Proteomic Analysis of INS-1 Rat Insulinoma Cells: ER Stress Effects and the Protective Role of Exenatide, a GLP-1 Receptor Agonist

Mi-Kyung Kim1,2, Jin-Hwan Cho1, Jae-Jin Lee1, Moon-Ho Son2, Kong-Joo Lee1*

1 Graduate School of Pharmaceutical Sciences and College of Pharmacy, Ewha Womans University, Seoul 120–750, Republic of Korea, 2 Dong-A ST Research Institute, Yongin-si, Gyeonggi-do 446–905, Republic of Korea

* kjl@ewha.ac.kr

Abstract

Beta cell death caused by endoplasmic reticulum (ER) stress is a key factor aggravating type 2 diabetes. Exenatide, a glucagon-like peptide (GLP)-1 receptor agonist, prevents beta cell death induced by thapsigargin, a selective inhibitor of ER calcium storage. Here, we report on our proteomic studies designed to elucidate the underlying mechanisms. We conducted comparative proteomic analyses of cellular protein profiles during thapsigargin-induced cell death in the absence and presence of exenatide in INS-1 rat insulinoma cells. Thapsigargin altered cellular proteins involved in metabolic processes and protein folding, whose alterations were variably modified by exenatide treatment. We categorized the proteins with thapsigargin initiated alterations into three groups: those whose alterations were 1) reversed by exenatide, 2) exaggerated by exenatide, and 3) unchanged by exenatide. The most significant effect of thapsigargin on INS-1 cells relevant to their apoptosis was the appearance of newly modified spots of heat shock proteins, thimet oligopeptidase and 14-3-3β, ε, and θ, and the prevention of their appearance by exenatide, suggesting that these proteins play major roles. We also found that various modifications in 14-3-3 isoforms, which precede their appearance and promote INS-1 cell death. This study provides insights into the mechanisms in ER stress-caused INS-1 cell death and its prevention by exenatide.

Introduction

Recent reports suggest that hyperglycemia results in both oxidative and endoplasmic reticulum (ER) stresses [1], suggesting that ER stress and oxidative stress are endogenous aggravating factors for type 2 diabetes. ER stress is a cellular state involving accumulation of unfolded proteins, perturbation of calcium ions, or disturbances of redox state [2,3]. Inhibitors of ER stress might therefore serve as antidiabetic agents. Thapsigargin, a selective inhibitor of endoplasmic reticulum Ca2+/ATPase causing the depletion of ER Ca2+ store, is widely used in studies of ER stress-caused beta cell death. When cells fail to adapt to ER stress, beta cell apoptosis is initiated by diverse signaling molecules.
A progressive increase in beta cell apoptosis and decrease in beta cell mass in the pancreas, occur with the progression of type 2 diabetes. Because pancreatic beta cell loss cannot be readily recovered, there has been a heightened interest in agents such as exenatide that preserve beta cell mass in addition to lowering glucose. Exenatide, a dipeptidyl peptidase 4-resistant glucagon-like peptide (GLP)-1 analogue, has been approved for therapy of type 2 diabetes [4], because it effectively blocks beta cell death caused by various diabetogenic agents via cAMP-dependent, beta arrestin-mediated and PI3-kinase-mediated signaling pathways [5]. Recently, it has been reported that a component of beta arrestin-mediated signaling pathway, 14–3–3 scaffold protein, binds to the proapoptotic protein, BAD, and inhibits beta cell apoptosis [6]. 14–3–3 proteins are 28–30 kDa signaling proteins, that inhibit apoptosis by regulating over 200 partner proteins [7,8]. 14–3–3 proteins appear in seven isoforms (β, γ, ε, ζ, η, θ, and σ), but little is known on the modulation of these isoforms under stress conditions [9].

We previously reported that isoform-specific changes in 14–3–3 proteins are crucial to INS-1 rat insulinoma cell death caused by stress and that decreases in 14–3–3θ is a causative factor in INS-1 cell death [10]. We also suggested that exenatide prevents stress-induced INS-1 cell death by modulating 14–3–3 isoforms. Several questions remain in this regard: Are other entities in addition to 14–3–3 isoforms affected by ER stress?; what are the effects of GLP-1R signaling on these entities?; and what modifications in these entities are responsible for INS-1 cell apoptosis in ER stress and type 2 diabetes?

In efforts to answer these questions, we comprehensively examined the protein changes occurred after treatment with thapsigargin with and without exenatide treatment based on our previous extensive studies [10]. For this, we performed proteomics combined with two-dimensional gel electrophoresis (2D-PAGE) and mass spectrometry (MS) to identify the proteins changed by ER stress as well as during prevention or reversal of those changes by exenatide. We sorted the proteins up- or down-regulated by thapsigargin and are thus presumably involved in ER stress, into three groups, based how they were further altered by exenatide: 1) those whose altered regulations were reversed; 2) those whose altered regulations were exaggerated; 3) those whose altered regulations were unchanged. We concluded that proteins in groups 1 and 2, in which exenatide up- or down-regulated thapsigargin initiated alterations, may be involved in cell death, and those in group 3 not affected by exenatide may be products of ER stress, but not involved in prevention by exenatide. We identified the modified proteins in the three groups by mass spectrometry and computer assisted predictions. Based on the identities of the key proteins, we attempted to infer the signaling pathways activated by ER stress and how exenatide may have influenced these pathways to reverse the effects of ER stress.

Materials and Methods

Materials

Exenatide was obtained from American Peptide (Sunnyvale, CA, USA). Unless otherwise specified, the cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA), and the chemical reagents from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Rat insulinoma cell line, INS-1, was kindly provided by Prof. Kang of Ajou University, Suwon, Korea [11]. The cells were maintained in RPMI 1640 (11 mM glucose), supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 10 mM HEPES, at 37°C in a humidified atmosphere (5% CO₂, 95% air). All experiments were performed with INS-1 cells between the 15th and 23rd passages.
Glucose-stimulated insulin secretion

INS-1 cells were seeded at a density of $5 \times 10^4$ cells per well in a 96-well plate, and allowed to grow for 48 h. After two washes with phosphate buffered saline, the cells were serum-deprived for 1 h in Krebs–Ringer bicarbonate HEPES (KRKH) buffer (11.5 mM NaCl, 24 mM NaHCO$_3$, 5 mM KCl, 1 mM MgCl$_2$, 25 mM HEPES, pH 7.4) including 1% bovine serum albumin (BSA), followed by treatment with exenatide in KRKH buffer containing 1% BSA, 11.1 mM glucose, and 100 $\mu$M Ro-201724 (Calbiochem, San Diego, CA, USA) for 0.5 h and centrifuged. The secreted insulin was measured in the supernatants using Ultrasensitive Rat Insulin ELISA kit (ALPCO, Windham, NH, USA). The cellular protein levels were measured using bichinchonic acid kit (Pierce, Rockford, IL, USA) after lysing the cells with 0.1 M hydrogen chloride. Insulin levels were presented as ng insulin/µg protein.

INS-1 cell viability assay

INS-1 cells were plated at $7 \times 10^4$ cells per well in a 96-well plate, and at $7.8 \times 10^6$ cells in 150 mm dish, and incubated for 48 h in serum-deprived in RPMI media with 5.6 mM glucose. The resulting cells were maintained in serum-free, glucose (5.6 mM) containing media and then treated with 0.3 $\mu$M thapsigargin, to promote ER stress, and apoptosis [12], in the absence or presence of exenatide, for 6 h at 0.2% final dimethyl sulfoxide concentration. Cell viability was assessed by determining intracellular ATP levels with Celltiter-Glo reagent (Promega, Madison, WI, USA) using a luminometer (LmaxII384, MDC, Sunnyvale, CA, USA), according to the manufacturer’s instructions and expressed as percentages of untreated control.

Caspase activity

This was assessed using Caspase-Glo 3/7 assay reagent (Promega Co., WI, USA) described as previously [10].

RT-PCR

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA). First strand cDNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA). PCR was performed for following rat target genes-thioredoxin interacting protein (TXNIP; Gene Bank Accession No. NM 001008767), insulin receptor substrate-2 (IRS-2; NM 001168633), immunoglobulin heavy chain binding protein (Bip, alternatively called GRP78; M14050), CCAAT/enhancer binding protein homologous protein (CHOP; U30186), glucagon-like peptide 1 receptor (GLP-1R; NM 012728) and beta actin (BC063166) in a Dyad Thermal Cycler (MJ Research, Watertown, MA). Each set of primers was designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). Primer sequences used, concentration of MgCl$_2$, cycle number and annealing temperature for each target gene are listed in S1 Table. Images of PCR products were captured using BioCapt v1.01 software and densitometric analysis was performed with Bio1D v1.01 software (Vilber-Loumat, Marne la Vallée, France).

Extraction of cellular proteins

Following harvest, the cells were frozen immediately and stored at −80°C until further use.

Soluble proteins were extracted from the cells as follows: Cells were solubilized with a lysis buffer containing 7 M urea, 2 M thiourea, 4% v/v CHAPS, 2% ampholine (1.5% for pH 3–10, 0.5% for pH 5–7) and 65 mM DTT. After centrifugation at 20,000 x g for 30 min at 25°C, the supernatants were left at room temperature for 1 h to ensure protein solubilization and
denaturation. The protein concentrations of the final extracts were determined using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ, USA).

Protein separation using two-dimensional polyacrylamide gel electrophoresis

Samples of the cellular protein extracts prepared as described above were subjected to two-dimensional-polyacrylamide gel electrophoresis (2D-PAGE). One hundred μg of each protein sample was loaded onto the strip gels and rehydrated for 12 h (18 cm, pH 4–7) with rehydration buffer (7 M urea, 2 M thiourea, 2% v/v CHAPS, 2% IPG buffer, pH 4–7) (GE Healthcare, Piscataway, NJ, USA). The samples were then electrofocused in a manifold cup-loading system with IPGphor (GE Healthcare, Piscataway, NJ, USA) in the following sequential steps; pre-separation at 100 V for 1 h, 200 V for 1 h, 500 V for 1 h; application of the samples to the strip gels initially at 1,000 V for 4 h; gradient focusing from 1,000 V to 8,000 V for 30 min; and finally steady-state focusing at 8,000 V for 8 h. The separated strip gels were equilibrated with equilibration buffer (6 M urea, 2% SDS, 50 mM Tris-Cl, pH 8.8, 30% glycerol) containing 65 mM DTT for 15 min. After a second equilibration with the same buffer containing 2.5% v/v iodoacetamide instead of DTT and a trace of bromphenol blue for 15 min; the equilibrated strip gels were applied to 1.0 mm thick 10% acrylamide gels and sealed with 0.25% (w/v) agarose. SDS-PAGE was carried out at 15 mA overnight using a PROTEAN IIx1 2-D Cell apparatus (BIO-RAD, Hercules, CA, USA).

Detection of protein spots and image analysis

All sets of gels were silver-stained simultaneously in the same tray. The stained gels were then scanned using an Image Scanner III (GE Healthcare, Piscataway, NJ, USA). Spot detection, matching, normalization and quantification were automatically carried out using the Progenesis SameSpots v5.0 (Nonlinear Dynamics, Newcastle, UK). For high reliability, same parameters, based on stringent criteria (fold difference in protein abundance > 1.5, p < 0.05) were applied to each set of analytical gels. The protein spots showing at least 1.5 fold difference in abundance in three replicates were subjected to MS/MS analysis for protein identification.

Identification of proteins and their post-translational modifications by UPLC-ESI-q-TOF tandem MS

In order to identify the proteins and post-translational modifications (PTMs), peptide sequencings were performed by nanoAcquity UPLC/ESI/MS (SYNAPT HDMS, Waters Co. UK). The gel spots on 2D-PAGE were destained and digested with trypsin and the resulting peptides extracted as previously described [13]. The peptide extracts were evaporated to dryness in SpeedVac and dissolved in 10% acetonitril solution containing 1.0% formic acid. The dissolved samples were desalted on line prior to separation using trap column (5 μm particle size, NanoEase dC18, Waters Co., Milford, MA, USA) cartridge. Peptides were separated by using a C18 reversed-phase 75 μm i.d. × 200 mm analytical column (1.7 μm particle size, BEH130 C18, Waters) with an integrated electrospray ionization PicoTip (±10 μm, New Objective, USA). Peptide mixtures (5 μL) were dissolved in buffer A (Water/formic acid; 100:0.1, v/v), injected on a column and eluted by a linear gradient of 5–60% buffer B (ACN/formic acid; 100:0.1, v/v) over 120 min. Initially, the flow rate was set to 250 nL/min and the capillary voltage (2.8 keV) was applied to the UPLC mobile phase before spray. Chromatography was performed on line to SYNAPT HDMS. The mass spectrometer was programmed to record scan cycles composed of one MS scan followed by MSMS scans of the 3 ~ 4 most abundant ions in each MS scan. MS
parameters for efficient data-dependent acquisition were intensity (>10), number of components (3 ~ 4) to be switched from MS to MS/MS analysis.

Raw data obtained from the mass spectrometer were converted to .pkl files using ProteinLynx Global Server (PLGS) 2.3 data processing software (Waters Co., Milford, MA, USA). MS/MS spectra were matched against amino acid sequences in NCBI (USA) and SwissProt using the database search program Mascot (global search engine), ProteinLynx Global SERVER (PLGS) 2.3 (Waters Co., UK).

In order to raise the MS coverage for PTM analysis, SEMSA methodology was employed [13]. The first run analysis, the 4 most abundant precursors were selected for MS/MS analysis. Following positive identification, all identified peptides from database search (Mascot) were non-redundantly excluded in the next run analysis until almost full sequence coverage was obtained. Large numbers and types of potential PTMs were considered. All reported assignments were verified by automatic and manual interpretation of spectra using the database search program Mascot (global search engine), ProteinLynx Global SERVER (PLGS) 2.3 (Waters Co., UK) and MODi (Korea, http://prix.hanyang.ac.kr/modi/) [14] in a blind mode. In addition, a minimum total score of 50, comprising at least a peptide match of ion score more than 20, was arbitrarily set as threshold for acceptance. All reported assignments were verified by automatic and manual interpretation of spectra. Each modification was assigned with an observed mass shift.

The functions of identified proteins determined using PANTHER classification system (www.pantherdb.org) are listed. The list includes proteins with a wide spectrum of cellular functions, not all which are known.

Statistical analysis
Statistical analysis was executed using SigmaStat v2.03 (SPSS Inc., Chicago, IL, USA). Statistical comparisons were performed using two-tailed Student’s t-test between two groups and using One-way ANOVA followed by Bonferroni’s post hoc analysis for multiple comparisons among over three groups. $P$ values under 0.05 were considered statistically significant. All data are presented as mean ± SE.

Results and Discussion
Effects of exenatide on thapsigargin-induced INS-1 cell death
Exenatide increased insulin secretion and cell viability in thapsigargin-treated INS-1 cells in a dose dependent manner. We first established the optimal concentration of exenatide that has maximal INS-1 cell protection against thapsigargin and increased insulin secretion in INS-1 cells. We found that 1 ~ 10 nM exenatide led to maximal efficacy in both effects (Fig. 1A, 1B). To determine whether exenatide has protective effects via inhibiting pro-apoptotic proteases caspase-3/7 activity, we examined the dose- and time-dependence to exenatide treatment on caspase activity. We found that levels higher than 10 nM of exenatide blocked caspase-3/7 activation caused by thapsigargin treatment (Fig. 1C, 1D). Since the response of insulin secretion induced by exenatide continuously increased up to 10 nM, and our previous study [10] showed that exenatide completely blocked all types of beta cell death at 10 nM, but not at 1 nM, we further used 10 nM exenatide as the optimal concentration at which exenatide can lead to the distinct effects in INS-1 cells.

We then examined whether exenatide affects the expression of the ER stress- or beta cell survival-related genes, during thapsigargin-induced beta cell death. Thapsigargin treatment by itself substantially increased the expression of Bip and CHOP genes, indicating that chaperone proteins were induced to overcome ER stress. This effect became larger when the treatment
was with a combination of thapsigargin and exenatide (Fig. 2A-C). This finding is in agreement with an earlier in vitro study [15]. Also, expression of insulin receptor substrate (IRS)-2, a signaling mediator of beta cell survival, which increased following thapsigargin treatment was also further enhanced by exenatide (Fig. 2D).

It was recently demonstrated that thioredoxin interacting protein (TXNIP), a glucotoxicity mediator and an endogenous inhibitor of thioredoxin, enhances ER stress-induced beta cell death through initiation of the inflammasome [16,17] and that exenatide protects beta cells from glucose-induced cell death, by reducing TXNIP protein levels [18]. The present study also demonstrated that thapsigargin-induced ER stress upregulated TXNIP mRNA and that exenatide blocked TXNIP up-regulation (Fig. 2E). Our results confirm that exenatide induces protein folding and replication, and decreases the inflammasome. But we found that exenatide does not affect the GLP-1R gene expression, although GLP-1R is a target molecule of exenatide.

**Fig 1. Effects of exenatide on glucose-stimulated insulin secretion and INS-1 cell death caused by thapsigargin-induced ER-stress.** (A) INS-1 cells were stimulated by exenatide at 11.1 mM glucose for insulin secretion. (B) Serum-deprived INS-1 cells were treated 0.3 μM thapsigargin in the absence or presence of various concentrations of exenatide for 6 h. At the end of experiment, cellular viability was assessed by determining intracellular ATP levels. (C) After 6 h treatment, cellular caspase-3/7 activity was determined as a sensitive apoptotic marker. (D) Caspase-3/7 activity was determined according to the treatment time of 0.3 μM thapsigargin in the absence or presence of 10 nM exenatide. ***p < 0.001 vs. untreated control by Bonferroni’s t-test. Each experiment was run in triplicate.

doi:10.1371/journal.pone.0120536.g001
Fig 2. Regulation of gene expression by exenatide treatment under thapsigargin-induced INS-1 cell death. Serum-deprived INS-1 cells were treated 0.3 μM thapsigargin in the absence or presence of 10 nM exenatide for 6 h. Total RNA was isolated and mRNA expression of five genes related to the INS-1...
cell death/survival was determined by RT-PCR. (A) Gel images of PCR products were obtained and quantified using densitometry. Expression levels of (B) Bip and (C) CHOP as ER stress-related chaperons, (D) IRS-2 as a signal mediator of INS-1 cell survival, (E) TXNIP as a glucotoxicity mediator, and (F) GLP-1 receptor genes as a target molecule of exenatide were assessed. Data were presented as mean ± SE from three individual determinants. #p < 0.05 vs. untreated control; *p < 0.05 vs. thapsigargin alone by Bonferroni’s t-test.

Fig. 2F). Our proteomic study thus confirms that thapsigargin induces cell death and that exenatide prevents the effects of thapsigargin and promotes cell survival.

Protein profiles of INS-1 cells treated with thapsigargin alone and with thapsigargin plus exenatide

We conducted comparative proteomic studies to explore the overall protein profile changes in INS-1 cells treated with thapsigargin alone and with thapsigargin plus exenatide, with the goal of understanding the molecular mechanisms underlying thapsigargin-induced ER stress and exenatide-induced protection or prevention of such ER stress. 2D-PAGE analysis of INS-1 cells treated with thapsigargin or thapsigargin plus exenatide revealed over 60 new protein spots that appeared in cells under thapsigargin-induced ER stress, but absent in control cells. We focused our comprehensive analysis on 58 protein spots that reproducibly appeared following these treatments (Fig. 3). Spot densities determined in triplicate runs are summarized in Table 1 and S2 Table. Of the 58 spots, 44 were statistically significantly down-regulated and 14 spots were up-regulated by thapsigargin treatment. These fifty eight spots came from 49 individual proteins, indicating multiple spots of the same protein possibly due to different modifications. As shown in Fig. 4, 24% of the 58 differentially altered proteins were associated with protein metabolic processes. These include thimet oligopeptidase and eukaryotic translation initiation factor 3 subunit 1, and 17% were concerned with protein folding. Most of the proteins associated with metabolic processes, tended to decrease and those involved in protein folding showed mixed responses. All protein spots related to carbohydrate and lipid metabolic processes were down-regulated. Structural proteins (12%), nucleic acid binding proteins (10%), and proteins having oxidoreductase activity in carbohydrate or lipid metabolic processes (9%) mostly changed in response to thapsigargin treatment.

String analysis (www.string-db.org) and other published information suggest that nineteen of the proteins altered by thapsigargin treatment may be part of a linked network. This protein network includes signaling molecules such as 14–3–3 isoforms that mediate signals to Hsps (chaperons), protein metabolism-related proteins and lipid-carbohydrate metabolism-related proteins (Fig. 5A). String map suggested that the network may be expanded, to include potential binding partners; i) stress-related Bad and Raf1; ii) cytoskeleton-related M-phase inducer phosphatase, nuclear distribution protein nudE-like 1, dynein heavy chain; iii) signaling-related casein kinase Iδ, 14–3–3 (Fig. 5B). The possible pathways involved in ER stress in insulinoma cells can be inferred from the combined findings from proteomic studies and informatic string analysis.

Comparison of protein expressions in cells treated thapsigargin and thapsigargin plus exenatide

To investigate how exenatide protects ER stress induced cell death, we compared the differentially expressed proteins in cells treated thapsigargin and thapsigargin plus exenatide. Treatment with thapsigargin resulted in changes in fifty eight protein spots. Results of treatment with thapsigargin plus exentide are as follows. 18 of the fifty eight spots were unaffected (Fig. 6A, S3 Table); 8 spots were exaggerated (Fig. 6B, S4 Table); and changes in 32 protein spots were reversed (Fig. 6C-D, S5 Table). The latter included twelve proteins that were up-
regulated by thapsigargin (Fig. 6D). The total list of proteins identified with MS/MS is shown in S6 Table. The appearance of $14-3-3\beta$, $\theta$, and $\varepsilon$ isomers after treatment with thapsigargin and its complete blocking by exenatide (Fig. 6E) is a significant feature of the manifold protein changes involved in the protective effect of exenatide on the cell death caused by thapsigargin induced ER stress.

We therefore tried to dissect the functions of the proteins subjected to thapsigargin induced changes but subsequently restored by exenatide treatment. We broadly classified them into four groups: i) proteins such as HSP90, HSP105, thimet oligopeptidase, ubiquilin-1, PAK2, and proteasome subunit $\alpha$ type-3 concerned with protein metabolic processes; ii) proteins such as GMP synthase, pirin, nucleophosmin, guanine-binding proteins, concerned with nucleotide metabolic processes; iii) proteins such as $14-3-3$ isoforms, related to signal transduction and iv) cellular structural proteins such as tubulin. We found it interesting that proteins related to translation machinery or glucose metabolism were not affected by thapsigargin plus exenatide treatment. Fourteen of the proteins discussed above contained different post-translational modifications (PTMs). Also, we noted in some cases that all spots representing the same protein were altered by the treatments. For example, various spots of HSP71, HSP105, $\alpha$-
Table 1. Proteins differentially altered by thapsigargin treatment (N = 3).

| Spot no. | Mascot score | Accession no. | Queries matched | Protein name                                           | Mass (Da) | pl | Fold difference vs. Control | Mean ± SE | P value |
|----------|--------------|---------------|----------------|-------------------------------------------------------|-----------|----|----------------------------|----------|--------|
|          |              |               |                |                                                       |           |    |                            |          |        |
| **Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process** | | | | | | | | | |
| D5       | 201          | Q4V7C6        | 14             | GMP synthase                                          | 76709     | 6.21 | 0.464 ± 0.029 | 1.6E-05 |
| D27      | 103          | Q3SWU3        | 2              | Heterogeneous nuclear ribonucleoprotein D-like        | 35272     | 9.14 | 0.710 ± 0.007 | 6.2E-09 |
| D30      | 165          | Q3SWU3        | 5              | Heterogeneous nuclear ribonucleoprotein D-like        | 35272     | 9.14 | 0.593 ± 0.018 | 7.4E-07 |
| D28      | 418          | P13084        | 12             | Nucleophosmin                                         | 32540     | 4.62 | 0.680 ± 0.025 | 1.4E-06 |
| D29      | 547          | P13084        | 20             | Nucleophosmin                                         | 32540     | 4.62 | 0.530 ± 0.007 | 2.7E-08 |
| D41      | 113          | Q5M827        | 7              | Pirin                                                 | 32158     | 6.22 | 0.616 ± 0.013 | 1.7E-07 |
| **Protein metabolic process** | | | | | | | | | |
| D3       | 409          | P24155        | 15             | Thimet oligopeptidase                                 | 78335     | 5.64 | 0.178 ± 0.003 | 1.8E-07 |
| D4       | 587          | P24155        | 26             | Thimet oligopeptidase                                 | 78335     | 5.64 | 0.300 ± 0.011 | 4.0E-06 |
| U47      | 385          | P24155        | 20             | Thimet oligopeptidase                                 | 78335     | 5.64 | 3.722 ± 0.516 | 6.2E-03 |
| D6       | 381          | Q8JJP9        | 12             | Ubiquilin-1                                           | 62032     | 4.87 | 0.752 ± 0.043 | 8.0E-06 |
| D7       | 179          | Q8JJP9        | 11             | Ubiquilin-1                                           | 62032     | 4.87 | 0.770 ± 0.017 | 1.4E-07 |
| D8       | 425          | Q64303        | 15             | Serine/threonine-protein kinase PAK 2                 | 57924     | 5.57 | 0.570 ± 0.008 | 3.7E-08 |
| D13      | 269          | Q3BBQ2        | 11             | Eukaryotic initiation factor 4A-III                   | 46811     | 6.30 | 0.670 ± 0.050 | 1.7E-05 |
| D14      | 179          | Q68FR6        | 10             | Elongation factor 1-gamma                              | 50029     | 6.31 | 0.647 ± 0.031 | 4.2E-06 |
| D15      | 256          | P62193        | 15             | 26S protease regulatory subunit 4                      | 49154     | 5.87 | 0.568 ± 0.008 | 3.6E-08 |
| D31      | 640          | B0BNA7        | 26             | Eukaryotic translation initiation factor 3 subunit I   | 36438     | 5.38 | 0.734 ± 0.009 | 1.2E-08 |
| D36      | 197          | P19945        | 10             | 60S acidic ribosomal protein P0                        | 34194     | 5.91 | 0.512 ± 0.058 | 1.2E-04 |
| D40      | 156          | P38983        | 6              | 40S ribosomal protein SA                               | 32803     | 4.80 | 0.529 ± 0.007 | 2.7E-08 |
| D43      | 324          | P18422        | 18             | Proteasome subunit alpha type-3                        | 28401     | 5.29 | 0.672 ± 0.020 | 6.1E-07 |
| U52      | 308          | Q01205        | 12             | Dihydrolipoyllysine-residue succinyltransferase        | 48894     | 8.89 | 1.434 ± 0.075 | 4.4E-03 |
|          |              |               |                | component of 2-oxoglutarate dehydrogenase complex, mitochondrial | | | | | |
| **Carbohydrate metabolic process** | | | | | | | | | |
| D35      | 239          | P11980        | 8              | Pyruvate kinase isozymes M1/M2                        | 57781     | 6.63 | 0.568 ± 0.017 | 9.6E-07 |
| D37      | 346          | P07943        | 13             | Aldose reductase                                       | 35774     | 6.26 | 0.709 ± 0.022 | 1.3E-10 |
| D44      | 564          | P25113        | 35             | Phosphoglycerate mutase 1                             | 28814     | 6.67 | 0.740 ± 0.038 | 5.0E-06 |

(Continued)
| Spot no. | Mascot score | Accession no. | Queries matched | Protein name | Mass | pl | Fold difference vs. Control | Mean | SE  | P value |
|----------|--------------|---------------|----------------|--------------|------|----|---------------------------|------|-----|---------|
| **Lipid metabolic process** | | | | | | | | | | |
| D10 | 348 | O35077 | 17 | Glycerol-3-phosphate dehydrogenase [NAD+], cytoplasmic | 37428 | 6.16 | 0.418 ± 0.051 | 4.0E-04 |
| D11 | 146 | O35077 | 11 | Glycerol-3-phosphate dehydrogenase [NAD+], cytoplasmic | 37428 | 6.16 | 0.222 ± 0.019 | 1.4E-04 |
| D25 | 351 | O35077 | 16 | Glycerol-3-phosphate dehydrogenase [NAD+], cytoplasmic | 37428 | 6.16 | 0.540 ± 0.018 | 1.4E-06 |
| U53 | 48 | O35077 | 4 | Glycerol-3-phosphate dehydrogenase [NAD+], cytoplasmic | 37428 | 6.16 | 1.816 ± 0.200 | 1.5E-02 |
| **Protein folding** | | | | | | | | | |
| D1 | 446 | Q66HA8 | 21 | Heat shock protein 105 kDa | 96357 | 5.4 | 0.383 ± 0.033 | 7.1E-05 |
| D2 | 94 | Q66HA8 | 3 | Heat shock protein 105 kDa | 96357 | 5.4 | 0.622 ± 0.042 | 1.7E-05 |
| U49 | 62 | Q66HA8 | 2 | Heat shock protein 105 kDa | 96357 | 5.4 | 2.703 ± 0.038 | 1.5E-06 |
| D9 | 686 | P18418 | 43 | Calreticulin | 47966 | 4.33 | 0.649 ± 0.040 | 1.1E-05 |
| D20 | 410 | P34058 | 20 | Heat shock protein HSP 90-beta | 83229 | 4.97 | 0.443 ± 0.003 | 4.6E-09 |
| U46 | 203 | P34058 | 8 | Heat shock protein HSP 90-beta | 83229 | 4.97 | 2.084 ± 0.312 | 2.5E-02 |
| U54 | 133 | P34058 | 6 | Heat shock protein HSP 90-beta | 83229 | 4.97 | 5.087 ± 0.586 | 2.2E-03 |
| U55 | 313 | P34058 | 16 | Heat shock protein HSP 90-beta | 83229 | 4.97 | 7.797 ± 0.576 | 3.0E-04 |
| D21 | 299 | P63018 | 14 | Heat shock cognate 71 kDa protein | 70827 | 5.37 | 0.719 ± 0.009 | 1.5E-08 |
| D39 | 178 | P63018 | 16 | Heat shock cognate 71 kDa protein | 70827 | 5.37 | 0.564 ± 0.024 | 3.0E-06 |
| **Tricarboxylic acid cycle** | | | | | | | | | |
| D12 | 139 | P16638 | 11 | ATP-citrate synthase | 1E +05 | 6.96 | 0.340 ± 0.072 | 4.5E-03 |
| D34 | 278 | P42123 | 19 | L-lactate dehydrogenase B chain | 36589 | 5.7 | 0.755 ± 0.013 | 5.6E-08 |
| **Signal transduction** | | | | | | | | | |
| D38 | 138 | P54311 | 5 | Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 | 37353 | 5.6 | 0.745 ± 0.022 | 4.4E-07 |
| U56 | 238 | P35213 | 11 | 14–3–3 protein beta/alpha | 28037 | 4.81 | 15.693 ± 0.175 | 1.2E-02 |
| U57 | 205 | P68255 | 8 | 14–3–3 protein theta | 27761 | 4.69 | 7.464 ± 0.341 | 4.6E-05 |
| U58 | 317 | P62260 | 22 | 14–3–3 protein epsilon | 29155 | 4.63 | 13.220 ± 1.034 | 2.9E-04 |
| **Transport** | | | | | | | | | |
| D16 | 69 | P10719 | 2 | ATP synthase subunit beta, mitochondrial | 56318 | 5.18 | 0.380 ± 0.009 | 5.4E-07 |
centractin, nuclear ribonucleoprotein D-like, nucleophosmin, and ubiquilin-1 represented different alterations produced by exenatide treatment. In the case of HSP90, ATP synthase subunit β, glycerol-3-phosphate dehydrogenase, thimet oligopeptidase and 14–3–3 isoforms, exenatide altered only some spots significantly changed by thapsigargin (S1 Fig.; Fig. 7). Exenatide blocked thapsigargin caused alterations in only three (D3, D4, and U47) of the four spots representing thimet oligopeptidase involved in intracellular protein metabolism (Fig. 7A, 7B).

We further examined the PTMs of thimet oligopeptidase employing proteomics and identified 4 different modifications including oxidative modifications with the conversion of cysteine (Cys) to dehydroalanin (Dha) or serine (Ser) at Cys175 residues as described previously [19] (Table 2 and S2 Fig.). Several phosphorylations at Ser172, Thr656, Ser89 of spot D4 and at The656 of U47 were identified. This indicates that phosphorylations of Ser172 and Ser89 that were not detectable in spot U47, appeared only in response to thapsigargin as more basic spot.

| Spot no. | Mascot score | Accession no. | Queries matched | Protein name | Mass | pI | Fold difference vs. Control |
|----------|--------------|---------------|----------------|--------------|------|----|---------------------------|
|          |              |               |                |              |      |    | Mean ± SE | P value |
| D17      | 323          | P10719        | 11             | ATP synthase subunit beta, mitochondrial | 56318 | 5.18 | 0.788 ± 0.011 | 3.9E-09 |
| D18      | 70           | P85515        | 1              | Alpha-centractin | 42587 | 6.19 | 0.777 ± 0.010 | 2.0E-08 |
| D19      | 239          | P85515        | 9              | Alpha-centractin | 42587 | 6.19 | 0.740 ± 0.012 | 4.2E-08 |

**Cellular organization**

| Spot no. | Mascot score | Accession no. | Queries matched | Protein name | Mass | pI | Fold difference vs. Control |
|----------|--------------|---------------|----------------|--------------|------|----|---------------------------|
| D22      | 64           | P62738        | 3              | Actin, aortic smooth muscle | 41982 | 5.24 | 0.352 ± 0.012 | 2.3E-06 |
| D23      | 186          | P63259        | 10             | Actin, cytoplasmic 2 | 41766 | 5.31 | 0.432 ± 0.004 | 7.1E-09 |
| D32      | 414          | Q6P9V9        | 25             | Tubulin alpha-1B chain | 50120 | 4.94 | 0.772 ± 0.013 | 4.3E-08 |
| D33      | 414          | Q6P9V9        | 25             | Tubulin alpha-1B chain | 50120 | 4.94 | 0.718 ± 0.057 | 3.4E-05 |
| U48      | 116          | Q62871        | 6              | Cytoplasmic dynein 1 intermediate chain 2 | 71134 | 5.11 | 3.407 ± 0.194 | 2.4E-04 |
| U50      | 331          | P68370        | 14             | Tubulin alpha-1A chain | 50104 | 4.94 | 3.377 ± 0.262 | 8.2E-04 |
| U51      | 361          | P68370        | 21             | Tubulin alpha-1A chain | 50104 | 4.94 | 6.029 ± 0.353 | 1.4E-04 |

**Apoptosis**

| Spot no. | Mascot score | Accession no. | Queries matched | Protein name | Mass | pI | Fold difference vs. Control |
|----------|--------------|---------------|----------------|--------------|------|----|---------------------------|
| U45      | 448          | Q9QZA2        | 16             | Programmed cell death 6-interacting protein | 96570 | 6.15 | 1.467 ± 0.025 | 4.8E-05 |

**Biological process unclassified**

| Spot no. | Mascot score | Accession no. | Queries matched | Protein name | Mass | pI | Fold difference vs. Control |
|----------|--------------|---------------|----------------|--------------|------|----|---------------------------|
| D24      | 50           | P04764        | 1              | Alpha-enolase | 47098 | 6.16 | 0.296 ± 0.005 | 1.6E-07 |
| D26      | 206          | Q6JE36        | 6              | Protein NDRG1 | 42927 | 5.77 | 0.377 ± 0.031 | 5.1E-05 |

From comparative differential 2D-PAGE analysis between untreated control and thapsigargin alone, 58 protein spots which were significantly altered by thapsigargin-induced INS-1 cell death were classified according to the biological process. Data were denoted as mean ± SE of fold differences vs. control from three individual determinants. Its statistical significance against untreated control was analyzed by Student’s t-test. Symbols in front of spot number present the up (U) or down (D) regulation of spot change, and same color spots denote same protein having several spots on 2D-PAGE.

doi:10.1371/journal.pone.0120536.t001
suggests that thimet oligopeptidase is regulated by phosphorylation and its phosphorylated form is dephosphorylated in ER stress induced by thapsigargin. This suggested regulatory mechanism for thimet oligopeptidase during ER stress and its protection by exenatide, should be verified experimentally. If confirmed, phosphorylation changes in thimet oligopeptidase may serve as biomarkers of ER stress.

Appearance of 14–3–3 protein isoforms is prominently associated with thapsigargin-induced INS-1 cell death

In our previous studies, we demonstrated that reduction in 14–3–3ζ causes for ER stress in INS-1 cells [10]. Moreover our data suggested that the appearance of specific 14–3–3 isoform spots correlates with the appearance of the apoptotic signal and cell death under ER stress. Previous studies reported changes in 14–3–3 proteins in INS-1 cells exposed to alternate ER stress inducers, but these changes included only alterations in 14–3–3β and ζ levels, but not their PTMs status [20]. In this study we attempted to identify the specific isoforms as well as the modifications of 14–3–3 proteins that might be involved in ER stress-caused INS-1 cell death. As shown in Fig. 8, 16 spots representing isoforms of 14–3–3 protein were identified; six
Fig 5. Protein networks; (A) proteins altered by thapsigargin-induced INS-1 cell death, (B) predicted protein interaction by String analysis (www.string-db.org). Proteins altered by thapsigargin in our experiment were denoted as bold and italic.

doi:10.1371/journal.pone.0120536.g005
of 14–3–3ε; three of 14–3–3θ; two of 14–3–3β, ζ and γ each and one of 14–3–3η. Of the seven isoforms of 14–3–3 proteins, 14–3–3σ was not detected on 2D-PAGE, because it is absent in INS-1 cells, but exists only in pancreatic ductal adenocarcinoma cells [21]. Significantly, β, θ and ε isoforms of 14–3–3 protein respectively appeared in the thapsigargin changed spots: U56, U57 and U58 (Fig. 6E, Table 1).

Exenatide completely blocks modifications of 14–3–3 proteins occurring under thapsigargin-induced ER stress

The spots of 14–3–3 isoforms appearing when INS-1 cells were treated with thapsigargin alone or thapsigargin plus exenatide, were analyzed. As shown in Fig. 9, 14-3-3ε spot no. 8 significantly increased during thapsigargin-induced cell death without discernible changes in

Fig 7. Changes in post-translational modifications of thimet oligopeptidase in response to thapsigargin and thapsigargin plus exenatide. Protein spots at a different modification status were quantified and data were presented as mean ± SE from three individual determinants. *p < 0.05 vs. control; #p < 0.05 vs. thapsigargin alone by Bonferroni’s t-test.

doi:10.1371/journal.pone.0120536.g007
Table 2. Modifications of thimet oligopeptidase identified on 2D-PAGE (Fig. 7) using peptide sequencing with MS/MS analysis.

| Spot no. | a.a | m/z     | Mr (cal.) | Mascot score | Observed modification                                      |
|----------|-----|---------|-----------|--------------|------------------------------------------------------------|
| D3       |     | not determined | not determined |              |                                                            |
| D4       | 79–93 | 729.8090 | 1457.7102 | 33 | RSLCIFDFNK + Phospho (ST); Carbamidomethyl (C) |
|          | 169–181 | 566.2813 | 1130.6213 | 56 | LSLLCIFDFNK + Dehydroala (C) |
|          | 170–181 | 575.2861 | 1148.6213 | 60 | LSLLCIFDFNK + Cys to Ser (C) |
|          | 170–181 | 794.8166 | 1587.7446 | 24 | NILDFPQHSVSPNK + Phospho (ST) |
|          | 642–660 | 929.8585 | 1857.8543 | 33 | TSLRPGGSEDASTMLK + Phospho (ST); Oxidation (M) |
| U47      | 170–181 | 566.2836 | 1130.6213 | 68 | LSLLCIFDFNK + Dehydroala (C) |
|          | 170–181 | 575.2897 | 1148.6213 | 45 | LSLLCIFDFNK + Cys to Ser (C) |
|          | 642–660 | 929.8628 | 1587.7446 | 23 | TSLRPGGSEDASTMLK + Phospho (ST); Oxidation (M) |

Differentially modified peptides between spot D4 and U47 were indicated as bold characters.

D1 modified peptides between spot D4 and U47 were indicated as bold characters.

doi:10.1371/journal.pone.0120536.t002

unmodified spot 5 or modified spot 6. 14–3–3β also showed no changes in its unmodified abundant form in spot no. 1, while spot no. 2 considerably increased with INS-1 cell death. 14–3–3ζ also significantly increased as spot no. 13 compared to spot no. 12. 14–3–3ζ tended to increase on treatment with thapsigargin alone or thapsigargin plus exenatide. Both the spots of 14–3–3ζ changed similarly. 14–3–3γ showed no significant change following either treatment.

Spots which could not be separated from other spots (e.g. spot no. 14), or remained unchanged (e.g. spot no. 11 of 14–3–3η), or were too faint (e.g. spots no. 3, 4 and 7) were not included in this study. These results suggest that 14–3–3 isoforms were altered both during thapsigargin induced cell death and during its reversal by exenatide.

Identification of ER stress related post-translational modifications of 14–3–3 proteins

Among the 14–3–3 protein isoform spots, spots 2, 13, and 8, respectively identical to U56, U57, and U58 shown in Table 1, differently changed in response to thapsigargin and restored by exenatide treatment (Fig. 9) suggesting that they play critical roles in ER stress. We examined the PTMs of these 14–3–3 isoforms employing MS methodology with SEMSA and the searching algorithm MOD3. As shown in Table 3, a single isoform of 14–3–3 appears in several spots, showing different PTMs including acetylation at Lys residue, oxidation at Met and Cys, and phosphorylation at Ser, Thr and Tyr. Also, a single spot of 14–3–3ε showed several modifications. As examples, 14–3–3ε showed six spots with a broad range of molecular weights, probably products of cleavage by caspase [22]. Spot 6, 14–3–3ε, a more abundant and more acidic spot than spot 8, showed phosphorylation at Tyr20, Ser156 and Thr205, while spot 8, which appeared after thapsigargin treatment and restored after exenatide treatment, contained acetylation at Lys29 and phosphorylations at Thr28, Ser156 and Thr205. Spot 8 appeared with a lower molecular weight and more basic position because of acetylation at Lys29, dephosphorylation at Tyr20 and phosphorylation at Thr28. This indicates that PTMs of 14–3–3 are changed during ER stress with thapsigargin and recovered with exenatide. Spot 2 is presumed to result from phosphorylations at Thr32 and Tyr106 of 14–3–3β and phosphorylation at Ser92 of spot no. 13 (Table 3, underlined; S2–4 Fig.). These results suggest that specific PTMs of 14–3–3 β, ε, and θ including phosphorylations, contribute to signaling in thapsigargin-induced ER stress. Protein spots no. 8 and 2 of 14–3–3ε and β, which were produced by thapsigargin treatment, reflect the heterogeneity in each modification, which makes it difficult to decide which particular
modification contributed to the position shift. On the other hand, protein 14–3–3ζ (spot no. 16) phosphorylated at Ser190 and 14–3–3γ (spot no. 10) phosphorylated at Thr31 were homogeneous entities. We ruled out 14–3–3ζ and γ for further study because they changed minimally during ER stress. Intriguingly, 14–3–3θ showed identical modification in each spot, indicating that phosphorylation at its Ser92 contributed to the shift of 14–3–3θ because spot no. 12 and spot no. 13 were both acetylated at Lys3 (Table 3, Fig. 9, S5–7 Figs.). These modifications also appeared in palmitate-induced (unpublished data) and in streptozotocin-induced INS-1 cell death [10]. It appears that phosphorylations of 14–3–3β, ε and θ may contribute to the signal pathway in INS-1 cell death, but how this pathway is regulated in ER stress and recovery is not clear.

Prediction of potential kinases involved in phosphorylation 14–3–3
In order to determine which kinases are phosphorylating 14–3–3 in response to ER stress, we employed in silico prediction using various available software listed below. 14–3–3β, ε, and ζ
Fig 9. Changes in post-translational modifications of 14–3–3 proteins during thapsigargin and thapsigargin plus exenatide treatments. Protein spots of each 14–3–3 isoform at a different modification status were quantified and data were presented as mean ± SE from three separate experiments. ***p < 0.001 vs. control by Bonferroni’s t-test.

doi:10.1371/journal.pone.0120536.g009
have a common JNK phosphorylation site (Ser-Pro) [9], while 14–3–3θ has no consensus sequences (Ser-Pro or Thr-Pro) for JNK phosphorylation. We tried to predict kinases potentially phosphorylating rat 14–3–3θ at Ser92 which was identified by MS/MS (Fig. 10).

For identification of cognate protein kinases, three softwares were applied; scansite available at http://scansite.mit.edu/motifscanseq.phtml, NetPhosK 1.0 at http://www.cbs.dtu.dk/services/NetPhosK/, and GPS 2.1 at http://gps.biocuckoo.org/ [23]. NetPhosK predicted ribosomal s6 kinase (RSK), protein kinase G (PKG), and cell division control protein 2 (cdc2; also known as cyclin-dependent kinase (CDK)-1) for Ser63, casein kinase (CK) II for Ser88 and Ser156, and CKI/II for Ser232; but it did not predict phosphorylation at Ser92 of 14–3–3θ. Scan-site also did not predict phosphorylation for Ser92 (data not shown). On the other hand, GPS 2.1 predicted cognate kinases for five Ser phosphorylations except for one at Ser63 among five sites which were predicted by NetPhos. Cognate kinases with highest score were Akt2 for Ser88, NimA related kinase (NEK)-2 for Ser156, mitogen-activated protein kinase kinase kinase 8 (MAP3K8; also called as COT) for Ser210, and Ca2+/calmodulin-dependent protein kinases (CaMK)1a for Ser232. For Ser92 of 14–3–3θ, predicted cognate kinases are listed with score in Table 4. CaMK1a was ranked with the highest priority. Protein kinase B or protein kinase C

Table 3. Modifications of 14–3–3 isoforms identified on 2D-PAGE (Fig. 8) using peptide sequencing with MS/MS analysis.

| Protein | Spot no. | a.a | m/z     | Mr (cal.) | Mascot score | Observed modification                      |
|---------|----------|-----|---------|-----------|--------------|--------------------------------------------|
| 14–3–3β/α | spot 1  | 30–43 | 839.8474 | 1,677.70  | 34            | AVTEQGHESNEER + Phospho (ST)               |
|         | spot 2  | 106–117 | 720.3335 | 1,438.69  | 21            | YLILNATHAESK + Phospho (ST)               |
| 14–3–3ε | spot 3  | 13–28 | 696.9818 | 2,087.80  | 20            | LAEQAERYDEMVESMK + Phospho (ST); Phospho (Y) |
|         | spot 4  | 197–215 | 1084.468 | 2,166.92  | 34            | AAFDDIAEELDLSEESYK + Phospho (ST)         |
|         | spot 5  | 154–170 | 958.4717 | 1,914.89  | 61            | AASDIAMTELPPTHPIR + Oxidation (M); Phospho (ST) |
| 14–3–3γ | spot 6  | 154–170 | 958.4717 | 1,914.89  | 39            | AASDIAMTELPPTHPIR + Oxidation (M); Phospho (ST) |
| 14–3–3θ | spot 9  | 153–162 | 607.7217 | 1,213.50  | 20            | AYSEAEHISK + Phospho (ST)                 |
|         | spot 10 | 29–42  | 862.4017 | 1,722.75  | 50            | NVTELNEPLSEER + Oxidation (M); Phospho (ST) |
|         | 153–62  | 607.7471 | 1,213.50  | 47          | AYSEAEHISK + Phospho (ST)                 |
| 14–3–3η | spot 11 | 92–106 | 877.9100 | 1,753.82  | 36            | ELETV/CNDVLALLDK + Oxidation (M); Phospho (ST) |
|         | 29–52  | 832.9036 | 1,665.72  | 34          | AYSEAEHISK + Phospho (ST)                 |
| 14–3–3ζ | spot 12 | 1–9    | 589.2384 | 1,176.61  | 20            | MEKTELIQ + Acetyl (K); Oxidation (M)       |
| 14–3–3δ/δ | spot 13 | 1–9    | 589.2384 | 1,176.61  | 26            | MEKTELIQ + Acetyl (K); Oxidation (M)       |
| 14–3–3ζ | spot 15 | 1–9    | 581.7730 | 1,161.57  | 29            | MDKNELVOK + Acetyl (K); Oxidation (M)      |
| 14–3–3δ/δ | spot 16 | 1–9    | 581.7884 | 1,161.57  | 23            | MDKNELVOK + Acetyl (K); Oxidation (M)      |
|         | 92–103  | 721.3329 | 1,440.66  | 21          | DlC/NDVL/SLEK + Phospho (ST)              |
| 14–3–3δ/δ | 92–103  | 749.8460 | 1,497.68  | 27          | DlC/NDVL/SLEK + Carbamidomethyl (C); Phospho (ST) |
|         | 140–158 | 1124.9850 | 2,247.96  | 62          | GIVDQSOQAYQAEFEISK + Phospho (ST)         |
|         | 188–212 | 711.3508 | 2,841.27  | 46          | ACS/LKATFDEAIELDLSEESYK + Carbamidomethyl (C); Phospho (ST) |

Differentially modified peptides in each isoform were indicated as bold characters.

doi:10.1371/journal.pone.0120536.t003
was also proposed, but with relatively lower scores. Other kinases were predicted as potential kinases phosphorylating spots no. 2 and 8 of 14–3–3β/α and 14–3–3ε. Mitogen-activated protein kinase kinase (MAPKK) known as a survival signal, was predicted with a high score as the phosphorylating kinase for both spots no. 2 and 8; MAP2K1 for phosphorylation at Thr32 of 14–3–3β/α, MAP2K1 or MAP2K7 for phosphorylation at Thr38 and MAP2K2 at Ser156 of 14–3–3ε. These results suggest that i) CaMK1a might be the kinase involved in phosphorylation of 14–3–3θ under ER stress-induced INS-1 cell death, and ii) MAPKK might play a role in phosphorylation of 14–3–3β/α and 14–3–3ε for survival signaling.

Conclusion

In this study, we employed comparative proteomic analyses of cellular protein profiles during thapsigargin-induced INS-1 rat insulinoma cell death in the absence and presence of exenatide, a GLP-1 receptor agonist, in INS-1 cell line, to identify the proteins involved in ER stress caused INS-1 cell death and its protection by exenatide. We found that thapsigargin caused a variety of alterations in a number of cellular proteins involved in metabolic processes and protein folding. These alterations were reversed by exenatide treatment. While most of the proteins up- and down-regulated by thapsigargin can be assumed to be involved in ER stress, our most significant finding relevant to INS-1 cell apoptosis is the appearance of modified spots of heat shock proteins, thimet oligopeptidase and three 14–3–3 protein isomers, 14–3–3β, ε, and θ. Treatment with exenatide completely blocked their appearance, suggesting that modifications of these proteins are major events in ER-stress induced INS-1 cell death. We further found that various other modifications including phosphorylation of 14–3–3 isoforms precede its appearance and promotion of INS-1 cell death. This study provides the following insights into INS-1 cell death during ER stress and the anti-apoptotic effect of exenatide: 1) Phosphorylations of 14–3–3 protein may be critical modification in the process of ER stress-induced INS-1 cell death. 2) Alteration of 14–3–3 phosphorylation status by exenatide may play a key role in
its INS-1 cell protective effect. 3) 14–3–3 and thimet oligopeptidase are selectively modified in response to INS-1 cell death caused by thapsigargin and restored by exenatide, caused prevention of INS-1 cell death in ER stress. This study is the first to report on the possible involvement of 14–3–3 and thimet oligopeptidase and their PTMs in the survival and function of INS-1 rat insulinoma cells. Our finding that phosphorylation of 14–3–3β, ε to the appearance of 14–3–3 protein spots after thapsigargin treatment, warrants further studies to confirm and extend the role of phosphorylations in 14–3–3 in ER stress and its protection. Also needed is further information identifying the interacting partners of these phosphorylated proteins, their up-stream cognate kinases and their mutants that could provide further insights into the mechanisms underlying ER stress induced INS-1 cell death and anti-apoptotic effect of exenatide.

Table 4. Prediction of kinases possibly promoting the phosphorylation of 14–3–3 using GPS v2.1 (http://gps.biocuckoo.org/).

| Isoform | Spot # | Peptide | Position | Code | Kinase | Score | Cutoff |
|---------|--------|---------|----------|------|--------|-------|--------|
| β/α     | 2      | AAAMKAVTEQGHELS | 32 | T | CK1 | 1.156 | 1.091 |
|         |        |         | 32 | T | AGC/GRK | 1.131 | 0.583 |
|         |        |         | 32 | T | CK1/CK1 | 1.575 | 1.521 |
|         |        |         | 32 | T | STE/STE-Unique | 2.286 | 1.571 |
|         |        |         | 32 | T | Other/PEK | 2.188 | 2.000 |
|         |        |         | 32 | T | AGC/GRK/GRK | 1.958 | 1.583 |
|         |        |         | 32 | T | CAMK/DAPK/DAPK3 | 2.944 | 2.889 |
|         |        |         | 32 | T | CK1/CK1/CK1a | 1.882 | 1.118 |
|         |        |         | 32 | T | STE/STE7/MAP2K1 | 11.000 | 5.000 |
|         |        |         | 32 | T | STE/STE7/MAP2K2 | 4.000 | 4.000 |
|         |        |         | 32 | T | Other/NEK/NEK2 | 5.667 | 4.333 |
|         |        |         | 32 | T | Other/PEK/PKR | 2.062 | 2.062 |
|         |        |         | 32 | T | Other/PLK/PLK1 | 1.149 | 1.021 |
|         |        |         | 32 | T | AGC/PKC/Alpha/PKCg | 2.000 | 1.600 |
|         |        |         | 32 | T | AGC/DMPK | 1.385 | 1.256 |
|         |        |         | 32 | T | AGC/PKC/Iota | 3.077 | 2.769 |
|         |        |         | 32 | T | STE/STE7/MAP2K1 | 7.000 | 5.000 |
|         |        |         | 32 | T | STE/STE7/MAP2K7 | 5.400 | 1.800 |
|         |        |         | 38 | T | TKL/MLK/ILK | 3.000 | 2.444 |
|         |        |         | 38 | T | Other/PLK/PLK1 | 1.277 | 1.021 |
|         |        |         | 38 | T | AGC/GRK/GRK/GRK-5 | 2.091 | 1.545 |
|         |        |         | 38 | T | AGC/PKC/Iota/PKCz | 2.769 | 2.692 |
|         |        |         | 156 | S | AGC/PKB | 1.588 | 1.118 |
|         |        |         | 156 | S | CK1/CK1/CK1e | 0.857 | 0.643 |
|         |        |         | 156 | S | STE/STE7/MAP2K2 | 5.000 | 4.000 |
|         |        |         | 156 | S | AGC/GRK/GRK/GRK-1 | 3.500 | 2.875 |
|         |        |         | 92 | S | AGC/PKB | 2.91 | 1.12 |
|         |        |         | 92 | S | CAMK/CAMK1 | 2.00 | 1.88 |
|         |        |         | 92 | S | CAMK/CAMKL | 2.58 | 1.87 |
|         |        |         | 92 | S | STE/STE-Unique | 1.57 | 1.57 |
|         |        |         | 92 | S | Other/IKK | 1.06 | 0.53 |
|         |        |         | 92 | S | AGC/PKB/PDK1 | 2.09 | 1.06 |
|         |        |         | 92 | S | CAMK/CAMK1/CAMK1a | 8.00 | 3.75 |
|         |        |         | 92 | S | CAMK/CAMKL/LKB | 2.41 | 2.06 |
|         |        |         | 92 | S | AGC/PKC/Eta/PKCh | 4.25 | 3.75 |

doi:10.1371/journal.pone.0120536.t004
These studies pave the way for further understanding of the role of ER stress in diseases associated with protein folding.

Supporting Information

S1 Fig. Changes of multiple spots of same protein by thapsigargin alone or thapsigargin plus exenatide. Left panel indicates quantitative amount of each spot on 2D-PAGE stained with silver staining (right panel).

S2 Fig. MS/MS spectra of oxidized thimet oligopeptidase.

S3 Fig. MS/MS spectrum of phosphorylated 14–3–3β/α.

S4 Fig. MS/MS spectra of phosphorylated 14–3–3ε.

S5 Fig. MS/MS spectrum of phosphorylated 14–3–3θ.

S6 Fig. MS/MS spectra of phosphorylated 14–3–3γ.

S7 Fig. MS/MS spectra of phosphorylated 14–3–3δ/ζ.

S1 Table. List of primer sequence and polymerase chain reaction conditions for rat target genes.

S2 Table. Comparative proteomic analysis of protein alterations during thapsigargin-induced beta cell death. Total protein spots significantly altered during thapsigargin-induced beta cell death are listed in the order of spot number.

S3 Table. Classification of 18 protein spots whose thapsigargin-induced changes were unaffected by exenatide treatment.

S4 Table. Classification of 8 protein spots whose thapsigargin-induced changes are augmented by exenatide treatment.

S5 Table. Classification of 32 protein spots whose thapsigargin-induced changes are reversed by exenatide treatment.

S6 Table. List of peptide sequences for each protein assignment.
Author Contributions
Conceived and designed the experiments: MKK MHS KJL. Performed the experiments: JHC JJL. Analyzed the data: MKK KJL. Contributed reagents/materials/analysis tools: JHC JJL. Wrote the paper: MKK JJL KJL.

References
1. Sheikh-Ali M, Sultan S, Alamir AR, Haas MJ, Mooradian AD. Hyperglycemia-induced endoplasmic reticulum stress in endothelial cells. Nutrition. 2010; 26: 1146–1150. doi:10.1016/j.nut.2009.08.019 PMID: 20080028
2. Xu C, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: cell life and death decisions. J Clin Invest. 2005; 115: 2656–2664. PMID:16200199
3. Cnop M, Ladriere L, Igoillo-Esteve M, Moura RF, Cunha DA. Causes and cures for endoplasmic reticulum stress in lipotoxic beta-cell dysfunction. Diabetes Obes Metab. 2010; 12 Suppl 2: 76–82. doi: 10.1111/j.1463-1326.2010.01279.x PMID: 21029303
4. Furman BL. The development of Byetta (exenatide) from the venom of the Gila monster as an anti-diabetic agent. Toxicol. 2012; 59: 464–4671. doi: 10.1016/j.toxicol.2010.12.016 PMID: 21194543
5. Dalle S, Burcelin R, Gourdy P. Specific actions of GLP-1 receptor agonists and DPP4 inhibitors for the treatment of pancreatic beta-cell impairments in type 2 diabetes. Cell Signal. 2013; 25: 570–579. doi: 10.1016/j.cellsig.2012.11.009 PMID: 21029303
6. Dalle S, Ravier MA, Bertrand G. Emerging roles for beta-arrestin-1 in the control of the pancreatic beta-cell function and mass: new therapeutic strategies and consequences for drug screening. Cell Signal. 2011; 23: 522–528. doi: 10.1016/j.cellsig.2010.09.014 PMID: 20849951
7. Obsilova V, Silhan J, Boura E, Teisinger J, Obsil T. Proteins: a Family of Versatile Molecular Regulators. Physiol Res. 2008; 57 Suppl 3: S11–S21. PMID:18481918
8. Shikano S, Coblitz B, Wu M, Li M. Proteins: Regulation of Endoplasmic Reticulum Localization and Surface Expression of Membrane Proteins. Trends Cell Biol. 2006; 16: 370–375. PMID:16769213
9. Tsuruta F, Sunayama J, Mori Y, Hattori S, Shimizu S, Tsujimoto Y, et al. JNK promotes Bax translocation to mitochondria through phosphorylation of Proteins. EMBO J. 2004; 23: 1889–1899. PMID: 15071501
10. Cho JM, Jang HW, Cheon H, Jeong YT, Kim DH, Lim YM, et al. A novel dipeptidyl peptidase IV inhibitor DA-1229 ameliorates streptozotocin-induced diabetes by increasing beta-cell replication and neogenesis. Diabetes Res Clin Pract. 2011; 91: 72–79. doi: 10.1016/j.diabres.2010.10.012 PMID: 21093089
11. Kono H, Suzuki T, Ohtsuka T, Ohara T, Nakamura S, Tanaka H, et al. Inhibition of nuclear factor-kappaB or Bax prevents endoplasmic reticulum stress- but not nitric oxide-mediated apoptosis in INS-1E cells. Endocrinology. 2009; 150: 4094–4103. doi:10.1210/en.2009-0029 PMID: 19556421
12. Seo J, Jeong J, Kim YM, Hwang N, Paek E, Lee KJ. Strategy for comprehensive identification of post-translational modifications in cellular proteins, including low abundant modifications: application to glyceraldehyde-3-phosphate dehydrogenase. J Proteome Res. 2008; 7: 587–602. doi:10.1021/pr700657y PMID: 18183946
13. Kim S, Na S, Sim JW, Park H, Jeong J, Kim H, et al. MODi: a powerful and convenient web server for identifying multiple post-translational peptide modifications from tandem mass spectra. Nucleic Acids Res. 2006; 34: W258–W263. PMID:16845006
14. Yusta B, Baggio LL, Estall JL, Koehler JA, Holland DP, Li H, et al. GLP-1 receptor activation improves beta cell function and survival following induction of endoplasmic reticulum stress. Cell Metab. 2006; 4: 391–406. PMID: 17084712
15. Chen J, Saxena G, Mungrue IN, Lusis AJ, Shalev A. Thioredoxin-interacting protein: a critical link between glucose toxicity and beta-cell apoptosis. Diabetes. 2008; 57: 938–944. doi:10.2337/db07-0715 PMID: 18171713
16. Oslowski CM, Hara T, O’Sullivan-Murphy B, Kanekura K, Lu S, Hara M, et al. Thioredoxin-interacting protein mediates ER stress-induced beta cell death through initiation of the inflammasome. Cell Metab. 2012; 16: 265–273. doi:10.1016/j.cmet.2012.07.005 PMID: 22893234
17. Chen J, Couto FM, Minn AH, Shalev A. Exenatide inhibits beta-cell apoptosis by decreasing thioredoxin-interacting protein. Biochem Biophys Res Commun. 2006; 346: 1067–1074. PMID: 16782054
19. Jeong J, Jung Y, Na S, Jeong J, Lee E, Kim MS, et al. Novel oxidative modifications in redox-active cysteine residues. Mol Cell Proteomics. 2011; 10: M110.000513. doi: 10.1074/mcp.M110.000513 PMID: 21148632

20. D’Hertog W, Maris M, Thorrez L, Waelkens E, Overbergh L, Mathieu C. Two-dimensional gel proteome reference map of INS-1E cells. Proteomics. 2011; 11: 1365–1369. doi: 10.1002/pmic.201000006 PMID: 21365744

21. Tian R, Wei LM, Qin RY, Li Y, Du ZY, Xia W, et al. Proteome analysis of human pancreatic ductal adenocarcinoma tissue using two-dimensional gel electrophoresis and tandem mass spectrometry for identification of disease-related proteins. Dig Dis Sci. 2008; 53: 65–72. PMID: 17492507

22. Nomura M, Shimizu S, Sugiyama T, Narita M, Ito T, Matsuda H, et al. 14–3–3 Interacts directly with and negatively regulates pro-apoptotic Bax. J Biol Chem. 2003; 278: 2058–2065. PMID: 12426317

23. Xue Y, Ren J, Gao X, Jin C, Wen L, Yao X. GPS 2.0, a tool to predict kinase-specific phosphorylation sites in hierarchy. Mol Cell Proteomics. 2008; 7: 1598–1608. doi: 10.1074/mcp.M700574-MCP200 PMID: 18463090