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Bottom-up proteomics analysis of the secretome of murine islets of Langerhans in elevated glucose levels

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Abstract

Glucotoxicity is a causative agent of type-2 diabetes, where high glucose levels damage the islets of Langerhans resulting in oxidative damage and endoplasmic reticulum stress. We evaluated the secretomes of healthy CD-1 murine islets. Three experimental conditions were investigated in biological triplicate: a control incubated with 11 mM glucose, 1-day incubation with 25 mM glucose, and 2-day incubation with 25 mM glucose. An SDS-based, filter-aided sample preparation protocol was used to prepare secretomes for analysis. A total of 428 protein groups were identified across the nine samples. Each condition generated between 328–349 protein IDs and intracondition protein overlap was between 66–90% for the biological triplicates. 232 protein groups were identified in all three conditions with 184 quantified at least once in each condition. Significant expression changes were observed for proteins associated with the unfolded protein response, such as proteases, chaperones, and elongation factors, as well as proteins associated with peptide hormone processing and small molecule metabolism.

Graphical abstract

Supporting information

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

It has been estimated that 422 million people suffer from diabetes,¹ a disease characterized by persistent high blood sugar levels. Islets of Langerhans are clusters of cells responsible for blood glucose regulation via hormone secretion. The peptide hormones insulin and glucagon work antagonistically, and dysregulation or insensitivity towards these hormones can cause type 2 diabetes, which accounts for roughly 90% of diabetes cases.²⁻³ It is believed that glucotoxicity is at least partially responsible for damage to islets, which in turn causes blood sugar dysregulation.⁴⁻⁵

In vitro, islets exhibit glucotoxicity at glucose concentrations between ~20-40 mM.⁶⁻⁹ Glucotoxic conditions have been used to study insulin¹⁰⁻¹¹ and glucagon¹² release, endoplasmic reticulum (ER) stress,¹³ and oxidative stress.¹⁴ Oxidative and ER-stress elicit the unfolded protein response (UPR), where unfolded or misfolded proteins are degraded and overall protein synthesis is reduced.¹⁵⁻¹⁶ Recent findings have implicated the UPR to be a causal agent in β -cell failure and diabetes.¹⁷ Herein, we study the effect of elevated glucose levels corresponding to glucotoxicity on the secretome of Islets of Langerhans.

Secretomes are the total proteinaceous content secreted from a sample. Mammalian secretomes are moderately complex, typically containing 250-700 protein groups.¹⁸ However, the study of secretomes is challenging. Components can span a wide range in abundance, and samples can be contaminated by growth media and cell lysis.¹⁹⁻²⁰ Secretomes have been found to be rich in biomarkers and signaling molecules.²¹⁻²² Moreover, the secretome has been shown to be altered in response to the UPR.²³

We previously described a filter-aided sample preparation (FASP) protocol for the bottom-up proteomic analysis of the secretome of the islets of Langerhans.²⁴ Insulin and other peptides are depleted using a 30-kDa molecular-weight cutoff filter. This depletion allows a deeper analysis of higher molecular weight components of the secretome by removing highly abundant insulin and C-peptide. We now report the use of this protocol to study islets in

three conditions: a control incubated with 11 mM glucose, 1-day incubation with 25 mM glucose (1 day elevated glucose), and 2-day incubation with 25 mM glucose (2 day elevated glucose). This glucose concentration falls within the glucose toxic range of 20-40 mM.²⁵

Materials and Methods

Islet Isolation and Culture

Islets were isolated from 6-7 week old CD-1 mice as described previously.²⁴ Pancreata were injected with 2-3 mL of collagenase and placed in 1 mL of G solution. Islets were isolated from the interface of a Histopaque-1077 and HBSS gradient separation. Islets were separated in groups of 50 and incubated overnight in RPMI-1640 medium with 10% FBS and 1% penicillin/streptomycin.

Islets were incubated in biological triplicate in three conditions: control, 1-day elevated glucose (1EG), and 2-day elevated glucose (2EG). Control islets were incubated for 24 h in FBS-free RPMI 1640 media with 11 mM glucose. 1EG islets were incubated for 24 h in FBS-free RPMI media with 25 mM glucose, and 2EG islets were incubated for 48 h in FBS-free RPMI media with 25 mM glucose. After incubation, media was collected for analysis.

FASP

Media was mixed with a protease inhibitor (Roche Diagnostics) and vacuum concentrated to ~500 μ L. Stock sodium dodecyl sulfate (SDS) and dithiothreitol (DTT) was added to make the final solution 1% SDS and 5 mM DTT in 600 μ L of solution. Samples were sonicated on ice for 5 min with a duty cycle of 50%. Samples were incubated at 60 °C for 30 min to ensure reduction. Alkylation was subsequently performed by incubating samples with 15 mM iodoacetamide (IAA) in the dark for 30 min. Alkylation was quenched with excess DTT. Samples were stored at -80 °C.

Samples were thawed, mixed with equal volume of freshly prepared UA (8 M urea and 100 mM Tris-HCl, pH 8.5) solution, loaded into 30 kDa molecular weight cut-off spin columns (Millipore), and centrifuged at 16,000 g for 20 min. All subsequent centrifugation steps used the same parameters. Samples were washed five times with 300 μ L of UA followed by three washes of 300 μ L of 25 mM NH_4HCO_3 (pH 8.5). Samples were incubated overnight at 37 °C using 4 μ g of sequencing grade trypsin in 25 mM NH_4HCO_3 . Peptides were eluted the next morning followed by two washes of LC grade water. Samples were dried using a speed vacuum and then resuspended in 20 μ L 2% acetonitrile 0.1% formic acid.

LC-MS/MS

An AQUITY BEH peptide UPLC column (1.7 $\mu\text{m} \times 100 \mu\text{m} \times 100 \text{ mm}$) coupled to a nanoACQUITY UPLC system (Waters) was used for peptide separation. LC solvents were 0.1% FA (A) and 0.1% FA in ACN (B). For each run, 1.5 μ L of sample was loaded onto the column with 5% B at a flow rate of 0.7 μ L/min for 14 minutes followed by a three step gradient: 1 min from 5-10% B and 10-30% B over 59 min both with flow rates of 0.600 μ L/min, followed by 1 min to 80% B at a flow rate of 0.700 μ L/min. The column was

equilibrated at 5% B for 15 min before the running the next sample. Technical triplicates were taken from each sample.

A Q Exactive HF hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) was used to collect mass data. Full MS scans were acquired across a range of 350-1800 m/z at a resolution of 60,000 (m/z 200) and automatic gain control (AGC) set to 3.00×10^6 . Higher energy collisional induced dissociation was performed on the 10 most intense ions with a charged state 2 with a normalized collisional energy setting of 28. MS/MS resolution was 60,000 (m/z 200) with an isolation window of 1.4 m/z, and an AGC value set to 1×10^5 . A 40 sec dynamic exclusion was set.

Data Analysis

MaxQuant²⁶ version 1.5.3.3 was used to process the spectra. The murine reference proteome database (*Mus musculus*/Uniprot: UP000000589) was searched using Andromeda.²⁷ Mass spectra were searched using the default setting including a 20 ppm mass tolerance. Cysteine carbamidomethylation was used as a fixed modification and acetylation (K and N-terminus), deamidation (NQ), phosphorylation (ST) and oxidation (M) were used as variable modifications. Only acetylation (N-terminus) and oxidation (M) modifications were considered for quantification. The selected protease was trypsin/p with up to two missed cleavages allowed. Protein and peptide false discovery rates (FDR) were set to 0.01 and minimum peptide length was set to 7 residues. iBAQ quantification and match between runs were enabled. Identifications were filtered to remove possible contaminants and reverse identifications.

Proteomic Analysis

Statistical tests including column correlation and one-way ANOVA were performed using Perseus.²⁸ A one-way ANOVA test was run using proteins quantified in all nine experiments. Biological triplicates were grouped and significance ($p < 0.05$) was tested for each protein. Column correlation was performed by comparing individual samples to the mean value for each protein. Sample list was reduced to proteins that were identified at least once in every condition. Missing values were imputed from a normal distribution using default values and the total matrix as the mode.

Protein visualization software GProX²⁹ was used to perform cluster analysis. Log₂ transformed iBAQ value normalized to our control condition were utilized. For cluster analysis, the number of clusters was set to 5 with fixed threshold values of +1 and -1, corresponding to a two-fold change. All other parameters remained at default settings. STRING 10.0 was used for gene ontology analysis and molecular interaction mapping with the whole genome being used as the statistical background.³⁰

Results and Discussion

Protein Identifications

Biological triplicates were generated for each of three conditions: control (11 mM glucose), one day elevated glucose (25 mM glucose, 1EG), and two-day elevated glucose (25 mM

glucose, 2EG) (Figure 1). 50 islets were used in each experiment, and islets were pooled from several mice. 70-100 islets were typically isolated from each mouse.

428 protein groups were identified across the nine samples (Figure 2). The numbers of protein IDs ranged between 328-349 for each sample. The average intercondition protein overlap was 85%, and the average intracondition protein overlap was 68%. Eighty-six protein groups were found in all nine samples, and an additional 29 were found in eight of the nine samples. The highest overlap between two conditions was between normal and one-day elevated glucose (1EG). The shared protein identifications suggest that there is a conserved core that composes the islet secretome.

Earlier papers have characterized the proteome of the intracellular insulin secretory granule. In one study, insulin granules were isolated by centrifugation; that study identified ~150 protein groups.³¹ Another study used immunopurification with VAMP2 antibodies to generate 51 protein IDs.³² Disappointingly, only 15 protein groups were identified in common in these two studies.³³ In contrast, we identified 30 of 51 proteins from reference 31 in all of our conditions, and 8 more were found in some conditions (Table 2).

Significant Protein Secretion Change—The data set was reduced to 52 proteins that were quantified in all nine runs. A one-way ANOVA was performed on each protein to determine if there was a significant difference between the three conditions. Expression of seven proteins differed at $p < 0.05$: chymotrypsin-like elastase 1, 2a, and 3b, chymotrypsinogen B1, trypsin 4, pancreatic lipase, and pancreatic alpha-amylase. Abundances for these proteins tended to be similar between the control and 1EG, but dropped precipitously in 2EG samples.

A Pearson correlation was calculated for the protein expression in each of the runs compared to the mean expression for identified proteins. The mean column correlation for the biological triplicates within the sample was between 0.88 and 0.90 (Table 1). Every individual sample most closely resembled the mean of its own condition (Supplemental Information, Table S1 and Figure S1). Despite high variation in our samples, there are reproducible intercondition tendencies.

Cluster Analysis—A cluster analysis was used to determine trends in secretome profile across the three conditions (Figure 3 and Supplemental Information – Cluster Analysis).³⁴ Proteins were clustered using a two-fold increase or decrease. Proteins that did not change expression by more than two-fold across the three experimental conditions were assigned to cluster 0.

Traditional proteomics uses imputation to estimate missing values. The cluster analysis turned out virtually the same with and without imputation. Only 7 of 184 proteins changed with the gene ontology enrichments seen in both condition. For the following cluster analysis, imputed values were used.

Cluster 1—Cluster 1 proteins are upregulated in 2EG samples. Cluster 1 was enriched for proteins associated with the RAP1 signaling pathway as well as peptide hormone processing

(Table 3). Evidence suggests that RAP1 signaling is essential for the regulation of insulin granule dynamics,³⁵ which is consistent with cluster 1 being enriched in peptide hormone processing proteins.

Cluster 2—Proteins that markedly increased in secretion in 1EG and 2EG are in cluster 2. Cluster 2 was found to be enriched for proteins associated with small molecule metabolism, specifically oxoacid (ketoacid) metabolism. Many of the proteins associated with oxoacid metabolism are known to be dysregulated with diabetes.³⁶⁻⁴⁰ Additionally, four of the 17 proteins are associated with the modulation of Ca²⁺ signaling, which is important for secretory vesicle release, including chromogranin A, secretogranin 1 and 3, and regucalcin. Secretogranin 2 has a similar expression profile to the other secretogranins, but fell below the significance threshold. Secretogranins are commonly found in secretory vesicles, the function of these proteins remains unknown, and they are known to be cosecreted.⁴⁰⁻⁴¹

Insulin-1 and carboxypeptidase E were found in cluster 2, whereas insulin-2, and neuroendocrine convertase 1 and 2 were found in cluster 1. Previous work has shown differential response in insulin gene expression to high glucose levels.⁴²⁻⁴³ It is not clear why insulin-1 and carboxypeptidase E have a quicker response to glucose than insulin-2 and the prohormone convertases. One possibility is the content of insulin granules alters in response to glucose.

Cluster 3 and 5—Clusters 3 and 5 show a drop in expression during the first 24 hours of high glucose incubation, but return after longer exposure. Cluster 3 protein expression did not returned to normal level despite the rebound whereas cluster 5 protein expression returned to or increased when compared to normal. Cluster 3 was enriched for proteins associated with apoptosis signaling pathway and cluster 5 was enriched in protein associated with translational elongation and protein folding, which are notable protein classes whose expression is altered during the UPR. The prevailing thought is that when stressed, chaperones bind ER-associated proteins.⁴⁴⁻⁴⁵ Secretion of ribosomal proteins and chaperones appeared to be upregulated after prolonged exposure to 25 mM glucose, but decreased in short-term exposure.

There are three main ER stress sensors that trigger the UPR: IRE1, PERK, and ATF6. We found several proteins in cluster 5 that interact with these stress sensors including two heat shock protein 90 proteins that interact with IRE1 and PERK⁴⁶ and Ppp2r1a, which is activated by IRE1.⁴⁷ However, HSP90 proteins were also found in the insulin secretory granule so that the higher expression of HSP90 proteins could also be an alteration in granule dynamics. Despite a global decrease in translation a few key proteins have increased expression during UPR. Several of these proteins were found in cluster 5 including Calm1, Eef1a, Eef1g, and Hspa8,⁴⁸⁻⁴⁹ Prdx1 and Prdx6,⁵⁰ and Fkbp2.⁵¹

Three additional chaperones (Ppie, Ptges3, and Cct4), three additional protein associated with elongation (Rps5, Eif3h, and Srp9), and three additional proteins associated with ER-transport (Copz1, Copb2, and Sec13) were found exclusively in 2EG samples. The observation of these proteins suggests that prolonged exposure to high glucose concentrations causes an increase in secretion of elongation and folding proteins. Our mass

spectrometry based data does not discriminate between lysosomal waste and active secreted protein. Presumably, there is an increase in lysosomal secretion as Hspa8, a protein that binds specific proteins and directs them to lysosomes,⁵² was found to have the highest expression in 2EG. The identification of additional ER-transport and elongation may be causative or symptomatic of the UPR. However, the identified chaperones may active as overall chaperone expression is increased during the UPR.⁵³

14-3-3 proteins are phosphoprotein-binding proteins that are implicated in many regulatory roles. Three of the four 14-3-3 proteins were associated in clusters 3 and 5. 14-3-3 proteins beta and eta (Ywhab, Yhah) were only quantified in 2EG samples. In general, 14-3-3 proteins were most abundant in 2EG, which is consistent with earlier data that showed 14-3-3 proteins were only found in glucotoxic β -cell incubations.⁹ 14-3-3 proteins are thought to help attenuate insulin signaling as well as being associated with oxidative stress.⁵⁴⁻⁵⁶ New evidence also suggests that 14-3-3 proteins may help protect against ER-stress.⁵⁷

Cluster 4—Expression of Cluster 4 proteins is similar for control and 1EG Islets but is downregulated in 2EG. Cluster 4 is enriched in pancreatic secretions, specifically those involved in amylase and protease activity. Nine more proteases (Psmc1, Psma5, Psma7, Blmh1, Cpa1, Ctrl, Klk, Ctsl, and Stambp) were identified in both normal and 1EG samples, but not 2EG samples. Proteosomal degradation is associated with the UPR, and our secretome profiling indicates that secretion of proteases precipitously drops following prolonged incubation at high glucose levels, which suggests that proteases are retained intracellularly during UPR. Noticeable exception to this observation are the increase in expression of carboxypeptidase E and proprotein convertase 1/2, which directly process insulin.

Desmoplakin, an obligate component of functional desmosomes, was found in cluster 4. Several additional proteins associated with desmosomes (Dsg1b, Pkp1, Dsc1) were not found in any of the 2EG samples. Literature has also shown a loss of desmosomes in association with type 2 diabetic pancreatic tissue.⁵⁸

Additional Observations

Insulin-like growth factor—Four insulin-like growth factor or insulin-like growth factor-binding proteins were found. Typically, insulin-like growth factor II is associated with growth-promoting activity, whereas insulin-like growth factor-binding proteins inhibit insulin-growth factor mediated growth and development. Three of the four proteins were found exclusively in elevated glucose samples. Moreover, intensities were highest in 2EG samples for all four proteins, which suggest a link between elevated glucose levels and the secretion of these proteins. The data suggests that supraphysiological glucose incubation causes the release some insulin-like growth factors, which is subsequently mediated by the release of insulin-like growth factor-binding proteins.

Oxidative Stress Markers—Glucotoxicity causes oxidative stress in islets through reactive oxygen species such as peroxides, superoxides, and hydroxyl radicals.⁵⁹ Islets are particularly susceptible to oxidative stress due to low concentrations of antioxidants. Our

data showed no clear secretion trend in known antioxidant proteins such as peroxiredoxins, thioredoxins, and superoxide dismutases.

Conclusions

As expected, the protein identifications generated in this work are similar to those observed in our earlier method development paper.²⁴ We have now extended that earlier study to consider the effect of high glucose treatments on the secretome. The CD-1 mouse islets generate highly reproducible secretomes when analyzed by the FASP protocol. This protocol can be considered for future work employing longer-term glucose, lipid, and other treatments.

In addition, STRING network analysis was used to find pathway and functional enrichment, Figure 4. STRING analysis of the proteins present in the secretome generated clusters corresponding to insulin processing, proteolytic enzymes, and protein folding, including proteins associated with the UPR. The expression of only a handful of proteins changed in response to 48 hour high-glucose treatment, and the role of the secretome in normal islet function and in diabetes remains obscure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic of the experimental workflow for analysis of elevated glucose levels on murine islet secretome. Islets were excised from CD-1 mice and cultured in FBS-containing media for 24 h. Islets were then incubated media with either 11 mM glucose or 25 mM glucose for the prescribed period. Collected conditioned FBS-free media was processed using FASP. Peptides were subjected to LC-MS/MS and subsequent data analysis.

Figure 2.

Venn diagrams showing intra- (A, B, C) and intercondition (D) protein group overlap.

Individual sample identifications ranged from 190-305 with intraconditional overlap between 66-98% with a sample average of 85%. 428 proteins were identified between the nine samples, and 328-349 proteins were identified in each condition. More than half of all proteins were identified in all conditions.

Figure 3.

Cluster analysis of proteins quantified at least once in each condition. Proteins were sorted into five clusters with log₂ abundance change thresholds set to 1 and -1, corresponding to a two-fold change between conditions. Any missing values were imputed prior to averaging. Clusters were determined using log₂ (protein ratio).

Figure 4.

STRING interaction map of proteins used for cluster analysis. The map is separated into roughly three zones. The top of the map is predominately associated with protein production and folding, corresponds to clusters 3 and 5. The central zone is associated with either Rap1 signaling and peptide hormone processing or small molecule metabolism, which correspond to clusters 1 and 2 respectively. The bottom-most zone predominately consists of proteases and was typically found in cluster 4.

Table 1

Pearson correlation coefficients for the expression of 52 proteins quantified in all runs. Protein expression is compared to the mean expression for each condition.

	Normal-1	Normal-2	Normal-3	1EG-1	1EG-2	1EG-3	2EG-1	2EG-2	2EG-3
Mean Normal	0.87	0.90	0.93	0.80	0.62	0.67	0.56	0.65	0.66
Mean 1EG	0.71	0.75	0.76	0.92	0.79	0.92	0.63	0.62	0.60
Mean 2EG	0.69	0.48	0.53	0.62	0.57	0.53	0.79	0.94	0.94

Table 2

Gene names for proteins found within the insulin secretory granule by Hickey *et al.*³¹ and proteins found in our secretomic analysis.

All Conditions			Some Conditions	Not Identified	
Hspa5	Gapdh	Ins2	Hspa9	Gaa	Rpl23a
Pdia3	Aldoa	Ins1	Cct3	Cox5b	Atp1a1
Pdia6	Eno1	Pcsk2	Atp5a1	Rab1A	
Hsp90ab1	Actb	Cpe	Aco2	Acta1	
P4hb	Tuba1a	Ctsd	Got2	Rab3a	
Hyou1	Eef2	Ywhaz	Rab2a	Krt6a	
Ppia	Tmed10	Eef1a1	Rpl18	Vgf	
Atp5b	Scg2	Calr	Nucb2	Gnao1	
Pkm	Chgb	Hnrnpk		Gnal	
Acly	Chga	Npm1		Gnb4	

Table 3

Function or pathway enrichment values as determined by STRING analysis.

Cluster	GO/Pathway ID	Pathway Description	Gene Count	FDR
1	4015	Rap1 signaling pathway	4	5.47E-03
	0016486	Peptide hormone processing	4	1.84E-05
2	44281	Small molecule metabolic process	8	8.28E-03
	43436	Oxoacid metabolic process	6	2.37E-02
3	2001233	Regulation of apoptotic signaling pathway	6	2.07E-02
4	4972	Pancreatic secretion	9	1.27E-12
	4175	Endopeptidase activity	6	7.08E-03
5	6414	Translational elongation	5	1.24E-05
	6457	Protein folding	7	1.65E-04