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Effect Of Metformin On Global Phosphorylation Profiles Of Primary Skeletal Muscle Cells Derived From Overweight/obese Insulin Resistant Human Participants

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EFFECT OF METFORMIN ON GLOBAL PHOSPHORYLATION PROFILES OF PRIMARY SKELETAL MUSCLE CELLS DERIVED FROM OVERWEIGHT/OBESE INSULIN RESISTANT HUMAN PARTICIPANTS

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To the World beloved

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CHAPTER 1 INTRODUCTION

1.1 DIABETES MELLITUS AND INSULIN

Diabetes describes a group of metabolic disorders in which high blood glucose along with relative insulin deficiency is observed with concomitant disturbances of carbohydrate, fat, and protein metabolism [1]. In addition to hyperglycemia, diabetes contributes to the dysfunction and failure of various organs, such as heart and kidney disease [1].

1.1.1 TYPES OF DIABETES

There are three main types of diabetes, type-I, type-II (T2D) and gestational diabetes [2]. Type-I and type-II diabetes are also known as Insulin Dependent Diabetes Mellitus (IDDM) and Non-Insulin Dependent Diabetes Mellitus (NIDDM), respectively [3]. Type-I diabetes is an autoimmune disorder that leads to destruction of the insulin producing pancreatic beta cell[2]. Type-II diabetes is the most common form of diabetes in which the body cannot properly utilize insulin secreted by the pancreas. Type-II diabetes occurs due to insulin resistance and relative insulin deficiency [2]. Initially the pancreas produces more insulin to compensate for insulin resistance but over time, the beta cells cannot produce enough insulin to keep the blood sugar levels within normal range [2]. Gestational diabetes occurs in women during the pregnancy. According to a 2014 analysis by the Center for Disease Control and Prevention, 9.2% of all pregnant women face gestational diabetes [2]. The causes behind gestational

diabetes is not known but based on some clues, it is believed that the actions of hormones from the placenta block insulin action in the mother's body [2].

In the United States 29.1 million Americans had diabetes in 2012, which is 9.3% of the total population. Among all cases of diabetes, approximately 95% of cases were Type-II Diabetes (T2D) in the US [2]. The major reasons behind type-II diabetes are the include insulin resistance and dysfunction of insulin secretion [4].

1.1.2 INSULIN SENSITIVITY AND INSULIN RESISTANCE

Type-II Diabetes is one of the most common increasing diseases at an alarming rate of the current era [5]. Insulin resistance is one of the primary contributors behind T2D [6]. Insulin is a hormone which is produced by beta cells of islets present in the pancreas [7]. Insulin contains two polypeptide chains containing 51 amino acids, with a molecular weight of ~6 kDa. The two chains, A and B, have 21 and 30 amino acids, respectively [3].

Among different mediators for insulin resistance, a key factor is reduced capacity of skeletal muscle to oxidize fatty acids and impairs mitochondrial function [8]. Fat deposition in skeletal muscle is more than usual in insulin resistance. One of the reasons behind it is that less free fatty acid (FFA) oxidation by leg tissues is observed in obese people compared to lean people [9]. However, the uptake of FFA is more in obese people than lean people which simultaneously develop insulin resistance [9]. Different organs, such as the liver, possess specific transporters which might be negatively affected in the diabetes

state or during obesity [10]. Insulin sensitivity has been shown to be ameliorated by acute exercises and/ or physical training in obese people or T2D patients, in addition weight loss also increases insulin sensitivity [11].

1.2 TREATMENT OF DIABETES

In the early stages of T2D, blood sugar levels within range may be attained with just diet and exercise. However, most T2D might need oral medication or insulin therapy which depends on many factors. Oral medications available on the market today, belong to different classes that control the blood sugar level with different mechanisms such as by reducing hepatic glucose production [12], increase insulin sensitivity [13], increasing glucose absorption [12], raising more glucose excretion [14] and stimulating insulin secretion [15]. Different classes of oral drugs are available in market today, such as Sulfonylureas, Metglitinides, Thiazolidinediones, Alpha-glucosidase inhibitors, DPP-4 inhibitors, SGLT2 inhibitors, and Biguanides.

Sulfonylureas stimulate insulin secretion from pancreatic β-cells through inhibition of K_{ATP} channels. Sulfonylureas primarily act on closing of K_{ATP} channels and indirectly cause degranulation of insulin containing secretory granules and increase the insulin concentration which is not dependent on blood sugar levels [15]. Drugs from the Metglitinides class work similar to the drugs from the sulfonylureas class. Metglinides bind on “sulfonylurea receptor” binding sites of β-cells in pancreas and stimulate insulin release [16]. Metglitinides do have a shorter half-life compared to Sulfonylureas [17]. Thiazolidinediones such

as pioglitazone, rosiglitazone, troglitazone, increase insulin sensitivity in muscle and fat, and decrease hepatic glucose production [13]. Thiazolidinediones work through activation of gamma isoform of the peroxisome proliferator-activated receptor (PPAR-γ) [18].

Drugs from the alpha-glucosidase inhibitor class do not result in more insulin production and also do not result in low blood sugar levels [19]. Alpha-glucosidase inhibitors help to maintain the blood sugar level within range by slowing down the digestion of complex carbohydrates [20]. Drugs from the Dipeptidyl Peptidase IV (DPP-4) inhibitor class exert their effect by inhibiting the degradation of incretins, glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1) [21].. Sodium-glucose cotransporter 2 (SGLT2), present in the proximal tubule of the kidney, is responsible for approximately 90% of renal glucose reabsorption. SGLT2 inhibitors lead to more glucose excretion in urine [14].

In addition to insulin, several injectable anti-diabetic medications have been developed, such as Pramlintide, and incretin mimetics (e.g., Exenatide), Pramlintide exerts it’s effect by increasing gastric emptying time, decreasing glucagon release and also by reducing food intake [22]. Exenatide is a GLP-1 receptor agonist and exerts its effects through inhibition of food intake and glucose-dependent stimulation of insulin secretion [23].

Biguanides (such as metformin, phenformin) act by increasing glucose absorption, insulin secretion, and by reducing hepatic glucose production but no

clear mechanism how biguanides work is yet completely understood [12]. Metformin is the first line of drug to treat T2D and ~30% of T2D patients are taking metformin [24]. Apart from T2D, metformin is useful for cancer treatment, gestational diabetes, and for prevention of T2D in pre-diabetic population [24]. Metformin is still under investigation for its potential anti-neoplastic activity and has been observed to reduce cardiac ischemia/ reperfusion injury [25]. Early treatment with metformin in rats in adolescence shows inhibition of tumor growth [26].

1.2.1 METFORMIN AND IT'S RELATED KNOWN FACTS

Chemical Formula: $C_4H_{11}N_5$

IUPAC Name: 3-(diaminomethylidene)-1,1-dimethylguanidine

Metformin's sugar lowering action was first discovered in 1929 by Slotta and Tschesche in rabbits but it was forgotten because its other analogues became popular [27]. In 1950, more research on metformin took place and after passing through clinical trials, it became available on market only in the UK in 1958. Later, metformin was approved by Canada in 1972 and by US-FDA in 1994 [28]. The structure of metformin is shown in Figure 1.

Metformin is absorbed slowly when given orally and has 50-60% of bioavailability under fasting conditions [29]. The elimination half-life of metformin is around 6 hours in plasma and 18 hours in red blood cells [29]. Metformin exists in its cationic form at physiological pH as it has acid dissociation constant values (pKa) of 2.8 and 11.5 [29].

Metformin has an effect on different tissues and/or organs such as skeletal muscle, adipose tissue, liver and kidney [24]. Multiple mechanisms might be involved individually or in combination [25].

The marked effect of metformin is that it decreases hepatic glucose production through inhibition of the mitochondrial respiratory-chain complex 1 [24]. Some research studies have suggested that the primary action of metformin is through activation of the AMP-activated protein kinase (AMPK) pathway [30]. Recent studies suggest that the primary effect of metformin is on the respiratory chain complex-1 instead of AMPK [24]. However, the molecular interactions between metformin and mitochondrial respiratory chain complex 1 are still not clear. Additional mechanisms include inhibition of adenylate cyclase in response to energetic stress, and the direct inhibition of mitochondrial glycerophosphate dehydrogenase. The above mechanisms might be involved individually or in combination [25]. Metformin activates the AMPK pathway in skeletal muscle which increases glucose uptake through SLC2A4 (Solute Carrier Family 2 (Facilitated Glucose Transporter), Member 4) [31]. Some research studies also suggested reduced activity of NADH:O_2 oxidoreductase when skeletal muscle tissue is treated with metformin [32].

Additionally, the major effect of metformin or any other drugs from the biguanide class is inhibition of glucose production through glucagon signaling rather than glucose uptake by activated AMPK [33]. Glucagon signaling in the liver is responsible for hepatic glucose production. Events such as activation of

adenylyl cyclase, production of second-messenger cyclic AMP (cAMP) and stimulation of protein kinase A (PKA) take place, when glucagon binds to its receptors on the hepatocyte plasma membrane. Stimulation of PKA further phosphorylates protein targets which increase or support the hepatic glucose production [33]. Metformin or any other biguanide drug reduces production of cAMP by inactivating adenylyl cyclase which further results in termination of stimulation and phosphorylation of PKA and related target proteins, respectively. Figure 4 shows a flow of how metformin reduces glucose production in liver. It is also suggested that metformin disrupts respiratory chain complex-I in mitochondria which results in reduction of hepatic glucose output and activation of AMPK pathway [34].

Metformin uptake in cells appears through Organic Cationic Receptor (OCT) 1, 2 and 3 [35]. Metformin occurs in its cationic form at physiological pH. Accumulation of metformin has been observed in mitochondria which results in change of membrane potential. Aggregation of positively charged metformin acts as hydrophobic inhibitors of complex-1 [36]. An increase in AMP/ATP ratio has been observed as a resulting effect of inhibition of complex-1 which further supports phosphorylation of AMPK.

Due to inhibition of the mitochondrial respiratory chain complex-1, ATP/AMP ratio decreases which supports the activation of AMPK. However, few studies suggest that activation of AMPK is not because of energy change related to inhibition of respiratory chain complex-1 [30]. AMPK is composed of three

different subunits called alpha, beta and gamma. Among the three subunits, the alpha subunit is a catalytic unit of AMPK and the regulatory site (Thr-172) on the catalytic alpha subunit of AMPK has been stimulated through phosphorylation by metformin in intact cells [30]. Metformin treated cells have shown more AMPK activity. AMPK is activated without any changes in the ADP-to-ATP ratio when treated with metformin in Chinese Hamster Ovary (CHO) cells and the rat hepatoma cells (H4IIE) [30].

1.3 SKELETAL MUSCLE INSULIN RESISTANCE

Insulin stimulates glucose uptake in different organs and tissues in the body such as adipose tissue, and skeletal muscle. Skeletal muscle is the major site of glucose disposal and skeletal muscle insulin resistance is considered a primary metabolic defect in the pathogenesis of type 2 diabetes (T2D) [37, 38]. It has been shown that under the hyperinsulinemic euglycemic clamp condition, glucose uptake between T2D patients and non-diabetic controls is not different in liver, brain and adipose tissue but is markedly reduced in skeletal muscle. Figure 7 describes the observed decrease in glucose uptake in skeletal muscle compared to liver, brain and adipose tissue.

There are several methods available clinically to determine insulin sensitivity or insulin resistance, such as Insulin Tolerance Test (ITT), Hyperglycemic Clamp, the Insulin Modified frequently Sampled Intravenous Glucose Tolerance Test (FSGIT), the Oral Glucose Tolerance Test (OGTT), Fasting Surrogates, and the Hyperinsulinemic-Euglycemic Clamp. All the

methods have their importance and validity but Hyperinsulinemic-Euglycemic Clamp to determine insulin sensitivity is considered as the golden standard [39].

1.4 PHOSPHORYLATION IN INSULIN SIGNALING AND INSULIN RESISTANCE

Reversible phosphorylation plays a fundamental role in insulin signaling [37, 40]. Abnormal phosphorylation of insulin signaling proteins, such as the insulin receptor and insulin receptor substrate 1, in skeletal muscle is reproducibly observed in T2D [37, 40]. Nonetheless, most studies on phosphorylation-mediated signaling in T2D are focused on a few down targets.

1.5 MASS SPECTROMETRY (MS) BASED PROTEOMICS

Many research projects today involve extremely complex mixtures, which increases the demand for instruments with better performance characteristics such as high sensitivity, mass accuracy, resolution, reproducibility [41]. Mass spectrometry has emerged as one of the best approaches for proteomics [42] due to recent development of mass spectrometer with high sensitivity, mass accuracy, resolution, reproducibility (such as Orbitrap) [43].

Emerging as a key technology in exploring signal-transduction, quantitative phosphoproteomics has mapped many differential phosphorylation events in signaling networks and cascades [44-46]. Phosphoproteome studies have generated valuable data, which are unattainable by genomics and transcriptomics studies, and provided insights into molecular mechanisms of various disease pathogenesis, and aided in the discovery of drug targets for

disease treatment [44-46]. Nonetheless, no large-scale phosphoproteome studies on the effect of metformin on primary human skeletal muscle cells have been reported.

In the present study, we performed the first large scale differential phosphoproteome analysis of primary skeletal muscle cells derived from obese/overweight insulin resistant non-diabetic participants under various conditions (with/out metformin treatment) by a combination of phosphopeptide enrichment technique, Titanium dioxide (TiO2), and the Universal-SILAC approach recently developed in our laboratory [47]. The goal of the study is to determine effect of metformin on global phosphorylation profiles in insulin resistant primary skeletal muscle cells with the emphasis on kinases and/or phosphatases that may be regulated by metformin.

CHAPTER 2 RESEARCH DESIGN AND METHODS

This project involves clinical study and proteomics study. In clinical study, after successful phone screening with participants, the clinical visit was scheduled. The research consent form was explained to them in detail during visit-1 and screening tests were performed to check their eligibility for the study (following the IRB protocol). After a thorough review by physicians, eligible participants were scheduled for the in-patient clinical test (visit-2) in which hyperinsulinemic-euglycemic clamp and skeletal muscle biopsy were performed and tissue sample of skeletal muscle from the thigh were collected and was used to perform primary cell culture. The cultured skeletal muscle cells were treated with different conditions. Cells were harvested and collected to perform proteomics study. Proteomics experiments started with spike-in standards (stable isotope labeled protein lysates obtained by stable isotope labeling with amino acids in cell culture (SILAC)) to minimize experimental variation, followed by in-solution trypsin digestion to generate tryptic peptides, TiO2 to enrich phosphorylated peptides, and mass spectrometry to identify and quantify phosphorylation sites using the Universal-SILAC approach recently developed in our laboratory [47] (See Figure 8A).

2.1 MATERIALS

2.1.1 REAGENTS

For the primary cell culture, Dulbecco's Modified Eagle Medium (including dialyzed DMEM), Phosphate Buffer Saline, Fetal Bovine Serum (including

dialyzed FBS), isotopes for labeling aspect were purchased from Thermo Fisher (Fair Lawn, NJ). Sequencing grade trypsin was purchased from Promega (Madison, WI). Metformin-hydrochloride was purchased from Sigma- Aldrich (St. Louis, MO) and TiO2 beads were purchased from GL Sciences (Tokyo, Japan).

2.2 SUBJECTS

Four overweight or obese participants were recruited for clinical research study. The motive and possible risks were explained in detail to all participants. The research consent form which was approved by the Institutional Review Board of Wayne State University was described thoroughly and signed by participants for further participation in the study. After going through vitals, bio-impedence test, electrocardiogram (ECG), and urine test, the Oral Glucose Tolerance Test (OGTT) was performed. In the OGTT, a sugar drink containing 75 g of sugar was given to the participant to determine their glucose tolerance. Figure 5 describes how the group of participant had been selected. None of the participants had any medical problems or any other issues that might affect the research results including any heavy exercises. All participants were asked not to perform any kind of exercise for at least two days before the OGTT. Participants were asked to fast for at least 10 hours before the OGTT.

2.3 HYPERINSULINEMIC-EUGLYCEMIC CLAMP

To determine or measure insulin sensitivity, hyperinsulinemic- euglycemic clamp study was performed [4]. Participants were asked to fast overnight for at least 10 hours. The study began in the morning at around 8:30 am. After taking

vitals, a catheter was inserted in an antecubital vein and maintained during the study for the infusion of insulin and glucose. A second catheter was inserted in a vein in the contra lateral arm for sampling blood to measure glucose level. The arm was covered with a heating pad (120°F or 48.89°C). At 9 am, skeletal muscle tissue samples from the vastus lateralis of human subjects were obtained through standard biopsy procedure using lidocaine as a local anesthetic agent. The collected samples (basal samples) of skeletal muscle were immediately washed with saline containing protease inhibitors and were used for primary cell culture immediately. Infusion of insulin and glucose was started at 9:30 am and kept constant up to 2 hours (120 minutes). Infusion of human regular insulin (Humulin R; Elily, Indianapolis, IN) was started at the rate of 80 mU m-2 minute-1. Infusion of 20% d-glucose was started simultaneously and adjusted the infusion to maintain the blood sugar level of 90 mg/dl. After 120 minutes, another biopsy was performed at contralateral vastus lateralis muscle. Samples of skeletal muscle were washed using the same saline and protease inhibitor solution as before and primary cell culture was performed. Overall procedure of hyperinsulinemic-euglycemic clamp is shown in Figure 6.

2.4 PROTEOMICS EXPERIMENTAL PROCEDURES

2.4.1 PRIMARY CELL CULTURE

Human Skeletal Muscle (HSkM) biopsies were washed twice with Phosphate Buffer Saline (PBS) (from Glibco) with careful handling. The HSkM tissues were placed in petri plate and washed with PBS. HSkM tissues were cut

into small or fine pieces using sterilized scissors. The cut tissues were transferred to 50 ml tube with PBS and allowed to settle down. Supernatant was removed without disturbing tissues. Trypsin with the concentration of 0.05 (mg/ml) was added and kept in a water bath for one hour with shaking every 10 minutes. Meanwhile, the growth media for cells was prepared. Growth media was prepared using 0.22 µm filter units in sterilized condition (under the hood). Fetuin (50 µg/ml), dexamethasone (0.4 µg/ml), Endothelial Growth Factor (EGF) (10 ng/ml), sodium pyruvate (1%), Non-Essential Amino Acids (NEAA) (1%), Penicillin-Streptomycin-Glutamine (PSG) (1%), Fetal Bovine Serum (FBS) (20%) was added to Dulbecco's Modified Eagle Medium (DMEM) (from life technologies). Tubes containing HSkM tissues were centrifuged at the speed of 1000 rpm for 5 min and allowed cells to settle down. The supernatant from tubes were removed under the hood and 10 ml of growth media was added to the tubes. Using filter units, it was transferred to 55 mm^2 petri dishes after pipetting up and down several times. Maintenance and splitting of cells were accomplished until each cell line reached to six 150 mm large culture dishes.

Each cell line was treated with six different conditions: 1) without metformin treatment and without 15 minutes insulin stimulation (No_Met_BAS), 2) without metformin treatment and with 15 minutes insulin stimulation (No_Met_INS), 3) with 1 day low metformin treatment (50 µM) and without 15 minutes insulin stimulation (Low_Met_BAS), 4) with 1 day low metformin treatment (50 µM) and with 15 minutes insulin stimulation (Low_Met_INS), 5)

with 1 day high metformin treatment (800 μM) and without 15 minutes insulin stimulation (high_Met_BAS), 6) with 1 day high metformin treatment (800 μM) and with 15 minutes insulin stimulation (high_Met_INS). Lysis buffer was used to collect the cell lines. Lysis buffer was made up of 50 mM HEPES (pH 7.6), 150 mM NaCl, 20 mM sodium pyrophosphate, 10 mM NaF, 20 mM beta-glycerophosphate, 1% Triton in required amount of HPLC grade water including protease inhibitors such as 1% leupeptin, 1% aprotinin, 1mM sodium vanadate, 1 mM PMSF.

2.4.2 BRADFORD PROTEIN MEASUREMENT

Cell lysates were centrifuged at 4°C, 13,000 rpm for 10 minutes followed by homogenizing lysates for 10 minutes. Protein concentrations from the lysates were fractioned out for measurement. Protein concentrations were determined through Bradford Protein Assay (Bio-Rad, Hercules, CA) using BSA as the standard [48].

2.4.3 PROTEIN DIGESTION

Protein from each sample was mixed with SILAC protein sample in a 4:1 ratio. Protein with 1.5 mg amount from each sample was mixed with 0.375 mg of SILAC protein and further used for protein digestion and TiO2 phosphopeptide enrichment experiment. The total amount of protein in each sample was 1.875 mg. DTT was added to each sample in the final concentration of DTT would be 50 mM and kept in hot-bath at 95°C for 5 min. After 10 min, IDA was added to each sample in the final concentration of 150 mM and kept in dark for 30

minutes. Each sample was filtered at speed of 13,000 rcf, 4°C temperature in centrifugation using 1.5 µm Amicon filter units after shaking on vertex shaker. Pellets were also washed twice with 200 µL of 40 mM Ammonium Bicarbonate (ABC). Amicon filter units were reversed in fresh tubes and centrifuged at speed of 10,000 rcf, 4°C temperature for 2 minutes to collects the pellets. The pellets were dissolved using 500-1000 µL of ABC. Trypsin was added to each sample at 1:100 ratio and kept on shaker with 800 rpm speed at room temperature. Trypsin with the same concentration was added to each sample again after 4 hours and kept on the shaker with 350 rpm speed at room temperature overnight. The next day, each sample was filtered using 1.5 µm Amicon filter units. The filtrate was saved. The filter units were washed twice with 100 µL ABC and filtrates saved. All filtrate for each sample was combined. Filtrate was evaporated till complete dryness, but not over dried.

2.4.4 TITANIUM DIOXIDE PHOSPHOPEPTIDE ENRICHMENT

The phosphopeptides were enriched using Titanium dioxide (TiO_2) beads essentially as described elsewhere with some modifications [49, 50]. Briefly, the TiO2 beads (GL Sciences, Tokyo, Japan) were mixed with 200 µL 65% ACN, 2% TFA with saturated by glutamic acid for 15 min. The peptide samples were then mixed with TiO2 beads and incubated with gentle rotation for 30 min. The incubated beads were then washed with 65% ACN, 0.5% TFA twice and 65%ACN, 0.1% TFA twice. The bound peptides were eluted once with 300 mM NH_4OH, 50% ACN and twice with 500 mM NH_4OH, 60% ACN. The eluates were

dried down by vacuum centrifugation and reconstituted in 0.1% TFA for MS analysis. The experimental flow chart is shown in Figure 8A and 8B.

2.4.5 HPLC-ESI-MS/MS ANALYSIS

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 C_{18}-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).

2.4.6 PHOSPHOPEPTIDE IDENTIFICATION

Peptides/protein identification and quantification were performed using the MaxQuant, one of the popular quantitative proteomics software packages [51, 52]. 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. Two missing trypsin cleavage site was allowed, carbamidomethylation was searched as a fixed modification, and 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 phosphorylation sites with a localization probability greater than 0.75 (classified as class I phosphosites, a commonly used threshold in phosphoproteome studies [53, 54]) were considered.

2.4.7 PHOSPHOPEPTIDE QUANTIFICATION

To minimize the experimental variation during sample preparation and HPLC-ESI-MS/MS data acquisition, we have 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 an universal standard for quantification purpose [47]. 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 [47]. In the present study, we applied Universal-SILAC to the quantification of the phosphoproteome in primary skeletal muscle cells derived from overweight/obese human participants in the absence and presence of metformin, and calculated the modified SILAC ratio as described in our manuscript [47]: an individual peak area for a phosphorylation

site (PA*i*) was normalized against the sum of the peak area for the heavy labeled phosphorylation sites in the same sample:

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

The normalized peak area (i.e., modified SILAC ratio) for each phosphorylation site was log2 transformed and compared to assess effects of insulin or metformin on phosphorylation levels among the 6 sets of samples (as described 6 different conditions above: No_Met_BAS, No_Met_INS, Low_Met_BAS, Low_Met_INS, high_Met_BAS, and high_Met_INS) of primary skeletal muscle cells derived from muscle biopsies from 4 obese/overweight human participants.

2.4.8 STATISTICAL ANALYSIS

Although a large number of phosphorylation sites were assigned in at least one of the 24 samples that were studied, a series of filters were used to narrow the number of phosphorylation sites that were used in comparisons among groups as described in Figure 9. Statistical significance was assessed using paired *t* tests. Differences were considered statistically significant at $p<0.05$.

CHAPTER 3 RESULTS

As can be seen from Table 1, four participants were included for the phosphorylation study including 2 male and 2 female. Body Mass Index (BMI) and M-values indicate that they were obese/overweight and insulin resistant. Other parameters such as Fasting Blood Sugar (FBS) levels, 2 hour OGTT values, and HBA1c values confirmed that they are not diabetic.

Phosphoproteomics analysis of primary skeletal muscle cells derived from muscle biopsies from 4 obese/overweight human participants identified 2930 phosphorylation sites assigned to 1085 proteins.

Among 2930 phosphorylation sites, 750 sites were identified in more than half of the 24 samples (i.e. more than 12 samples). Among the 750 sites, 325 sites showed significant changes among the 6 sets of samples, including 6 sites in 4 phosphatase subunits and 17 sites in 9 kinases subunits which are showed in Table 3.

CHAPTER 4 DISCUSSION

After performing proteomics experiments on cells from primary cell culture, we found 325 phosphorylation sites which showed significant changes upon metformin treatment and/or insulin stimulation. Among 325 phosphorylation sites, we focused on 6 phospho sites in 4 phosphatase subunits and 17 phosphorylation sites in 9 kinase subunits as described in table 3. Few research studies have been reported on these phosphorylation sites for metformin studies.

Protein Phosphatase inhibitor 2 restrains the catalytic subunit of protein phosphatase 1 [55]. phosphatase inhibitor-2 (S121) is regulated/ phosphorylated by Casein Kinase-2 (CK2) [55]. Phosphorylation is necessary for the regulation at T73 or T72 by Glycogen Synthase Kinase-3 (GSK-3) in human spermatozoa [55] and COS-7 cells (kidney cells from monkeys) [55], respectively. Phosphorylation at S121, S120, S86 and T72/T73 is necessary for the localization of protein phosphatase inhibitor-2 during S phase of mitosis [56]. Moreover, research has been reported on S121 phosphorylation using hepatic cell lines, renal cell lines, lymphocytes, spermatozoa, cancer and leukemia cell lines, but not skeletal muscle tissue or cells. In our experiment, S121 phosphorylation has shown significant change. S121 phosphorylation was higher in cells treated with both low and high metformin compared to no metformin under insulin stimulation. In addition, S122 phosphorylation was higher in cells treated with high metformin compared to no metformin under insulin stimulation (See Figure 10A and 10B).

PPP2R5E (S33 and S34) (PP2A R epsilon subunit) & PPP2R5D (S88) (PP2A R delta subunit) are a subfamily of PP2A Protein Phosphatase 2A). PPP2 (or PP2A) accounts for almost 80% of serine/threonine Phospho Protein Phosphatases (PPP) in mammalian cells and considered to be involved in most of the serine/threonine phosphatase activity in cells. PP2A is a trimeric holoenzyme consists of three subunits, scaffold A subunit, catalytic C subunit and regulatory B subunit [57]. In mammals, the function of catalytic subunit is dephosphorylation. There are several families of the B subunit which are believed to be involved in stabilization of holoenzyme via binding with the A or C subunit. The B subunit is felt to be involved in regulation of the A or C subunit for its further activities [58]. The δ form of B subunit is expressed in cytoplasm and nucleus where ε form is expressed in cytoplasm only [58]. Nuclear form may be involved in regulation of transcription by CREB and AP-1, in controlling the activity of the retinoblastoma proteins and in the dephosphorylation of p53 [58, 59]. The B regulatory subunit is believed to be involved in modulations of substrate selectivity and catalytic activity [59]. The B subunit is involved in cell growth and apoptosis [57]. For all three specified phosphorylation sites, no studies on their role in skeletal muscle cells have been reported. Significant changes that are observed in our experiment in comparison with different treatment has been shown in Figure 12A, 12B and 12C in S88, S33 and S34 sites, respectively.

PPP1R12A (Protein Phosphatase 1 regulatory subunit 12A) (S299) - Studies for this particular site has been reported for cancer but not for any other diseases in human. It has never been studied in human skeletal muscle tissue or cells before. It has been found that PPP1R12A and the catalytic subunit of PP1 (PP1cδ) were identified as interaction partners of IRS-1 in L6 cells [60]. PPP1R12A/PP1cδ may dephosphorylate IRS-1 in L6 cells to maintain proper insulin action through IRS-1. The kinases and phosphatase has effect on p85 [60]. Observed significant changes in phosphor-S299 in the experiment have been shown in Figure 11.

DCLK1 or doublecortin-like kinase 1(4 isoforms) is a serine-threonine kinase of the calmodulin kinase (CAMK) family. There are 6 phosphorylated sites were observed on serine/threonine protein kinase DCLK1 at S330, S334, T336, S337, S353, S362. Most of the research on DCLK1 within human and other vertebrates has been done on nervous tissue, which is primarily thought to be involved in cell differentiation and neurogenesis. DCLK1 has been shown to interact with calmodulin 1 (CALM1), doublecortin (DCX), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHAE) in mouse brain [61, 62]. Phosphorylation of DCLK1 S330, S334, T336, S337, S353, S362/3 have been identified in multiple cell types including embryonic stem cells [63-65] or in brain tissue; thus, this is the first identification of these phosphorylation sites within skeletal muscle cells as well as associated with insulin resistance in human or any other organism. Notably, DCLK1 along with

multiple phosphorylation sites observed here have been detected in mouse cardiac muscle [66]. However, the role of DCLK1 in skeletal muscle and/or insulin signaling is yet to be determined. Phosphorylation of S330, S334, S337, was observed in 3T3-L1 adipocytes upon insulin stimulation [67]; however, the regulation of these phosphosites have not been studied. All of the phosphorylation sites of DCLK1 observed within this study fall between the 2nd doublecortin domain and the protein kinase domain, which suggests phosphorylation may regulate the binding of DCX and/or kinase activity/specificity. Figure 13A, 13B, 13C, 13D, 13E, and 13F reflects phosphorylation sites in DCLK1 with different treatment comparison for S330, S334, T336, S337, S353 and S362 sites, respectively.

Serine/threonine-protein kinase or p21-activated kinase (PAK4, S181). PAK4 is regulated by Rho GTPase activity during cytoskeletal reorganization and nuclear signaling in apoptosis and cell cycle regulation [68]. PAK4 has been shown to interact with, but not limited to, CDC42, CAS8, and GRB2; known substrates of PAK4 include LIM kinase, Slingshot phosphatase, GEF-H1, Integrin beta 5, BAD, Myosin light chain 9, Ran, Paxillin, Raf-1, Beta-Catenin, p120-Catenin, and PDZ-RhoGEF [69]. Within humans and rodents, S181 has been identified within multiple cancer-related cell types but not within skeletal muscle cells. The role of S181 is unknown but may regulate auto inhibition, kinase activity or binding of GTPase. Phosphorylation of S181 was observed in 3T3-L1

adipocytes upon insulin stimulation [67]. Figure 14 illustrated significant changes in phosphor-S181 in PAK4 upon metformin and insulin treatment.

Phosphatidylinositol 4-kinase type 2-alpha (PI4K2A, S47 and S51) PI4K2A has been shown to interact with and promote VAMP3, part of the SNARE complex with GLUT4 involved in glucose uptake [70]. Phosphorylation of S47 and S51 was observed in 3T3-L1 adipocytes upon insulin stimulation [67]. S47 and S51 have not been mutated and don't fall within any well-defined domain. PI4K2A S47 and S51 phosphorylation has never been studied for skeletal muscle cells before. Figure 15A and 15B shows significant changes in S47 and S51 phosphorylation, respectively.

5'-AMP-activated protein kinase subunit beta-1 (PRKAB1, S108) and 5'-AMP-activated protein kinase subunit beta-2 (PRKAB2, S108) also known as AMPK. S108 site on both proteins has been observed as phosphorylated. AMPK contains three subunit, alpha, beta and gamma. There are different mechanisms of metformin have been proposed for its effect on AMPK. It has been proposed that metformin primarily affects hepatic glucose production through activating AMPK but our results support its effect in skeletal muscle also. It has been found that metformin has effect on alpha subunit of AMPK but effect on beta subunits of AMPK has been observed in our study. In liver AMPK is expected to be involved in reducing glucose production but in skeletal muscle is involved in glucose uptake. Research study also suggests that AMPK has an effect on alpha subunit of AMPK [30]. No research study has been done on PRKAB1 (S108) and

PRKAB2 (S108) for diabetes study using skeletal muscle. Significant increase of S108 phosphorylation in PRKAB1 (AMPK-beta-1) and PRKAB2 (AMPK-beta-2) upon high metformin treatment is shown in Figure 16 and 17 respectively.

cAMP-dependent protein kinase type-II alpha regulatory subunit (PRKARA2, S78 and S80). Activated PRKARA2 binds to four cAMP molecules. Research on S78 and S80 phosphorylation has been reported on pulmonary cell lines, skeletal muscle, liver, spermatozoa, stem cells but regulation of S78 and S80 phosphorylation for metformin has not been reported. Figure 18A and 18B shows the significant changes in PRKARA2 S78 and S80 phosphorylation in our experiments.

Protein kinase PRP4 homolog (S277). More study for this phospho site has been done on different types of cancer but study on skeletal muscle has not been reported yet and also it has not been studied for metformin's action. Figure 19 shows the significant change in this phosphorylation site in our experiments.

Protein kinase-10 (STK10, S450 and S454). STK10 is a kinase from STE20 family. Kinases from STE20 family are important in cellular functions such as apoptosis and morphogenesis. STK10 has an effect on PIK1 (Polo-like kinase kinase). STK10 is believed to be involved in phosphorylation of PIK1 [71]. It is mostly expressed in hematopoietic tissues. Functions of STK10 in human is more complex than in mice [71]. Site specific regulation study has not been performed in skeletal muscle and/or for metformin. Figure 21A and 21B show

significant changes in S450 and S454 phosphorylation in our experiments, respectively.

CHAPTER 5 CONCLUSION & FUTURE STUDIES

In summary, we report the 1st global analysis of the effect of metformin on phosphorylation profiles in primary skeletal muscle cells derived from muscle biopsies from 4 obese/overweight insulin resistant human participants. We identified 2930 phosphorylation sites assigned to 1085 proteins. More importantly, 325 sites showed significant changes among the 6 sets of samples, including 6 sites in 4 phosphatase subunits and 17 sites in 9 kinases/kinase subunits (Table 3). We demonstrated that metformin has significant effect on some of the phospho sites present in phosphatases and kinases which are important for insulin signaling or glucose uptake. We also noticed that there is very little known about the involvement of these 23 phosphorylation sites in the development of diabetes or in the action of metformin, because of few reported site-specific regulation studies. These results may shed new lights into molecular mechanisms for metformin's action.

We will perform literature search on the other phosphorylation sites with a significant changes among the 6 sets of samples. In addition, we will quantify the total corresponding protein abundance through western blot and/or through HPLC-ESI-MS/MS. Moreover, we will perform the same experiments in primary cell culture from lean/healthy and T2D people to identify metformin stimulated or suppressed phosphorylation sites. Furthermore, we will use a variety of techniques, such as site-specific mutagenesis, in vitro kinase assays, in cell kinase inhibitor assays, as well as the generation of several phospho-specific

antibodies to study the biological function of novel phosphorylation sites regulated by metformin.

Table 1. Clinical characteristics of participants in the metformin study.

Results were shown as mean ± SEM values.

	4 Participants in OB/OW group
Gender (M/F)	(2/2)
Age (years)	36 ± 9.7
BMI (kg/m^2)	29.7 ± 2.8
FBS (mg/dl)	92.72 ± 2.3
2h OGTT (mg/dl)	149.6 ± 20.6
HBA1c (%)	5.4 ± 0.1
M values (mg/kg/min)	**4.2 ± 0.8**

Table 2. Different conditions and/or treatments of cells

Sr. No.	Condition/ Treatment (No insulin stimulation)
1	Low Glucose + No Metformin (3d)
2	Low Glucose (2d) + Low Metformin (1d)
3	Low Glucose (2d) + High Metformin (1d)
With 15 minutes insulin stimulation	
4	Low Glucose + No Metformin (3d)
5	Low Glucose (2d) + Low Metformin (1d)
6	Low Glucose (2d) + High Metformin (1d)

The above table describes the conditions that are cells treated with. Each condition was treated with 15 minutes insulin stimulation and without insulin stimulation.
1d- 1 day, 2- 2 day, 3d- 3 day

Table 3. Significantly changed phospho sites among the 6 sets of samples

Protein names	Protein IDs	Gene names	Phosphorylation site
PHOSPHATASE SUBUNITS			
Protein phosphatase inhibitor 2	P41236	PPP1R2	S121
Protein phosphatase inhibitor 2	P41236	PPP1R2	S122
Protein phosphatase 1 regulatory subunit 12A	O14974	PPP1R12A	S299
Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform	Q14738	PPP2R5D	S88
Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit epsilon isoform	Q16537	PPP2R5E	S33
Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit epsilon isoform	Q16537	PPP2R5E	S34

KINASES/KINASE SUBUNITS			
Serine/threonine-protein kinase DCLK1	Q5VZY9	DCLK1	S330
Serine/threonine-protein kinase DCLK1	Q5VZY9	DCLK1	S334
Serine/threonine-protein kinase DCLK1	Q5VZY9	DCLK1	T336
Serine/threonine-protein kinase DCLK1	Q5VZY9	DCLK1	S337
Serine/threonine-protein kinase DCLK1	Q5VZY9	DCLK1	S353
Serine/threonine-protein kinase DCLK1	Q5VZY9	DCLK1	S362
Serine/threonine-protein kinase PAK 4	O96013	PAK4	S181
Phosphatidylinositol 4-kinase type 2-alpha	Q9BTU6	PI4K2A	S47
Phosphatidylinositol 4-kinase type 2-alpha	Q9BTU6	PI4K2A	S51
5'-AMP-activated protein kinase subunit beta-1	Q9Y478	PRKAB1	S108
5'-AMP-activated protein kinase subunit beta-2	O43741	PRKAB2	S108
cAMP-dependent protein kinase type II-alpha regulatory	P13861	PRKAR2A	S78

subunit			
cAMP-dependent protein kinase type II-alpha regulatory subunit	P13861	PRKAR2A	S80
Serine/threonine-protein kinase PRP4 homolog	Q13523	PRPF4B	S277
Serine/threonine-protein kinase SIK3	H0Y4E8	SIK3	S545
Serine/threonine-protein kinase 10	O94804	STK10	S450
Serine/threonine-protein kinase 10	O94804	STK10	S454

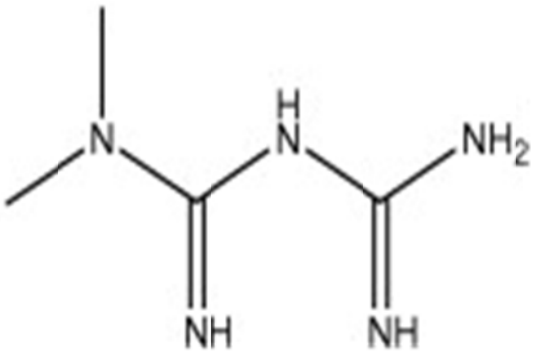

Figure 1. Structure of Metformin

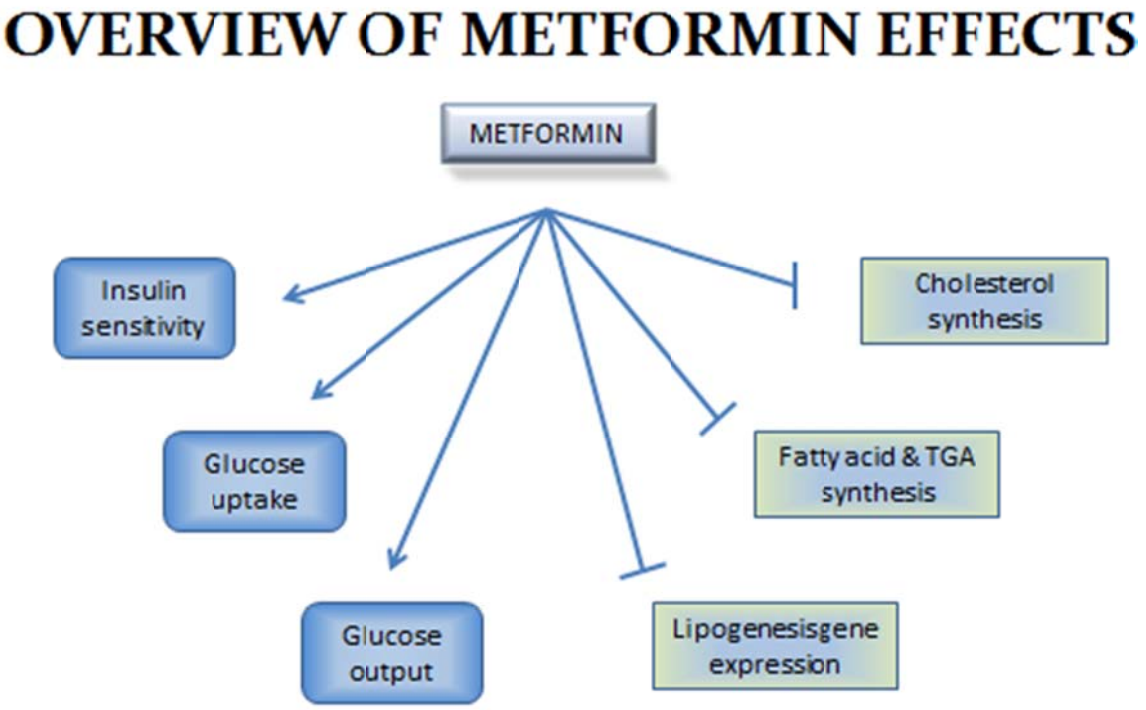

Figure 2. Possible effects of metformin

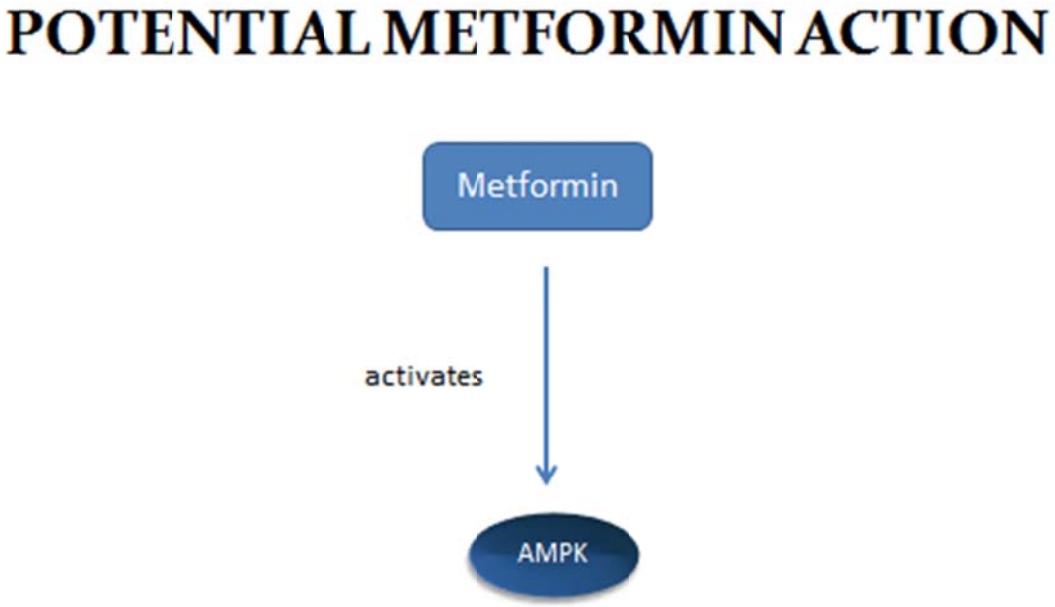

Figure 3. Potential action of metformin

It shows that metformin activates AMPK. AMPK is involved further in glucose uptake.

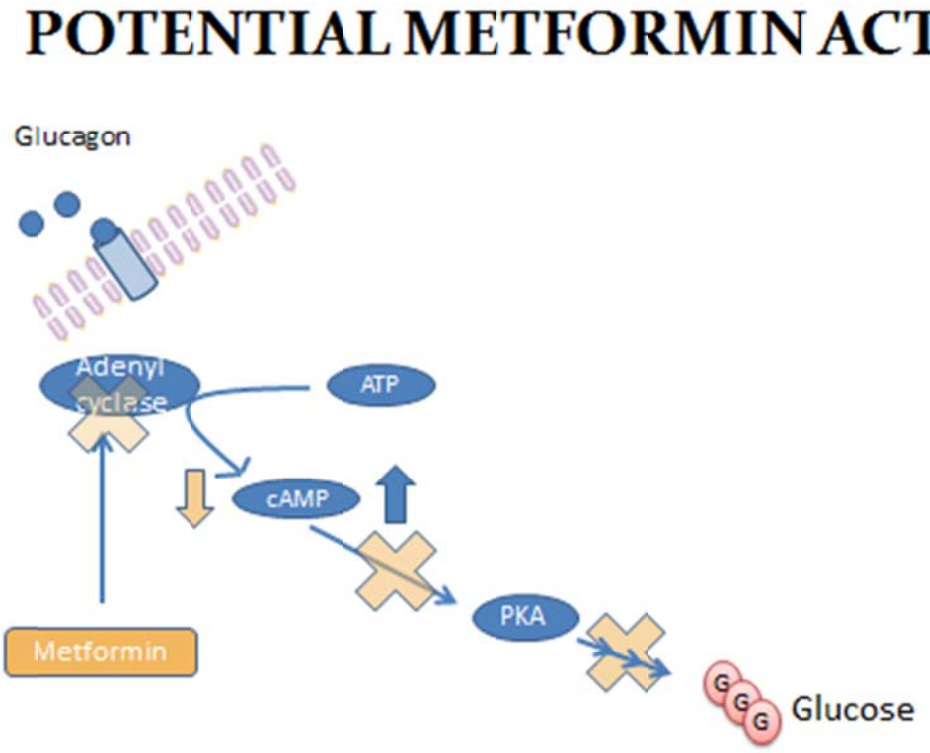

Figure 4. Potential action of metformin in glucose production

The above figure explains that metformin reduces cAMP formation by inactivating adenyl cyclase in liver which reduces glucose production [33].

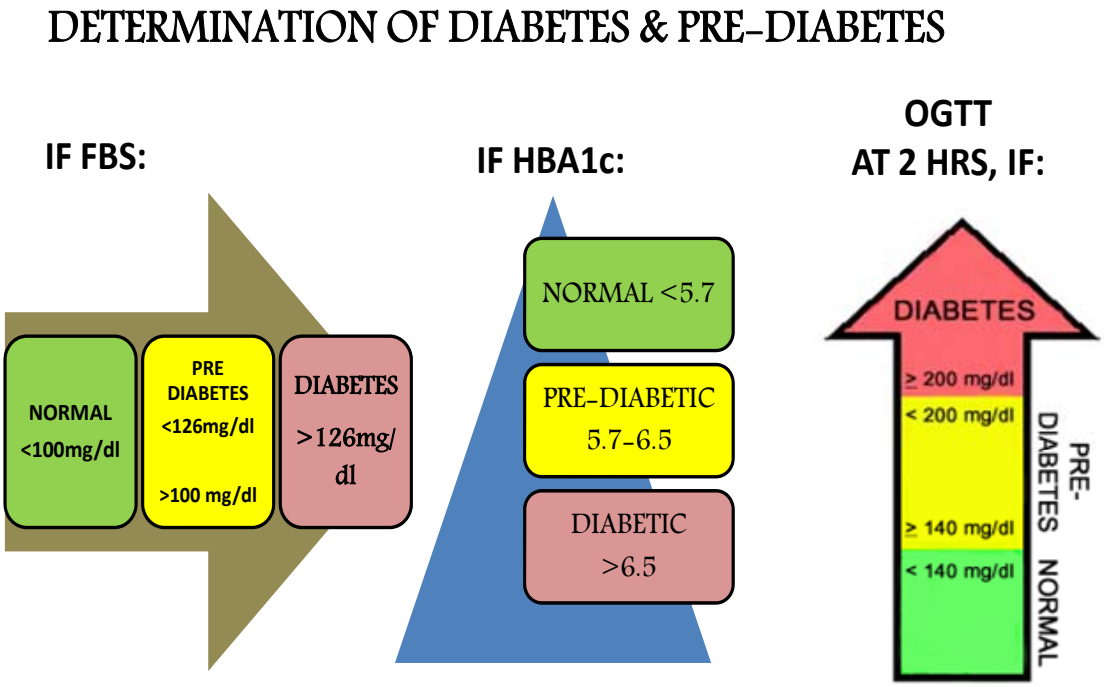

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Figure 5. Parameters with their range to determine diabetes & pre-diabetes.

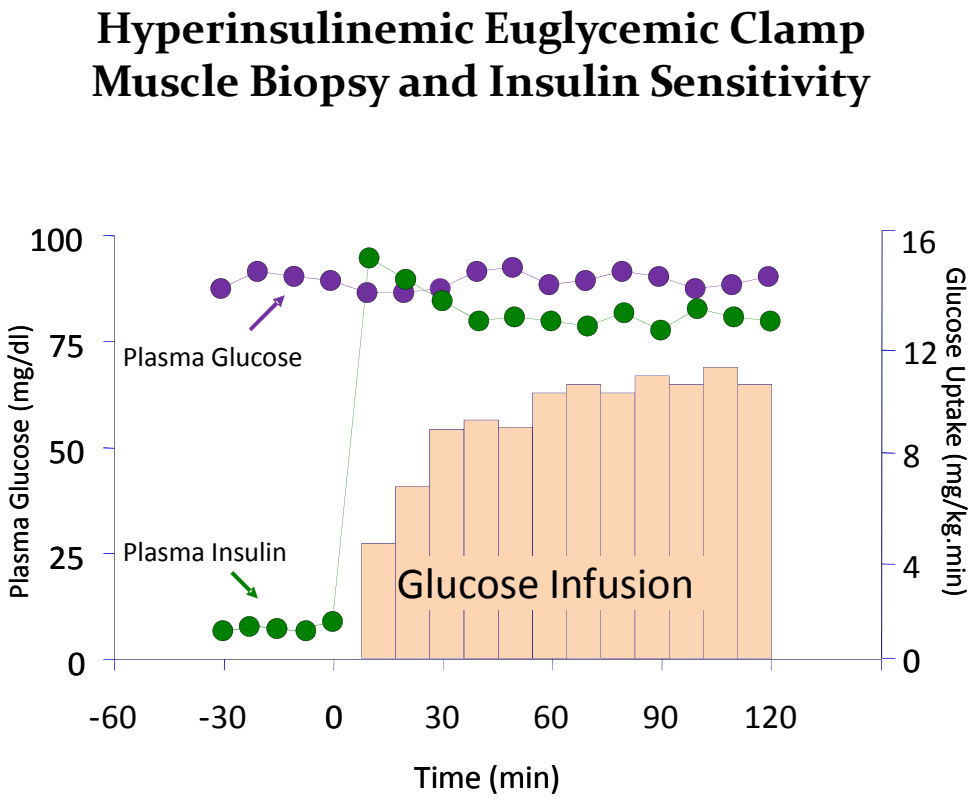


Figure 6. Hyperinsulinemic-euglycemic clamp

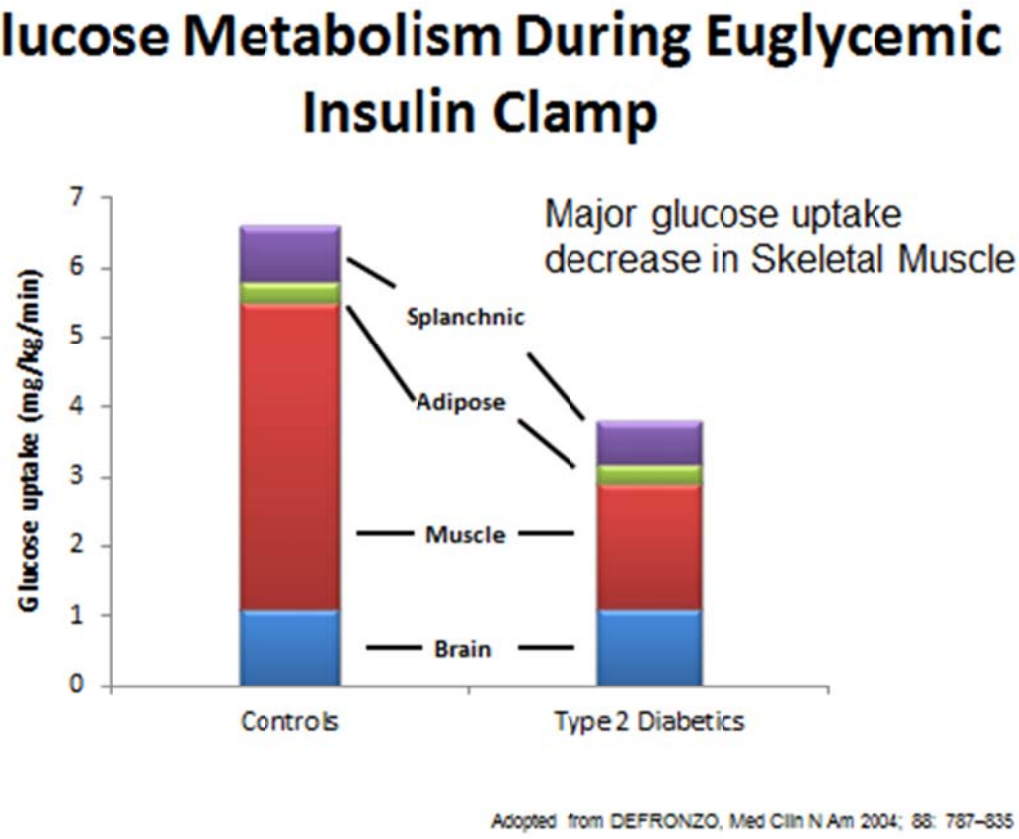
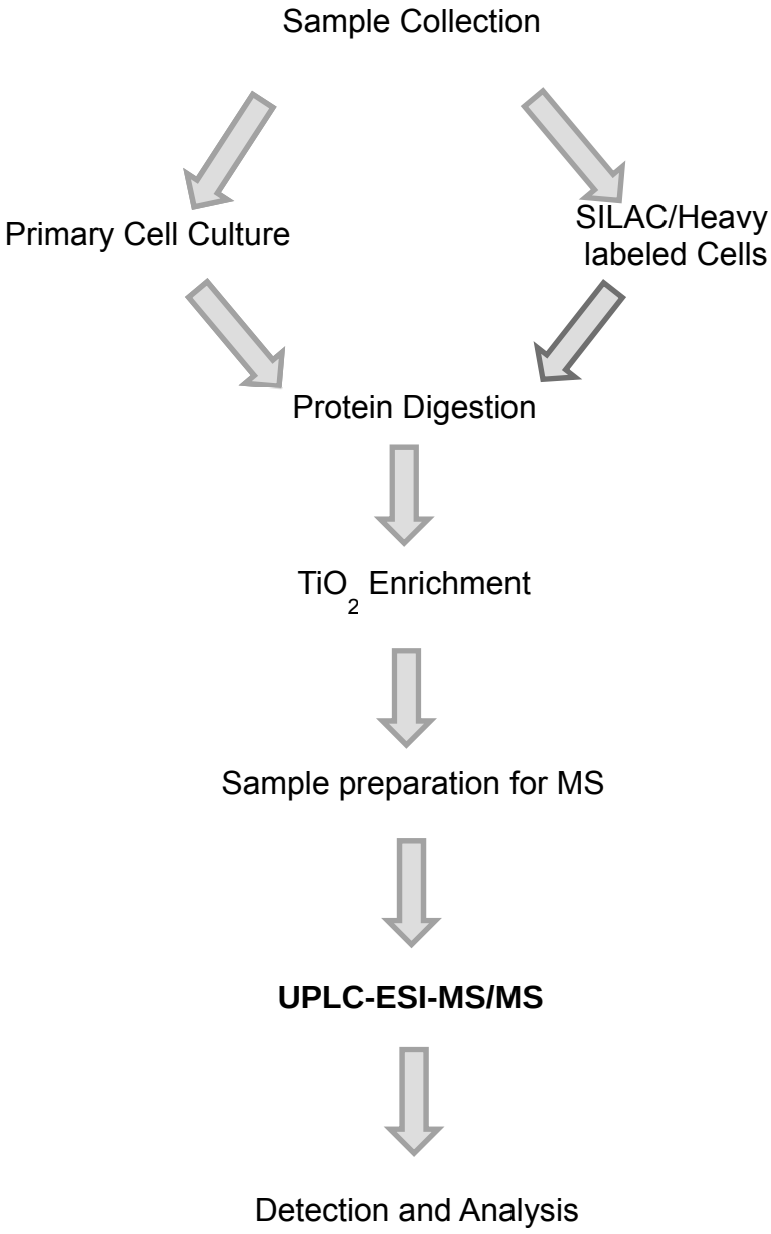


Figure 7. Comparison of glucose uptake in different organs/tissues under hyperinsulinemic-euglycemic clamp condition between non-diabetic controls and T2D patients.


Sample Collection
Primary Cell Culture
SILAC/Heavy labeled Cells
Protein Digestion
TiO2 Enrichment
Sample preparation for MS
UPLC-ESI-MS/MS
Detection and Analysis

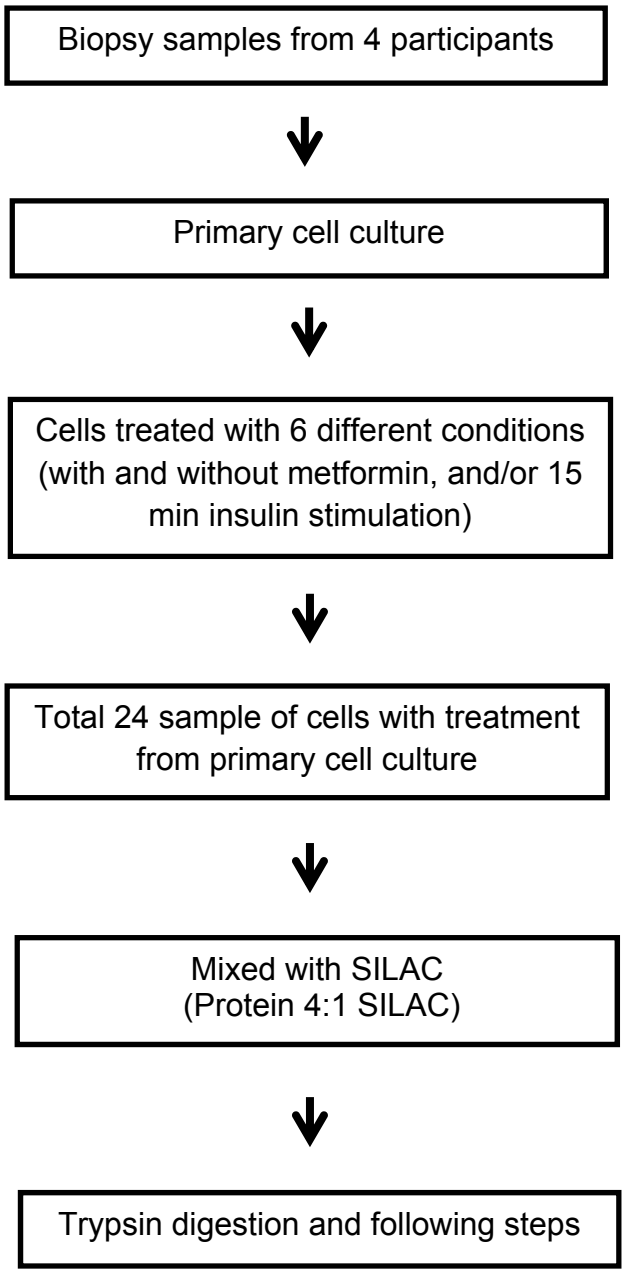


Figure 8B. Sample preparation for proteomics experiments

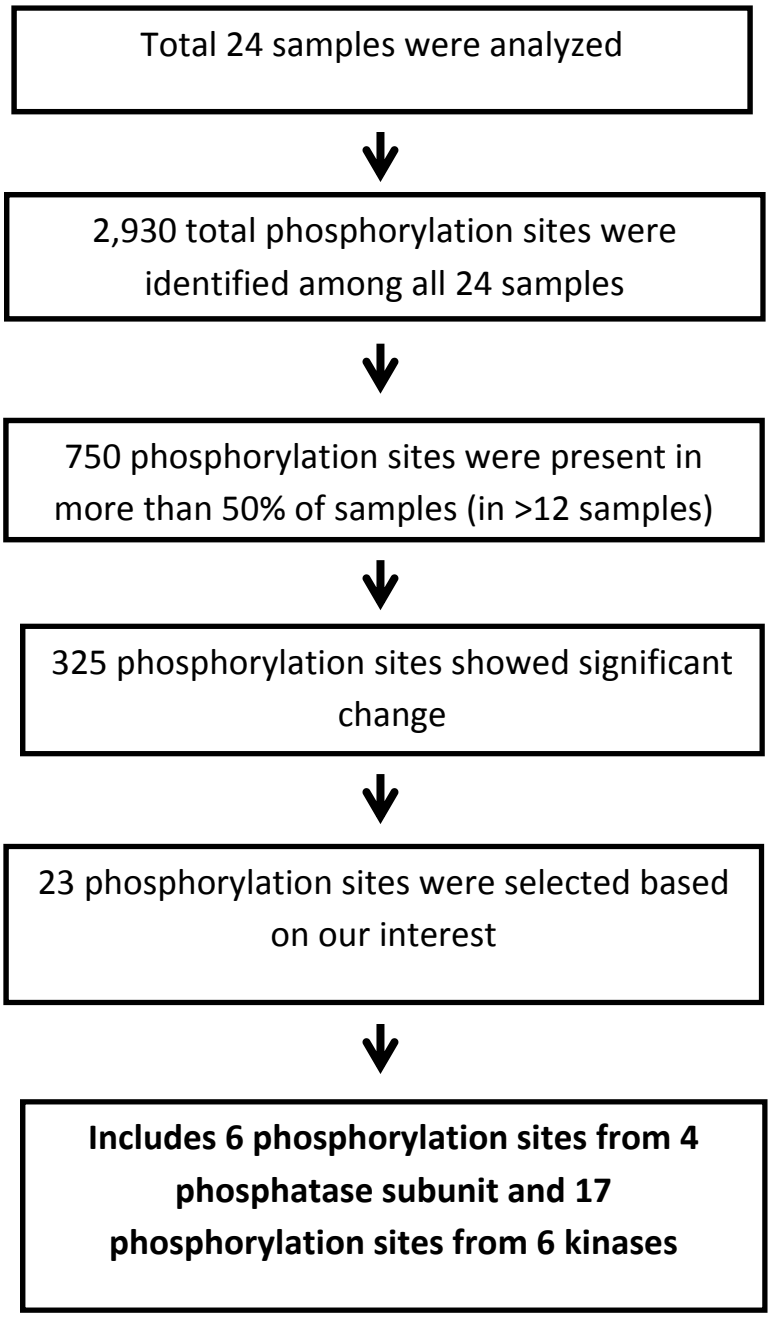


Figure 9. The statistical proteomics analysis workflow

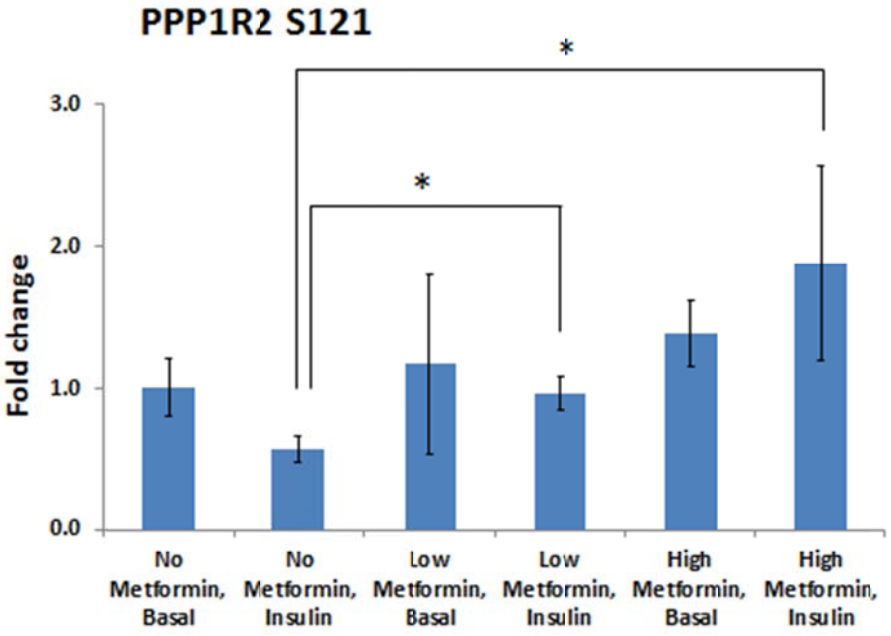


Figure 10A.

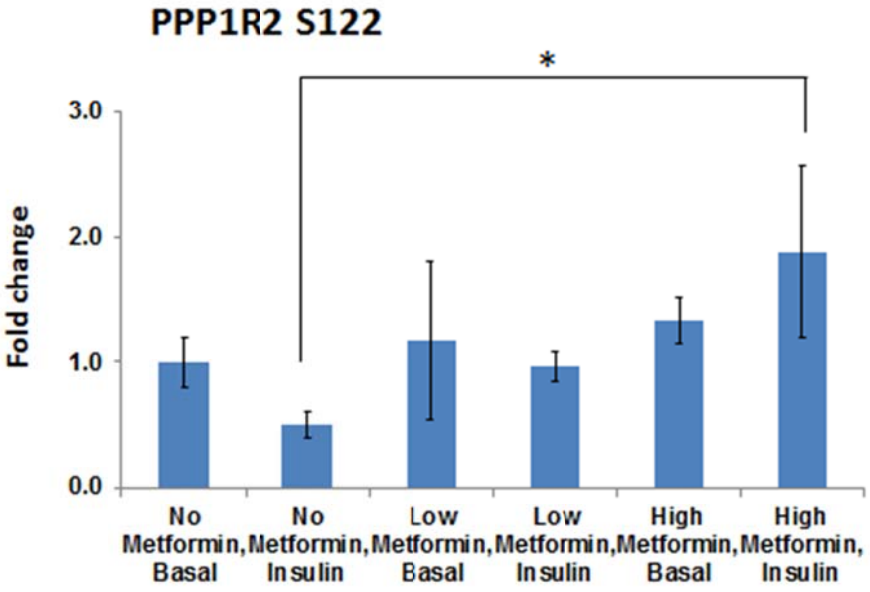


Figure 10B.

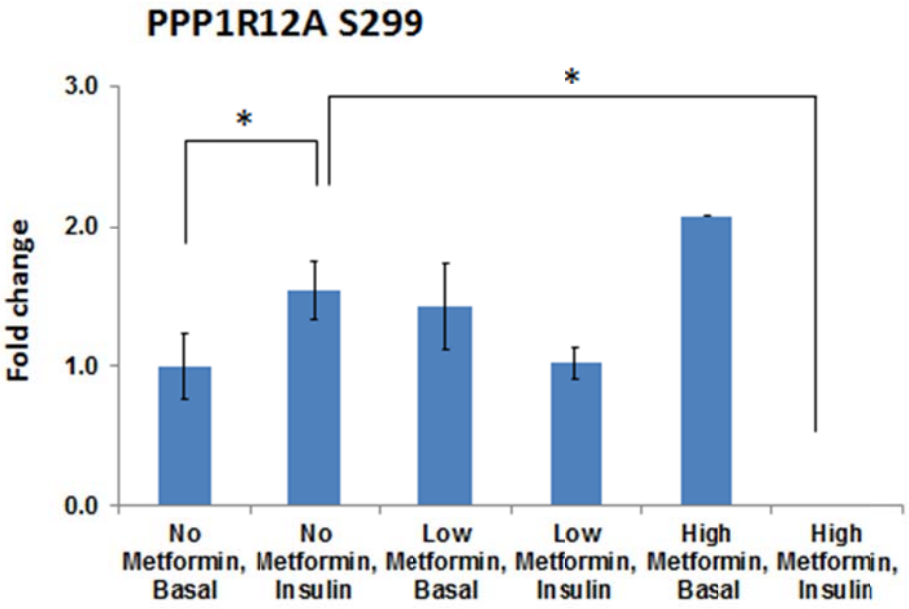


Figure 11.

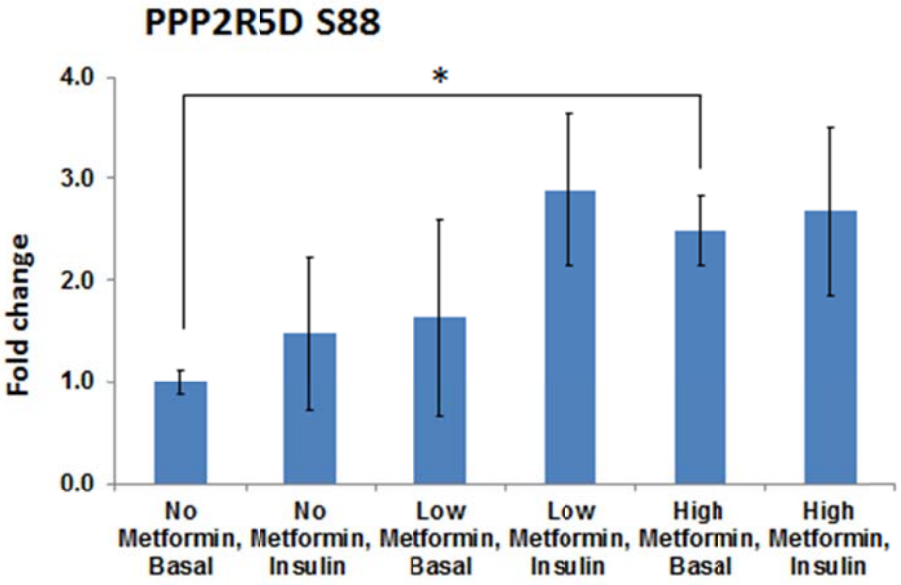


Figure 12A.

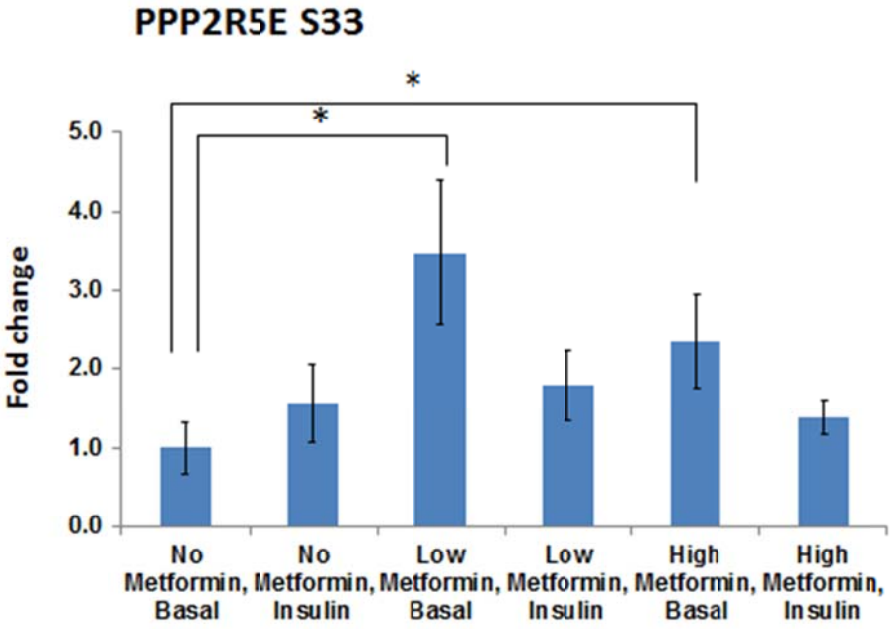


Figure 12B.

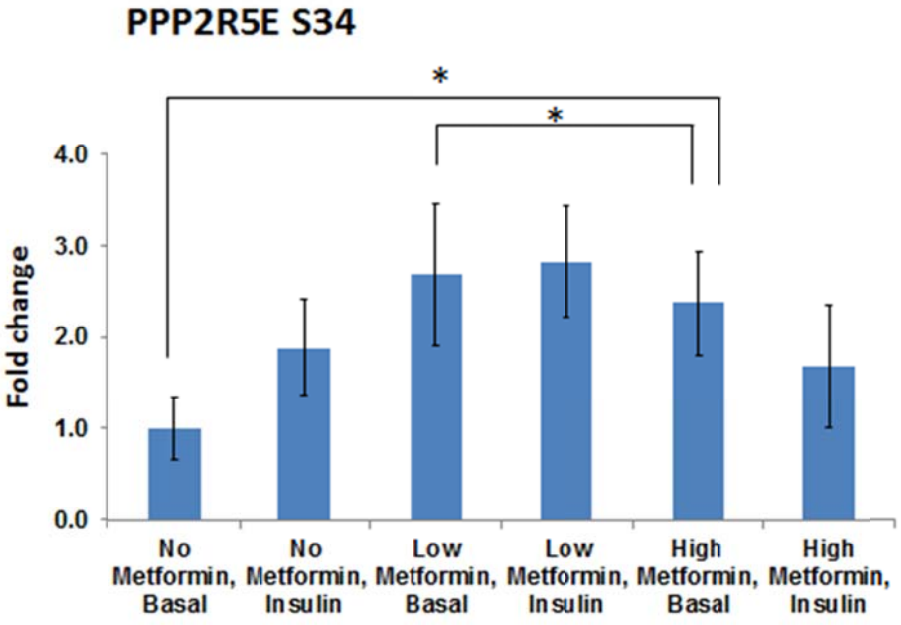


Figure 12C.

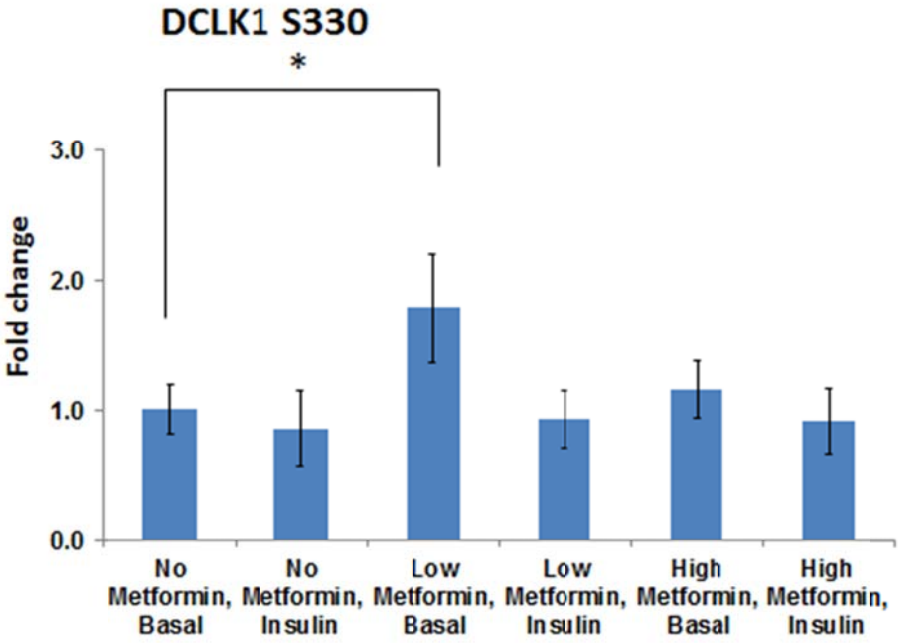


Figure 13A.

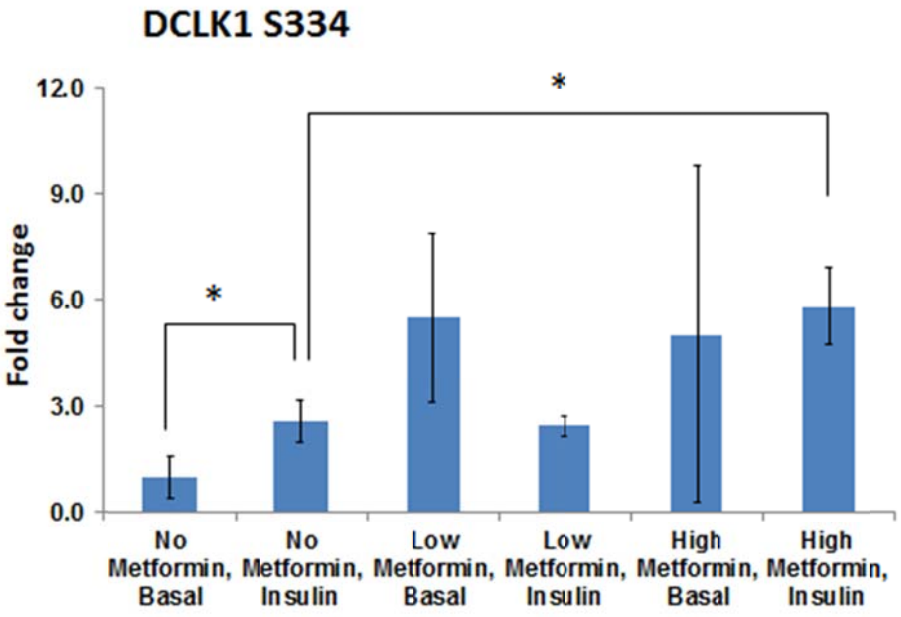


Figure 13B.

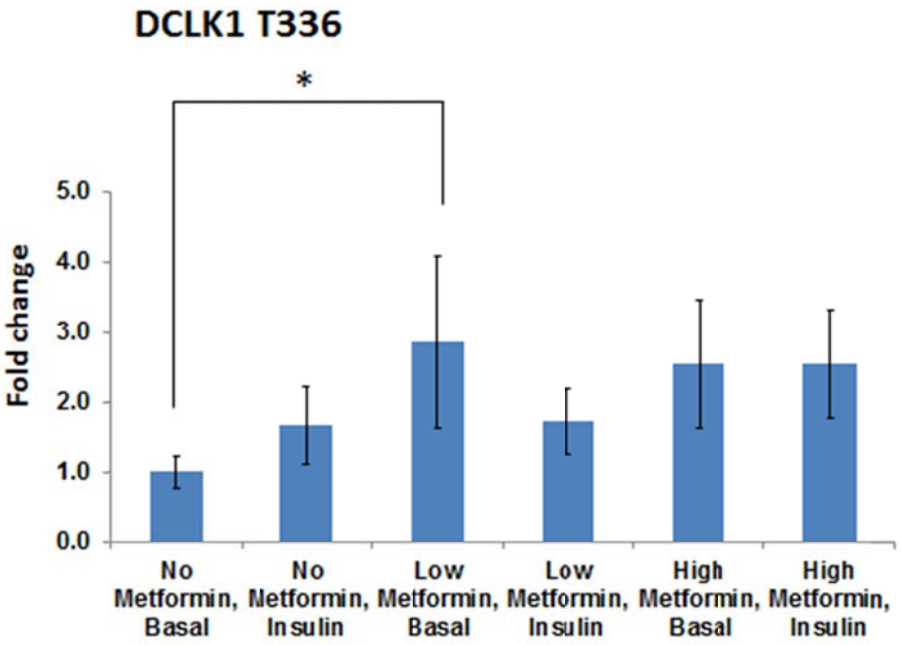


Figure 13C.

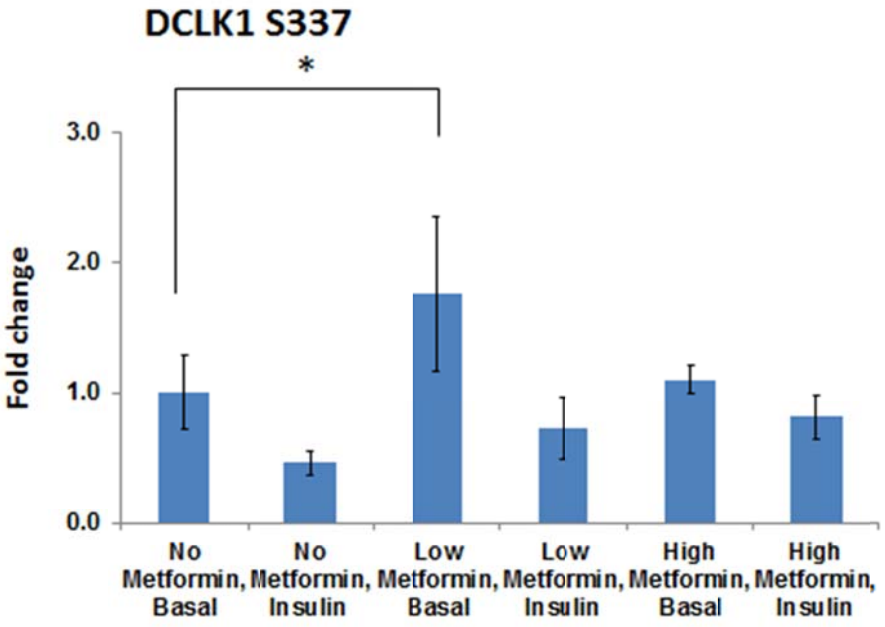


Figure 13D.

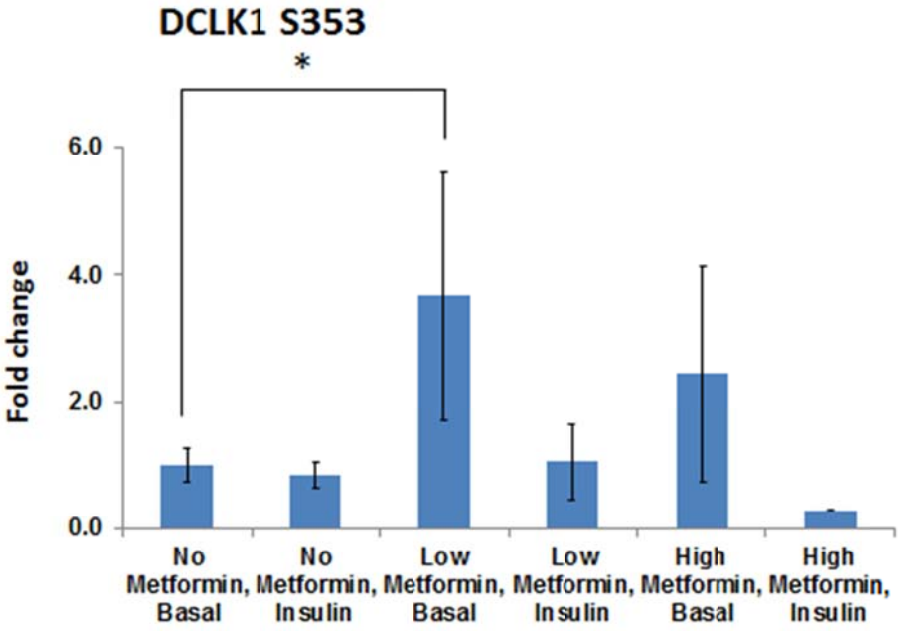


Figure 13E.

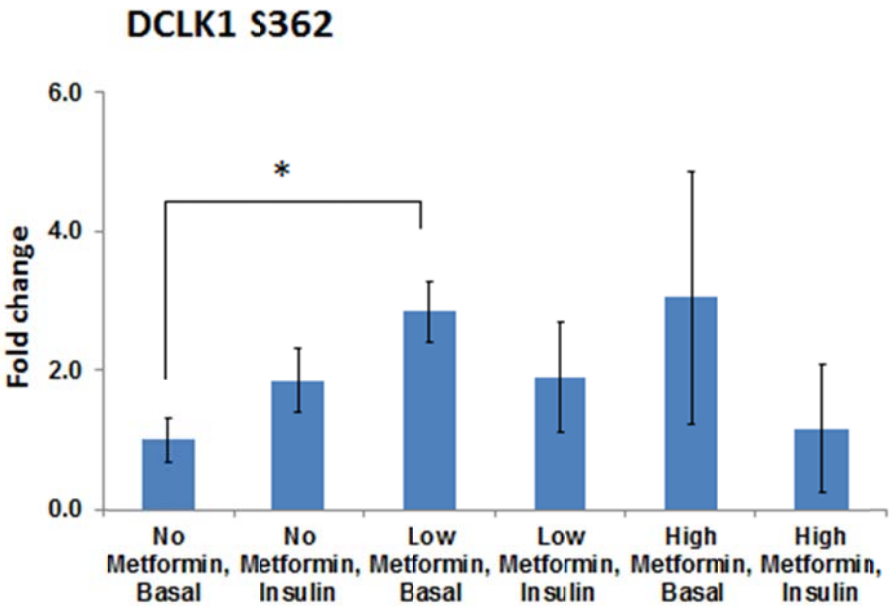


Figure 13F.

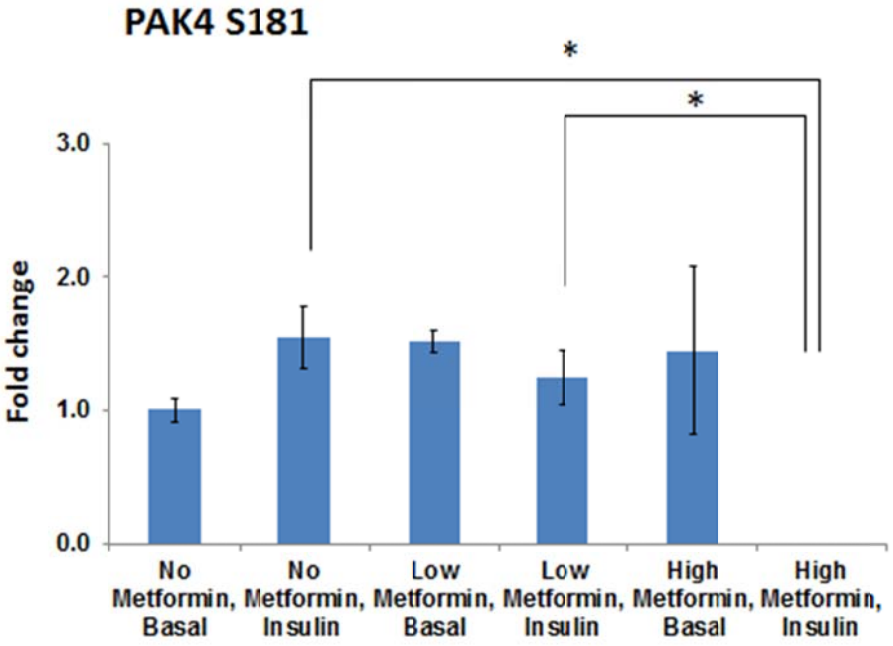


Figure 14

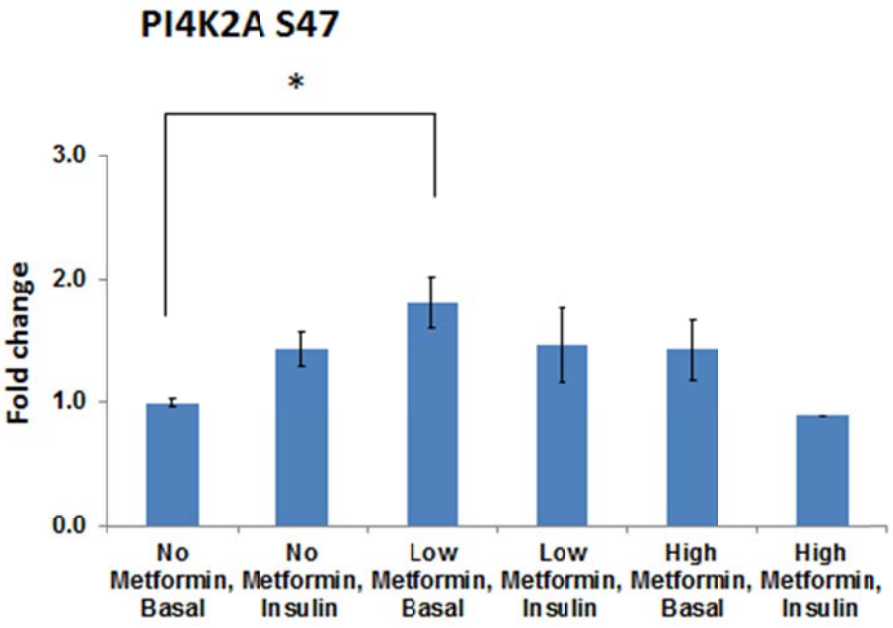


Figure 15A

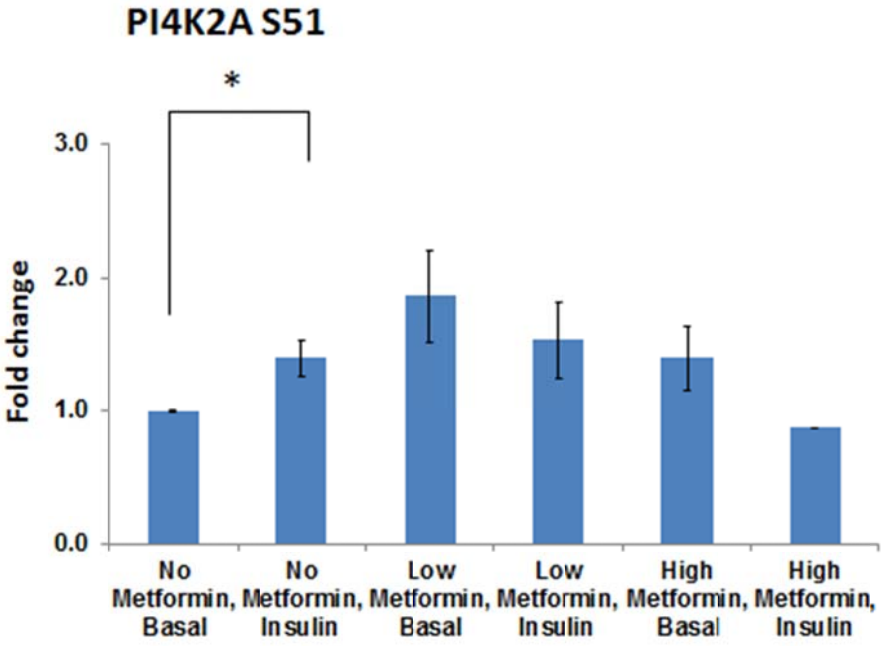


Figure 15B.

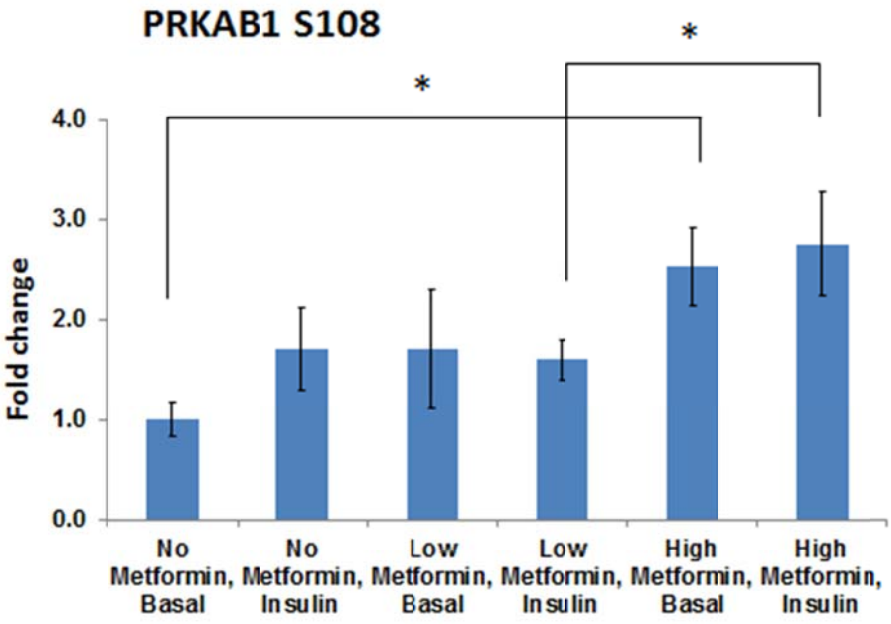


Figure 16.

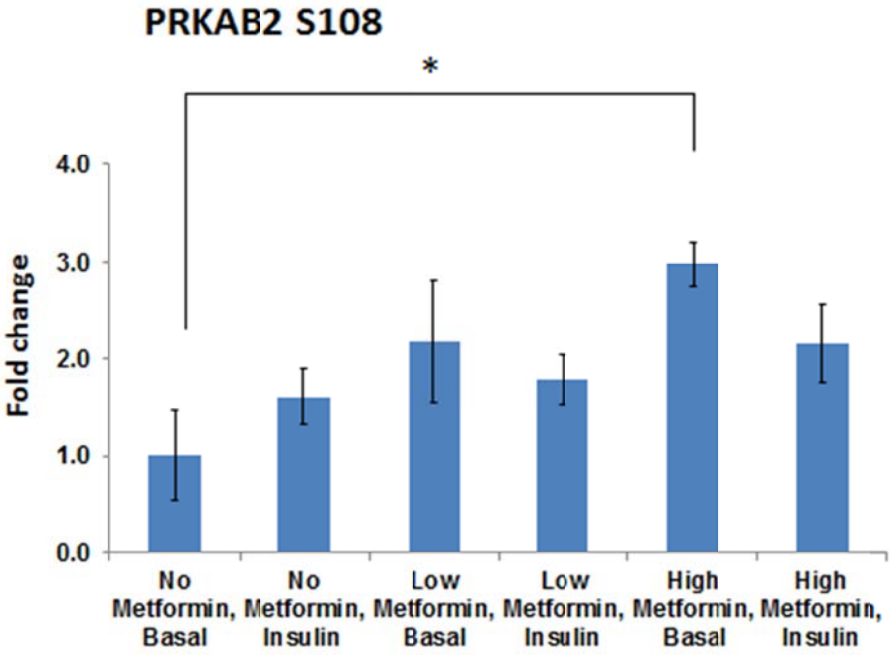


Figure 17.

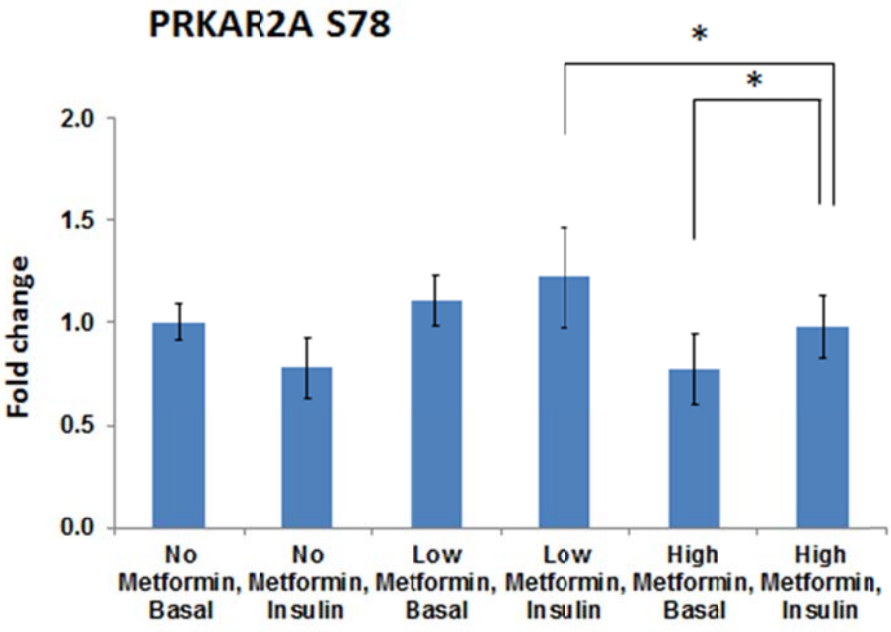


Figure 18A.

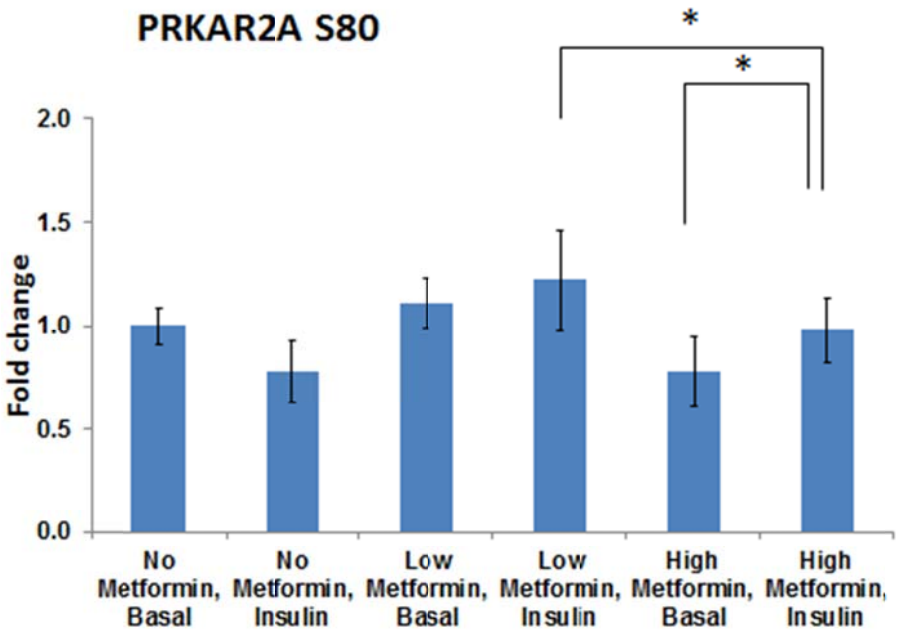


Figure 18B.

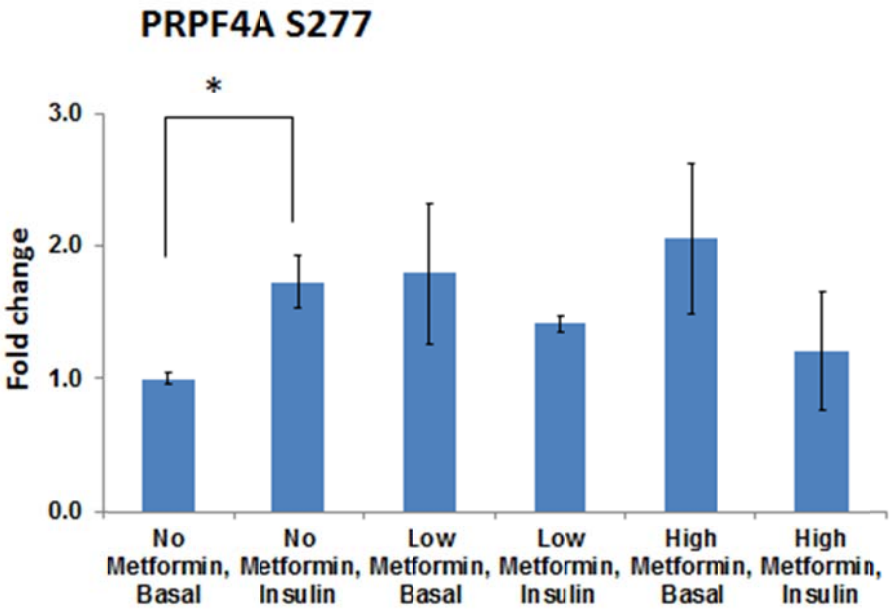


Figure 19.

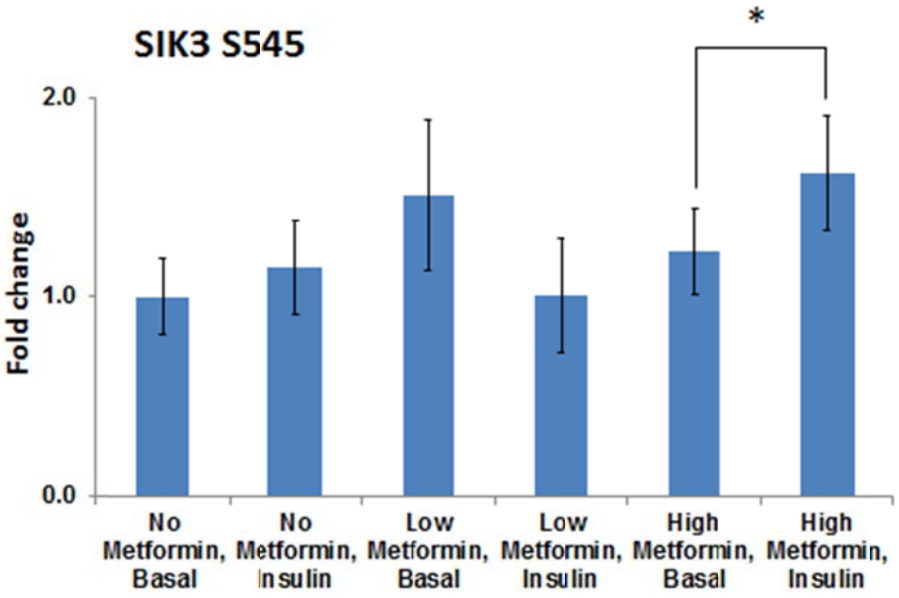


Figure 20.

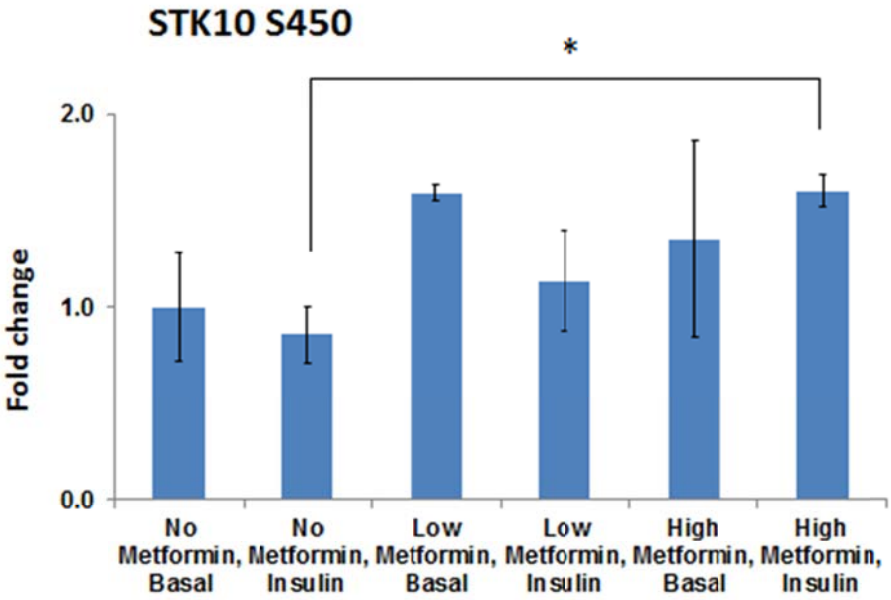


Figure 21A.

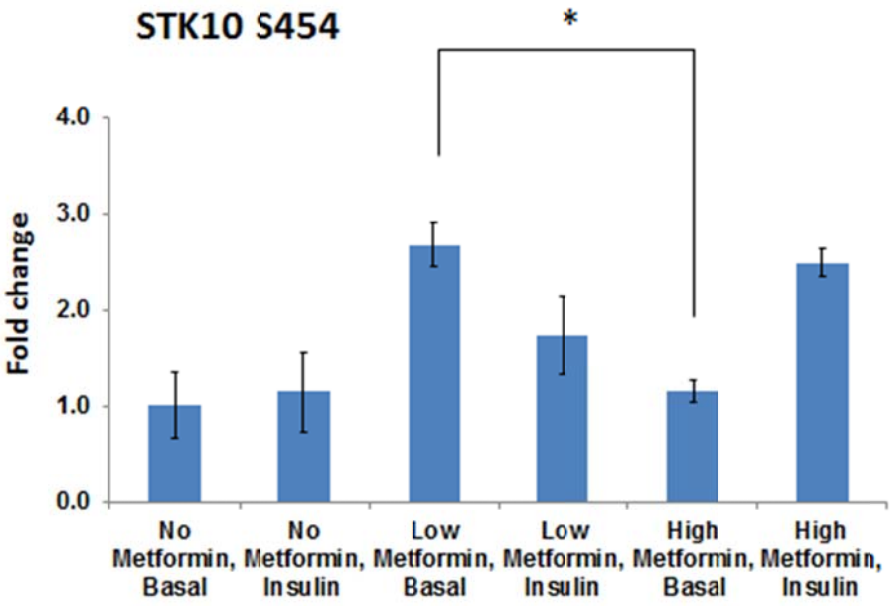

STK10 S454
*
Fold change
4.0
3.0
2.0
1.0
0.0
No Metformin, Basal
No Metformin, Insulin
Low Metformin, Basal
Low Metformin, Insulin
High Metformin, Basal
High Metformin, Insulin

Figure 21B.

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ABSTRACT

EFFECT OF METFORMIN ON GLOBAL PHOSPHORYLATION PROFILES OF PRIMARY SKELETAL MUSCLE DERIVED FROM OVERWEIGHT/OBESE INSULIN RESISTANT HUMAN PARTICIPANTS

by

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Metformin is a drug from the biguanide class and it has been in use for the treatment of type 2 diabetes for a long time, and it can improve insulin sensitivity in skeletal muscle. However, the mechanism for metformin's action is unclear. Phosphatases and kinases, and their subunits are the proteins required for dephosphorylation and phosphorylation of proteins in cells during various signaling pathways. Phosphorylation studies of proteins from primary cell culture derived from skeletal muscle tissue from obese/overweight insulin resistant participants will help to understand the regulation of phosphorylation in phosphatases and kinases by metformin.

In the current research, we used primary cell culture cells of human skeletal muscle tissue to identify and quantify the phosphorylation effects of metformin in obese/overweight participants. After treating cells with different

conditions which include different concentration of metformin, we identify 2930 phospho sites in 1085 proteins. Among 2930 phospho sites, 325 phospho sites showed significant change among 6 sets of samples (total 24 samples). Of particular interest, metformin treatment significantly changed phosphorylation levels of 23 sites including 6 phosphorylation sites in 4 phosphatase subunits and 17 phosphorylation sites in 9 kinases/kinase subunits. These results provide new information on how metformin works in skeletal muscle cells.

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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.
2. Danjun Ma, Yue Qi, Abdullah Mallisho, Michael Alexander. Caruso, Divyasri Damacharla, Xiangmin Zhang, Rebecca Tagett, Sorin Draghici, Rodney O. Berry, **Nishit Shah**, Majed Abdullah. Alharbi, Berhane Seyoum, Zhengping Yi, “Abnormal Protein Phosphorylation In Plasma From Type 2 Diabetic Patients”. Accepted as a poster presentation in the 75th American Diabetes Association conference, June 5 - 9, 2015, Boston, Massachusetts.

3. Yue Qi; Abdullah Mallisho; Danjun Ma; Xiangmin Zhang; Michael Caruso; Divyasri Damacharla; **Nishit Shah**; Majed Abdullah Alharbi; Berhane Seyoum; Zhengping Yi, "Comparison of ATP Affinity Probe-based kinome enrichment at the protein and peptide levels". Accepted as a poster presentation in the 63rd ASMS Conference on Mass Spectrometry and Allied Topics, May 31 - June 4, 2015 - America's Center, St. Louis, Missouri.
4. **Nishit Shah,** et al. Stability Indicating RP-HPLC Method for Determination of Metformin Hcl and Nateglinide in Bulk and Tablet at 63rd IPC (Indian Pharmaceutical Congress) at Bangalore, 2012.
5. **Nishit Shah,** et al. Formulation and In-vitro Evaluation of Sustained Release Multiparticulate Drug Delivery System at ‘Avishkar 2010’ (Zonal level Research Competition), Pune, 2010.