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**PROTEIN UBIQUITINATION IN PRIMARY HUMAN SKELETAL MUSCLE CELLS
UNDER HYPERINSULINEMIC HYPERGLYCEMIC CONDITIONS**

by

MAJED ALHARBI

THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

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APPROVED BY:

Advisor

Date

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CHAPTER 1 INTRODUCTION

1.1 Diabetes Mellitus

Metabolism is a sequence of biochemical reactions that take place in every single cell and organ to maintain the organism growth, reproduction, damage repair, and adaptation to the surrounding environment [1]. These reactions involve catabolism and anabolism using the major sources of energy: proteins, lipids, and carbohydrates. Furthermore, any disruption in the regulation of the metabolic processes affects the individual's morbidity and mortality [1]. Accordingly, in this chapter, we are going to discuss diabetes mellitus, which is one of the main metabolic disorders.

1.1.1 Pancreatic Regulation of Blood Glucose

The regulation of the cornerstone of the entire body's energy, glucose, in the blood stream by the pancreas, specifically the islets of Langerhans, is very critical through the secretion of insulin and glucagon. Insulin, which is secreted from beta cells of the islets of Langerhans in response to high blood glucose levels, has an anabolic effect via its stimulation of glucose uptake in insulin sensitive tissues, such as skeletal muscle and adipose tissue to be stored in the form of glycogen and fat [2]. On the other hand, low glucose levels enhance the secretion of glucagon from pancreatic alpha cells, and this plays the catabolic role through stimulation of stored glycogen breakdown and formation of glucose from non-carbohydrate sources via glycogenolysis and hepatic gluconeogenesis, respectively [2]. This mechanism is well controlled through the opposite secretion

of these hormones in response to the blood glucose level and their physiological efficacy in the central as well as peripheral tissues. Any disruption in this mechanism leads to abnormal glucose levels, either hyperglycemia or hypoglycemia, that result in serious complications [3]. For instance, prolonged uncontrolled hyperglycemia results in microvascular and macrovascular complications. The common microvascular complications of chronic hyperglycemia are nephropathy, neuropathy, and retinopathy. And, the macrovascular complications that may result from uncontrolled high blood glucose levels are coronary artery disease, peripheral arterial disease, and stroke [4]. These complications are the main source of increased morbidity and mortality in people who suffer from disrupted glucose regulation, i.e., diabetes.

1.1.2 Diabetes Background and Epidemiology

The most well-known form of glucose regulation disturbance is the epidemic disease “diabetes mellitus”. This disease is generally characterized by abnormal insulin levels or activities in response to blood glucose that consequently affect the glucose uptake into tissues [5]. There are two major types of diabetes mellitus, Type I, which is manifested by insufficient insulin secretion from beta cells, and Type II, which is characterized by relative insulin deficiency and decreased insulin sensitivity in the target tissues (i.e., insulin resistance) [5, 6]. Type I Diabetes is also known as Insulin Dependent Diabetes since it could be simply managed by exogenous insulin administration. In contrast, Type II Diabetes, which is known as Non-Insulin-Dependent Diabetes, is one of the greatest challenges that human beings face nowadays due to its serious complications that threaten human lives.

Even though the mechanism by which Type II Diabetes is developed is still unclear, studies show that there are many risk factors, such as genetic factors, unhealthy lifestyle, and some diseases, that increase the likelihood of developing insulin resistance and further Type II diabetes mellitus [7-9]. Statistically, according to the Centers for Disease Control and Prevention, there were 29.1 million diabetic people in the United States in 2012. This represents about 9.3% of the United States population. Moreover, 90-95% of these diabetics are Non-Insulin Dependent (Type II) patients. In addition, the hospital admission rate of heart attack cases in people aged 20 or older was 1.8 higher in diabetic patients than non-diabetics in 2010. In the same year, 60% of amputation cases of lower limbs that did not result from trauma in people aged 20 or older people were from patients diagnosed with diabetes. Furthermore, in 2005-2008, 4.2 million diabetic people aged 40 or older were diagnosed with retinopathy. In 2011, 44% of individuals with newly diagnosed renal failure were diabetics. Moreover, diabetes was the primary cause of 10-15% death cases in 2010. In addition to all of the previously mentioned data about diabetes and its complications, 37% of United States adults were prediabetics, those who have high fasting blood glucose or HbA1c levels but not high enough to be classified as T2D, in the period 2009-2012. These individuals are likely to become Type II diabetics in 10 years or less and the long term complications may have already begun. Economically, the total estimated cost of diabetes in the United States in 2012 was \$245 billion. These physiological and statistical data make diabetes, especially T2D, one of the most serious epidemic

diseases that require further investigations to identify the etiologies and mechanisms behind their pathogenesis.

1.1.3 Insulin Resistance and T2D

Insulin insensitivity or “insulin resistance” is a major manifestation that is denoted in nearly all Type II diabetes patients. In addition, as mentioned previously, studies have proposed several predisposing factors behind the development of insulin resistance, including: (1) genetic defects of protein-protein interactions in insulin signaling pathways (2) some diseases, such as Polycystic Ovarian Syndrome (3) unhealthy lifestyle, e.g. improper eating habits that result in obesity [7-10]. This in turn, opens up new questions about Type II Diabetes Mellitus and leads to increasing the focus of the extensive diabetes research on insulin signaling pathways to point out the detailed sequence of biochemical cascades of insulin actions.

There are three major energy storage tissues, skeletal muscle, liver, and adipose tissue, where insulin anabolic activity mainly occurs. Skeletal muscle, in particular, plays a major role in glucose homeostasis through its insulin- and exercise-induced glucose uptake to maintain euglycemia [11].

During the last few decades, insulin sensitivity in the main glucose utilizing tissues has been a flash point in diabetes research, and many studies have successfully pointed out the impact of impaired insulin signaling in these tissues on the development of T2D [11]

1.2 Post-Translational Modifications and Type 2 Diabetes

1.2.1 Post-Translational Modifications

After translation, proteins such as enzymes, receptors, and cell-signaling messengers undergo numerous reversible and irreversible cellular modifications [12]. Single or different reactions of a specific-signal-triggered and/or a time-induced modification could take place in a given protein and mandate the protein structure and function, control its locations, and affect the protein-protein interactions [12-15].

Many proteins PTMs have been reported in the past few decades. However, only a handful of them have been studied reasonably, e.g., phosphorylation, acetylation, and ubiquitination.

One of the well-known reversible PTMs that is involved in controlling protein-protein interactions in signaling pathways and regulating enzymes activity, activation and inactivation, is protein phosphorylation through the counter-regulatory actions of kinase or phosphorylase, that catalyze the transfer of a phosphate group from ATP/GTP to a specific residue on a protein, and phosphatase, which catalyzes the removal of phosphate groups from phosphoproteins [16]. In response to a particular cellular condition, kinases/phosphorylases and phosphatases may switch a protein on or off. For instance, in case of hyperglycemia, insulin binds to its receptor on the muscle cells triggering IRS phosphorylation that results in activation of the kinase Akt, which eventually facilitates the translocation of GLUT4 to the plasma membrane to induce glucose uptake [17]. Therefore, it is necessary to identify specific

phosphorylated/dephosphorylated proteins under a certain cellular condition to understand the role of phosphorylation in cellular homeostasis in response to that condition.

Acetylation is another common PTM that is involved in cellular regulation. Studies have shown that the reversible acetylation of N-terminal and lysine residues antagonizes the action of protein phosphorylation. The best example to illustrate the phosphorylation-acetylation switch is the regulation of the transcription factor STAT1 signaling. Deacetylation of STAT1 allows the cytokines-stimulated STAT1 phosphorylation to take place and further switch STAT1 to the active form. Then, histone acetyltransferase catalyzes the phosphorylated STAT1 acetylation and facilitates the binding of phosphatase (TCP45) to phospho-STAT1 [18]. Upon the complex formation, TCP45 inactivates STAT1 through dephosphorylation [18]. In addition, acetylation plays a key role in protein stability, DNA recognition, and protein-protein interactions. Particularly, lysine acetylation has a major role in regulating cellular metabolic activity [19]. Studies have shown that all the enzymes that are involved in glycolysis, gluconeogenesis, the urea cycle, the TCA cycle, lipolysis, and glycogenolysis are acetylated in hepatocytes [20].

Addition and removal of a small (8 kDa) protein, ubiquitin, which is made up of 76 amino acids to proteins, is another pattern of PTMs. This protein, ubiquitin, is highly conserved from yeast to human and its conjugation to a substrate can be singly (mono-) or in a chain (polyubiquitination) [21]. It was discovered in the early 1980s within research studying ATP-dependent proteolysis [22]. Three classes of

enzymes, (E1) ubiquitin activating enzymes, (E2) ubiquitin conjugating enzymes, and (E3) ubiquitin ligating enzymes, which catalyzes the addition of the activated ubiquitin to the targeted substrate, facilitate the binding of ubiquitin to the N-terminal amino acid or a lysine residue [21, 23] (See Fig 2). On the other hand, the deubiquitinase class of enzymes counteracts the ubiquitin conjugation and catalyzes the ubiquitin removal from substrates [21, 23]. The great number of enzymes, almost 1000 enzymes, which are associated with ubiquitination/deubiquitination reactions suggested that this reversible reaction has a significant physiological role. Recently, many ubiquitination-targeted studies have successfully addressed some of its regulatory functions, such as regulation of receptor signaling and proteolysis of unwanted proteins through ubiquitin proteasome system (UPS) [24, 25]. Apparently, a dysfunction in the ubiquitination regulatory roles may interfere in the development of different diseases due to abnormal cell signaling or degradation of essential proteins [24-26].

1.2.2 Ubiquitin Proteasome System

In a normal state, all proteins, intracellular and extracellular, are constantly subjected to a process called “turn over” [27]. The proteins are selectively degraded in a timely or a signal-induced manner and are replaced by newly synthesized proteins in an accurately balanced rate. Proteolysis takes place in different compartments in each cell, i.e. cell membrane, cytosol, lysosome, and endoplasmic reticulum [28]. While extracellular proteins are degraded via lysosomes after endocytosis, the breakdown of the vast majority of intracellular proteins is carried out by the action of ATP-dependent ubiquitin proteasome

system (UPS) [27, 28]. The UPS is composed of two complementary subsystems: (1) Ubiquitination system, which is made up of ubiquitinating enzymes, E1, E2, and E3, which tag the targeted proteins to activate the proteolytic processing, and (2) Proteasome system, which is responsible for protein degradation into smaller polypeptides and amino acids [29]. The ubiquitin-marked proteins are recognized and degraded by the 26S proteasome. The 26S proteasome is made up of a central subunit, 20S proteasome, and a peripheral regulatory subunit, 19S, that precisely select the ubiquitinated substrate, unfold it, and translocate it to the 20S subunit where the proteolysis takes place [27, 29].

UPS has many crucial homeostatic functions such as quality control, regulation of cell cycle, and activation of some physiological responses. The misfolded or unfolded proteins that result from mutations, abnormal synthesis, and free radicals are precisely degraded and removed from the cells representing the UPS quality control role [27]. For example, many misfolded proteins in the endoplasmic reticulum are removed to the cytoplasm via ubiquitination to be processed by UPS.

Since in each cell cycle phase there are different proteins to control it, these proteins levels are essential to trigger and terminate that particular phase. UPS plays a major role in the regulation of cell cycle through controlling some proteins levels. For instance, UPS induces the transition from metaphase to anaphase by the proteolysis of Cyclin B, a cell-cycle regulatory protein, marking the end of cell mitosis [30].

Regarding its role in the activation of immune response, UPS acts on major histocompatibility complex (MHC) class I to develop and display antigenic fragments on the cell surface to be detected by CD8⁺ T cells. When a foreign fragment is detected, the displaying cell is rapidly killed by cytotoxic T cell [31].

1.2.3 Druggability of the UPS

As mentioned previously, the 20S subunit of the 26S proteasome is where the proteins' breakdown is carried out [27]. Unlike other proteases, this subunit ensures that nearly every peptide bond in the targeted polypeptide, except the repeated glutamine sequence, is prone to degradation due to the multidimensional active proteolytic sites in this subunit [32]. Many different research projects have been carried out to study the 20S proteasome genetically, structurally, and functionally. In general, the 20S proteolysis takes place specifically after a particular amino acid. It involves three different classes of proteolytic active sites, six catalytic β -subunits, two active sites degrade the polypeptide after the hydrophobic amino acids, two sites breakdown the protein after the acidic amino acids, and two act on the bond after the basic amino acids [29]. In addition, several functional studies have successfully proved the catalytic role of the N-terminal Thr in the 20S proteasome activity and found that mutations of the N-terminal threonine inactivate this subunit, suggesting the major role that the Thr residue plays in the proteolysis reaction [33]. Accordingly, further studies have come up with the proposed mechanism of the 20S proteasome action: (1) The (-OH) group of the N-terminal threonine attacks the amide group of the targeted peptide bond forming a covalent acyl-enzyme intermediate with transferring the (-OH) proton to the

dissociated (-NH) group, releasing the first fragment. (2) Hydrolysis of the acyl-enzyme intermediate occurs to re-form a free enzyme and the second fragment [29, 33]. Since this mechanism is unique for the 20S proteolysis, many molecules have been discovered to inhibit this process.

Bortezomib, an important antineoplastic medication, is one of the synthetic UPS inhibitors. In 2003, this drug was approved by US FDA to treat multiple myeloma and became the first proteasome inhibition-based drug. Its mechanism of action generally is through blocking NF- κ B activation [34]. NF- κ B is a transcription factor, which is essential for cell proliferation and cytokines synthesis in response to inflammations and was found persistently active in cancer cells displaying anti-apoptotic activity [35]. Moreover, it was revealed that myeloma cells depend on NF- κ B in growth factor synthesis [34, 35]. Therefore, due to the important role of the ubiquitin proteasome system in activation of NF- κ B, Bortezomib (Valcade[®]) induces apoptosis in myeloma cells [34].

1.2.4 UPS and Insulin Resistance

Inflammation-induced insulin-signaling disturbance leads to insulin resistance, which is one of the manifested characteristics of Type II diabetes [36, 37]. Accordingly, many studies have shown that ubiquitination plays a critical role in inflammation-mediated insulin resistance development in mice [38]. A chronic low-grade inflammation induces the abundant formation of cytokines. These cytokines stimulate the expression of suppressors of cytokines signaling (SOCS) that target IRS1 to initiate its ubiquitination and breakdown by UPS [38].

Normally, PI3k/Akt pathway regulates IRS1 via its dual role, positive and negative feedback [39]. A downstream messenger, mechanistic target of rapamycin (mTOR), is activated by the action of protein Ras homolog enriched in brain (Rheb) that is stimulated by Akt in response to insulin to stimulate protein synthesis [40]. mTOR binds to and activates S6 kinase (translation factor) that binds to the large subunit of ribosomes and triggers the translation of mRNA into a protein [40, 41]. A study has shown that the hyperactivated mTOR and S6 kinase result in phosphorylation of specific serine residues (Ser-307/Ser312 and Ser-527) on IRS1 [41]. This phosphorylation promotes the binding of Ubiquitin ligase-7 to IRS1 to be degraded by UPS [41].

1.3 Skeletal Muscle in T2D Research

As mentioned previously, insulin has many critical metabolic activities such as glucose and lipid metabolism, protein synthesis, and regulation of gene expression [7]. And, insulin resistance in the major glucose-consuming tissues is involved in T2D pathogenesis [6, 8, 10]. Accordingly, obtaining extensive research studying the deviation in insulin molecular signaling networks during the development of insulin resistance may help unveil the mysterious mechanism of T2D pathogenesis.

1.3.1 Proteomics

After the impressive results of the human genome project of identifying almost 20,000-25,000 genes that led to the identification of many disease-associated genetic mutations, a relatively new phase in molecular biology has

been established to study structures and functions of the resultant proteins of each gene to understand/predict their role in the development of different diseases [42-44]. Unlike genome, which mostly remains unchanged during a person's life, proteome reflects the cellular changes in a particular phase [42]. Consequently, proteome provides a critical tool that could be targeted to study the impact of a specific endogenous or exogenous factor on a disease's development. Many novel discoveries in molecular biology have been concluded through comparing different states of a specific cell, i.e., studying the changes in a cell line under stress and comparing that with a normal state cell line. In proteomics, scientists also study the changes of a specific protein posttranslational modification, protein-protein interactions, and a particular organelle under different cellular states [42]. This, in turn, could result in many clinical benefits, e.g., identifying a novel molecule that can be targeted to treat a particular disease and recognizing many biomarkers that indicate the progression of diseases at their early stages, i.e., Type II Diabetes Mellitus [45]. Due to the capability of proteomics to identify and quantify hundreds of proteins, protein-protein interactions, and protein posttranslational modifications, its employment to diabetes research provides a promising discipline to unveil the pathophysiological processes behind the disease progression and to identify novel pathways that could be targeted in drug development.

1.3.2 Skeletal Muscle Targeted Proteomics Research

Many research projects have been studying the posttranslational modifications in main energy storage tissues (e.g., liver, adipose tissue, and skeletal muscle). However, only a handful of them are focusing on skeletal muscle,

which is responsible for the uptake of nearly 80% of blood glucose in response to insulin [46] (Fig 3). This represents the apparent demand of skeletal muscle-targeted research studies. In addition, the significantly accelerated technological improvements in proteomics-based research have generated interesting information on posttranslational modifications that are unattainable by genomics approaches. The application of proteomics to human skeletal muscle will expand the scientific understanding of insulin resistance and T2D pathogenesis.

1.3.3 Hyperinsulinemic-Euglycemic Clamp

In order to investigate the human body response to the circulating insulin *in vivo*, several different methods have been invented. The most widely used clinical methods are Oral Glucose Tolerance Test, Insulin Tolerance Test, and Glucose clamps [47]. In 1979, Dr. DeFronzo's group developed a unique method that has the capability to test the tissues' insulin sensitivity *in situ*, Hyperinsulinemic-Euglycemic Clamp. Basically, this method includes infusion of insulin and glucose into the human body according to the participant's body mass index (BMI) and measuring blood glucose in constant time intervals to monitor their infusion accordingly to keep the blood glucose level in a steady state. Any change in glucose infusion rate is represented as a value called (M-value) that reflects the body's sensitivity to insulin [48].

1.4. Mass Spectrometry-Based Proteomics

Two major kinds of protein information can be obtained from proteomics experiments, classical and functional data. Classical data provides basic

information about the protein identity and abundance. On the other hand, functional data provides more information about the protein activity and interactions [49]. Accordingly, several techniques can be used in proteomics to quantify and/or characterize proteins such as two-dimensional gel electrophoresis, multidimensional protein identification technology, protein chips, isotope-coded affinity tagging, activity-based probe, and mass spectrometry [49]. Each method has its pros and cons. For instance, using two-dimensional gel electrophoresis, researchers can detect up to thousands of proteins at one run. However, its low sensitivity to the low abundance proteins, which represent about 50% of cellular proteins, is a major drawback [49]. Thus, researchers have ranked the proteomics methods according to many factors: the sensitivity to low abundance proteins, the ability to investigate proteins activities, the ability to detect protein interactions, and the method speed [49]. Only a few methods fulfill all these characteristics. The golden method, mass spectrometry, is one of the most widely used methods in proteomics due to its ability to detect and quantify proteins at a high throughput fashion [50]. The extensive technological improvements in mass spectrometry have significantly enhanced proteomics research [51].

In this present study, we performed the first large-scale differential lysine ubiquitination analysis of primary skeletal muscle cells derived from lean, insulin-sensitive, non-diabetic participants under the hyperinsulinemic hyperglycemic condition. Pan ubiquitination antibodies were used to quantify overall protein ubiquitination under low glucose no insulin or high glucose and high insulin conditions. Furthermore, a combination of ubiquitinated-peptide enrichment

technique and the Universal-SILAC approach recently developed in our laboratory was employed [52] to identify and quantify site-specific lysine ubiquitination sites under these conditions. The goal of the study is to determine the effect of the treatment of high glucose and high insulin on global ubiquitination in insulin-sensitive primary skeletal muscle cells.

CHAPTER 2 EXPERIMENTAL DESIGN

The experimental results of this project were obtained from two complementary studies, clinical research and bench-side investigation (Fig 4). Human clinical research was carried out to recruit, screen, assess, and classify the participants. Each participant was scheduled for two visits to the Clinical Research Center at Wayne State University. In addition to the phone screening prior to the first visit (V-1), a comprehensive assessment was performed to assign the participants to the corresponding classes and to confirm their eligibility to be included in the study. After the confirmation of the participants' eligibility, they were scheduled for a second visit (V-2) to perform the hyperinsulinemic-euglycemic clamp and collect the skeletal muscle biopsies (Fig 5). Then, the obtained muscle biopsies were sent to the laboratory to conduct the proteomics investigations. Basically, the proteomics study workflow was as follow: skeletal muscle cell culture, cell collection, cell homogenization, in-solution trypsin digestion, desalting and peptide extraction, ubiquitinated peptide enrichment, and HPLC-ESI-MS/MS analysis. Afterwards, molecular pathway and functional assessment were performed using bioinformatics analysis and literature search to interpret the proteomic data.

2.1. Materials

2.1.1 Antibodies

We purchased a PTMScan® Pilot Ubiquitin Remnant Motif (K-e-GG) Kit (from Cell Signaling Technology; Catalogue number: 14482S) containing Ubiquitin

monoclonal anti-diglycine Remnant (K- ϵ -GG) antibody, IAP buffer, and trypsin digested control peptides for immunoprecipitation. For western blot, we purchased Anti-Ubiquitin antibody (Abcam; Catalogue number: ab7780) containing Rabbit polyclonal to Ubiquitin. The secondary antibody was Anti-rabbit IgG, peroxidase-linked species-specific whole antibody from donkey.

2.1.2 Reagents

In this study, the following supplies were purchased: MS Grade Trypsin protease (Fisher scientific; Catalogue numbers: 90057 and 90058); iodoacetamide (Sigma, St Louis, MO; Catalogue number: I1149-5G); C18 ZipTip (Millipore, Billerica, MA); Alltech™ Reversed-Phase Extract-Clean™ Columns (High Capacity C18; 500mg; 8ML, High capacity C-18; Column size: 1.5mL; Bed weight: 100mg; Fisher scientific; Catalogue numbers: AT255350 and AT255100, respectively); Insulin ELISA Jumbo (AIPCO, Salem, NH, Catalogue number: 80-INSHU-E10.1); and IAP buffer (PTMScan® IAP Buffer (10X); Cell Signaling; Catalogue number: 9993S)

2.2 Subjects

Four lean, healthy participants were enrolled in the study at the C. S. Mott Clinical Research Center at Wayne State University. The purpose, procedures, and the possible risks of the study were described to all volunteers, and their agreement to take part in the study was obtained in the form of a written consent prior to their involvement in the study. Primary screening, including vital signs, ECG, bioimpedance, and urine analysis, was done on the same day when the

written consent was obtained. In addition, participants' fasting blood glucose levels were measured and an oral glucose tolerance test (OGTT) was performed to measure their ability to tolerate a 75 g glucose drink and to assign them to the corresponding class, either healthy or insulin resistant. None of the participants was involved in any form of intense exercise. The study protocol was approved by the Institutional Review Board of Wayne State University.

2.3 Hyperinsulinemic-Euglycemic Clamp with Muscle Biopsies

Insulin sensitivity was assessed using hyperinsulinemic-euglycemic clamp, and skeletal muscle was exposed to insulin *in vivo*, as previously described [53, 54]. On the day of the 2nd visit, the participants came in at approximately 08:15 a.m., after a minimum of a 10-hour overnight fast, and their vital signs were measured. Then, at approximately 08:30 a.m. (time -60 min) the study began with placing a catheter in an antecubital vein. This catheter was maintained throughout the study for insulin and glucose infusions. A second catheter for sampling of arterialized venous blood was placed in a vein in the contralateral arm. Baseline blood samples were drawn for plasma glucose and insulin concentrations determination. At 09:00 a.m. (time -30 min) under local anesthesia using Lidocaine, a biopsy was obtained from the vastus lateralis muscle using a percutaneous needle [55]. Protease inhibitors were immediately added to the extracted muscle biopsy that were cleaned from connective tissue and fat (~30 sec), and muscle biopsy was immediately transferred in ice-cold PBS to the laboratory to extract skeletal muscle cells through a process called primary cell

culture. At 09:30 a.m. (time 0 min), at a rate of 80 mU/m²/min, a continuous infusion of human regular insulin (Humulin R; Eli Lilly, Indianapolis, IN) was started, and continued for 2 hrs. Plasma glucose level was measured at 5-min intervals and monitored throughout the clamp to maintain euglycemia, ~90 mg/dL, by variable infusion of D-glucose.

2.4 Plasma Insulin Concentration Determination

Plasma insulin level was measured using the ALPCO Insulin ELISA Jumbo.

2.5 Proteomics Sample Preparation and Analysis

2.5.1 Muscle Cells Processing and Protein Measurement

The extracted skeletal muscle cells were maintained to reach 70-80% confluence for each experimental condition in 150 mm culture dishes. Then, the myoblasts were fused into multi-nucleated fibers, myotubes, through a process called differentiation. During differentiation, each cell line was divided into 4 conditions: (1) Low glucose (1 g/L) without insulin stimulation, LGNI_BAS; (2) Low glucose (1 g/L) with insulin stimulation (100 nM) for 15 min after 4 hrs starvation, LGNI_INS; (3) High glucose (4.5 g/L) /High insulin (100nM) for 2 days without 15 min insulin stimulation, HGHI_BAS; (4) High glucose (4.5 g/L) /High insulin (100nM) for 2 days with 15 min insulin (100 nM) stimulation after starvation, HGHI_INS. All cells were washed with ice-cold PBS 3 times before harvesting them. All dishes from each condition were harvested using the same amount of ice-cold lysis buffer A (50 mmol/l Hepes, pH 7.6, 150 mmol/l NaCl, 20 mmol/l

Na₄P₂O₇, 20 mmol/l β-glycerophosphate, 10 mmol/l NaF, 2 mmol/l sodium vanadate, 2 mmol/l EDTA, 1% Triton, 10% glycerol, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 1 mmol/l MgCl₂, 1 mmol/l CaCl₂, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and a scraper. After that, all cell lysates were homogenized using a Next Advance Bullet Blender (Model BBY5E) at 4°C until no visible contents remained (~4×5min). The lysates were cleared by centrifugation at 20,000 rcf (g) for 15 min at 15°C or room temperature and the protein extracts (supernatants) were transferred into new tubes. Protein concentrations in the supernatant samples were measured by the Bradford protein assay (Nanodrop 2000c spectrophotometer; Thermo Scientific). A small volume of the lysates was saved to perform western blot, and SILAC lysates were added in a ratio of 1:4 to every condition's lysate that underwent immunoprecipitation.

2.5.2 Western Blot

To detect the level of protein ubiquitination among all conditions, whole cell lysates prepared from human skeletal muscle cell lines were loaded and the gels were run under denaturing conditions. 5% milk in TBS-Tween was used for blocking. Anti-Ubiquitin antibodies were used at a 1/1000 dilution, incubated for 16 hours at 4°C in 5% milk in TBST. The secondary antibody was peroxidase-linked species-specific whole antibody donkey anti-rabbit IgG, used at a 1/10,000 dilution in 5% milk in TBST for 1 hr at RT. Immunoreactive bands were detected according to their chemiluminescence and the bands intensities were quantified using the ImageJ software (<http://imagej.nih.gov/ij/>).

2.5.3 Immunoprecipitation

2.5.3.1 Reduction and Alkylation of Proteins

1/278 volume of 1.25 M DTT was added to the extracted proteins from each condition, mixed well, and the tubes were placed into a 55°C incubator for 30 min. The solutions were cooled on ice briefly until they had reached room temperature. 1/10 volume of 1M iodoacetamide solutions were added to the protein extracts, mixed well, and incubated for 15 min at room temperature in the dark. Then, the fractions were subjected to ultracel 10k filters to purify the denatured proteins from salts.

2.5.3.2 Trypsin Digestion and Peptide Purification

1/50 volume of 1 mg/ml trypsin in 40 mM NH₄HCO₃ was added to the samples and incubated overnight at room temperature with mixing. The digested peptides were acidified with 1/20 volume of 20% TFA to the peptides for a final concentration of 1% TFA before loading them on the Sep-Pak C18 columns for desalting. The samples were placed on ice for 15 min. After centrifugation for 15 min, the acidified digested peptides were loaded to the columns that were prewashed with (1x 100% ACN and 3x 0.1% TFA). The samples were washed 3x 0.1 TFA and 1x 5% ACN/0.1% TFA. They were collected after elution with 40% ACN/0.1% TFA. The purified samples were subjected to SpeedVac to remove the solvents.

The beads were washed four times with PBS, and the samples were resuspended in 1.4 ml IAP buffer and pH was adjusted to pH=7.8 before centrifugation at 10,000 rcf (g) for 5 min at 4°C. Then, the peptides were

transferred into the bead-containing tubes and incubated for 2 hours on a rotator at 4°C. The bead-bound peptides were washed with IAP buffer and HPLC-grade water before elution with 0.15% TFA three times. The eluates were dried off by vacuum centrifugation and reconstituted in 0.1% TFA for HPLC-ESI-MS/MS analysis.

2.5.3.3 Mass Spectrometry

The peptide mixture was separated with a linear gradient of 5-35% buffer B (100% ACN and 0.1% FA) in 180 minutes at a flow rate of 250 nL/min on a C₁₈-reversed phase column (75µm ID, 15 cm length) packed in-house with ReproSil-Pur C18-AQ µm resin (Dr. Maisch GmbH) in buffer A (0.1% FA). A nanoflow Ultimate 3000 RSLCnano system (Thermo Scientific) was on-line coupled to a Thermo Finnigan LTQ-Orbitrap Elite fitted with a nanospray flex Ion source (Thermo Fisher, San Jose, CA). MS data were acquired in a “Top-20-RCID” data-dependent strategy selecting the fragmentation events based on the precursor abundance in the survey scan (400-1650 Th).

2.5.4 Data Analysis

Peptides/protein identification and quantification were performed using the MaxQuant, one of the popular quantitative proteomics software packages [56, 57]. Briefly, raw MS files were processed using the MaxQuant (ver.1.3.0.5) against a database with forward and reversed Uniprot Human protein sequences, downloaded from www.uniprot.org. Standard settings in the MaxQuant were applied. Parent mass tolerance was 5 p.p.m., and fragment mass tolerance was 0.5 Da. Four missing trypsin cleavage site was allowed, carbamidomethylation was

searched as a fixed modification, and lysine ubiquitination (as reflected by GG-lysine), methionine oxidation (M_{ox}), phosphorylation (STY), Acetylation (Protein N-term), isotope-labeled lysine (+4.00 Da) and isotope-labeled arginine (+10.00 Da) were allowed as a variable modification. The FDR for both proteins and peptides (with minimum 6 amino acids) was set to 0.01. Only ubiquitination sites with a localization probability greater than 0.75 [58, 59]) were considered.

To minimize the experimental variation during sample preparation and HPLC-ESI-MS/MS data acquisition, we developed and validated a modified Super-SILAC approach, in which SILAC-labeled protein lysates were spiked-in to each experimental sample and were used as a universal standard for quantification purposes [52]. This modified Super-SILAC approach (we now term it as Universal-SILAC) provides quantitative information for more phosphorylation sites than the traditional Super-SILAC quantification. Using this approach, we identified 3876 phosphorylation sites (620 were novel), and found that knockdown of Protein phosphatase 1 regulatory subunit 12A in L6 cells resulted in increased overall phosphorylation in L6 cells at the basal condition, and changed phosphorylation levels for 698 sites (assigned to 295 phosphoproteins) at the basal and/or insulin-stimulated conditions [52]. In the present study, we applied Universal-SILAC to the quantification of the ubiquitination in primary skeletal muscle cells derived from lean healthy human participants in the absence and presence of high glucose/high insulin treatment, and calculated the modified SILAC ratio as described in our manuscript [52]: an individual peak area for a ubiquitination site (PA_i) was

normalized against the sum of the peak area for the heavy labeled ubiquitination sites in the same sample:

$$\text{Norm: } i = \frac{PA_i}{\text{Sum of the peak area for the heavy labeled ubiquitination sites}}$$

The normalized peak area (i.e., modified SILAC ratio) for each ubiquitination site was log₂ transformed and compared to assess effects of acute insulin or relatively long-term high glucose/high insulin treatment on ubiquitination levels among the 4 sets of samples (LGNI_BAS, LGNI_INS, HGHI_BAS, and HGHI_INS) of primary skeletal muscle cells derived from muscle biopsies from 4 lean insulin-sensitive human participants.

2.5.5 Statistical Analysis

Although a large number of ubiquitination sites were assigned in at least one of the 16 samples that were studied, a series of filters were used to narrow the number of ubiquitination sites that were used in comparisons among groups as described in Figure 11. Statistical significance was assessed using paired *t* tests. Differences were considered statistically significant at $p < 0.05$.

2.5.6 Bioinformatics Analysis

Pathway analysis was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery) (<https://david.ncifcrf.gov/>), which assigns each identified gene to its corresponding biological process. DAVID is a web-accessible database that provides an extensive functional annotation tool to a

gene list. A pathway was considered as significantly enriched if p-value < 0.01
(See Fig 12 and Table 2).

CHAPTER 3 RESULTS

The clinical information of participants involved in the project is shown in Table 1. Criteria classifying the state of diabetes are shown in the table, namely 2h OGTT Glucose level, HBA1c percentage, and Fasting plasma glucose level, manifesting the values within healthy, non-diabetes range.

Hyperinsulinemia and hyperglycemia are known to have unfavorable effects on many cells types [60]. To understand the effect of the high glucose/high insulin condition on the skeletal muscle system, the levels of proteins ubiquitination in human skeletal muscle cells was evaluated under low and high concentrations. In addition, the impact of an acute 15-minute insulin treatment (after serum starvation) on the ubiquitination pattern was investigated.

3.1. Total Ubiquitination in Primary Human Skeletal Muscle Cells under Hyperinsulinemic Hyperglycemic Conditions

First, the effect of hyperglycemia on the total protein conjugation to ubiquitin moieties in skeletal muscle cells was examined. The cells were incubated with low glucose/no insulin (LGNI) or high glucose/high insulin (HGHI) culture media for 48 h, and then the average intensities of total ubiquitinated proteins in each cell line were measured.

Bands in Fig. 6 show that HGHI treatment caused a significant decrease (fold change 0.72 ± 0.058 vs. LG, $P < 0.05$) in total protein ubiquitination compared to LGNI treated cells.

Next, whether the exposure of skeletal muscle cells to acute 15-minute insulin treatment affects the reduction pattern of total ubiquitinated proteins level

with hyperglycemia-Hyperinsulinemia was assessed. As shown in Fig. 7, the reduction in total proteins ubiquitination with HGHI treatment was significantly decreased (fold change 0.71 ± 0.056 vs. LG, $P < 0.01$) compared to LGNI treated cells. This suggests that insulin does not change the marked reduction in total protein ubiquitination after 2-day HGHI treatment.

Furthermore, the 15-minute-insulin-stimulated cells were normalized to their corresponding basal cells to investigate the effect of insulin stimulation on the total conjugation of human skeletal muscle proteins to ubiquitin. As shown in Fig. 8, there is no effect for 15-minute insulin stimulation on the total ubiquitination level in skeletal muscle cells treated with HGHI.

Next, the effect of HGHI treatment on individual bands shown in Fig. 6 was examined, separately. Fig. 9.A shows a significant reduction in the ubiquitination level in the proteins with molecular weight around 75 KD in the case of hyperglycemia-hyperinsulinemia (fold change 0.68 ± 0.06 vs. LG, $P < 0.05$). After insulin stimulation (Fig. 9.B), fold change was similar (0.75 ± 0.05 vs. LG, $P < 0.005$). Similar to the total ubiquitination, Fig. 9.C shows no effect for 15-minute insulin stimulation on the accumulation of ubiquitinated proteins in the 75 KD band with HGHI treatment.

Moreover, as shown above in Fig. 10, the abundance of ubiquitinated proteins in 50KD and 37KD bands was not different between LGNI and HGHI treated cells except for ubiquitinated proteins in 50KD band upon 15-minute insulin stimulation (Fig. 10.B, fold change 0.50 ± 0.09 vs. LG, $P < 0.05$).

3.2. Site-Specific Ubiquitination in Primary Human Skeletal Muscle Cells under Hyperinsulinemic Hyperglycemic Conditions

In order to identify the changes in site-specific ubiquitination, ubiquitin remnant motif (K- ϵ -GG) antibodies were used to enrich ubiquitinated peptides from all 4 skeletal muscle primary cell cultures used for the study. In total, 339 ubiquitination sites assigned to 171 proteins were detected. Among the 339 sites, 110 sites were identified in more than half of the samples. 21 out of the 110 sites showed significant change in their ubiquitination in the 4 sets of samples (LGNI_BAS, LGNI_INS, HGHI_BAS, and HGHI_INS). Out of these 21 sites, 5 were previously unreported in human. Please see (See Fig 13 A-U and Table 3 for details)

DAVID analysis of the ubiquitination sites indicated that multiple biological processes are significantly enriched, such as pathways related to interleukin-1 receptor-associated kinase 1, regulation of activated PAK-2p34, protein synthesis and degradation, and cytoskeleton dynamics (Fig 12 and Table 2). These results imply that ubiquitination participates in regulation of these biological processes.

Furthermore, as mentioned above, we have observed a significant reduction in ubiquitinated protein level at the 75 KD band using WB (Figure 6). Combining the WB results with the IP data, we identified 21 candidate proteins with molecular weight around 75 KD after ubiquitination based on the number of possible ubiquitination sites in each protein. Additional experiments are warranted to determine which of these 21 proteins contribute to the decreased ubiquitination in the 75 KD bands upon HGHI treatment.

CHAPTER 4 DISCUSSION

Recently, genomics along with transcriptomics studies have unveiled valuable information regarding mechanisms underlying insulin resistance and T2D. However, the abnormalities in protein posttranslational modifications may not be investigated using genomics and transcriptomics [61]. Mass spectrometry-based proteomics has emerged as a powerful method for investigating protein post-translational modification in the development of insulin resistance. Ubiquitination along with phosphorylation represents a crucial regulatory modification that controls intracellular signaling [62]. However, no ubiquitinome study in primary human skeletal muscle cells has been reported yet. The present project determined overall ubiquitination and site-specific ubiquitination in skeletal muscle primary cells derived from 4 lean healthy participants using immunoblotting technique and Mass spectrometry-based proteomics approach, respectively. The immunoblotting technique includes using anti-ubiquitin polyclonal antibody to detect the ubiquitination level change in the cells under different conditions. On the other hand, the proteomics approach includes ubiquitin remnant motif (K- ϵ -GG) immunoprecipitation followed by HPLC-ESI-MS/MS. Using this strategy, we have identified 21 ubiquitination sites that are significantly changed among the 4 sets of samples (LGNI_BAS, LGNI_INS, HGHI_BAS, and HGHI_INS). The involvement of these sites in multiple functional pathways in skeletal muscle cells is discussed below (See Fig 13 A-U and Table 3 for more details).

4.1. Total Ubiquitination in Primary Human Skeletal Muscle Cells under Hyperinsulinemic Hyperglycemic Conditions

In this study, we have expanded the findings of physiological changes in primary human skeletal muscle cells under different glucose/insulin concentrations. Although insulin involvement in the regulation of protein degradation was reported previously [63, 64], its impact on ubiquitin-proteasome system in different cells remains unclear.

In HepG2 and red blood cells, insulin showed a significant inhibitory effect on UPS activity through blocking the proteasomal degradation of ubiquitinated proteins and further increased the accumulation of ubiquitin conjugates [65]. In contrast, we did not observe increased total ubiquitination in primary human skeletal muscle cells, suggesting insulin affects ubiquitination in a tissue/cell-type specific manner.

Interestingly, we observed a significant reduction in the total ubiquitinated proteins in cases of hyperglycemia and hyperinsulinemia in primary human skeletal muscle cells (Fig 6-10). One possible explanation is that hyperglycemia and hyperinsulinemia increase the activity of de-ubiquitination enzymes.

4.2 Site-Specific Ubiquitination in Primary Human Skeletal Muscle Cells under Hyperinsulinemic Hyperglycemic Conditions

4.2.1 ACTA1

α -actin is a member of the actin family that also includes β - and γ -actin. It is a highly conserved protein that forms a major part of the cytoskeleton. Within the myocytes, actin along with myosin forms the sarcomere, which is the contractile structure in muscles. Also, they are important in maintaining the

structural framework of the muscle cells, the cytoskeleton. Recent studies suggested that insulin receptor signaling is important for the development of actin organization in neuronal cells through insulin-PI3K-Akt or DHA-Akt pathways [66]. In this study, we showed that the ubiquitin-conjugation with α -actin at **K193** is reduced upon 15-minute insulin stimulation (Fig. 13A). This result suggests that ubiquitin-dependent degradation is a part of insulin regulation of actin.

4.2.2 ANTXR1

ANTXR1, which is a coding gene for a protein named as Anthrax toxin receptor 1, showed a decreased ubiquitination at **K453** with hyperinsulinemia-hyperglycemia under after 15-minute insulin stimulation (Fig. 13B). This protein has a major role in cell adhesion and migration via its binding to extracellular matrix proteins, and it facilitates cells attachment to type 1 collagen and gelatin and cell spreading. Moreover, Anthrax toxin receptor 1 is important for angiogenesis. A recent study showed that upon ANXTR1 blockade, tumor cells become more responsive to antineoplastic agents and there is a reduction in patient mortality [67].

4.2.3 CCDC50

Coiled-coil domain-containing protein 50, also known as Ymer, is a cytoplasmic, soluble protein that has several ubiquitination sites. This protein is involved in the regulation of many pathways as a positive or negative regulator. As a negative regulator, it is involved in NF- κ B signaling. During the beginning stages of TNF-Receptor-1 signaling, ubiquitination takes place in Receptor-interacting protein 1 (RIP-1) to activate IK-kinase and NF- κ B. IK-kinase inactivates the de-

ubiquitinase enzyme (Cylindromatosis) via phosphorylation. Then, NF- κ B induces the over-expression of proteins A20 and I κ B α and serves as a negative feedback. Therefore, A20 binds to many proteins, including YMER, to induce RIP1 degradation. In contrast to its role in NF- κ B signaling, YMER facilitates JNK phosphorylation as a positive regulator in this pathway [68]. The ubiquitin-conjugation with CCDC50 at lysine **152** was significantly increased with low glucose/no insulin condition after 15 min insulin stimulation (Fig. 13C).

4.2.4 DAG-1

In normal muscle, dystroglycan forms a complex to stabilize the connection between the cytoskeleton and the extracellular matrix. A downregulation of dystroglycan in skeletal muscle may be involved in the muscle fiber weakness, sarcolemmal signaling disruption and/or cytoprotective mechanism disturbance in diabetics. Studies suggested that insulin effect in diabetic skeletal muscle might be altered due to impaired sarcolemmal signaling with GLUT4 transporters, which might be a key step in the development of skeletal muscle insulin resistance [69]. We observed a significant change in the ubiquitination of lysine **794** in this protein upon different treatment conditions (Fig. 13D).

4.2.5 EPN-1

Epsin is a protein that is detected in cytoplasm, cell membrane, and nucleus. It is necessary in controlling the activity of the epidermal sodium channel, which regulates sodium and water reabsorption at the cell surface [70]. Also, it is involved in the EGFR signaling pathway, and studies showed that epsin-1 is critical for EGFR endocytosis [71]. It facilitates the trafficking of ubiquitinated EGFR

across the cell membrane. This study showed a significant reduction in the ubiquitin binding to **K107** under different conditions (Fig. 13E).

4.2.6 ERH

The enhancer of rudimentary homolog is a highly conserved protein that is involved in transcription and cell cycle regulation. Many studies showed a critical role for ERH in mitosis and genomic stability [72]. In this study, there was a significant increase in ubiquitination at lysine **12** with hyperglycemia (Fig. 13F).

4.2.7 ITM2B

ITM2B belongs to the Bcl-2 family that has a crucial role in apoptotic processes by regulating the outer mitochondrial membrane permeability. This family is composed of anti-apoptotic and pro-apoptotic members. ITM2B is one of the pro-apoptotic proteins that are induced by IL-2 deprivation in T lymphocytes undergoing apoptosis [73]. We observed a significant reduction in ubiquitination of **K13** with hyperglycemic-hyperinsulinemic condition after insulin stimulation (Fig. 13H).

4.2.8 SPG20

Spartin is a mitochondrial protein that contains a domain of microtubule interacting and trafficking molecule (MIT). Spartin activity is controlled by ubiquitination/de-ubiquitination. This protein is involved in regulating mitochondrial functions and endocytosis. Recent studies have shown an important mechanism for SPG20 in the regulation of lipid molecules' turnover [74]. Ubiquitination of lysine **362** was significantly increased with hyperinsulinemic-hyperglycemic condition

(Fig. 13M).

4.2.9 VCP

VCP, also known as transitional endoplasmic reticulum ATPase, is involved in the assembly of Golgi stacks during mitosis. It also plays a major role in the transfer of membranes from the endoplasmic reticulum to the Golgi apparatus. Transitional endoplasmic reticulum ATPase drives misfolded-ubiquitinated proteins from the ER to the cytoplasm, for the proteasomal degradation [75]. In this study, ubiquitin binding to **K668** was increased with hyperglycemia-hyperinsulinemia (Fig. 13O).

4.2.10 Ribosomal subunits:

Although ubiquitination has been recognized as a critical regulatory modification in many biological processes, its activity was mostly known for protein degradation. With the increased awareness about ubiquitination's role, it was reported that ubiquitin conjugation to some molecules has a key role in their activation. Ubiquitin attachment to ribosomes, which have been well defined as the large molecular machineries for protein synthesis, in a cell-cycle dependent manner results in improved ribosomal efficiency in protein synthesis [76]. In this study, we observed a significant reduction in ubiquitin conjugation to ribosomal subunits at specific sites (**RPL9-K21**, **RPS27A-K6**, **RPS27A-K33**, and **RPS27A-K48**) in the case of hyperglycemia/hyperinsulinemia. On the other hand, there was a significant increase in **RPS20** ubiquitination at the site **K8** with high glucose/high insulin treatment. These sites need further investigation to unveil the impact of their

different ubiquitin conjugation on the protein synthesis in skeletal muscle cells (Fig. 13Q-U).

Summary

In this project, we observed a significant reduction in the total ubiquitinated proteins in cases of hyperglycemia and hyperinsulinemia in primary human skeletal muscle cells (Fig 6-10). Furthermore, 339 ubiquitination sites assigned to 171 proteins have been identified in human primary skeletal muscle cells from lean, healthy subjects. Among the 339 sites, 110 sites were identified in more than half of the samples. In addition, 21 sites among them significantly changed with different in-vitro treatments (including ACTA1, ANTXR 1, CCDC50, DAG1, VCP, as well as ribosomal proteins RPL9, RPS20, and RPS27A). Because of the increased recognition of the importance of ubiquitin proteasomal pathway in cellular processes, our data provides a list of ubiquitination sites in human skeletal muscle cells as a reference for future research in protein ubiquitination as well as diabetes research.

The future directions of this study will be validating these sites with different biochemical approaches, and studying the most interesting sites showed significant changes upon different treatment conditions to determine their functional roles in insulin signaling. To further investigate the potential involvement of the UPS in the development of insulin resistance in skeletal muscle, future efforts will be focused on the effect of proteasome inhibitors or Bortezomib on the reduced glucose uptake in skeletal muscle cells from type 2 diabetic individuals.

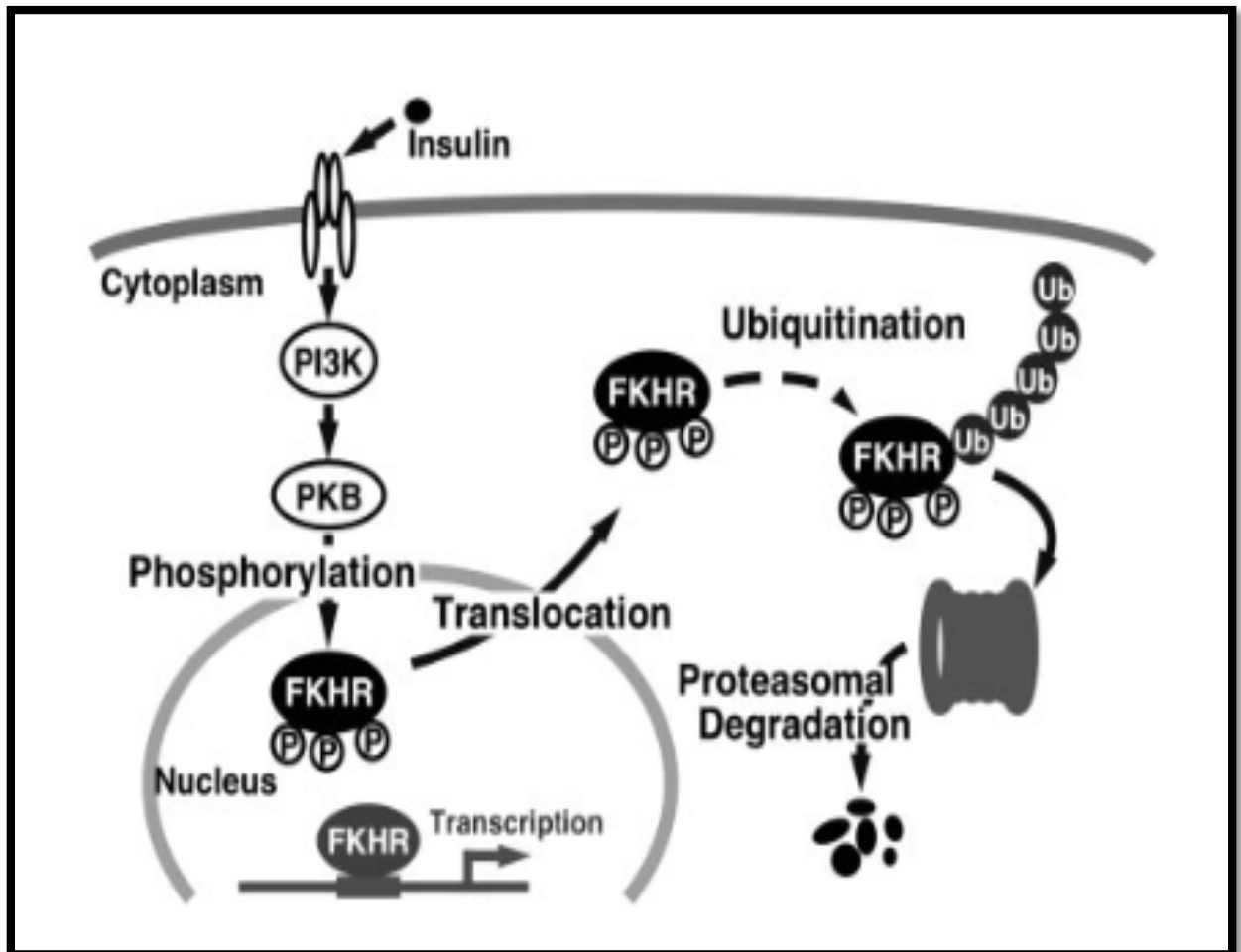


Figure 1. PI3k-Akt-FOXO pathway. The figure illustrates the role of ubiquitin-dependent proteasomal degradation of FOXO1 upon insulin stimulation [77]. (PKB: Akt; FKHR: FOXO1)

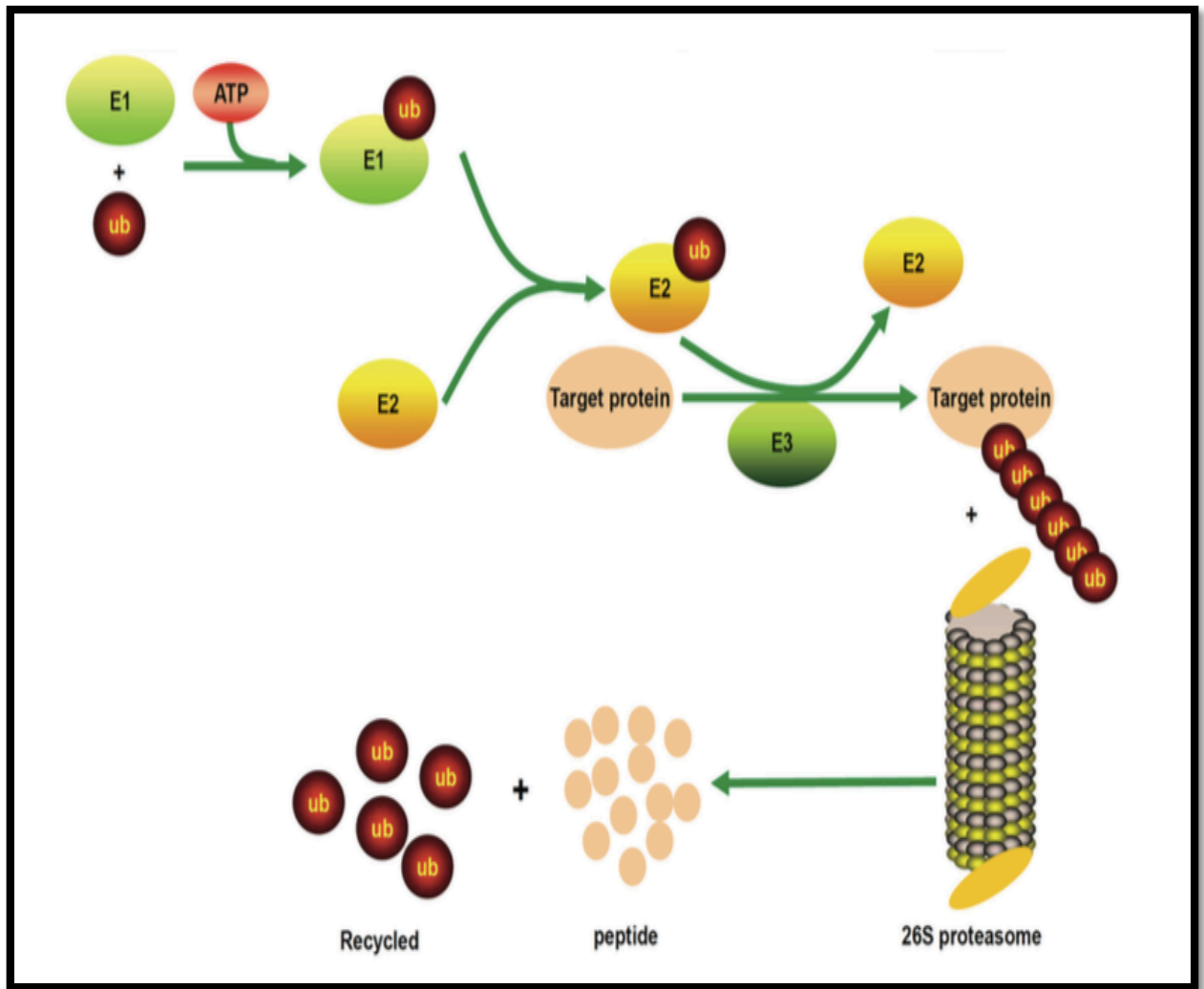


Figure 2. The ubiquitin-dependent proteasomal degradation pathway. [78]

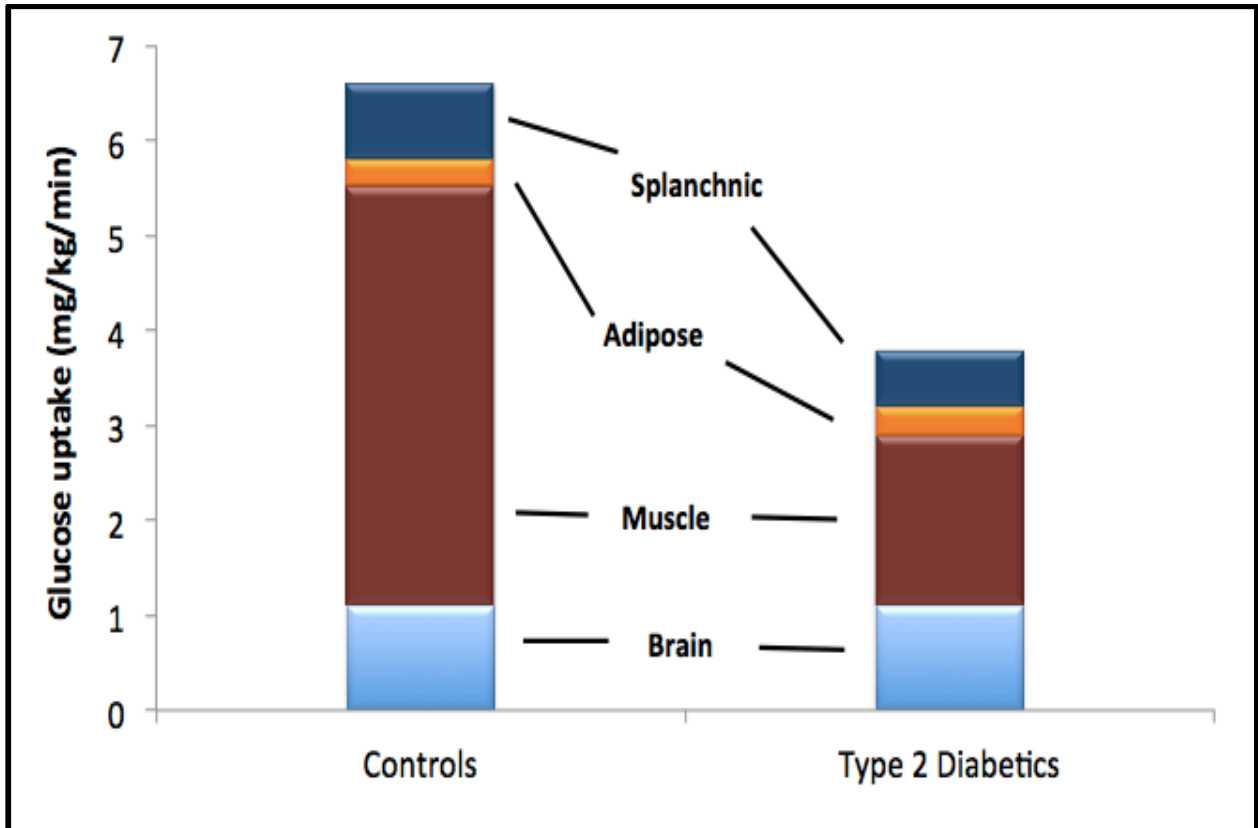


Figure 3. Glucose Metabolism during Hyperinsulinemic-Euglycemic Clamp. The figure above shows the significant reduction in glucose uptake by skeletal muscle in diabetics, while other major glucose consuming organs remain unaffected. [79]

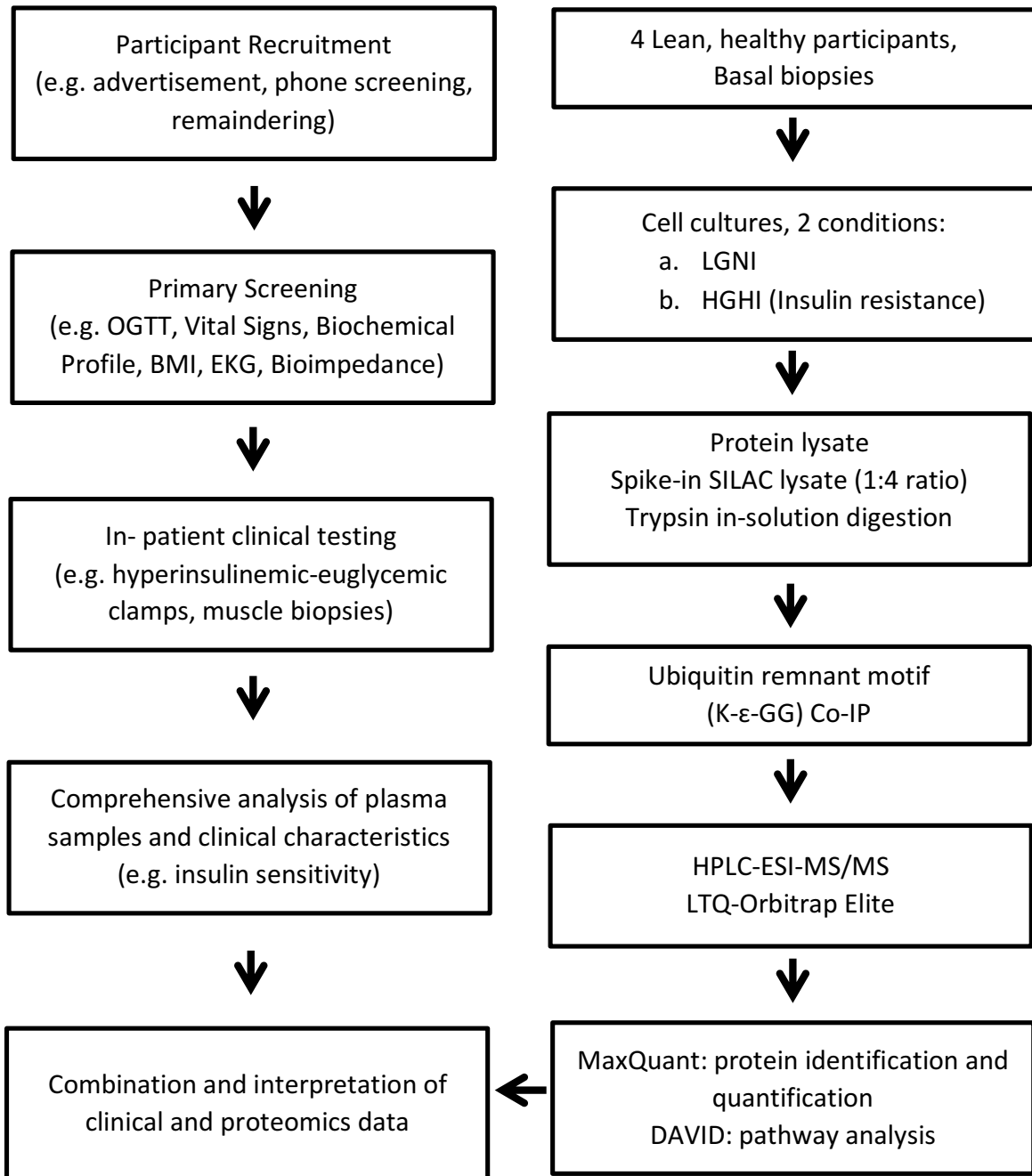


Figure 4. General flow chart of clinical and proteomics study.

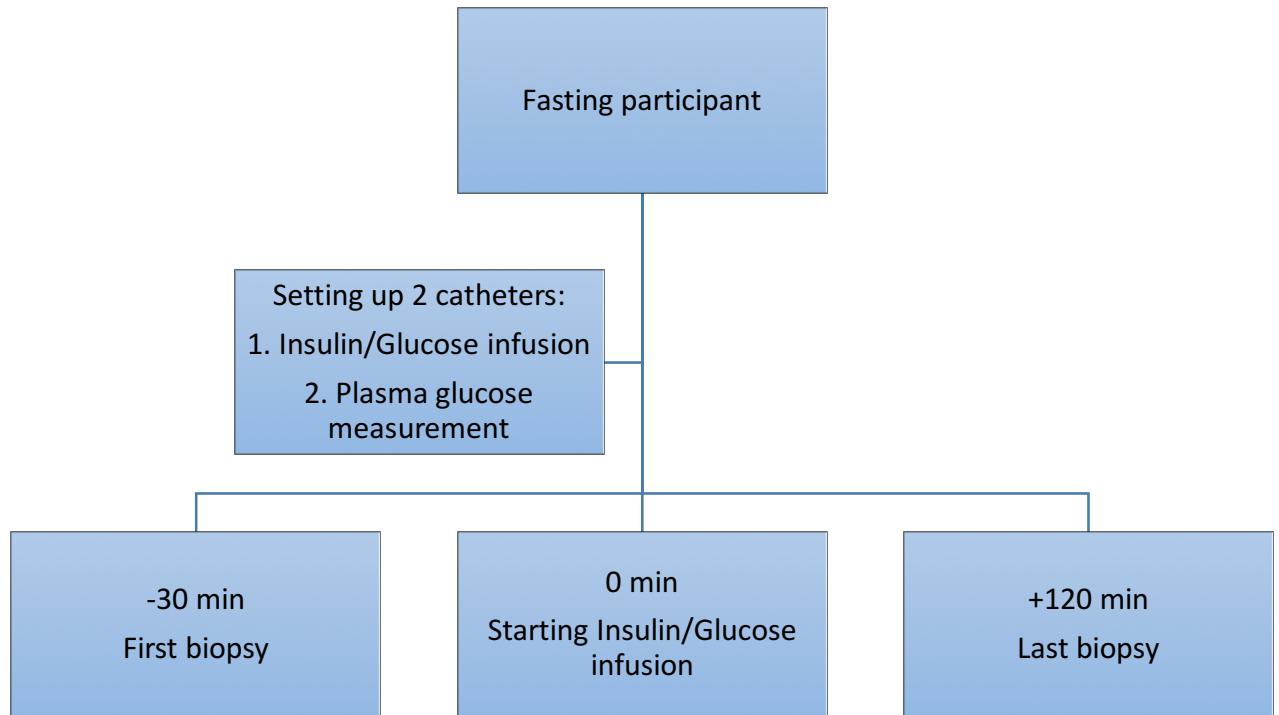


Figure 5. Overview of clinical visit 2 procedures

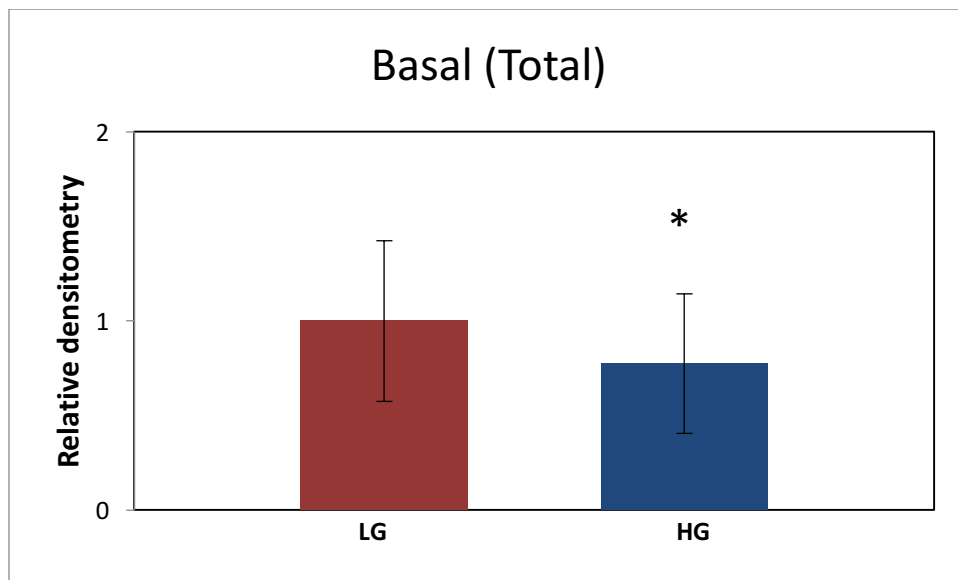
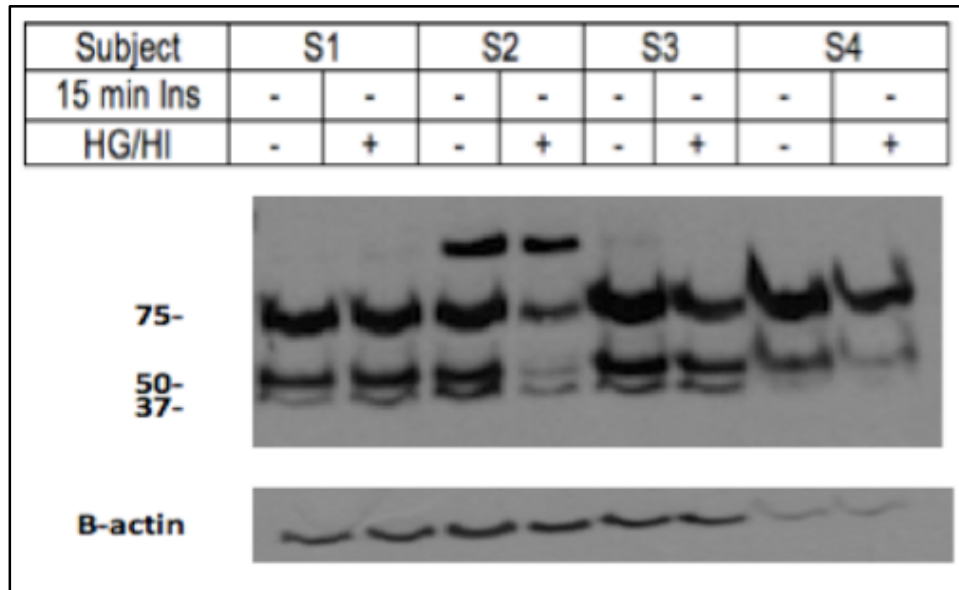


Figure 6. Effect of HG on total ubiquitination in the basal condition.

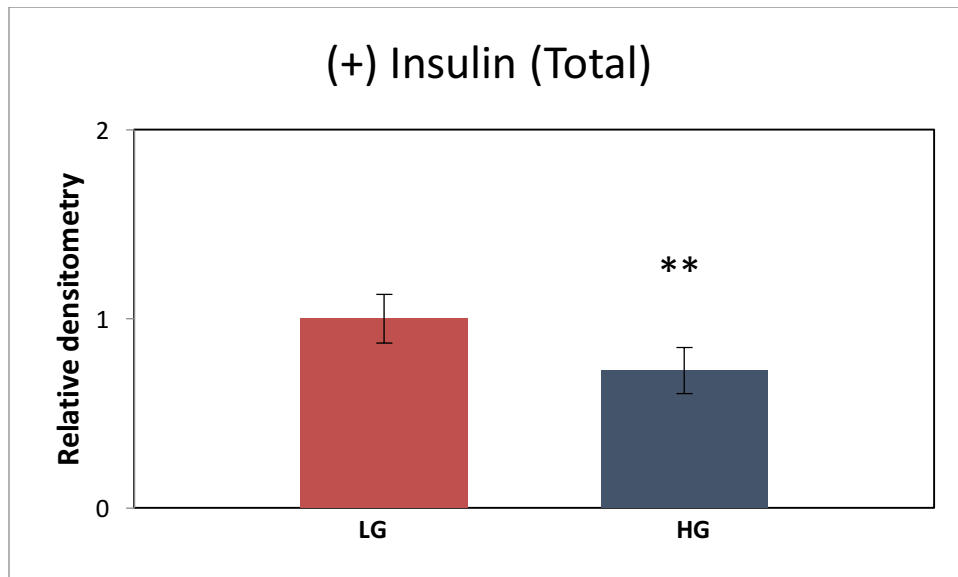
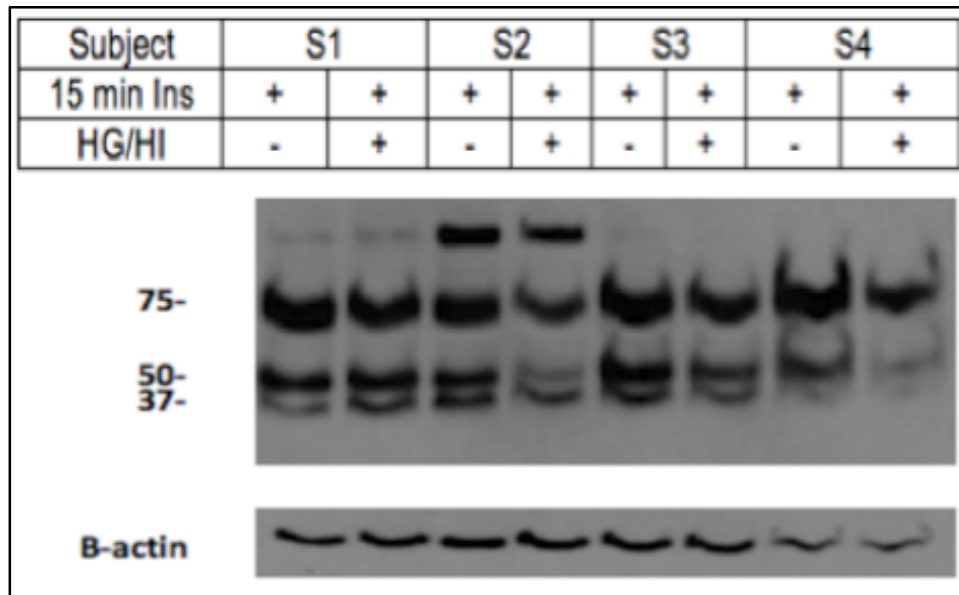


Figure 7. Effect of HG on total ubiquitination after insulin stimulation

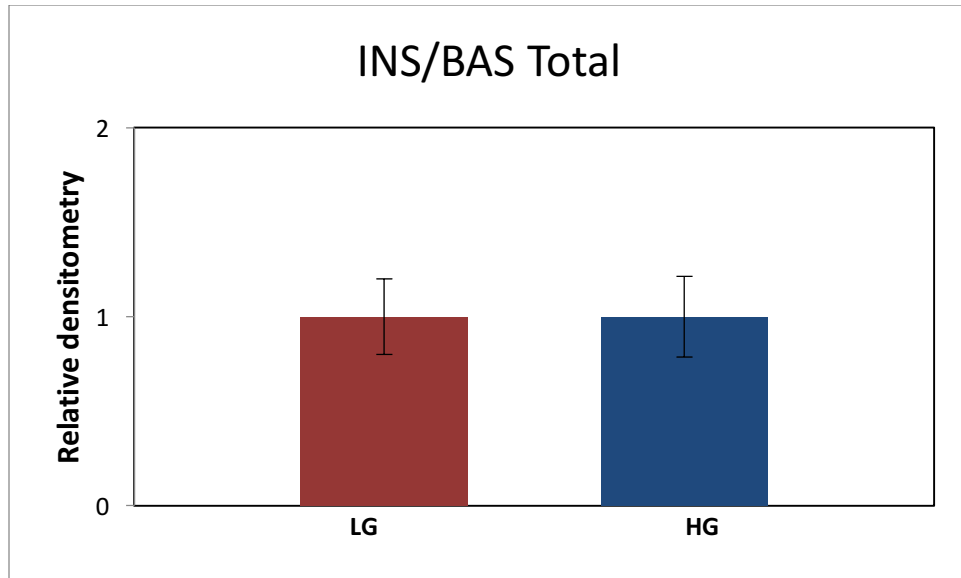


Figure 8. Effect of insulin on total ubiquitination level in LG and HG conditions

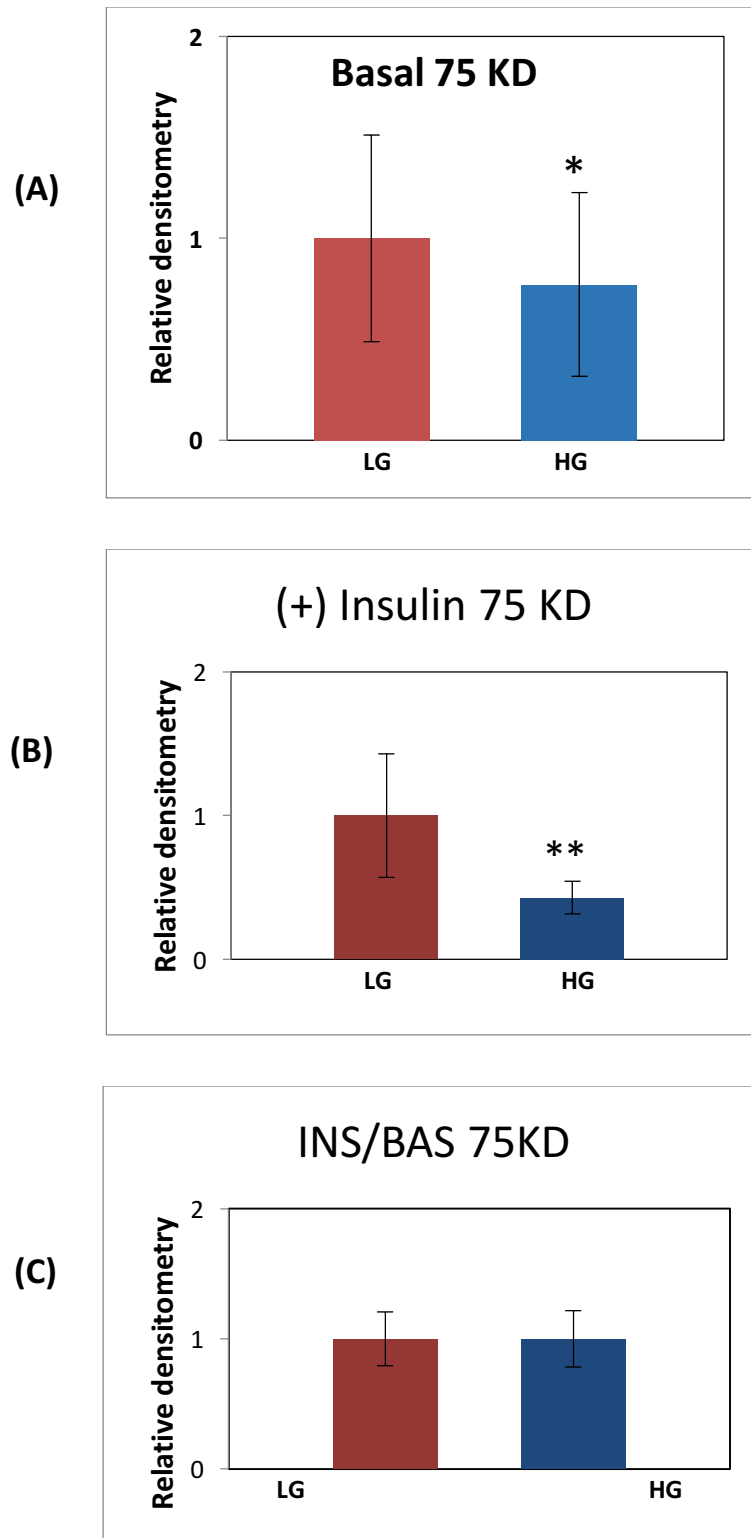


Figure 9. Ubiquitination level at 75 KD: (A, B) Effect of HG in the basal and insulin stimulated conditions. (C) Effect of insulin in LG and HG conditions.

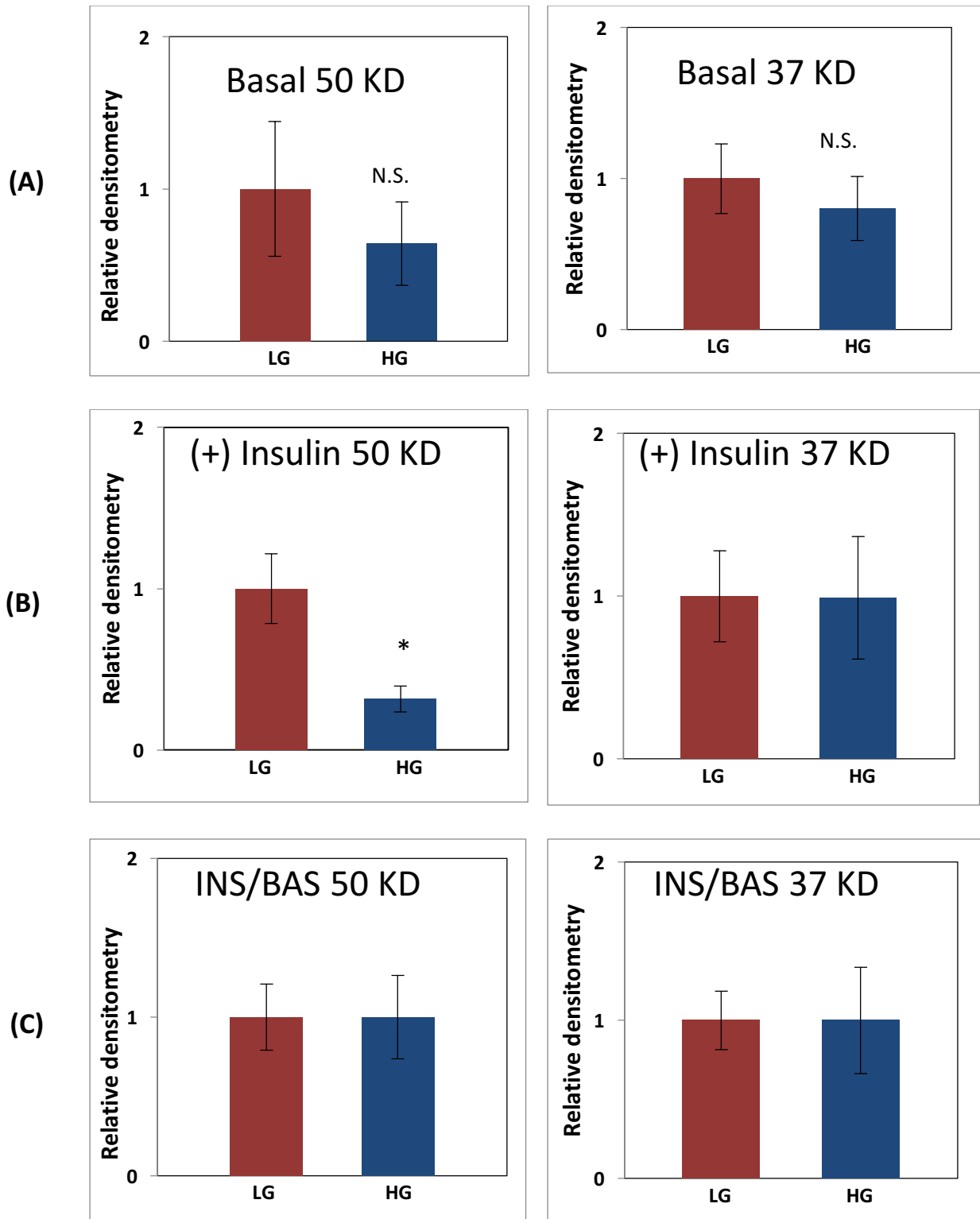


Figure 10. Ubiquitination level at 50 KD and 37 KD: (A, B) Effect of HGHI in the basal and insulin stimulated conditions. (C) Effect of 15-minute insulin treatment in LGNI and HGHI conditions.

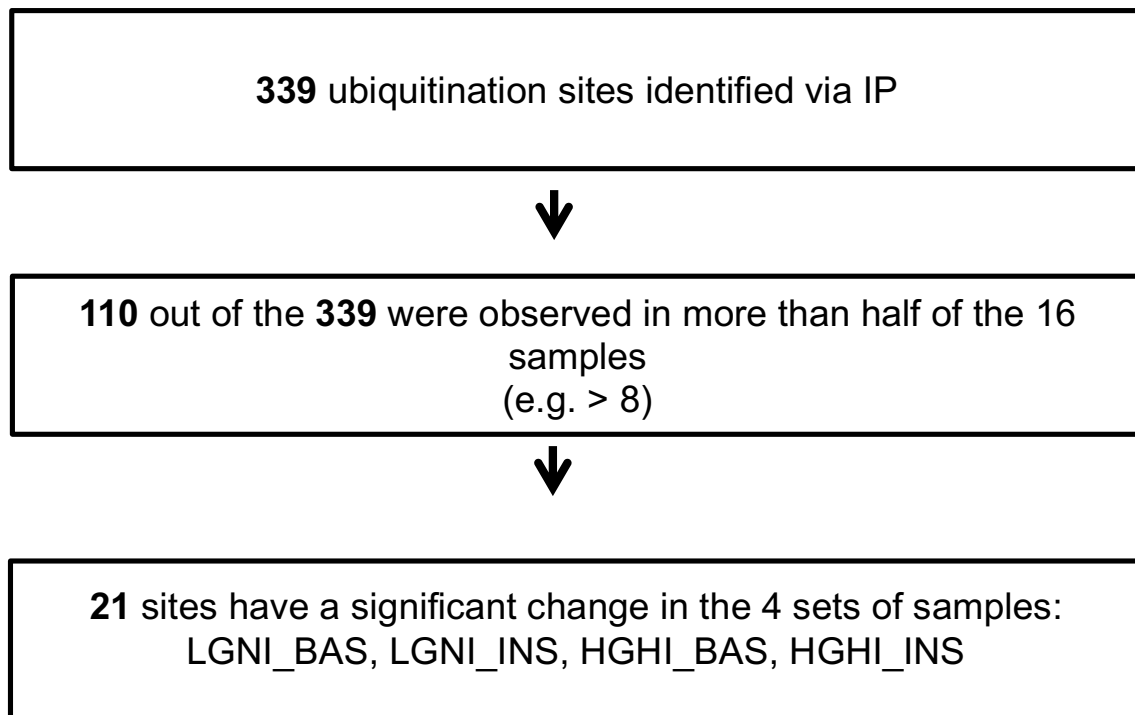


Figure 11. Detailed Proteomic Analysis workflow

LGNI_BAS: low glucose no insulin and without 15 minutes' insulin treatment before harvesting

LGNI_INS: low glucose no insulin and with 15 minutes' insulin treatment before harvesting
HGHI_BAS: high glucose high insulin for 2-days and without 15 minutes' insulin treatment before harvesting

HGHI_INS: high glucose high insulin for 2-days and with 15 minutes' insulin treatment before harvesting

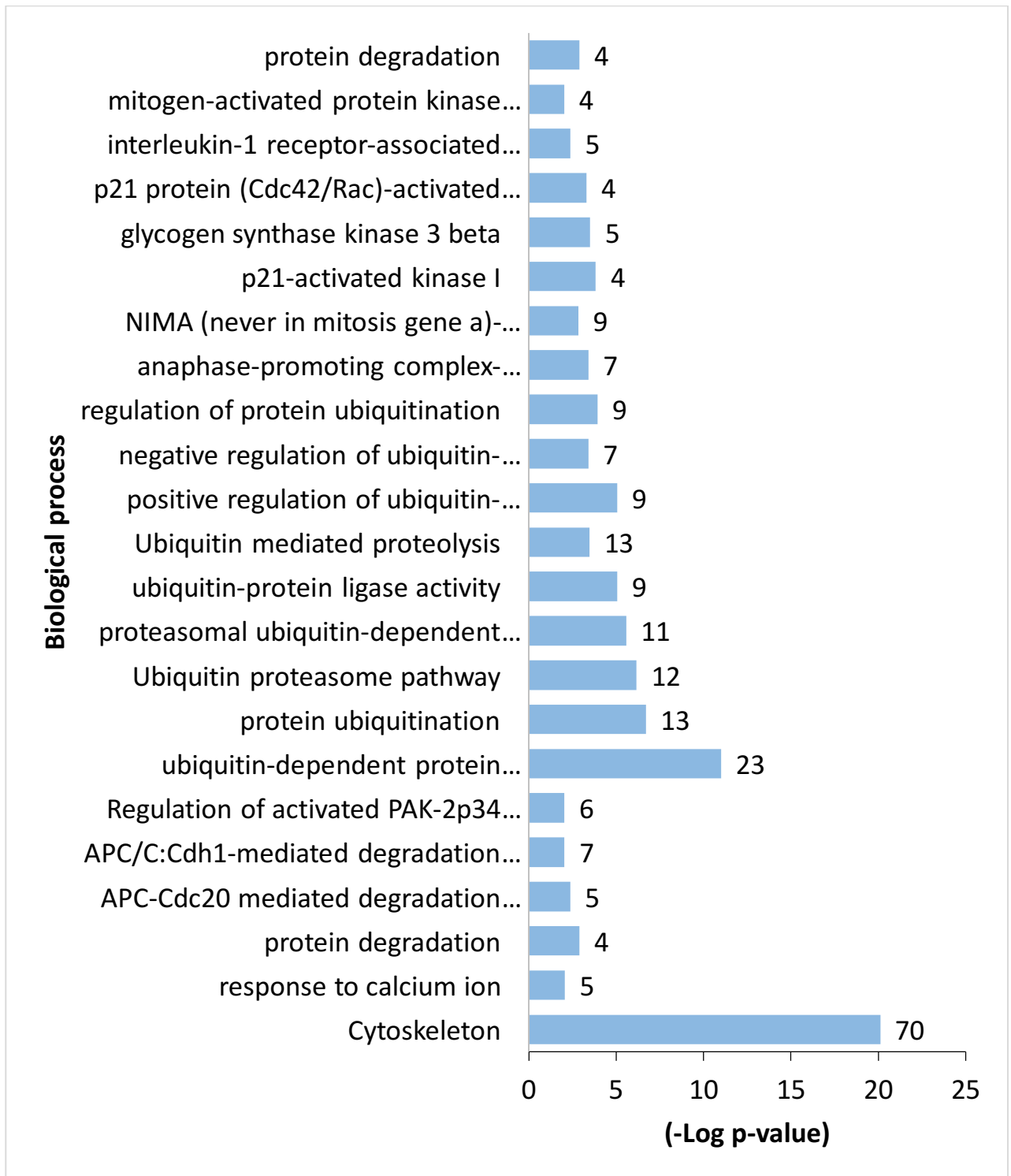


Figure 12. Significantly enriched pathways identified in this study revealed by Database for Annotation, Visualization and Integrated Discovery (DAVID) (<https://david.ncifcrf.gov/>). The total number of identified proteins for every pathway is denoted beside the corresponding bar. See (Table 2) for more details.

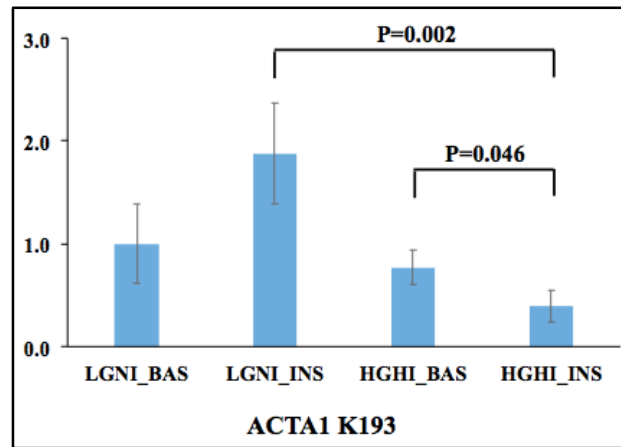
Figure 13. Significantly changed ubiquitination sites among the 4 sets of samples:

LGNI_BAS: low glucose no insulin and without 15 minutes' insulin treatment before harvesting

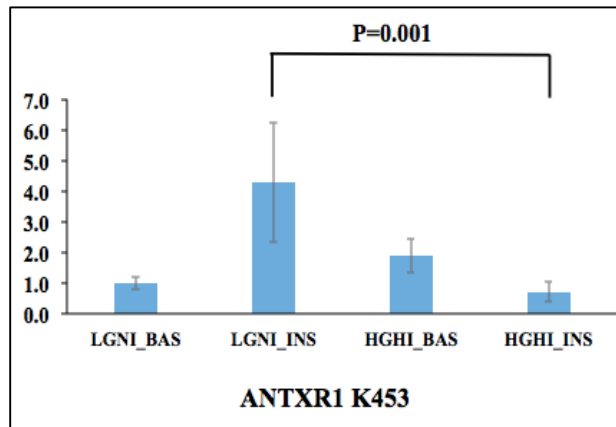
LGNI_INS: low glucose no insulin and with 15 minutes' insulin treatment before harvesting

HGHI_BAS: high glucose high insulin for 2-days and without 15 minutes' insulin treatment before harvesting

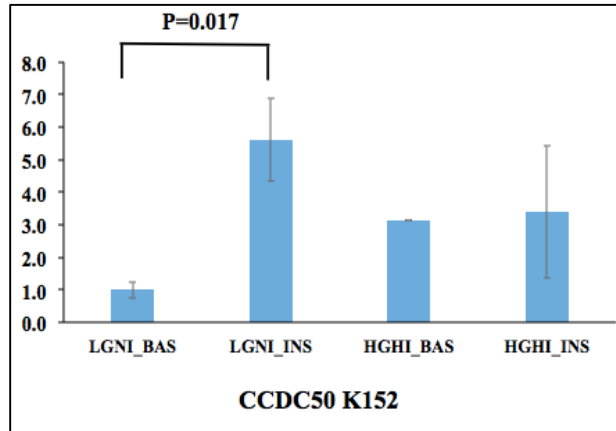
HGHI_INS: high glucose high insulin for 2-days and with 15 minutes' insulin treatment before harvesting



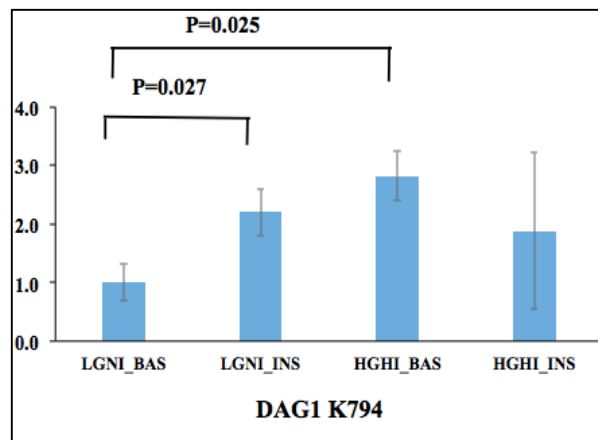
(A)



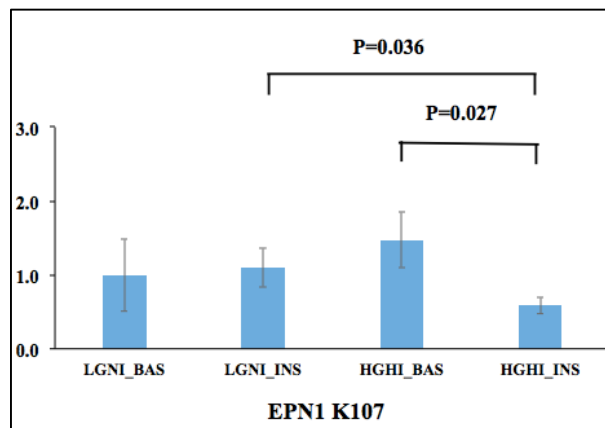
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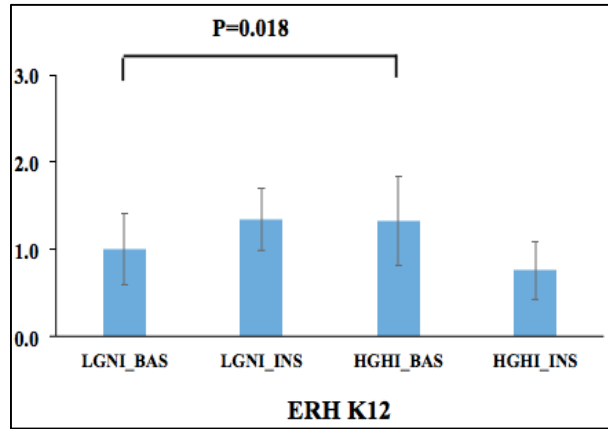
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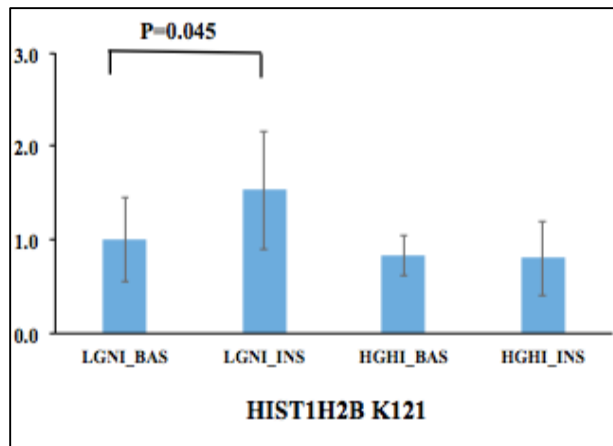
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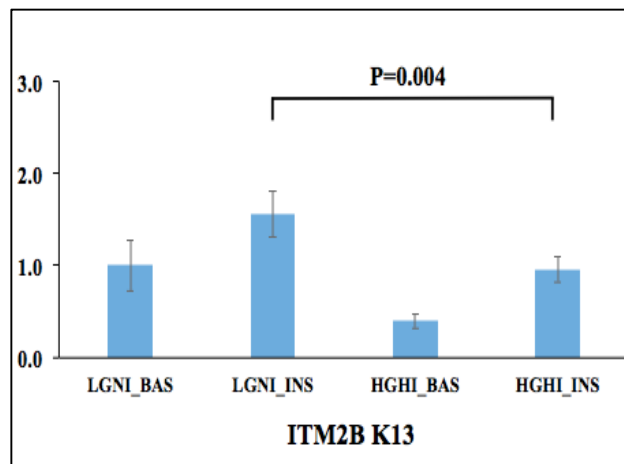
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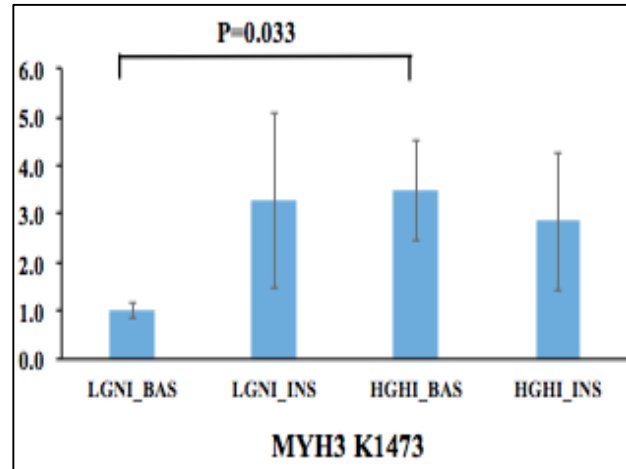
(F)



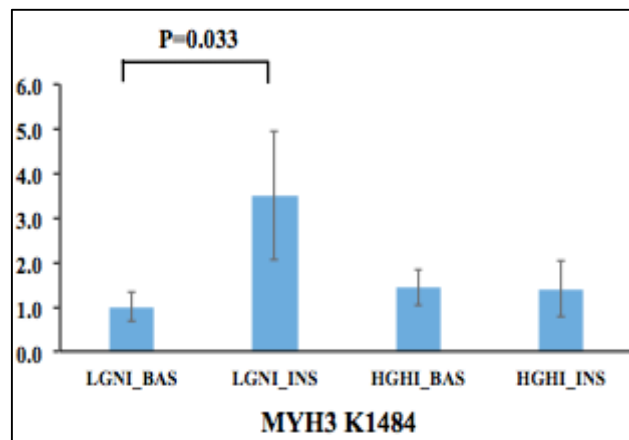
(G)



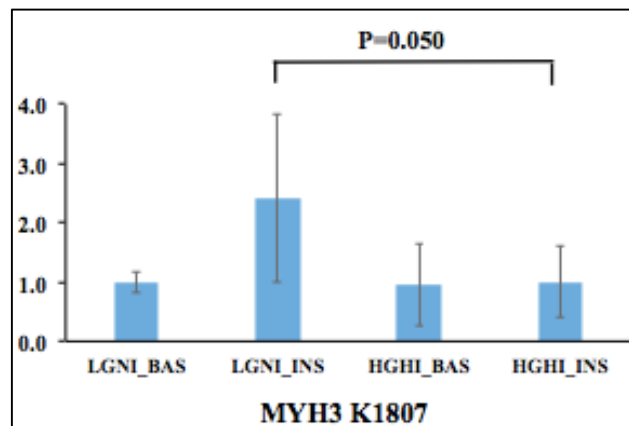
(H)



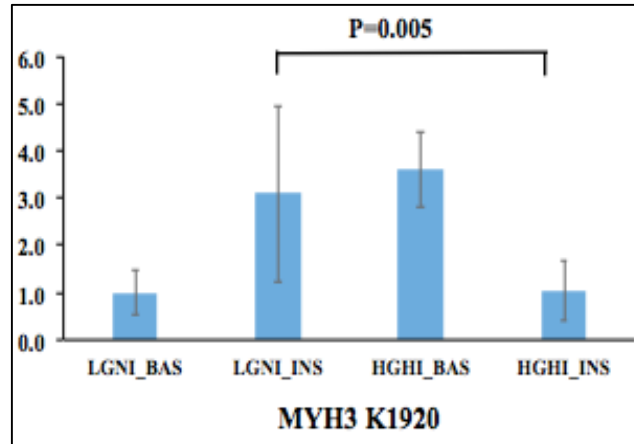
(I)



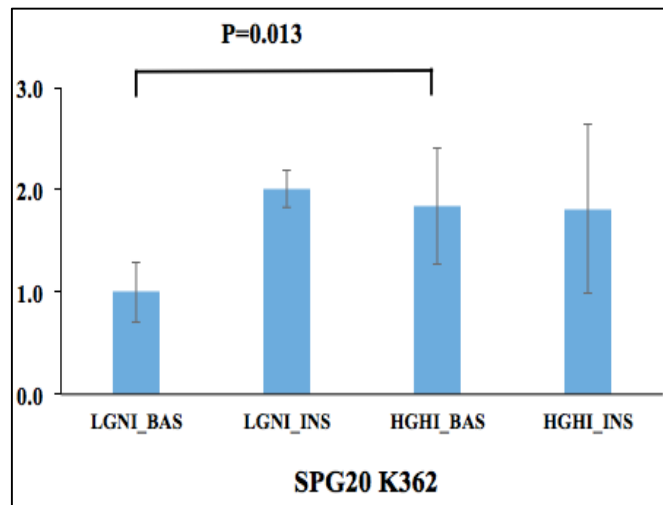
(J)



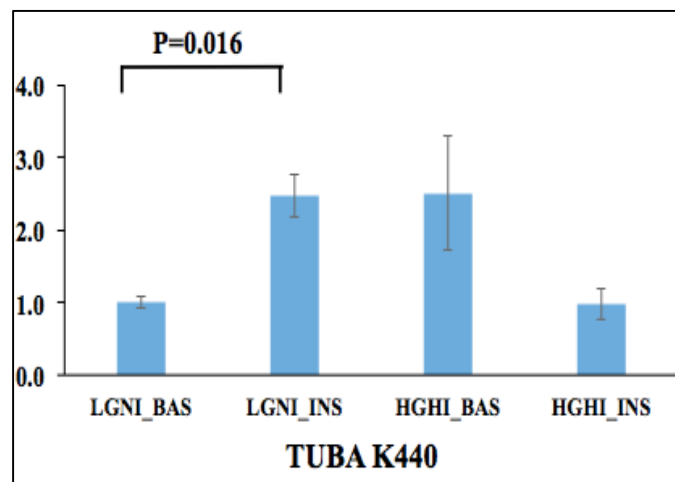
(K)



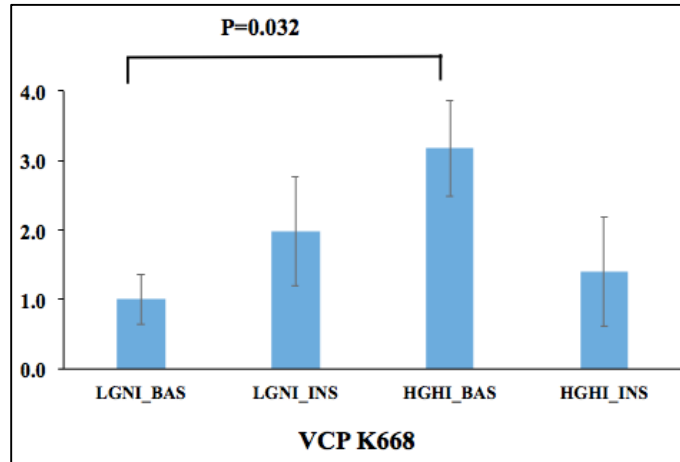
(L)



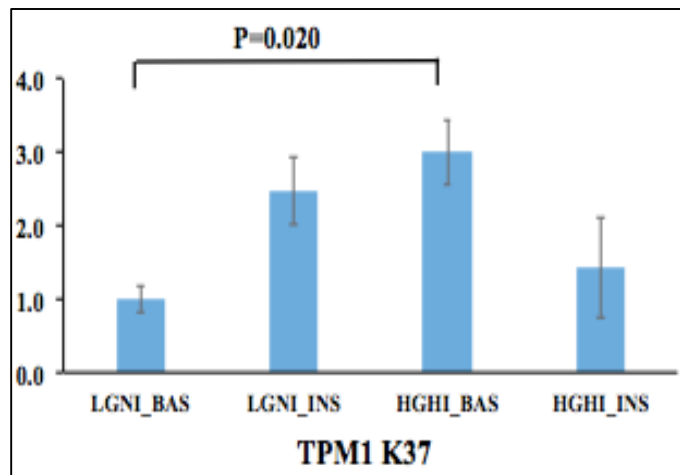
(M)



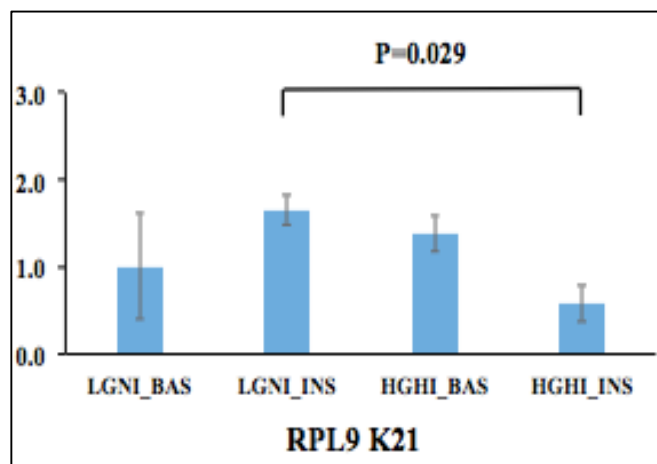
(N)



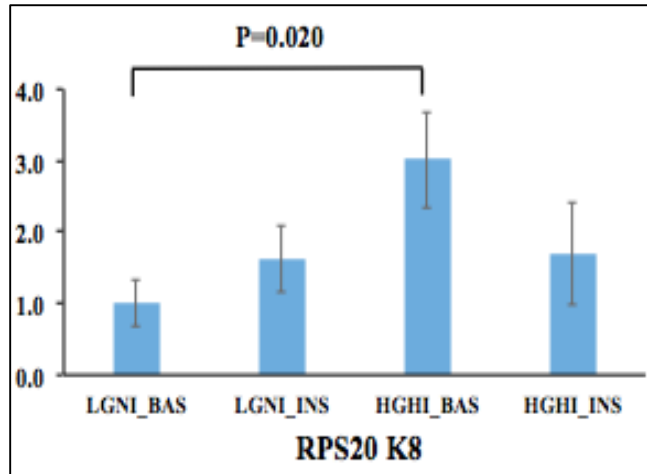
(O)



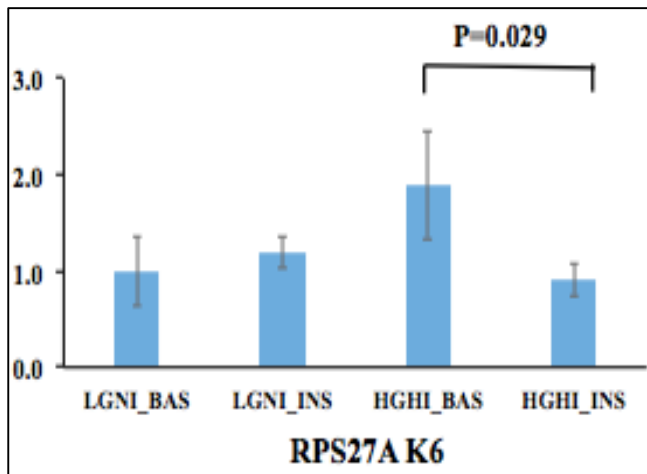
(P)



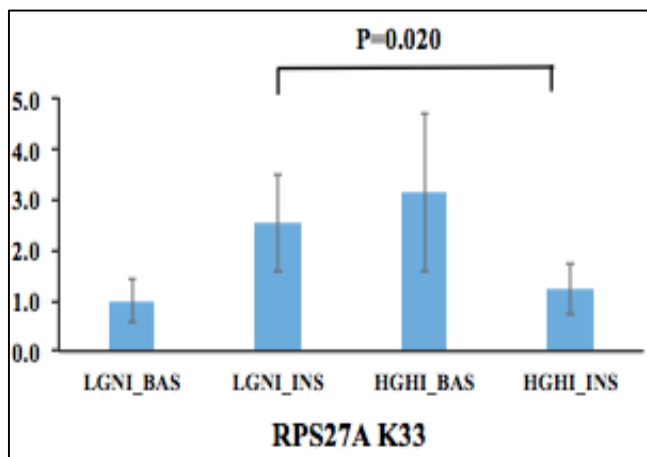
(Q)



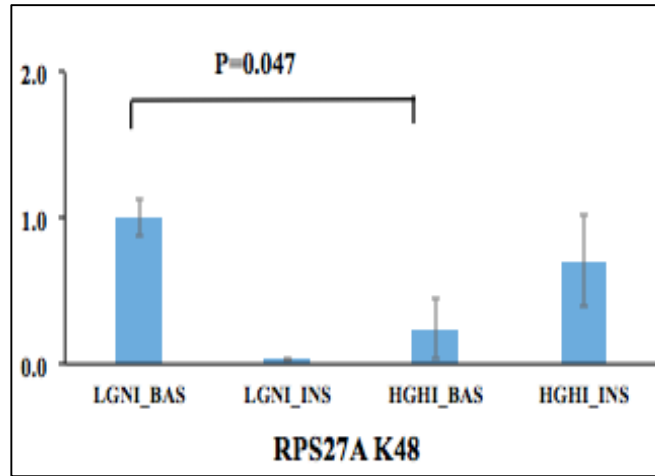
(R)



(S)



(T)



(U)

Table 1. Clinical characteristics of participants in the study.

Results were shown as mean \pm SEM. Normal values are in **Bold** after BMI, 2h OGTT glucose, HBA1c, and Fasting plasma glucose values.

	4 participants in lean group
Gender (M/F)	(3/1)
Age (years)	33.75 \pm 10.45
BMI (kg/m^2)	22.25 \pm 1.09 (< 25)
2h OGTT Glucose (mg/dl)	107.78 \pm 10.03 (<140)
2h OGTT Glucose (mmol/l)	5.98 \pm 0.56
HBA1c (%)	5.4 \pm 0.14 (<5.7)
Fasting plasma glucose (mg/dl)	93.75 \pm 2.52 (<100)
Fasting plasma glucose (mmol/l)	5.21 \pm 0.14
M-value (mg/kg/min)	9.46 \pm 1.12

Table. 2. A list of the identified genes for the significantly enriched proteins by immuno-precipitation in this study assigned to their biological processes according to DAVID (<https://david.ncifcrf.gov/>): The tables below show the pathway, number of identified genes in the pathway, (-log p-value), and the gene names.

Biological Process	Number of Genes	(-log p-value)	Gene Names
Regulation of activated PAK-2p34 by proteasome mediated degradation	6	2.022	PSMC3,PSMC6,RPS27A,UBA52,UBB,UBC
Cdc20:Phospho-APC/C mediated degradation of Cyclin A	7	1.886	PSMC3,PSMC6,RPS27A,UBA52,UBB,UBC,UBE2D1
Ubiquitin-dependent protein catabolic process	23	11	RAD23A,RAD23B,SKP1,UBXN1,CUL5,DDB1,ITCH,NEDD4,NEDD8,PSMC3,PSMC6,RPS27A,SQSTM1,UBA52,UBB,UBC,USP13,USP5,UBE2D1,UBE2D2,UBE2D3,UBE2NL,VCP
Protein ubiquitination	13	6.698	DDB1,ITCH,LRSAM1,NEDD4,RPS27A,UBA52,UBB,UBC,UBE2D1,UBE2D2,UBE2D3,UBE2N,VCP
Response to metal ion	7	1.886	ACTB,ACTG1,CAV1,GSN,NEDD4,TTN,TNNC1
Protein degradation	4	2.886	RPS27A,UBA52,UBB,UBC
Ubiquitin proteasome pathway	12	6.161	ITCH,NEDD4,PIP5K1A,PSMC3,PSMC6,UBE2D1,UBE2D2,UBE2D3,UBE2D4,UBE2N,UBE2NL,UBA1
Proteasomal ubiquitin-dependent protein catabolic process	11	5.568	RAD23A,RAD23B,UBXN1,PSMC3,PSMC6,RPS27A,UBA52,UBB,UBC,UBE2D1,VCP
Ubiquitin-protein ligase activity	9	5.050	BIRC6,ITCH,LRSAM1,NEDD4,UBE2D1,UBE2D2,UBE2D3,UBE2D4,UBE2N,UBE2NL

Biological Process	Number of Genes	(-log p-value)	Gene Names
Anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	7	3.397	PSMC3,PSMC6,RPS27A,UBA52,UBB,UBC,UBE2D1
NIMA (never in mitosis gene a)-related kinase 2	9	2.823	RPS27A,TUBA1A,TUBA4A,TUBB,YWHAG,UBA52,UBB,UBC,UBE2D1
p21-activated kinase 1	4	3.823	RPS27A,UBA52,UBB,UBC
Glycogen synthase kinase 3 beta	5	3.508	SKP1,RPS27A,UBA52,UBB,UBC
p21 protein (Cdc42/Rac)-activated kinase 2	4	3.292	RPS27A,UBA52,UBB,UBC
Interleukin-1 receptor-associated kinase 1	5	2.366	RPS27A,SQSTM1,UBA52,UBB,UBC
Mitogen-activated protein kinase kinase kinase 7	4	2.013	RPS27A,UBA52,UBB,UBC
Ubiquitin mediated proteolysis	13	3.468	SKP1,BIRC6,CUL5,DDB1,ITCH,NEDD4,UBE2D1,UBE2D2,UBE2D3,UBE2D4,UBE2N,UBE2NL,UBA1
Positive regulation of ubiquitin-protein ligase activity	9	5.050	SKP1,PSMC3,PSMC6,RPS27A,UBA52,UBB,UBC,UBE2D1,UBE2N
Negative regulation of ubiquitin-protein ligase activity during mitotic cell cycle	7	3.397	PSMC3,PSMC6,RPS27A,UBA52,UBB,UBC,UBE2D1
Regulation of protein ubiquitination	9	3.920	SKP1,PSMC3,PSMC6,RPS27A,UBA52,UBB,UBC,UBE2D1,UBE2N
Cyclin-dependent kinase 2	6	1.744	SKP1,RPS27A,UBA52,UBB,UBC,UBE2D1

Biological Process	Number of Genes	(-log p-value)	Gene Names
Cytoskeleton	70	20.113	ARL2,,ARL8B,JAK1,ACTA1,ACTA2,ACTC1,ACTB,ACTBL2,ACTG1,ACTG2,ACTN1,ACTN2,ACTN4,ANXA1,CFL1,CFL2,DES,DYNLRB1,DYNLRB2,DAG1,GSN,HSPB1,TOM1,TUBA1B,EZR,KRT7,KRT8,LMNA,MSN,MYOM1,MYLPF,MYH1,MYH13,MYH2,MYH3,MYH4,MYH7,MYH7B,MYH8,MYL1,MYL12A,MYL12B,MYL4,MYL6,MYL6B,NES,PRPH,PEA15,PFN1,RDX,TMSB4X,TTN,TRIM54,TRIM55,TPM1,TPM2,TPM3,TPM4,TNNC1,TNNI1,TUBA1A,TUBA1C,TUBA3C,TUBA3E,TUBA4A,TUBA8,TUBB,YWHAZ,VIM,VCL
Calcium ion binding	22	1.537	ATP2B1,S100A11,S100A6,ACTN1,ACTN2,ACTN4,ANXA1,ANXA2P2,ANXA2,DAG1,GSN,MYLPF,MYL1,MYL12A,MYL12B,MYL4,MYL6,MYL6B,TTN,TPM4,TNNC1,TTYH3
Response to calcium ion	5	2.050	ACTB,ACTG1,CAV1,NEDD4,TTN
Protein degradation	4	2.886	RPS27A,UBA52,UBB,UBC
APC-Cdc20 mediated degradation of Nek2A	5	2.356	RPS27A,UBA52,UBB,UBC,UBE2D1
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	5	1.721	SKP1,RPS27A,UBA52,UBB,UBC
Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	5	1.721	SKP1,RPS27A,UBA52,UBB,UBC
3-phosphoinositide dependent protein kinase-1	4	1.408	RPS27A,UBA52,UBB,UBC
APC/C:Cdh1-mediated degradation of Skp2	7	2.036	PSMC3,PSMC6,RPS27A,UBA52,UBB,UBC,UBE2D1

Table 3. Ubiquitinated lysine sites with significant changes in the 4 sets of samples; low glucose no insulin without 15 minutes' insulin treatment before harvesting (LGNI_BAS), low glucose no insulin with 15 minutes' insulin treatment before harvesting (LGNI_INS), high glucose high insulin without 15 minutes' insulin treatment before harvesting (HGHI_BAS), high glucose high insulin with 15 minutes' insulin treatment before harvesting (HGHI_INS)

Gene names	Protein ID	Protein names	Positions Within Proteins	Localization probability	GlyGly (K) modified peptide sequence	Position in peptide	Charge	Mass error [ppm]
ACTA1	P68133	Actin	193	1.00	DLTDYLMKILTER	8	2	0.122
ANTXR1	Q9H6X2-5	Anthrax toxin receptor 1	453	1.00	GKLDALWVLLR	2	3	-1.401
CCDC50	Q8IVM0	Coiled-coil domain-containing protein 50	152	1.00	AYADSYYYEDGGMKP R	14	3	-0.115
DAG1	Q14118	Dystroglycan	794	0.91	LTLEDQATFIKK	12	3	0.122
EPN1	Q9Y6I3-3	Epsin-1	107	1.00	ENMYAVQTLKDFQY VDR	10	3	-0.178
ERH	G3V279	Enhancer of rudimentary homolog	12	1.00	SHTILLVQPTKRPEGR	11	3	-0.280
HIST1H2B	U3KQK0	Histone H2B	121	1.00	AVTKYTSSK	4	2	0.154
ITM2B	Q9Y287	Integral membrane protein 2B	13	1.00	VTFNSALAQKEAK	10	2	0.318
MYH3	P11055	Myosin-3	1473	1.00	TKCEESQAELEASLKE SR	15	3	-0.266

Gene names	Protein ID	Protein names	Positions Within Proteins	Localization probability	GlyGly (K) modified peptide sequence	Position in peptide	Charge	Mass error [ppm]
MYH3	P11055	Myosin-3	1484	1.00	SLSTELFKLK	8	3	-0.871
MYH3	P11055	Myosin-3	1807	0.99	LDEAEQLALKGGK	10	2	-0.070
MYH3	P11055	Myosin-3	1920	1.00	ADIAESQVNKLR	10	3	-0.387
RPL9	E7ESE0	60S ribosomal protein L9	21	1.00	TILSNQTVDIPENV DITL KGR	19	3	-0.109
RPS20	E5RIP1	40S ribosomal protein S20	8	1.00	DTGKTPVEPEVAIHR	4	3	-0.426
RPS27A	P62979	Ubiquitin-40S ribosomal protein S27a	6	1.00	MQIFVKLTGK	6	2	-0.141
RPS27A	P62979	Ubiquitin-40S ribosomal protein S27a	33	1.00	IQDKEGIPPDQQR	4	3	-0.544
RPS27A	P62979	Ubiquitin-40S ribosomal protein S27a	48	1.00	LIFAGKQLEDGRTLSDY NIQK	6	3	-0.0925
SPG20	Q8N0X7	Spartin	362	1.00	TRPSSDQLKEASGTDVK	9	3	-0.468

Gene names	Protein ID	Protein names	Positions Within Proteins	Localization probability	GlyGly (K) modified peptide sequence	Position in peptide	Charge	Mass error [ppm]
TUBA	F5H5D3	Tubulin alpha	440	1.00	VGINYQPPTVVPGGDL AKVQR	18	3	-0.343
VCP	P55072	Transitional endoplasmic reticulum ATPase	668	1.00	SPVAKDVDLEFLAK	5	3	0.737
TPM1	P09493	Tropomyosin alpha-1 chain	37	1.00	SKQLEDELVSLQK	2	3	0.181

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ABSTRACT

PROTEIN UBIQUITINATION IN PRIMARY HUMAN SKELETAL MUSCLE CELLS UNDER HYPERINSULINEMIC HYPERGLYCEMIC CONDITIONS

by

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Ubiquitin proteasome system is a relatively newly discovered pathway for protein degradation. Many studies have successfully pointed out the critical functions that UPS plays in regulating many physiological processes. On the other hand, recent studies suggested that abnormal UPS activities might be involved in the pathophysiology of several disorders including type 2 diabetes. However, the specific changes in UPS during the development of insulin resistance and consequently T2D are still unclear.

UPS is composed of two major steps, a reversible ubiquitin conjugation to the targeted substrate followed by proteasomal degradation of the ubiquitinated proteins. In this study, we examined the changes in the total ubiquitination as well as the site-specific ubiquitin conjugation under hyperinsulinemic-hyperglycemic conditions in primary skeletal muscle cells derived from lean, healthy people. We

observed a significant decrease in the total ubiquitination in case of glucotoxicity, which is a simulation for insulin resistance in cell culture. In addition, we identified 21 ubiquitination sites that showed significant changes upon treatment with different glucose and insulin concentrations. This study provides a list of ubiquitination as a reference for future research in ubiquitination and diabetes in human skeletal muscle cell.

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PRESENTATIONS

1. Yue Qi, Abdullah Mallisho, Danjun Ma, Xiangmin Zhang, Michael Caruso, Divyasri Damacharla, Rodney Berry, Nishit Shah, Majed Abdullah. **Alharbi, Majed Abdullah.** Alharbi, Berhane Seyoum, Zhengping Yi, "Global Kinome Interactome in Human Skeletal Muscle Revealed by ATP Affinity Probes and Proteomics". Accepted as a poster presentation in the 75th American Diabetes Association conference, June 5 - 9, 2015, Boston, Massachusetts.
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