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Amino Acids are Necessary for the Insulin-Induced Activation of mTOR/S6K1 Signaling and Protein Synthesis in Healthy and Insulin Resistant Human Skeletal Muscle

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Abstract

Background—Amino acids (AA) activate the mammalian target of rapamycin (mTOR) signaling pathway but overactivation has a negative feedback effect on insulin signaling which may lead to insulin resistance and type 2 diabetes (T2DM). Purpose: To determine the effect of reduced AA concentrations on mTOR and insulin signaling during increased nutrient and insulin availability.

Methods—Six control and six T2DM subjects were studied at baseline and following a 5h AA lowering high energy and insulin clamp. Stable isotopic techniques in combination with femoral catheterizations were used to measure AA kinetics across the leg while muscle biopsies were used to measure mTOR and insulin signaling proteins using immunoblotting techniques.

Results—AA concentrations decreased by ~30–60% in both groups ($P < 0.05$). Phospho-mTOR, S6K1, eEF2, and eIF2 α were unchanged in both groups following the clamp ($P > 0.05$). In T2DM subjects, IRS-1 serine phosphorylation was unchanged while phospho-AMPK α decreased and phospho-Akt, phospho-AS160 and glucose uptake increased following the clamp ($P < 0.05$). In comparison, AA concentrations were maintained in a separate group during an insulin infusion. In this group, phospho-Akt, mTOR and S6K1 ($n=4$) increased.

Conclusion—Amino acids are necessary for insulin-induced activation of mTOR signaling and protein synthesis in both healthy and insulin resistant skeletal muscle.

Keywords

mammalian target of rapamycin; muscle protein synthesis; amino acid availability; adenosine 5'-monophosphate-activated protein kinase

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Introduction

Obesity is a worldwide health concern affecting nearly 300 million people with approximately 90% of type 2 diabetes (T2DM) patients being obese¹. It has been known for quite some time that nutrient overload is the primary factor causing obesity, insulin resistance and eventually T2DM^{2, 3}. Furthermore, obesity is associated with an increase in circulating amino acid concentrations and in particular the branched chain amino acids^{4, 5}.

A large majority, but not all^{6, 7}, of these studies have shown that increasing blood amino acid availability resulted in an inhibition of glucose disposal at the whole body level and across the forearm⁸⁻¹⁵. It is now proposed that increased amino acid availability induces insulin resistance by inhibiting insulin signaling within muscle cells. Patti et al. elucidated the cellular mechanisms responsible for the inhibition in glucose transport when they found that cultured myotubes exposed to high physiological levels of amino acids activated ribosomal protein S6 kinase 1 (S6K1) and 4E binding protein 1 (4E-BP1), downstream targets of the mammalian target of rapamycin (mTOR)¹⁶. In particular, an increase in amino acid availability reduced insulin-stimulated tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1) and reduced the activity of the class 1 phosphatidylinositol 3-kinase (PI3K)¹⁶. Additional *in vitro* work in muscle and adipocytes confirmed that amino acid overactivation of the mTOR nutrient signaling pathway was responsible for the increase in serine phosphorylation of IRS-1 and the subsequent inhibition of insulin signaling^{17, 18}.

Amino acids are potent activators of the mammalian target of rapamycin (mTOR) nutrient signaling pathway^{19, 20}. The effect of amino acids, and in particular leucine, on mTOR activation appear to be independent of insulin signaling mechanisms and apparently operate through a class 3 PI3K called hVps34^{21, 22} upstream of mTOR. A recent study by Um et al.²³ found that genetically engineered mice deficient in the mTOR downstream target S6K1 were more insulin sensitive and did not demonstrate IRS-1 serine phosphorylation on a high fat diet as did their wild type counterparts. More recently, Tremblay et al.²⁴ have confirmed in humans that an acute infusion of amino acids increased serine phosphorylation of IRS-1 and inhibited insulin signaling by overactivation of S6K1.

We have recently shown that an increase in nutrient and insulin availability can stimulate protein synthesis in human skeletal muscle if amino acid concentrations are maintained by using a local infusion of insulin into the femoral artery^{25, 26}. On the other hand, a systemic infusion of insulin significantly reduces circulating amino acid concentrations^{25, 27, 28}. Therefore, we hypothesized that reduced amino acid availability during an acute, systemic infusion of insulin and nutrients would not increase protein synthesis because mTOR/S6K1 signaling would not be activated in either healthy or insulin resistant skeletal muscle. A secondary aim was to assess whether a reduction in amino acids during increased insulin and nutrient availability would alter insulin signaling and glucose uptake in obese, T2DM skeletal muscle. To address our hypotheses we analyzed several cell signaling protein in muscle tissue from several of our recent metabolic studies^{25, 26, 29, 30}.

Methods

Subjects

We recruited 9 men and 3 women to participate in the study. The characteristics of the six controls (35 ± 3 y; 81 ± 6 kg; 23 ± 1 body fat %) and the six T2DM (40 ± 4 y; 79 ± 6 kg; 28 ± 2 body fat %) are expanded in previous publications^{29, 30}. The T2DM subjects were eligible for this study if their fasting blood glucose levels were ≥ 7 mmol/L and/or 2 h blood glucose levels were ≥ 11 mmol/L (200 mg/dl) following the oral glucose tolerance test and if they were not taking any medications. T2DM subjects were classified as having poorly controlled blood

glucose values as indicated by nonenzymatically glycated hemoglobin levels >7% ($8.7 \pm 1.5\%$). Both healthy controls and T2DM groups were sedentary at the time and gave their written consent to participate. The study was approved by the Institutional Review Board at the University of Southern California.

Study design for Reduced AA groups

The study protocol was designed to assess amino acid and fatty acid kinetics in obese T2DM under postabsorptive (Basal) conditions and during a 5 h amino acid (AA) lowering, hyperglycemic-hyperinsulinemic-hyperlipidemic (Reduced AA) clamp. Therefore, blood glucose levels were increased to a similar extent above baseline in both groups during the clamp so that the total amount of nutritional energy was similar in the two groups. Following admission to the General Clinical Research Center, the evening before the study, subjects were given a Dual-Energy X-Ray Absorptiometry scan (Hologic QDR 4500W, Bedford, MA) to measure body composition and consumed a standardized meal (41.9 kJ/kg of body weight), after which only ad libitum water was allowed until the end of the experiment.

The study began the next morning with the insertion of femoral arterial and venous polyethylene catheters in the leg. The arterial catheter was also used for the infusion of indocyanine green (Akorn, Inc., Buffalo Grove, IL) to measure blood flow. Additional catheters were placed in the forearm vein for systemic isotope infusion and in a contralateral wrist vein, which was heated in order to obtain arterialized blood samples. Background blood and breath samples were obtained, and a ^{13}C bicarbonate prime of $3 \mu\text{mol/kg}$ and a L-[ring- $^2\text{H}_5$]phenylalanine prime of $2 \mu\text{mol/kg}$ was given followed by a continuous infusion of L-[ring- $^2\text{H}_5$] phenylalanine ($0.05 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) and U- ^{13}C -palmitate ($0.04 \mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) (Cambridge Isotope Laboratories, Andover, Massachusetts). At 80 min after the tracer infusion was initiated, an infusion of indocyanine green was initiated to measure blood flow, and four breath and blood samples were obtained at 10 min intervals (approximately 90, 100, 110, and 120 min). At 120 min, using a 5 mm Bergström biopsy needle (Stille, Stockholm, Sweden) a first skeletal muscle biopsy was taken from the lateral portion of the *vastus lateralis* muscle, about 20 cm above the knee, using local anesthesia with 1% lidocaine injected subcutaneously and on the fascia. The muscle sample was immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Immediately following the first skeletal muscle biopsy, the palmitate tracer was stopped and the 5 h Reduced AA clamp was initiated. Plasma insulin concentrations were elevated with a systemic insulin infusion ($0.5 \text{mU}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) into the forearm vein, and blood samples (0.5 ml) were taken every 5–10 min to monitor the plasma glucose concentration. A 20% dextrose infusion was also initiated and varied to increase glucose concentration by approximately 2200 $\mu\text{mol/L}$ above baseline and clamp it at the new concentration until the end of the experiment in both groups. During the infusion period, Intralipid[®] ($0.7 \text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) and heparin ($7 \text{U}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) (Baxter, Deerfield, Illinois, USA) were also infused to prevent the expected decrease in plasma free fatty acids.

Three hours after the beginning of the clamp, the palmitate tracer infusion was restarted. Indocyanine green infusion was restarted at 380 min, indirect calorimetry measures were taken, and breath and blood samples were obtained at 390, 400, 410, 420 min. A final skeletal muscle biopsy was obtained at 420 min.

Maintained AA group

We have recently performed a local insulin infusion (i.e., into the femoral artery) in six young (23 ± 1 yrs), insulin sensitive subjects in order to prevent large decreases in circulating amino acids as previously described²⁵. The study design was similar to the design as described for

the Reduced AA groups and consisted of using stable isotopic techniques (L-ring- $^{13}\text{C}_6$ -phenylalanine), femoral catheterization, and collection of muscle biopsies from the vastus lateralis before and after an insulin infusion. However, slight differences in study design are acknowledged such as less energy was infused (lipid, glucose) and a palmitate tracer was not used. Each subject was studied at baseline (following an overnight fast) and again following a 3 h hyperinsulinemic-euglycemic clamp (20% dextrose) ²⁵.

Metabolic parameters

We measured phenylalanine, and palmitate blood, breath, and muscle enrichments as previously described ^{25, 29, 30}. We calculated the concentrations of the branch-chained amino acids (BCAA) in the plasma using gas chromatography-mass spectrometry (GCMS) followed by use of the external standard technique as previously described ³¹. In addition, the equations used to calculate muscle protein synthesis, breakdown, amino acid and fatty acid kinetics have been described in detail elsewhere ^{25, 29, 30}. Insulin concentrations were measured via a radioimmunoassay commercial kit (Diagnostic Products Corp., Los Angeles, California, USA). Plasma glucose was determined by the glucose oxidase method using a YSI 2700 analyzer (Yellow Springs Instruments, Yellow Springs, Ohio, USA), immediately after each blood draw. Plasma free fatty acid concentration was determined enzymatically (Wako NEFA; Wako Chemicals GmbH, Neuss, Germany). To determine leg blood flow, serum samples were analyzed in a spectrophotometer (805 nm) following a continuous infusion of indocyanine green. Glucose uptake across the leg was determined by calculating the product between blood flow and the arterial-venous difference of glucose.

Immunoblot analysis

40 mg of frozen tissue was homogenized (1:9, wt/vol), centrifuged for 10 min at 4°C followed by the removal of the supernatant. Total protein concentrations were determined by using the Bradford assay analyzed by a spectrophotometer (Smartspec Plus; BioRad, Hercules, CA). The supernatant was diluted (1:1) in a sample buffer mixture containing 125 mM Tris, pH 6.8; 25% glycerol; 2.5% sodium dodecyl sulfate 2.5%; β -mercaptoethanol and 0.002% bromophenol blue then boiled for 3 min at 100°C. One hundred micrograms (50 μg total protein for mTOR) of total protein was loaded into each lane and the samples were separated by electrophoresis (150 V for 60 min) on a 7.5% polyacrylamide gel (BioRad, Criterion). Each gel consisted of Basal and Reduced AA muscle samples of two subjects (Control and T2DM) with each sample loaded in duplicate. An internal loading control (standard) and a molecular weight ladder (Precision Plus; BioRad, Hercules, CA) were also included on each gel. Following electrophoresis, protein was transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA) at 50V for 60 min. Blots were incubated in a single primary antibody overnight at 4°C (antibody concentrations are described below). The next morning, blots were incubated in secondary antibody for 1 h at room temperature. Chemiluminescent solution (ECL plus; Amersham BioSciences, Piscataway, NJ) was applied to each blot. After a 5 min incubation, optical density measurements were obtained with a phosphoimager (BioRad, Hercules, CA) and densitometric analysis was performed using Quantity One software (Ver 4.5.2) (BioRad, Hercules, CA). Phosphorylation and total protein were normalized to a standard. Final data was expressed as normalized protein phosphorylation relative to normalized total protein. However, phosphorylation status of acetyl-CoA carboxylase beta (ACC β) and eukaryotic initiator factor 2 alpha (eIF2 α) were expressed relative to a standard.

Antibodies

The phospho and total primary antibodies used were purchased from Cell Signaling (Beverly, MA) unless otherwise noted: phospho-mTOR (Ser2448; 1:1000); phospho-p70 S6K1 (Thr389; 1:500); phospho-IRS-1 (Ser312; Ser636/639; 1:500); phospho-eEF2 (Thr56; 1:1000);

phospho-eIF2 α (Ser52; 1:1000; purchased from BioSource, Camarillo, CA); phospho-Akt (Ser473; 1:500); phospho-AMPK α (Thr172; 1:1000); phospho-ACC β (Ser79; 1:500); phospho-Ser/Thr Akt substrate (1:500). Total protein for each of these molecules were detected using an antibody dilution of 1:1000. AS160 (Rab-GAP) was purchased from Upstate Signaling (Charlottesville, VA). Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was purchased from Amersham Bioscience (Piscataway, NJ) (1:2000).

Statistics

All data was statistically analyzed using NCSS (Kaysville, UT) software. Comparisons of groups at baseline were performed using a Student's t-test and comparisons with the treatment period were performed using a two-way ANOVA (2×2) with factors being treatment (Control and T2DM) and time (Basal and Reduced AA). Under conditions of a main effect, a Tukey post hoc analysis was used to determine interactions. Data comparing AA concentrations in the blood and delivered to the leg at Basal and Reduced AA and mTOR signaling proteins at Basal and Maintained AA were conducted using a paired t-test. Significance was determined to be $P < 0.05$. Values were expressed as mean \pm SE.

Results

In order to put the signaling data in the proper metabolic context the relevant baseline parameters of plasma amino acid, glucose, insulin, glucose uptake across the leg, muscle protein synthesis and breakdown for Control, T2DM, and Maintained AA groups are found in Table 1.

To further verify a reduction in AA, we measured the branched chain amino acids (BCAA) before and after the Reduced AA clamp (Fig. 1A-C). A) Leucine, B) isoleucine, and C) valine concentration were significantly reduced by ~30–60% in both groups ($P < 0.05$). In the same figure, we report delivery of AA to the leg (blood flow \times AA concentration) before and after Reduced AA clamp. We found that the delivery to the leg was significantly reduced for leucine, isoleucine and valine in both groups ($P < 0.05$, Fig. 1). Although the percentage reduction before and after treatment was similar between groups, T2DM demonstrated significantly greater levels of delivery to the leg at Basal and Reduced AA clamp for all BCAA ($P < 0.05$).

mTOR signaling

The phosphorylation status of mTOR at Ser2448 (Fig. 2A) was unchanged in Control and T2DM subjects following the Reduced AA clamp. The total mTOR protein level was significantly reduced at Basal and Reduced AA time points for T2DM compared to Control (Table 2) ($P < 0.05$). There were no significant differences for S6K1 phosphorylation (Thr389; Fig. 2B), eEF2 phosphorylation (Thr56; Fig. 3A) and their total protein abundance (Table 2) at the Basal or Reduced AA time points for Control or T2DM subjects. Interestingly, phosphorylation status of eIF2 α at Ser52 (Fig. 3B) was significantly lower for T2DM subjects compared to Control at baseline ($P < 0.05$).

Insulin signaling

Phosphorylation of IRS-1 at Ser312 (Fig. 4A) and Ser636/639 (Fig. 4B) was not different at baseline or following the Reduced AA clamp in either the Control or T2DM group. There was no significant change in IRS-1 total protein content in either group (Table 2). The phosphorylation status of Akt at Ser473 (Fig. 5A) increased in both groups following the Reduced AA clamp ($P < 0.05$) while total Akt protein abundance (Table 2) was lower only in T2DM as a result of Reduced AA ($P < 0.05$). AS160 phosphorylation (Fig. 5B) increased in the T2DM subjects following the Reduced AA clamp as compared to the Control subjects

($P < 0.05$). There was no difference in total AS160 protein content (Table 2) within or between the two groups at Basal or as a result of the Reduced AA clamp.

AMPK signaling

AMPK α phosphorylation at Thr172 (Fig. 6A) was significantly reduced in both groups following the Reduced AA clamp ($P < 0.05$). Total AMPK α protein content was not different between the Control or T2DM subjects at baseline and did not change following the Reduced AA clamp (Table 2). There was no difference in ACC β phosphophorylation at Ser79 (Fig. 6B) before or after the clamp in either the Control or T2DM subjects. Malonyl-CoA increased ($P < 0.05$) to a similar extent in both groups following the Reduced AA clamp²⁹.

Maintained AA group

The metabolic and amino acid kinetic data from the six subjects in the Maintained AA group have been previously published²⁶ and are summarized in Table 1. To determine whether phosphorylation status was altered in the muscles of these subjects, we performed new experiments with the remaining muscle tissue ($n=4$). As shown in Figure 7, the phosphorylation status of Akt, mTOR and S6K1 were significantly increased following the insulin infusion in which AA concentrations were maintained ($P < 0.05$; Fig. 7A–C).

Discussion

Our primary finding was that reduced amino acid concentrations during increased nutrient and insulin availability blunted mTOR/S6K1 signaling and muscle protein synthesis in both Control and T2DM subjects compared to a group that maintained AA concentrations during an insulin infusion. Interestingly, following the Reduced AA clamp, insulin signaling (e.g., Akt phosphorylation and IRS-1 serine phosphorylation) and glucose uptake were similar between the insulin-sensitive Control subjects and the insulin-resistant T2DM subjects. The T2DM subjects also had an increased phosphorylation of AS160 following the Reduced AA clamp. These findings imply that amino acid availability is an important regulator of not only mTOR signaling and muscle protein synthesis, but may be playing a role in insulin signaling. Other novel findings were that total mTOR protein and eIF2 α phosphorylation were reduced in the T2DM subjects.

Amino acid availability (particularly the essential amino acids) plays a pivotal role in the regulation of protein synthesis^{20, 32}. Fasting in humans and animals results in a net negative protein balance^{33, 34} while ingesting amino acids increases the rate of muscle protein synthesis^{35–37}. As shown in the current study, an increase in nutrient and insulin availability decreased amino acid concentrations, reduced delivery of AA to the leg (Fig. 1A–C), and tended to reduce muscle protein synthesis (Table 1; $P=0.08$) in both Control and T2DM subjects. On the other hand, a local infusion of insulin maintained amino acid concentrations, delivery to the leg, and increased muscle protein synthesis^{25, 26}. Although insulin alone can increase muscle protein synthesis in animals³⁸ and humans^{26, 39}, amino acids (particularly leucine) appear to be a much more potent anabolic agent^{20, 40, 41}. Furthermore, insulin cannot stimulate muscle protein synthesis if amino acid concentrations are not maintained^{25, 26}. Thus, when the necessary building blocks are not available to the cell, protein synthesis is ineffective regardless of the presence of insulin and nutritional energy.

Protein synthesis is modulated at the cellular level primarily by the mTOR signaling pathway³². Recent studies by Byfield et al.²¹ and Nobukuni et al.²² indicate that amino acids can activate mTOR through hVps34, a class 3 PI3K. We report that mTOR phosphorylation at Ser2448 (Fig. 2A) was not significantly elevated nor its downstream effector S6K1 (Thr389; Fig. 2B) in response to reduced amino acids even though insulin and nutrient energy was

substantially increased. This corresponded nicely with the attenuated muscle protein synthesis in both groups following the Reduced AA clamp (Table 1). Other markers of either translation elongation, (eEF2; Fig. 3A), or translation initiation, (eIF2 α ; Fig. 3B) phosphorylation were not different following the clamp. In our maintained AA group, protein synthesis was increased as previously reported²⁵. As a follow-up, we measured the phosphorylation status of Akt, mTOR and S6K1 in these same subjects²⁵. We found an increased phosphorylation of all three proteins (Fig. 7A–C) when AA are maintained further supporting the notion that amino acids must be adequately present to stimulate protein synthesis. We have also recently increased essential amino acid and insulin availability in young subjects and show that mTOR and S6K1 phosphorylation are increased to a very large extent in combination with a rapid and potent increase in muscle protein synthesis⁴². Therefore, blunted mTOR/S6K1 signaling appears to be a plausible cellular mechanism which may explain why muscle protein synthesis did not increase in our subjects even though insulin and abundant nutrient energy was present.

Recent reports have also indicated that excess amino acids can cause insulin resistance in humans^{14, 15, 24, 43} and thereby influence glucose uptake into the cell. We demonstrate that after a continuous infusion of energy and insulin, absent of amino acids, glucose uptake increased to a similar extent in both Control and T2DM groups (Table 1). Although we increased the glucose concentration above basal levels in both groups during the clamp, simply increasing the glucose availability does not completely explain why our T2DM subjects had similar glucose uptake values as compared to the Control group. Our data show that in addition to increasing glucose uptake during the clamp, insulin signaling in the muscles of our T2DM group improved. Specifically, Akt phosphorylation at Ser473 was increased (Fig. 5A) followed by an increased phosphorylation of AS160 (Fig. 5B). This occurred concurrently with a blunted IRS-1 serine phosphorylation response (Fig. 4A and B). We refer to a blunted response here because we have recently shown that ingestion of 20 grams of essential amino acids and 35 grams of carbohydrate tends to increase muscle IRS-1 serine phosphorylation ($P=0.08$)⁴². It is difficult to explain why Akt phosphorylation increased in the Control group to a similar magnitude as T2DM without the paralleled increase in AS160 phosphorylation. One possibility is that AS160 phosphorylation may have occurred earlier in the insulin sensitive control subjects and thus we missed the peak increase in AS160 phosphorylation. Since AS160 has been associated with an increased GLUT4 translocation to the cell surface⁴⁴ and our T2DM subjects were insulin resistant, another possible explanation may be that it took longer for AS160 to be phosphorylated in the T2DM group which we were able to detect at 5 h following the initiation of the insulin clamp. Although the data presented in the current paper do not provide overwhelming evidence to support a role for mTOR signaling in regulating negative feedback to insulin signaling, it is apparent that components of the insulin signaling pathway and the end result (glucose uptake across the leg) appear to be improved during the Reduced AA clamp in the insulin resistant subjects. We also acknowledge that glucose and fatty acids can influence aspects of insulin signaling and therefore it is possible that these nutrients may have influenced our insulin signaling results as well. However, much more work is needed to verify the specific role of mTOR and amino acid availability in controlling insulin sensitivity.

Previous work strongly supports a link between amino acids and reduced insulin signaling. For instance, Terruzzi et al.⁴⁵ noted that rat hearts infused with insulin and amino acids have reduced PI3K activity and glucose uptake compared to insulin alone. In addition, Patti and colleagues¹⁶ demonstrated that L6 myotubes stimulated with amino acids in the presence of insulin inhibit critical components of glucose transport such as PI3K activity and tyrosine phosphorylated IRS-1. Finally, recent work indicates that mTOR and/or S6K1 are primarily responsible for inducing IRS-1 serine phosphorylation and inhibiting insulin signaling^{18, 23}. Furthermore, Tremblay et al.²⁴ have also shown that an acute infusion of amino acids into human subjects resulted in an overactivation of S6K1 and an inhibition of insulin signaling. On the other hand, in the current study we found that when amino acid availability is reduced

during an increase in insulin and nutrient infusion (in comparison to subjects in which AA's are maintained), that AA's ability to stimulate mTOR signaling was blunted, which seems to be associated with improved insulin signaling for glucose uptake in both Control and T2DM subjects. Our data, along with others, support the hypothesis that amino acids may play a role in the regulation of insulin signaling via the mTOR nutrient signaling pathway.

Our data support the notion that blood amino acid availability plays a regulatory role in regulating muscle protein synthesis, and perhaps muscle protein breakdown, which is especially important in conjunction with elevated insulin concentrations. However, it also appears that supraphysiological doses of insulin can override the effect of lowered amino acids on protein synthesis with a slight decrease in protein breakdown in humans⁴⁶.

In summary, we demonstrate that a reduction in amino acid concentrations during an increase in nutrient and insulin availability in Control and T2DM subjects results in a blunted mTOR/S6K1 signaling and reduced muscle protein synthesis in comparison to when AA concentrations are maintained during an insulin infusion. In addition, the T2DM subjects had similar rates of glucose uptake across the leg as compared to the Control group which corresponded with a stimulation of key components of the insulin signaling pathway (i.e., increased phosphorylation of Akt and AS160). Thus, a reduction in amino acid availability prevents mTOR activation despite an apparent improvement in insulin signaling. Therefore, we conclude that amino acids are necessary for the insulin-induced activation of mTOR/S6K1 and protein synthesis in both healthy and insulin resistant human skeletal muscle. Whether the blunted mTOR signaling is directly linked to improved insulin signaling in the T2DM subjects still needs to be confirmed. Therefore, future studies are required to determine whether chronic exposure of excess nutrients and amino acids lead to insulin resistance in human skeletal muscle and whether mTOR is a potential therapeutic target for improving insulin sensitivity in skeletal muscle.

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Non-standard abbreviations

4E-BP1	4E binding protein 1
AA	amino acids
ACCβ	acetyl CoA carboxylase beta
Akt	protein kinase B
AMPK	adenosine 5' -monophosphate-activated protein kinase
AS160	Akt substrate 160

BCAA	branch-chained amino acids
eEF2	eukaryotic elongation factor 2
eIF2α	eukaryotic initiation factor 2 alpha
IRS-1	insulin receptor substrate-1
mTOR	mammalian target of rapamycin
PI3K	phosphatidylinositol 3-kinase
S6K1	ribosomal S6 kinase 1
T2DM	type 2 diabetes mellitus

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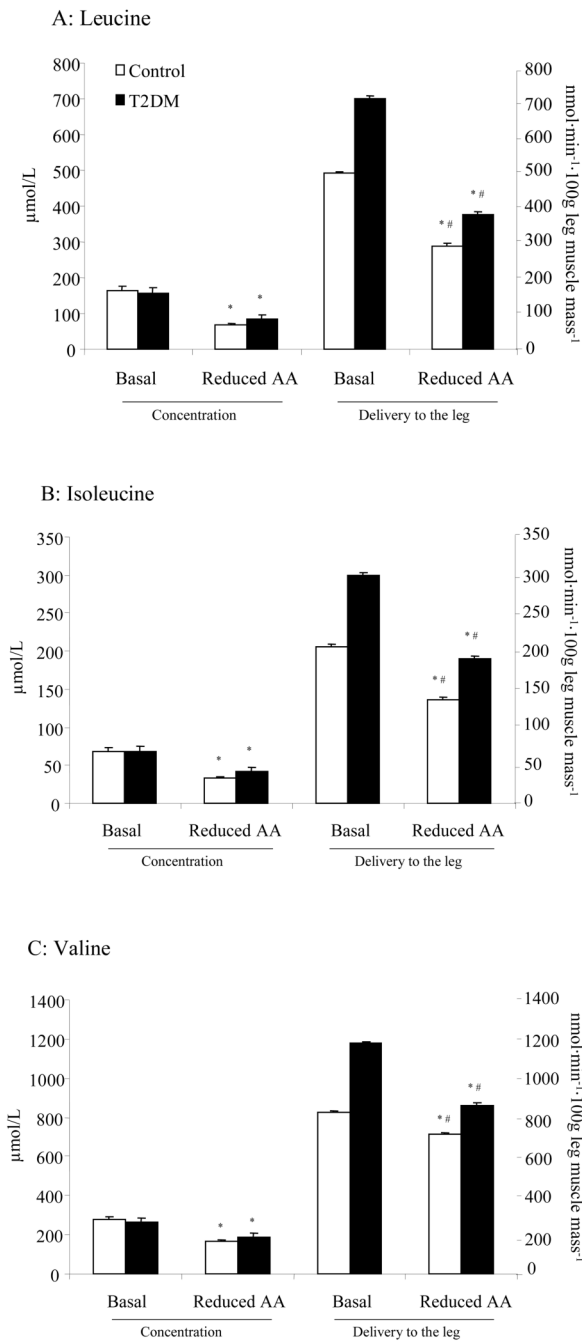


Figure 1. Histograms represent arterial AA concentrations on left Y-axis and AA delivered to leg on right Y-axis (n=6) for A) Leucine (n=6), B) Isoleucine (n=6), and C) Valine (n=6) at Basal and following Reduced AA clamp in Control and T2DM subjects. All values are expressed as mean \pm SE. *Significantly different than Basal (P<0.05). #Significantly different from Control (P<0.05).

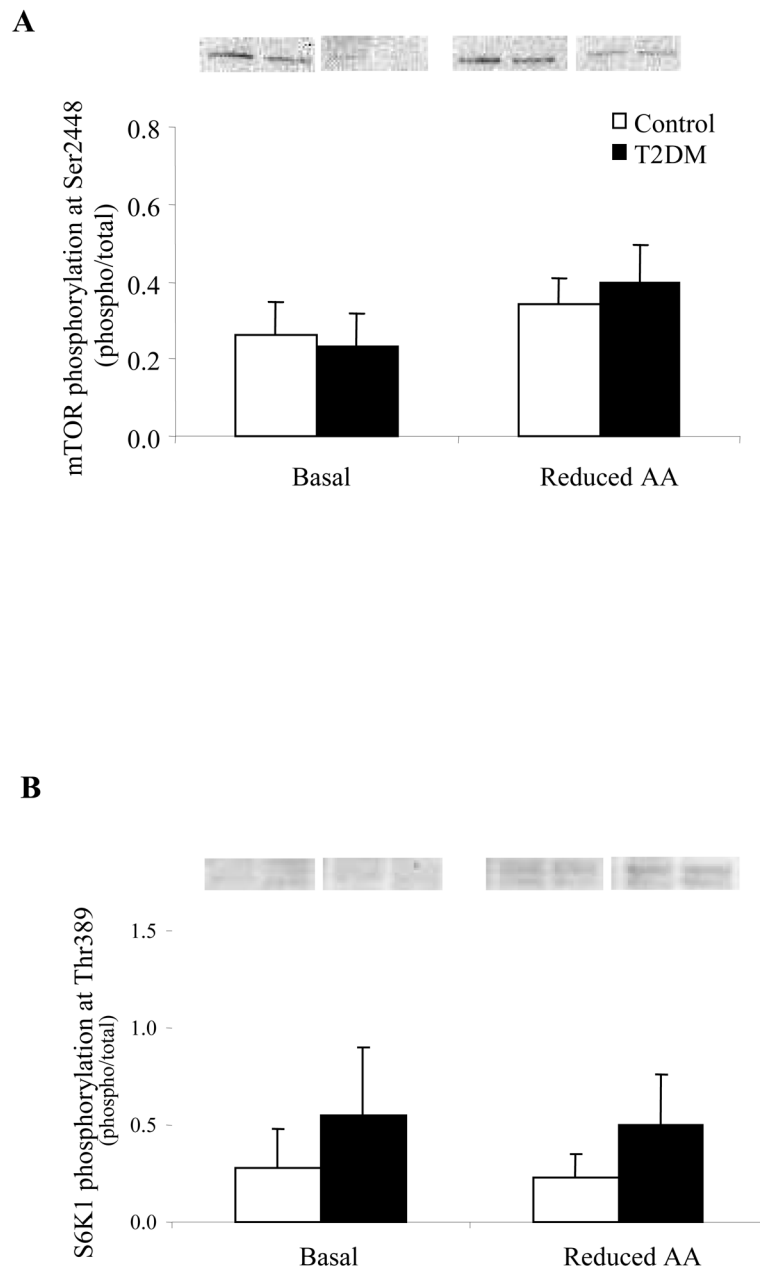


Figure 2. Phosphorylation status of A) mTOR at Ser2448 (n=6) and B) S6K1 at Thr389 (n=5) at Basal and following a Reduced AA clamp in Control and T2DM subjects. Phosphorylation values are relative to total protein. All values are expressed as mean \pm SE.

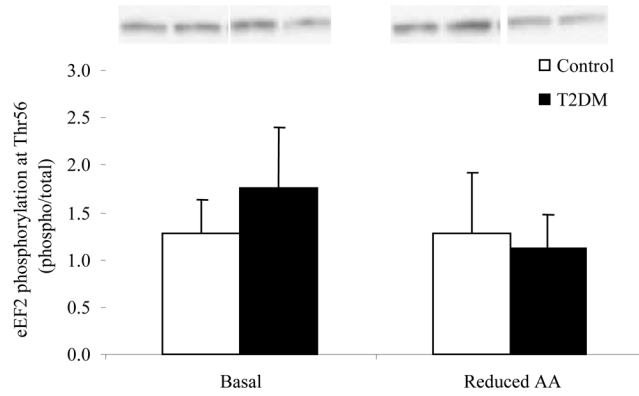
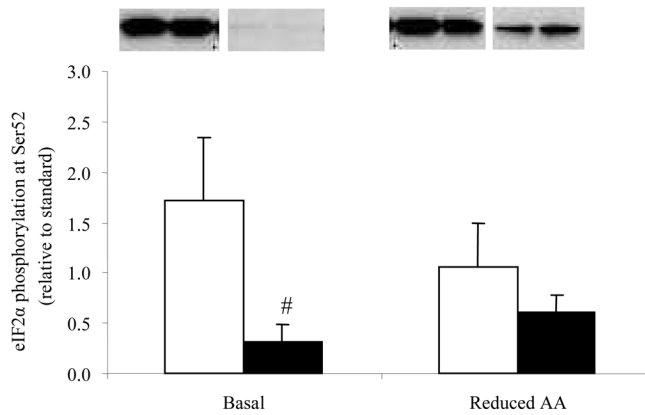
A**B**

Figure 3. Phosphorylation status of A) eEF2 at Thr56 (n=5) and B) eIF2 α at Ser52 (n=6) at Basal and following a Reduced AA clamp in Control and T2DM subjects. Representative immunoblot images are shown above each figure. Phosphorylation values are relative to total protein in Fig. 3A and relative to standard in Fig. 3B. All values are expressed as mean \pm SE. [#]Significantly different from Control (P<0.05).

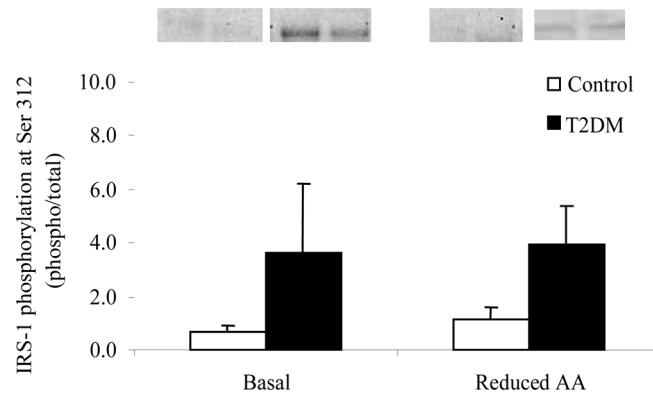
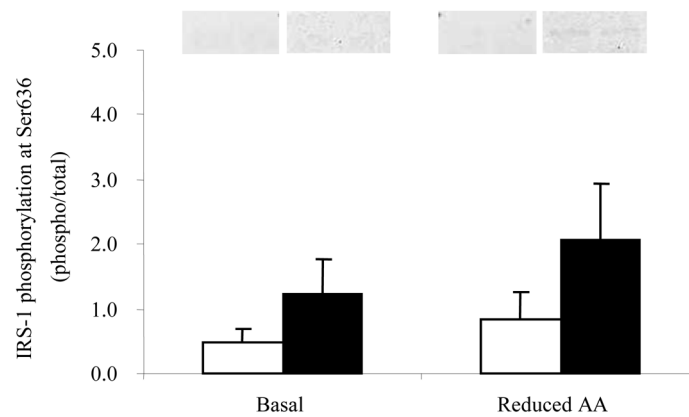
A**B**

Figure 4. Phosphorylation status of IRS-1 at Basal and following a Reduced AA clamp in Control and T2DM subjects. A) IRS-1 at Ser312 (n=5) and B) Ser636/639 (n=6). Representative immunoblot images are shown above each figure. Phosphorylation values are relative to total protein. All values are expressed as mean \pm SE.

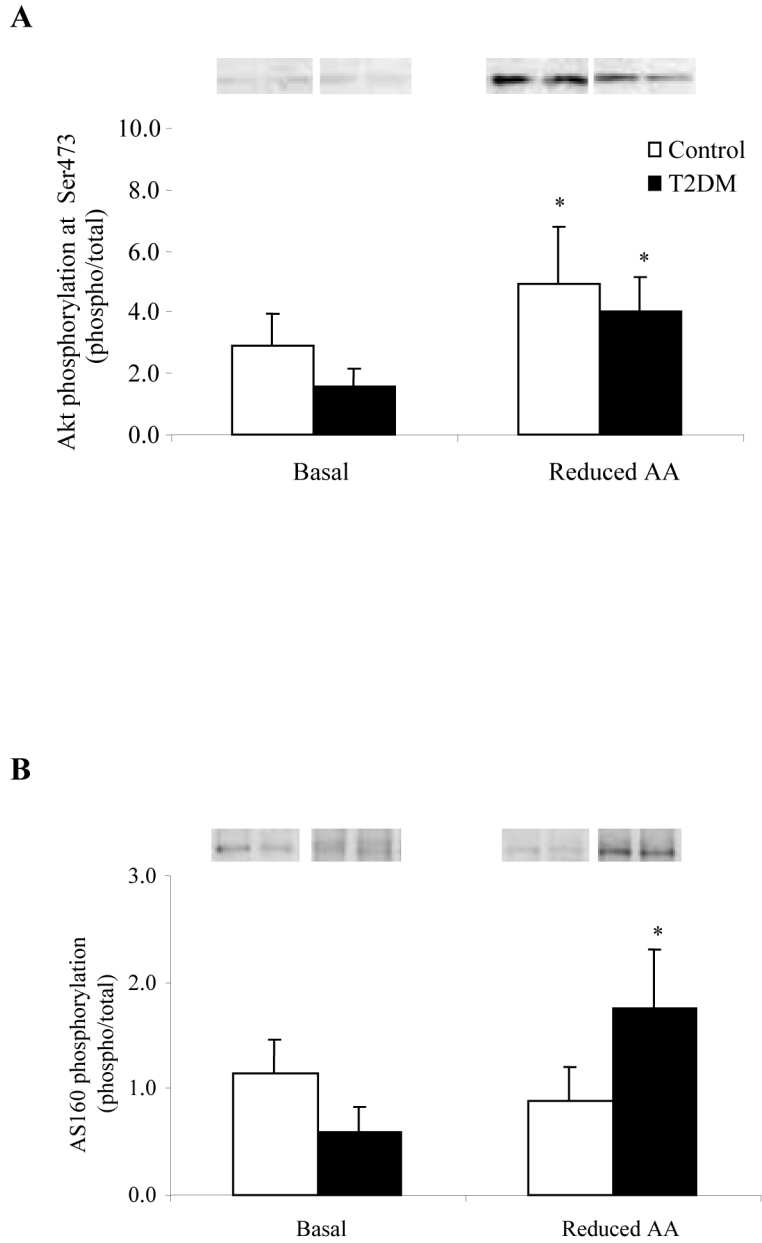


Figure 5. Phosphorylation status of A) Akt at Ser473 (n=5) and B) AS160 (n=6) at Basal and following a Reduced AA clamp in Control and T2DM subjects. Representative immunoblot images are shown above each figure. Phosphorylation values are relative to total protein. All values are expressed as mean \pm SE. *Significantly different than Basal ($P<0.05$).

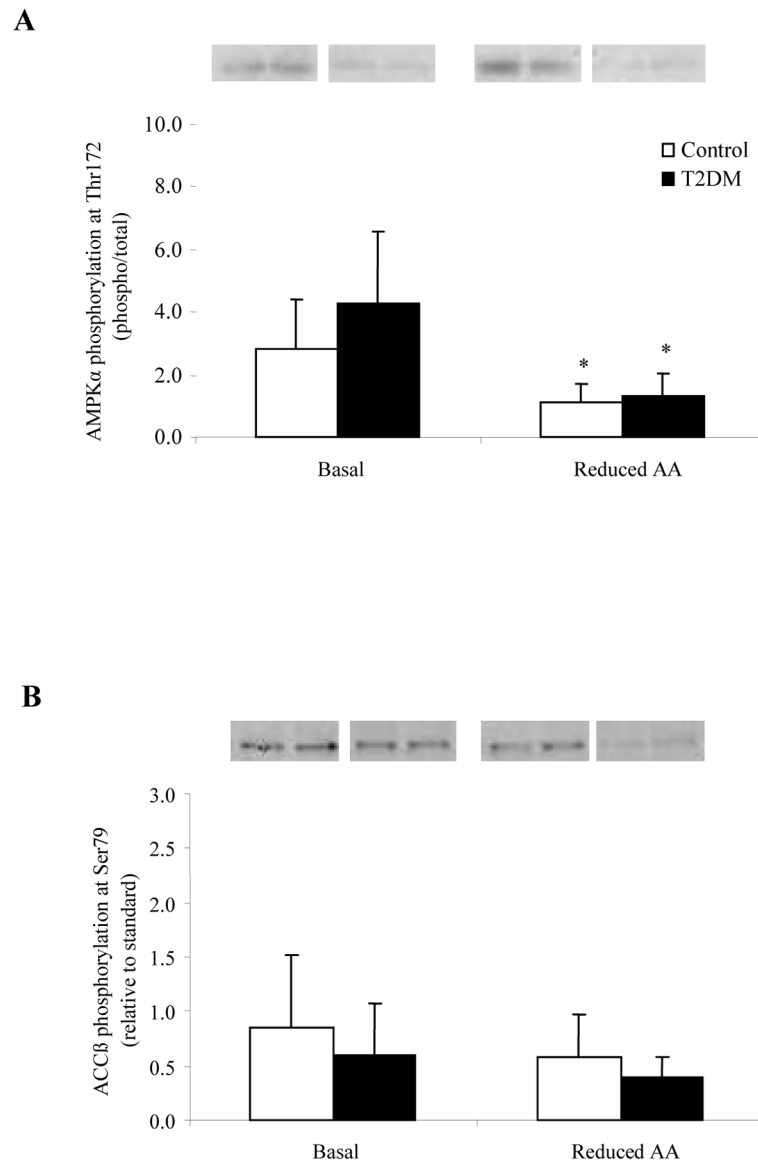
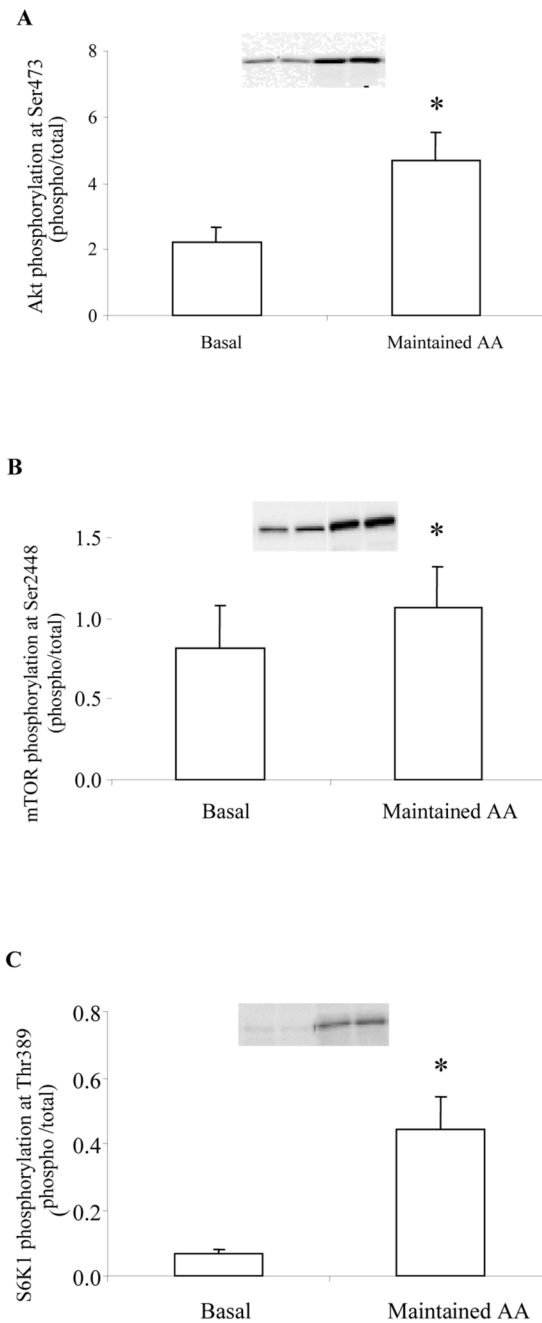


Figure 6. Histograms represent the phosphorylation status of A) AMPK α (n=5) and B) ACC β at Ser79 (n=5) at Basal and following a Reduced AA clamp in Control and T2DM subjects. Representative immunoblot images are shown above each figure. Phosphorylation values are relative to total protein in Fig. 6A and relative to standard in Fig. 6B. All values are expressed as mean \pm SE. *Significantly different than Basal (P<0.05).

**Figure 7.**

Histograms represent the phosphorylation status of A) Akt at Ser473 (n=4), B) mTOR at Ser2448 (n=4), and C) S6K1 at Thr389 at Basal and following treatment with Maintained AA in young, healthy subjects. Representative immunoblot images are shown above each figure. Phosphorylation values are relative to total protein. All values are expressed as mean \pm SE. *Significantly different than Basal (P<0.05).

Table 1Metabolic measurements taken after clamp^{1,2,3,4}

Metabolic measurements	Control	T2DM	Maintained AA
Blood amino acid concentration	↓	↓	—
Blood glucose concentration	↑	↑ ^a	—
Blood insulin concentration	↑	↑ ^b	↑
Glucose uptake across the leg	↑	↑	↑
Muscle protein synthesis	↓*	↓*	↑
Muscle protein breakdown	↓	↓	—

¹ Arrows are a summary of results from Ref 25 and ²⁶. All arrows that point up or down are significantly different from Basal levels ($P < 0.05$). No arrow represents no change from Basal ($P > 0.05$).

^{2a} a indicates significantly higher than Control.

^{3b} b indicates significantly lower than Control.

^{4*} * indicates P-value of $P = 0.08$.

Table 2Total protein content at Basal and after Reduced AA clamp^{1,2,3,4}

	Basal		Reduced AA	
	Control	T2DM	Control	T2DM
mTOR (n=6)	1.26 ± 0.15	0.38 ± 0.11 [#]	0.90 ± 0.11	0.49 ± 0.10 [#]
S6K1 (n=5)	1.04 ± 0.15	0.64 ± 0.16	0.96 ± 0.16	0.78 ± 0.19
eEF2 (n=5)	4.33 ± 0.35	2.61 ± 0.30	4.53 ± 0.36	2.28 ± 0.27
IRS-1 (n=6)	1.69 ± 0.13	1.13 ± 0.16	1.49 ± 0.17	1.33 ± 0.22 [*]
Akt (n=5)	0.39 ± 0.11	0.48 ± 0.12	0.43 ± 0.09	0.27 ± 0.11 [*]
AS160 (n=6)	1.21 ± 0.14	1.18 ± 0.16	1.64 ± 0.16	1.17 ± 0.14
AMPK (n=5)	2.72 ± 0.32	1.66 ± 0.24	2.49 ± 0.26	1.41 ± 0.18

¹ Subject total (n) is representative for both treatments and time points.

^{2*} indicates significantly different than Basal (P < 0.05).

^{3#} indicates significantly different than Control at corresponding time point (P < 0.05).

⁴ All values are expressed as mean ± SE.