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Dynamin-related protein 1 mediates high glucose induced pancreatic beta cell apoptosis

Xiuli Men[a], Haiyan Wang[b], Mi Li[c], Hanqing Cai[a], Shiqin Xu[a], Wenjian Zhang[a], Yaping Xu[a], Liya Ye[a], Wenying Yang[a], Claes B. Wollheim[b], Jinning Lou[a,*]

[a] Institute of Clinical Medical Sciences, China-Japan Friendship Hospital, Beijing 100029, PR China
[b] Department of Cell Physiology and Metabolism, University Medical Center, 1 Rue Michel-Servet, CH-1211 Geneva 4, Switzerland
[c] The Fifth Hospital of Sun Yat-Sen University, Zhuhai 519000, PR China

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ABSTRACT

The pancreatic beta cell dysfunction is critical cycle in the pathogenesis of diabetes. Hyperglycemia is one of factors that induce pancreatic beta cell dysfunction, but the underlying mechanisms have not been well elucidated. In this study, we reported that a mitochondrial fission modulator, Dynamin-related protein 1 (Drp-1), plays an important role in high glucose induced beta cell apoptosis. Drp-1 expressed in islet beta cells was increased drastically under hyperglycemia conditions. Induction of Drp-1 expression significantly promoted high glucose induced apoptosis in Drp-1WT (Drp-1 wild type) inducible beta cell line, but not in Drp-1K38A (a dominant negative mutant of Drp1) inducible beta cell line. We further demonstrated that mitochondrial fission, cytochrome C release, mitochondrial membrane potential decreased, caspase-3 activation and generation of reactive oxygen species were enhanced by induction of Drp-1WT, but prevented by Drp-1K38A in pancreatic beta cells under high glucose condition. These results indicated that Drp-1 mediates high glucose induced pancreatic beta cell apoptosis.

1. Introduction

Type 2 diabetes is characterized by both insulin resistance and beta cell dysfunction (Maedler et al., 2002; Donath et al., 1999). It has been demonstrated that hyperglycemia is one of the factors that induced beta cell dysfunction, but the underlying mechanisms have not been well elucidated. It has been proposed that impaired mitochondrial function might be involved cell apoptosis (Pick et al., 1998; Koyama et al., 1998; Fine et al., 1999). Mitochondria play a critical role in the process of cell apoptosis, since the release of pro-apoptotic proteins from the organelle is a pivotal event. It has been reported that a striking morphological change during apoptosis is disintegration of the semi-reticular mitochondrial network into small punctiform organelles, even resulting in their disappearance (Youle and Karbowski, 2005; Martinou and Youle, 2006).The GTP-binding protein, dynamin-related protein 1 (Drp-1) is a mitochondrial fission protein. It has been found that expression of Drp-1 promotes mitochondrial fragmentation, while the expression of a dominant-negative form of Drp-1 inhibits mitochondrial fission and thereby apoptosis (Youle and Karbowski, 2005; Martinou and Youle, 2006).

Recently, Leinninger et al. found that high glucose increase Drp-1 expression and yield Drp1-induced mitochondrial fission resulting in mitochondrial fragmentation in dorsal root ganglia neurons (Leinninger et al., 2006). Drp-1 dominant-negative mutant impedes fission and causes assembly of elongated mitochondria in networks (Smirnova et al., 2001, 1998). However, to our knowledge, the effect of Drp-1 on high glucose-induced beta cell apoptosis has not been investigated so far.

In order to clarify the role of Drp-1 in pancreatic beta cell glucotoxicity, we firstly checked whether the expression of Drp-1 affected by high glucose either in vivo in islets of GK rats or in vitro in pancreatic beta cell line (INS-1). We then established two stable pancreatic beta cell lines, permitting respectively, inducible expression of wild-type Drp-1 (Drp-1WT) and its dominant-negative mutant (Drp-1K38A) to investigate the role of Drp-1 on high glucose induced beta cell apoptosis. Finally, the related mechanisms of Drp-1 mediating pancreatic beta cell apoptosis were further analyzed through the assessment of various morphological and functional parameters.

* Corresponding author. Tel.: +86 10 84250016; fax: +86 10 64206643.
E-mail address: Lou.j@mail.com (J. Lou).

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2. Materials and methods

2.1. Real-time PCR and immunohistochemistry

A 8- and 16-week old, male diabetic GK and age- and sex-matched non-diabetic Wistar rats were obtained from Shanghai SLAC laboratory animal Co. Ltd. The Goto-Kakizaki (GK) rat is a spontaneously diabetic animal model of non-insulin-dependent diabetes mellitus, which is characterized by progressive loss of beta cell mass and function in the pancreatic islets. Nonfasting blood glucose concentrations were measured in 8-, 16-week old diabetic GK and non-diabetic Wistar rats respectively. Pancreatic tissues and islets were isolated respectively from the three type rats for immunohistochemistry and RNA extraction. The animal experimental procedure is in agreement with the animal ethical committee of our institution.

Total RNA samples respectively from isolated islets of diabetic GK rats and non-diabetic Wistar rats were analyzed by real-time PCR. PCR reactions were carried out in a volume of 25 μl consisting of 2.5 μl cDNA, 12.5 μl reaction mix (SYBR Green I dye, Toyobo) and 0.5 μl 25 pmol/l oligonucleotide primers. All reactions were performed in an Applied Biosystems (ABI) in which samples underwent 40 cycles of PCR with an annealing temperature of 55 °C. The following primers were used (forward and reverse, respectively): ATTGCGGATTCACTACTC and GATTTCTACTGCGACCATA (Drp-1), GACATCCGTAAAGACCTCTATGCC and AATAGAGCCACCAATCCACACAGAG (β-actin). The value obtained for each specific product was normalized to a control gene (β-actin) and expressed as a percentage of the value in control gene.

The pancreas were excised, fixed in 4% paraformaldehyde, and processed for paraffin embedding. Pancreatic tissue sections (5 μm) were immunostained using mouse anti-Drp-1/DLP1 (BD, Transduction Laboratories; USA. 1:100 dilution), biotinylated secondary antibodies and horseradish peroxidase–conjugated antibiotin using an ABC-Peroxidase kit (Vector Laboratories, Burlingame, CA) according to the manufacture's protocols. The results were viewed under a light microscope (Olympus, IX71 Japan).

2.2. Establishment of INS-1 stable cell lines allowing inducible expression of Drp-1WT and Drp-1K38A

The plasmids were constructed by subcloning the cDNAs encoding, respectively, Drp-1WT and Drp-1K38A. The dominant-negative mutant Drp-1K38A, changes the critical lysine in the G1 consensus motif of the GTPase domain into an alanine, thereby presumably inhibiting GTP binding of Drp-1. This mutation was used in a previous study of mammalian Drp-1 (Smirnova et al., 1998). The rat insulinoma cell line, INS-1-rβ (also referred to as r9), which carry the reverse tetracycline/doxycycline-dependent transactivator (Gossen et al., 1995), were generated using the procedures described previously (Wang and Iynedjian, 1997; Wang et al., 2001).

The r9, Drp-1WT and Drp-1K38A cell lines were cultured in complete medium composed of RPMI1640 (GIBCO) supplemented with 10 mM HEPES, 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol. Cells were incubated in 37 °C and 5% CO_2 incubator (Asfari et al., 1992) and changed medium every 3 days.

2.3. Immunofluorescence staining and Western blotting

The cells were planted in a 96-well plate and cultured in 37 °C and 5% CO_2 incubator for 48 h, and then treated with or without 500 ng/ml doxycycline for induction of Drp-1WT and Drp-1K38A expression, respectively. After 72 h incubation, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS containing 0.5% BSA (PBS–BSA) for 30 min. The cells were subsequently incubated with anti-Drp-1/DLP1 antibody (BD, Transduction Laboratories; USA. 1:100 dilution) for 30 min, followed by labeling with FITC-conjugated rat anti-mouse IgG antibody. The cells were viewed under a fluorescent microscope (Olympus, IX71 Japan).

For Western blotting, cells were cultured in flasks for 48 h before treated with indicated concentrations of doxycycline at specified time under either 11.2 mmol/l or 30 mmol/l glucose conditions. All these cells were collected and lysed by sonication. The protein was extracted in buffer containing 20 mM Tris–HCl, pH 7.4, 2 mM EDTA, 150 μM NaCl, 10 mM NaP, 1% NP-40, and 1 mM phenylmethylsulfonyl fluoride. Total cellular proteins were fractionated by 10% SDS–PAGE. Immunoblotting was performed with anti-Drp-1 antibody as described previously (Wang et al., 1998) using enhanced chemiluminescence (Millipore, Billerica, USA) for detection.

2.4. Confocal microscopy

The cells were treated with or without 500 ng/ml doxycycline for 96 h in medium containing 11.2 mmol/l or 30 mmol/l glucose in 37 °C and 5% CO_2 incubator and then incubated with 1 μM MitoTracker Red at 37 °C for 15 min. The cells were fixed with 4% formaldehyde for 15 min at room temperature. The cells were stained with anti-cytochrome C antibody and FITC conjugated antibody as previously described (Santel and Fuller, 2001). The cells were viewed under a confocal microscope (Olympus, IX81 Japan). Multitrack scanning mode was used to record single-labeled cells and double-labeled cells.

2.5. Electron microscopy

Drp-1WT cells and Drp-1K38A cells were cultured to confluence in cell culture inserts in 37 °C, 5% CO_2 incubator. The cells were treated with or without 500 ng/ml doxycycline for 4 days in medium containing 11.2 mmol/l or 30 mmol/l glucose. The cells were fixed with 2.5% (v/v) glutaraldehyde for 30 min. The cells were dehydrated, embedded, sectioned and then visualized by a JEM-1010 transmission electron microscopy (JEOL, Tokyo, Japan).

2.6. Measurements of insulin secretion and cellular insulin content

Drp-1WT and Drp-1K38A cells were planted in 24-well plates and cultured at a 37 °C and 5% CO_2 incubator in medium with or without 500 ng/ml doxycycline containing 11.2 mmol/l or 30 mmol/l glucose for 96 h. The cells were stimulated with Krebs–Ringer–Bicarbonate–HEPES buffer containing 2.5 mmol/l or 20 mmol/l glucose. After 30 min, the supernatant was collected and insulin content was determined after extraction with acid ethanol following the procedures described by Wang et al. (1998). Insulin concentration was determined by ELISA kits (Linco, St. Charles, MO, USA).

2.7. DNA fragmentation

r9, Drp-1WT and Drp-1K38A cells were cultured in medium containing either 11.2 mmol/l or 30 mmol/l glucose at a 37 °C and 5% CO_2 incubator for 96 h, in the presence or absence of 500 ng/ml doxycycline. The cells were washed twice with PBS, re-suspended in the lysis buffer (10 mM Tris–HCl, pH 8.0 and 10 mM EDTA, 10 mM NaCl, 0.5% SDS, 100 μg/ml proteinase K) and stored at 50 °C for 2 h.