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Effect of vasodilation on the response of muscle protein synthesis to insulin in aging

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Effect of vasodilation on the response of muscle protein synthesis to insulin in aging

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Dedication

I would like to dedicate this thesis to Jayson Jay, Polly Lee, Billy Lee, Rebecca Lee, and Joan Jay. Thank you all for your love and support!

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Effect of vasodilation on the response of muscle protein synthesis to insulin in aging

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The loss of muscle with aging (sarcopenia) is an important contributor to disability and physical dependence in older people. Studies have recently found that aging is associated with a relative resistance of muscle proteins to the anabolic action of insulin, even in subjects who do not have diabetes. This alteration impairs the response of older muscle to the anabolic action of a mixed meal, and may contribute to muscle loss with aging.

The hypothesis is that aging reduces the vascular sensitivity to insulin, which prevents the physiological increase in blood flow and muscle perfusion in response to exogenous or endogenous insulin, thereby decreasing the response of muscle protein synthesis and net balance to the anabolic action of insulin. This study looked at whether the response of muscle protein synthesis and net balance to insulin improves in older subjects when blood flow and muscle perfusion are pharmacologically restored to youthful values.

Six elderly subjects were assigned to a control group which received an insulin infusion in one leg during the intervention period. Another six subjects in the experimental group received insulin as well as the vasodilator, sodium nitroprusside (SNP). Blood flow increased significantly ($P<0.001$) with the addition of SNP. Using stable isotope methodologies, it was determined that there were also increases in muscle protein anabolism with the addition of SNP. The muscle protein synthesis (mTOR-associated) cell signaling pathways also showed increased phosphorylation of specific insulin-related proteins when vasodilation was increased with SNP.

Therefore, this study showed that pharmacologically restoring blood flow and nutrient delivery to youthful values, did improve the response of muscle protein synthesis to insulin in older subjects. Decreases in blood flow and muscle perfusion are clearly important contributors to the insulin resistance of skeletal muscle in aging. Understanding the mechanisms behind the loss of muscle with aging will serve as a basis for developing effective treatments for sarcopenia in older individuals.

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BACKGROUND AND SIGNIFICANCE

Human life expectancy is steadily increasing, but aging is also associated with a progressive loss of physical independence, which significantly worsens quality of life. A fundamental cause of and contributor to disability in older people is sarcopenia, the involuntary loss of skeletal muscle mass, strength, and function found in older individuals, generally aged 65 and older. This degeneration of muscle leads to reduced function (1-3), thus increasing the risk of falls and vulnerability to injury (4,5). Sarcopenia can also lead to a reduction in physical activity, which may have other significant metabolic effects including decreased bone density, obesity and diabetes (1,6).

Muscle mass is determined by the balance between muscle protein synthesis and breakdown (7). Hence, the measurement of muscle protein synthesis and breakdown allows for the determination of the mechanisms underlying muscle loss, and for the testing of the efficacy of potential treatments. Although previous studies had suggested that sarcopenia may be due to a reduced basal rate of muscle protein synthesis (8-12), Dr. E. Volpi at UTMB has recently reported in the largest cohort of healthy men to date that despite a decline in muscle mass, basal muscle protein synthesis and breakdown are not reduced with healthy aging (13). Instead, the response of muscle proteins to the anabolic action of insulin is impaired in healthy older adults as compared to younger controls. This occurs despite normal glucose tolerance and normal muscle glucose metabolism.

We began focusing on the potential role of insulin resistance in the development of sarcopenia following a series of nutritional studies. This work highlighted that despite older subjects exhibiting a normal anabolic response by muscle proteins to an amino acid load (14-16), the addition of carbohydrate to the amino acid meal was not beneficial (17).

A subsequent study highlighted that insulin is unable to stimulate muscle protein synthesis and net muscle protein deposition in older subjects as opposed to younger controls in whom insulin exerted a significant anabolic effect (18). Specifically, muscle protein synthesis and breakdown were measured in young and older glucose-tolerant volunteers using stable isotope-labeled phenylalanine, femoral arteriovenous blood sampling, and muscle biopsies in the basal state, and during local insulin infusion in the femoral artery (0.15 mU/min/100ml leg volume), which increased insulin concentration in the femoral vein to post-prandial levels while avoiding a post-prandial systemic hyperinsulinemia and the consequent decrease in amino acid concentrations. To compare our data with those obtained by others in younger subjects, we used three different methods to estimate muscle protein turnover: a two-pool model, a three-pool model, and the precursor-product model (for synthesis only). Phenylalanine net balance across the leg increased both in young and elderly, but the response was significantly blunted in the elderly. This is because insulin increased muscle protein synthesis in the young but not in the elderly. Muscle protein breakdown did not change in young or elderly using both the two- and the three-pool model. Amino acid delivery to the leg was significantly increased only in the young due to increased blood flow during insulin in the young. In the same study, it was found that the responsiveness of muscle protein synthesis to insulin administered locally in one leg was associated with insulin-induced changes in amino acid delivery to the muscle tissue, which increased in the younger subjects while was unchanged in the older subjects. This, in turn, was due to a differential stimulation of blood flow by insulin in young and older subjects, with insulin increasing blood flow in the young but not in the elders (18).

This suggests that blood and nutrient supply may play an important role in the response of muscle proteins to insulin, and that this mechanism is impaired with aging. Since insulin-stimulated glucose uptake was not different between groups, it could be concluded that healthy aging induces an insulin resistance of muscle proteins regardless of glucose tolerance, and is probably due, at least in part, to an impaired response of blood flow to the vasodilatory effects of insulin.

The finding that leg blood flow did not increase in older subjects during either exogenous or endogenous hyperinsulinemia (17) was consistent with previously published data, and suggests an impairment in the ability of insulin to modulate blood flow in normal aging (19). Insulin increases blood flow by inducing a nitric oxide-dependent vasodilation of the precapillary arterioles in skeletal muscle (20,21), which is accompanied by capillary recruitment resulting in an increased and more homogenous tissue perfusion (5,22,23). This mechanism allows for a greater and more diffuse capillary exchange of substrates during hyperinsulinemia in skeletal muscle. Thus, the response of muscle perfusion may become rate-limiting for the action of insulin on muscle, and it is possible that in aging muscle protein is more sensitive than glucose turnover to changes in muscle perfusion.

In another study designed to assess whether changes in nutrient delivery to the muscle tissue affect the physiological response of muscle protein synthesis to insulin, muscle protein turnover was measured in 19 young healthy subjects at baseline and during insulin infusion at three physiological doses, chosen in order to expose the muscle tissue to increasing insulin levels while maintaining or reducing amino acid concentrations. This was accomplished with the administration of insulin in the femoral artery with no exogenous amino acid infusion. The lowest dose (0.05 mU/min/100ml leg

volume, corresponding to ~0.06 mU/kg/min) increased insulin concentrations in the femoral vein to insulin resistant fasting levels (30±8 μU/ml) while avoiding a significant systemic hyperinsulinemia, a reduction in amino acid concentration and an increase in blood flow. The middle dose (0.15 mU/min/100ml leg volume, corresponding to ~0.2 mU/kg/min) increased the insulin concentration in the femoral vein to prandial values (48±7 μU/ml) while avoiding a significant systemic hyperinsulinemia, and increasing blood flow and amino acid delivery to the leg. The high dose (0.3 mU/min/100ml leg volume, corresponding to ~0.4 mU/kg/min) increased the insulin concentration in the femoral vein to insulin resistant prandial values (85±5 μU/ml), induced a three-fold increase in the systemic insulin concentrations, increased blood flow, and significantly decreased amino acid concentrations in the femoral artery and delivery to the leg. As a result, muscle protein turnover was unaffected by the lowest and highest insulin doses, while significant increases in muscle protein synthesis and net balance were found at the middle dose.

Moreover, confirming the previous findings in young and older subjects given insulin, it was also found that insulin-stimulated changes in protein synthesis were strongly correlated to changes in amino acid delivery to the leg ($R=0.7353$, $P=0.0003$) and blood flow ($R=0.7276$, $P=0.0004$), but not to changes in arterial amino acid concentration or insulin concentration. Thus, it was concluded that insulin modulation of blood flow and amino acid availability seems to play an important role in the regulation of muscle protein synthesis by insulin in healthy glucose tolerant young subjects.

Following this conclusion, we have reanalyzed the previous data obtained in young and older subjects given oral amino acids with or without glucose (16,17), and assessed whether changes in amino acid delivery and/or blood flow, might have been

related to changes in muscle protein synthesis. When data from all subjects, young and old, were analyzed together, we found that there was a significant positive correlation between changes in blood flow (R=0.5015, P=0.0106) or amino acid delivery to the leg (R=0.4584, P=0.0212) and the changes in muscle protein synthesis. No correlations were found between amino acid concentrations in the arterial blood (R=0.1143, P=0.59) and muscle protein synthesis. When data from young and older subjects were analyzed separately, we found that the correlations between changes in blood flow or amino acid availability and muscle protein synthesis held only for the elderly (blood flow and muscle protein synthesis: R=0.7077, P=0.0046; amino acid delivery and muscle protein synthesis: R=0.5491, P=0.0420) but not for the young (blood flow and muscle protein synthesis: R=0.2723, P=0.39; amino acid delivery and muscle protein synthesis: R=0.3912, P=0.21). This suggests that in the presence of abundant exogenous amino acids, blood flow and nutrient flow to the muscle may be rate limiting factors for the response of muscle protein synthesis to anabolic stimuli in older individuals. It is likely that these relationships between blood flow, amino acid delivery, and muscle protein synthesis are a reflection of the relationship between muscle protein anabolism and muscle perfusion.

It also important to underscore that the anabolic effect of insulin on muscle proteins occur through the activation of intracellular signaling pathways increasing the activity of the mammalian target of rapamycin (mTOR) signaling cascade, which is also activated by amino acids. Thus, insulin's anabolic effect may be mediated by increased amino acid availability, secondary to increase muscle perfusion, which then stimulates mTOR activity. Specifically, insulin stimulates protein synthesis by activating the insulin-signaling pathway leading to an increase in phosphatidylinositol 3-kinase (PI3K)

and protein kinase B (Akt/PKB) activity. Akt phosphorylates and inhibits the tuberous sclerosis complex (TSC2), which increases mTOR kinase activity. mTOR signaling to its downstream effectors ribosomal S6kinase 1 (S6K1) and 4E-binding protein 1 (4E-BP1) is an important mechanism for stimulating translation initiation. Recent work has shown that S6K1 can phosphorylate eukaryotic elongation factor 2 (eEF2) kinase, leading to a reduced phosphorylation/activation of eEF2 and a stimulation of translation elongation. Within the past few years, the work in cells and rodents has been translated into human studies. It has now been shown that a 2 hour hyperinsulinemic-euglycemic clamp or a 6 hour hyperinsulinemic infusion of insulin can increase the phosphorylation status of S6K1 (24).

mTOR regulates a variety of processes involved in cell growth through phosphorylation of downstream targets. Of particular relevance to mRNA translation, mTOR phosphorylates eukaryotic initiation factor (eIF)4E-binding protein 1 (4E-BP1) and S6K1. 4E-BP1 binds to eIF4E and prevents it from binding to eIF4G to form the eIF4F complex. During translation initiation, eIF4F binds to mRNA through the association of eIF4E with the m^7GTP cap structure of the mRNA. The eIF4F-mRNA complex then binds to the 40S ribosomal subunit through the association of eIF4G with the eIF3-40S ribosomal subunit complex. Phosphorylation of 4E-BP1 by mTOR is a precursor even that allows subsequent phosphorylation by other, as yet unidentified, protein kinases that ultimately result in release of 4E-BP1 from the inactive 4E-BP1-eIF4E complex, permitting eIF4E to bind to eIF4G and form the active eIF4F complex. Phosphorylation of S6K1 on T389 by mTOR generates a docking site for another protein kinase, phosphoinositide-dependent kinase 1 (PDK1), which subsequently phosphorylates S6K1 on T229, resulting in its activation. S6K1 phosphorylates at least

three proteins that have been implicated in regulating mRNA translation, including eIF4B, ribosomal protein S6 (rpS6) and eukaryotic elongation factor 2 (eEF2) kinase. As a consequence, phosphorylation of S6K1 and mTOR indirectly modulates both the initiation and elongation phases of mRNA translation. Signaling through mTOR is enhanced in response to signaling through the Akt/PKB signaling pathway (25).

Insulin rapidly activates protein synthesis by activating components of the translational machinery including eIFs (eukaryotic initiation factors) and eEFs (eukaryotic elongation factors). In the long term, insulin also increases the cellular content of ribosomes to augment the capacity for protein synthesis. The rapid activation of protein synthesis by insulin is mediated primarily through PI3K. This involves the activation of PKB. In one case, PKB acts to phosphorylate and inactivate glycogen synthase kinase 3, which in turn phosphorylates and inhibits eIF2B. Insulin elicits the dephosphorylation and activation of eIF2B. Since eIF2B is required for recycling of eIF2, a factor required for all cytoplasmic translation initiation events; this will contribute to overall activation of protein synthesis (26).

In a recent study, Fujita et al. have found that a bout of aerobic exercise can restore the anabolic response of skeletal muscle protein to insulin in healthy, non-diabetic older subjects by increasing blood flow, and Akt/mTOR signaling (27).

Thus, we hypothesize that the absence of changes in muscle protein synthesis during hyperinsulinemia in older subjects may be due to the unresponsiveness of the vascular bed to insulin. This, in turn, could hamper the physiological increase in muscle perfusion and nutrient flow to the muscle cells, and blunted the insulin induced increase in mTOR signaling and muscle protein synthesis.

For this thesis, the specific hypothesis tested was whether insulin-induced increases in blood flow and muscle perfusion were necessary for the physiological stimulation of muscle protein synthesis and net balance by insulin. To test this hypothesis, an experiment was devised to determine if the response of muscle protein synthesis and net balance to insulin improved in older subjects when blood flow and muscle perfusion were pharmacologically restored to youthful values by sodium nitroprusside (SNP). SNP is a salt compound that serves as a source of nitric oxide which directly diffuses into the vascular smooth muscle, thus it is frequently used to test endothelium-independent vasodilation. The expectation is that muscle protein synthesis will increase in the older subjects only during insulin infusion with pharmacological vasodilation.

Determining the mechanisms underlying sarcopenia, will allow us to define specific interventions to target this defect and provide the scientific basis for the prevention and treatment of this disease. A year 2000 study published in the Journal of the American Geriatric Society estimated that the direct healthcare cost attributable to sarcopenia in the United States was $18.5 billion, which represented about 1.5% of total healthcare expenditures. In fact, a 10% reduction in sarcopenia prevalence would result in savings of $1.1 billion (dollars adjusted to 2000 rate) per year in U.S. healthcare costs (28). From a clinical perspective it is encouraging that sarcopenia cannot be explained by an age-related reduction in basal muscle protein synthesis, as it would be difficult to target such a fundamental response with an appropriate therapeutic intervention. As such, it may be more feasible to impact alterations stemming from altered response of muscle to anabolic stimuli, undernutrition, or inactivity, which have been identified as potentially preventable contributing factors to sarcopenia (17,29-31) (Figures 1&2).

MATERIALS AND METHODS

Chapter 1: Subject characteristics

The Institutional Review Board of the University of Texas Medical Branch approved the study and all subjects gave written, informed consent before participation in the study. Twelve healthy older volunteers were recruited through the Sealy Center on Aging Volunteers Registry of the University of Texas Medical Branch. Six healthy older volunteers were randomly assigned to the control group receiving insulin alone. The remaining six healthy older volunteers were assigned to the experimental group receiving insulin and sodium nitroprusside (SNP). Eligibility of the volunteers was determined by clinical history, a physical examination, electrocardiogram, blood count, coagulation profile, plasma electrolytes, fasting blood glucose concentration, oral glucose tolerance test (OGTT), thyroid panel, liver and renal function tests, hepatitis B and C screening, human immunodeficiency virus (HIV) test, and drug screening. Inclusion criteria were subjects aged 65 – 85 years, ability to undergo informed consent (score >23 on the 30-item Mini Mental State Examination (MMSE)), and a stable body weight for at least three months. Exclusion criteria were physical dependence or frailty; defined as impairment in any of the Activities of Daily Living (ADL), history of falls (≥2 per year), or significant weight loss in the past year. In addition, those who had recently undergone exercise training (≥2 weekly sessions of moderate to high intensity aerobic or resistance exercise), had significant medical conditions (heart, liver, kidney, blood, or respiratory disease), had peripheral vascular disease, had diabetes mellitus or other untreated endocrine disease, had active cancer, had recent (within 6 months) treatment with

anabolic steroids or corticosteroids, had alcohol or drug abuse, or had severe depression (score >5 on the 15-item Geriatric Depression Scale (GDS)) were excluded (Table 1).

Chapter 2: Study Design

All subjects were instructed to eat their normal diets and to refrain from strenuous activity for the week before participation in the study. The evening before the study, the subjects were admitted to the General Clinical Research Center (GCRC) of the University of Texas Medical Branch. Upon admission, a Dual-Energy X-Ray Absorptiometry (DEXA) scan (Hologic QDR 4500W, Bedford, MA, USA) was obtained for all of the subjects in order to measure leg muscle volume and body composition. They were given a regular standardized dinner (10kcal/kg of body weight; 60% carbohydrate, 20% fat and 20% protein) and a snack at 11:00pm, after which they were only allowed water until the end of the experiment the next day.

In both groups, the study protocol was identical with the exception of the addition of SNP to the experimental group. In the morning, a polyethylene catheter was inserted into a forearm vein for stable isotope tracer and glucose infusion. A retrograde catheter was placed in the contralateral hand and kept at ~60°C for arterialized blood sampling to measure systemic insulin and indocyanine green (ICG) concentrations. Using sterile procedure and local anesthesia, femoral arteriovenous catheters were placed into one leg for blood sampling. The catheter in the femoral artery was also used for the infusion of ICG, insulin and/or SNP.

After collecting a background blood sample, a primed-continuous infusion of L-[ring-$^{13}C_6$]phenylalanine (Cambridge Isotope Laboratories, Andover, MA, USA) was

started to measure muscle protein kinetics. The priming dose for the tracer was 2 μmol/kg, while the continuous infusion rate was 0.05 μmol/kg/min. At 2 hours, the first muscle biopsy was taken from the lateral portion of the vastus lateralis muscle of the leg bearing the femoral catheters, approximately ~20cm above the knee, using a 5-mm Bergström biopsy needle. The tissue was immediately frozen in liquid nitrogen and stored at -80°C until analyzed. A second biopsy was taken at 4 hours from the same site but at a different angle so the muscle biopsies were taken at least 5cm apart from each other. The third and fourth biopsies, at 5.5 and 7 hours respectively, were taken from a new incision about 5-cm away from the first incision, following the same techniques as for the first biopsy.

Between 2 and 3 hours and 5.5 to 6 hours, ICG dye (0.5 mg/mL) was infused into the femoral artery and four blood samples were obtained from the femoral vein and the peripheral vein to measure leg blood flow for both the basal and intervention periods. Following ICG in both periods, four blood samples were obtained from the femoral artery and vein to measure the concentrations of amino acids, glucose, insulin, and the enrichments of free phenylalanine and glucose.

During the intervention period, both groups received an insulin infusion (0.15 mU/min/100mL of leg) directly into the femoral artery. This dose was chosen based on previous studies indicating this is the most anabolic dose in the absence of amino acid replacement, and for this reason it will be easier to detect any changes induced by the inhibition of vasodilation. Insulin was infused locally in order to increase the leg insulin concentration to a postprandial level while avoiding excessive systemic hyperinsulinemia. During insulin infusion, 20% dextrose was also infused in the forearm catheter at a variable rate in order to keep the arterial blood glucose concentration at their

preinsulin infusion values. To maintain the hyperinsulinemic-euglycemic clamp, 0.5mL of blood was drawn every 5 – 10 minutes for measurement of blood glucose concentrations. Those subjects in the experimental group, also received an infusion of SNP (dose 2 mg/min) administered at a variable rate, starting at 0.1µg/min/100mL of leg volume, in order to achieve the insulin-stimulated youthful values of about 6mL/min/100mL leg volume (Figure 3).

Chapter 3: Analytical methods

Blood flow was measured using a dye dilution method including ICG infusion in the femoral artery, and spectrophotometrical measurement (Beckman Coulter, Fullerton, CA, USA) of ICG concentration (λ=805 nm) in the femoral vein and in a wrist vein. Leg plasma flow was calculated from the steady state dye concentration values in the femoral and wrist vein as previously described (32,33). The calculation for blood flow is as follows:

$$BF = i_{ICG} / \{([FV] - [PV]) \cdot (1 - Ht)\}$$

Where i_{ICG} is the ICG infusion rate, [FV] and [PV] are the average ICG dilution in the femoral vein and peripheral vein, respectively, and Ht is the hematocrit.

Free phenylalanine $^{13}C_6$ enrichment and concentration in blood and tissue fluid were measured by gas chromatography-mass spectrometry (GCMS, 6890 Plus GC, 5973N MSD, 7683 autosampler, Agilent Technologies, Palo Alto, CA, USA) after addition of an appropriate internal standard (L-^{15}N-phenylalanine), extraction, purification and tert-butyldimethylsilyl (t-BDMS) derivatization (34). Muscle tissue samples were ground, and intracellular free amino acids and muscle proteins were extracted as described previously (34). The incorporation of labeled phenylalanine in the

mixed and myofibrillar muscle proteins were measured by GCMS, after protein hydrolysis and amino acid extraction, using the external standard curve approach (35). Glucose concentrations will be measured using an automated enzymatic colorimetric method (YSI Glucose Analyzer, Yellow Springs Instrument Co., Yellow Springs, OH, USA) (34).

Muscle tissue samples were also used to measure cell signaling, specifically the phosphorylation of mTOR/Akt, 4E-BP1, S6K1, and eEF2. The muscle tissue samples were immediately quick-frozen in liquid nitrogen (i.e., within seconds) following the biopsy, and kept under liquid nitrogen until analyzed. Then ~30 – 50mg of frozen tissue was homogenized (1:9, w/v) in a buffer containing: 50mm Tris-HCl, 250mm mannitol, 50mm NaF, 5mm sodium pyrophosphate, 1mm EDTA, 1mm EGTA, 1% Triton X-100, pH 7.4, 1mm DTT, 1mm benzamidine, 0.1mm PMSF and 5 μgml−1 soybean trypsin inhibitor (SBTI). DTT, benzamidine, PMSF, and SBTI were added to the buffer immediately prior to use. The homogenate was centrifuged for 10 min at 4°C, followed by removal of the supernatant. Total protein concentrations were determined by using the Bradford assay (Smartspec plus spectrophotometer, Bio-Rad, Hercules, CA, USA). The supernatant was diluted (1:1) in a 2x sample buffer mixture (125mM Tris, pH 6.8, 25% glycerol, 2.5% SDS, 2.5% β-mercaptoethanol, 0.002% bromophenol blue) and then boiled for 3 min at 100°C. 50μg of total protein homogenate were loaded onto each lane in duplicate and the samples were separated by electrophoresis (150V for 1 hour) on a 7.5% polyacrylamide gel (Criterion, Bio-Rad, Hercules, CA, USA), except for 4E-BP1 which was run on a 15% gel. A molecular weight ladder (Precision Plus protein standard, Bio-Rad, Hercules, CA, USA), and a rodent internal loading control were also included on each gel. Following electrophoresis, protein was transferred to a

polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) at 50V for 1 hour. Once transferred, PVDF membranes were placed in blocking buffer, 5% non-fat dry milk (NFDM) in Tris-buffered saline and 0.1% Tween-20 (TBST) for 1 hour. Blots were then rinsed twice in deionized water and twice in TBST before being incubated in a single primary antibody overnight at 4°C with constant agitation. The primary phospho-antibodies (Cell Signaling, Beverly, MA, USA) used were phospho-mTOR (Ser 2448, 1:1000), phospho-Akt (Ser 473, 1:1000), phospho-4E-BP1 (Thr 37/46, 1:1000), phospho-p70 S6K1 (Thr 389, 1:500), and phospho-eEF2 (Thr 56, 1:5000). The next morning, the blots were washed in TBST twice and incubated in secondary antibody for 1 hour in 5% NFDM in TBST at room temperature with constant rocking. The secondary antibody used for all of the primary phospho-antibodies was anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody in a ratio of 1:2000 (Amersham Biosciences, Piscataway, NJ, USA). After secondary incubation, the blots were washed for 15 min then 3 washes of 5 min with TBST. Chemiluminescent solution (ECL plus, Amersham BioSciences, Piscataway, NJ, USA) used to detect horseradish peroxidase activity was applied to each blot. After a 5 min incubation, optical density measurements were obtained with a CCD camera mounted in a ChemiDoc XRS phosphoimager (Bio-Rad, Hercules, CA, USA) and densitometric analysis was performed using Quantity One software (Version 4.5.2, Bio-Rad, Hercules, CA, USA). Following quantification of each phosphorylated protein, the membrane was stripped and incubated with a specific antibody to determine total protein content of each protein. Thus, the final data was expressed as phosphorylated protein divided by total protein (24,36).

Chapter 4: Calculations

To simultaneously assess in skeletal muscle the kinetics of intracellular free amino acids and the rates of muscle protein synthesis and breakdown in response to exogenous or endogenous hyperinsulinemia and pharmacological manipulations of blood flow and muscle perfusion, the kinetics of muscle phenylalanine were calculated using the two-pool model method. With the two-pool model, the phenylalanine enrichments and concentrations in the femoral artery and vein are measured to estimate muscle protein synthesis, breakdown, and net balance. These parameters are based on the extraction of the labeled phenylalanine from the femoral artery, the appearance of unlabeled phenylalanine from the muscle in the femoral vein, and the net arteriovenous difference of phenylalanine concentrations, respectively (37). Thus, this model provides data regarding the kinetics of plasma phenylalanine across the leg, with no consideration for intracellular recycling of the amino acids from breakdown to synthesis. In other words, this method allows for the measurement of the effect of the treatments on the net kinetics of plasma phenylalanine across the leg, while not offering any insight into the intracellular amino acid kinetics.

The two-pool model parameters are as follows:

Amino acid delivery to the leg = Fin = $CA \cdot BF$

Amino acid output from the leg = Fout = $CV \cdot BF$

Leg net balance = NB = $(CA - CV) \cdot BF$

Total rate of appearance = Total Leg Ra = $(CA \cdot EA / EV) \cdot BF$

Release from the leg = Leg Ra =Total Ra – Fin = $BF \cdot CA [(EA / EV) - 1]$

Rate of disappearance in the leg = Leg Rd = Ra + NB = $BF \cdot [(CA \cdot EA / EV) - CV]$

CA and CV are the blood amino acid concentrations in the femoral artery and vein, respectively. EA and EV are the amino acid enrichments, expressed as tracer/tracee ratio, in the femoral arterial and venous blood, respectively. BF is leg blood flow as calculated from the steady state ICG concentration values in the femoral and wrist veins, as described previously (32,33). Data were expressed per 100 ml of leg volume.

Leg glucose utilization was calculated as net glucose uptake across the leg:

$$\text{Leg glucose uptake} = (CA - CV) \cdot BF$$

To determine the degree of muscle tissue exposure to insulin, we calculated the insulin delivery rate to the leg. This is because a small portion of the insulin directly infused in the leg was recycled through the systemic circulation back into the leg, thus increasing the amount of insulin delivered to the muscle. Additionally, changes in leg blood flow can significantly affect insulin concentration when the exogenous infusion is constant, and for this reason, insulin concentration alone may not reflect the actual insulin availability for the muscle tissue. The arterial insulin concentration was not measurable during insulin infusion because the infusion was administered through the arterial catheter; therefore, insulin delivery to the leg was estimated by calculating the insulin outflow from the femoral vein multiplying the insulin concentration in the femoral vein (InsFV) by the blood flow:

$$\text{Insulin delivery} = \text{InsFV} \cdot BF$$

Although this method may slightly underestimate the insulin delivery rate because some insulin is taken up by the muscle cells after binding the insulin receptor and will not return in the venous blood, for the reasons listed above, we found it preferable to rely only on the calculated insulin dose as assessed at the time of infusion.

Leg glucose utilization will be calculated as net glucose uptake across the leg:

$$\text{Glucose Uptake} = (GA - GV) \cdot BF$$

GA and GV are blood glucose arterial and venous concentrations, respectively. Data will be reported per 100 mL of leg volume or 100 mg of leg muscle mass by DEXA (38) (Figure 4).

Chapter 5: Statistical analysis

Statistical analyses were performed using the statistical software, SigmaStat version 3.5 (Systat Software Inc., San Jose, CA, USA). The primary endpoints were measures of blood flow, muscle perfusion, amino acid delivery, muscle protein synthesis, and net muscle phenylalanine balance. Secondary endpoints were all remaining measures of muscle amino acid turnover, glucose kinetics, and phosphorylation/activity of intracellular signals. To determine the effects of vasodilation on the response of muscle protein turnover to insulin in aging, comparisons were carried out using ANOVA for repeated measures, the factors being subject, group (control, experimental) and time (basal, intervention). Post hoc comparisons were carried out using the Tukey-Kramer test. Linear relationships between measures of blood flow and muscle perfusion and measures of muscle protein synthesis and net muscle protein balance were tested using the Pearson product-moment correlation coefficient. For those data which did not pass the normality test, the values were transformed using natural log (ln). All values are listed as means ± SEM. Differences were considered significant at $P<0.05$.

RESULTS

Chapter 1: Leg volume

Leg volume as measured in the 6 control subjects and the 6 experimental subjects was not significantly different between the two groups. The average leg volume was 9489.28 ± 800.86 mL for the control subjects and 9122.05 ± 727.06 mL for the experimental subjects. The P value as calculated using a Student's t-test was 0.371 (Table 1).

Chapter 2: Glucose uptake

Glucose uptake across the leg (mmol/min/100 mL of leg) in the control group was 8.61 ± 2.77 at baseline and 63.04 ± 30.79 after the insulin was given. In the experimental group, glucose uptake was 4.33 ± 1.61 at baseline and 34.26 ± 7.89 after insulin + SNP were given. There was no significant difference between the baseline and intervention period as well as no significant difference between the control and experimental groups.

Chapter 3: Blood flow

Baseline blood flow (mL/min/100 mL of leg) across the leg was not significantly different between the control group (2.62 ± 0.47) and the experimental group (2.63 ± 0.33), P = 0.985. After the administration of insulin, the blood flow for the control group did not increase (3.12 ± 0.55) significantly (P = 0.588). In the experimental group, blood flow significantly increased from baseline to 6.79 ± 1.01 (P<0.001), after the administration of insulin + SNP. In addition, there was a significant increase in blood

flow (P<0.001) for the subjects who were given insulin + SNP over the subjects who were given insulin alone (Table 2, Figure 5).

Chapter 4: Phenylalanine concentrations and enrichments

The arterial concentrations of phenylalanine (μmol/L) were not different between baseline and intervention periods for both the control (64 ± 3 vs 61 ± 4) and experimental (62 ± 3 vs 62 ± 4) groups. Also, there were no significant differences between the groups for the two time periods. Venous concentrations of phenylalanine (μmol/L) followed much the same pattern as the arterial concentrations of phenylalanine. There were no differences between groups and time with baseline control being 71 ± 4, intervention control being 63 ± 5, baseline experimental being 68 ±4, and intervention experimental being 60 ± 4.

Phenylalanine enrichment in the femoral artery showed a time effect (P = 0.003), but no group effect. Therefore, there was not a statistically significant interaction between group and time (P = 0.611). Enrichment in the femoral vein showed a similar trend, with a significant time effect (P<0.001) but no interaction between group and time (P = 0.471) (Table 2).

Chapter 5: Muscle amino acid and protein kinetics

Phenylalanine net balance across the leg showed a significant time effect (P = 0.007), however there was no significant interaction between group and time (P = 0.170). There was a trend toward a higher net balance in the experimental group during the intervention period, P = 0.064. In addition, the net balance only became positive during

the SNP infusion, therefore only SNP caused a switch from net muscle protein catabolism to net muscle protein anabolism.

Phenylalanine delivery to the leg (Fin) showed a significant increase in the experimental group between the baseline (164 ± 24) and intervention (417 ± 61) periods, $P<0.001$. There was also a significant difference between the intervention periods of the control group and the experimental group (180 ± 25 vs 417 ± 61), $P<0.001$. Thus SNP improved phenylalanine delivery to the leg. Phenylalanine release from the leg (Fout) followed the same trends as delivery to the leg. The experimental group showed a significant increase between baseline and intervention (181 ± 27 vs 400 ± 60, $P = 0.002$) as well as an increase in release over the control group (400 ± 60 vs 184 ± 26, $P = 0.002$).

The rate of phenylalanine appearance (Ra) in the blood is an indicator of protein breakdown, while the rate of phenylalanine disappearance (Rd) is an estimate of protein synthesis. Ra showed no significant differences between the groups in both the baseline and intervention periods. Also, there were no differences in proteolysis between the two time periods for either group. Rd showed a significant group effect ($P = 0.042$) as well as a significant time effect ($P = 0.017$). However, the effect of the different levels of group did not depend on the level of time, so there was no significant interaction between group and time ($P = 0.276$). (Table 3, Figures 6&7).

Chapter 6: Westerns

Akt/PKB phosphorylation at Ser 473 showed a significant group effect ($P = 0.017$) as well as a significant time effect between biopsies ($P<0.001$). There was also a trend for interaction between group and time ($P = 0.10$). The phosphorylation of mTOR at Ser 2448 showed a significant difference between the different biopsy times.

However, there was no difference between groups and interaction between the group and biopsy times (P = 0.889). S6K1 phosphorylation at Thr 389 showed no difference among the groups, though there was a time effect between biopsies. There was also a trend for interaction between group and biopsy times (P = 0.068). The phosphorylation of 4E-BP1 at Thr 37/46 was not significantly increased between the baseline biopsy and the biopsies at 1.5 and 3 hours in both the control and experimental group. eEF2 phosphorylation at Thr 56, which normally inhibits protein synthesis, showed significant differences between the groups (P = 0.006), but no differences in biopsy times. Therefore, there was no significant interaction between group and biopsy times (P = 0.539) (Figures 8-12).

DISCUSSION

This study shows that pharmacologically increasing blood flow and muscle perfusion during hyperinsulinemia in non-diabetic older adults positively impacts muscle protein synthesis, thereby overcoming the age-related insulin resistance of muscle protein anabolism. Specifically, the induction of a three-fold increase in blood flow with SNP during insulin infusion increased amino acid delivery to the muscle, which, in turn, increased the rate of phenylalanine disappearance (Rd), an index of muscle protein synthesis. Since protein breakdown did not change significantly, the net protein balance across the leg became positive, indicating muscle net protein accretion. On the other hand, in the control group, the infusion of insulin alone did not induce vasodilation or an increase in amino acid delivery to the leg and, consequently, muscle protein synthesis did not increase. This is consistent with previous reports published in the literature by our groups and others (18,27).

The cell signaling results showed a pattern of response consistent with the kinetics data. Insulin signaling, specifically Akt/PKB phosphorylation, increased in both groups during hyperinsulinemia, but the increase was larger in the SNP-treated group after 3 hours of insulin infusion. Since it is unlikely that SNP directly stimulated insulin signaling, we speculate that such an improvement in Akt/PKB phosphorylation may have been due to increased muscle perfusion, which augmented the amount of muscle tissue exposed to insulin. The enhanced Akt/PKB phosphorylation, however, did not result in an increased glucose uptake in the SNP group. Thus, it is possible that the relatively modest improvement in Akt/PKB phosphorylation was insufficient to activate downstream signals in the glucose uptake pathway, which were not measured in this study, or, conversely, that glucose uptake was already maximal in these two groups of

healthy, non diabetic subjects. This is not surprising, as we have already shown that glucose metabolism is much more insulin-sensitive than protein metabolism in healthy, non diabetic subjects (24) and that the age-related insulin resistance of muscle protein metabolism does not correlate with the insulin sensitivity of glucose metabolism (18).

Interestingly, also mTOR phosphorylation at Ser 2448 increased in both groups with no differences between groups, suggesting that the increase in Akt/PKB phosphorylation was again insufficient to further activate this central signal in the translation initiation cascade. However, it is important to underscore that mTOR is a very large protein with many phosphorylation sites, and that we measured only the Ser 2448, which typically responds to changes in amino acid availability. Thus it is possible that either Akt/PKB had a weak effect on mTOR, or that it phosphorylated a different site. The second hypothesis appears more likely due to the finding that S6K1 phosphorylation mirrored Akt/PKB phosphorylation during insulin, i.e. it increased with insulin in both groups with a larger increase in the SNP group after 3 hours of insulin infusion. S6K1 is in fact a known downstream signal in the mTOR signaling pathway and its enhanced phosphorylation in the SNP group can well justify the increased muscle protein synthesis observed in this group. The absence of differences between groups in eEF2 and 4E-BP1 phosphorylation is not an impediment to this interpretation as S6K1 can directly stimulate translation initiation independent of these two other signals.

Overall, these results suggest that pharmacological restoration of NO-dependent vasodilation during hyperinsulinemia can normalize the anabolic response of skeletal muscle proteins to insulin. This effect appears mediated by enhanced Akt/PKB and S6K1 phosphorylation, possibly through increased exposure of skeletal muscle to insulin, while it seems less likely due to increased amino acid availability, as there were no differences

between the two groups in the phosphorylation of the amino acid sensitive site on mTOR Ser 2448. Nonetheless, due to the complexity of the mTOR protein, we cannot completely exclude that the increased amino acid availability was involved in the measured improvement in muscle protein synthesis in this elderly subjects. Further studies involving limitations or provision of additional amino acids during hyperinsulinemia are necessary to clarify this point.

Moreover, while blood flow and muscle perfusion emerge as important contributors to the age-related insulin resistance of muscle protein metabolism, other elements may also be involved. In a paper regarding the transport of insulin across the endothelial barrier (39), it has been reported that insulin may promote its own transport across the endothelial barrier to then bind its receptor on the end target tissue, such as skeletal muscle, and that this process may be saturable. In that study, insulin increased the microvascular surface available for uptake, thus facilitating its own delivery to the skeletal muscle. It was also found that after a short period of time there was a decline in insulin clearance from the blood, suggesting a saturation point. If insulin transport is a self-mediating and saturable process, then increasing vasodilation and muscle perfusion may contribute to overcome the insulin resistance of muscle protein metabolism seen in aging. This theory fits well our data, as the increase in Akt/PKB phosphorylation may be due to increased insulin delivery to the muscle. Additional studies are warranted to test this hypothesis.

In conclusion, pharmacological restoration of blood flow and muscle perfusion to youthful values by sodium nitroprusside, did improve the response of muscle protein synthesis to insulin in older subjects. This is particularly encouraging due to the availability of a number of drugs and lifestyle interventions, such as exercise, that can

improve muscle perfusion and may be also useful to prevent and treat sarcopenia. Future research is necessary to better understand the mechanisms underlying sarcopenia and find simple, safe and widely applicable means to treat this major contributor to disability and morbidity with aging.

Tables

Table 1: Subject characteristics

	Control	Experimental	P value
n	6 (5 males, 1 female)	6 (4 males, 2 females)	NA
Age (years)	74 ± 3	69 ± 1	0.100
Weight (kg)	74.85 ± 5.18	73.53 ± 6.88	0.441
Height (m)	1.68 ± 0.05	1.71 ± 0.05	0.343
BMI (kg/m^2)	26.55 ± 2.01	24.80 ± 1.15	0.233
Leg Volume (mL)	9489.28 ± 800.86	9122.05 ± 727.06	0.371

Table 2: Concentrations and enrichments of free phenylalanine in the femoral artery and vein of the control and experimental subjects in the basal state and during the intervention period in the femoral artery of one leg. Control intervention = addition of insulin, Experimental intervention = addition of insulin+SNP.

	Control Basal	Control Intervention	Experimental Basal	Experimental Intervention
Blood Flow (mL/min/100mL leg)	2.62 ± 0.47	3.12 ± 0.55	2.63 ± 0.33	6.79 ± 1.01†‡
Arterial Concentration (µmol/L)	64 ± 3	61 ± 4	62 ± 3	62 ± 4
Venous Concentration (µmol/L)	71 ± 4	63 ± 5	68 ±4	60 ± 4
Arterial Enrichment (tracer/tracee ratio)	8.93% ± 0.42%	10.15% ± 0.31%	9.15% ± 0.34%	10.04% ± 0.10%
Venous Enrichment (tracer/tracee ratio)	6.80% ± 0.30%	7.94% ± 0.17%	6.91% ± 0.28%	8.40% ± 0.18%

† P<0.05 basal vs intervention (insulin or insulin+SNP)

‡ P<0.05 control vs experimental

Table 3: Phenylalanine kinetics (2-pool model) across the leg in control and experimental groups in the basal state and during the intervention period in the femoral artery of one leg. Control intervention = addition of insulin, Experimental intervention = addition of insulin+SNP.

	Control Basal	Control Intervention	Experimental Basal	Experimental Intervention
NB – net balance across the leg	-16 ± 3	-4 ± 5	-17 ± 4	17 ± 13
Fin – delivery to the leg	161 ± 24	180 ± 25	164 ± 24	417 ± 61†‡
Fout – release from the leg	178 ± 26	184 ± 26	181 ± 27	400 ± 60†‡
Ra – release in blood	50 ± 11	50 ± 8	52 ± 7	85 ± 18
Rd – disappearance from the blood	34 ± 10	45 ± 10	35 ± 4	102 ± 26

† P<0.05 basal vs intervention (insulin or insulin+SNP)

‡ P<0.05 control vs experimental

Figures

Figure 1: Insulin signaling pathway in muscle protein synthesis. Akt/PKB = protein kinase B, mTOR = mammalian target of rapamycin, S6K1 = ribosomal S6 kinase 1, 4E-BP1 = 4E-binding protein 1, eEF2 = eukaryotic elongation factor 2 kinase

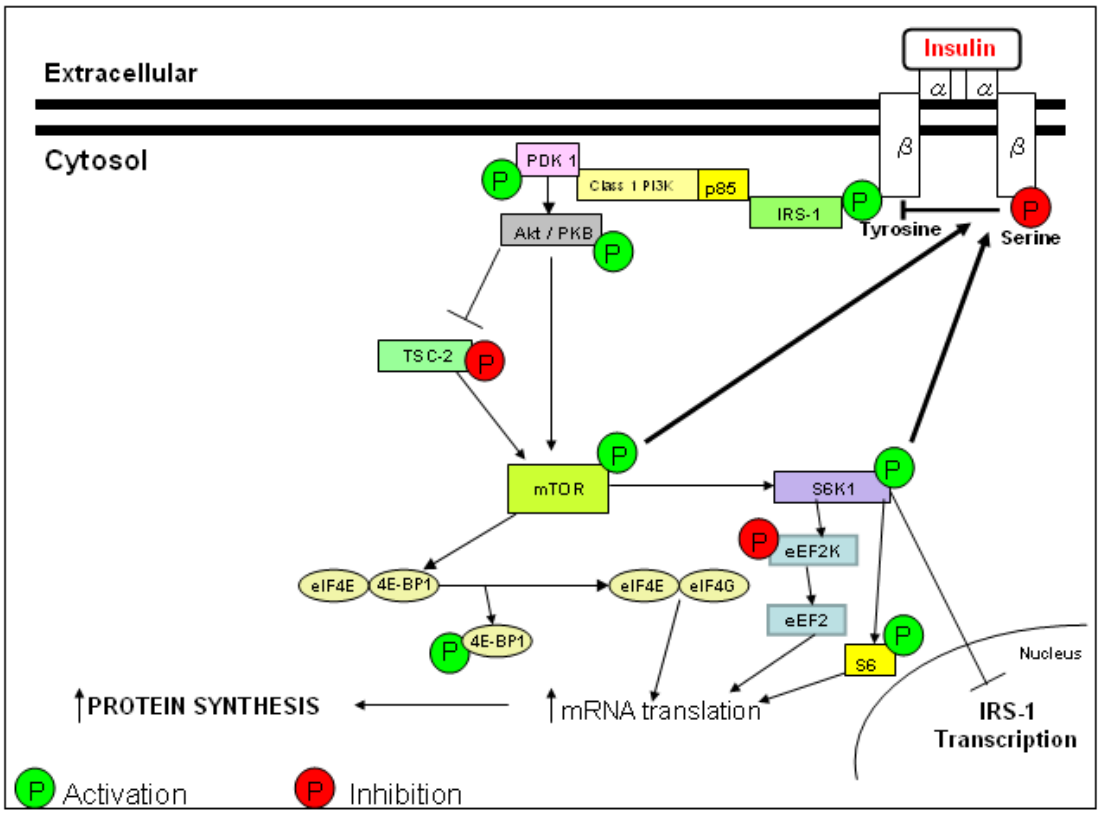


Figure 2 (Modified from 40): Schematic representation of the sites of action of sodium nitroprusside (SNP) and L-arginine (L-arg) on nitric oxide (NO) production and release by the endothelium. Cyclic guanine monophosphate (cGMP), nitric oxide synthase (NOS).

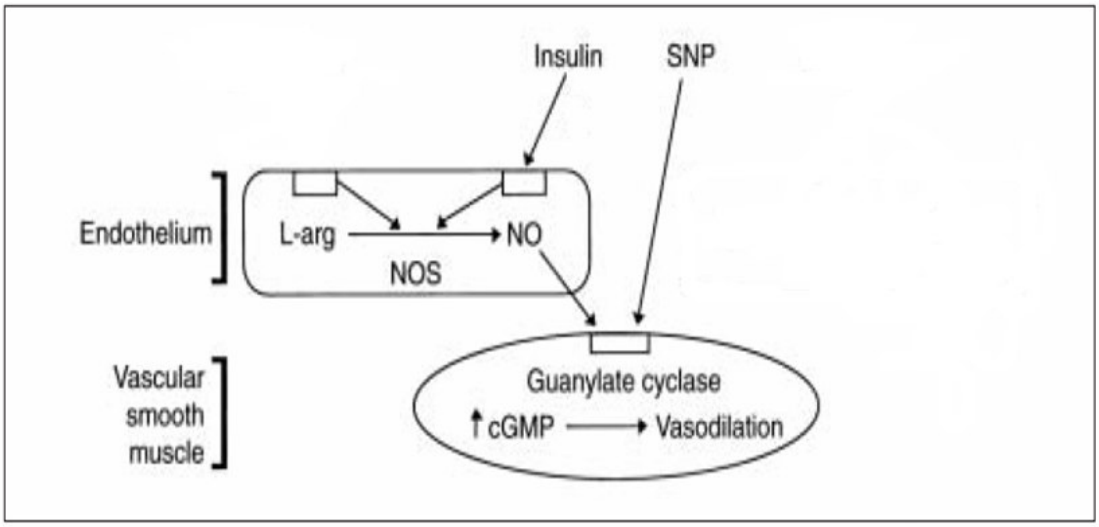


Figure 3: Muscle protein turnover (labeled phenylalanine) and blood flow (ICG) will be measured in older subjects in the basal period and during intraarterial infusion of insulin +/- SNP. ICG: indocyanine green infusion. SNP: sodium nitroprusside.

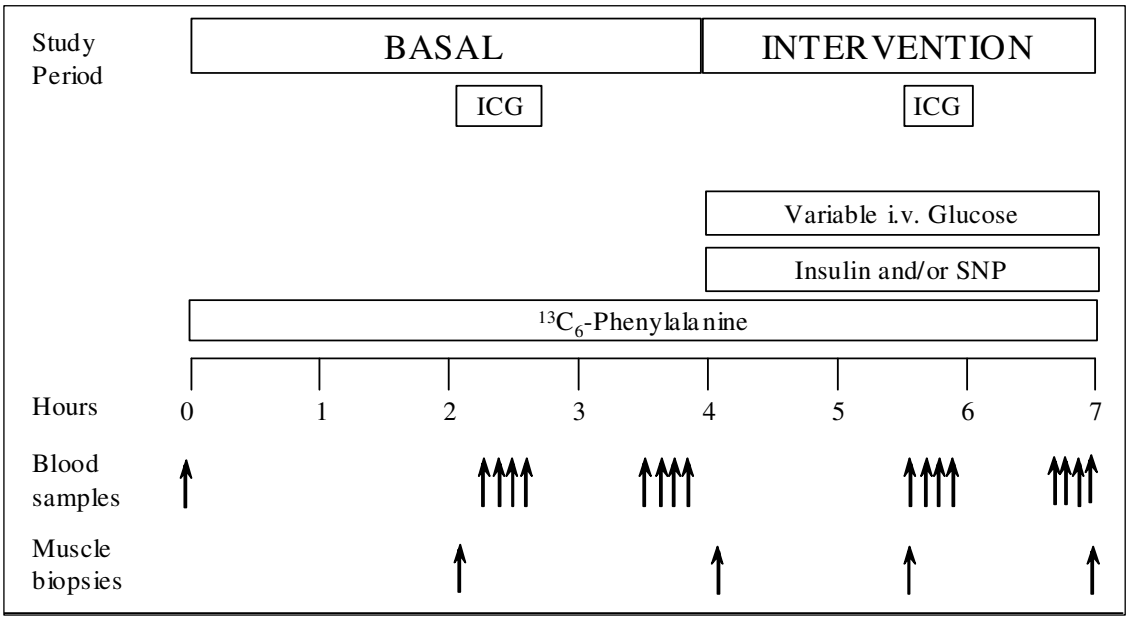


Figure 4 (Modified from 38): Two pool model of leg phenylalanine (phe) kinetics. Free phe pools in femoral artery (A), femoral vein (V) and muscle (M) are connected by arrows indicating unidirectional phe flow between each compartment. Phe enters the leg via femoral artery (Fin) and leaves the leg via femoral vein (Fout). Rd, rate of phe disappearance (estimate of protein synthesis); Ra, rate of phe appearance from breakdown

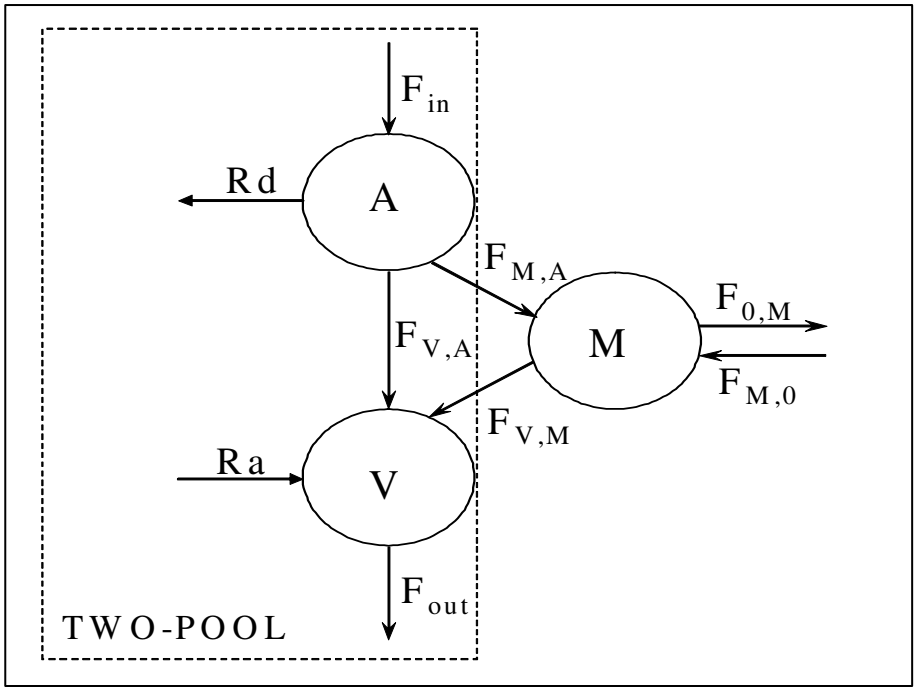
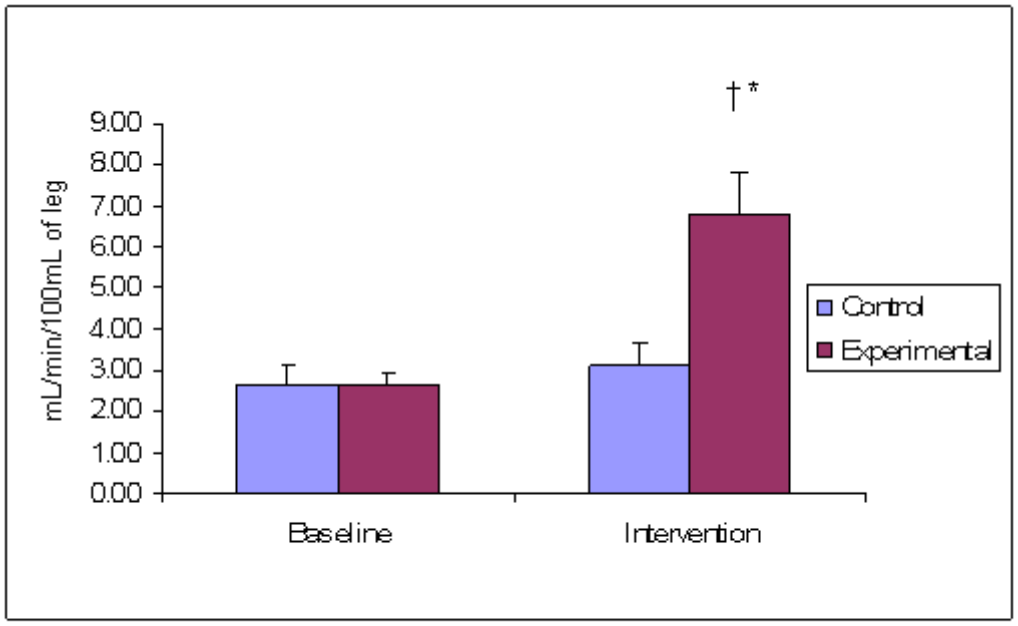


Figure 5: Blood flow



† P<0.05 basal vs intervention (insulin or insulin+SNP)

* P<0.05 control vs experimental

Figure 6: Net balance

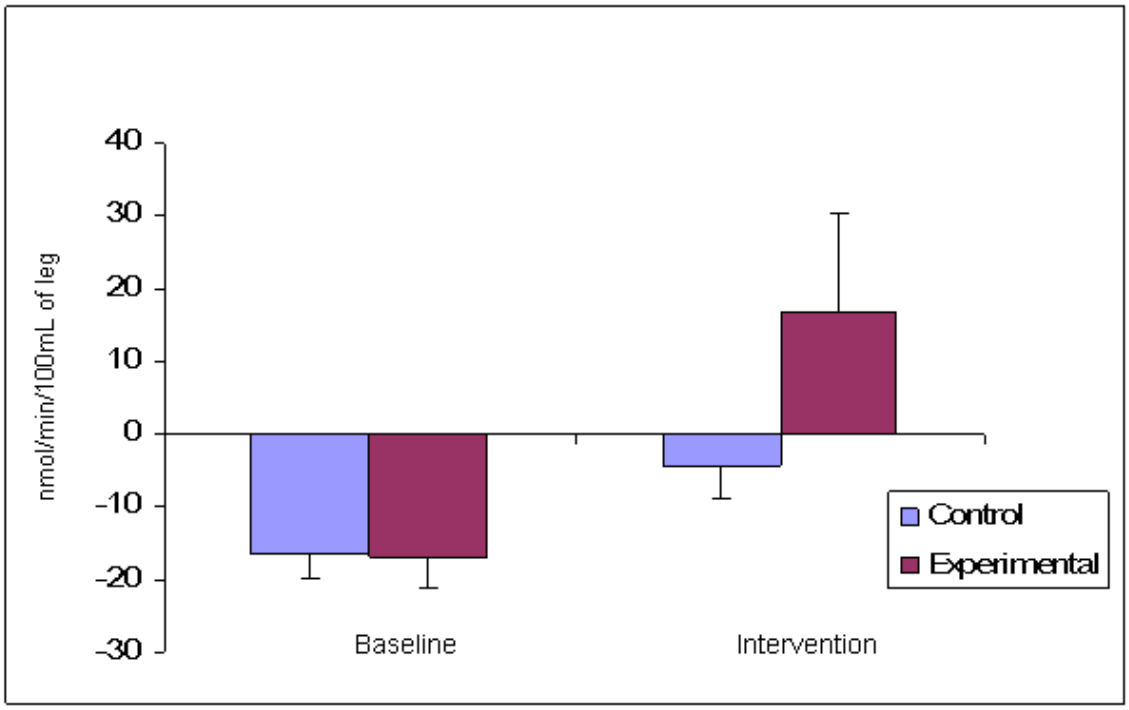


Figure 7: Protein synthesis (Rd)

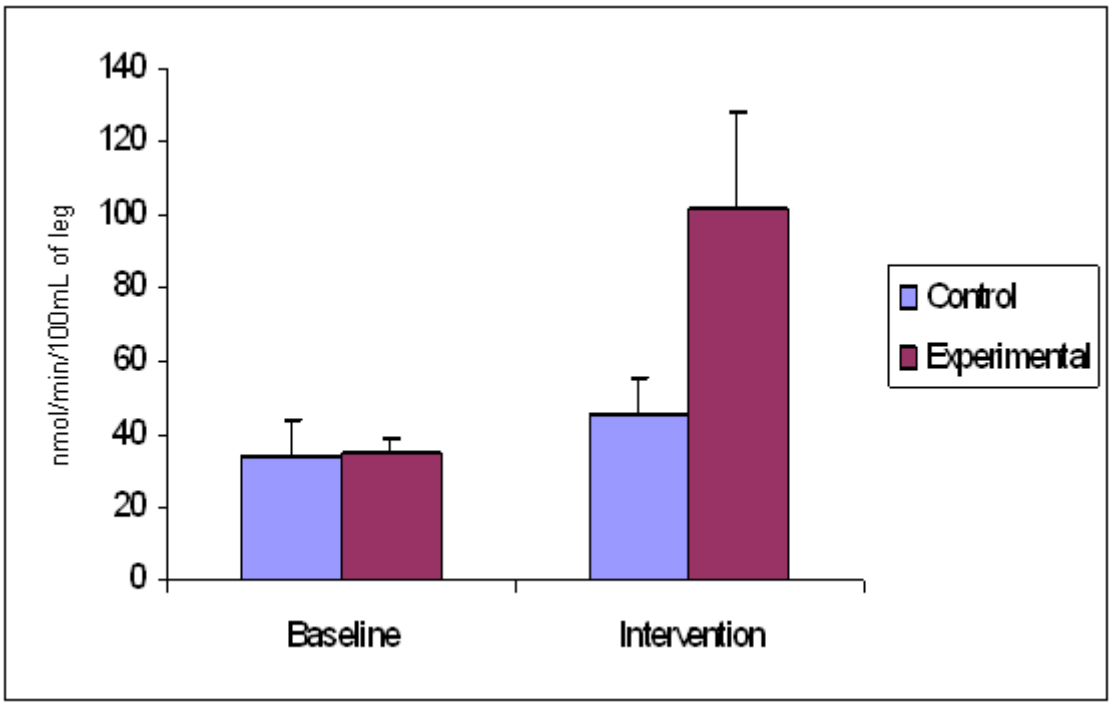


Figure 8: Akt/PKB (Ser 473) Phosphorylation

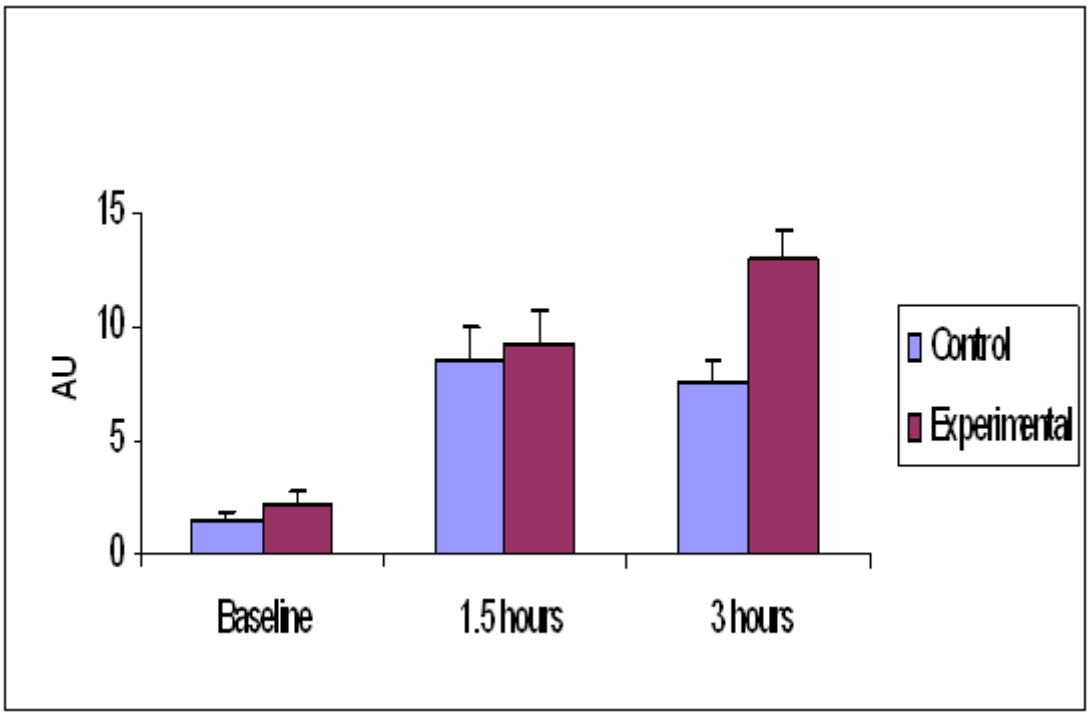


Figure 9: mTOR (Ser 2448) Phosphorylation

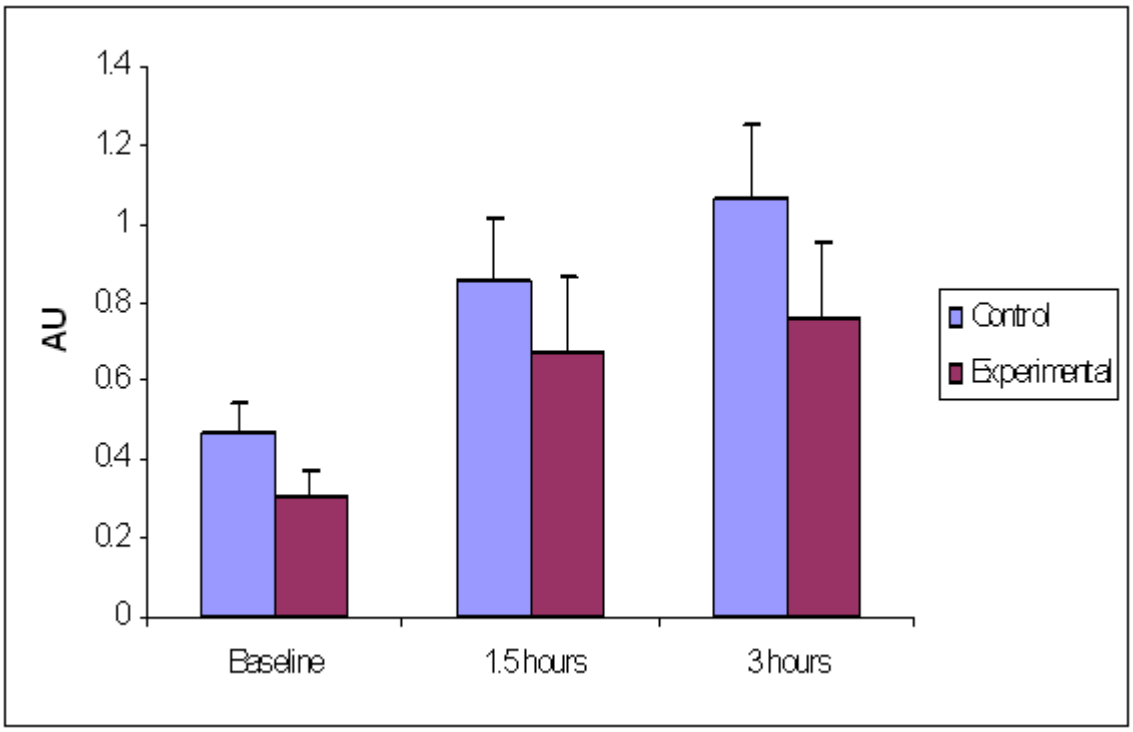


Figure 10: S6K1 (Thr 389) Phosphorylation

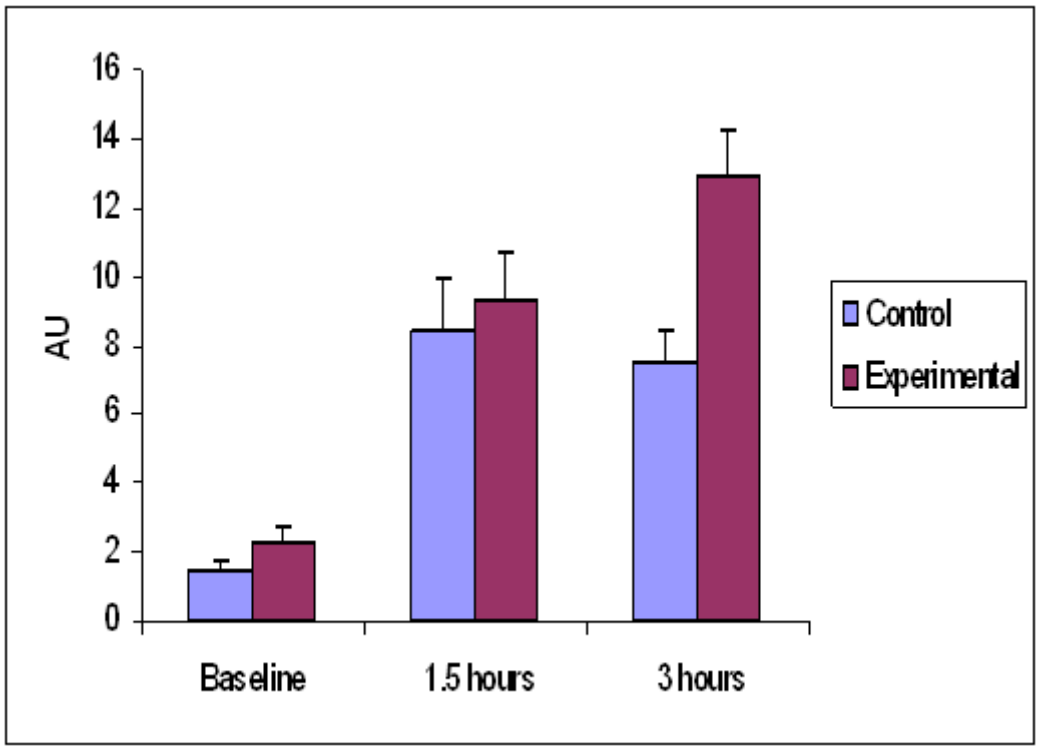


Figure 11: 4E-BP1 (Thr 37/46) Phosphorylation

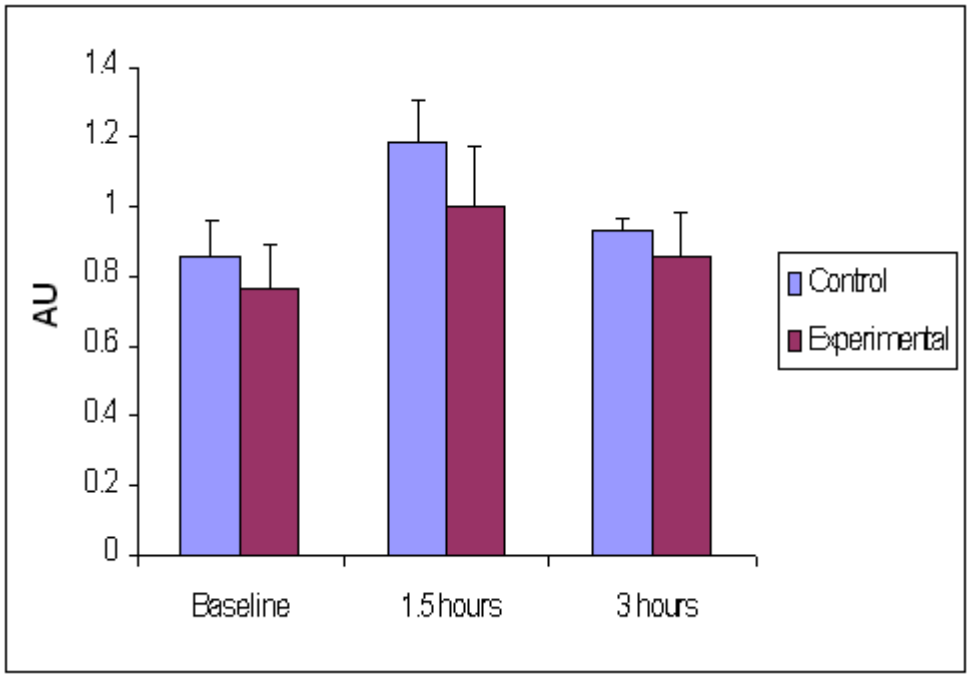
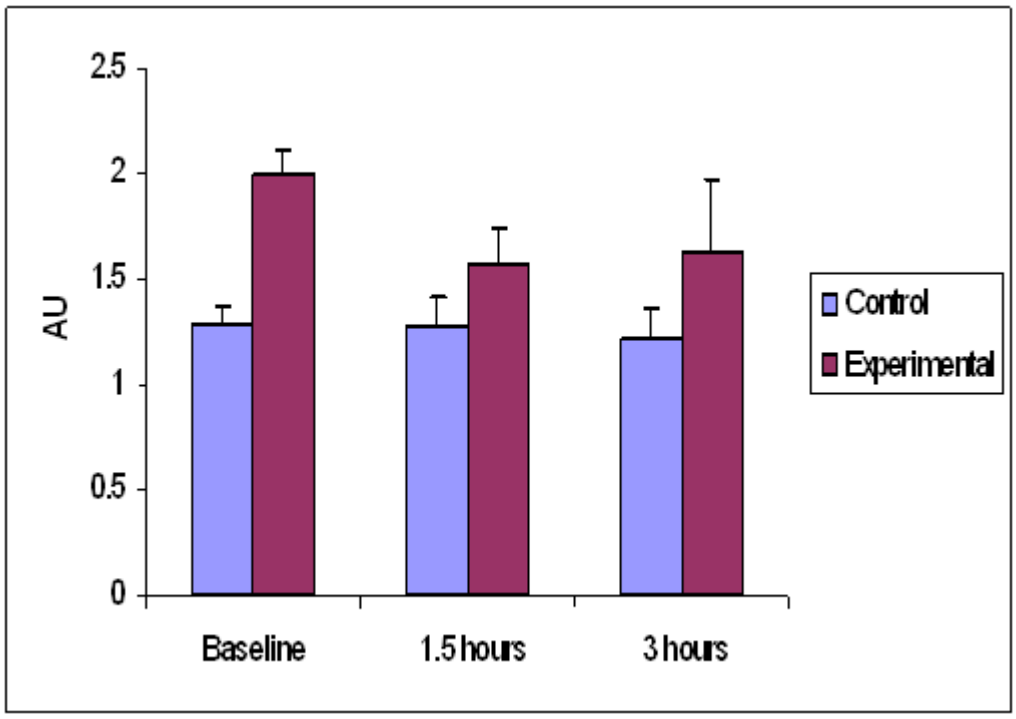


Figure 12: eEF2 (Thr 56) Phosphorylation



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Vita

Jessica Lan Lee was born in 1982 in Houston, Texas to Polly and Billy Lee. After graduating from Clear Lake High School in Houston, she entered The University of Texas at Austin in 2000. In 2004, she completed her Bachelor of Science degree in Nutrition, earning Special Honors. During the fall of 2004, she enrolled at The University of Texas Medical Branch's School of Medicine. Between her third and fourth years of medical school she took a year off in order to obtain a Master's degree in Clinical Science from The University of Texas Medical Branch's Graduate School of Biomedical Sciences. Following her graduation from the graduate school, she will re-enter the medical school in order to complete her medical degree.

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