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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Tat-DJ-1 inhibits oxidative stress-mediated RINm5F cell death through suppression of NF-κB and MAPK activation

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Abstract Oxidative stress is highly involved in the development of diabetes mellitus by destruction of pancreatic β-cells. DJ-1 is an antioxidant protein and DJ-1 expression levels are known to be reduced in diabetes mellitus. Thus, we examined the effects of DJ-1 protein against oxidative stress-induced pancreatic β-cell (RINm5F) death using cell permeable wild-type and mutant-type (C106A) Tat-DJ-1 proteins, which both efficiently transduced into RINm5F cells. Intracellular stability of wild-type Tat-DJ-1 persisted two times longer than C106A Tat-DJ-1. Wild-type Tat-DJ-1 protein markedly protected cells from hydrogen peroxide-induced toxicities such as cell death, reactive oxygen species generation, and DNA fragmentation. Further, wild-type Tat-DJ-1 protein significantly inhibited hydrogen peroxide-induced activation of mitogen-activated protein kinases and NF-κB signaling. On the other hand, C106A Tat-DJ-1 protein did not show the same protective effects. These results indicate that wild-type Tat-DJ-1 inhibits oxidative stress-induced cellular toxicity and activation of mitogen-activated protein kinases and NF-κB signals in RINm5F cells. These results suggest that wild-type Tat-DJ-1 protein may be a potential therapeutic agent against diabetes mellitus or toward the prevention of pancreatic β-cell destruction.

Keywords WT Tat-DJ-1 · Mutant DJ-1 · Oxidative stress · Cytotoxicity · Protein therapy

Introduction

Oxidative stress-induced cellular reactive oxygen species (ROS) are highly associated with pancreatic β-cell death, since antioxidant protein expression levels are very low in pancreatic β-cells which leaves them more susceptible to ROS in comparison to other tissues (Lenzen et al., 1996; Suarez-Pinzon et al., 1997; Tabatabaie et al., 2003). Other studies have demonstrated that increased cellular ROS levels lead to pancreatic β-cell destruction, while overexpressed antioxidant proteins inhibit oxidative stress-induced pancreatic β-cell death (Hohmeier et al., 1998; Tiedge et al., 1998; Tiedge et al., 1999; Lee et al., 2015). In a previous study, we demonstrated that antioxidant proteins transduce into pancreatic β-cells where they deliver a significant protective effect against oxidative stress-induced pancreatic β-cell death. Delivery of proteins into cells was achieved using protein transduction domains (PTDs), which, when fused with therapeutic proteins, can deliver those proteins into cells (Kim et al., 2013; Lee et al., 2015). These studies suggest that the regulation of ROS production plays a crucial role against oxidative stress-induced pancreatic β-cell death. In addition, several studies including those by our group have shown that PTD fusion proteins transduced into various cells and prevent oxidative stress-induced cell death (Wadia and Dowdy, 2002; Kubo et al., 2008; Dietz, 2010; van den Berg and Dowdy, 2011; Zhang et al., 2014; Kim et al., 2015a, b). Among the PTDs, Tat PTD is a short peptide consisting of 11 basic amino acids. Tat PTD have widely been used to deliver therapeutic

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proteins into a variety cells (Fawell et al., 1994; Koren and Torchilin, 2012; Jay and Lee, 2013).

DJ-1 is a homodimeric protein, belonging to the Thi/Pfp1 superfamily and is abundant in most living things from humans to bacteria (Bonifati et al., 2003). The results of other studies suggest that DJ-1 protein may have an antioxidant function by protecting against oxidative stress-induced neuronal cell death (Shendelman et al., 2004; Taira et al., 2004). However, DJ-1 mutations are known to cause autosomal-recessive hereditary Parkinson disease (Bonifati et al., 2003). The antioxidant function of DJ-1 protein depends on its cysteine residues (C46, C53, and C106). Among these cysteine residues, C106 is well characterized by being the most sensitive to oxidative stress and its mutation leads to loss of antioxidant function (Kinumi et al., 2004). Although several studies have shown that DJ-1 has neuroprotective effects against neuronal diseases (Aleyasin et al., 2007; Yanagida et al., 2009), there has been little study of the role the DJ-1 protein plays against oxidative stress-induced pancreatic β-cell death.

In this study, we prepared both cell permeable wild-type (WT) and mutant-type (C106A) Tat-DJ-1 proteins to examine the effects of DJ-1 protein against oxidative stress-induced pancreatic β-cell (RINm5F) death. Our results show that WT Tat-DJ-1 protein significantly protected against oxidative stress-induced pancreatic β-cell death, while C106A Tat-DJ-1 protein did not show the same protective effects. These results suggest a potential role as a therapeutic agent against pancreatic β-cell death for WT Tat-DJ-1 protein.

Results and discussion

Several studies have shown that WT DJ-1 protein plays an important role in neuronal cell survival as an antioxidant protein in Parkinson's disease. However, mutant DJ-1 protein is highly associated with one of the risk factors and onset of PD (Bonifati et al., 2003; Kinumi et al., 2004; Shendelman et al., 2004; Taira et al., 2004). Although several studies have demonstrated that DJ-1 protein plays important roles in neuronal cell survival, the functions of WT DJ-1 and mutant C106A DJ-1 are not fully understood in pancreatic β-cells. Since antioxidant protein expression levels are very low in pancreatic β-cells, antioxidant proteins are considered to be potential therapeutic agents for pancreatic β-cell survival. Numerous studies have revealed that therapeutic proteins, when fused with PTDs, transduce into various cells and prevent against oxidative stress-induced cell death in vitro and in vivo (Wadia and Dowdy, 2002; Kubo et al., 2008; Dietz, 2010; van den Berg and Dowdy, 2011; Zhang et al., 2014; Kim et al., 2015a, b). We have also demonstrated that Tat-GLO protein protects against SNP-induced pancreatic cell death in a previous study (Kim et al., 2013). Thus, we prepared cell permeable WT and mutant-type (C106A) Tat-DJ-1 proteins in order to examine the effects of Tat-DJ-1 proteins on pancreatic RINm5F cells. We constructed a Tat-DJ-1 plasmid (Fig. 1a), which contained human DJ-1 complementary DNA (cDNA) (WT and C106A), Tat peptide, and 6His, while we also constructed control DJ-1 plasmid without Tat peptides. These DJ-1 proteins were overexpressed in *E. coli* and purified using Ni-NTA and PD-10 affinity chromatography. Purified proteins were confirmed by SDS-PAGE and Western blotting analysis (Figs. 1b, c).

To assess the levels of transduced Tat-DJ-1 proteins in RINm5F cells, cells were treated with various concentrations of WT and C106A Tat-DJ-1 proteins (1–3 μM) for 1 h or treated with WT and C106A Tat-DJ-1 protein (3 μM) for a range of times (10–60 min) after which transduced Tat-DJ-1 protein levels were determined by Western blotting. As shown in Fig. 2, WT and C106A Tat-DJ-1 proteins transduced into RINm5F cells time and concentration dependently. In contrast, control DJ-1 protein did not show the same transduction levels in the cells.

We examined the intracellular stability of transduced WT and C106A Tat-DJ-1 proteins in RINm5F cells. Significant levels of transduced WT Tat-DJ-1 protein persisted in the cells for 24 h, compared to C106A Tat-DJ-1 protein, which was markedly reduced, persisting in the cells for 6 h (Fig. 3a). Using fluorescence staining, we also determined the intracellular stability of transduced WT and C106A Tat-DJ-1 proteins under same experimental conditions. Fluorescence signals show similar patterns to those of Western blot data (Figs. 3b, c).

In addition, we confirmed the transduction of Tat-DJ-1 protein in RINm5F cells using immunofluorescence staining. As shown in Fig. 4a, immunofluorescence signals strongly detected in Tat-DJ-1 protein-treated cells. However, immunofluorescence signals were not detected in control or control DJ-1 or Tat peptide-treated cells.

Several studies have demonstrated that ROS play a crucial role in pancreatic β-cell death. Since pancreatic β-cells contain very low levels of antioxidant proteins, pancreatic β-cells are more susceptible to oxidative stress (Lenzen et al., 1996; Suarez-Pinzon et al., 1997; Tabatabaie et al., 2003). We examined the potential effects of Tat-DJ-1 protein against oxidative stress-induced RINm5F cell death using a 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) assay. Figure 4b shows how transduced WT Tat-DJ-1 protein significantly increased cell viability against oxidative stress-induced cell death compared to the cells treated with hydrogen peroxide (H_2O_2) only. In contrast, C106A Tat-DJ-1 protein did not affect cell viability compared to H_2O_2-treated cells or control DJ-1 protein-treated cells. In agreement with our results, other

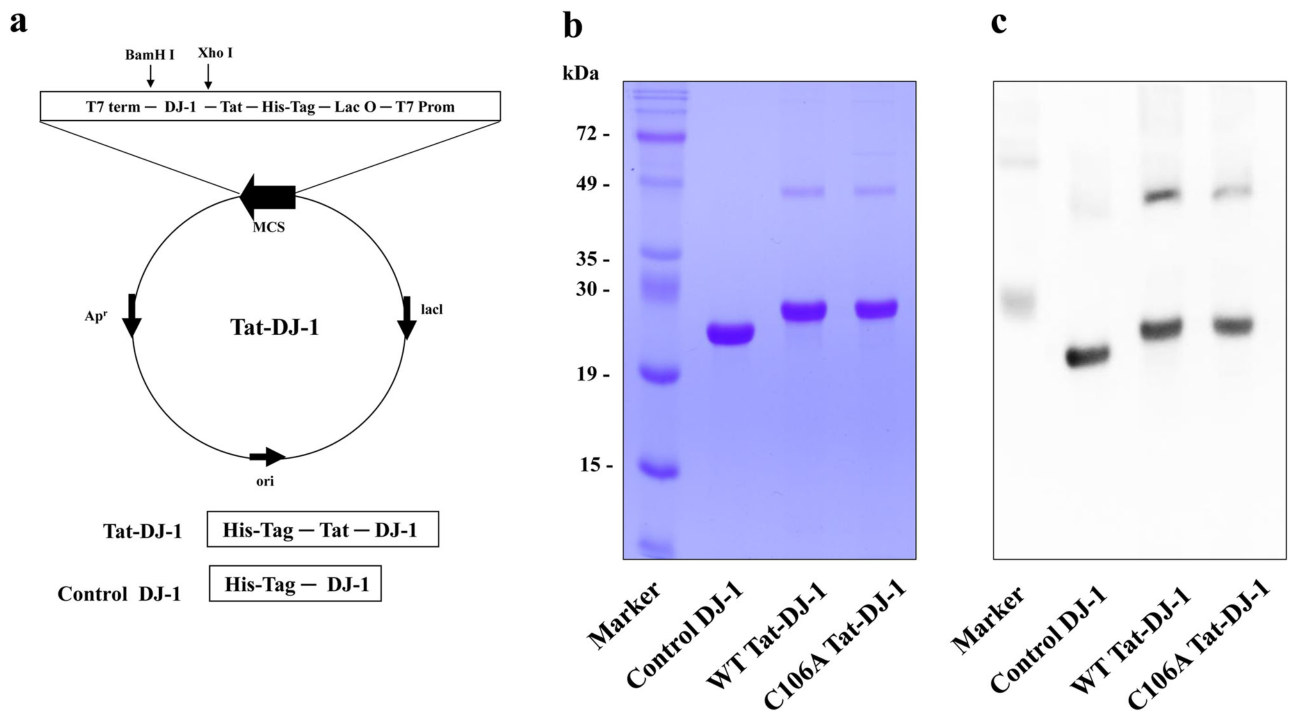


Fig. 1 Purification of WT and C106A Tat-DJ-1 protein. A schematic representation of the WT and C106A Tat-DJ-1 protein (**a**). Expressed and purified fusion proteins were analyzed by 12 % SDS-PAGE (**b**) and subjected to Western blot analysis (**c**) with an anti-polyhistidine antibody

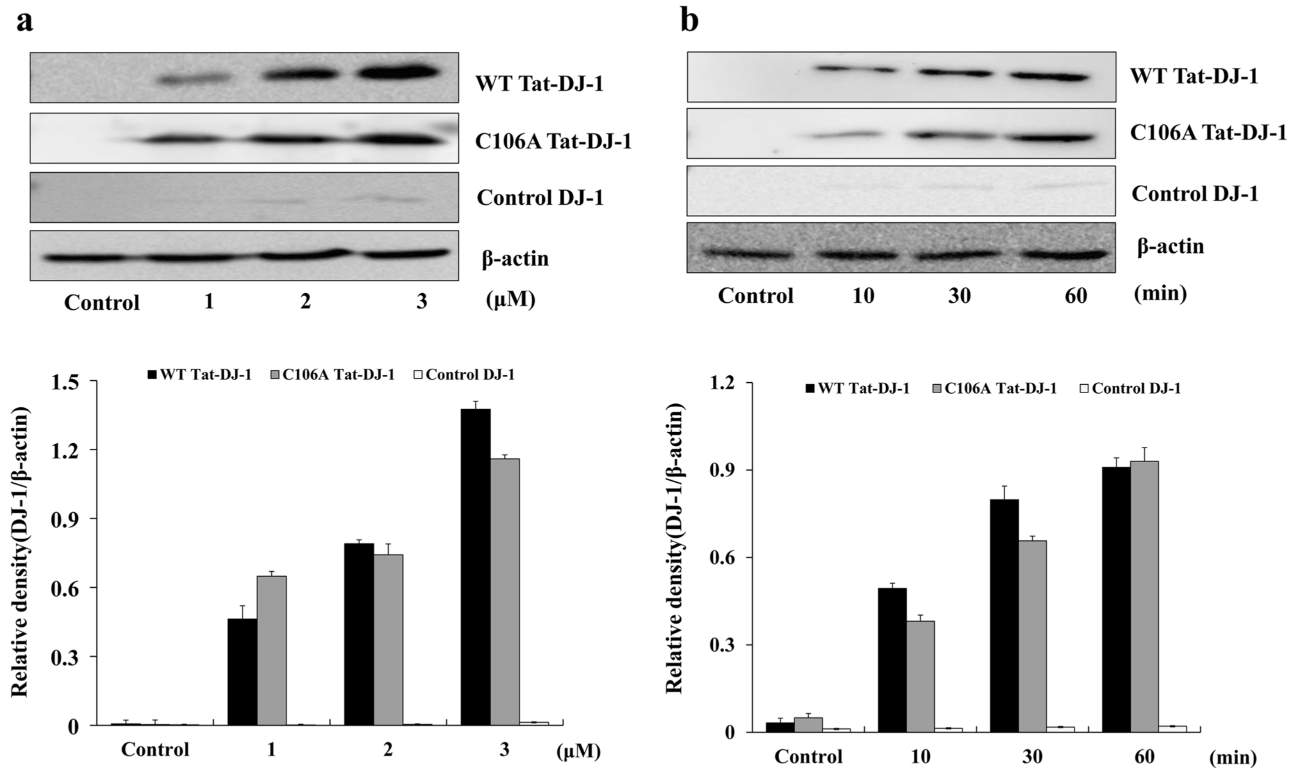


Fig. 2 Transduction of Tat-DJ-1 proteins into pancreatic RINm5F cells. WT and C106A Tat-DJ-1 and control DJ-1 proteins (1–3 μM) were added to the RINm5F culture media for 1 h (**a**). WT and C106A Tat-DJ-1 and control DJ-1 proteins (3 μM) were added to the RINm5F culture media for 10–60 min (**b**). Then, transduced Tat-DJ-1 protein levels were determined by Western blotting using an anti-polyhistidine antibody. The band intensities were measured by a densitometer

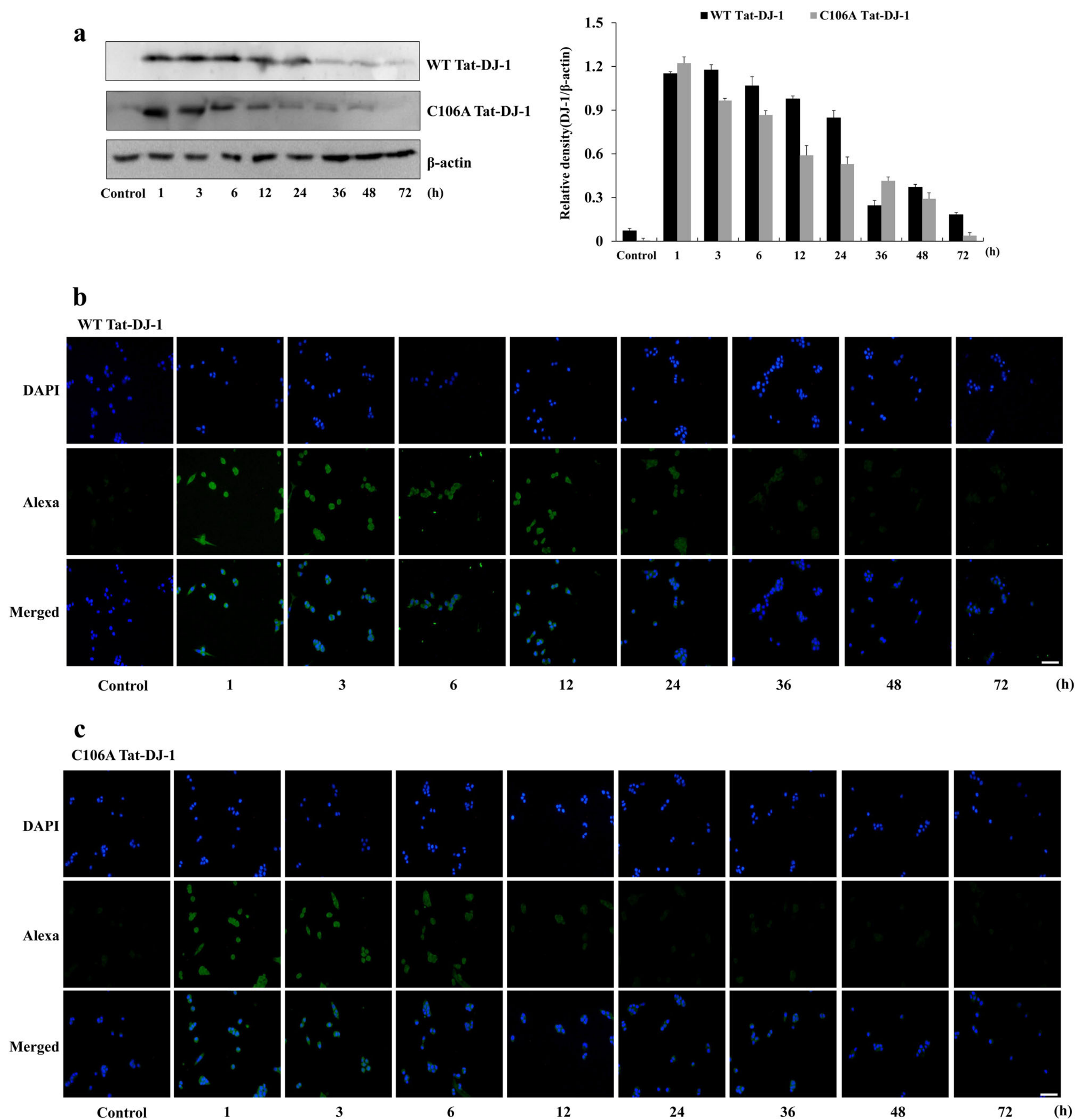
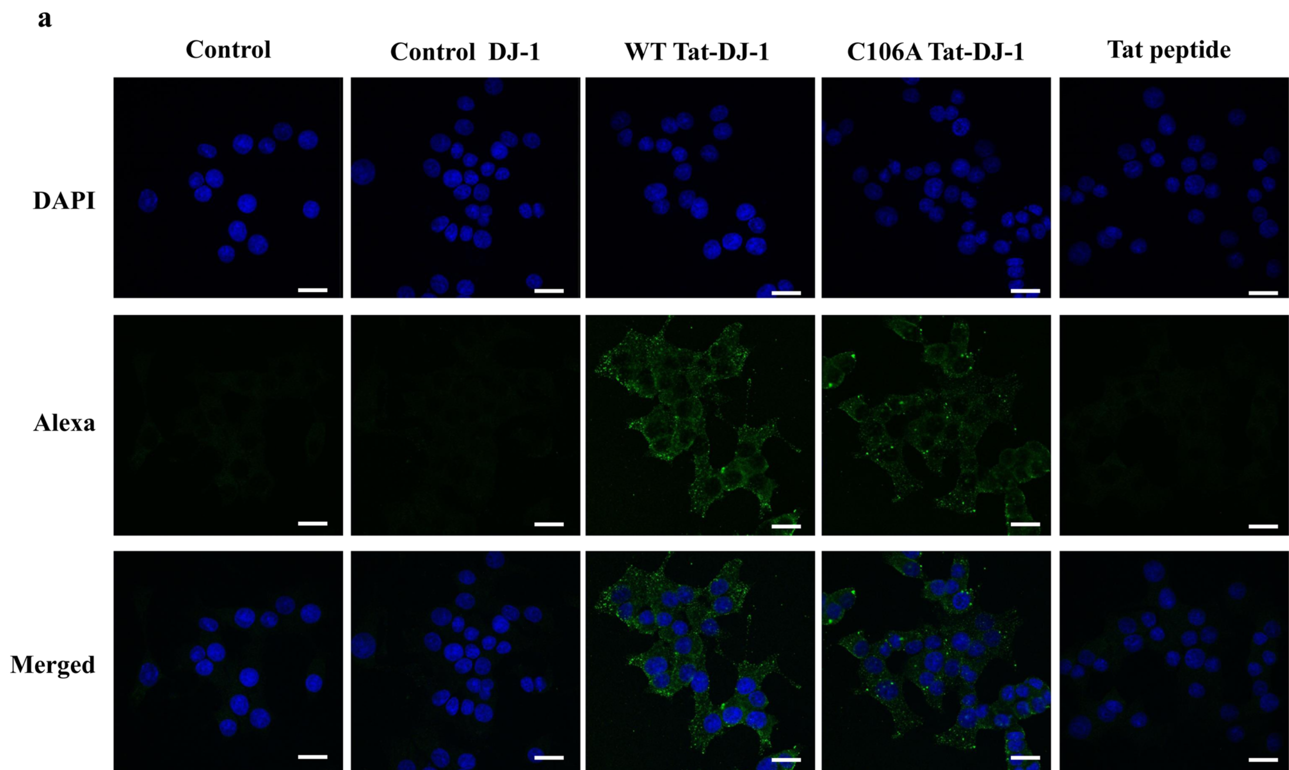


Fig. 3 Stability of transduced WT and C106A Tat-DJ-1 proteins into pancreatic RINm5F cells. RINm5F cells pretreated with 3 μM WT and C106A Tat-DJ-1 proteins incubated for 1–72 h, and analyzed by Western blotting and band intensity was measured by densitometer (**a**) and by fluorescence microscopy (**b**–**c**). Scale bar = 20 μm

studies have demonstrated that overexpression of DJ-1 protein in MIN6 cells caused by DJ-1 gene transfection significantly increased cell survival in a dose-dependent manner in the H_2O_2-induced cells. In contrast, knockdown of DJ-1 in the cells by the small interfering RNA markedly reduced cell survival under same experimental conditions (Inberg and Linial, 2010). Other reports have shown that DJ-1 protein significantly protected against STZ-induced or cytokine-induced pancreatic β-cell death in DJ-1 knockout mice, leading the authors to suggest that the DJ-1 is required to protect against oxidative stress including STZ and cytokine-induced β-cell death (Inberg and Linial, 2010; Jain et al., 2015).

The effects of Tat-DJ-1 protein against oxidative stress-induced insulin secretion levels, intracellular ROS production, and DNA fragmentation were also explored. As shown in Fig. 5, insulin secretion levels in H_2O_2 exposed RINm5F cells



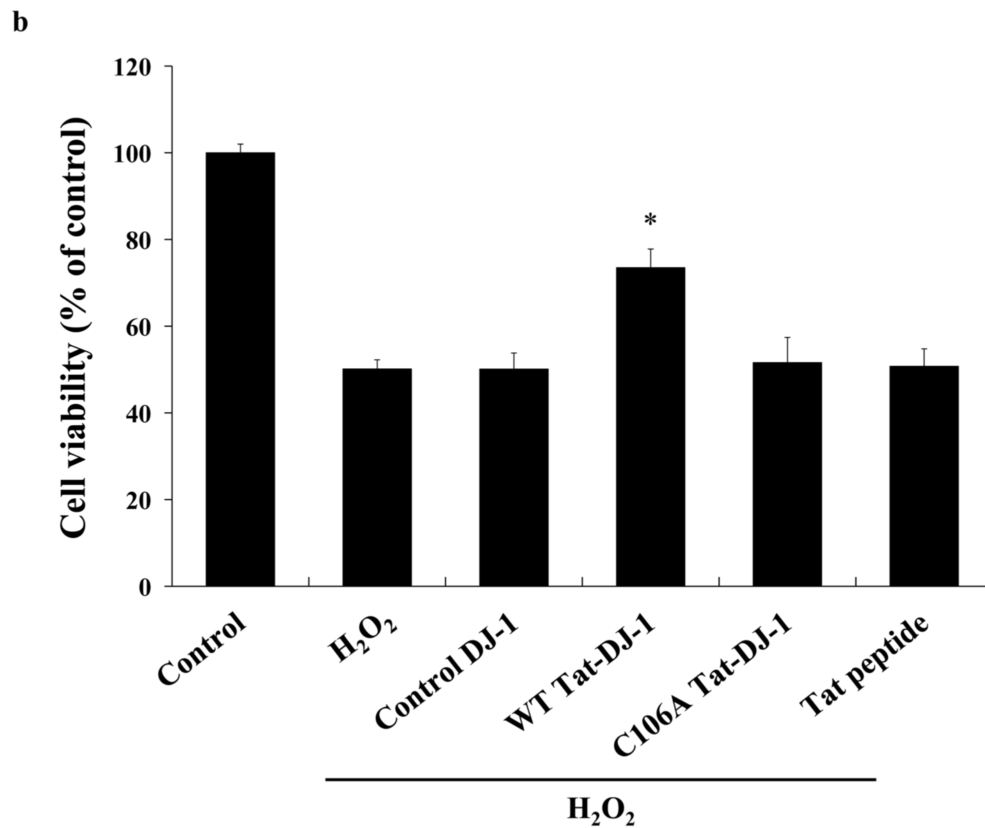


Fig. 4 Effects of transduced WT and C106A Tat-DJ-1 protein against oxidative stress-induced RINm5F cell death. The distribution of transduced Tat-DJ-1 into RINm5F cells was observed by fluorescence microscopy (**a**). Scale bar = 20 µm. H_2O_2 (0.3 mM) was added to RINm5F cells pretreated with WT and C106A Tat-DJ-1 (3 µM) for 1 h. Cell viabilities were estimated using a colorimetric assay using MTT (**b**). $^{*}P < 0.05$, compared with H_2O_2-treated cells

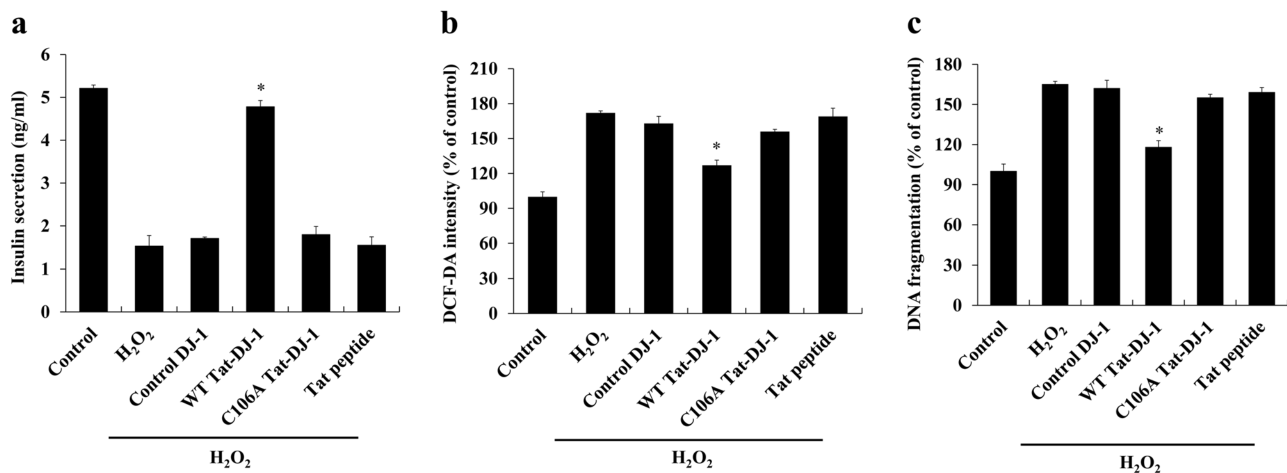


Fig. 5 Effects of WT and C106A Tat-DJ-1 protein against oxidative stress-induced insulin secretion, ROS generation, and DNA fragmentation in RINm5F cells. H_2O_2 (0.3 mM) was added to RINm5F cells pretreated with WT and C106A Tat-DJ-1 (3 μM) for 1 h. Insulin secretion levels (**a**), intracellular ROS levels (**b**), and DNA fragmentation (**c**) were determined. $^{*}P < 0.05$, compared with H_2O_2-treated cells

were reduced in comparison to those of control cells; however, when the cells were treated with WT Tat-DJ-1 protein, the insulin secretion levels were markedly increased. Moreover, intracellular ROS production and DNA fragmentation were markedly increased in the cells treated with H_2O_2 only. Compared to cells treated with the H_2O_2 alone, cells treated with WT Tat-DJ-1 protein showed significantly reduced intracellular ROS production and DNA fragmentation. In contrast, C106A Tat-DJ-1 and control DJ-1 protein did not show any protective effects against oxidative stress-induced insulin secretion, intracellular ROS production, and DNA fragmentation. Taken together our results indicate that WT Tat-DJ-1 protein inhibits oxidative stress-induced cell death and plays an antioxidant role in cells. However, C106A Tat-DJ-1 protein lost its antioxidant function under oxidative stress conditions. Consistent with our results, other studies have demonstrated that Cys-106 mutations, including C106A, abolish the antioxidant functions of DJ-1 protein and suggest that Cys-106 residues in DJ-1 protein is of critical importance to the central redox site for DJ-1 function as a cytoprotective protein against oxidative stress (Blackinton et al., 2009; Waak et al., 2009). In addition, several studies have demonstrated that WT DJ-1 protein protected against oxidative stress-induced neuronal cell death by reducing intracellular ROS levels (Yanagisawa et al., 2008; Yanagida et al., 2009).

NF-κB, which is known as a transcription factor, plays a critical role in regulating the expression of a number of genes and involves the various diseases, including inflammation, tumor development, and cellular proliferation and survival (Sun and Zhang, 2007; Liu et al., 2008; Vallabhapurapu and Karin, 2009; Mitchell et al., 2016). In resting cells, NF-κB consists of p65 and p50 proteins and binds to IκBα protein in the cytoplasm. By various external stimuli, IκBα changed to phosphorylated IκBα and subsequently dissociated from complex of NF-κB/IκBα and then NF-κB translocated from the cytosol to the nucleus (Kim et al., 2016; Mitchell et al., 2016; Yang and Wang, 2016).

Mitogen-activated protein kinases (MAPKs) consist of p38, c-Jun NH_2-termimal kinase (JNK), and extracellular signal-regulated kinase (ERK), and associates with cell proliferation, differentiation, survival, and cell death (McCubrey et al., 2006; Dhillon et al., 2007; Kim and Choi, 2010). Since activated MAPK signaling pathways induced by various cellular stress has been associated with various human diseases, NF-κB and MAPK singling pathways are considered as targets for the development of therapeutic drugs (Cowan and Storey, 2003; Kim and Choi, 2010; Mitchell et al., 2016; Yang and Wang, 2016). Therefore, we examined the effects of Tat-DJ-1 proteins against oxidative stress-induced MAPK activation. Figure 6 demonstrates the marked increase of phosphorylated p38, JNK, and ERK expression levels in H_2O_2-treated cells. However, the levels were drastically reduced in WT Tat-DJ-1-treated cells, while the levels were unchanged in C106A Tat-DJ-1 and control DJ-1 protein-treated cells compared to cells exposed to H_2O_2 only.

Next, we examined the effects of Tat-DJ-1 protein on oxidative stress-induced NF-κB activation by Western blotting. Our results show that phosphorylated p65 and IκBα expression levels were increased by H_2O_2. In contrast, transduced WT Tat-DJ-1 protein significantly decreased phosphorylated p65 and IκBα expression levels. The expression levels of phosphorylated p65 and IκBα were unaltered in C106A Tat-DJ-1 and control DJ-1 protein-treated cells compared to H_2O_2-treated cells (Fig. 7). The combined results of our experiments suggest that WT Tat-DJ-1 protein, and not C106A Tat-DJ-1 protein, inhibit oxidative stress-induced pancreatic RINm5F cell death via regulation of NF-κB and MAPK activation.

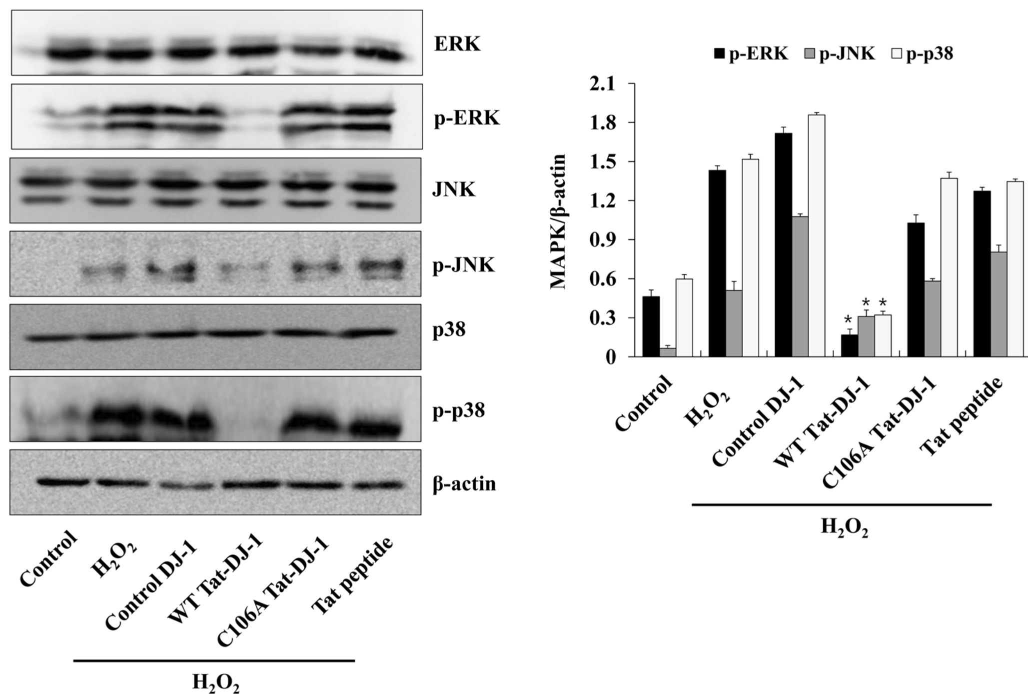


Fig. 6 Effects of WT and C106A Tat-DJ-1 protein against oxidative stress-induced MAPK activation. RINm5F cells were treated with WT and C106A Tat-DJ-1 (3 μM) for 1 h. MAPKs activation was detected using Western blot analysis and band intensity was measured by densitometer. $^{*}P < 0.05$, compared with H_2O_2-treated cells

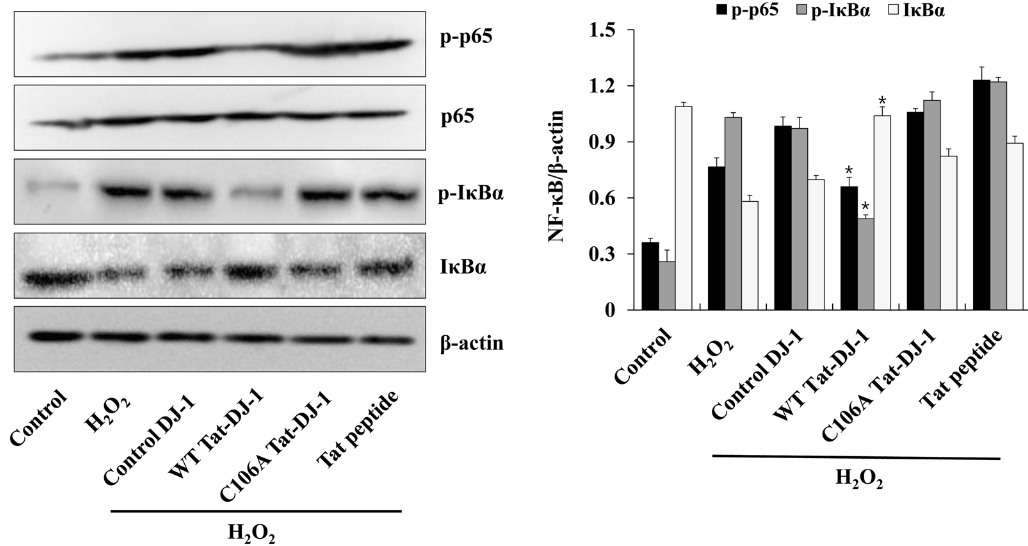


Fig. 7 Effects of WT and C106A Tat-DJ-1 protein against oxidative stress-induced NF-κB activation. RINm5F cells were treated with WT and C106A Tat-DJ-1 (3 μM) for 1 h. NF-κB activation was detected using Western blot analysis and band intensity was measured by densitometer. $^{*}P < 0.05$, compared with H_2O_2-treated cells

Conclusions

We have shown that WT Tat-DJ-1 protein transduced into RINm5F cells and markedly inhibited oxidative stress-induced cell death through a reduction in ROS generation and DNA fragmentation, as well as the activation of NF-κB and MAPK. In contrast, C106A Tat-DJ-1 and control DJ-1 protein did not show any protective effects against oxidative stress-induced cell death. Although further studies are needed in vivo, our results suggest a potential role for WT

Tat-DJ-1 protein as a therapeutic agent for oxidative stress-induced diseases including diabetes mellitus.

Materials and methods

Materials

Primary, secondary, and β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mutant C106A DJ-1 cDNA was obtained from Dr. Eun-Hye Joe (Ajou University). RINm5F cells, a pancreatic β-cell line, were purchased from the ATCC (Manassas, VA, USA). Tat peptides were purchased from PEPTRON (Daejeon, Korea). All other agents were of the highest grade available unless otherwise stated.

Purification and transduction of Tat-DJ-1 proteins into RINm5F cells

A Tat expression vector was prepared as described previously (Kim et al., 2013). After human DJ-1 cDNA were amplified by polymerase chain reaction using the sense primer 5′-CTCGAGGCTTCCAAAAGAGC-3′ and the antisense primer 5′-GGATCCCTAGTCTTTAAGAA-3′, the product was cloned in a TA-cloning vector and ligated into the Tat expression vector. Tat-DJ-1 proteins were cultured in *E. coli* and overexpressed with Isopropyl-b-D-thiogalactoside (IPTG). Then the cells were lysed and purified using Ni-NTA affinity and PD-10 column chromatography. Purified protein concentrations were estimated by the Bradford assay (Bradford, 1976).

RINm5F cells were grown in RPMI1640 medium containing 10 % fetal bovine serum and antibiotics (100 µg/ml streptomycin, 100 U/ml penicillin) at 37 °C in a humidity chamber with 5 % CO_2 and 95 % air. To examine Tat-DJ-1 protein transduction into RINm5F cells, the cells were treated with Tat-DJ-1 protein (1–3 µM) for 1 h or treated with Tat-DJ-1 protein (3 µM) for various times (10–60 min). The cells were then treated with trypsin-EDTA and washed with phosphate-buffered saline (PBS) and harvested for the preparation of cell extracts to perform Western blot analysis.

Western blot analysis

Equal amounts of proteins were separated with 12 % SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5 % nonfat dry milk in tris-buffered saline including Tween 20 (TBST) buffer (25 mM Tris-HCl, 140 mM NaCl, 0.1 % Tween 20, pH 7.5) for 1 h and incubated with the primary and secondary antibodies at room temperature. The indicated protein bands were detected using chemiluminescent reagents (Amersham, Franklin Lakes, NJ, USA) (Kim et al., 2013; Kim et al., 2015b).

Cell viability assay

To determine whether transduced Tat-DJ-1 protein has a protective effect against oxidative stress-induced cell death, RINm5F cells were treated with Tat-DJ-1 proteins (3 µM) for 1 h and H_2O_2 (0.3 mM) was added to the culture medium for 12 h. Then, cell viability was analyzed by a colorimetric assay using MTT as described in previous studies (Seo et al., 2015). Cell viability was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Labsystems Multiskan MCC/340) and cell viability was defined as the percentage of untreated control cells.

Insulin secretion measurement

We examined whether Tat-DJ-1 protein ameliorates insulin secretion in RINm5F cells under oxidative stress. The cells were treated with Tat-DJ-1 proteins (3 µM) for 1 h and H_2O_2 (0.3 mM) was added to the culture medium for 12 h. Then, insulin secretion levels were measured using an insulin ELISA kit (Shibayagi Co. Ltd, Japan).

ROS measurements

Intracellular ROS levels were measured using a ROS detection kit according to the manufacturer's instructions (Kim et al., 2015b; Shehzad et al., 2015). RINm5F cells were pretreated with Tat-DJ-1 protein (3 µM) for 1 h and treated with H_2O_2 (0.3 mM) for 30 min. Then, the cells were washed twice with PBS and fluorescence intensity was measured at 485 nm excitation and 538 nm emission using a Fluoroskan ELISA plate reader (Labsystems Oy, Helsinki, Finland).

Measurement of DNA damage

RINm5F cells were cultured in the presence or absence of Tat-DJ-1 proteins (3 µM) for 1 h and the cells were incubated with H_2O_2 (0.3 mM) for 6 h. Then, DNA damage was assessed using a Cell Death Detection kit (Roche Applied Science, Basel, Switzerland) as previously described (Kim et al., 2015b). Fluorescence-positive cells were counted by phase-contrast microscopy (×200 magnification) of the cells.

Analysis of NF-κB and MAPK activation

RINm5F cells were cultured in the presence or absence of Tat-DJ-1 proteins (3 μM) for 1 h and the cells were incubated in H_2O_2 (0.3 mM) for 4 h. The activation of MAPK and NF-κB were analyzed by Western blotting using indicated specific antibodies. The expression bands were quantified using Image J software (NIH, Bethesda, MD, USA).

Statistical analysis

Data represent as means ± standard error of the mean. Comparison between each groups were performed using one-way analysis of variance followed by the Dunnett's test using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was set at $P < 0.05$.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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