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Transduced Tat-glyoxalase protein attenuates streptozotocin-induced diabetes in a mouse model

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ABSTRACT

Diabetes mellitus (DM) is characterized by hyperglycemia. Glyoxalase 1 (GLO) has considerable potential as a possible therapeutic agent for DM. However, the precise action of GLO remains unclear in DM. In this study, we examined the protective effects of GLO protein in a streptozotocin (STZ)-induced diabetes animal model using cell-permeable Tat-GLO protein. Purified Tat-GLO protein was efficiently transduced into RINm5F cells in a time- and dose-dependent manner and protected cells against sodium nitroprusside (SNP)-induced cell death and DNA fragmentation. Furthermore, Tat-GLO protein significantly inhibited blood glucose levels and altered the serum biochemical parameters in STZ-induced diabetic mice. These results demonstrate that transduced Tat-GLO protein protects pancreatic cells by the inhibition of STZ-mediated toxicity. Therefore, Tat-GLO protein could be useful as a therapeutic agent against DM.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disease that is characterized by hyperglycemia resulting from the destruction of insulin-producing pancreatic β cells by a number of causes, including viruses, chemical toxins, diet, and autoimmune responses [1–3]. DM is a significant global health concern and associated with disturbances in carbohydrate, protein and fat metabolism leading to metabolic imbalances in tissues especially the pancreas [4,5]. Diabetic animal models exhibit high oxidative stress in pancreatic islets and oxidative stress plays a role in DM. Streptozotocin (STZ), an antibiotic produced by *Streptomyces achromogenes*, is a commonly used agent in experimental diabetes and the diabetogenic capacity of STZ may depend on its ability to damage β-cells and induce ROS [6–8].

Methylglyoxal (MG) is known to be the most important precursor in the formation of advanced glycation end products (AGEs) which play an important role in the pathogenesis of diabetes [9–11]. MG is detoxified by the glyoxalase system which is formed from GLO1 and GLO2 components. MG reacts with reduced glutathione to a hemithioacetal adduct and then to *S*-D-lactoylglutathione, which is then catalyzed by GLO1. This product is converted into D-lactate by GLO2, thereby reforming the consumed GSH [12–14]. Overexpression of GLO prevents MG and AGE formation in endothelial cells exposed to high doses of glucose suggesting that GLO plays an important role in AGE formation under hyperglycemic conditions [15]. Other groups have also reported that GLO overexpression decreased hyperglycemia-induced AGE formation and oxidative stress in *Caenorhabditiselegans* and diabetic rats [16–19].

Protein transduction technology allows the successful delivery of exogenous full-length fusion proteins into living cells *in vitro* and *in vivo* by protein transduction domains (PTDs) or cell penetrating peptides (CPPs). Among the cell-permeable peptides, Tat peptide is well-known for its ability to deliver exogenous proteins into cells [20,21]. Although Tat fusion proteins have been used to deliver therapeutic proteins *in vitro* and *in vivo*, the exact mechanism remains unclear. In previous studies, we have shown both *in vitro* and *in vivo* that various transduced fusion proteins efficiently protect against cell death [22–29].

In the present study, we examined the protective effect of Tat-GLO in oxidative stress-induced insulin producing RINm5F cells and STZ-induced diabetic mice. Our results demonstrated that Tat-GLO protein directly transduced and protected against cell death *in vitro* and *in vivo* leading us to suggest that Tat-GLO protein may be a potential therapeutic agent for diabetes mellitus.

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2. Materials and methods

2.1. Materials

RINm5F cells, an insulin-producing cell line, were purchased from the American Type Culture Collection (ATCC; USA). Sodium nitroprusside (SNP) and streptozotocin (STZ) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Ni^{2+}-nitrilotri-acetic acid Sepharose Superflow was purchased from Qiagen (Valencia, CA, USA). Human GLO cDNA were isolated using the polymerase chain reaction (PCR) technique. All other chemicals and reagents were of the highest analytical grade available.

2.2. Expression and purification of Tat-GLO proteins

A Tat expression vector was prepared in our laboratory as described previously [22]. The cDNA sequence for human GLO was PCR-amplified using the following sense and antisense primers: GLO sense primers, 5′-CTCGAGATGGCAGAACCGCAGCCCCCGTCC-3′; GLO antisense primer, 5′-GGATCCCTACATTAAGGTTGC-CATTTTGTT-3′. The resulting PCR product was sub-cloned in a TA cloning vector and ligated into the Tat expression vector in frame with six histidine open-reading frames to generate the expression vector, and was cloned into *Escherichia coli* DH5α cells.

The recombinant Tat-GLO plasmid was transformed into *E. coli* BL21 cells and induced with 0.5 mM IPTG at 37 °C for 3–4 h. Harvested cells were lysed by sonication and the recombinant Tat-GLO was purified using a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column and PD-10 column chromatography (Amersham, Piscataway, NJ, USA). The protein concentration was estimated by the Bradford procedure using bovine serum albumin as a standard [30].

2.3. Transduction of Tat-GLO protein into RINm5F cells

The RINm5F cells were cultured in RPMI1640 medium containing 2 mM of glutamine, 10% fetal bovine serum (FBS) and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37 °C under humidified conditions of 95% air and 5% CO_2.

For the transduction of Tat-GLO, RINm5F cells were treated with various concentrations of Tat-GLO (0.5–3 μM) for various durations (10–120 min). The cells were treated with trypsin–EDTA, washed with phosphate-buffered saline (PBS) and harvested for the preparation of cell extracts to perform Western Blot analysis.

2.4. Fluorescence microscopic analysis

RINm5F cells were grown on coverslips treated with 3 μM of Tat-GLO. Following incubation for 2 h at 37 °C, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 5 min at room temperature. The cells were permeabilized and blocked for 30 min with 3% bovine serum albumin, 0.1% Triton X-100 in PBS (PBS-BT) and washed with PBS-BT. The cells were then exposed to a primary antibody (His-probe, 1:2000; Santa Cruz Biotechnology) for 1 h at room temperature. The secondary antibody (Alexa fluor 488, 1:15,000; Invitrogen) was applied for 45 min at room temperature in the dark. Nuclei were stained for 5 min with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI; Roche, Basel, Switzerland). The distribution of fluorescence was analyzed by confocal microscopy using a model FV-300 microscope (Olympus, Tokyo, Japan).

2.5. Viability assay

The biological activity of the transduced Tat-GLO was assessed by measuring the cell viability of RINm5F cells treated with SNP. The cells were seeded into wells of six-well plates and grown to 70% confluence. The cells were then pretreated with Tat-GLO (0.5–3 μM) for 2 h before SNP (0.5 mM) was added to the culture medium for 5 h. Cell viability was estimated by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT). The absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay microplate reader (Labsystems Multiskan MCC/340), and cell viability was defined as the percentage of untreated control cells.

2.6. TUNEL assay

RINm5F cells were incubated in the absence or presence of Tat-GLO (3 μM) for 2 h, and then treated with SNP (0.5 mM) for 5 h. Terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated UTP nick end labeling (TUNEL) staining was performed using a Cell Death Detection kit (Roche Applied Science) according to the manufacturer's instructions. Images were taken using an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan).

2.7. Animals and experimental design

Male 6-week-old ICR mice were purchased from the Experimental Animal Center, at Hallym University, Chunchon, Korea. The animals were housed at a constant temperature (23 °C) and relative humidity (60%) with a fixed 12 h light:12 h dark cycle and 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 Hallym Medical Center Institutional Animal Care and Use Committee.

We examined whether Tat-GLO ameliorates the diabetic status of STZ-induced diabetic mice. Male ICR mice were divided into four groups (n = 7 per group). The experimental groups were as follows: Group 1, non-diabetic control mice; Group 2–4, STZ-induced diabetic mice; Group 3, pre-treated with Tat-GLO; Group 4, post-treated with Tat-GLO. Mice in Group 2 were given a single intraperitoneal (i.p.) injection of 120 mg/kg STZ freshly dissolved in 50 mM citrate buffer (pH 4.5) to induce diabetes, whereas mice in Group 1 were injected with an equivalent volume of citrate buffer. Mice in Group 3 were given a single injection of Tat-GLO (4 mg/kg) beginning 12 h prior to the induction of diabetes. Mice in Group 4 were injected three times (1, 3, and 5 days) after STZ injection with Tat-GLO (4 mg/kg). The mice were killed by cervical dislocation 7 days after STZ injection, and the pancreata were dissected for histological examinations. For observation of pancreatic β-cells, tissue sections were incubated with an anti-mouse insulin IgG (InnoGenex, USA; dilution 1:300) and stained with a peroxidase/DAB system kit (Dako EnVision kit, Glostrup, Denmark).

2.8. Blood analytical measurement

Blood glucose from the tail plexus was assayed using AccuChek glucose strips and a refractance meter (Roche, Germany). To minimize the effects of diurnal fluctuations, blood samples were collected at the same time every day. The serum insulin (Shibayagi, Japan), alkaline phosphatase (ALP; Asan Pharmaceutical, Korea), and free fatty acid (FFA; Bioassay system, USA) levels were measured using a commercially available assay kit.

2.9. Statistical analysis

Statistical analysis was performed using the student's t-test and ANOVA. P values of <0.05 were considered statistically significant.

3. Results

3.1. Construction and purification of Tat-GLO fusion protein

To generate a cell permeable expression Tat-GLO vector, a human GLO cDNA was subcloned into a pET-15b plasmid that had been reconstructed to contain the Tat peptide. The Tat-GLO expression vector contained consecutive cDNA sequences encoding human GLO, Tat peptide and six histidine residues at the amino-terminus. A GLO expression vector was also constructed to produce control GLO without Tat transduction peptides (Fig. 1A).

Following the induction of expression, Tat-GLO proteins were purified using a Ni^{2+}-nitrilotriacetic acid Sepharose affinity column and PD-10 column chromatography. SDS–PAGE and Western Blot analysis of the purified Tat-GLO protein were performed (Fig. 2). The purified Tat-GLO was confirmed by Western Blot analysis using an anti-rabbit polyhistidine antibody.

3.2. Transduction of Tat-GLO into RINm5F cells

We analyzed the transduction of Tat-GLO protein by adding 3 μM of Tat-GLO protein to a RINm5F cell culture medium at for various periods of time (10–120 min), and assessed the levels of transduced proteins by Western Blotting. The intracellular concentration of transduced Tat-GLO proteins in the cells was detected within 10 min and gradually increased to 120 min. The dose-dependency of the transduction of Tat-GLO was also analyzed. Various concentrations (0.5–3 μM) of Tat-GLO proteins were added to a culture medium for 120 min and the levels of transduced proteins were measured by Western Blotting. As shown in Fig. 2C, Tat-GLO proteins efficiently transduced into RINm5F cells in time- and dose-dependent manners. However, the control GLO protein did not transduce into the cells. Also, the intracellular stability of transduced Tat-GLO protein in RINm5F cells is shown in Fig. 2C. Tat-GLO proteins were added to the culture medium at a concentration of 3 μM for various times and the resulting levels of transduced protein were analyzed by Western Blotting. The intracellular level of transduced Tat-GLO proteins in cells was initially detected after 1 h. The level declined gradually over the period of observation. However, significant levels of transduced Tat-GLO proteins persisted in the cells for 48 h.

To further clarify the cellular localization of transduced proteins in the cells, the transduced cells were double stained with the nucleus-specific marker DAPI. As shown in Fig. 2D, Tat-GLO protein was detected in the cytoplasm and in the nucleus of transduced cells.

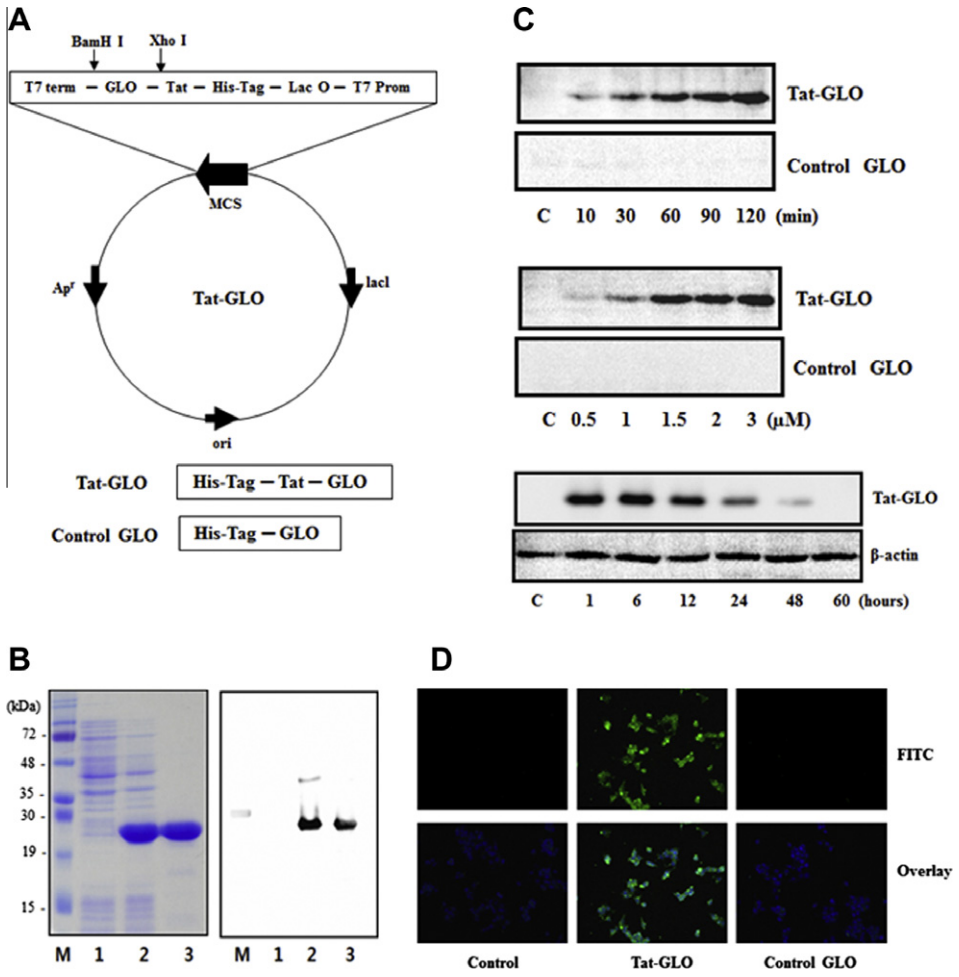

Fig. 1. Purification and transduction of Tat-GLO protein. (A) Construction of the Tat-GLO expression vector system based on the vector pET-15b. Diagram of expressed control GLO and Tat-GLO proteins. The coding frame of human GLO is represented by an open box along with 6 His and the Tat peptide. Expression was induced by adding IPTG. (B) After induction, purified Tat-GLO proteins were analyzed by 12% SDS–PAGE and subjected to Western Blot analysis with an anti-rabbit polyhistidine antibody. Lanes are as follows: lane 1, non-induced Tat-GLO; lane 2, induced Tat-GLO; lane 3, purified Tat-GLO. (C) Transduction of Tat-GLO proteins into RINm5F cells. Tat-GLO and control GLO (3 μM) were added to the culture media for 10–120 min, Tat-GLO and control GLO (0.5–3 μM) were added to the culture media for 2 h, and cells pretreated with 3 μM Tat-GLO were incubated for 1–60 h and analyzed by Western blot. (D) Cellular localization of Tat-GLO protein. The distribution of transduced Tat-GLO was observed by microscopy. After transduction of Tat-GLO, the cells were examined by DAPI and Alexa staining.

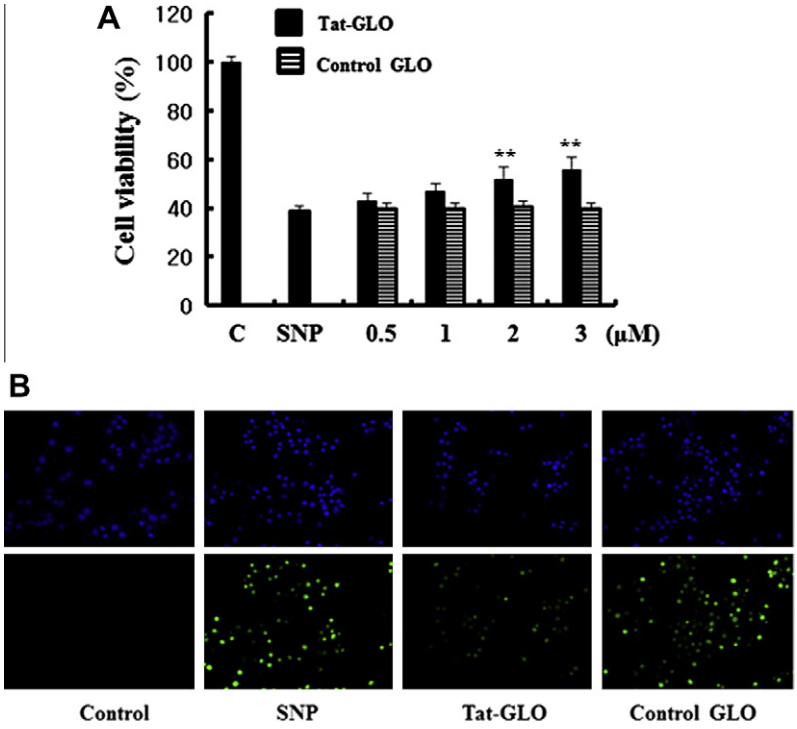

Fig. 2. Effect of transduced Tat-GLO protein on cell viability. (A) SNP (0.5 mM, 5 h) was added to RINm5F cells pretreated with Tat-GLO and control GLO (0.5–3 μM) for 2 h. Cell viabilities were estimated by with a colorimetric assay using MTT. $^{**}P < 0.05$ compared with SNP-treated cells. (B) Protective effect of Tat-GLO protein against SNP-induced DNA fragmentation in RINm5F cells. The cells were treated with Tat-GLO (3 μM) for 2 h, and then exposed to SNP (0.5 mM) for 5 h and 24 h respectively, and then DNA fragmentation was detected by TUNEL staining.

3.3. Transduced Tat-GLO protein protects RINm5F cells from SNP-induced cytotoxicity

To determine the cytoprotective effects of Tat-GLO protein on RINm5F cells, we examined the effect of Tat-GLO protein on sodium nitroprusside (SNP) induced cell death. As shown in Fig. 2A, when the cells were exposed to SNP (0.5 mM) for 5 h, only 39% of cells were viable. However, the cell viability of cells pretreated with Tat-GLO protein increased in a dose-dependent manner, reaching >56% at the maximum concentration used. However, control GLO protein showed no protective effects under the same conditions, indicating that transduced Tat-GLO protein plays a defensive role against SNP-induced cell death without a cytotoxic effect.

We further examined whether Tat-GLO protein inhibits DNA fragmentation. The protective effect of transduced Tat-GLO protein against DNA fragmentation was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. As shown in Fig. 2B, the number of stained cells among SNP treated cells increased markedly compared with that in the control group, whereas the number of cells treated with Tat-GLO protein that were stained decreased significantly. However, control GLO protein treated cells were similarly stained as SNP-treated cells.

3.4. Effect of transduced Tat-GLO protein in STZ-induced diabetic mice

The blood glucose levels of control mice in a fasting state were about 85 mg/dl throughout the week-long experimental period. In the STZ-induced diabetic groups, a single injection of 120 mg/kg streptozotocin (STZ) markedly increased blood glucose levels from day 1, reaching a maximum level of about 410 mg/dl at 7 days after the initial STZ injection. These high glucose levels were maintained throughout the experimental period. However, these increased glucose levels were considerable lowered by single or multiple injections of Tat-GLO protein. The blood glucose levels of the Tat-GLO-treated mice were significantly decreased compared to those of the STZ-injected diabetic mice (Fig. 3A). Also, morphological studies showed that Tat-GLO protein efficiently protected pancreatic β-cells in STZ-induced diabetic mice. As shown in Fig. 3B, a single injection of STZ (120 mg/kg) induced severe pancreatic β-cell destruction. As a result, the masses of Langerhans' islet were markedly reduced and their overall shape became non-spherical and irregular in appearance. This cellular destruction was prevented by injections of Tat-GLO protein.

Next, the effects of Tat-GLO protein on insulin, alkaline phosphatase (ALP), and free fatty acid (FFA) secretion were examined in STZ-induced diabetic mice. As shown in Fig. 4, serum insulin levels of STZ-induced diabetic mice groups were significantly lower than those of the control group. However, serum insulin levels of the Tat-GLO protein-treated groups were markedly increased than compared to STZ-induced diabetic group. ALP and FFA levels of STZ-induced diabetic groups were significantly higher than those of the control group. The Tat-GLO protein-treated groups demonstrated markedly decreased levels ALP and FFA levels.

4. Discussion

Recent studies have indicated that reactive dicarbonyls or α-oxoaldehydes, such as methylglyoxase (MG), glyoxal (GO) and 3-deoxyglucose (3-DG), are important precursors of AGEs, and high levels of which have been identified in diabetic tissues [32,33]. Furthermore, α-oxoaldehydes are known to be elevated in diabetic patients [34]. GLO enzymes play an important role in cells by suppressing AGE formation in various chronic diseases including diabetes, hyperglycemia and Alzheimer's disease, as well as in aging [12,13,16]. Although these enzymes are considered to be potential therapeutic agents for diabetes mellitus, the inability of the enzymes to transduce into cells has limited their use for this purpose.

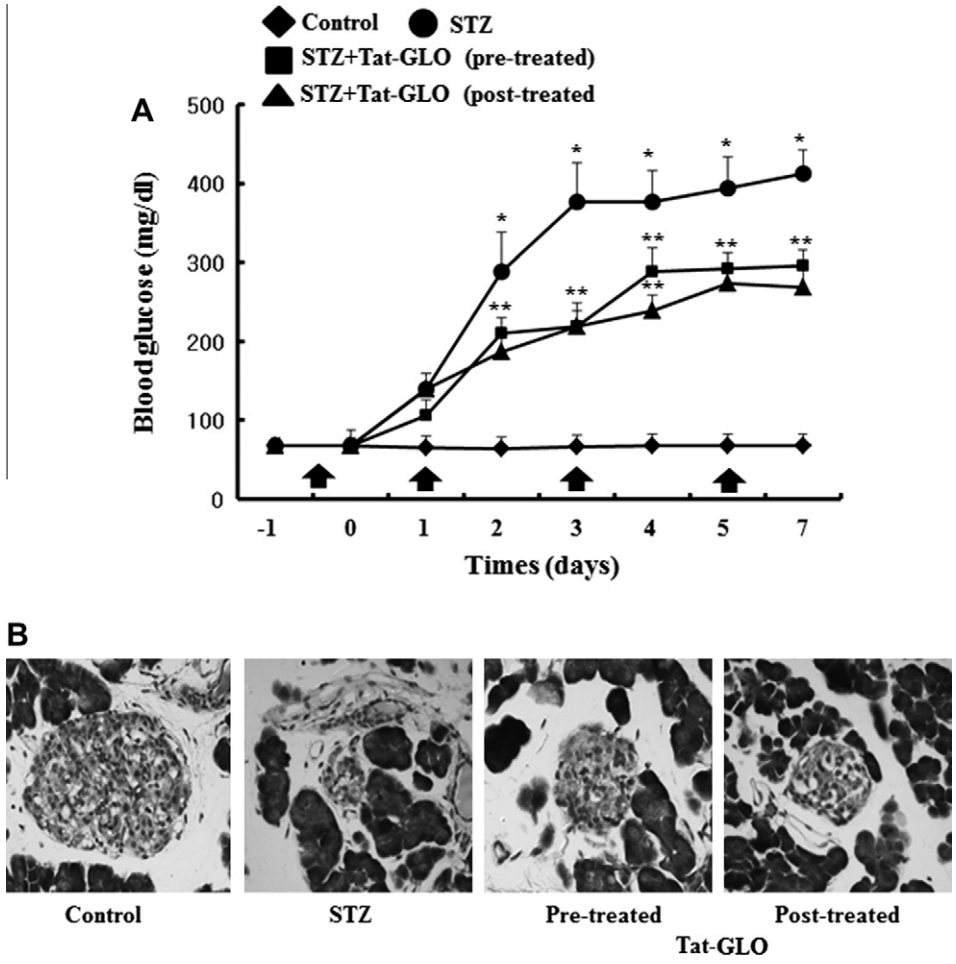

Fig. 3. Protective effect of Tat-GLO protein in STZ-induced diabetic mice. (A) Changes in blood glucose levels of control, STZ-induced diabetic mice, and Tat-GLO treated mice. Diabetes was induced by a single i.p. injection of 120 mg/kg STZ in mice. STZ-induced diabetic mice were injected single (pre-treated) or three (post-treated) times with 4 mg/kg Tat-GLO protein. $^{*}P < 0.01$ versus untreated control group. $^{**}P < 0.01$ versus the relevant STZ-induced diabetic group. (B) Light micrographs of mouse pancreas showing insulin positive cells. Tissue sections were incubated with an anti-mouse insulin antibody, stained with a peroxidase/DAB system, and observed under light microscopy.

Therefore, we investigated their protective effects against SNP-induced cell death and STZ-induced diabetic mice using cell permeable Tat-GLO protein.

The Tat-GLO protein was highly expressed as a major component of the total soluble proteins in cells; the expressed protein was nearly homogeneous and more than 95% pure, as determined by SDS–PAGE. Purified Tat-GLO protein was efficiently transduced into RINm5F cells in a time- and dose-dependent manner.

We examined the effect of transduced Tat-GLO protein on the cell viability of SNP-treated RINm5F cells. Cell viability increased when the cells were pretreated with Tat-GLO protein. Also, we examined the ability of transduced Tat-GLO protein to inhibit SNP-induced DNA fragmentation. Transduced Tat-GLO protein efficiently protected against SNP-induced DNA fragmentation. Brouwers et al. (2011) have demonstrated that overexpression of GLO protein decreased MG, GO, AGE, and reactive oxygen species (ROS) levels under hyperglycemic conditions in animal models thus suggesting that GLO overexpression and functions could be important targets in the prevention of diabetes [13]. Schlotterer et al. (2009) has shown that overexpression of GLO in *C. elegans* decreased hyperglycemia-induced accumulation of AGEs and oxidative stress and enhanced lifespan [19]. Also, Kim et al. (2012) have shown that GLO overexpression exerts protective effects on high glucose-induced AGE formation, causing an interruption of events leading to oxidative stress [14]. Our results were similar to those obtained by other experiments involving the overexpression of GLO.

Streptozotocin (STZ) has a β-cell cytotoxicity and it is one of the most commonly used substances to induce diabetes in experimental animals [35,36]. STZ does irreversible damage to pancreatic β-cells and causes loss of insulin secretion [37]. To examine the ability of transduced Tat-GLO protein to protect against STZ-induced diabetes in animal models, we injected Tat-GLO protein in STZ-induced diabetic animals. The protective effects of Tat-GLO protein were confirmed by blood glucose levels, immunohistochemistry and insulin secretion levels. Blood glucose levels in Tat-GLO protein treated diabetic mice were about half those of STZ-induced diabetic mice. Immunohistochemistry revealed that Tat-GLO protein ameliorates pancreatic β-cell damage in STZ-injected mice. Also, insulin secretion levels were enhanced by treatment with Tat-GLO protein. These results indicate that Tat-GLO protein attenuates pancreatic β-cell destruction induced by STZ. Chronic hyperglycemia is the main factor in the development and progression of DM. High glucose levels lead to the function of insulin secretion and many proteins [38–40]. In addition, several studies have shown that overexpression of GLO reduces the hyperglycemia, AGEs and oxidative stress in diabetic animal models [13,14,31,41,42]. These protective effects are in agreement with our results. Although the detailed mechanism remains to be further elucidated, Tat-GLO protein may have value as a therapeutic agent against DM progression.

In summary, we have demonstrated that Tat-GLO protein inhibits its SNP-induced RINm5F cell death and STZ-induced pancreatic

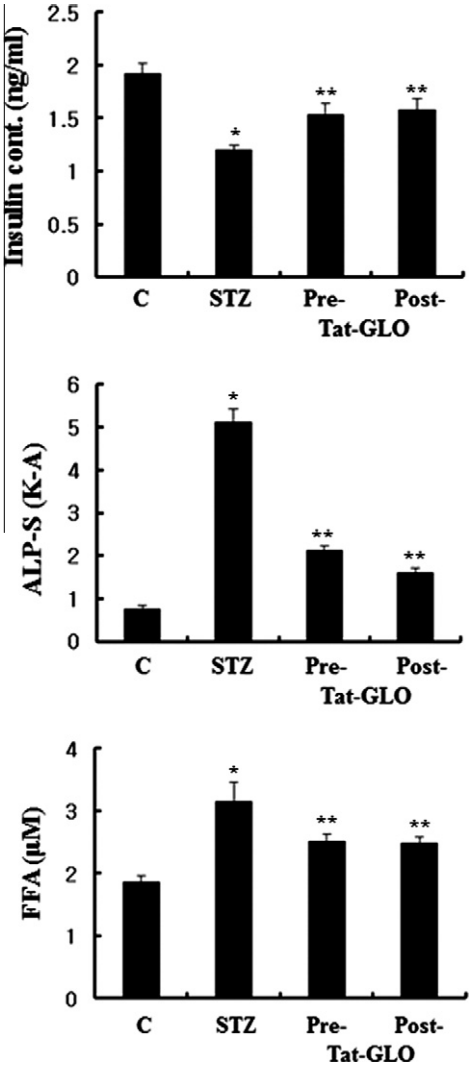

Fig. 4. Effect of Tat-GLO protein on blood parameters in STZ-induced diabetic mice. Serum levels of insulin, ALP, and FFA were determined using assay kit, respectively. $^{*}P < 0.01$ versus untreated control group. $^{**}P < 0.01$ versus the relevant STZ-induced diabetic group.

β-cell destruction in diabetic mice. Therefore, our results indicate that Tat-GLO protein may provide a new strategy for protecting against cell destruction and may provide a therapeutic agent for the treatment of DM and diabetic complications.

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