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Dynamic changes in the protein localization in the nuclear environment in pancreatic β-cell after brief glucose stimulation

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ABSTRACT

Characterization of molecular mechanisms underlying pancreatic β-cell function in relation to glucose-stimulated insulin secretion is incomplete, especially with respect to global response in the nuclear environment. Here, we focus on the characterization of proteins in the nuclear environment of β-cells after brief, high glucose-stimulation. We compared purified nuclei derived from β-cells stimulated with 17mM glucose for 0, 2, and 5 minutes using quantitative proteomics, a time frame that most likely does not result in translation of new protein in the cell. Among the differentially regulated proteins we identified 20 components of the nuclear organisation processes, including nuclear pore organisation, ribonucleoprotein complex, and pre-mRNA transcription. We found alteration of the nuclear pore complex, together with calcium/calmodulin-binding chaperones that facilitate protein and RNA import or export to/from the nucleus to the cytoplasm. Putative insulin mRNA transcription-associated factors were identified among the regulated proteins and they were cross-validated by Western blotting and confocal immunofluorescence imaging. Collectively, our data suggests that protein translocation between the nucleus and the cytoplasm is an important process, highly involved in the initial molecular mechanism underlying glucose stimulated insulin secretion in pancreatic β-cells.

Key Words: pancreatic beta-cells, glucose-stimulated insulin secretion, SILAC, proteomics, mass spectrometry, nucleus, protein translocation, nuclear pore complex, signalling pathway
INTRODUCTION

Pancreatic β-cell (PBC) is the most abundant and largest cell in the islets of Langerhans that is distributed throughout the islets of Langerhans. The biological function of PBCs is mostly to maintain the glucose level in the blood by secreting the peptide hormone insulin in response to glucose, thereby controlling the energy balance and glucose homeostasis through molecular regulation of metabolic pathways in the body.\(^1\) The normal PBCs rapidly and abundantly secrete insulin in response to high blood glucose concentrations to maintain blood glucose levels.\(^2\) However, chronic high blood glucose levels eventually promote the progressive failure of insulin action, accompanied by irreversible PBC dysfunction, thereby triggering the pathogenesis of type 2 diabetes (T2D).\(^3;4\) Indeed, the deterioration of PBC function, caused by prolonged glucose exposure, is a major determinant of T2D, which is an important reason for studying the molecular mechanisms underlying glucose-stimulated insulin-secretion (GSIS) in PBCs in details. Pathophysiological and biochemical studies have contributed to our knowledge of PBC functions for many decades. Nonetheless, the systemic GSIS-related signal transduction pathways associated with the first-phase, compensatory GSIS processes are still incomplete and poorly understood.\(^5\)

High glucose levels affects diverse cellular and molecular events on various levels in PBCs, such as regulation of glucose uptake, glycolysis, ion and amino acid transport, lipid metabolism, gene transcription, DNA synthesis, mRNA modulation and protein synthesis and degradation.\(^2\) For instance, the forkhead transcription factors, FOXO1, plays a key role in the metabolic processes of PBCs, including glucose homeostasis, alteration of PBC mass, insulin secretion and regulation of downstream genes, such as pancreas/duodenum homeobox protein 1 (PDX1) and transcription factor 7-like 2 (TCF7L2).\(^6;8\) In this regard, the nuclear transcription factors and its associated nuclear proteins transmit signals from the nucleus and translate them into metabolic or molecular responses. High glucose concentrations are sensed by the glucose transporter GLUT2 that transport the glucose over the plasma membrane and into the cell. The glucose is degraded by glycolysis resulting in an increase in the cytoplasmic ATP concentration that trigger the closure of the ATP-sensitive potassium channel and a depolarization is taking place. The depolarization triggers
voltage-gated calcium channels and subsequently the influx of \( \text{Ca}^{2+} \). The increase in the \( \text{Ca}^{2+} \) concentration initiate a multitude of signalling pathways leading to pre-insulin processing, maturation of secretory granules, regulation of the focal adhesion, and subsequently the release of insulin by exocytosis. Many of these processes have previously been characterized using molecular biological techniques. However, it remains challenging to identify signalling mechanism underlying GSIS in PBCs using physiological, molecular biological or biochemical assays. In contrast, a global proteomics approach using biological mass spectrometry (MS) will be able to characterise molecular signalling networks associated with short time GSIS in PBCs with a depth, accuracy and complexity in the analysis, including post-translational modifications (PTMs), that surpass most other assays.

In the present study, we explored signalling networks involving proteins associated with the nuclear environment of PBCs (the INS1E cell line), based on their differential appearance in the nucleus after short time stimulation with glucose. For this purpose, MS-based quantitative proteomics was applied to the subcellular nuclear fraction of INS-1E cells using stable isotope labelling with amino acids in cell culture (SILAC) combined with peptide separation using hydrophilic interaction chromatography (HILIC) and an Orbitrap MS. We quantitatively compared the nuclear proteome of INS-1E cells stimulated with 17mM of glucose for 0 min, 2 min, and 5 min. We systematically investigated the nuclear environment as well as the alteration of the nuclear pore complexes (NPCs) that facilitate the export or import of proteins and RNA in the nuclear envelope of these cells. The quality of the dataset was validated for the cytoplasm and nuclear fraction by employing Western blotting, Immunofluorescence imaging, and bioinformatics analyses. The findings support the potential nuclear-cytoplasmic translocation of proteins through the NPCs, revealing the initial GSIS mechanism in the nucleus in PBCs. The dynamic nuclear proteome could be used as a resource to improve our understanding of the mechanisms of high glucose regulated-transcription processing, post-transcriptional modifications, translocation and the physiological role of \( \text{Ca}^{2+} \)-mediated signal transduction pathway in the nuclear environment of PBCs.
EXPERIMENTAL SECTION

Cell culture and stable isotope labelling using amino acids in cell culture (SILAC)

The rat insulin-producing INS1E cell line (passages 71) were grown for 7 days in vitro on 10 cm non-coated petri dishes (Nunc™) in RPMI-1640 with glutamax and supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (PenStrep)(Gibco®), 1% sodium pyruvate, 0.1% β-mercaptoethanol, and 200 µg/ml Proline (Sigma-Aldrich) at 37 °C and 5% CO₂. The medium was changed 2 times a week. For the metabolic labelling, The cells were separately acclimated in culture media that were supplemented with distinctive stable isotope-labelled arginine and lysine amino acids (‘light’: arginine (R0) and lysine (K0); ‘medium’: arginine (R6) and lysine (K4); ‘heavy’: arginine (R10) and lysine (K8)) for several cell divisions to achieve > 95% incorporation of labeled amino acid and verified incorporation efficiency using MS analysis.

Glucose-stimulated insulin secretion

After SILAC experiment, INS1E cells were stimulated with high glucose for 0 min (control;light), 2 min (medium), or 5 min (heavy) in Krebs-Ringer HEPES buffer (KRHB; 115 mM NaCl, 4.7 mM KCl, 2.6 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 20 mM HEPES, 2 mM glutamine, 5 mM NaHCO₃, 1% (v/v) PenStrep and 0.2% (w/v) BSA; pH 7.4). For high glucose stimulation, equal numbers of INS1E cells (approximately 5 x 10⁵ cells per group) were stimulated with fresh KRHB (0.5 mM glucose;0 min) or 17 mM glucose for 2 or 5 min in non-coated 10 cm petri dishes. Subsequently, KRHB was removed and INS1E cells collected in ice-cold PBS, centrifuged 1 min at 300 x g and washed once in ice-cold PBS. After the final wash and discarding PBS, we collected the samples 0 minute (light), at 2 minutes (medium) and at 5 minutes (heavy) after high glucose treatment in biological triple experiments on a time courses (0 min, 2 min, and 5 min). Each INS1E samples were stored in ice at cold room until further processing.
Cell lysis and nuclei fractionation

To lyse the cells without damaging the nuclei, the samples were resuspended with 1 ml of ice-cold lysis buffer (10 mM HEPES; pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM dithiothreitol (DTT)) and then gently passed the cell suspension through a pre-chilled narrow-opening syringe needle (20 G) for 20 strokes. The samples were centrifuged at 228 x g for 5 minutes at 4 °C. The supernatant was stored at -80 °C as a cytoplasmic fraction, and the pellet was resuspended with 3 ml of sucrose buffer 1 (0.25 M sucrose, 10 mM MgCl₂, and EDTA-free protease inhibitor cocktail (Complete™, Roche)) and layer over a 3 ml cushion of sucrose buffer 3 (0.88 M sucrose, 0.5 mM MgCl₂, and EDTA-free protease inhibitor cocktail (Complete™, Roche)). The samples were centrifuged at 2800 x g for 10 minutes at 4 °C. At this point, we combined the nuclei samples equally in 1:1:1 proportion. The nuclei pellets can be frozen at -80 °C until needed.

Nucleus lysis, protein purification, and trypsin digestion

To extract pure nuclear proteins from the pellet, 2,2,2-Trifluoroethanol (TFE)-based lysis buffer was added to the samples for a final concentration of 50% TFE. The sample volume should be at least 50 µl in a 1.5 ml tube (Eppendorf). Samples were sonicated in an ice-cold water bath for 1 min followed by incubation in a thermomixer (Eppendorf) at 60°C for 2 hrs with gentle shaking at 300 rpm, first minute at 1400 rpm for proper mixing. After incubation, the samples were treated with 10 mM DTT for reduction of protein disulfide bonds. The extracts were sonicated in an ice-water bath for 1 min. Then, samples were incubate in a thermomixer (Eppendorf) at 37°C for 1 hr with gentle shaking at 300 rpm (first minute at 1400 rpm for proper mixing). After incubation, samples were diluted 5-fold with 50 mM NaHCO₃ buffer and 20 mM iodoacetamide was added followed by incubation for 30 minutes at room temperature in the dark in order to alkylate cysteines. At this point, the quantification achieved from Qubit® Fluorometric Quantitation (Thermo Fisher Scientific, USA). For protein cleavage, trypsin (Sigma) was added 1:50 w/w trypsin-to-protein ratio. The samples were incubated in a thermomixer (Eppendorf) for 3 hrs at 37 °C at 300 rpm or overnight. The digested samples can be stored at -80 °C or -20 °C until further processing.
**HILIC fractionation**

HILIC fractionation protocol was previously described in the literature. Briefly, all digested peptides were separately resuspended in Buffer B (90% ACN/0.1% TFA). The samples were loaded onto an in-house-packed TSKgel Amide-80 HILIC 320 µm × 170 mm capillary HPLC column using an Agilent 1200 capillary HPLC system. Samples were separated using a gradient from 100% to 60% buffer B (Buffer A: 0.1% TFA) over 35 min, then 60% to 0% over 7 min at a flow rate of 6 µL/min. The fractions were automatically collected in a 96 well plate at one minute intervals after UV detection at 210 nm, and the fractions were pooled according to the UV detection to a total of 16-18 fractions in triple experiments. The fractions were dried by vacuum centrifugation and subsequently stored at −20 °C.

**Nanoflow liquid chromatography-mass spectrometry (nLC-MS/MS) analysis**

All fractions were redissolved in A buffer (0.1% FA) and analyzed using a nLC-MS/MS system consisting of an Easy-nLC (Thermo Fisher Scientific, Odense, Denmark) and a Q exactive HF mass spectrometer (Thermo Fisher Scientific, Germany). The samples were loaded either onto 15 cm fused silica capillary column (75 µm inner diameter) or onto a 2 cm trap column (100 µm inner diameter) and separated on a 15 cm analytical column. All columns were homemade and packed with ReproSil-Pur C18 AQ 3 µm reverse phase material (Dr. Maisch, Germany). The peptides were eluted using 103–153 min gradients from 1 to 40% B buffer (95% ACN, 0.1% FA) via nanoelectrospray introduced into an MS according to the intensity of each fraction. A full MS scan in the mass area of 400–1650 Da was performed in the Orbitrap with a resolution of 120,000, an AGC target value of $3 \times 10^6$, and a maximum injection time of 100 ms. Dynamic exclusion was set to 30 s. For each full scan, the 15 most intense multiply charged ions ($4 \geq z \geq 2$) were sequentially isolated and fragmented by higher energy collision induced dissociation (HCD) with a fixed injection time of 100 ms and a resolution of 15,000. The settings for the HCD were as follows: AGC target value of $1 \times 10^5$, maximum injection time of 100 ms, isolation window of 1.4 Da, and normalized collision energy of 29.
Proteins identification and quantification

The raw MS datasets from INS1E cells with high glucose-induced different time-points (0 minute (light), 2 minutes (medium), and 5 minutes (heavy)) were processed for protein identification using the Proteome Discoverer (v2.0, Thermo Scientific) and the Sequest HT algorithm with a peptide mass tolerance of 2 ppm, fragment m/z tolerance of 0.05 Da, and a false discovery rate (FDR) of 1% for proteins and peptides. All peak lists were searched against both the UniProtKB/Swiss-Prot database (2015_08, 7,928 entries) of rat sequences with decoy using the parameters as follows: enzyme, trypsin; maximum missed cleavages, 2; fixed modification, carbamidomethylation (C); variable modifications, oxidation (M), ‘light’: arginine (R0) and lysine (K0), ‘medium’: arginine (R6) and lysine (K4), ‘heavy’: arginine (R10) and lysine (K8). Datasets with raw MS values were filtered to remove potential errors using several criteria. Protein relative expression values from the respective unique or razor peptides were calculated by summing all unique/razor peptides intensity of each protein and normalized to the number of total intensity of each group (0 min, 2 min and 5 min) estimating the relative amounts of the different protein within the relative sample. The resulting ratios were logarithmized (base = 2) to achieve a normal distribution. Ratios were averaged, an accession number was accepted as differentially expressed with a ratio of 1.5 folds or greater for medium/light and heavy/light and beyond ±1.5 fold changes, coefficient of variation (CV%) of 30% or smaller, and same expression direction (positive or negative) were defined as significantly regulated in triple experiments data. To further assess the individual statistical significance of the expression level, we showed the statistically regulated proteins by z-test for p-value (p-value<0.05, 95% confidence level) in standard normal distribution. The resulting p-values were adjusted for multiple testing with the Benjamini and Hochberg correction\(^\text{11}\). Raw data are available via ProteomeXchange with identifier PXD008988.

Functional analysis of regulated proteins

Gene Ontology annotation enrichment analysis was performed using DAVID Bioinformatics Resources (version 6.7) developed by the National Institute of Allergy and Infectious Diseases, US National Institutes of Health \(^\text{12}\). DAVID analysis made possible the enrichment of biological process, cellular components, and
KEGG pathway enrichment. The differentially expressed proteins were searched against the STRING database (version 10) for protein–protein interactions.

**Western blot analysis**

Protein extracts containing high glucose-stimulated INS1E cells lysate of either nuclei fractions or cytoplasmic fractions on a time-course (0 minute, 2 minutes, and 5 minutes) in protein loading buffer (10% glycerol, 10% sodium dodecyl sulfate, 5% β-mercaptoethanol, 0.05% bromophenol blue, and 0.5 M Tris HCl, pH 6.8) were heated at 95 °C for 5 min. Proteins were then separated using a Bis-Tris 4-12% gel (Invitrogen) and transferred onto a Hybond ECL nitrocellulose membrane (GE Healthcare) using a Trans-Blot® SD cell (Bio-Rad). After transfer, the membranes were incubated for 1 h with 5% skimmed milk powder in 50 mM Tris–HCl (pH 7.4) and 150 mM NaCl (TBS) at room temperature to prevent nonspecific binding. Blots were then incubated overnight with primary antibodies [monoclonal rabbit anti-YBX1 (36 kDa, 1:1,000, Abcam), monoclonal mouse anti-COX6C2 (9 kDa, 2 µg/ml, Abcam), monoclonal rabbit anti-SNRPF (10 kDa, 1:1,000, Abcam), monoclonal mouse anti-ARCN1 (57 kDa, 1:1,000, Novus biologicals), polyclonal rabbit anti-BEND7 (46 kDa, 1:1,000, Novus biologicals), polyclonal rabbit anti-RCC2 (60 kDa, 1:1,000, Cell signaling), monoclonal mouse anti-CREB (46 kDa, 1:1,000, Cell signaling), monoclonal rabbit anti-GAPDH (37 kDa, 1:1,000, Cell signaling), and monoclonal mouse anti-β-actin (43 kDa, 1:1,000, Santa Cruz)] and diluted in 5% skimmed milk powder in TBS at 4 °C. After three washes for 10 min in 0.1% Tween–TBS, primary antibodies were detected using an anti-rabbit IgG peroxidase conjugate (1:3,000, Cell signaling) or an anti-mouse IgG peroxidase conjugate (1:3,000, Abcam) in a solution containing 3% skimmed milk powder and 0.1% Tween–TBS at room temperature. After three washes, immune complexes were revealed using ECL detection reagent (GE Healthcare).

**Confocal immunofluorescence imaging**

For immunofluorescence staining, INS1E cells were grown on coverslips in 24-well plate without coating, and then exposed to high glucose with 500 µl of fresh KRHB (0.5 mM glucose;0 min) or 17 mM glucose for
2 or 5 min at 37 °C. For cells fixation, ice-cold methanol was added and incubated for 15 minutes at -20 °C. After washing in PBS, the cells were stored at 4 °C until they were analyzed by confocal microscopy. Fixated cells slides were then incubated overnight with primary antibodies [monoclonal rabbit anti-YBX1 (1:100, Abcam), polyclonal rabbit anti-BEND7 (1:50, Novus biologicals), monoclonal rabbit anti-SNRPF (1:100, Abcam)] diluted in 5% BSA in Tris-buffered saline (TBS) at 4 °C. Primary antibodies were incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen) secondary antibody at RT. Finally the cells were mounted using ProLong™ Diamond Antifade Mountant with DAPI (ThermoFisher, Slangerup, Denmark). Confocal imaging was performed on the fixated cells using a Zeiss LSM 510 META (Carl Zeiss). Images were analyzed using LSM Image Browser software (Carl Zeiss).

RESULTS

Proteome-wide screening for nuclear proteins in response to high glucose stimulation in PBCs

To identify the glucose-regulated nuclear proteome in INS1E cells, we stimulated SILAC labelled cells with 17mM glucose. The cells were separately acclimated in culture media that were supplemented with distinctive stable isotope-labelled arginine and lysine amino acids (‘light’: arginine (R0) and lysine (K0); ‘medium’: arginine (R6) and lysine (K4); ‘heavy’: arginine (R10) and lysine (K8)) for 6-7 cell divisions. We collected the samples 0 minute (light), at 2 minutes (medium) and at 5 minutes (heavy) after high glucose treatment. We performed a total of three separate experiments. After gently lysing cells harvested from each time point, mixed in a 1:1:1 proportion, we fractionated the nuclei using sucrose gradients and digested the nuclear proteins into peptides using trypsin. SILAC-labelled peptides were fractionated using HILIC prior to liquid chromatography-tandem mass spectrometry (LC-MSMS), and we identified the unique proteins using the SEQUEST software (Figure 1A). We identified a total of 2,123 proteins (1% false discovery rate (FDR)). We examined the temporal protein expression changes in a total of 1,413 unique proteins (at least one unique peptide or more), and 1,103 proteins (at least two unique peptides
or more). Above all, 1,037 unique proteins overlapped in at least two out of the three experiments and 74.6% (774/1,037) had the same direction of change (positive or negative) in all experiments (Figure 1B and Table S1). Following this conservative analysis, we confidently investigated the high glucose-regulated proteins (±1.5 fold changes and coefficient of variation (CV%<30%). This approach is much more conservative than most proteomic analyses but provides maximal accuracy in peptide/protein quantitation. Interestingly, after only 2 minutes of high glucose stimulation, 39 proteins increased and 14 proteins decreased in the nuclear fraction. After 5 minutes, 12 proteins increased and 57 proteins decreased (Figure 1C, Table S2, and Figure S1). Among the 774 proteins with known subcellular localisation in the Uniprot database, around 61% were known to localise in the nucleus, 41% in the nuclear lumen and 17% were co-localised with the cytoplasm (Figure 1D). We used the distinct nuclear fraction in the INS-1E cells and verified the separation of the nucleus by Western blotting with cyclic AMP-responsive element-binding protein (CREB; nuclear marker) (Figure 1E).

**A global and temporal view of the function of changing nuclear proteins**

To explore the biological meaning of the regulated proteins in a mammalian system, we systematically assigned them to the Kyoto Encyclopedia for Genes and Genomes (KEGG) pathway database and the Functional Annotation Bioinformatics Microarray Analysis (DAVID). Potential high glucose-regulated signalling pathways of the nuclear proteins were mapped for enrichment of genes by probability (p<0.05). We evaluated the signalling pathways with significantly activated or inhibited genes responding to high glucose treatment at 2 minutes and at 5 minutes compared to the control. We found 20 significantly enrichment categories for gene-sets known to play vital cellular roles in nuclear-associated functions in the cells, including ribonucleoprotein complex biogenesis, ribosome, spliceosome, ATP metabolic process, ncRNA metabolic process, oxidative phosphorylation, post-transcriptional regulation of gene expression, citrate cycle (TCA cycle), biosynthesis of antibiotics, protein processing in endoplasmic reticulum, carbon metabolism, RNA transport, synaptic vesicle cycle, ribosome biogenesis in eukaryotes, mRNA surveillance pathway, valine, leucine and isoleucine degradation, insulin secretion, endocrine and other factor-regulated
calcium reabsorption, fatty acid degradation, and tight junction (Figure 2). Interestingly, our data revealed that nuclear-related biological processes and their dynamic activity changes corresponded to classified genes in protein expression levels 2 minutes and 5 minutes after high glucose treatment. Strikingly, after only 2 minutes of high glucose stimulation, high glucose-regulated signalling pathways were mostly activated, but after 5 minutes of high glucose treatment, they were contrastively inhibited presumably due to protein translocation (Table S3).

**Rapid proteome changes suggest a reversible translocation of the nuclear proteins after high glucose stimulation**

In addition to the rapid changes of proteins in the nucleus mentioned above, we asked whether the protein translocation might be associated with the dynamic in the NPC, as it can rapidly mediate protein and RNA transport for molecular exchanges between the nucleus and the cytoplasm\(^4\). To find protein-protein interaction networks among NPC-linked proteins, we employed STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis. Interestingly, we found that the regulated proteins of the NPC participated strongly in protein and RNA export from the nucleus and protein import into the nucleus, after high glucose stimulation in our time scale (Figure 3 and 4). For protein export from the nucleus, four NPC proteins (Ran GTPase-activating protein 1 (RANGAP1), nuclear pore complex protein Nup214 (NUP214), nuclear pore complex protein Nup88 (NUP88), and Nucleoprotein TPR (TPR)) were increased after 2 min. Further, these NPC component proteins interact with calreticulin (CALR), which is a \(\text{Ca}^{2+}\)/calmodulin-binding chaperone (Figure 3). For RNA export from the nucleus, seven NPC proteins (NUP214, NUP88, nuclear envelope pore membrane protein POM 121 (POM121), nuclear pore complex protein Nup155 (NUP155), nuclear pore complex protein Nup93 (NUP93), nuclear pore complex protein Nup107 (NUP107), and TPR) were upregulated in the nucleus after 2 min high glucose stimulation, but were downregulated after 5 min glucose stimulation. We also found that eleven nucleoplasm or nuclear envelope proteins (nuclear RNA export factor 1 (NXF1), UAP56-interacting factor (FYTTD1), THO complex subunit 6 homolog (THOC6), THO complex subunit 4 (ALYREF), SAP domain-containing ribonucleoprotein
(SARNP), heterogeneous nuclear ribonucleoproteins A2/B1 (HNRNPA2B1), KH domain-containing, RNA-binding, signal transduction-associated protein 1 (KHDRBS1), ribosome biogenesis regulatory protein homolog (RRS1), nucleolar protein 6 (NOL6) and 40S ribosomal protein S28 (RPS28)) were differentially regulated after high glucose treatment compared to that control (0 min) (Figure 3). For protein import into the nucleus, nine NPCs proteins (NUP214, NUP88, POM121, NUP93, NUP98, NUP155, NUP107, nuclear pore complex protein Nup35 (NUP35) and TPR) were regulated in response to high glucose concentrations. Using STRING analysis (Figure 4), we predicted that importin subunit beta-1 (KPNB) might be a key mediator of NPC functions responding to the increase in the glucose-induced Ca$^{2+}$ concentration. In addition, we found that five mitochondrial inner membrane proteins (mitochondrial import inner membrane translocase subunit TIM16 (PAM16), mitochondrial import inner membrane translocase subunit Tim21 (TIMM21), mitochondrial import inner membrane translocase subunit Tim9 (TIMM9), sorting and assembly machinery component 50 homolog (SAMM50) and mitochondrial import receptor subunit TOM40 homolog (TOMM40)), seven nuclear envelope proteins (Cofilin-1 (CFL1), heat shock protein HSP 90-beta (HSP90AB1), heat shock cognate 71 kDa protein (HSPA8), poly [ADP-ribose] polymerase 1 (PARP1), prelamin-A/C (LMNA), heterogeneous nuclear ribonucleoprotein M (HNRNPM), and transcription intermediary factor 1-beta (TRIM28)) and Ca$^{2+}$ homeostasis-related protein (AFG3-like protein 2 (AFG3L2)) showed Ca$^{2+}$ dependent and independent associations after high glucose stimulation. As shown in Figure 3 and 4, one network is centred on NPCs and their protein and RNA export processing machinery at the nuclear membrane. Another protein import network of mainly NPCs and mitochondrion-localised proteins is shown in Figure 4.

**Relationship between the mitochondria electron transport chain and nucleus in GSIS**

To investigate a potential link between the mitochondria and the nucleus in response to brief high glucose stimulation in PBCs, we used STRING analysis in conjunction with KEGG pathway analysis. We monitored the dynamic changes in proteins belonging to the electron transport chain (ETC) in the inner mitochondrial membrane, together with a Ca$^{2+}$ binding carrier (calcium-binding mitochondrial carrier protein
Aralar1 (SLC25A12)) and nuclear proteins (e.g., methyl-CpG-binding protein 2 (MECP2) and myb-binding protein 1A (MYBBP1A)) (Figure 5). Our results showed that high glucose stimulation rapidly increased the level of four proteins in the ETC such as NADPH dehydrogenase, cytochrome b-c, cytochrome oxidase and ATP synthase together with ubiquinone, and the phosphate/calcium carrier, after 2 minutes of high glucose stimulation. Interestingly, after 5 minutes, the protein levels were simultaneously decreased (Figure 5). In our data, MECP2 and MYBBP1A were independently upregulated in the nucleus corresponding to the alteration of subunits of ETCs in the mitochondrion in our temporal data, whereas these were not downregulated after that elevated intracellular Ca\(^{2+}\) concentrations. In previous studies, MECP2 or MYBBP1A were known to be upstream regulators of mitochondrial functions, such as ATP synthesis, reactive oxygen species (ROS) generation, unfolded protein response and proteasome processes.\(^{15-17}\) These findings are highly consistent with the alteration of proteins in the nucleus in our temporal data set, indicating that high glucose concentrations may concurrently regulate the activities of both ETCs and nuclear functions according to intracellular Ca\(^{2+}\) accumulation in GSIS.

**Temporal regulations and translocation between nucleus and cytoplasm in GSIS**

To further elucidate the nuclear-cytoplasmic translocation of proteins in GSIS, we selected six differentially regulated and/or putative translocated proteins (nuclease-sensitive element-binding protein 1 (YBX1), small nuclear ribonucleoprotein F (SNRPF), cytochrome c oxidase subunit 6C-2 (COX6C2), coatamer subunit delta (ARCN1), RCC1-like protein TD-60 (RCC2) and BEN domain-containing protein 7 (BEND7)), that have not been previously studied in relation to GSIS. To seek evidence of high glucose-induced nuclear protein translocation, we performed Western blotting analysis in the cytoplasmic and nuclear fractions 2 and 5 min after high glucose treatment, revealing the initial high glucose-mediated molecular events occurring between nucleus and the cytoplasm in PBCs. As shown in Figure 6A, exposure to high glucose levels had an effect on nuclear-cytoplasmic trafficking. YBX1 gradually decreased in the nuclear fraction after 2 minutes of high glucose treatment in INS1E, and elevated levels of YBX1 was also found in the cytoplasmic fraction after 2 minutes of high glucose stimulation. Four proteins (SNRPF, COX6C2, ARCN1, and BEND7) were predominantly increased in the nucleus at 2 minutes of high glucose treatment.
stimulation, but the proteins levels were significantly decreased after 5 minutes compared with that of the control (0 minutes). While RCC2 showed an equal expression at all the time points in the nuclear preparation, the cytoplasmic fraction of the RCC2 protein was decreased only at 2 minutes of high glucose stimulation. As shown in Figure 6B, the SILAC results showed a good correlation with the Western blotting results. As shown in Figure 6C, the tryptic peptide of full C-peptide was increased after 2 minutes of high glucose treatment. The C-peptide of proinsulin is very well known to be a hallmark of insulin synthesis processes and mature insulin secretion in response to glucose in the PBCs of pancreatic islets.\textsuperscript{18}

**High glucose-induced translocation of YBX1, BEND7, and SNRPF in PBCs**

To further evaluate the nuclear-cytoplasmic translocation upon high glucose stimulation in a short-term time scale (0, 2, and 5 minutes), we assessed three proteins (YBX1, BEND7 and SNRPF) in INS-1E cells using immunofluorescence labeling combined with confocal microscopy. As shown in Figure 7, INS-1E cells expressed the YBX1, BEND7, and SNRPF proteins, which can have nuclear and/or cytoplasmic localisations (Figure 7). The YBX1 protein was mostly localised in a nucleus before high glucose stimulation, and its fluorescence staining indicated a translocation to the cytoplasm after glucose stimulation (Figure 7A). On the other hand, BEND7 and SNRPF proteins were highly expressed in the nucleus at 2 minutes of high glucose stimulation (Figure 7B and 7C). After 5 minutes, the localisation of BEND7 and SNRPF proteins was shown to be mainly in the cytoplasm of PBCs (Figure 7B and 7C). We demonstrate that YBX1, BEND7 and SNRPF proteins could, dependent on a time scale, be either translocated into the nucleus or exported from the nucleus to the cytoplasm, as a result of the glucose stimulation. The accumulation of proteins in the nucleus correlates with our temporal SILAC proteomics data.
DISCUSSION

Our results demonstrated that the observed dynamic changes of nuclear proteins in response to high glucose stimulation were not only due to an overall regulation in proteins expression but also probably resulted from an elevated import/export of nuclear proteins to the cytoplasmic localisation through NPCs at short-term time points (0, 2, and 5 minutes). We molecularly characterised the molecular mechanisms underlying GSIS in PBCs in the nuclear environment using a commonly used model system in PBC biology, the INS-1E cells. We applied SILAC-based quantitative proteomics on the isolated nuclei fraction from INS-1E cells after 0, 2 and 5 min of high glucose stimulation and cross-validated our results using Western blotting and confocal immunofluorescence imaging. Accordingly, we generated the first quantitative and, we believe, reliable resource about the proteome-wide nuclear environment in conjunction with GSIS in PBCs. We expect that our temporal proteome data will be a useful resource for improving our knowledge of the nuclear environment and the importance of protein translocation between the nucleus and cytoplasm in PBCs exposed to high glucose concentrations.

Most interestingly, our temporal data revealed spectacular changes in proteins belonging to the nuclear organisation (e.g., RNA polymerase I&II and active genes) and the NPC-mediated protein and RNA import and export machinery, which occurred after short time glucose exposure. These changes affected the regulation of transcription from RNA polymerases promoters, non-coding RNA metabolic processes, spliceosome and ribosomal RNA processing, post-transcriptional modification of gene expression and ribonucleoprotein complex biogenesis (Figure 8). All the transcriptional regulations are known to be activated along with Ca\(^{2+}\) accumulation in the cell at only 2 minutes of high glucose treatment.\(^{19,20}\) After 5 minutes of high glucose stimulation, these biological processes were reversed, whereas, the amount of NPC component proteins was relative higher after that time. As such, the NPC-associated proteins and nucleo-cytoplasmic trafficking factors imply transcriptional regulation in the cell\(^{21,22}\). Taken together, high glucose stimulation affected the proteome-wide temporal regulations and reversible nucleo-cytoplasmic transport through the modulation of the NPCs organisation. The temporal- and nucleo-proteome regulations are
coming together in a cohesive view of how this alteration of protein localisation enables communication that
instructs GSIS-related gene expression, controls proinsulin synthesis, and modulates other major biological
processes in the nuclear environment in PBC during GSIS. These findings are also consistent with the
crosstalk between nucleus and mitochondrion including ATP synthesis, via the regulation of the ETC.\textsuperscript{23}

To narrow down our data mining, we selected proteins that had not previously been examined
in association with GSIS and validated our proteomic targets by Western Blotting and immunofluorescence
imaging analysis. We chose to further investigate YBX1, which is known to be a Y-box transcription factor
that can bind and stabilise cytoplasmic mRNA and shuttle between the nucleus and cytoplasm upon specific
conditions, and localise in cytoplasmic granules containing untranslated mRNAs in proliferating cells\textsuperscript{24-27}.
Furthermore, it has been shown to regulate the MEK/ERK pathway-dependent genes.\textsuperscript{28} In the current study,
we found that the YBX1 level was higher in the nucleus before high glucose treatment and its level was
lower in the nucleus after glucose stimulation due to translocation into the cytoplasm. Our data suggest that
YBX1 could be implicated in cytoplasmic mRNA processing, triggering glucose-dependent short-term
regulation of new preproinsulin transcription. Another protein of interest is SNRPF, which plays an
important role in the splicing of cellular pre-mRNAs. The survival of motor neuron (SMN)-mediated
assembly into spliceosomal small nuclear ribonucleoproteins (snRNPs) occurs in the cytosol before SMN-
mediated transport to the nucleus to be included in spliceosomes.\textsuperscript{29} In response to elevated glucose levels (2,
5, 7, or 25 mM), the insulin gene pre-mRNA species elevated within 60 minutes only at 25 mM glucose
concentration, whereas increases in mature mRNA did not occur until after 48 hours, implying the early
increase of the insulin gene pre-mRNA transcription respond to high glucose, but more chronic exposure of
islet to glucose eventually resulted in an increase in mature mRNA.\textsuperscript{30} As shown in Figure 6, SNRPF
increased at 2 minutes of high glucose stimulation, and it decreased after 5 minutes of high glucose treatment,
presumably through translocation into the cytoplasm. These data suggest that SNRPF might play a key role
in the changes of the insulin gene transcriptional rates and that high glucose acutely enhances insulin
transcription by recruitment of the pre-mRNA species machinery within a relatively short time. Another
protein involved in protein translocation between nucleus and cytoplasm in GSIS is BEND7, which is a BEN
domain containing proteins 7. There is no information about the biological function of BEND7. A protein containing four BEN domains (BEND3) has been identified; it is thought to be involved in chromatin functions and transcription.\textsuperscript{31} We used Western blotting and immunofluorescence to confirm that BEND7 was highly expressed specifically at 2 minutes of high glucose stimulation in the nucleus, and after 5 minutes, it is translocated into the cytoplasm.

There were a number of protein changes that were unknown in relation with GSIS. One of these proteins, COX6C2, is the nuclear-coded polypeptide chains of cytochrome c oxidase, the terminal oxidase in the mitochondrial ETC.\textsuperscript{32} ARCN1 is associated with mRNA binding proteins and ADP-ribosylation factors (ARFs),\textsuperscript{33} which is further associated with ER-to-Golgi biosynthetic protein transport. Another protein of interest, RCC2, is known to be a guanine nucleotide exchange factor for the small GTPase RAC1.\textsuperscript{34} According to our Western blotting results, levels of COX6C2, ARCN1, and RCC2 were significantly higher in the nucleus at 2 minutes of high glucose treatment compared to the control (0 minutes). After 5 minutes, the levels were lower compared to the control. This difference between the time points was also consistently observed in our SILAC data.

Although we have started to address the evidence of brief high glucose-mediated translocation of proteins in INS-1E cells in conjunction with NPCs organisation, developing a detailed understanding of how it initially progresses and functions associated with the proinsulin synthesis and production to maintain blood glucose levels would be far more complicated. In this study, we did not investigate PTMs like phosphorylation and O-GlcNAcylation, which are known to affect protein localization and function. Several studies have proposed how the regulation of O-GlcNAcylation and its interplay with phosphorylation might play a key role in protein translocation between the nucleus and the cytoplasm, in response to nutrients like glucose.\textsuperscript{35-37} Subcellular-based PTMomics is extremely difficult to perform, but it may contribute to increase our knowledge of the mechanisms of GSIS and its initial signal transduction pathways at the molecular level with an advanced combination of phosphorylation and O-GlcNAcylation enrichment methods.\textsuperscript{38-39}
To this end, many studies have characterized the GSIS molecular events using long-term high glucose stimulation, over several hours or days. However, from a physiological point of view, PBCs are exposed to high glucose levels for several minutes rather than hours after food uptake.\textsuperscript{40-41} For example, Leibiger et al. demonstrated that short-term glucose stimulation led to a destabilisation of the insulin gene pre-mRNA, thereby triggering the transient increase of cytoplasmic steady-state insulin gene pre-mRNA levels and protein translocation.\textsuperscript{40} Wang et al. has described that glucose stimulation lead to splicing of the insulin gene pre-mRNA and the occurrence of the matured transcript in the cytoplasm within minutes in mouse islets.\textsuperscript{42} NPC is a large protein complex and normally stable with a function as a structural scaffold. A change in NPC composition can dynamically regulate export or import proteins/mRNA rapidly within ~0.5 second in response to extracellular stimulus, maintain their functions such as protein/mRNA targeting, translocation, recycling, and release transport between the cytoplasm and the nucleus as the long-lived protein complex.\textsuperscript{43-45} Thus, dynamic behaviour of NPC is a critical molecular machinery for nucleocytoplasmic transport.\textsuperscript{46} In our study, we showed evidence of molecular events occurring in association with the dynamics of proteins translocation between nucleus and cytoplasmic localisation through cytoplasmic pre-mRNA transcription factors, such as YBX1 and SNRPF. Taken together, our data suggest that the subcellular crosstalk between the nucleus and the cytoplasm could be a key mechanism of GSIS in PBCs, triggering the transportation of newly synthesised insulin gene pre-mRNA to the cytoplasm together with various proteins. Consequently, our short-term nucleo-proteome data could be a useful resource for improving our understanding of the mechanisms of short time GSIS related to protein and mRNA translocation between the nucleus and cytoplasmic localisations, spliceosomes, NPCs, post-transcriptional modifications and biosynthesis of preproinsulin in PBCs. Altogether, our proteome-wide data for the nuclear environment and bioinformatics analyses first provide a global roadmap of the major molecular components and signalling pathways potentially involved in PBC functions and initial nuclear responses for GSIS.
ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

The following files are available free of charge at ACS website http://pubs.acs.org:

Table S1. The list of overlapping proteins in at least two out of the three replicates (excel)

Table S2. The list of regulated proteins (excel)

Table S3. The list of significant 20 enrichment categories for regulated proteins (excel)

Figure S1. The observed MS/MS spectra of the regulated proteins containing single unique peptide (word)

AUTHOR CONTRIBUTIONS

M.R.L conceived research. M.R.L and T.K. designed experiments. T.K. and P.J. performed experiments. T.K. performed mass spectrometry and bioinformatics analysis. P.J., V.S., J.R.B. carried out confocal microscopy. T.K. and M.R.L wrote the paper. All authors proofread the manuscript.

REFERENCES


**FIGURE LEGENDS**

**Figure 1.** Characterization of the proteome of nuclear environment responding to high glucose (17 mM) in INS1E cells. (A) SILAC-based proteomics workflow for nuclear proteome profile. (B) Venn diagram of quantified proteins in the three biological replicates, resulted in 1,037 proteins containing unique peptides for 1,249 overlapping proteins. (C) Bar graph representing the distribution of regulated proteins between 2 minute of high glucose stimulation (left) and 5 minutes of high glucose stimulation (right) compared to that control (0 min). (D) Showing the efficiency of the applied subcellular fractionation among the proteins with known subcellular localization in the Uniprot database. (E) Immunoblotting of CREB, verification of nuclei fractions in comparison with cytosol fractions.

**Figure 2.** A schematic representation of global and temporal view of changing nuclear proteins. Heatmap of regulated proteins (green) of the nuclear dynamics, with the probability of KEGG pathways analysis (orange
The expression of regulatory proteins with the corresponding signaling pathways as indicated their activation (blue) or inhibition (yellow).

Figure 3. Assembly of nuclear pore complexes from differentially modulated proteins, as revealed in our temporal data. Node colors were indicated the expression levels according to a time scale (2 min and 5min) in protein export from nucleus (left) and RNA/RNS export from nucleus (right).

Figure 4. Dynamic alteration of proteins in protein import into nucleus, including nuclear pore complexes. Node colors were indicated the expression levels according to a time scale (2 min and 5min), as revealed in our temporal data.

Figure 5. Schematic representation of the relationship between electron transport chain and nucleus, as revealed in our temporal data. Node colors were indicated the expression levels according to a time scale (2 min and 5min).

Figure 6. Altered proteins expression or localization between nucleus and cytoplasmic fractions on a time scale (0, 2, and 5 min) after high glucose (17 mM) treatment. (A) Western blot analysis of six regulated proteins (YBX1, SNRPF, COX6C2, ARCN1, RCC2, and BEND7) and control markers (beta-actin and GAPDH), and shows the different expression levels between fractions according to a short-term time scale. (B) Bar graph represent the SILAC-based proteome data consistent with our Western blot results. (C) MS/MS spectra of the C-peptide from insulin protein.

Figure 7. Immunofluorescence imaging analysis of INS1E cells imaged after 0, 2 and 5 minutes of high glucose stimulation show altered protein localization. (A) YBX1 (green) and DAPI (blue) are co-localized at 0 minutes of high glucose stimulation in the nucleus of pancreatic β-cells but not at later time points. (B) BEND7 (green) and DAPI (blue) co-localize at 0 min and 2 min after high glucose stimulation. However after 5 minutes most of the BEND7 is observed outside of the nucleus. (C) SNRPF (green) and DAPI (blue) are seen to be more highly co-localized at 2 minutes of high glucose stimulation then at 0 and 5 minutes. The labeled proteins are displayed in the green while DAPI (blue) was used to stain the nuclei (scale bar=2 μm).
Figure 8. Schematic representation of the nuclear environment in INS1E exposed to high glucose levels (17 mM). High glucose affected the regulation of nuclear functions and biological processes for the indicated time points with reported Ca$^{2+}$ levels in our time course data.
Figure 1

(A) Pancreatic beta-cells were cultured in light (ROK0) or medium (R6K4) and heavy (R10K8) conditions with 17 mM glucose. Light cells were harvested at 0 min, medium cells were harvested at 2 min, and heavy cells were harvested at 5 min. Cells were mixed lysates 1:1 and fractionated to nuclei.

(B) Venn diagram showing the overlap of unique proteins across replicates. Replicate 1: 1,381 proteins; Replicate 2: 1,625 proteins; Replicate 3: 1,094 proteins. Overlapping: 1,249 unique peptides (1,037 unique proteins).

(C) Graph showing the number of proteins identified over time. 2 min: Replicate 1 has 39 upregulated proteins, 14 downregulated proteins, and 984 not regulated proteins. 5 min: Replicate 1 has 12 upregulated proteins, 57 downregulated proteins, and 966 not regulated proteins.

(D) Same direction of expression changes (n): 774. List total genes (n): 704.

(E) Western blot showing the expression of CHLB in different conditions.
Figure 2

[Image of a heatmap and bar graph showing regulated proteins in KEGG pathways, with bars indicating probability and ratio (log2) for 'down' and 'up' regulated proteins for 2 min and 5 min time points.]
Figure 3
Figure 4
Figure 5

High glucose concentration

Cytoplasm

Nucleus

Calcium-binding carrier

Matrix

SLC25A12

NCUFA7

NCUFA2

NCUFA3

NCUFA4

NCUFA5

NCUFA6

SLC25A3

COX10

COX11

ETTOH

NDUFA1

NDUFA2

NDUFB2

NDUFG2

Fumarate

Succinate

Ubiquinone

Cytochrome c

Cytochrome b-c1

Cytochrome oxidase

ATP synthase

Time scales

2 min 5 min

Up-regulation
Down-regulation
Figure 6

A

<table>
<thead>
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<th>17 mM glucose (+)</th>
<th>Nuclei</th>
<th>Cytosol</th>
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<tbody>
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<td>YBX1</td>
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B

- **YBX1**
  - Relative intensity (%)
  - Time (minutes)
  - 0, 2, 5

- **SNRPF**
  - Relative intensity (%)
  - Time (minutes)
  - 0, 2, 5

- **COX6C2**
  - Relative intensity (%)
  - Time (minutes)
  - 0, 2, 5

- **ARCN1**
  - Relative intensity (%)
  - Time (minutes)
  - 0, 2, 5

- **RCC2**
  - Relative intensity (%)
  - Time (minutes)
  - 0, 2, 5

- **BEND7**
  - Relative intensity (%)
  - Time (minutes)
  - 0, 2, 5

C

- **C peptide**
  - Intensity (pM)
  - Mix
  - Lights, Minutes, Hours
  - 0, 2, 5, 10, 15, 30, 60
Figure 7

A

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<th>YBX1</th>
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B

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C

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Figure 8

High Glucose concentration

GLUT2

Mostly decreased

Mostly increased

0 min

2 min

5 min

Cytosol

Nuclear pore

Nucleus

RNP complex

Post-transcriptional modifications

Finished-mRNA

Spliceosome

rRNA

Pre-mRNA

ncRNA

Transcription

DNA

Nuclear pore organization

Ribonucleoprotein (RNP) complex biogenesis

Posttranscriptional regulation of gene expression

rRNA processing

Spliceosome

ncRNA metabolic process

Negativeregulation of transcription from RNA polymerase II promoter

Transcription of nuclear large rRNA transcript from RNA polymerase I promoter
for TOC only