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Involvement of chronic stresses in rat islet and INS-1 cell glucotoxicity induced by intermittent high glucose

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ABSTRACT

In order to investigate the toxic effect of intermittent high glucose (IHG) and sustained high glucose (SHG) on rat pancreatic beta cell functions and the potential involved mechanisms, isolated rat islets and INS-1 beta cells were exposed to SHG (25 mmol/l) or IHG (11.1 and 25 mmol/l glucose alternating every 12 h) for 72 h. The results showed that IHG induced a more significant impairment of insulin release response in rat islets and INS-1 cell than SHG. Simultaneously, the intracellular levels of endoplasmic reticulum and oxidative stress were more markedly increased in islets and INS-1 cells exposed to IHG. However, there was no significant difference between reducing cell viability, insulin content and gene expression induced by SHG and IHG. Taken together, this study suggested the more serious toxic effect on rat pancreatic beta cell function induced by IHG treatment may be due to excessive activation of cellular stress.

1. Introduction

Pancreatic islet beta cells play a crucial role in maintaining glucose homeostasis through secretion of insulin in response to changes in the extra-cellular glucose. Overwhelming body of evidence has suggested chronic elevated glucose concentrations results in beta cell dysfunction and ultimately death, a state called beta cell glucose toxicity. Except chronic persistent hyperglycemia, repeated fluctuation from euglycemia to hyperglycemia is another general and important phenomenon existed in patients with diabetes which is more excessive than in health individuals (Cho et al., 2006; Ogata et al., 2007). Recently, several studies demonstrated the effects of fluctuating high glucose on development of diabetic cardiovascular complications, via enhancement of monocyte adhesion to endothelial cells, generation of reactive oxygen species and other mechanisms (Azuma et al., 2006; Otsuka et al., 2005; Yano et al., 2004). However, there is few published reports throw light on the toxic effect of fluctuating high glucose on islet beta cell functions and survival. Previous data suggested that chronic exposure to daily alternating high glucose decreased insulin release, store and gene expression in INS-1 beta cells, compared with control (Brock et al., 2002). More recently, Del Guerra and colleagues found that incubation with intermittent high glucose (IHG) induced significant impairment of insulin secretion in human pancreatic islets compared with sustained high glucose (SHG), even triggers beta cell apoptosis (Del Guerra et al., 2007). However, the potential mechanisms involved in islet beta cell glucose toxicity induced by intermittent high glucose remain to be clarified.

With the development of researches on beta cell dysfunction induced by stable high glucose, it suggested that excessive or prolonged endoplasmic reticulum (ER) stress had a close association with beta cell glucose toxicity. The ER is one of the most important organelles to beta cell which is responsible for several cellular functions, including the synthesis, initial post-translational modification, proper folding, and maturation of newly synthesized pro-insulin, as well as regulation of intracellular calcium homeostasis. Various biochemical and physiological stimuli can induce ER dysfunction, which causes proteotoxicity in the ER that is defined as ER stress (Zhang et al., 2004). Islet beta cells are particularly susceptible to ER stress owing to high rate of insulin synthesis and high development of intercellular ER (Scheuner et al., 2005). Although several studies indicated the physiological role of moderate ER stress in regulation of insulin synthesis and secretion in beta cell (Harding et al., 2001; Scheuner et al., 2001; Wu et al., 2006), recent evidence supported strong and long-term ER stress induced by chronic high glucose has harmful effects, leading to beta cell dysfunction and death (Cnop et al., 2007; Fonseca et al., 2007; Kaneto et al., 2005; Lipson et al., 2006; Wang et al., 2005). For example,

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Previous evidence supported that elevated free fatty acids initiated the excessive activation of pancreatic ER stress kinase and eukaryotic translation initiation factor 2alpha (*PERK-eIF2α*) of the ER stress responses, and exacerbated apoptosis of beta cell through markedly up-regulation the expressions of activating transcription factor-4 (*ATF4*) and C/EBP homologous protein (*CHOP*/*GADD153*) (Cnop et al., 2007). Moreover, in the states of SHG, the inositol requiring 1 (IRE1) signaling branch was hyper-activated, and then reduced insulin store and gene expression in rat beta cells (Lipson et al., 2006). However, little is known whether IHG can also effectively trigger activation of these ER stress responses, leading to deterioration of the beta cell functions.

On the other hand, pancreatic beta cell is vulnerable to changes in redox status because of the relative lack of antioxidant enzymes. Therefore, oxidative stress is considered as one of the important mechanisms involved in beta cell glucose toxicity (Robertson et al., 2007, 2006). Studies in vivo and in vitro have indicated that increased oxidative stress is closely associated with induction of pancreatic beta cell dysfunction and death under high glucose condition, as shown by higher generation of reactive oxygen species (ROS) and expression of markers, such as nitrotyrosine (Del Guerra et al., 2007; Marchetti et al., 2004; Miyazaki et al., 2007; Tang et al., 2007; Zraika et al., 2006). However, few reports illuminate whether intermittent high glucose triggers the oxidative stress within pancreatic beta cell in vitro.

Therefore, in order to investigate the effect of IHG on pancreatic beta cells function and the potential involved mechanisms, we detected the insulin synthesis and secretion in isolated rat pancreatic islets and INS-1 cells exposed to SHG or IHG, as well as the expression of intracellular ER and oxidative stress indicators, such as *eIF2α*, PKR-like endoplasmic reticulum kinase (*PERK*), *ATF4*, *CHOP*, 8-hydroxy-2′-deoxyguanosine (*8-OHdG*) and *nitrotyrosine*.

2. Materials and methods

2.1. Reagents and antibodies

Male Sprague–Dawley rats were purchased from Animal Center of China–Japan Friendship Hospital, Ficoll-400 from Pharmacia Biotech Inc. (Piscataway, NJ) and collagenase P from Roche Products Limited (Welwyn Garden City, Herts, UK). RPMI 1640 medium, fetal bovine serum, Trizol and DNAzol reagents, and nitrocellulose membranes were obtained from Invitrogen, (Grand Island, NY, USA). Insulin RIA kit was from DSL (Webster, TX, USA). BCA kit was from Bio-Rad, (Hercules, CA, USA). RT-PCR kit and SYBP Green PCR master mix were from TOYOBO (Osaka, Japan). Antibody for phosphorylated *eIF2α* was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for phosphorylated *PERK* was from Cell Signaling Technology (Beverly, MA, USA). ECL chemiluminescence kit was purchased from Amersham Pharmacia Biotech (UK). Mouse monoclonal anti-nitrotyrosine antibody was from Upstate Biotechnology (Lake Placid, NY, USA). *8-OHdG*-EIA kit was from OXIS Health Products (Portland, OR, USA). DNase I was from Qiagen (Chatsworth, CA, USA). The phospatase inhibitor mixture I was from Sigma (USA).

2.2. Isolation of rat pancreatic islets and culture of INS-1 cells

Pancreatic islets were isolated from male Sprague–Dawley rats weighing about 200 g. Islets were isolated following digestion of total pancreas with collagenase P and subsequent separation of the total digested pancreas on a discontinuous Ficoll gradient, according to the method of Gotoh et al. (1987). Just after the isolation, islets were handpicked and cultured in RPMI 1640 medium containing 11.1 mmol/l glucose, supplemented with 20% fetal calf serum, 10 mmol/l HEPES, 1 mmol/l sodium pyruvate and 50 μmol/l beta-mercaptoethanol, pH 7.4.

The INS-1 cell line was donated by Nanjing Medical University, China. The INS-1 cells from passage 10 to 20 were seeded at equal density and cultured in RPMI 1640 medium containing 11.1 mmol/l glucose, supplemented with 10% fetal calf serum, pH 7.4, as described by Olson et al. (1998). Isolated islets and INS-1 cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C.

Isolated islets and INS-1 cells were rinsed twice with PBS, and then randomly divided into three groups, and exposed to 11.1 mmol/l glucose (control), or 25.0 mmol/l glucose (SHG), or 11.1 and 25.0 mmol/l glucose alternating every 12 h (IHG), and maintained for 72 h, respectively. All media were changed every day.

2.3. Glucose stimulated insulin secretion (GSIS) and insulin content assay

After exposure for 24, 48 and 72 h, isolated islets and INS-1 cells were rinsed twice with PBS, followed by pre-incubation in Krebs–Ringer bicarbonate HEPES buffer (KRBH buffer: 115 mmol/l NaCl, 24 mmol/l $NaHCO_3$, 5 mmol/l KCl, 1 mmol/l $MgCl_2$, 25 mmol/l HEPES, 0.5% BSA, pH 7.4) containing 3 mmol/l glucose at 37 °C for 30 min. After aspiration of the buffer, islets and INS-1 cells were incubated in fresh KRBH buffer supplemented with 3 mmol/l or 27.8 mmol/l glucose at 37 °C for 20 min. The insulin secreted into the medium was collected and determined using an insulin RIA kit. Finally, the value of insulin was standardized to that of total protein content measured using the BCA kit, and insulin secretion index (27.8 mmol/l *GSIS* over 3 mmol/l *GSIS*) was calculated.

According to the method as described by Hamid et al. (2002), total intracellular insulin content was extracted by the acid/ethanol method. Briefly, islets and cells were incubated in 1% hydrochloric acid alcohol (ethanol/H_2O/HCl, 14:57:3) overnight at 4 °C. The insulin in the supernatant was detected by RIA, and normalized to total protein content.

2.4. Quantitative real-time PCR analysis

After exposure for 72 h, total cellular RNA of rat islets and INS-1 cells was extracted using Trizol reagent according to the manufacturer's protocol. For RT-PCR, 2 μg RNA was reverse transcribed with RT-PCR kits using random primers in a total volume of 20 μl. The real-time PCR amplification reaction was performed in a volume of 20 μl containing 2.5 mmol/l $MgCl_2$, 0.5 mmol/l forward/reverse primers, 2 μl SYBP Green PCR master mix, and 2 μl cDNA. The PCR was performed for 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The relative quantification of gene expression was analyzed by the $2^{-\Delta\Delta C_t}$ method (Livak et al., 2001) and was presented as fold over the control. The expression level of the gene of interest was corrected with that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The specific primers for *insulin* were: forward (5′-TCT TCT ACA CAC CCA TGT CCC-3′) and reverse (5′-GGT GCA GCA CTG ATC CAC-3′), fragment (149 bp); for pancreatic duodenal homeobox-1 (*PDX-1*) forward (5′-AAA CGC CAC ACA CAA GGA GAA-3′) and reverse, (5′-AGA CCT GGC GGT TCA CAT G-3′), fragment (150 bp); for *CHOP* forward (5′-CCA GCA GAG GTC ACA AGC AC-3′) and reverse (5′-CGC ACT GAC CAC TCT GTT TC-3′), fragment (125 bp); for *ATF4* forward (5′-GTT GGT CAG TGC CTC AGA CA-3′) and reverse (5′-CATT CGA AAC AGA GCA TCG A-3′), fragment (109 bp); for *GAPDH* forward (5′-AGT TCA ACG GCA CAG TCA G-3′) and reverse (5′-TAC TCA GCA CCA GCA TCA CC-3′), fragment (118 bp).

2.5. Western blotting analysis of eIF2α and PERK phosphorylation in INS-1 cells

Following the 72-h exposure, INS-1 cells were rinsed twice with PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer, supplemented with protease inhibitors (1 mmol/l phenylmethanesulfonyl fluoride, PMSF) and phosphatase inhibitors (phosphatase inhibitor mixture I). The protein samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for *eIF2α* or 7% SDS-PAGE for *PERK* and transferred onto nitrocellulose membranes. After blocking with 5% non-fat milk, the membranes were probed with primary antibodies, rabbit-antiphosphorylated *eIF2α* (1:500 dilution), rabbit-antitotal *eIF2α* (1:1000 dilution), rabbit-antiphosphorylated *PERK* (1:1000 dilution), rabbit-antitotal *PERK* (1:1000 dilution) at 4 °C overnight. Subsequently, the membranes were incubated with donkey anti-rabbit HRP-conjugated antibody (1:2000 dilution) as secondary antibody for 1 h at room temperature. The bands on X-ray film were developed by using the ECL chemiluminescence method. The membranes were re-used after stripping the primary antibody by incubation in 2% SDS, 50 mmol/l Tris/HCl, 150 mmol/l NaCl, and 100 mmol/l beta-mercaptoethanol at pH 7.4 for 1 h at 50 °C in a shaking water bath.

2.6. Determination of nitrotyrosine concentration

Nitrotyrosine concentration in isolated islets and INS-1 cells was determined by ELISA, as preciously described (Del Guerra et al., 2005; Quagliaro et al., 2003). After exposure for 72 h, cultured islets and INS-1 cells were lysed, and protein content was measured as described for Western blotting analysis. ELISA plates were coated with lysates or BSA standard samples in 50 mmol/l Na_2CO–$NaHCO_3$ buffer at pH 9.6, and allowed to bind overnight at 4 °C. Non-specific binding was blocked by 1% BSA in PBS plus 0.05% Tween 20 for 1 h at 37 °C, and the wells were incubated with 5 μg/ml purified mouse monoclonal anti-*nitrotyrosine* IgG for 1 h at 37 °C, and then for 45 min at 37 °C with a peroxidase-conjugated goat anti-rat IgG secondary antibody. The peroxidase reaction product was generated using peroxidase substrate. Plates were incubated for 10 min at room temperature, and optical density (OD) was read at 492 nm in a microplate reader.

2.7. Determination of 8-hydroxy-2′-deoxyguanosine (8-OHdG) concentration

8-OHdG content was measured using the Bioxytech 8-OHdG-EIA Kit (Del Guerra et al., 2005; Quagliaro et al., 2003). Islets and INS-1 cells DNA was isolated using DNAzol reagent according to the manufacturer's instructions, and quantified using