Biochemical and Biophysical Research Communications 430 (2013) 294–300

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 27 October 2012
Available online 14 November 2012

Keywords:
Tat-GLO
Blood glucose
Diabetes mellitus
Insulin
Protein therapy

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.

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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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http://dx.doi.org/10.1016/j.bbrc.2012.10.134

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′-GGATCCCTACATTAAGGTTGCCATTTTGTT-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.

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