells (SMC), overexpression of AR protein is involved in the methylglyoxal (MG)- and hydrogen peroxide-induced p38 and ERK signaling pathways [39]. Another study also showed that transfection of AR gene reduced the ultraviolet-B (UVB)-mediated activation of MAPKs (p38 and JNK) in HaCaT cells while the ERK was not affected in HaCaT cells [38]. Those studies showed coincidence with our data suggesting that AR protein inhibits the activation of MAPKs signaling pathways.

In a previous studies have shown that excessive ROS generation leads to DNA damage and cell death by mediating apoptotic signaling pathways [40, 41]. Thus, we examined the effect of Tat-AR protein on apoptotic-related protein expression including p53, Bax, Bcl-2, and Caspase-3 under oxidative stress condition. Our data showed that transduced Tat-AR protein reduced the phosphorylation of p53, Bax, and cleaved Caspase-3 expression, while Bcl-2 expression were significantly increased in the oxidative stress-induced HT-22 cells. Kang et al (2011) demonstrated that AR protein prevents activation of p53 expression levels in keratinocytes which are exposed to UVB. This protein also suppressed

the activation of Caspase-3 expression levels and markedly attenuated Bax and Bcl-2 expression in UV-B treated cells, suggesting that overexpression of AR protein inhibits UVB-induced apoptotic cell death via the regulation of apoptotic protein expression [38]. Although further studies are necessary to clarify the precise role of AR in MAPK and apoptotic signaling pathways, those results suggested that AR protein plays a detoxification role and increases cell survival via regulation of MAPKs and apoptotic signaling pathways under oxidative stress conditions.

Oxidative stress-induced ROS plays a crucial role in brain injury following ischemia-reperfusion [42-45]. It has been reported that oxidative stress induced accumulation of 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA) during ischemic injury [46, 47]. Thus, these authors suggest that the balance between antioxidant and oxidative stress in organism might be the best approach for protection against ischemic damage. As *in vivo* study, we examined whether transduced Tat-AR protein protects against oxidative stress-induced brain damage and attenuates ischemic injury. Our results showed that Tat-AR protein transduced into the hippocampal CA1 region, passing through the BBB. In addition, transduced Tat-AR protein markedly increased hippocampal neuronal cell survival and suggest that transduced Tat-AR protein protected neuronal cell damage during ischemic injury. It has been reported that overexpression of AR in cardiac myocytes prevents damage caused by ischemia-reperfusion *via* the inhibition of lipid peroxidation derived aldehydes such as 4-HNE [48]. Some PTD-fused antioxidant proteins have been shown to transduce into the brain and significantly reduce ischemic injury by reducing MDA and 4-HNE levels in the hippocampus [18, 49]. 4-HNE is metabolized by AR and aldehyde dehydrogenase (ALDH) and ALDH and decreasing of 4-HNE prevented PC12 cell death [50-53]. Thus, AR is responsible for the beneficial effects of the late phase of ischemic preconditioning by inhibition of 4-HNE accumulation [54]. In agreement with these results, we showed that transduced Tat-AR protein inhibited hippocampal neuronal cell death and responsible for beneficial effects in ischemic injury.

In a previous study, Cho et al (2008) have demonstrated that cell permeable PTD-GFP fusion protein transduced into ischemic hippocampal neurons and transduced PTD-GFP fusion protein levels were persisted over 4 days in ischemic animal model [55]. Usually the stability of most transduced proteins in the cells is about 24~72 hours depending on the cell types or proteins, however the stability of transduced proteins in brain tissues is about 4~7 days [17, 55-58]. The differences of stability between *in vitro* and *in vivo* are not understood yet. However, as you see in Fig.6A, the protein remains lower level at 7 days compared to 4 days which means the transduced protein degrades very slowly.

It is known that Iba-1 and GFAP are generally expressed in microglia and astrocytes of the intact brain and its expression under ischemic injury is increased. Thus, the enhanced expressional patterns of Iba-1 and GFAP in the brain are considered as markers of microglia and astrocyte activities under ischemic injury [59, 60]. In previous studies we have demonstrated that the changes of glias activation after administration of various proteins were observed in ischemic animal model [17, 18, 57].

Other studies have shown that activation of microglia protects neuronal cell death by inhibition of inflammation at early stage of neuronal diseases [61-63]. Microglia are the principal immune cells in the central nervous system and microglial activation has dual effects (pro-inflammatory; M1-like or anti-inflammatory/protective; M2-like) by the release of a number of inflammatory mediators in neuronal diseases including ischemic injury [64, 65]. In the mild activated microglial cells contributes to restore the tissue homeostasis by clearing pathogens, necrotic cells and suppressing the inflammation, and facilitating the brain repair [66, 67]. However, over-activated microglial cells may exacerbate tissue damage and neuronal cell death by excessive production of neurotoxic substances including cytokines, nitric oxide (NO), and ROS [68, 69]. Thus, since microglial activation has the dual roles like promoting beneficial and detrimental effects on neurons, regulation of balance between beneficial and detrimental effects of microglial responses may be important in ischemic injury.

NF- κ B and MAPK signaling pathways considered to be one of critical regulators and plays a major role in the inflammatory responses by controlling the activation of microglia [70, 71]. In the inactivated state of microglia, NF- κ B is present in the cytoplasm in inactive form. However, NF- κ B translocates into the nucleus when microglia are activated and regulates the gene expression of pro-inflammatory mediators. Some studies have shown that the expression of pro-inflammatory cytokines increases within hours and elevates at several weeks later in ischemic stroke [72-74]. MAPK is a crucial signaling pathway that mediates inflammation and participates in cytokine control [75]. Dong et al. (2019) have shown that oxymatrine (OMT) alleviates neuronal damage and improves hippocampal neuronal states by inhibition of microglia activation and MAPK signal pathways activation in rat brain tissues and primary microglia cells [76]. Furthermore, there are many evidences suggest that inhibition of microglia activation is important for neuroprotection in ischemic injury [77-80], therefore how to reduce of pro-inflammatory mediators from activated microglia is one of factors to find out the therapeutic molecules against ischemic injury [81-86].

In this study, after treatment of Tat-AR protein to ischemic animal model, the patterns of the changes of microglia and astrocyte

activation showed coincidence with the protection of the hippocampal neuronal cells obtained by CV and FJB staining experiments. These our results indicate that transduced Tat-AR protein play an important role for hippocampal neuronal cell survival as an antioxidant function. However, further studies are necessary to understand the precise protective molecular mechanisms and functions of AR protein during ischemic injury.

In summary, we demonstrated that Tat-AR protein transduced into HT-22 cells and significantly protected cell death caused by oxidative stress via inhibition of DNA fragmentation and regulation of MAPK and apoptotic signaling pathways. In addition, Tat-AR protein transduced into hippocampal neuronal cell and prevents cell death in an ischemic animal model. Thus, we suggested that Tat-AR protein can be a potential therapeutic protein agent for ischemic injury.

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