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RESEARCH ARTICLE

Chronic high glucose induced INS-1β cell mitochondrial dysfunction: A comparative mitochondrial proteome with SILAC

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As glucose-stimulated insulin secretion of pancreatic β cell is triggered and promoted by the metabolic messengers derived from mitochondria, mitochondria take a central stage in the normal function of β cells. β cells in diabetics were chronically exposed to hyperglycemia stimulation, which have been reported to exert deleterious effects on β-cell mitochondria. However, the mechanism of the toxic effects of hyperglycemia on β-cell mitochondria was not clear. In this study, we characterized the biological functional changes of rat INS-1β cells and their mitochondria with chronic exposure to hyperglycemia and created a research model of chronic hyperglycemia-induced dysfunctional β cells with damaged mitochondria. Then, SILAC-based quantitative proteomic approach was used to compare the mitochondrial protein expression from high glucose treated INS-1β cells and control cells. The expression of some mitochondrial proteins was found with significant changes. Functional classification revealed most of these proteins were related with oxidative phosphorylation, mitochondrial protein biosynthesis, substances metabolism, transport, and cell death. These results presented some useful information about the effect of glucotoxicity on the β-cell mitochondria.

Keywords:
β-Cell / Cell biology / Diabetes / High glucose / Mitochondria / SILAC

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1 Introduction

Type 2 diabetes is characterized by hyperglycemia that results from insufficient insulin to metabolize the glucose, which in turns, destroys the balance between the insulin secretion and metabolic demand. Although there are debates on whether β-cell abnormality is the cause or the result of the type 2 diabetes [1], it is well known that hyperglycemia exerts toxic effects on β cell and results in the impairment of insulin secretion of β cell and even cell death, which is referred as glucotoxicity [2]. To elucidate the complex molecular mechanism underlying hyperglycemia-induced β-cell dysfunction, quantitative SILAC strategy was applied to investigate the

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Abbreviations: GSIS, glucose-stimulated insulin secretion; **HAX-1**, HS1 binding protein; **ISG**, insulin secretory granules; **ΔΨm**, mitochondrial membrane potential; **MudPIT**, multidimensional protein identification technology; **OXPHOS**, oxidative phosphorylation; **PI**, propidium iodide

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deleterious effect of glucose on the expression of proteins from INS-1E cells [3] and INS-1E cells insulin secretory granules (ISGs) [4], and meaningful results were obtained to improve our understanding the mechanism of glucotoxicity [3, 4].

As many studies have proposed that glucotoxicity is mainly induced by the excess production of ROS from β-cell mitochondria [5], and mitochondria play key roles in the consensus model of glucose-stimulated insulin secretion (GSIS) of pancreatic β cells [6, 7], it is intriguing to explore how both the configuration and function of mitochondria changed under such supraphysiological blood glucose level. Nyblom and colleagues have found the peak intensity of many mass spectra, obtained with SELDI-TOF-MS from the mitochondrial fractions of rat INS-1E cells exposed to high glucose, were changed greatly [8]. However, they did not determine these changed spectra to specific mitochondrial proteins due to the limitation of SELDI-TOF-MS. In this study, we simulated the glucose status in physiologic and diabetic conditions to find out the effect of glucotoxicity on β-cell mitochondria. The SILAC-based proteomics strategy was applied to compare the expression of mitochondrial proteins after chronic exposure of β cell to high glucose. The expression of some mitochondrial proteins related to oxidative phosphorylation (OXPHOS), mitochondrial protein biosynthesis, substance metabolism, substance transport, and apoptosis was dysregulated. These results presented a systematic overview of the effects of glucotoxicity on the β-cell mitochondria.

2 Materials and methods

2.1 Materials

Analytical grade chemicals were obtained from Sigma (St. Louis, MO). Normal RPMI 1640 media, FBS, dialyzed FBS, PBS, penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA). RPMI 1640 SILAC medium deficient in arginine and lysine was purchased from Sigma. Heavy $^{13}C_6{}^{15}N_4$-L-arginine and $^{13}C_6{}^{15}N_2$-L-lysine were obtained from Cambridge Isotope Laboratories (Andover, MA). Protease inhibitor cocktail tablets were obtained from Roche (Mannheim, Germany). Sequence grade modified trypsin was purchased from Promega (Madison, WI). HPLC-grade ACN and formic acid were obtained from J. T. Baker (Phillipsburg, PA).

2.2 Cell culture, glucose treatment, and isotopic labeling

Rat INS-1β cells were cultured in RPMI 1640 media containing 11 mM glucose and supplemented with 10 mM HEPES, 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 50 mM 2-mercaptoethanol, 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37°C, 5% CO_2 [9]. The media were changed every 2 days. For high glucose treatments, INS-1β cells were cultured for 5 days in the identical medium except that the glucose concentration was elevated to 27 mM. For SILAC experiments, INS-1β cells were cultured in the customed RPMI 1640 media (10% dialyzed FBS, 11 mM glucose, and light $^{12}C_6{}^{14}N_4$-L-arginine/$^{12}C_6{}^{14}N_2$-L-lysine or heavy $^{13}C_6{}^{15}N_4$-L-arginine/$^{13}C_6{}^{15}N_2$-L-lysine) for at least seven cell population doubling times to achieve complete labeling (98% labeling efficiency). After complete isotopic labeling, the glucose concentration in "heavy" media was elevated to 27 mM and the glucose concentration in the "light" media was kept unchanged, then the two groups of cells were cultured for another 5 days before harvested.

2.3 Measurement of insulin secretion and insulin content

Insulin secretion analysis was performed as described previously [8]. Briefly, two groups of INS-1β cells were seeded in 24-well dishes and cultured in media containing 11 or 27 mM glucose for 5 days, respectively. For insulin release measurements, two groups of cells were cultured for another 60 min under identical conditions except a lowered the glucose concentration (1 mM). Then two groups of cells were washed twice with Krebs-Ringer bicarbonate buffer (KRBB) and incubated in 2 mL of KRBB for 30 min separately. The glucose-free buffer was replaced by the same buffer supplemented with either 5.5 or 15 mM glucose and the cells were incubated for 30 min at 37°C. The supernatants were collected for insulin release measurement. For insulin content measurements, cells were detached by trypsinization and counted with cell counter (MIDSCI, St. Louis, USA), the cell pellets were washed with PBS twice and lysed with buffer (10 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Triton 100, 1% DOC, 5 mM EDTA, pH 7.2). After centrifuged at 800 × *g* for 5 min, the supernatants were collected for insulin content measurement. The samples of insulin release and content were analyzed with an insulin ELISA assay kit (Mercodia, Sweden).

2.4 Detection of cell apoptosis

Cell apoptosis was detected following the procedure recommended by cell apoptosis AnnexinV-FITC assay kit (Jingmei, Shenzhen, China). Briefly, after cultured as described previously, two groups of INS-1β cells were washed twice with PBS and collected by trypsinization and centrifugation. Then cell pellets were resuspended in 100 μL of staining solution (20 μL FITC-labeled annexin-V (10 μg/mL) and 20 μL propidium iodide (PI) (50 μg/mL) in 1 mL HEPES buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$)) and incubated in the dark for 15 min at room temperature. After incubation, 400 μL HEPES buffer was added and the samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.5 Measurement of cellular ATP content

To clear away the effect of death cells on the cellular ATP content, after cultured in media containing 11 or 27 mM glucose for 5 days, two groups of cells were collected and incubated with 10 μg/mL PI for 10 min, then PI-negative cells were sorted with FACSAriaII flow cytometer (Becton Dickinson). Then, ATP content was measured with a luciferase ATP determination kit (Beyotime, China).

2.6 Measurement of ROS

ROS production was measured with fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (DCDHF-DA) as describe previously [10]. After cultured in media containing 11 or 27 mM glucose for 5 days, two groups of INS-1β cells were washed twice with PBS, collected by trypsinization and centrifugation. Then the cell pellets were resuspended in 10 μM DCDHF-DA for 30 min in incubator at 37°C, 5% CO_2. After washed twice with PBS, cells were incubated with 10 μg/mL PI for 10 min. The DCF (2′,7′-dichlorofluorescein) fluorescence intensity of PI-negative cells was analyzed with a FACSCalibur flow cytometer (Becton Dickinson).

2.7 Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi m$) was measured with the fluorescent probe Rhodamine 123 (Rho123) as described previously [11] with some modifications. Briefly, after cultured in media containing 11 or 27 mM glucose for 5 days, two groups of INS-1β cells were harvested, washed twice with PBS, and incubated with the Rhodamine 123 (2 μM in PBS) for 30 min in incubator at 37°C, 5% CO_2. After washed twice with PBS, cells were incubated with 10 μg/mL PI for 10 min. The Rho123 fluorescence intensity of PI-negative cells was analyzed with a FACSAriaII flow cytometer (Becton Dickinson).

2.8 Assessment of the mitochondrial morphology with electron microscope

INS-1β cells were cultured in low glucose and high glucose separately, and prepared for electron microscope analysis as described previously [9].

2.9 Purification of mitochondria

For mitochondria isolation from SILAC labeling cells, INS-1β cells cultured in low glucose (11 mM) and labeled with "light" amino acids, and INS-1β cells cultured in high glucose (27 mM) and labeled with "heavy" amino acids were washed three times with ice-cold PBS (pH 7.4), respectively. Then two groups of cells were detached with trypsin and harvested with centrifugation at 700 × *g* for 3 min at 4°C. After that, two groups of cells were resuspended in ice-cold PBS and aliquots of two cell suspensions were collected for cell counting with hemocytometer. After determined the cell concentrations, equal number of two groups of cells (about 1.2×10^8 cells totally) was combined for mitochondria isolation. Then the mitochondria were isolated by Nycodenz density gradient centrifugation as described previously [9]. This entire process was repeated twice.

For Western blot, mitochondria were purified separately from INS-1β cells cultured in low glucose and high glucose without labeling. In these experiments, INS-1β cells were scraped into 10 mL ice-cold homogenization buffer and mitochondria were prepared as described previously [9].

2.10 Western blot analysis to assess the mitochondrial purity

Total cell lysate (designated TL) from INS-1β cells cultured in low and high glucose was extracted separately as describe previously [12]. The supernatants (designated S) collected during the cellular fractionation in mitochondria isolation process was precipitated with methanol/chloroform and solubilized in 8 M urea. Then protein concentrations of three fractions (TL, S, and M) were determined by Brandford method. For Western blot, equal amount of three fractions were separated by 12% SDS-PAGE and electroblotted onto 0.45-μm PVDF membranes (GE Healthcare, USA) by the semidry method. The membrane were then probed with primary antibody anti-cytochome c (BD, San Jose, CA), anti-mitochondrial ATP synthase subunit β, sodium potassium ATPase, KDEL, Rb, tubulin beta (Abcam, Cambridge), anti-prohibitin 2 (Millipore Upstate, Billerica, MA), anti-VDAC-1 (Santa Cruz, CA), and HRP-conjugated primary antibody to GADPH (Kangcheng, Shanghai, China). Then HRP-conjugated anti-mouse, rabbit, goat IgG antibody was used as the secondary antibody. Then immunoreactive proteins on the membranes were detected by SuperSignal® west Femto trial kit and exposed to X-ray film. Western blots for every selected protein were repeated three times for every batch of samples.

2.11 In-solution digestion of mitochondrial proteins

In-solution digest of SILAC-labeled mitochondrial proteins was carried out with an optimized MS-compatible surfactant and organic solvent method as described previously [9].

2.12 2D-LC-MS/MS analysis

The tryptic peptide mixture solutions were centrifuged at 13 000 × *g* for 10 min to get rid of the particles prior to

analysis. 2D-LC-MS/MS analysis were performed using an Eksigent HPLC system (Dublin, C, USA) interfaced to an LTQ-Orbitrap XL (Thermo Fisher Scientific, Waltham, USA) equipped with an in-house built nanoelectrospray device. For a single analysis, 100 μg of peptide mixtures were pressure-loaded onto a 2D silica capillary column (200 μm id) packed with 3 cm of C_{18} resin (sunchrom 5μ, Friedrichsdorf, Germany) and 3 cm of strong cation exchange resin (Luna 5μ SCX 100A, Phenomenex, USA) [13]. The buffer solutions used were 0.1% formic acid (buffer A), 100% ACN/0.1% formic acid (buffer B), and 700 mM ammonium acetate/5% ACN/0.1% formic acid (buffer C). The 2D column was first desalted with buffer A and then eluted with a 12-step salt gradient ranging from 0 to 700 mM ammonium acetate. The effluent peptides of the two-phase column in each step were directed into a 10 cm of C_{18} analytical column (100 μm id) with a 3–5 μm spray tip. Step 1 consisted of a 100-min gradient from 0 to 95% buffer B. Steps 2–12 had the following profile: 3 min of 95% buffer A, 8 min of X% buffer C, an 87-min gradient from 5 to 40% buffer B, 10-min gradient from 40 to 80% buffer B, and then kept the buffer B for 10 min. The 3-min buffer C percentages (X) were 5, 10, 15, 20, 25, 30, 40, 50, 60, 80, and 100, respectively, for the other 11-step analysis. Nanoelectrospray ionization was accomplished with a spray voltage of 2.2 kV and a heated capillary temperature of 200°C. A cycle of one full-scan mass spectrum (350–1700 m/z) followed by seven data-dependent tandem mass spectra was repeated throughout each step of the multidimensional separation. All tandem mass spectra were collected using normalized collision energy (a setting of 35%). Mitochondrial protein samples were analyzed three times under identical conditions.

2.13 MS/MS data analysis

The MS/MS raw spectra were searched with MaxQuant (v 1.1.1.14) [14, 15] against a concatenated database combining proteins from IPI rat protein database version 3.75, 27 commonly observed contaminations (forward database), and the reversed sequences of all proteins (reverse database). A fixed modification of 57.02 on cysteines, variable modification of 15.99 on methionines and *N*-acetylation (protein) were set. Full tryptic specificity was required. Up to two missed cleavages and three labeled amino acids (arginine and lysine) were allowed. Initial mass deviation of precursor ion and fragment ions were up to 10 ppm and 0.5 Da, respectively. The minimum required peptide length was set to six amino acids. To pass statistical evaluation, posterior error probability for peptide identification (MS/MS spectra) must be below or equal to 0.01; the maximum false discovery rate of proteins was set at 0.01 to ensure the false identification hits was lower than 1%. The data from the three replicates were analyzed with unified criteria, and then the data list was combined. For the protein quantified more than twice, average ratio was used, however, if the proteins quantified twice or thrice that displayed ratios with RSD greater than 1, the quantifications were excluded from further analysis and not included in the list of quantified proteins.

2.14 Bioinformatics analysis of quantitative proteomic data

The subcellular locations of the quantified proteins were annotated with the GO database and Uniprot protein database. The proteins annotated to be located in mitochondria in the GO database or Uniprot protein database were accepted as mitochondrial proteins. For the proteins without subcellular annotations, protein predicted by at least two programs mentioned below—TargetP1.1, mitoprot, predator, and subLoc [16] as to be in mitochondria or possibly in mitochondria were also accepted as mitochondrial proteins. The functional annotation of the dysregulated mitochondrial proteins were performed with Uniprot protein database and BiNGO [17].

3 Results

3.1 Functional changes of INS-1β cells and their mitochondria after chronic hyperglycemia stimulation

Insulin secretion and contents of INS-1β cells cultured in 11 and 27 mM glucose were determined and normalized to the cell number (Fig. 1A and B). As shown in Fig. 1A, insulin secretion at 5.5 mM glucose stimulation was minimal and almost the same in both groups of cells, which indicated that the basal release of insulin was similar for the two groups of cells. However, insulin secretion was increased dramatically in both groups of cells when the stimulated glucose was

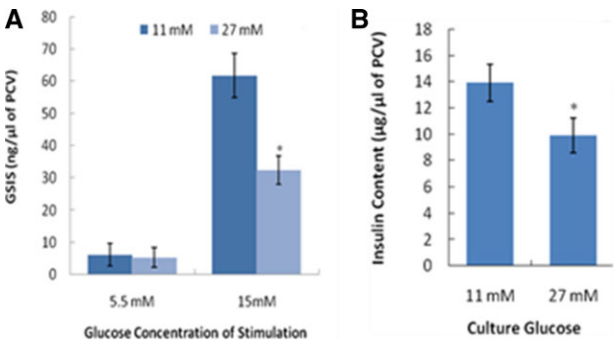

Figure 1. Measurements of glucose-stimulated insulin secretion (GSIS) and insulin contents after chronic high glucose stimulation. (A) GSIS from INS-1β cells cultured in media with 11 or 27 mM glucose for 5 days. After preincubation in KRBB containing 1 mM glucose, INS-1β cells were incubated in KRBB containing 5.5 or 15 mM glucose and insulin secretion was measured with ELISA. (B) Insulin content from INS-1β cells cultured in media with 11 or 27 mM glucose for 5 days.

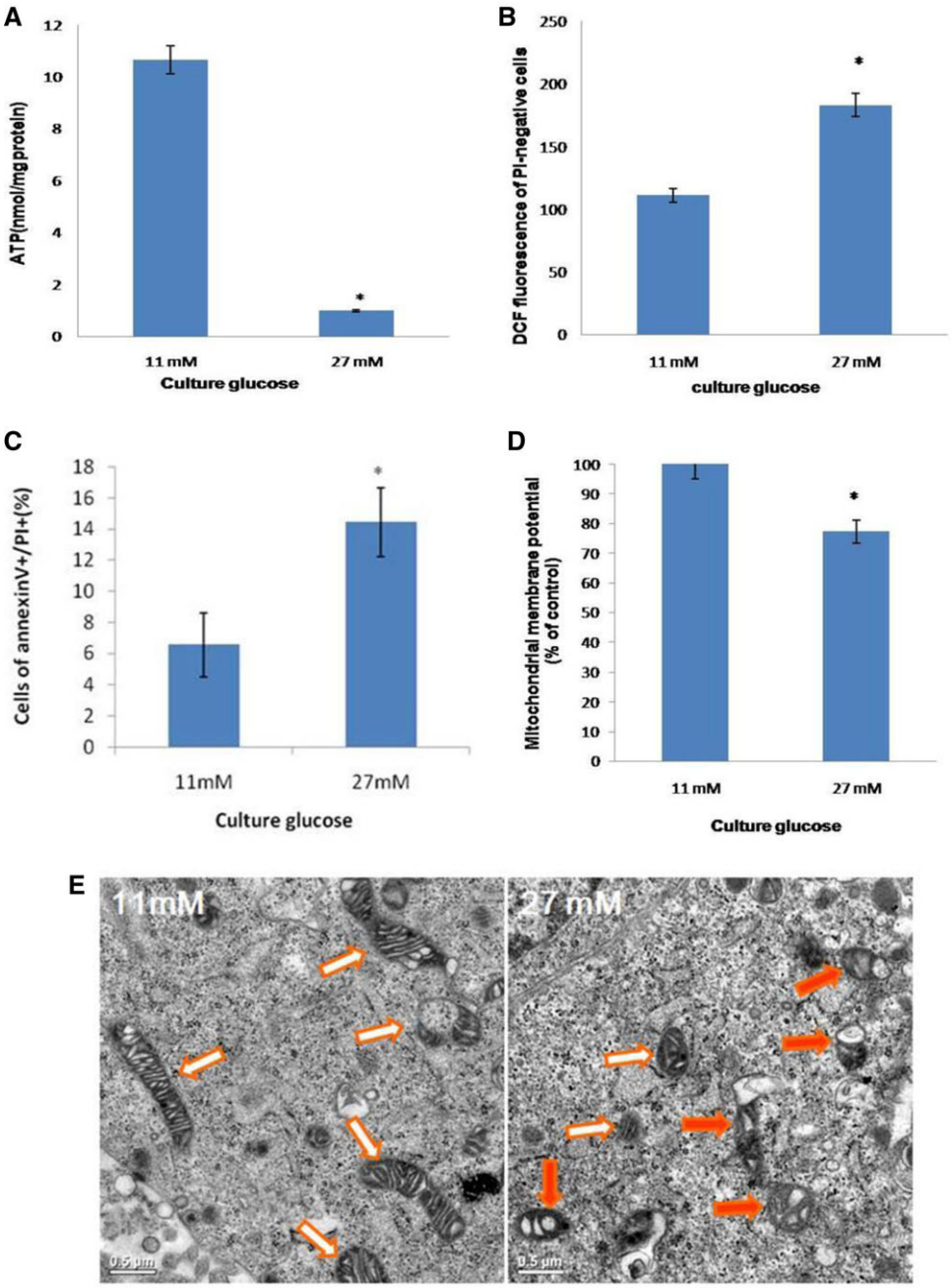

Figure 2. Measurements of biological functional parameters of INS-1β cells mitochondria after chronic high glucose stimulation. (A) INS-β cells were cultured in 11 or 27 mM glucose for 5 days and stained with PI for 10 min, then PI-negative cells were sorted and ATP contents were measured with ATP-dependent luciferin-luciferase luminometric method. (B) ROS of INS-1β cells cultured in 11 or 27 mM glucose for 5 days was measured with DCDHF-DA and PI. The DCF fluorescence of PI-negative cells was determined. (C) Cell apoptosis of INS-1β cells cultured in 11 or 27 mM glucose for 5 days was measured with annexin V-FITC and PI. (D) Mitochondria membrane potential (ΔΨm) of INS-1β cells cultured in 11 or 27 mM glucose for 5 days were measured with Rho123 and PI. The Rho123 fluorescence of PI-negative cells was determined. Results are expressed as the means ± SD from three experiments (*$p < 0.05$ compared to the control group). (E) Mitochondrial morphology were observed with electron microscope in INS-1β cells cultured in low glucose (11 mM) (left) and high glucose (27 mM) (right). The hollow arrow indicates mitochondria and the solid arrow indicates mitochondrial with vacuoles.

elevated to 15 mM, but the stimulated insulin secretion level in cells cultured in 27 mM glucose was significantly lower than cells cultured in 11 mM glucose, indicating GSIS was decreased by prolonged hyperglycemia stimulation. Moreover, insulin production also decreased significantly in INS-1β cells cultured in 27 mM glucose compared to that cultured in 11 mM glucose (Fig. 1B).

As mitochondria play important roles in GSIS of pancreatic β cells, several functional parameters of β-cells mitochondria including ATP content, ROS production, cell apoptosis, ΔΨm, and mitochondrial morphology were investigated. As mitochondria are the major source of ATP, luciferase assay was employed to quantify the cellular ATP content. The result indicated that ATP level showed an obvious reduction in cells cultured in 27 mM glucose than cells cultured in 11 mM glucose (Fig. 2A), indicating hyperglycemia disturb energy production in INS-1β cells. ROS production in cells cultured in hyperglycemia condition increased 1.6-fold compared with that of cells cultured in normal condition, which indicated that high glucose induced a significant increase of intracellular ROS production (Fig. 2B). The number of β cells cultured in high glucose media that displayed apoptosis

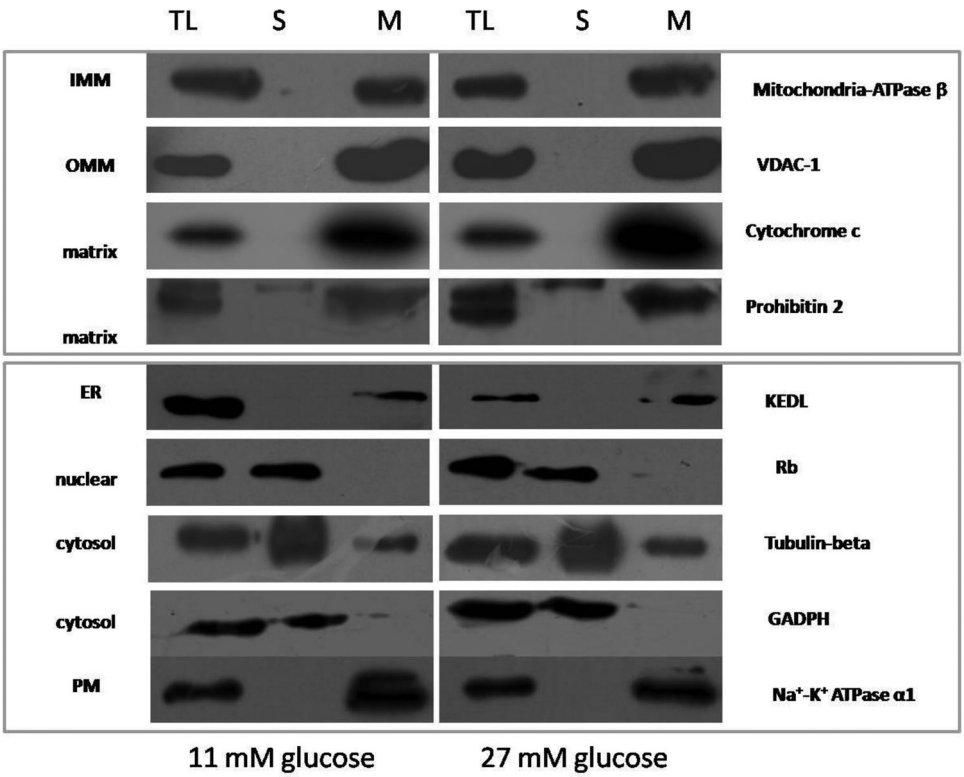

Figure 3. Assessment of mitochondrial purity with Western blot mitochondrial purity was assessed with Western blot against marker proteins from different cellular parts (mitochondria, plasma membrane, ER, and cytosol) with samples from total cell lysate (TL), soluble protein fraction (S), and mitochondrial fraction (M). The upper panel showed that all the mitochondrial proteins located in every mitochondrial compartment have been identified, the bottom panel showed contaminations of the mitochondrial factions.

characteristics was increased significantly compared with cells cultured in low glucose media (Fig. 2C), indicating high glucose induced β-cell apoptosis. $\Delta\Psi m$ was reduced in hyperglycemia-treated cells (Fig. 2D). Mitochondrial morphology was also significantly altered in high glucose treated cells (Fig. 2E). Mitochondria in the control cells were long-shaped and had fine interior structures, while mitochondria in high glucose treated cells appeared round-shaped and hypertrophic, with great vacuoles in some mitochondria.

In summary, chronic hyperglycemia could cause β-cell mitochondrial dysfunction, as characterized by metabolic, functional, and morphological abnormalities. Based on above results, a research model of chronic hyperglycemia-induced dysfunctional β cells with damaged mitochondria was created. This model paved the way for the subsequent experiment to quantitatively analyze the expression of mitochondrial proteins in β cells after chronic exposure to high glucose using SILAC-based proteomic approach.

3.2 Mitochondria isolation and quality control

After mitochondrial fractionation, Western blot was carried out to assess the purity of the isolated mitochondria. Several subcellular maker proteins including mitochondrial ATP synthase β, VDAC-1, prohibitin 2, and cytochrome c for mitochondria, GADPH and tubulin-beta for cytosol, Na^+-K^+ ATPase α1 for plasma membrane, KDEL for ER, and Rb for nuclear were detected by Western blot. As shown in Fig. 3, all of the mitochondrial markers were detected in the mitochondrial fraction, and cytosol and nuclear proteins were effectively removed by the purification process, but plasma membrane marker Na^+-K^+ ATPase α1 and ER marker KDEL were detected in mitochondria fraction, which indicate that plasma proteins and ER proteins were the main contaminations in the preparations. As all mitochondrial markers were detected by Western blot, we conclude that mitochondrial proteins were highly enriched in the mitochondrial fraction.

3.3 Identification and quantification of the mitochondrial proteins

SILAC-based quantitative proteomic approach was applied to quantitatively compare mitochondrial proteins between chronic high glucose treated INS-1β cells and control cells. After in-solution digestion of the SILAC-labeled mitochondrial proteins, tryptic peptide mixture was analyzed with MudPIT [18]. Then these spectra were analyzed and quantified with MaxQuant against the target-decoy rat IPI database. A total of 832, 665, 841 proteins were quantified in three replicated experiments, respectively (Supporting Information Table 1). Nearly half the proteins were quantified by all the three replicates, and 67.8% proteins were quantified twice in the three replicates (Supporting Information Fig. 1), implying a good level of reproducibility in MS analysis. For the proteins quantified more than twice, average ratios were used as the ratios of the proteins. With above analysis, a

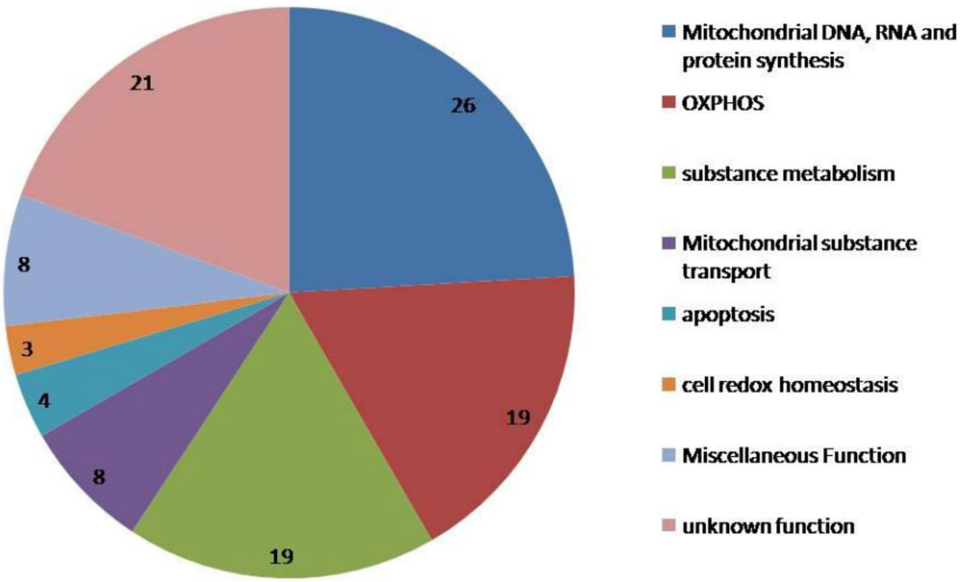

Figure 4. Functional annotations of dysregulated mitochondrial proteins after chronic hyperglycemia stimulation.

total 1054 proteins were quantified in the study (Supporting Information Table 1). For selecting the significant cutoff for differentially expressed proteins, protein ratios with tenfold change was defined as outliers and thus excluded from the analysis. As the average ratio of all other proteins was around 1, and the average RSD of these proteins was 0.65, a cutoff value of 2.5-fold was chosen as the threshold for screening the significantly changed proteins. With these criteria, 193 proteins were accepted as dysregulated proteins (including 132 downregulated and 61 upregulated). Among the 193 dysregulated proteins, 108 proteins were annotated in protein knowledge database or predicted with bioinformatics tools as mitochondrial proteins, including 24 proteins upregulated (ratio $>$ 2.5) and 84 proteins downregulated (ratio $<$ 0.4) (Supporting Information Table 2). Functional annotation of these 108 dysregulated mitochondrial proteins with Uniprot database revealed that most of these proteins were related to OXPHOS, mitochondrial protein biosynthesis, substance metabolism, transport, and apoptosis regulation (Fig. 4).

4 Discussion

SILAC-based quantitative proteomic approach was previously applied to compare the protein expression from high glucose treated INS-1E cell ISGs, and 1082 proteins were identified, including 143 mitochondrial proteins [4]. This study identified 116 of these 143 mitochondrial proteins, indicating a good reproducibility between these studies. Here, the expression of 108 mitochondrial proteins was found with significant changes. Functional classification of these mitochondrial proteins showed that most of them were related to the key functions of mitochondria, such as ATP production (OXPHOS and substances metabolism), mitochondrial protein biosynthesis, substance transport, and cell apoptosis regulation. Consistent with previous literature, some proteins identified were reported to link with high glucose induced mitochondrial dysfunction and type 2 diabetes, some proteins were shown to be associated with mitochondrial dysfunction for the first time. Herein, we summarized some classes of proteins that may play important roles in hyperglycemia-induced mitochondria dysfunction.

4.1 Oxidative phosphorylation

The key function of mitochondria is to produce ATP through electrons passing along a series of electron transport chain complexes [19]. In this study, 13 components of mitochondrial electron transfer chain were downregulated and six components were upregulated. These results were consistent with Mootha's observation that the expression of some OXPHOS genes was changed in type 2 diabetes [20]. Among these 13 downregulated proteins, 11 proteins were components of mitochondrial complex I (NADH:ubiquinone oxidoreductase), which indicated that mitochondrial complex I was a major target for hyperglycemia stimulation. As mitochondrial oxidative capacity is determined by the expression levels of OXPHOS subunits, activity of OXPHOS and the number and size of mitochondria [21], dysregulation of these oxidative phosphorylation proteins would decrease mitochondrial oxidative capacity, in turn reduce cell ATP production (Fig. 2A).

As mitochondrial ATP production is a prerequisite for GSIS [22], attenuated ATP production would lead to defective insulin secretion (Fig. 1A). So, decreased cellular energy output may be one of the mechanisms for the high glucose induced β-cell dysfunction.

4.2 Mitochondrial DNA, RNA, and protein synthesis

Twenty-one proteins involved in mitochondrial protein biosynthesis were dysregulated by the chronic hyperglycemia stimulation, including 14 proteins that were downregulated and seven proteins that were upregulated. Several components of mitochondrial protein synthesis apparatus, including amino acid-tRNA synthetases and mitochondrial ribosomal proteins were downexpressed. Downexpression of this mitochondrial protein synthesis apparatus suggested an impaired mitochondrial translational activity. As defects in mitochondrial protein synthesis could cause several mitochondrial disorders [23], decreased expression of these mitochondrial biogenesis-related proteins may be involved in the mechanism of hyperglycemia-induced mitochondrial dysfunction.

Besides that, the expression of five proteins related with mitochondrial DNA and RNA processing was dysregulated, including tRNA modification GTPase GTPBP3 (Gtpbp3), zinc phosphodiesterase ELAC protein 2 (Elac2), Peo1, endonuclease G-like 1 (EXOG), indicating hyperglycemia-disturbed DNA and RNA processing.

4.3 Substance metabolism

One important function of mitochondria is substance metabolism, such as fatty acid β-oxidation, carbohydrate, amino acid, and sulfur metabolism. In this study, 19 proteins related to tricarboxylic acid cycle, lipid, carbohydrate, steroid, and sulfur metabolism were dysregulated. Dysregulation of these substance metabolism proteins in part reflected defective energy metabolism in chronic hyperglycemia-treated β cells. BiNGO analysis also showed that metabolic process, including macromolecular metabolic process, primary metabolic process, and small molecular metabolic process were overrepresented in the dysregulated mitochondrial proteins compared with the whole proteome (Supporting Information Fig. 2).

4.4 Mitochondrial substance transport

It is well known that the vast majority of mitochondrial proteins are encoded by nuclear genome, synthesized in the cytosol, and imported into mitochondria [24]. Several translocases played important roles to transport proteins into mitochondria: TOM (translocase of the outer mitochondrial membrane) complex and TIM (translocase of the inner mitochondrial membrane) complex are the major translocases in the mitochondrial outer membrane and mitochondrial inner membrane, respectively [25]. In the study, the expression of Tim 17, Tim22, and Tom22 were downregulated and Tim23 were upregulated. The reason why chronic hyperglycemia selectively dysregulated these four translocases still need further investigations, but it may imply mitochondrial protein transport system defects in high glucose treated cells.

Besides mitochondrial translocases, the expression of two proteins related with mitochondrial calcium uptake: coiled-coil domain containing 109A (MCU) and calcium-binding atopy-related autoantigen 1 (Micu1), was downregulated. As accumulation of calcium into mitochondria has major functional consequences for the mitochondria and cells and alterations in mitochondrial calcium handling has implicated in many disease processes [26], downregulation of these proteins could decrease mitochondrial calcium uptake and lead to mitochondrial dysfunction. The decreased mitochondrial calcium uptake and ATP synthesis is also reflected by the reduced $\Delta\Psi m$ (Fig. 2D), as $\Delta\Psi m$ is essential for mitochondrial ATP synthesis [27], ROS production [28], as well as mitochondrial calcium uptake [29].

In summary, decreased protein import into mitochondria and deceased calcium uptake may be one of mechanisms for the high glucose induced β-cell dysfunction.

4.5 Cell death

One important role of mitochondria is to regulate cell apoptosis. In this study, four proteins related to cell apoptosis were downregulated, including HS1-binding protein (HAX-1), FK506-binding protein 8 (Fkbp8), HtrA serine peptidase 2 (Htra2), histidine triad nucleotide-binding protein 2 (Hint2). HAX-1 is a mitochondrial antiapoptotic protein and it has reported that degradation of HAX-1 is an early event of apoptotic process [27]. Fkbp8 inhibits apoptosis by interaction with Bcl-2 [28]. Htra2 is a serine protease that promotes cell survival when confined in the mitochondria [29]. Hint 2 is a mitochondrial apoptotic sensitizer [30], and it positively regulates mitochondrial lipid metabolism and glucose homeostasis [31]. The absence of Hint 2 could provoke the changes of mitochondrial morphology [31], which is consistent with our observation of decrease expression of Hint2 and mitochondrial morphology alterations (Fig. 2E). The decrease expression of these antiapoptotic proteins by chronic hyperglycemia could increase cell apoptosis (Fig. 2C).

4.6 Other important functions

Besides the proteins mentioned above, three proteins related with cell redox homeostasis were downregulated: glutathione *S*-transferase zeta 1 (Gstz1), microsomal glutathione *S*-transferase 1 (Gst12), and glutaredoxin 5 homolog (Glrx5). The decrease expression of these proteins may correlate with reduced ROS deactivating activity, thus increase ROS production (Fig. 2B). Some proteins that play important role in regulating mitochondrial morphology, such as ubiquitin-specific protease 30 (USP30) [32], mitochondrial fission factor (Mff), mitochondrial outer membrane protein 25 (Omp25), was downregulated, correlated with the alterations of mitochondrial morphology (Fig. 2E).

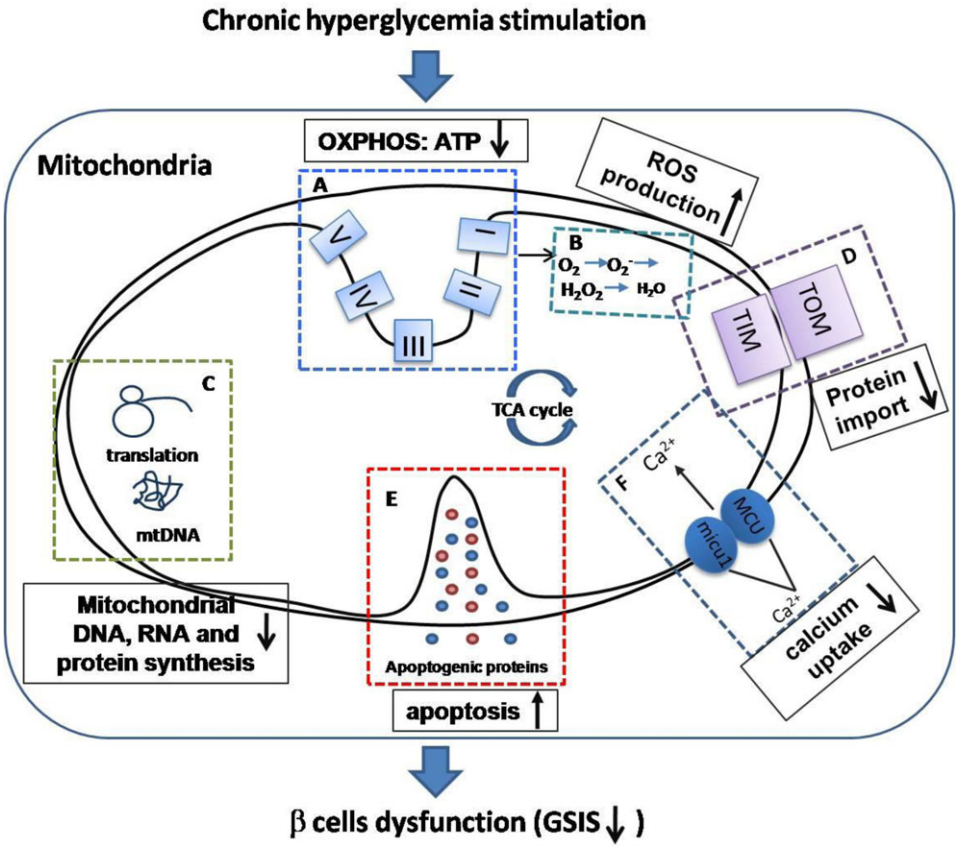

Figure 5. Integrative view of the molecular mechanisms of hyperglycemia-induced mitochondrial dysfunction on β-cell function.

In summary, the SILAC results revealed that chronic hyperglycemia stimulation could dysregulate the expression of some mitochondrial proteins involved in OXPHOS, substance metabolism, mitochondrial DNA, RNA and protein synthesis, substance transport and apoptosis, which in turn leads to decreased cellular ATP content and $\Delta\Psi m$, increased ROS production and cell apoptosis, and changed mitochondrial morphology. This ultimately leads to β-cell dysfunction (Fig. 5). These results presented an integrative view of the molecular mechanism of hyperglycemia-induced mitochondrial dysfunction and provided useful information to understand type 2 diabetes.

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The authors have declared no conflict of interest.

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