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Deficiency of voltage-gated proton channel Hv1 attenuates streptozotocin-induced β-cell damage

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ABSTRACT

Reactive oxygen species (ROS) impairs pancreatic β-cells and plays an important role in development of diabetes. Streptozotocin (STZ) can lead to β-cell dysfunction via inducing ROS production. The voltage-gated proton channel Hv1 contributes a majority of the charge compensation required for ROS production. Here, we investigated the effects of Hv1 on STZ-induced β-cell damage. We found that deficiency of Hv1 obviously inhibits STZ-induced glucose intolerance in mice, and prevents the decrease in β-cell mass and pancreatic insulin content from STZ-treatment. Further studies showed that loss of Hv1 significantly attenuates STZ-induced β-cell damage and ROS production in pancreatic β-cells. Our results suggest that Hv1 might contribute to development of diabetes through producing ROS.

1. Introduction

Diabetes is a serious metabolic disease resulting from absolute or relative insulin deficiency. As insulin-producing cells, the dysfunction of pancreatic β-cells is considered as a major factor contribute to type 1 diabetes (T1D) and type 2 diabetes (T2D). T1D is a cytotoxic T cell-mediated antigen-specific process, leading to increased cytokine and reactive oxygen species (ROS) production and destruction of β-cells [1]. T2D is mainly caused by the insufficient insulin to overcome insulin resistance, finally resulting in hyperglycemia [2]. Under high glucose conditions, a persistent state of excessive production of ROS has been proposed to be a contributor to the development of complications and further destruction of β-cells [3,4]. Streptozotocin (STZ) has been usually used as a reagent which induces diabetes in rodents and to study the mechanism of the development of diabetes [5]. STZ-treatment can induce direct β-cells destruction and the indirect β-cells destruction from T-cell-dependent immune reaction [6], both two ways generate ROS which may facilitate the destruction [7].

The voltage-gated proton channel Hv1 has been demonstrated to contribute to ROS production cooperated with NADPH oxidase, an enzyme which moves electrons across membranes, in many cell types [8]. In previous study [9], we found that Hv1 is expressed in pancreatic islet β-cells and regulates insulin secretion, but the role of Hv1 in the ROS production and in the development of diabetes remains to be determined.

In the present study, we used Hv1 knockout mice and INS-1(832/13) cell line to investigate the role of Hv1 in STZ-treated β-cell destruction. We demonstrated that Hv1-deficiency protects the β-cells from STZ damage through decreasing ROS production.

2. Materials and methods

2.1. Animals and treatments

Mice bearing a targeted disruption in the VSOP/Hv1 (VSOP/$Hv1^{-/-}$, backcrossed eight times) were kindly provided by Dr. Y. Okamura (School of Medicine, Osaka University), as previously described [10]. WT mice (VSOP/$Hv1^{+/+}$) were of the same genetic background (C57BL/6J). Animals were kept in a pathogen-free facility under a 12-h light-dark cycle with access to water and a standard mouse diet (Lillico Biotechnology). Genotyping was

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performed by PCR as described by Ramsey et al. [11]. Experiments were performed with 2 month-old male mice, unless indicated otherwise. All animal husbandry and experiments were approved by and performed in accordance with guidelines from the Animal Research Committee of Nankai University.

2.2. Isolation of pancreatic islets

Pancreatic islets were isolated according to the collagenase digestion method described by Lacy and Kostianovsky [12], with slight modifications. Krebs-Ringer bicarbonate HEPES (KRBH) buffer (in mM: 135 NaCl, 3.6 KCl, 5 $NaHCO_3$, 0.5 NaH_2PO_4, 0.5 $MgCl_2$, 1.5 $CaCl_2$, 10 HEPES, pH 7.4) was used for islet isolation. And the isolated islets were cultured overnight in RPMI 1640 (GIBCO) containing 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37 °C before handpicking for experiments.

2.3. Cell culture

Pancreatic islet β-cell line, INS-1 (832/13) cells were obtained from Dr. Hans Hohmeier (Duke University) and grown in RPMI 1640 medium (GIBCO) supplemented with 10% FBS, 2 mM glutamate, 1 mM sodium pyruvate and 55 μM β-mercaptoethanol in a humidified 5% CO_2 atmosphere at 37 °C.

2.4. siRNA silencing

To down-regulate Hv1 expression level in INS 1(832/13) cells, the sequences of the small interfering RNA (siRNA) targeting the Hv1 gene 5′-CTACAAGAAATGGGAGAAT-3′ and the scramble sense sequence 5′-TTCTCCGAACGTGTCACGT-3′, which were obtained from Ribobio (Guangzhou, China), were used, as described previously [9].

2.5. Measurement of ROS

Total ROS production in isolated islets and INS-1 (832/13) cells was determined by dichlorofluorescein diacetate assay (DCF). Islets and cell line were homogenized in 100 μl KRBH buffer and incubated with 50 μmol/l DCF at 37 °C 30 min. Fluorescence was measured by excitation (490 nm) and emission (525 nm) spectra with a fluorescent microscope (OLYMPUS).

2.6. STZ-treatment of mice and cell line

To induce diabetes, WT, $Hv1^{+/-}$ and $Hv1^{-/-}$ mice were injected with STZ (50 mg/kg body weight i.p. for 5 consecutive days). Additionally, some WT, $Hv1^{+/-}$ and $Hv1^{-/-}$mice were treated with saline as vehicle controls. Blood glucose levels were measured once every two days with fasting 6 h after the final injection of STZ. At the day 9, an i.p. glucose tolerance test (IPGTT) was performed using an i.p. injection of glucose at 2 g/kg body weight after 6 h fasting. Blood glucose was analyzed at 0, 15, 30, 60, and 120 min after introducing glucose. Diabetic hyperglycemia was defined as a fasting blood glucose concentration >11.1 mmol/l for two or more consecutive tests. The cell line was incubated in medium containing 0.5 mmol/l STZ.

2.7. Immunohistochemistry, β-cell mass, proliferation and apoptosis

Pancreases from $Hv1^{-/-}$ and WT mice after finally injected by STZ for 4 days were fixed in 4% paraformaldehyde for 4 h, embedded in paraffin, and cut into 5 μm sections. Immunohistochemistry was carried out with anti-mouse insulin monoclonal antibody (dilution 1:200, Abcom). Relative β-cell mass was determined as a ratio of total insulin-positive area to total pancreatic area. Proliferation was determined as a ratio of total Ki67-positive area to total pancreatic area. Apoptosis was determined as a ratio of total TUNEL-positive area to total pancreatic area by DeadEnd™ Colorimetric TUNEL System (Promege, USA), according to the manufacturer's protocol.

2.8. Cell survival rate

The survival rates of cell line were measured by MTT assay as described previously [13]. Briefly, cells were plated in 96-well plates at a concentration of 5×10^4 cells/ml at 100 μl per well in RPMI 1640 medium. The next day, the medium in 96-well plates was replaced by the KRBH buffer. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed after the cells were kept in culture at 37 °C for 30 min.

2.9. Insulin determinations

Total insulin content in isolated islets and INS-1 (832/13) cells were extracted with acidic ethanol and determined using rat/mouse insulin ELISA (Mercodia, Uppsala), according to the manufacturer's protocol.

2.10. Statistical analysis

All statistics were performed using SPSS 20.0 software. Measurement data were represented as mean ± SEM. Comparison of the mean between groups was performed by *t*-test. P values < 0.05 were considered significant.

3. Results

3.1. Deficiency of Hv1 attenuates STZ-induced β-cell damage

STZ is a natural compound which could destruct β-cells and cause hyperglycemia and glucose intolerance, so the STZ-induced diabetic mouse model is widely used to inspect protective properties of the proteins against β-cell death [14]. To investigate the effect of Hv1 on STZ-treated β-cell damage, we injected WT, $Hv1^{+/-}$ and $Hv1^{-/-}$ mice with STZ (50 mg/kg body weight for consecutive 5 days). On fifth day after STZ injection, the blood glucose levels were increased both in WT and $Hv1^{-/-}$ mice (Fig. 1A), but the WT mice appeared to more serious glucose intolerance compared to $Hv1^{-/-}$ mice (Fig. 1B).

In WT islets, the functional β-cell mass was greatly decreased, whereas, in $Hv1^{-/-}$ mice, the β-cell mass appeared to be protected from STZ treatment (Fig. 1C, left panel). The pancreatic β-cell masses in both $Hv1^{+/-}$ and $Hv1^{-/-}$ mice analyzed from immunohistochemistry were obviously higher than that of WT after STZ-treatment five days (Fig. 1C, right panel). Under a normal condition (without STZ), the pancreatic insulin contents of $Hv1^{+/-}$ and $Hv1^{-/-}$ mice were lower than that of WT mice (data not shown). However, after STZ-treatment, the pancreatic insulin content of WT mice was significantly lower than that of both $Hv1^{+/-}$ and $Hv1^{-/-}$ mice (Fig. 1D). Our results demonstrated that loss of Hv1 attenuates STZ-induced β-cell damage.

3.2. Deficiency of Hv1 prevents pancreatic β-cell apoptosis from STZ-treatment

To detect whether the loss of Hv1 prevents β-cell death from STZ-treatment, we measured β-cell apoptosis by TUNEL staining. Compared with WT mice, the apoptosis of β-cells in $Hv1^{-/-}$ mice was markedly reduced by 70% after STZ-treatment (Fig. 2A), while

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