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Tat-indoleamine 2,3-dioxygenase 1 elicits neuroprotective effects on ischemic injury

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Running title: Tat-IDO-1 inhibits hippocampal cell death

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Abstract

It is well known that oxidative stress participates in neuronal cell death causeds production of reactive oxygen species (ROS). The increased ROS is a major contributor to the development of ischemic injury. Indoleamine 2,3-dioxygenase 1 (IDO-1) is involved in the kynurenine pathway in tryptophan metabolism and plays a role as an anti-oxidant. However, whether IDO-1 would inhibits hippocampal cell death is poorly known. ~~The Our aim of in this study is to explore the effects of cell permeable Tat-IDO-1 protein against oxidative stress-induced HT-22 cells and in a cerebral ischemia/reperfusion injury model.~~ Therefore, we explored the effects of cell permeable Tat-IDO-1 protein against oxidative stress-induced HT-22 cells and in a cerebral ischemia/reperfusion injury model. Transduced Tat-IDO-1 reduced cell death, ROS production, and DNA fragmentation and inhibited mitogen--activated protein kinases (MAPKs) activation in H_2O_2 exposed HT-22 cells. In the cerebral ischemia/reperfusion injury model, Tat-IDO-1 transduced into the brain and passing ~~through~~ by means of the blood-brain barrier (BBB) ~~and~~ significantly ~~protected~~ prevented hippocampal neuronal cell death. These results suggest that Tat-IDO-1 may present an alternative strategy to improve from the ischemic injury.

Keywords: Tat-IDO-1, Oxidative stress, Ischemia, MAPKs, Protein therapy.

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메모 포함[오전1]: There two verbs here. I cannot deduce what grammar is intended.

메모 포함[오전2R1]: We edited the sentence.

메모 포함[오전3]: It is not logical to attribute volition to something, like a study, created by human effort.

메모 포함[오전4R3]: We logically edited this sentence.

메모 포함[오전5]: It does not make sense that something would "protect death". Do you mean "prevent"? Or what?

메모 포함[오전6R5]: Yes, 'prevent' is correct.

서식 있음: 들여쓰기: 첫 줄: 0.65 cm

INTRODUCTION

서식 있음: 왼쪽, 들여쓰기: 첫 줄: 0.65 cm

Indoleamine 2,3-dioxygenase 1 (IDO-1) is a heme-containing enzyme involved in the first step of the kynurenine pathway in tryptophan metabolism and is expressed in response to interferon gamma (IFN-γ) stimulation in the cortex, hippocampus, and various cells, including neurons, astrocytes, macrophages, and microvascular endothelial cells (1-3). The ~~Kynurenine~~ kynurenine pathway finally ~~generated~~ generates kynurenic acid and quinolinic acid. Quinolinic acid causes excitotoxicity and neuronal cell death, whereas kynurenic acid ~~plays~~ has antioxidant properties (4, 5). IDO-1, a unique cytosolic enzyme, exerts powerful antioxidant effects ~~through~~ by means of free radical scavengers (4, 6, 7). Over-expressed human IDO-1 by gene transfection significantly protects endothelial cell ~~damages~~ against damage from oxidative stress and lung transplant ischemia/reperfusion injury in an animal model (8). It ~~and~~ also protects against atherosclerosis by regulation of T cells in plasmacytoid dendritic cells (9). Although reactive oxygen species (ROS) ~~is~~ are important ~~to~~ for keeping balance in cellular redox signaling, overproduction of ROS is involved in neuronal diseases including ischemia (10-13). Since ROS play~~s~~ crucial roles in the pathogenesis of this disease,

antioxidant protein~~s~~ seems to be a potential therapeutic approach for ischemic injury (14, 15).

Mitogen-activated protein kinases (MAPKs) signaling pathways, such as extracellular-signal regulated kinase (ERK), c-Jun NH2 terminal kinase (JNK), and p38 are associated with cell differentiation, cell proliferation, cell survival, and cell death (16). Even though several studies have reported that oxidative-stress-mediated MAPKs activation plays an important role in death-receptor-initiated exogenous and mitochondrial apoptotic pathways as well as neuronal cell death or neurodegenerative disorders (17-19), little is known about the effects of IDO-1 on oxidative-stress-mediated neuroprotective effects in hippocampal cells and a cerebral ischemia/reperfusion injury model.

서식 있음: 왼쪽

It is recognized that small molecules ~~are able to~~can transduce into the cell, but larger macromolecules like protein cannot ~~be permeable~~ permeate owing to their physicochemical characteristics (20, 21). Thus, we fused IDO-1 protein with protein-transduction domains (PTD), such as Tat peptide, which can allow protein to transduce into cells. In previous studies, we showed that PTD fusion proteins transduced into cells and significantly protected them against various oxidative-stress-induced ~~various~~ diseases (14, 15, 22, 23). In this study, we investigated whether Tat-IDO-1 inhibits hippocampal cell death in HT-22 cells and a cerebral ischemia/reperfusion injury model.

RESULTS

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Construction, production, and transduction of recombinant Tat-IDO-1 fusion protein

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As shown in Fig. 1A, we constructed recombinant Tat-IDO-1 and control IDO-1 plasmid. Tat peptide is linked to a human IDO-1 gene to permit transduction of a fusion protein into cells, whereas a control IDO-1 gene ~~did~~ was not linked to the Tat peptide. Then, SDS-PAGE and Western blot analysis confirmed the purified fusion proteins, Tat-IDO-1 and control IDO-1, as shown in Fig. 1B. Purified fusion proteins appeared to have the expected molecular weights of 38 and 36 kDa, respectively.

~~—~~ To investigate whether a fusion protein ~~possesses the capacity to~~can transduce ~~tion~~ into HT-22 cells, we treated control IDO-1 and Tat-IDO-1 proteins ~~were treated~~ with cells for various times (10-180 min) and concentrations (0.5-3 μM). As expected, Tat-IDO-1 protein was detected in a dose- and time-dependent manner (Fig. 1C). Also, transduced protein detected not only stability for up to 36 h but also distributed both cytosol and nuclei in the cells. In contrast, control IDO-1 was not detected (Supplementary Fig. S1).

Effects of Tat-IDO-1 protein on cell death

To confirm the effect of Tat-IDO-1 proteins on HT-22 cell death, HT-22 cells were ~~treatment~~ treated with 1 mM H_2O_2 before a cell viability assay (Supplementary Fig. S2). We

found that Tat-IDO-1 protein increased cell viability up to 72% in the presence of the H_2O_2. To examine how the Tat-IDO-1 protein affects the cell viability, we investigated cellular toxicity, ROS generation, and DNA damage (Fig. 2A and 2B). We confirmed that Tat-IDO-1 protein significantly inhibits the cellular toxicities. However, there was no significant difference between H_2O_2 and control IDO-1 protein- ~~-~~treated cells.

서식 있음: 왼쪽, 들여쓰기: 첫 줄: 0.65 cm

Effects of Tat-IDO-1 protein on signaling pathways under oxidative stress

To explore whether there was an association between Tat-IDO-1 protein and signaling pathways, we ~~determined~~ investigated apoptosis and MAPK signaling pathways in H_2O_2-~~=~~exposed HT-22 cells. Tat-IDO-1 protein reduced Bax expression levels ~~compared to~~more than did the cells treated only with H_2O_2 ~~treated cells~~. In contrast, Bcl-2 expression levels were increased by Tat-IDO-1 protein. In addition, Tat-IDO-1 protein reduced cleaved Caspase-3 and -9 expression levels, whereas control IDO-1 proteins did not affect apoptotic protein~~s~~ expression levels (Fig. 3A and 3B).

메모 포함[오전7]: I think this may be a more accurate word. The primary meanings of "determine" are "cause" or "set the values for." Neither of which makes sense here. The word is often used as a vague academic buzzword with a dozen possible meanings, of course, but you need not follow the throng.

메모 포함[오전8R7]: Yes, your opinion is correct.

As shown in Fig. 3C, phosphorylation of MAPKs (p38, ERK and JNK) expression levels were increased by H_2O_2. However, Tat-IDO-1 protein reduced phosphorylation of MAPKs expression levels dose-dependently. Control IDO-1 protein showed ~~the similar~~ patterns similar ~~compared~~ to those of ~~to the~~ cells ~~only~~ exposed only to H_2O_2.

서식 있음: 왼쪽

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Effects of Tat-IDO-1 protein on a cerebral ischemia/reperfusion injury model

서식 있음: 왼쪽, 들여쓰기: 첫 줄: 0.65 cm, 단어 잘림 방지

We investigated whether Tat-IDO-1 protects against ischemic injury in a cerebral ischemia/reperfusion injury model. Cresyl violet (CV) staining showed that neuronal cell death was markedly increased in the vehicle- or control IDO-1-treated group. However, neuronal cell death was significantly inhibited in the Tat-IDO-1-treated group. Also, ionized calcium-binding adapter molecule 1 (Iba-1), Fluoro-Jade B (F-JB), and glial fibrillary acidic protein (GFAP) staining were drastically increased in the vehicle- or control IDO-1-treated group. In contrast, Iba-1, GFAP, and F-JB staining were significantly reduced in the Tat-IDO-1-treated group (Fig. 4).

서식 있음: 왼쪽, 들여쓰기: 첫 줄: 0.65 cm

DISCUSSION

IDO-1 is a key enzyme in tryptophan metabolism and is known to induce the production of metabolite kynurenic acid and quinolinic acid. Kynurenic acid protectmotes cell survival against oxidative stress, whereas quinolinic acid induces cell death (24, 25). Since many studies have demonstrated that IDO-1 protein expression is highly associated with various diseases, including Alzheimer's disease, cancer, and diabetes, IDO-1 is generally known as to be a marker of those diseases, and inhibition of IDO protein expression is considered to be a target for various diseases therapy therapies (26, 27). On the other hand, other studies have

shown that IDO-1 expression significantly inhibits oxidative-stress-induced cell death via by exerts exerting powerful antioxidant functions in cancer, inflammation, and neuronal diseases (28-30). Even though some studies have suggested that the IDO-1 protein can be a therapeutic agent for neuronal and immune-related diseases (2, 3, 9), the effects of IDO-1 protein in brain ischemia are not fully investigated yet.

PTD has been known as one of thea tools to overcome the delivery limit of a wide array of compounds, such as peptides and proteins *in vitro* and *in vivo* (20, 21, 31) and extensive experiments have shown that PTD fusion protein is transduced into cells and tissues (14, 15, 31-35). In this study, we showed that cell-permeable Tat-IDO-1 fusion protein is transduced into HT-22 cells. Although Tat-IDO-1 protein transduction ability showed similar patterns similar to those ofwith other Tat fusion protein studies, the transduction time of Tat-IDO-1 protein is longer than that of the other Tat fusion proteins. The difference of in transduction time may depend on various factors, such as protein size, polarity, and protein shape.

Excessive production of ROS induced by oxidative stress causes irreversible degeneration of proteins, nucleic acids, and lipids, and ultimately leads to cell death (36). In this study, we showed that transduced Tat-IDO-1 protein markedly inhibited H_2O_2-induced cell death, ROS generation, and DNA fragmentation. It has been reported that overexpressed IDO-1 protein inhibited against H_2O_2-induced cell death, DNA damage, and intracellular ROS levels in an ischemic-injury rat model, and prevented H_2O_2-induced HUVEC cell death, and also

prtectevented neuronal cell death by free-radical scavengers (8, 37). Our results finding that Tat-IDO-1 protein inhibited HT-22 cell death induced by oxidative stress are coincidesnce with those reports suggesting that IDO-1 acts as an antioxidant protein.

Oxidative stress altered the expression levels of Bax and Bcl-2 protein, and led to cell death, and expressed high levels of activated cleaved Caspase-9 and Caspase-3 (38, 39). In addition, it is well known that anti-apoptotic protein (Bcl-2) expression levels were reduced and pro-apoptotic protein (Bax) expression levels were increased under excessive oxidative stress conditions (38, 40). Our data also showed that Tat-IDO-1 protein regulated apoptotic protein expression levels, including Bax, Bcl-2, cleaved Caspase-9 and Caspase-3.

It is well known that MAPKs (p38, ERK, and JNK) signaling pathways are highly associated with oxidative- stress-induced cell death (41-43). O and other studies have shown that overexpression of IDO-1 protein inhibited the activation of Akt and MAPKs signaling pathways as well asand regulated apoptotic protein expression levels in neuronal cells under excessive oxidative stress conditions (44, 45). Our results showed the same patterns, indicating that Tat-IDO-1 protein inhibits neuronal cell death by regulation of apoptosis, Akt, and MAPKs signaling pathways under oxidative stress.

Since it has been reported that ROS is one of thea major risk factors in ischemic injury and plays crucial roles in the pathogenesis of ischemia/reperfusion injury (13, 36), we examined whether Tat-IDO-1 protein protects against ischemic injury in a cerebral ischemia/reperfusion

서식 있음: 왼쪽

injury animal model. Several studies have demonstrated that activated astrocytes and microglia cells are highly associated with ischemic injury~~;~~ ~~and~~ these cells were increased in brain ischemia, and their reactivities were increased in the hippocampus and led to neuronal cell death by release~~s~~ of pro-inflammatory cytokines and neuroinflammatory response (46-49). Also, Liu ~~et at.~~ (2007) demonstrated that overexpressed IDO-1 protein significantly ameliorates lung ischemia/reperfusion injury (8). In this study, we showed that Tat-IDO-1 protein markedly reduced activation of microglia and astrocytes and reduced neuronal cell damages significantly in an ischemic~~-~~ injury animal model. Therefore, we suggest that IDO-1 protein may represent a potential therapeutic strategy against lung ischemia/reperfusion injury as well as brain ischemic injury. However, further studies ~~remains~~ are needed to ~~be~~ elucidate~~d~~ the exact protective mechanism on ischemic injury.

In summary, we showed that transduced Tat-IDO-1 protein inhibited oxidative stress-induced HT-22 cell death by ~~reduction~~ reducing~~of~~ cellular cytotoxicity as well as ~~modulation~~ regulation of cellular signaling pathways, such as apoptosis and MAPKs and Tat-IDO-1 protein transduced into the hippocampal CA1 region of the brain, and markedly ameliorates neuronal cell death. Therefore, Tat-IDO-1 protein can be a candidate ~~of~~ as a useful therapeutic agent for ischemia.

MATERILALS AND METHODS

메모 포함[오전9]: "Et al." is short for "et aliis" and means "and others". The period is essential. It is not a good idea to try using these remnants of medieval Latin scholarship unless one knows exactly what they mean and how to use them correctly.

메모 포함[오전10R9]: We deleted 'et al'

서식 있음: 왼쪽, 들여쓰기: 첫 줄: 0.65 cm

메모 포함[오전11]: I am seeing this word being misused in many papers lately. It is used in the fields of electronics and music, but I have no idea what it is intended to mean here. Modifying, perhaps?

메모 포함[오전12R11]: We modify the word. Modulation to regulation.

서식 있음: 들여쓰기: 첫 줄: 0.65 cm

See **supplementary information** for this section

서식 있음: 왼쪽, 들여쓰기: 첫 줄: 0.65 cm, 단어 잘림 방지

CONFLICTS OF INTEREST

The authors have no conflicting interests.

서식 있음: 왼쪽, 들여쓰기: 첫 줄: 0.65 cm

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REFERENCES

메모 포함[오전13]: It is our policy to edit figure legends and table titles, but not the contents of the references, figures, and tables. Be sure to consult the style guide of the professional journal you have in mind; it might be quite idiosyncratic. It is standard that all book titles and names of periodicals should be in italics

메모 포함[오전14R13]: Thanks for the comments. The word was changed to italic.

서식 지정함: 글꼴: 기울임꼴

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서식 있음: 왼쪽, 단어 잘림 방지

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Figure legends

Fig. 1. Construction, purification, and transduction of Tat-IDO-1 protein. Diagrams of

Tat-IDO-1 and control IDO-1 protein (A). Purification of Tat-IDO-1 and control IDO-1 protein. We analyzed ~~Purified~~ purified Tat-IDO-1 and control IDO-1 protein ~~were analyzed~~ using 15% SDS-PAGE and Western blotting (B). Transduction of Tat-IDO-1 protein into HT-22 cells. The cells were treated with Tat-IDO-1 and control IDO-1 protein (0.5-3 μM) for 3 h or Tat-IDO-1 and control IDO-1 protein (3 μM) for various incubation~~s~~ times (10-180 min) (C). Then, transduced Tat-CIAPIN1 protein levels ~~was~~ were ~~determined~~ assessed by Western blotting, and the intensity of the bands was measured by a densitometer. Data are repressed as mean ± SEM (n = 3).

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Fig. 2. Effects of Tat-IDO-1 protein against oxidative ~~-~~stress ~~-~~induced HT-22 cell damage. Tat-IDO-1 (3 μM) and control IDO-1 protein (3 μM) were pretreated with HT-22 cells for 3 h before treatment with 1 mM of H_2O_2. Intracellular ROS levels were ~~determined~~ measured by DCF-DA staining. Fluorescence intensity was quantified using an ELISA plate reader (A). DNA fragmentation was ~~determined~~ assessed by TUNEL staining, and quantitative evaluation of TUNEL positive cells were confirmed by cell counting under a phase-contrast microscopy (×200 magnification) (B). Scale bar = 50 μm. *~~P~~ p < 0.05 and **~~P~~ p < 0.01 compared with H_2O_2-treated cells. Data are repressed as mean ± SEM (n = 3).

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Fig. 3. Effect of Tat-IDO-1 protein on signaling pathways in HT-22 cells. Tat-IDO-1

(0.5-3 μM) and control IDO-1 protein (3 μM) were treated with HT-22 cells for 3 h before H_2O_2 (1 mM). Expression levels Bax and Bcl-2 (A), cleaved Caspase-3 and -9 (B), and MAPKs (C) were detected by Western blotting with indicated specific antibodies. The protein band intensities were measured by densitometer. **~~P~~ $p < 0.01$ compared with H_2O_2-treated cells. Data are repressed as mean ± SEM (n=3).

Fig. 4. Effect of Tat-IDO-1 on ischemic injury in a cerebral ischemia/reperfusion model. Neuroprotective effects of transduced Tat-IDO-1 protein were analyzed by the CV, GFAP, Iba-1, and F-JB immunostaining in the CA1 region of the hippocampus of the gerbil brain 7 days (n =10 per groups) after ischemic injury. Relative numeric analysis of CV, GFAP, Iba-1, and F-JB-positive neurons in CA1 region. Scale bar = 400 um and 50 um (CV), 25 um (GFAP and Iba-1), and 50 um (F-JB). ~~Scale bar = CV; 400 μm and 50 μm, GFAP, Iba-1; 25 μm. F-JB; 50 μm.~~ **~~P~~ $p < 0.01$, significantly different from the vehicle group.

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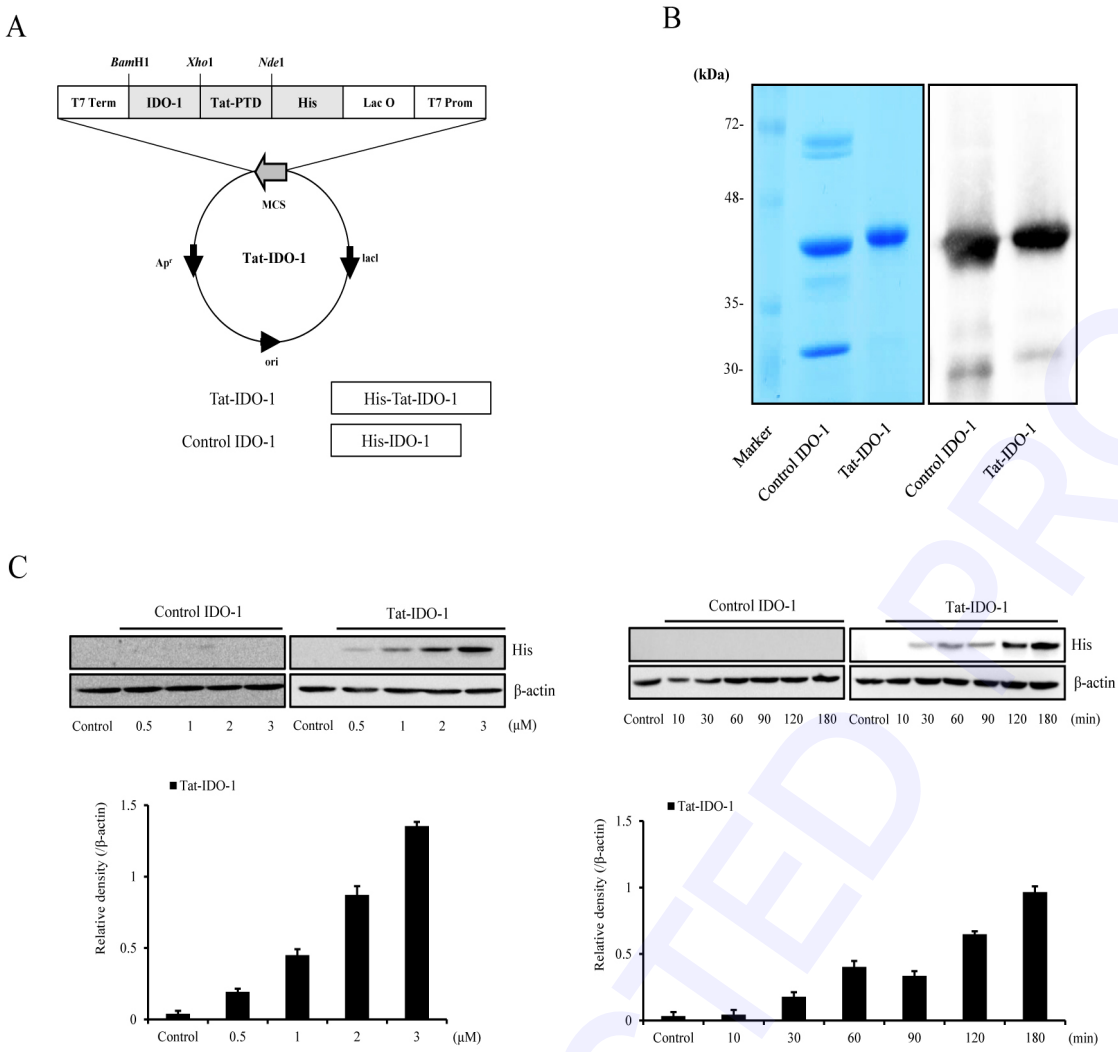

Figure. 1

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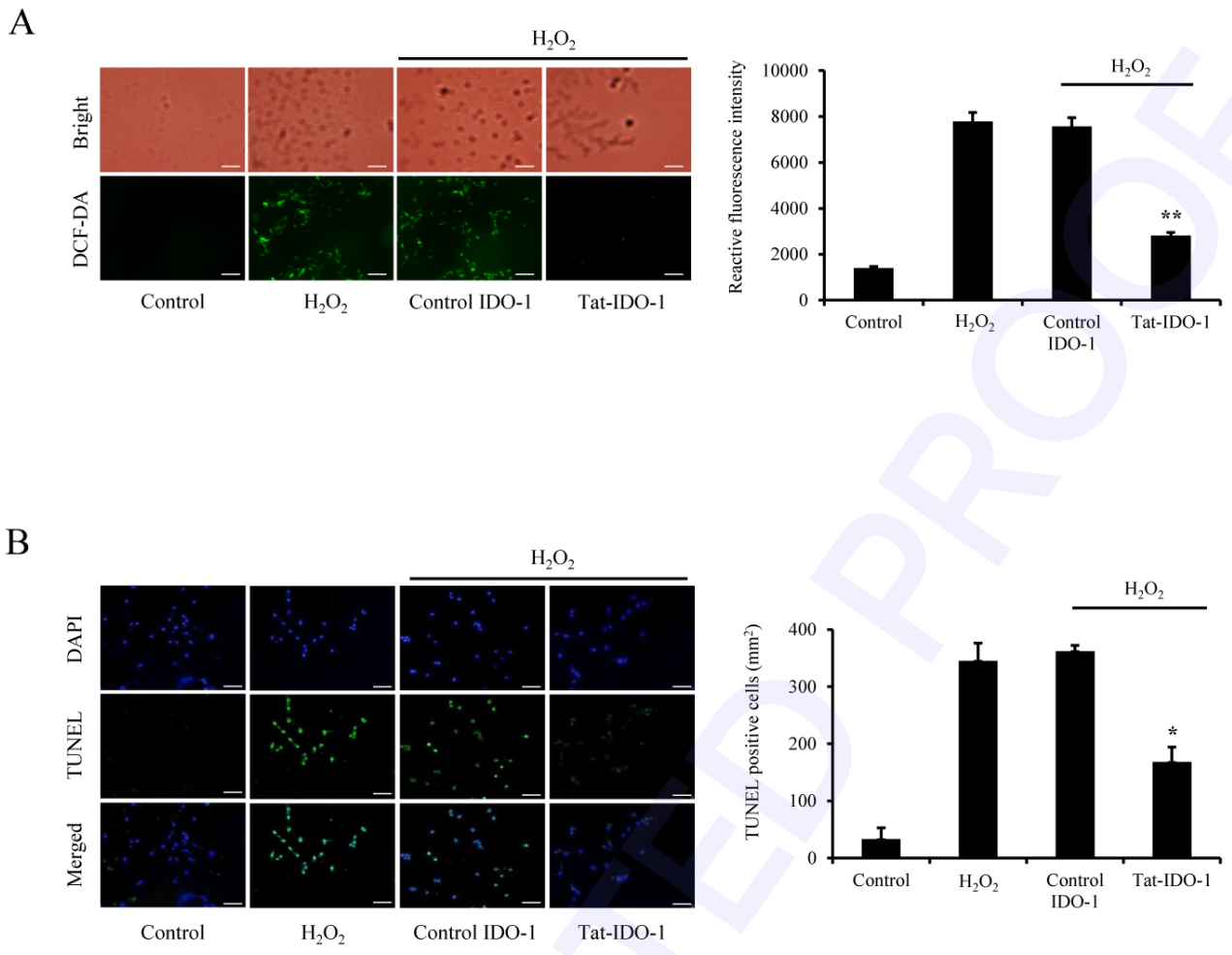

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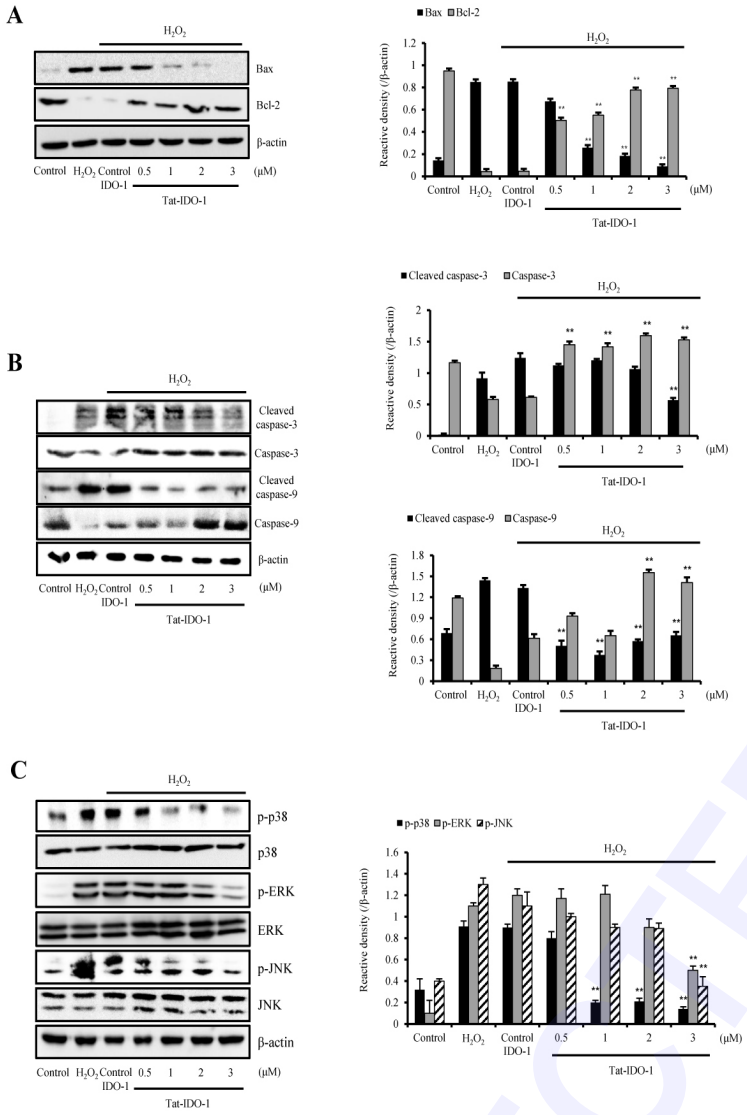

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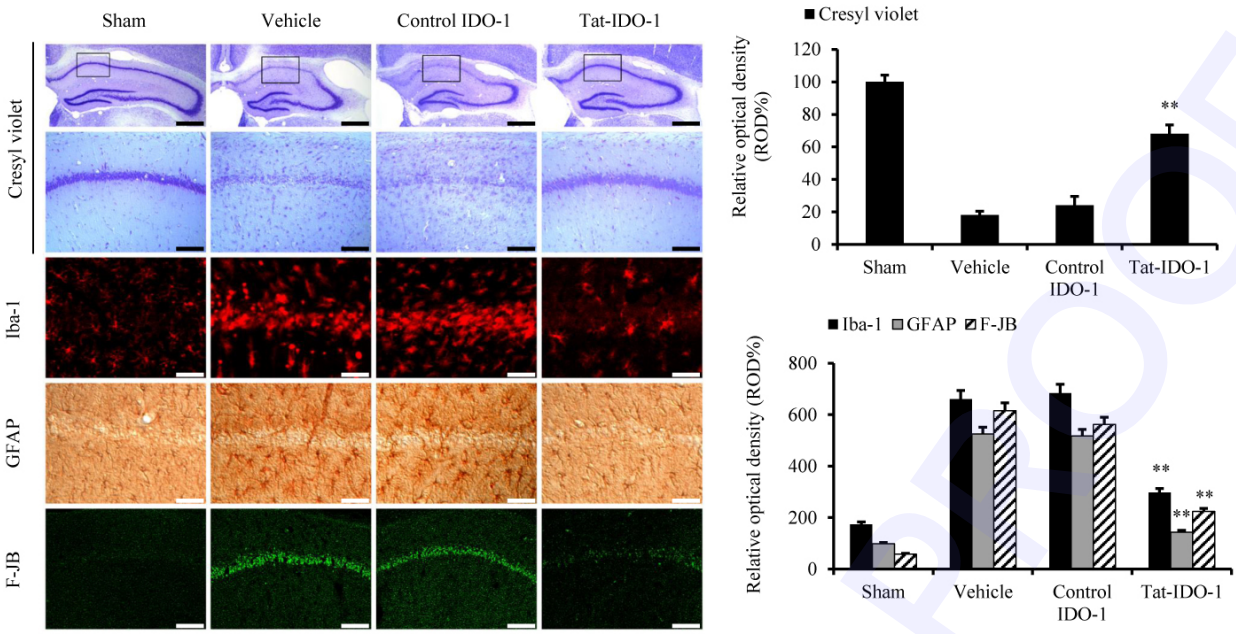

Figure. 4

Fig. 4.

Supplementary Information

Tat-indoleamine 2,3-dioxygenase 1 elicits neuroprotective effects on ischemic injury

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Running title: Tat-IDO-1 inhibits hippocampal cell death

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FIGURES

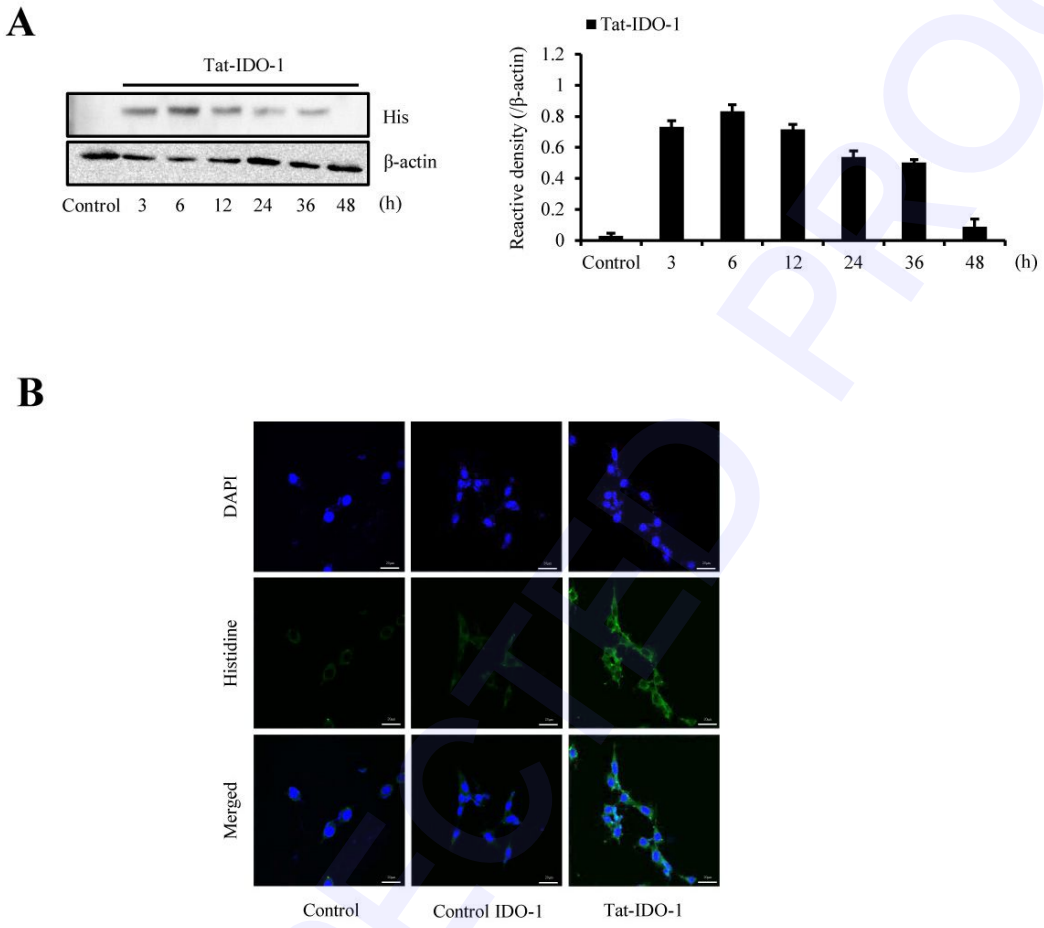

Figure S1. Transduction of Tat-IDO-1 protein into HT-22 cells. Intracellular stability of transduced Tat-IDO-1 protein. Tat-IDO-1 protein was transduced into HT-22 cells for 3 h and further incubated the cells for 48 h. Then, the intracellular level of Tat-IDO-1 protein was measured by Western blot analysis. The protein band intensities were measured by densitometer (A). Cellular localization of Tat-IDO-1 protein. Cellular localization of Tat-

IDO-1 protein was determined by immunofluorescence analysis using a histidine and Alexa 488 conjugated antibodies (B). Scale bar = 50 μm. Data are repressed as mean ± SEM (n=3).

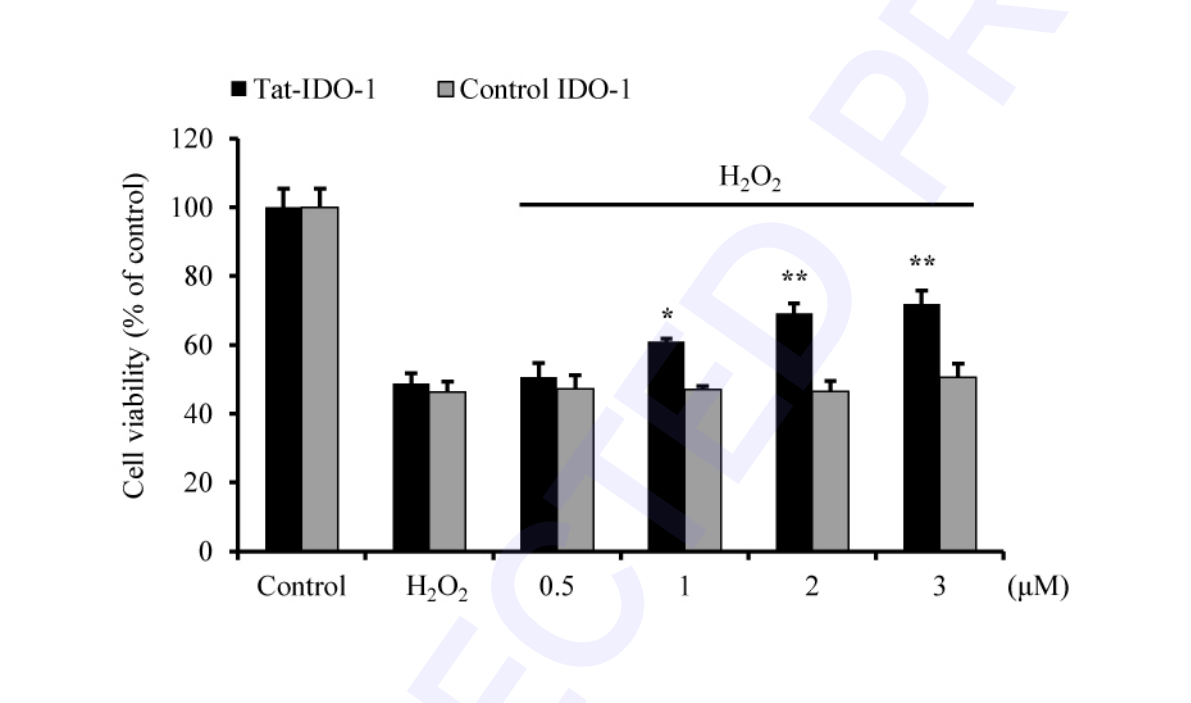

Figure S2. Effects of Tat-IDO-1 protein against oxidative stress induced HT-22 cell death. Tat-IDO-1 (0.5-3 μM) and control IDO-1 protein (0.5-3 μM) were pretreated with HT-22 cells for 3 h and H_2O_2 (1 mM) was added to the cells for 2 h. Cell viabilities were estimated using a colorimetric assay using WST-1. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with H_2O_2-treated cells. Data are repressed as mean ± SEM (n=3).

MATERILALS AND METHODS

Materials

All antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA) unless stated otherwise.

Tat-IDO-1 purification

Recombinant Tat-IDO-1 was constructed and purified as described previous studies (1, 2). Briefly, the PCR products was digested and ligated to the Tat expression vector. Then, the constructed Tat-IDO-1 plasmid was confirmed by restriction enzyme digestion and DNA sequence analysis. The constructed Tat-IDO-1 and control IDO-1 plasmids were transformed into *E.coli* BL21 (DE3; Novagen, Madison, WI, USA) cells and proteins expression were induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Duchefa, Haarlem, Netherlands) for 4 h. Then, harvested cells were purified using Ni-NTA affinity and PD10 chromatography (Amersham, Braunschweig, Germany). The protein concentration was determined by Bradford assay (Bio-Rad Lab., Hercules, CA, USA) (3).

Transduction of recombinant protein into cells

Analysis of recombinant proteins transduction into cells were performed as previously described (1, 4). HT-22 cells were treated with Tat-IDO-1 proteins (0.5-3 μM) for 3 h or

treatment with Tat-IDO-1 proteins (3 μM) for various times (10-180 min) and incubated in CO_2 incubator. After treatment of Tat-IDO-1 proteins (3 μM) for 3 h, the cells were further incubated for 48 h and washed with trypsin-EDTA (Gibco BRL; Grand Island, NY, USA) and phosphate-buffered saline (PBS). Then, the cells were prepared for analysis by Western blotting.

Western blot analysis

After the proteins were separated by 12% SDS-PAGE and transferred to PVDF membrane, the membranes were blacked with TBS buffer containing 0.1% Tween 20 and 5% non-fat milk and incubated with indicated primary antibodies (dilution 1: 10000). Then, the membrane was probed with appropriate secondary antibodies for 1 h and the band was detected using ECL solution (Amersham, Franklin Lakes, NJ, USA) (1, 5).

Cell viability assay

HT-22 cells were pretreated with Tat-IDO-1 (0.5-3 μM) proteins for 3 h and then treated with hydrogen peroxide (H_2O_2, 1mM) for 3 h. To examine the cell viability, a WST-1 cell viability assay kit (Daeil Lab service Co., Seoul, Korea) was used according to the manufacturer's protocol.

Analysis of ROS and DNA fragmentation levels

Measurement of ROS and DNA fragmentation level in HT-22 cells was performed with 2',7'-Dichlorofluorescein diacetate (DCF-DA; Sigma-Aldrich, St. Louis, MO, USA) and A terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick-end labeling (TUNEL; Roche Applied Science, Basel, Switzerland) staining as described previous studies (1, 4).

Experimental animals and treatment

Male gerbils (65-75 g, 6 months) were housed at a temperature of 23ºC, with humidity of 60%, and exposed to 12 h periods of light and dark with free access to food and water. All animal experimental were approved by the Institutional Animal Care and Use Committee of Soonchunhyang University.

Cerebral ischemia/reperfusion injury model was performed as described previously (6-8) and the animals were divided into 4 groups (each n=10); control sham, vehicle-, control IDO-1, and Tat-IDO-1-treated groups with ischemic surgery. Recombinant proteins (each 2 mg/kg) were administered intraperitoneally 30 min before ischemia/reperfusion. To examine the protective effects of Tat-IDO-1 protein against ischemic injury, we performed Cresyl violet (CV; Junsei Chemical Co. Ltd., Saitama, Japan), Fluoro-Jade B (F-JB; Millipore Co., Temecula, CA, USA), ionized calcium-binding adapter molecule 1 (Iba-1; Wako, Osaka,

Japan), and glial fibrillary acidic protein (GFAP; Millipore Co., Temecula, CA, USA) staining as described in previous studies (6-8).

Statistical analysis

Data are expressed as the mean ± SEM. The data were analyzed using one-way ANOVA to determine statistical significance. Bonferroni's test was used for post-hoc comparisons. A value of $P < 0.05$ was considered statistically significant.

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