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Involvement of iron depletion in palmitate-induced lipotoxicity of beta cells

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ABSTRACT

High levels of plasma free fatty acid are thought to contribute to the loss of pancreatic beta-cells in type 2 diabetes. In particular, saturated fatty acid such as palmitate or stearate can induce apoptosis in cultured beta cells (lipotoxicity). Endoplasmic reticulum stress is a critical mediator of free fatty acid-induced lipotoxicity. Recently, disorders in mitochondrial respiratory metabolism have been linked to lipotoxicity. Since iron is a critical component of respiratory metabolism, this study is initiated to determine whether abnormal iron metabolism is involved in palmitate-induced beta cell death. Immunoblotting analysis showed that treatment of INS-1 beta cells with palmitate reduced the level of transferrin receptor 1 (TfR1), but increased the level of heavy chain ferritin (FTH). In addition, palmitate reduced intracellular labile iron pool. Whereas iron depletion through treatment with iron-chelators deferoxamine or deferasirox augmented palmitate-induced cell death, iron supplementation with ferric chloride, ferrous sulfate, or holo-transferrin significantly protected cells against palmitate-induced death. Furthermore, overexpression of TfR1 reduced palmitate-induced cell death, whereas knockdown of TfR1 augmented cell death. In particular, treatment with deferoxamine increased the level of endoplasmic reticulum (ER) stress markers phospho-PERK, phospho-eIF2α, CHOP and phospho-c-Jun N-terminal kinase. Treatment with chemical chaperone significantly protected cells against deferoxamine-induced apoptosis. Iron supplementation also protected cells against palmitate-induced primary islet death. These data suggest that iron depletion plays an important role in palmitate-induced beta cell death through inducing ER stress. Therefore, attempts to block iron depletion might be able to prevent beta cell loss in type 2 diabetes.

1. Introduction

Type 2 diabetes mellitus is believed to be caused by relative deficiency of insulin and subsequent insufficient compensation against insulin resistance (Cnop et al., 2007; Kahn et al., 1993). Insulin deficiency results partly from progressive loss of pancreatic beta cells (Butler et al., 2003). High levels of circulating free fatty acid (FFA) have been suggested to contribute to the loss of beta cells in this disease (Unger, 1995). In particular, long exposure of beta cells in culture to saturated FFAs such as palmitate or stearate can induce cell death, a phenomenon termed FFA-induced lipotoxicity (Choi et al., 2007; Lupi et al., 2002; Shimabukuro et al., 1998). Death is mainly apoptotic with cytochrome c release, caspase 3 activation, and DNA fragmentation (Lupi et al., 2002; Maedler et al., 2001). Although the molecular and cellular mechanisms involved in FFA-induced beta cell death are not fully understood, it is accepted that unresolved endoplasmic reticulum (ER) stress is a central mediator of FFA-induced lipotoxicity (Cnop et al., 2008; Cunha et al., 2008; Karaskov et al., 2006).

Endoplasmic reticulum (ER) stress is a condition in which unfolded or mis-folded proteins are accumulated in the ER rumen due to disrupted balance between ER protein load and folding capacity to process the load. ER stress triggers unfolded protein response

Abbreviations: ATF6, activating transcription factor-6; CHOP, C/EBP homologous protein; DFO, deferoxamine; DS, deferasirox; ER, endoplasmic reticulum; FFA, free fatty acid; FTH, ferritin heavy chain; h-Tf, holo-transferrin; IRE1, Inositol-requiring enzyme-1; IRP, iron regulatory protein; JNK, C-Jun N-terminal kinase; LIP, labile iron pool; OCR, oxygen consumption rate; PA, palmitate; 4-PBA, 4-phenylbutyrate; PERK, protein kinase RNA (PKR)-like ER kinase; ROS, reactive oxygen species; SD, Sprague–Dawley; TfR, transferrin receptor; TCA, tricarboxylic acid; UPR, unfolded protein response.

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(UPR), a signaling network activated by three sensors in ER membrane: protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) (Hetz, 2012). PERK-dependent phosphorylation of eukaryotic initiation factor 2α (eIF2α) reduces the protein load to ER by attenuating general translation. ATF6-dependent transcriptional induction of ER chaperones can be enhanced by X-box-binding protein 1 (XBP1) transcription, which is synthesized upon IRE1-mediated splicing of a 26-base fragment from its precursor mRNA (Hetz, 2012). UPR is an initially adaptive response to resolve excessive folding load to the ER. However, if UPRs are insufficient to resolve the folding load, adaptive UPRs are able change to damaged responses, which ultimately induce cell death through activating death signals. Induction of CCAAT/enhancer binding protein homologous protein (CHOP) and activation of c-Jun N-terminal kinase (JNK) have been reported to be typical death signals for ER stress-induced cell death (Hetz, 2012). In addition, JNK-dependent serine phosphorylation of insulin receptor substrate-1 (IRS-1) is reported to be a linker between ER stress and obesity-induced insulin resistance (Hotamisligil, 2008). Insufficient activation of insulin signaling pathway was suggested to be another contributor to beta cell damage (Hennige et al., 2003). Although it remains to be determined on how FFA induces ER stress responses, FFA surplus may facilitate UPRs through ER Ca^{2+} depletion (Cunha et al., 2008; Hara et al., 2014; Mandl et al., 2009). Impaired ER-Golgi trafficking may be involved in palmitate-induced ER stress (Preston et al., 2009).

Iron homeostasis is critical for maintaining normal functions of cells, since iron is essential in mitochondrial respiratory metabolism. In fact, iron itself is an electron transfer component in oxidation/reduction reactions observed in several iron–sulfur cluster proteins of mitochondrial electron transport complex and tricarboxylic acid (TCA) cycle enzymes (Levi and Rovida, 2009; Ye and Rouault, 2010). Iron deficiency causes cell death and growth arrest, whereas iron in excess will generate free radicals that can structurally and functionally damage cellular biomolecules, such as DNA, membrane lipids and proteins, ultimately resulting in cell death (Anderson et al., 2012; Muckenthaler et al., 2008). Therefore, an appropriate level of cellular iron must be tightly regulated. Cellular iron homeostasis is achieved by balanced expression of proteins involved in iron uptake, storage, and export. The expression level of proteins is mainly regulated through iron regulatory proteins (IRPs) that are activated under iron-deficient conditions. They can bind to iron-responsive elements (IREs) of iron-related target mRNAs and positively or negatively modulate the expression of proteins that control iron level (Anderson et al., 2012; Muckenthaler et al., 2008; Pantopoulos, 2004). Transferrin receptor (TfR) as an iron uptake molecule (Chen et al., 2006) is positively regulated by IRPs binding to IREs in the 3′-untranslated region of its mRNA to improve its stability (Muckenthaler et al., 2008). On the other hand, IRPs can bind to the 5′-untranslated region of ferritin as iron storage proteins. Such binding negatively regulates the expression of ferritin by blocking its translation efficiency (Muckenthaler et al., 2008).

The objective of this study was to determine whether abnormal iron metabolism plays a role in palmitate-induced lipotoxicity of INS-1 beta cells. To determine whether palmitate treatment would affect intracellular iron level, the level of intracellular iron was determined by measuring palmitate-induced fluorescence change in calcein-AM-treated INS-1 cells. Expression levels of TfR and ferritin were investigated through immunoblotting analysis in palmitate-treated INS-1 cells. The effect of iron depletion or iron supplementation on palmitate-induced cell death was investigated by measuring cleaved caspase 3s and fragmented DNAs after co-treatment with iron chelators deferoxamine (DFO) or deferasirox (DS), or iron ions $FeCl_3$, $FeSO_4$, or holo-transferrin. The effect of TfR overexpression or knockdown on palmitate-induced cell death was investigated through caspase 3 activation. To determine the involvement of ER stress in iron depletion-induced INS-1 cell death, expression levels of ER stress markers phospho-PERK, phospho-eIF2α, CCAAT/enhancer binding protein homologous protein (CHOP), and phospho-c-Jun N-terminal kinase (P-JNK) were investigated by immunoblotting analysis in DFO-treated INS-1 cells. The effect of iron depletion on palmitate-induced ER stress responses was also investigated by measuring changes of ER stress markers. The protective effect of chemical chaperone on iron chelator-induced cell death was investigated by reduction of caspase 3 cleavage in DFO-treated cells after chemical chaperone 4-phenylbutyrate (4-PBA) co-treatment. The effect of iron depletion or supplementation on palmitate-induced primary islet cell death was also investigated.

2. Materials and methods

2.1. Cells and culture

INS-1 rat insulinoma cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS F4135, Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin (Duchefa, Haarlem, The Netherlands), and 100 μg/mL streptomycin (Duchefa) at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.

2.2. Reagents

Most chemicals, including glucose, palmitate, deferoxamine, ferric chloride ($FeCl_3$), ferrous sulfate ($FeSO_4$), holo-transferrin (h-Tf), and 4-phenylbutyrate (4-PBA), were purchased from Sigma-Aldrich. Deferasirox and Dp44mT were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Calcein-Am was obtained from BD Bioscience (San Diego, CA). Chemicals were dissolved in medium or in dimethyl sulfoxide (DMSO). Anti-caspase 3, anti-phospho-eIF2α, anti-CHOP, anti-FTH, and anti-phospho-PERK antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-actin, anti-XBP1 and anti-TfR1 antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho-JNK antibody was obtained from Invitrogen (Carlsbad, CA). Anti-BiP antibodies were obtained from Enzo Life Science (Farmingdale, NY).

2.3. Preparation of palmitate

Palmitate/bovine serum albumin (BSA) conjugates were prepared by soaping palmitate with sodium hydroxide and mixing with BSA. Palmitate (20 mM in 0.01 M NaOH) was incubated at 70 °C for 30 min. Fatty acid soaps were then complexed with 5% fatty acid-free BSA in phosphate-buffered saline (PBS) at a 1:3 volume ratio. Complexed fatty acids consisted of 5 mM palmitate and 3.75% BSA. The palmitate/BSA conjugates were diluted in RPMI 1640 medium containing 10% FBS and administered to cultured cells.

2.4. Isolation of islets

Islets were isolated from 10-week-old male Sprague–Dawley (SD) rats using a collagenase digestion method. Briefly, after injecting 10 ml of collagenase P (0.75 mg/ml) into bile ducts, each swollen pancreas was excised and incubated in a water bath at 37 °C for 7 min. After stopping the collagenase digestion with cold Hanks' balanced salt solution (HBSS), pancreatic tissues were disrupted by repetitive pipetting and subsequently by passing through a 400-μm mesh. Islets were separated by centrifugation on 25%, 23%, 21.5%, and 11.5% Ficoll gradients. Islets at the interface between the 21.5% and 11.5% fractions were collected and washed with HBSS. Healthy islets were hand-picked under a stereomicroscope. To obtain single beta cells, islets were treated with trypsin-EDTA for 2 min and dissociated by repetitive pipetting. The dissociated islet cells were then