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

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It is well known that oxidative stress participates in neuronal cell death caused 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 inhibit hippocampal cell death is poorly known. 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 by means of the blood-brain barrier (BBB) significantly prevented hippocampal neuronal cell death. These results suggest that Tat-IDO-1 may present an alternative strategy to improve from the ischemic injury. [BMB Reports 2020; 53(11): 582-587]

INTRODUCTION

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 pathway finally generates kynurenic acid and quinolinic acid. Quinolinic acid causes excitotoxicity and neuronal cell death, whereas kynurenic acid has antioxidant properties (4, 5). IDO-1, a unique cytosolic enzyme, exerts powerful antioxidant effects by means of free radical scavengers (4, 6, 7). Over expressed human IDO-1 by gene transfection significantly protects endothelial cell against damage from oxidative stress and lung transplant ischemia/reperfusion injury in an animal model (8). It also protects against atherosclerosis by regulation of T cells in plasmacytoid dendritic cells (9). Although reactive oxygen species (ROS) are important for keeping balance in cellular redox signaling, overproduction of ROS is involved in neuronal diseases including ischemia (10-13). Since ROS play crucial roles in the pathogenesis of this disease, antioxidant protein 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.

It is recognized that small molecules can transduce into the cell, but larger macromolecules like protein cannot permeate owing to their physicochemical characteristics (20, 21). Thus, we fused IDO-1 protein with protein transduction domains (PTD),

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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 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

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

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 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 can transduce into HT-22 cells, we treated control IDO-1 and Tat-IDO-1 proteins 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 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.

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 investigated apoptosis and MAPK signaling pathways in H_2O_2 exposed HT-22 cells. Tat-IDO-1 protein reduced Bax expression levels more than did the cells treated only with H_2O_2. In contrast, Bcl-2 expression levels were increased by Tat-IDO-1 protein. In

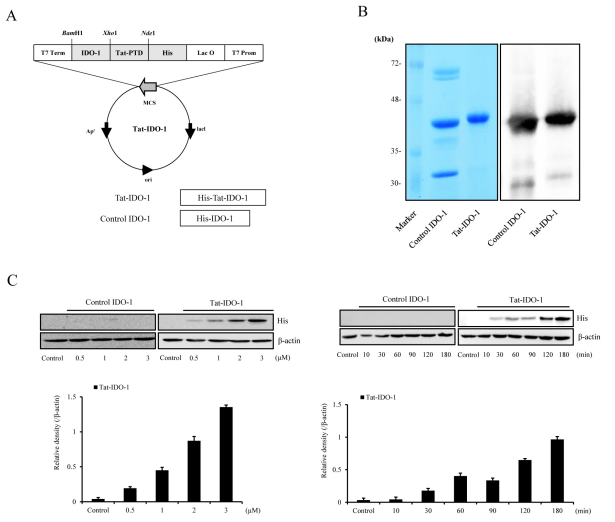

Fig. 1. Construction, purification, and transduction of Tat-IDO-1 protein. Diagrams of Tat-IDO-1 and control IDO-1 protein (A). Purification of Tat-IDO-1 and control IDO-1 protein. We analyzed purified Tat-IDO-1 and control IDO-1 protein using 15% SDS-PAGE and Western blotting (B). Transduction of Tat-IDO-1 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 times (10-180 min) (C). Then, transduced Tat-IDO-1 protein levels were assessed by Western blotting, and the intensity of the bands was measured by a densitometer. Data are repressed as mean ± SEM (n = 3).

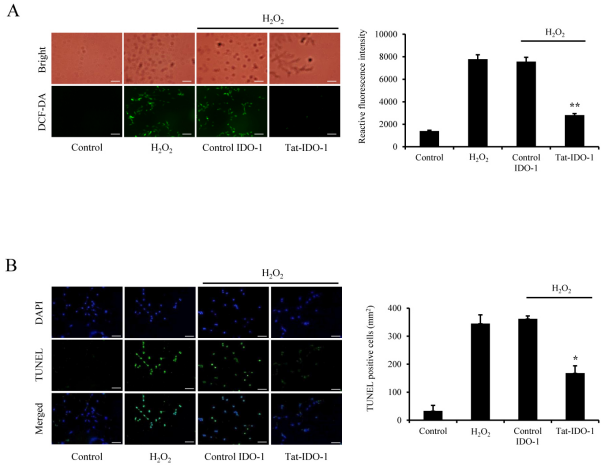

Fig. 2. Effects of Tat-IDO-1 protein against oxidative stress-induced HT-22 cell damage. Tat-IDO-1 (3 μM) and control IDO-1 protein (3 μM) were pretreated with HT-22 cells for 3 h before treatment with 1 mM of H_2O_2. Intracellular ROS levels were measured by DCF-DA staining. Fluorescence intensity was quantified using an ELISA plate reader (A). DNA fragmentation was 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 < 0.05 and **P < 0.01 compared with H_2O_2-treated cells. Data are repressed as mean ± SEM (n = 3).

addition, Tat-IDO-1 protein reduced cleaved Caspase-3 and -9 expression levels, whereas control IDO-1 proteins did not affect apoptotic protein expression levels (Fig. 3A and 3B).

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 patterns similar to those of cells exposed only to H_2O_2.

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

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).

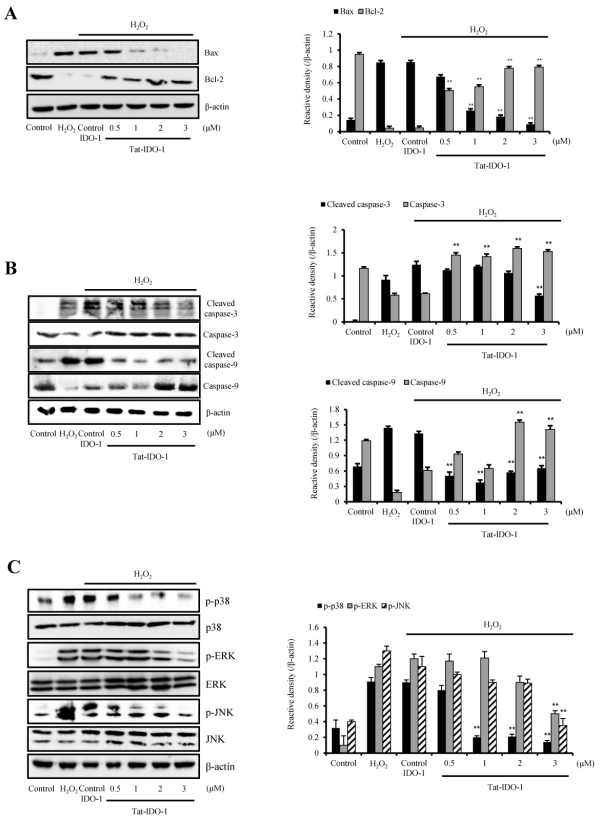

Fig. 3. Effect of Tat-IDO-1 protein on signaling pathways in HT-22 cells. Tat-IDO-1 (0.5-3 μM) and control IDO-1 protein (3 μM) were treated with HT-22 cells for 3 h before H_2O_2 (1 mM). Expression levels Bax and Bcl-2 (A), cleaved Caspase-3 and -9 (B), and MAPKs (C) were detected by Western blotting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

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 promotes 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 to be a marker of those diseases, and inhibition of IDO protein expression is considered to be a target for various disease therapies (26, 27). On the other hand, other studies have shown that IDO-1 expression significantly inhibits oxidative stress-induced cell death by 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 a tool 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 patterns similar to those of other Tat fusion protein

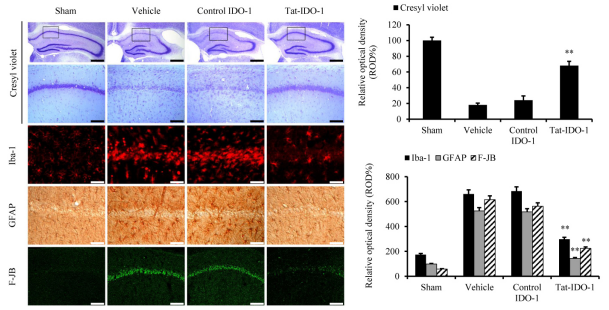

Fig. 4. Effect of Tat-IDO-1 on ischemic injury in a cerebral ischemia/reperfusion model. Neuroprotective effects of transduced Tat-IDO-1 protein were analyzed by the CV, GFAP, Iba-1, and F-JB immunostaining in the CA1 region of the hippocampus of the gerbil brain 7 days (n =10 per groups) after ischemic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

studies, the transduction time of Tat-IDO-1 protein is longer than that of other Tat fusion proteins. The difference 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 H_2O_2-induced cell death, DNA damage, and intracellular ROS levels in an ischemic injury rat model, prevented H_2O_2-induced HUVEC cell death, and prevented neuronal cell death by free radical scavengers (8, 37). Our finding that Tat-IDO-1 protein inhibited HT-22 cell death induced by oxidative stress coincides with those reports suggesting that IDO-1 acts as an antioxidant protein.

Oxidative stress altered the expression levels of Bax and Bcl-2 protein, 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 (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). Other studies have shown that overexpression of IDO-1 protein inhibited the activation of Akt and MAPKs signaling pathways and regulated apoptotic protein expression levels in neuronal cells under excessive oxidative stress (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 a major risk factor 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 injury animal model. Several studies have demonstrated that activated astrocytes and microglia cells are highly associated with ischemic injury; these cells were increased in brain ischemia, and their reactivities were increased in the hippocampus and led to neuronal cell death by release of pro-inflammatory cytokines and neuroinflammatory response (46-49). Also, Liu (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 are needed to elucidate 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 reducing cellular cytotoxicity as well as 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 as a useful therapeutic agent for ischemia.

MATERIALS AND METHODS

See supplementary information for this section.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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