

Title: Ingestion of lean meat elevates muscle inositol hexakisphosphate kinase 1 protein content independent of a distinct post-prandial circulating proteome in young adults with obesity

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Abstract

Background: We have recently shown that a novel signalling kinase, inositol hexakisphosphate kinase 1 (IP6K1), is implicated in whole-body insulin resistance via its inhibitory action on Akt. Insulin and insulin like growth factor 1 (IGF-1) share many intracellular processes with both known to play a key role in glucose and protein metabolism in skeletal muscle.

Aims: We aimed to compare IGF/IP6K1/Akt signalling and the plasma proteomic signature in individuals with a range of BMIs after ingestion of lean meat.

Methods: Ten lean [Body mass index (BMI) (in kg/m²): 22.7 ± 0.4; Homeostatic model assessment of insulin resistance (HOMA_{IR}): 1.36 ± 0.17], 10 overweight (BMI: 27.1 ± 0.5; HOMA_{IR}: 1.25 ± 0.11), and 10 obese (BMI: 35.9 ± 1.3; HOMA_{IR}: 5.82 ± 0.81) adults received primed continuous L-[ring-¹³C₆]phenylalanine infusions. Blood and muscle biopsy samples were collected at 0 min (post-absorptive), 120 min and 300 min relative to the ingestion of 170 g pork loin (36 g protein and 5 g fat) to examine skeletal muscle protein signalling, plasma proteomic signatures, and whole-body phenylalanine disappearance rates (R_d).

Results: Phenylalanine R_d was not different in obese compared to lean individuals at all time points and was not responsive to a pork ingestion (basal, *P* = 0.056; 120 & 300min, *P* > 0.05). IP6K1 was elevated in obese individuals at 120 min post-prandial vs basal (*P* < 0.05). There were no acute differences plasma proteomic profiles between groups in the post-prandial state (*P* > 0.05).

Conclusions: These data demonstrate, for the first time that muscle IP6K1 protein content is elevated after lean meat ingestion in obese adults, suggesting that IP6K1 may be contributing to the dysregulation of nutrient uptake in skeletal muscle. In addition, proteomic analysis showed no differences in proteomic signatures between obese, overweight or lean individuals.

Key words: insulin resistance, obesity, amino acids, anabolic resistance, IP6K1.

Abbreviations used: R_d , rate of disappearance; IGF-1, insulin like growth factor 1; IP6K1, inositol hexakisphosphate kinase 1; IRS-1, insulin receptor substrate 1; IGFR, insulin like growth factor receptor; mTORC1, mechanistic target of rapamycin complex 1; mTOR, mechanistic target of rapamycin; FFA, free fatty acids; BMI, body mass index; $HOMA_{IR}$, homeostatic model assessment of insulin resistance; MPS, muscle protein synthesis; PH, p130Cas homology; PIP3, phosphatidylinositol 3,4,5-trisphosphate; Akt, protein kinase B; LAT1, large neutral amino acid transporter-1; SNAT2, sodium dependent neutral amino acid transporter-2 ; OW, overweight; OB, obese.

Introduction

Obesity and increased fat mass are associated with impaired metabolic function in a variety of tissue types, which can result in a range of diseases including metabolic syndrome and type 2 diabetes (Golay et al., 1986; Stumvoll et al., 2005). In skeletal muscle, decreased insulin-stimulated glucose uptake in obese individuals, when in an inflammatory state, is partly caused by reduced insulin receptor substrate 1 (IRS-1) and post receptor activity (Golay et al., 1986; DeFronzo & Tripathy, 2009) and can spare protein and prolonging survival (Wu & Ballantyne, 2017). In healthy humans and animals, it has been shown that insulin and IGF-1 have important roles in the activation of Akt and mechanistic target of rapamycin complex 1 (mTORC1) (Svanberg et al., 1996; Greenhaff et al., 2008). The mTORC1 pathway has been shown to have an important role in the regulation of post-prandial (Dickinson et al., 2011), but not basal (Dickinson et al., 2013), muscle protein synthesis rates. Given that obesity contributes to insulin resistance and insulin and IGF-1 activate mTORC1 through a similar intracellular mechanism, there is likely a link between altered IGF-1 signalling, coupled with reduced insulin action, and the reduced post-prandial mTORC1 signalling previously observed in people with excess adiposity (Beals et al., 2016).

Using rodent models, O'Neill et al., (2015) demonstrated the synergistic roles of insulin-like growth factor 1 receptor (IGFR) and IRS-1 in the maintenance of skeletal muscle mass and function. Altered

expression of IRS-1/IGFR results in reduced glucose and protein synthesis, with the knockout of both receptors resulting in skeletal muscle atrophy whilst over expression of IGFR induces glucose intolerance (O'Neill et al., 2015). Currently, it is unknown if differences exist in muscle IGFR in insulin resistant obese when compared to healthy and overweight individuals. In addition, evidence in rodent and *in vitro* models suggests that inositol hexakisphosphate kinase 1 (IP6K1) reduces insulin sensitivity and inhibits glucose uptake (Bhandari et al., 2008; Chakraborty et al., 2010). This is supported by recent work from our laboratory which showed decreased IP6K1 skeletal muscle content and improved insulin sensitivity in response to muscle contraction in pre-diabetic humans (Naufahu et al., 2018). IP6K1 is known to synthesize IP6 to IP7 which binds to the pleckstrin homology (PH) domain of Akt, preventing translocation to the cell membrane (Chakraborty et al., 2010). This mechanism results in decreased Akt phosphorylation owing to reduced PIP3 binding to the PH domain (Chakraborty et al., 2010; Naufahu et al., 2018). However, the impact of increasing adiposity on whole body amino acid kinetics and muscle IGF-1/IP6K1/Akt signalling pathway in humans in the basal and postprandial-states has not been characterized.

Given that both IRS-1 and IGF-1 signalling pathways converge on Akt to induce numerous postprandial metabolic responses, we aimed to investigate the role IP6K1 may play on both muscle IGF-1/Akt signalling and skeletal muscle phenylalanine uptake in overweight and obese individuals, previously shown to have dysregulated mTORC1 signalling (Beals et al., 2016), in response to lean meat ingestion. Moreover, we examined whether there was an interaction between excess adiposity and changes in the postprandial proteome after high quality meat ingestion to improve our understanding of circulating protein factors that may contributed to dysregulated metabolism in adults with excess fat mass (Alfadda et al., 2017; Beals et al., 2017; Beals et al., 2016; Halton & Hu, 2004). We hypothesised that insulin-resistant humans would display a divergent postprandial circulating proteome and reduced whole body phenylalanine uptake in response to lean meat ingestion which would be coupled with altered muscle IGF-1/IP6K1/Akt signalling when compared to lean adults.

Materials and Methods

Participants and Ethical Statement

Ten normal weight (lean), 10 overweight (OW), and 10 obese (OB) young men and women volunteered to participate in this study. Inclusion criteria included males and females aged between 20-45 years, healthy, sedentary, BMI=18-24.9 kg/m² (normal weight), BMI=25-29.9 kg/m² (overweight), or BMI=30-39.9 kg/m² (obese). Exclusion criteria were set as the following: participants with cardiovascular disease, diabetes mellitus, fasting blood glucose \geq 126 mg/dL, postprandial blood glucose value \geq 200 mg/dL at 2 h during an oral glucose tolerance test, resting systolic blood pressure \geq 139 or diastolic blood pressure \geq 89 mmHg, chronic smoker (Marijuana or tobacco), irregular menstrual cycles, or on medications known to affect protein metabolism (i.e., corticosteroids, non-steroidal anti-inflammatories, or androgen/oestrogen containing compounds), or a high BMI that is not representative of being overweight or obese due to a high ratio of lean body mass to fat mass based on DEXA measurements were excluded from the study. All participants were classified as sedentary based on the Godin Leisure-Time Exercise Questionnaire and deemed healthy based on routine medical history screening questionnaire. These participants were part of a larger investigation being conducted in our laboratories to investigate the impact of adiposity on skeletal muscle mass regulation to anabolic stimuli (Beals et al., 2016). Participant characteristics have been described previously (Beals et al., 2016) and the groups were counterbalanced for age and sex (Beals et al., 2017; Beals et al., 2016). Prior to commencing experimental procedures, written informed consent was gained from all volunteers and ethical approval was obtained by the Institutional Review Board at University of Illinois. All procedures conformed to standards for the use of human participants in research studies as outlined in the Declaration of Helsinki.

Table 1.**Familiarisation and Pretesting**

Participants presented to the University of Illinois laboratory on two separate occasions and diabetes risk was assessed by oral glucose tolerance test as well as assessing height, weight, and body composition by dual energy X-Ray absorptiometry (Hologic QDR 4500A, Bedford, USA). Time was taken to familiarise volunteers to all procedures.

Infusion Protocol

For three days prior to the experimental infusion trial, participants were instructed to refrain from all physical activity, alcohol, and analgesic drugs. The evening prior to this trial, participants were required to consume a standardized meal of the same composition providing ~30% of estimated daily energy expenditure and containing 50% calories of carbohydrate, 25% calories of fat, and 25% calories of protein. Participants were instructed to fast overnight and arrive to the laboratory by car. Upon arrival, a cannula was inserted into an antecubital vein for baseline blood sample collection. Following this blood sample, a primed ($2 \mu\text{mol}\cdot\text{kg}^{-1}$), continuous infusion of L-[ring- $^{13}\text{C}_6$]phenylalanine ($0.05 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) was initiated ($t=-180 \text{ min}$) and maintained until the end of the trial. Using a heated blanket, repeated arterialized blood sampling was carried out from a second cannula in a contralateral dorsal hand vein which was kept patent with a 0.9% saline drip.

Muscle biopsy samples (~100 mg) of the vastus lateralis were collected with a Bergstrom needle under local anaesthesia in the post-absorptive state at 0 min. Post-absorptive biopsies were randomly obtained from one leg whilst the post-prandial biopsies from the contralateral leg. Participants then consumed 170 g of grounded cooked lean pork loin (36g protein, ~3g leucine, and 5g fat) with the addition of 300 mL of water supplemented with 4% with L-[ring- $^{13}\text{C}_6$]phenylalanine according to the phenylalanine content of pork to minimize disturbances in isotopic equilibrium during the infusion trial ($t=0$). Analysis of pork composition is described elsewhere (Beals et al., 2016). Additional

muscle biopsies were collected at $t=120$ and 300 min after pork ingestion. Arterialized blood samples (8 mL) were drawn every 30 or 60 min in EDTA – containing tubes and were spun at $3000 \times g$ at 4°C for 10 min then stored at -80°C . Plasma samples obtained before and after pork ingestion at $t=120$ and 300 min were used to assess the post-prandial proteome profile.

Western blot analysis

Muscle samples were homogenized using previously reported methodology (Beals et al., 2016). An aliquot of muscle homogenate representing the sarcoplasmic fraction was used for chemiluminescent Western blot analysis. Prior to blotting, total protein was quantified using the Lowry method (Bradford assay; Bio – Rad). Equal quantity of protein (20 μg) was loaded to 7.5% precast polyacrylamide gel. After blocking, membranes were incubated in primary antibodies at 4°C overnight to determine the total protein of IP6K1 (Abcam, USA), total and phosphorylated Akt Ser⁴⁷³ (Cell Signaling, USA) and total IGFR (Cell Signaling, USA). Following one-hour incubation with appropriate secondary antibodies, membranes were imaged using Li – Cor Fe and quantified using Image Studio Pro. Band normalization was quantified by measurement of total protein stain (Bio Rad, USA).

Plasma Analysis

Plasma IGF-1 were determined using commercially available enzyme-linked immunosorbent assays following manufacturer's instructions (IGF-1: Sigma Aldrich, USA). Plasma L-[ring-¹³C₆]phenylalanine enrichments were measured by GC/MS using electron impact ionization as previously described (Beals et al., 2016).

Plasma proteomics

Plasma samples (10 μL) were prepared for LC-MS/MS analysis by first depleting 12 commonly most abundant proteins using spin columns according to the manufacturer's instructions (Pierce Top 12,

Pierce Biotechnology, Rockford, IL USA) centrifuged at $1000 \times g$ for 2 min. Spin columns contained antibodies for α 1-Acid glycoprotein, α 1-Antitrypsin, α 2-macroglobulin, Albumin, Apolipoprotein A-I, Apolipoprotein A-II, Fibrinogen, Haptoglobin, Immunoglobulin A, Immunoglobulin G, Immunoglobulin M, and Transferrin. Subsequently, sample clean-up was performed using a commercially available sample preparation kit (G-Biosciences, St. Louis, MO, USA). Sample was digested with mass spectrometry grade-Trypsin (G-Biosciences, St. Louis, MO) at a w/w ratio of 1:10 using a microwave digestion system (CEM Discover, Mathews, SC) at 55°C and maximum power of 60 watts for 30 minutes. Digested peptides were lyophilized in a Virtis-55 lyophilizer to dryness. The digested peptides were dissolved in 5% acetonitrile + 0.1% formic acid for LC/MS.

LC-MS/MS was performed using a Thermo Dionex Ultimate RSLC3000 operating in nano mode at 300 microliters/min with a gradient from 0.1% formic acid to 100% acetonitrile + 0.1% formic acid in 120 minutes. The trap column used was a Thermo Acclaim PepMap 100 (100 μ m x 2 cm) and the analytical column was a Thermo Acclaim PepMap RSLC (75 μ m x 15 cm). 2 micrograms of the digested peptides were loaded per injection.

The raw data files from the Thermo Fusion Orbitrap were processed using Mascot Distiller using the built-in processing parameter set Orbitrap_low_res_MS2_1.opt to yield MGF peak lists that were subsequently submitted to Mascot server for analysis. The Mascot search parameters were 10 ppm precursor mass and 0.6 Da fragment pass tolerance with up to 5 missed cleavages using trypsin. SwissProt was used as the database with the taxonomy of *homo sapiens*. Two quantitation methods were used and results from both the T3PQ (Grossmann et al., 2010) and emPAI scores (Ishihama et al., 2005) were used for analysis. Only protein hits with Mascot scores that indicated a $P < 0.05$ were considered significant and used for further analysis. All proteins significantly identified in ≥ 3 samples were used for functional classification.

Calculations

Rate of disappearance (R_d), R_a and MCRs were calculated as previously described by the authors (Mackenzie et al., 2012; Gastaldelli et al., 1999; Bergman, 1989). Where f is isotope infusion rate ($\mu\text{mol}\cdot\text{kg}^{-1}$), V is the volume of distribution of phenylalanine assumed to be equal and constant (Kaufman et al., 1999; Gastaldelli et al., 1999). The concentration (C) of the tracee at time (t) can be calculated from the endogenous measured concentration (C_m) of phenylalanine and the enrichment at that time. C_1 and C_2 ; phenylalanine concentrations and time points 1 (t_1) and 2 (t_2) (Brooks et al., 1991). IE is tracer enrichment expressed as atoms percent excess (APE) at sampling times minus the background enrichment value determined from basal plasma samples (i.e. pre bolus and infusion) (Rosenblatt et al., 1992).

$$C = C_m / (1 + IE)$$

$$R_a(t) = \frac{f(t) - V \cdot C(t) \cdot \frac{dIE(t)}{dt}}{IE(t)}$$

$$R_d = R_a - V [(C_2 - C_1) / (t_2 - t_1)] \text{ (body wt}^{-1}\text{)}$$

$$MCR = R_d / [(C_1 + C_2) / 2]$$

***In Vitro* Experiment**

C2C12 myoblasts (ATCC # CRL – 1772) derived from mouse skeletal muscle were cultured in growth media (GM) of Dulbecco's modified Eagle's Media (DMEM; Gibco), supplemented with 10 % FBS, penicillin ($50 \text{ U}\cdot\text{mL}^{-1}$) and streptomycin ($50 \text{ U}\cdot\text{mL}^{-1}$) in standard culture conditions (37°C , 5

% CO₂, 100 % humidity) until 80% confluence. Cells were then trypsinized and seeded into 6 well plates. To induce differentiation from myoblast to myotube, cells were first washed in Dulbecco's PBS (dPBS) and cultured in differentiation media (DM) of DMEM with 2 % donor equine serum, penicillin (50U.mL⁻¹) and streptomycin (50 U.mL⁻¹) for 96hours with DM changed every 24 hours until experimental treatments were administered.

Following differentiation from myoblast to myotube, the mature C2C12 myotubes were incubated under control (DM) and IGF-1 (Long R³ IGF-1; Sigma Aldrich) in a serial concentration of 10, 30 and 50ng/mL. All treatments were contained in serum-free DMEM and passed through a 0.22µm Millipore filter to ensure sterility. At the 24hour point, treatments were aspirated, cells were washed on ice with ice cold PBS prior to 400µL of cell lysis buffer with phosphatase & protease (1:100; Cell Signalling) inhibitor was added. Following a 20 minute incubation, cells were scraped into 1.5mL Eppendorf tubes, spun for 6 minutes at 700G, supernant removed and used for later analysis of proteins by Western blot methodology (described above).

Statistics

Differences in plasma IGF-1, R_d, metabolic clearance rate (MCR), muscle IGFR, muscle IP6K1, muscle P/tAkt⁴⁷³, were examined by two factor (group x time) repeated measures analysis of variance (ANOVA). Tukey's *post-hoc* test located specific differences between means for all significant main effects and interactions. Participant characteristics, muscle signalling, and plasma variables were correlated with R_d using a Pearson's correlation coefficient. Calculations were performed using IBM SPSS Statistics Version 21. Plasma proteome analysis was conducted using two-factor (group x time) repeated measures ANOVA with sex as a covariate using log₂ (Average value + 0.5). The false discovery rate was set at 0.05. Statistical analysis of the plasma proteome was conducted using R (version 3.4.0) and the limma package (version 3.32.2). Functional classification of proteins was analysed using statistical overrepresentation tests (PANTHER classification system, Gene Ontology

database; Mi et al. Nature Protocol: 2013 July 18 Supplement 1551 – 66) with Bonferroni corrections applied. Data are expressed as means \pm SEMs. Significance was accepted at $P < 0.05$.

Results

Plasma Analysis

Concentration of IGF-1 in plasma was significantly greater in OW individuals compared to OB (Figure 1A, $P = 0.009$, +61%) but not lean at basal ($P = 0.088$). Following pork ingestion, lean individuals increased IGF-1 concentration at 120 min compared to basal ($P = 0.001$, +26%), whereas in OW individuals this decreased ($P = 0.02$, -17%) and OB remained unchanged ($P > 0.05$). Thus, lean ($P = 0.002$, +46%) and OW ($P = 0.046$, +34%) individuals had significantly greater IGF-1 concentration compared to OB during the post-prandial period. Plasma phenylalanine (data not shown; Beals et al., 2017) concentrations increased from basal ($P < 0.001$), but did not differ between groups after the ingestion of pork ($P > 0.05$).

Concentration of FFA between groups was not different at basal or 120 min ($P > 0.05$) of the post-prandial period. However, all groups had reduced concentration of FFA at 120 min after pork ingestion when compared to basal and 300 min (Figure 1C, $P = 0.001$). Similarly, 300 min after pork ingestion, lean ($P = 0.001$, +54%) and OW ($P = 0.046$, +21%), but not OB ($P = 0.091$) individuals had increased plasma FFA compared to basal. Finally, at the same time point, lean individuals had a significantly greater concentration of FFA compared to OW individuals ($P = 0.037$, +36%).

Figure 1.

Muscle IGF-1 Signalling

Basal IGFR protein content in skeletal muscle was not different between groups ($P > 0.05$), however only OB individuals increased IGFR protein content at 120 min of the post-prandial period ($P = 0.02$,

+20%; Figure 2A), with IGFR decreasing back to basal concentrations at 300 min (Figure 2A). IGFR remained constant in lean and OW at all time points ($P > 0.05$). However, OB adults had reduced IGFR at 300 min when compared to 120 min of the post-prandial period ($P = 0.033$, -22%). OW individuals showed reduced IGFR protein compared to the OB group ($P = 0.036$, -69%) at 120 min of the post-prandial period. There was no difference between OW and lean groups ($P > 0.05$) at all time points.

Relative IP6K1 protein content increased in OB individuals in the post-prandial state (120min; $P = 0.034$, +52%) but was not different between groups (Figure 2B, 120min; $P > 0.05$). A trend was present between OW individuals who showed greater IP6K1 protein content versus lean (120min; $P = 0.079$, +71%) and OB versus lean (120min; $P = 0.101$, +66%). No between group differences were observed at 300min post-prandial ($P = 0.265$). P/tAkt⁴⁷³ was not different among groups in both the post-absorptive and post-prandial states (Figure 2C, $P = 0.151$). OW individuals tended to demonstrate increased Akt⁴⁷³ phosphorylation compared to lean at basal ($P = 0.079$, +23%) whilst OW had a similar response compared to OB at 300 min ($P = 0.058$, +34%).

Figure 2.

Rate of Phenylalanine Appearance (R_a), Rate of Phenylalanine Disappearance (R_d) and Metabolic Clearance Rate (MCR)

Phenylalanine R_a was increased in lean individuals versus OB ($P=0.028$) in the basal state and lean individuals R_a was significantly reduced from basal to 300min ($P=0.038$). Basal phenylalanine R_d was not different between OB and lean individuals at basal (Figure 3A, $P = 0.056$), or OB and OW or OW and lean (all, $P > 0.05$). There was no effect of pork ingestion on skeletal muscle R_d across the groups ($P > 0.05$). MCR was not different at basal among groups, but decreased in response to the high protein food in lean, OW and OB at 120min whilst only lean and OW maintained the decreased MCR at 300 min (Figure 3B, $P < 0.05$).

Figure 3.***Basal Correlation Data***

In the post-absorptive state, phenylalanine R_d is positively correlated with plasma IL-6 ($R = 0.413$, $P < 0.05$) and MCR ($R = 0.680$, $P < 0.001$). MCR of lean body mass was not significantly correlated with any variable aside from R_d in the post-absorptive state ($P > 0.05$).

Table 2.***Post-Prandial Correlation Data***

Phenylalanine R_d was positively correlated with IGFR ($R = 0.400$, $P < 0.05$) and MCR at 120min ($R = 0.633$, $P < 0.05$), whilst negatively correlated with SNAT2 at the same time point ($R = -0.376$, $P < 0.05$).

Table 3.***Proteomics***

The relative abundance of 517 and 139 plasma proteins using emPAI scores and the Top 3 Protein Quantification (T3PQ) method, respectively were included in our analysis. Statistical analyses of results from both approaches yielded similar findings with respect to BMI, time, and sex. Therefore, we elected to focus on the results from the T3PQ method since it provided more conservative results. No group differences were found in any of the proteins detected (Figure 4, $P > 0.05$). Similarly, there was no effect of pork ingestion on the circulating proteome (Figure 5, $P > 0.05$). There was an effect of sex where proteins involved primarily in plasma lipid handling were detected at greater levels in men vs. women (Table 5, $P < 0.05$). KEGG pathway analysis depicted 28 functional classifications significantly represented by the proteins found ($P < 0.05$). In addition, GO overrepresentation analysis

of the proteins identified 1306 biological processes and 163 molecular functions significantly represented in the data ($P < 0.05$). The top 10 most overrepresented protein functional classifications from the PANTHER overrepresentation tests are presented in table 4.

Table 4.

Table 5.

Figure 4.

Figure 5.

Protein Signalling in C2C12 Muscle Cells

Low concentration of IGF-1 significantly increased IP6K1 content in C2C12 myotubes ($P = 0.021$; +75%). There was no difference between control, moderate ($P = 0.181$; + 69%) and high IGF-1 concentrations ($P = 0.388$; +72%; Figure 6A). Downstream, neither p/tAkt³⁰⁸ and p/tAkt⁴⁷³ was not different among treatments (Figure 6B & 6C).

Figure 6.

Discussion

High protein diets are commonly used for weight management and treatment of obesity-related co-morbidities. This is the first study to characterize IGF-1/IP6K1/Akt signalling in skeletal muscle and post-prandial circulating proteome after lean meat ingestion, a common component of a western dietary pattern, in sedentary young adults of various BMIs and fat masses. The novel finding here is that muscle IP6K1 protein content is elevated in individuals with obesity after the ingestion of lean meat when compared to lean adults, suggesting that IP6K1 may be implicated in the reduction of nutrient uptake in skeletal muscle in the post-absorptive state in obese individuals (Naufahu et al., 2018). Our data also showed that IGFR signalling in skeletal muscle is elevated in the obese phenotype which is present in the face of reduced plasma IGF-1 in the same population compared to lean controls. Indeed, we hypothesised that adults with obesity would demonstrate differentially expressed circulating proteins yet our data showed no clear differences in the postprandial plasma

proteome between groups. Hence, this high-throughput analysis did not provide insight into postprandial circulating events that may contribute to dysregulated molecular signalling in insulin resistant obese muscle.

IP6K1 is thought to inhibit Akt phosphorylation via the secondary messenger IP7 with its subsequent binding to Akt's PH domain (Chakraborty et al., 2010). IP6K1 is elevated in the post-prandial state, and this may be an important cause or at least a consequence of poor amino acid metabolism in skeletal muscle as supported in the obese rodent (Friedman et al., 1990), obese humans (Liebau et al., 2014) and our recent work showing reduced peripheral glucose uptake post intravenous glucose administration in insulin resistant pre-diabetic individuals (Naufahu et al., 2018). Given that one of IP6K1 proposed actions is to inhibit Akt activity, we would have expected a reduction in phosphorylated Akt⁴⁷³ at the same time point, yet our current data does not support this. The lack of change in p/tAkt⁴⁷³ may be due to the increase in IP6K1 whereby p-Akt³⁰⁸, not p-Akt⁴⁷³, is downregulated in response to increased IP6K1 muscle content (Chakraborty et al., 2010; Naufahu et al., 2018). With the ingestion of a sole food source, glucose handling is not a particular relevant variable in this study. However, it is worth acknowledging that the pork contained ~3.3 g leucine, which is a particularly strong insulin stimulator (van Loon et al., 2000). Furthermore, a protein feed without carbohydrate has the ability to cause acute insulin resistance due to increased glucagon secretion (Lee et al., 2016; Calbet & MacLean, 2002). To probe additional upstream mechanisms that may underpin the obesity-related dysregulation of mTORC1 signalling (Beals et al., 2016), we examined muscle IGFR protein content (an upstream regulator of Akt and mTORC1) in the volunteers of different BMIs. Like IP6K1, muscle IGFR protein content increased at 120 min in the post-prandial period from basal in the obese group, and was greater in the obese compared to overweight group. This finding could demonstrate a compensatory mechanism due to decreased plasma IGF-1 in the obese individuals and/or hyperinsulinemia, and not IGF-1, contributes to increased IGFR-IP6K1 muscle content. Indeed, O'Neill et al. (2015) showed that IRS-1 and IGF-1Rs are required for hypertrophic muscle protein remodelling in mice, which highlights the protein interaction between these otherwise separate pathways in a rodent model.

It appears that IP6K1 muscle content in overweight men and women is increased after protein ingestion, however large variation within this data set may have contributed to a lack in statistical significance ($P = 0.084$). This is also the first set of data that has investigated the IP6K1 response to meat ingestion. Lean and overweight groups had similar glycaemic and insulinemic responses to a protein-dense feed. We would therefore not expect IP6K1 to be different between these groups (Chakraborty et al., 2010; Ghoshal et al., 2016). However, obese individuals in this study were insulin resistant and displayed hyperinsulinemia (data presented Beals et al., 2016), which may have contributed to the increased IP6K1 at 120 minutes post pork ingestion given that the hyperinsulinemia is known to drive IP6K1 activity (Chakraborty et al., 2010). It is hypothesised that over stimulation of the insulin signalling pathway by insulin, not IGF-1, in the obese group results in increased IP6K1 activity that maybe contributing to a reduction in protein synthesis (Beals et al., 2016). We see indirect support for this notion with a previously documented decrease in muscle IP6K1 content with an increase in phosphorylation of Akt and subsequent improvement in insulin sensitivity (Naufahu et al., 2018). IP6K1 inhibition using TNP in insulin-stimulated hepatic cell lines and IP6K1 KO mice show hyper Akt activation (Ghoshal et al., 2016; Chakraborty et al., 2010), suggesting that IGF-1 and insulin may act independent of each other in the potential IP6K1–Akt signalling pathway. In a separate *in vitro* experiment using the C2C12 muscle cell line we investigated the effects of IGF-1 on IP6K1. Low IGF-1 concentration (10 ng/mL) was sufficient to increase muscle IP6K1 content while increasing IGF-1 to 30 and 50 ng/mL showed no difference from control cells (Figure 6A). Interestingly, our *in vitro* and *in vivo* data agree whereby obese individuals who had the lowest plasma IGF-1 concentration, also increased their muscle IP6K1 at 120 and 300min which suggests that whilst higher concentrations of IGF-1 may not affect long term muscle function (Morton et al., 2016), it may be favourable for reducing IP6K1 muscle content and therefore increasing insulin sensitivity. Yet this is a notion that requires further investigation.

We have previously shown that mTORC1 is hyper-phosphorylated in a post-absorptive-state in obese individuals, yet in response to a large oral protein load mTORC1 phosphorylation is blunted compared to lean controls (Beals et al., 2016). However, there was an increase in the muscle protein

content of the large neutral amino acid transporter-1 (LAT1) and sodium dependent neutral amino acid transporter-2 (SNAT2) at 5 hr of the post-prandial period after a high protein food ingestion irrespective of BMI score in sedentary young adults (Beals et al., 2016). It has been suggested that these amino acid transporters may play a key role in the activation of the mTORC1 signaling pathway via an amino acid transporter/receptor (Gran & Cameron-Smith, 2011; Drummond et al., 2011; Beals et al., 2016). As such, we tested the relationship with these amino acid transporters with phenylalanine uptake in skeletal muscle and its MCR, observing a negative correlation between phenylalanine uptake in skeletal muscle and SNAT2 muscle content at 120 minutes. Moreover, it is worth highlighting that the total protein content of amino acid transporters as determined by Western Blot does not provide information into the intracellular location of these transporters nor their activity and/or transport capacity (Hodson et al., 2017). Thus, we are unable to conclude that there are no defects in amino acid transport capacity in people with excess fat mass.

We have previously established a clear defect in the post-prandial myofibrillar protein synthetic response in obese adults (Beals et al., 2016), yet skeletal muscle phenylalanine R_d is not different in this group compared to lean individuals (Figure 3B). Phenylalanine R_d is known to reflect skeletal muscle amino acid uptake, oxidation and protein and hormone/metabolite synthesis which drives the utilization of ingested nutrients to deposit them to various tissues (Matthews, 2007). The current study demonstrated a trend towards lower R_d in obese individuals at basal ($P = 0.056$), suggesting that insulin resistant obese individuals may suffer from reduced skeletal muscle amino acid uptake in the post-absorptive state (Liebau et al., 2014). Obese individuals have elevated mTORC1 signalling in the post-absorptive state, and therefore this exacerbated activation of this protein complex of mTOR may be already over stimulated which makes the muscle insensitive to further uptake of remaining amino acids (Beals et al., 2016). Contrary to this, there was no difference within, or between groups for skeletal muscle R_d of phenylalanine, suggesting that the ability of obese individuals to dispose of amino acids in skeletal muscle is normal and that the mechanism driving anabolic resistance may be explained by hyperinsulinemia (data not shown), intracellular mTORC1 and associated protein signalling (Beals et al., 2016), particularly given the finding that plasma IGF-1

is suppressed in this population (Figure 1B). We acknowledge that there is a gap between the skeletal muscle uptake of amino acids and molecular signalling in skeletal muscle, however ingested amino acids predominantly deposit into skeletal muscle versus other tissues (Chang & Goldberg, 1978).

To help examine skeletal muscle phenylalanine R_d and muscle anabolic response further, we investigated whether obese insulin resistant individuals display a different circulating proteome in both the basal and post-prandial states compared to our healthy lean and overweight sample. This is the first work of its kind to profile the plasma proteome in response to a typical dinner amount of dietary protein (36 g) across various BMI scores. We observed no differences in the postprandial circulating proteome in the basal and post-prandial states in obese individuals. It is worth noting that previous work has demonstrated that fasting-state proteomic signatures in an overweight female adolescent population correlated with $HOMA_{IR}$ (Rothwell et al., 2011). However, our work is generally consistent with studies in adults that showed the post-prandial plasma proteome is unchanged after the ingestion of a low protein, but high in fat, meal (Pellis et al., 2012), and consistent with the notion that changes in the plasma metabolome are more sensitive to an acute meal setting (Pellis et al., 2012).

Some limitations are present in the current study which must be acknowledged. Firstly, the small sample size between groups is important when drawing conclusions from the data. Secondly, due to lack of sample, IRS-1 muscle protein data is missing from our data set and would have been a useful protein target for our interpretation of IGF-1 and IGFR signalling in the overweight and obese adults. Finally, we acknowledge that there is a gap between the skeletal muscle uptake of amino acids and molecular signalling in skeletal muscle, however ingested amino acids predominantly deposit into skeletal muscle versus other tissues (Chang & Goldberg, 1978).

To conclude, we characterized IGF-1/IP6K1/Akt signalling in individuals with various levels of adiposity and BMI scores before and after lean meat ingestion and showed that IP6K1 was acutely elevated in obese individuals post feeding. This finding, in combination with the hyperinsulinemia (Beals et al., 2016) and the trend for reduced phenylalanine R_d , and our previous findings (Naufahu et al., 2018) suggest that IP6K1 may be contributing to regulation of nutrient uptake of skeletal muscle

in the post-prandial state. Our data showed no clear differences in plasma proteomic signatures between groups despite overt differences in muscle nutrient signalling. We therefore conclude that acute changes in the post-prandial plasma proteome does not contribute significantly to altered molecular signalling in skeletal muscle and that the mechanism(s) driving anabolic resistance may be explained by intracellular mTORC1 and associated protein signalling.

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Statement of Authorship

RDB, JWB, NAB, and RWM contributed to the conception and the design of the experiment. RDB, JWB, JD, BSI, PMY, AVU, MVM, SAP, NAV and RWM contributed to collection, analysis, and interpretation of data. RDB, JWB, NAT, PW, NT, NAB, and RWM contributed to drafting or revising intellectual content of the manuscript. RB, NAB, and RWM had primary responsibility for final content. All authors read and approved the final manuscript.

Conflict of Interest Statement

There are no conflicts of interests to disclose.

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Figure 1. Plasma concentration of insulin like growth factor 1 (IGF-1) in the basal and in post-prandial state (A) ($n = 10/\text{group}$). Plasma concentration of free fatty acids (FFA) in the basal and in post-prandial state (B). * $P < 0.05$ vs basal. # $P < 0.05$ vs OB. + $P < 0.05$ vs OW. ^ $P < 0.05$ 120min vs 300min. Data are mean \pm standard error of the mean.

Figure 2. Skeletal muscle protein content for insulin like growth factor receptor (IGFR; A) and inositol hexakisphosphate kinase 1 (IP6K1; B) content in the basal state and after ingestion of pork (A, $n = 6/\text{group}$; B, $n = 10/\text{group}$). Skeletal muscle protein content for phosphorylated/total Akt Ser473 (P/tAkt⁴⁷³; C) in the basal state and after ingestion of pork ($n = 10/\text{group}$). * $P < 0.05$ vs basal. # $P < 0.05$ vs 120min. ^ $P < 0.05$ OW vs OB. Data are mean \pm standard error of the mean.

Figure 3. Rate of disappearance (R_d) of L-[ring-¹³C₆]phenylalanine (A), metabolic clearance rate (MCR; B) and rate of appearance (R_a) of L-[ring-¹³C₆]phenylalanine (C) in the basal state and after pork ingestion ($n = 10/\text{group}$) for lean body mass (LBM). * $P < 0.01$ vs lean. # $P < 0.05$ vs basal. ^ $P < 0.05$ vs 300min. ^ $P < 0.05$ vs OB. Data are mean \pm standard error of the mean.

Figure 4. Plasma proteomic signature organized by BMI category.

Figure 5. Plasma proteome in response to protein-dense food ingestion.

Figure 6. C2C12 muscle protein content for inositol hexakisphosphate kinase 1 (IP6K1; A), Phosphorylated/total Akt Thr³⁰⁸ (B), Phosphorylated/total Akt Ser⁴⁷³ (C), and respective blot images for protein targets (D) ($n=6/\text{treatment}$). * $P < 0.05$ vs Control. Data are mean \pm standard error of the mean.

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Table 1. Participant Characteristics.

Variable	Lean	Overweight	Obese
Participants (males)	10 (5)	10 (5)	10 (5)
Age (years)	24 ± 1	26 ± 2	27 ± 3
Height (m)	1.73 ± 0.03	1.70 ± 0.02	1.71 ± 0.03
Weight (kg)	68.5 ± 3.5	78.6 ± 2.2	106.0 ± 5.0* [‡]
BMI (kg/m ²)	22.7 ± 0.4	27.1 ± 0.5	35.9 ± 1.3
Waist: Hip	0.79 ± 0.02	0.83 ± 0.02	0.92 ± 0.01* [‡]
Body Fat (%)	22.2 ± 1.8	29.1 ± 1.4*	35.3 ± 1.8* [‡]
Lean Body Mass (kg)	51.3 ± 3.7	53.8 ± 1.3	65.5 ± 3.2* [‡]
Fasting Glucose (mmol/L)	4.33 ± 0.12	4.35 ± 0.07	4.55 ± 0.12
120min Glucose (mmol/L)	4.36 ± 0.34	4.47 ± 0.23	5.51 ± 0.43* [‡]
Fasting Insulin (pmol/L)	49.31 ± 6.25	45.14 ± 3.47	175.01 ± 26.39* [‡]
HOMA _{IR}	1.36 ± 0.17	1.25 ± 0.11	5.82 ± 0.81* [‡]

Glucose values were obtained from the oral-glucose-tolerance test. Demographic, body-composition, and HOMA_{IR} data were analysed by 1-factor ANOVA. Fasting glucose and 120-min glucose data were analysed by using 2-factor repeated-measures ANOVA. A Tukey's post hoc test was used to locate differences between group means when indicated by significant group effects or group - time interactions. * $P < 0.05$ vs Lean group; [‡] $P < 0.05$ vs OW group. Glucose: group effect, $P = 0.59$; time effect, $P < 0.001$; group x time, $P < 0.001$; OB, obese; OW, overweight. Values are means ± standard error of the mean.

Table 2. Fasting and Post-prandial Phenylalanine Disappearance Rate Correlation analysis.

Variable	Rate of Disappearance (R _d) Basal (μmol.min ⁻¹ .kg ⁻¹)	Rate of Disappearance (R _d) 120min (μmol.min ⁻¹ .kg ⁻¹)	Rate of Disappearance (R _d) 300min (μmol.min ⁻¹ .kg ⁻¹)
Plasma IGF-1 (ng/mL)	.319	.074	.232
Muscle IGFR (A/U)	.145	.400*	-.113
Muscle IP6K1 (A/U)	-.082	.081	-.044
p/t Akt ⁴⁷³ (A/U)	-.045	-.262	.051
p/t Akt ³⁰⁸ (A/U)	-.102	.268	.282
Body Fat (%)	-.182	-.017	-.078
Lean Body Mass (g)	-.180	-.253	-.381

BMI (kg/m ²)	-.298	-.150	-.227
Waist: Hip	.220	.182	.211
HOMA _{IR}	-.182	.018	-.173
QUICKI	.265	-.101	.256
FISI	-.182	.017	-.430
Insulin (pmol/L)	-.178	.061	-.242
Blood Glucose (mmol/L)	-.134	.200	-.081
FFA (mM)	.244	-.87	.235
IL-6 (mg/dL)	.413*	-.063	.012
SNAT2 (A/U)	.146	-.376*	-.095
LAT1 (A/U)	-.138	.043	-.175

Values are *R* values obtained by Pearson's correlation coefficient to measure the strength and direction of correlation between variables with significance (two-tailed) indicated by **P* < 0.05 or ***P* < 0.01.

Table 3. Fasting and Post-prandial Phenylalanine Metabolic Clearance Rates Correlation Analysis.

Variable	Metabolic Clearance Rate (MCR) Basal (mL.min ⁻¹ .kg ⁻¹)	Metabolic Clearance Rate (MCR) 120 min (mL.min ⁻¹ .kg ⁻¹)	Metabolic Clearance Rate (MCR) 300min (mL.min ⁻¹ .kg ⁻¹)
Plasma IGF-1 (ng/mL)	-.011	-.102	.137
Muscle IGFR (A/U)	.282	.156	-.080
Muscle IP6K1 (A/U)	-.054	-.163	-.080
p/t Akt ⁴⁷³ (A/U)	-.225	-.251	.242
p/t Akt ³⁰⁸ (A/U)	.006	.128	.347
Body Fat (%)	.006	.192	.024
Lean Body Mass (g)	-.298	-.266	-.190

BMI (kg/m ²)	-.213	-.016	-.051
Waist: Hip	.143	.132	.125
HOMA _{IR}	-.196	.009	-.065
QUICKI	.193	-.023	.070
FISI	-.197	.007	-.308
Insulin (pmol/L)	-.189	.017	-.059
Blood Glucose (mmol/L)	-.116	.056	-.111
FFA (mM)	.059	.333	.061
IL-6 (mg/dL)	.110	.176	.152
SNAT2 (A/U)	-.184	-.028	-.146
LAT1 (A/U)	-.164	-.050	-.299

Values are *R* values obtained by Pearson's correlation coefficient to measure the strength and direction of association between variables with significance (two-tailed) indicated by **P* < 0.05 and ***P* < 0.01.

Table 4. Top 10 GO protein classifications.

Protein categories	Term	Representative protein(s)	<i>P</i> -value
Biological processes	Platelet degranulation	Platelet basic protein	2.56×10^{-53}
	Protein activation cascade	Complement C1 subunits	2.78×10^{-52}
	Acute inflammatory response	Complement factors	1.34×10^{-45}
	Defense response	LPS-binding protein	1.16×10^{-38}
	Regulation of proteolysis	Carboxypeptidase B2	4.67×10^{-38}
	Complement activation	Immunoglobulin	1.56×10^{-37}
	Proteolysis	Plasma serine protease inhibitor	9.36×10^{-37}
	Vesicle-mediated transport	Apolipoprotein D	2.77×10^{-36}
	Response to stress	Galectin-3-binding protein	2.46×10^{-35}
	Inflammatory response	Serum amyloid A	2.72×10^{-35}
Molecular function	Endopeptidase inhibitor activity	Kallistatin	1.37×10^{-31}
	Endopeptidase regulator activity	Protein AMBP	3.94×10^{-31}
	Peptidase inhibitor activity	Inter-alpha-trypsin inhibitor	6.59×10^{-31}
	Peptidase regulator activity	Kininogen-1	6.85×10^{-30}
	Enzyme inhibitor activity	Alpha-1-antichymotrypsin	4.55×10^{-27}

Serine-type endopeptidase inhibitor activity	Protein Z-dependent protease inhibitor	1.27×10^{-25}
Enzyme regulator activity	Angiotensinogen	2.23×10^{-18}
Serine-type endopeptidase activity	Prothrombin	1.53×10^{-16}
Glycosaminoglycan binding	Thrombospondin	5.25×10^{-16}
Serine-type peptidase activity	Plasminogen	8.45×10^{-16}

*LPS; lipopolysaccharide

Table 5. Top 10 GO terms differentially represented in males vs. females.

Protein categories	Term	P-value
Biological processes	Negative regulation of very-low density lipoprotein particle remodeling	1.20×10^{-03}
	Regulation of very-low density lipoprotein particle remodeling	2.10×10^{-05}
	Negative regulation of very-low density lipoprotein particle clearance	2.83×10^{-03}
	regulation of very-low-density lipoprotein particle clearance	2.83×10^{-03}
	Chylomicron remodeling	5.22×10^{-09}
	Chylomicron assembly	9.80×10^{-09}
	Positive regulation of cholesterol esterification	1.18×10^{-06}
	Complement activation, alternative pathway	2.53×10^{-10}
	Triglyceride-rich lipoprotein particle remodeling	2.53×10^{-10}
	Triglyceride-rich lipoprotein particle clearance	1.37×10^{-04}
Molecular function	High-density lipoprotein particle receptor binding	4.37×10^{-4}
	Phosphatidylcholine-sterol O-acyltransferase activator activity	1.59×10^{-5}
	Hemoglobin binding	2.01×10^{-03}
	Lipase inhibitor activity	1.01×10^{-05}
	Complement binding	3.93×10^{-07}
	Phosphatidylcholine binding	4.53×10^{-05}
	Cholesterol transporter activity	1.55×10^{-03}
	lipoprotein particle receptor binding	8.30×10^{-05}
	sterol transporter activity	9.63×10^{-03}
	cholesterol binding	1.10×10^{-03}

Highlights:

- IP6K1 muscle protein is increased in adults with obesity after feeding
- Proteomic profiles are not different between lean and obese adults
- Phenylalanine disappearance (R_d) is not different between lean and obese adults

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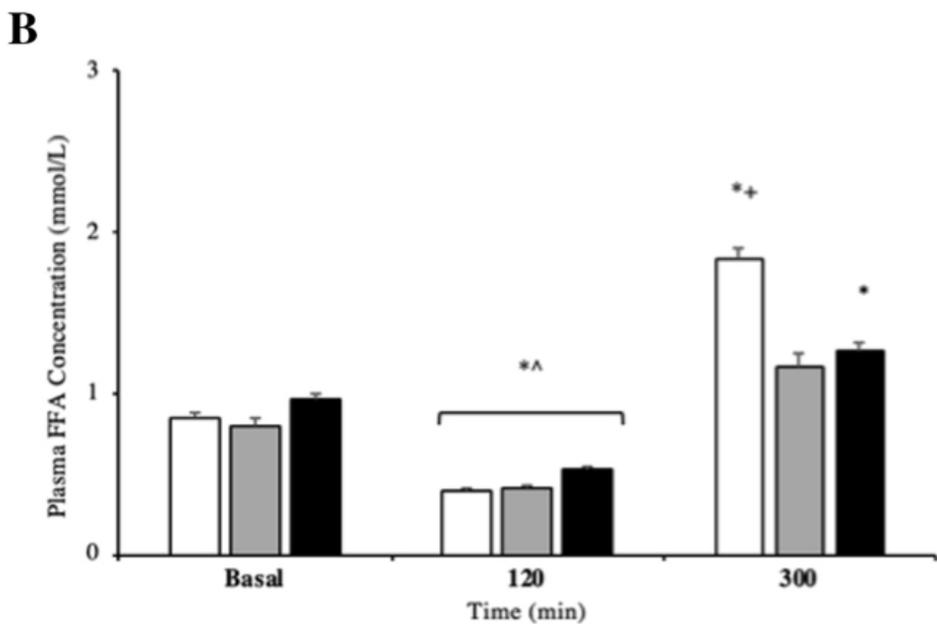
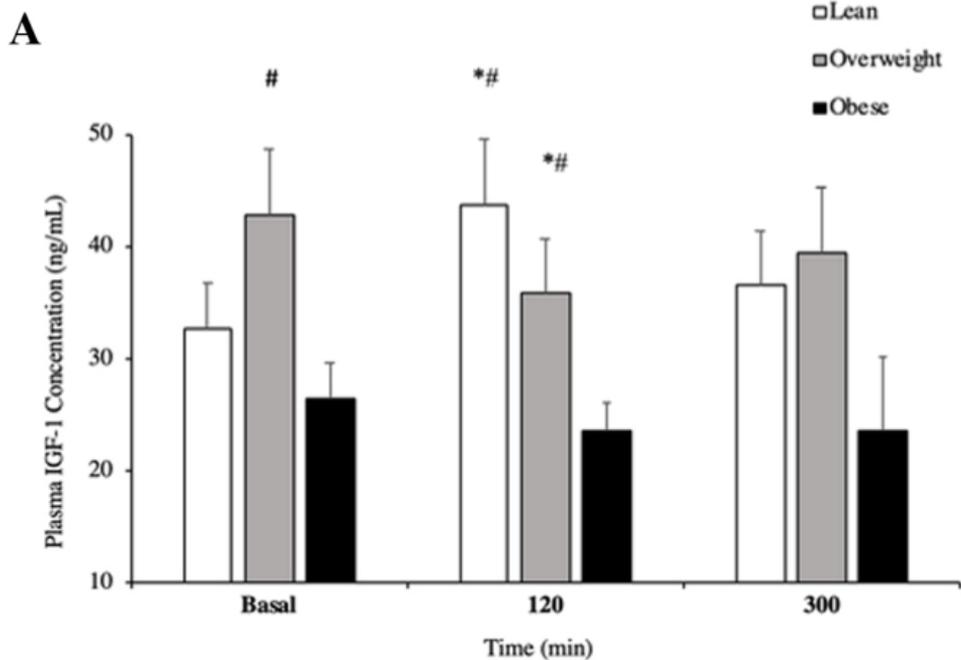


Figure 1

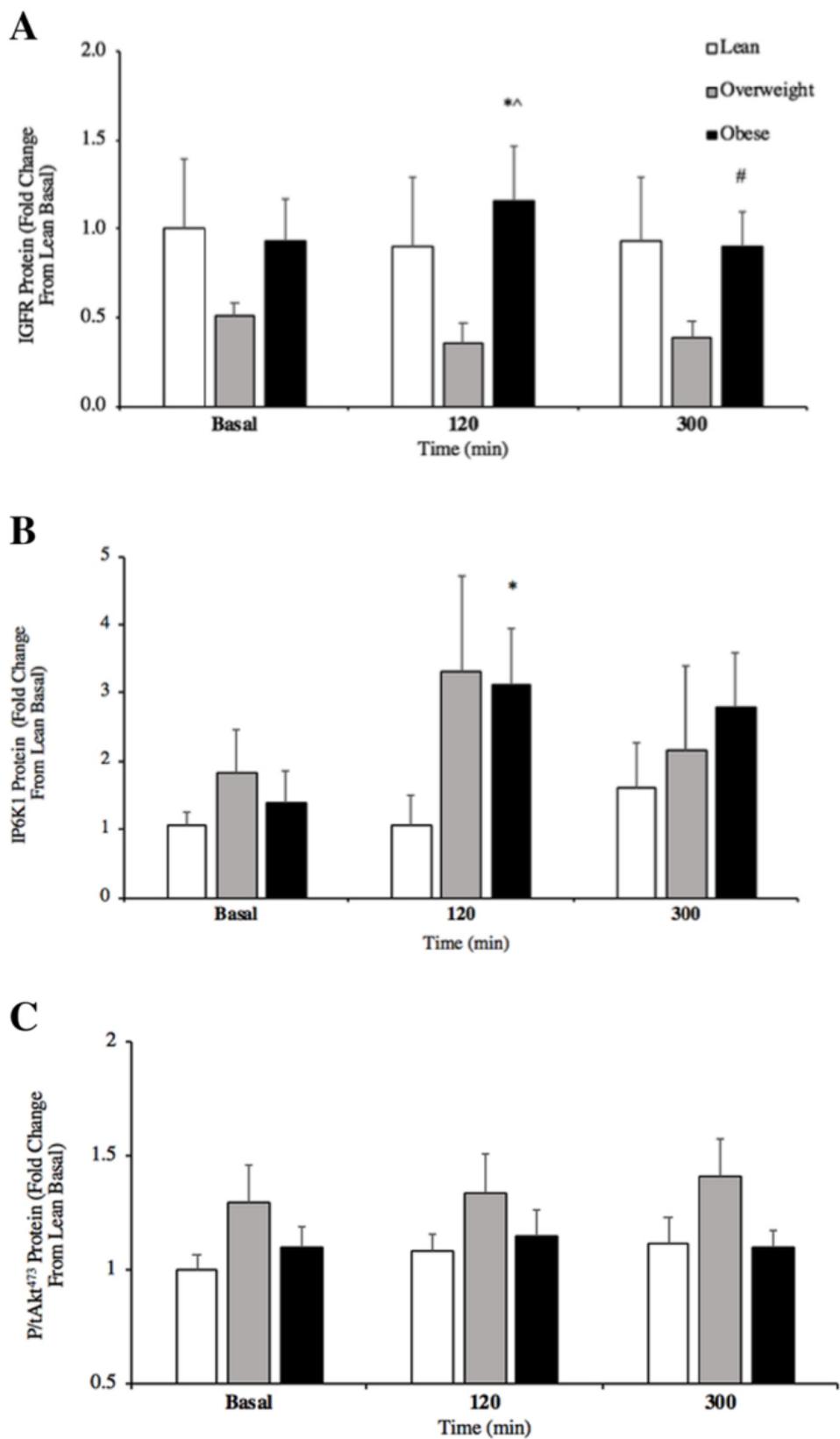


Figure 2

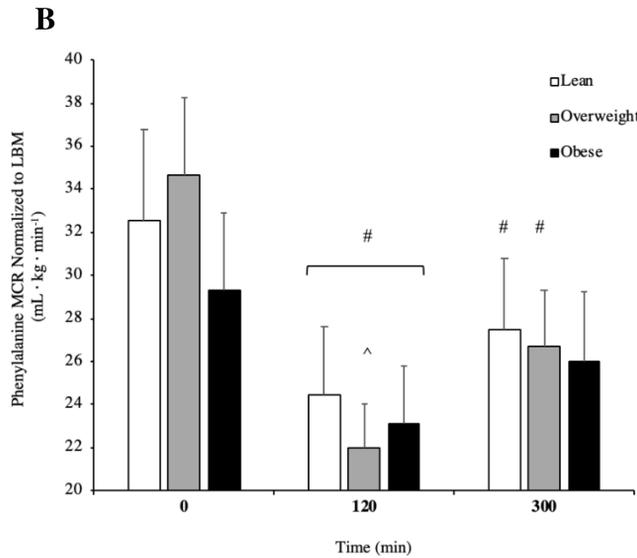
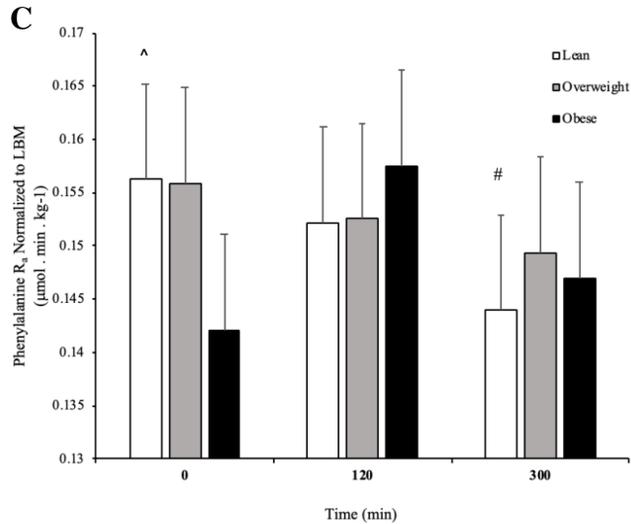
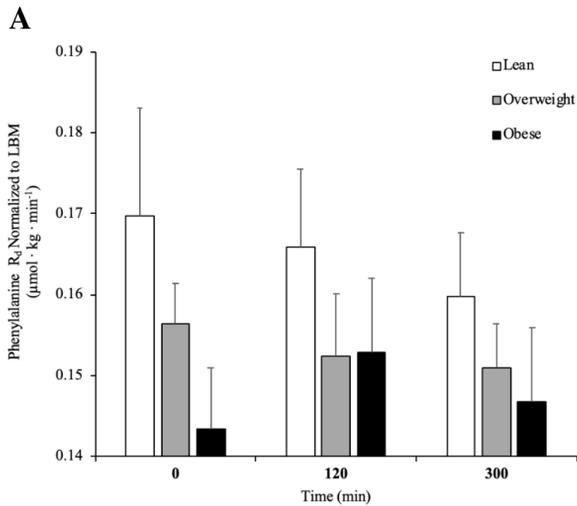


Figure 3

Color Key

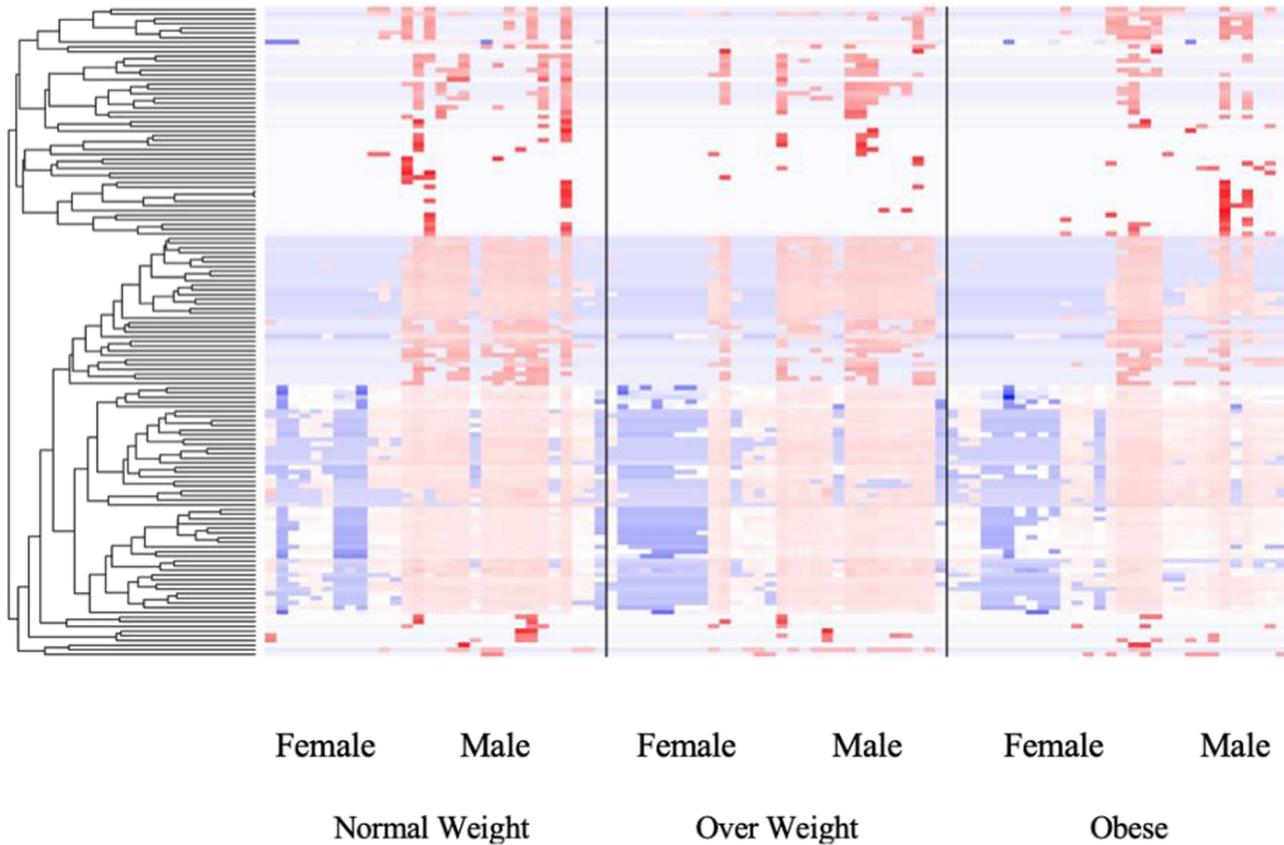
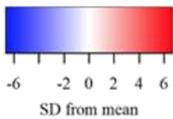


Figure 4

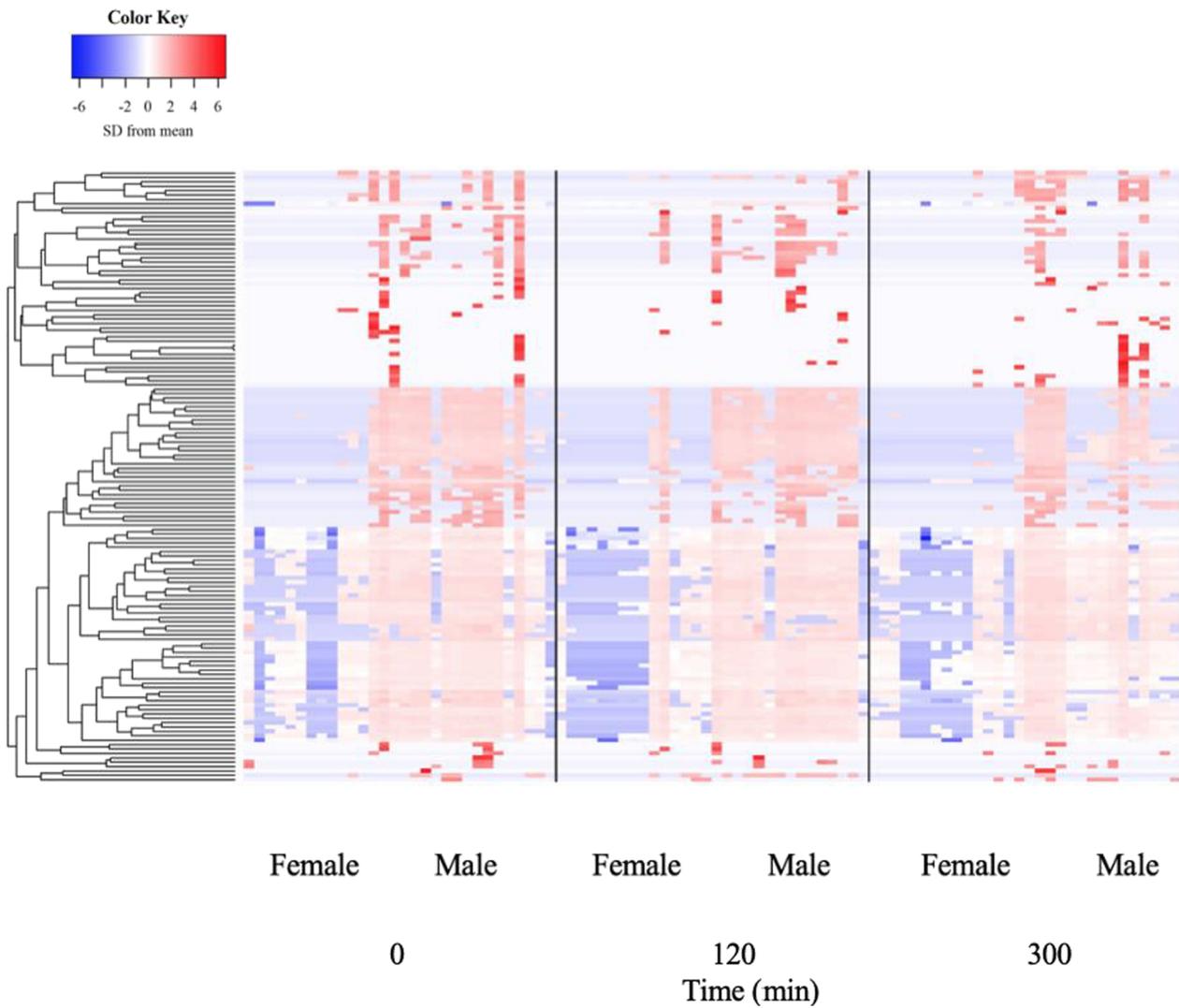


Figure 5

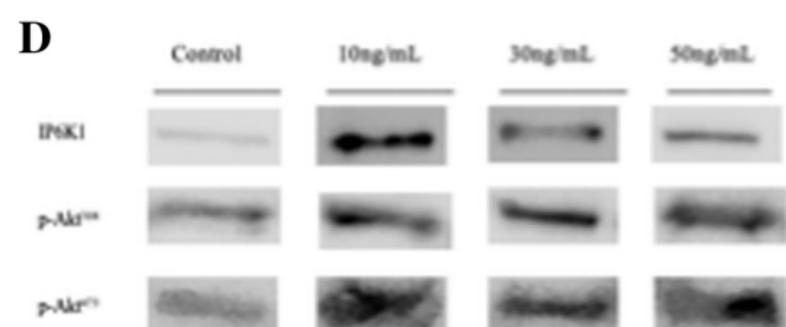
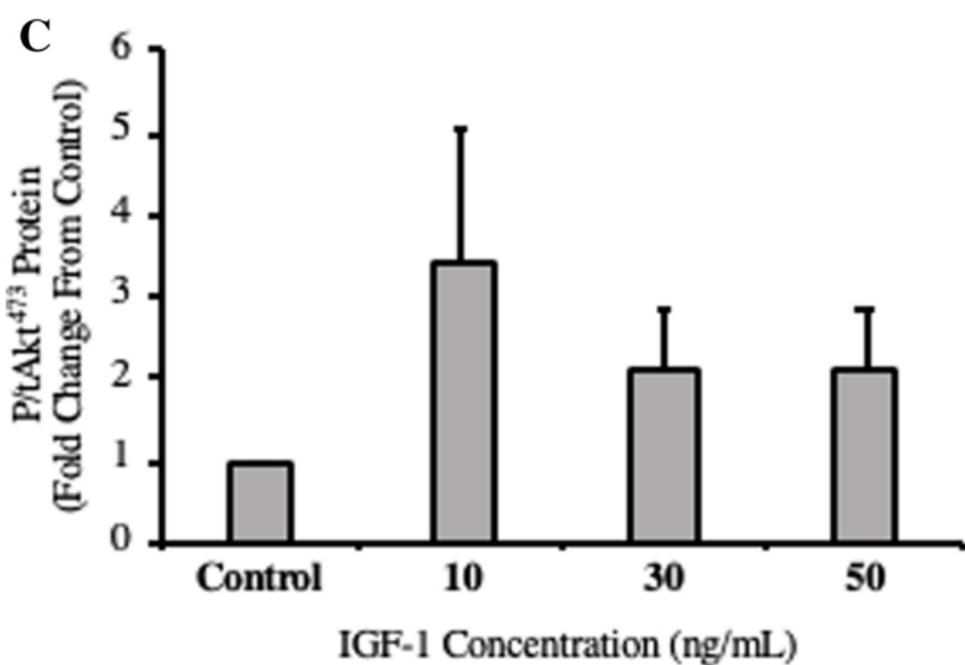
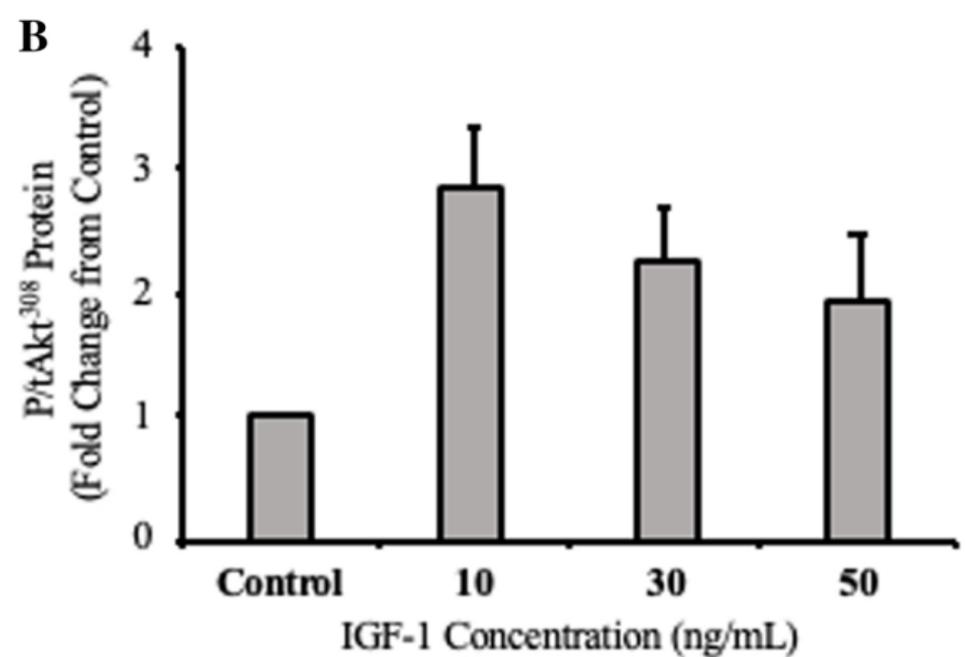
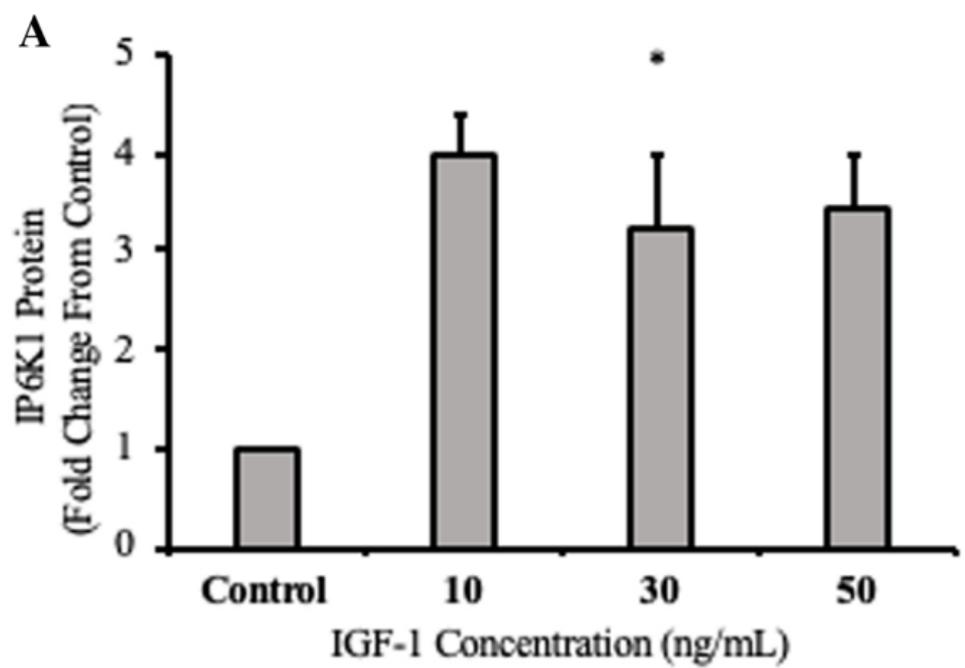


Figure 6