Supplementary Files

Title: Lower Fasted State but Greater Change in Plasma Amino Acid-Induced Rise in Muscle Protein Synthesis in People with Obesity

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Methods

Statistical Power Calculations

Main end-points were the rate of synthesis of mixed-muscle and mitochondrial proteins, and we assumed comparable fasted-state responses with respect to both muscle protein pools. Our previous study (1) showed a difference of 1.43 SD for mixed-muscle protein synthesis between subjects with obesity and lean controls. A sample of 9 subjects per group had 80% power ($\alpha = 0.05$) to detect a difference between groups in mixed-muscle protein synthesis. The rate of amino acid infusion was chosen based on previous report showing stimulation of both mixed-muscle and mitochondrial proteins (2). Due to the different amino acid mixture used, the rate of infusion of the amino acid solution in the current study was adjusted to reflect a rate of leucine infusion comparable to that in the previous report (2), and given that leucine has primary role in the stimulation of muscle protein synthesis (3, 4). The leucine infusion rate was further adjusted to the fat free mass (FFM) with the goal to achieve comparable increase in the plasma leucine concentrations between the two subject populations during the amino acid mixture infusion, and given that the two groups were expected to differ considerably in FFM. Based on earlier evidence (2) we expected that the amino acid infusion will change/increase mixed-muscle protein synthesis in the lean subjects, and that the change in mixed-muscle protein synthesis rate in the subjects with obesity would be greater (i.e., sum of the change previously observed in the lean subjects (2) and the difference in the fasted-state mixed-muscle protein synthesis between groups (1)). A sample of 7 subjects per group had 80% power ($\alpha = 0.05$) to detect a difference (1.6 SD) in the change resulting from the amino acid infusion of mixed-muscle protein synthesis between groups.

Processing of Muscle for Isolation of Amino Acids from Mixed-muscle Protein

Muscle was processed for mixed-muscle protein amino acid enrichment using procedures we have previously described (5). These procedures were as follows: 10-15 mg of muscle tissue was weighted, and 500 uL of 5% sulfosalicylic acid (SSA) was added to the tissue to precipitate the

muscle proteins. Following homogenization of the muscle, the homogenate was centrifuged at 2,500 x g for 45 min at 4^{o}C. After discarding the supernatant, 500 uL of 5% SSA was added, the muscle was homogenized, and the homogenate was centrifuged again. This procedure was repeated one more time (total of three times). The final muscle pellet was washed initially with 500 uL of 5% SSA, then with 1 ml of ethanol, followed by a wash with 1 ml of ethyl ether. The resulting pellet was placed in an oven overnight at 50^{o}C, and until dry. Proteins in the pellet were hydrolyzed the following day by adding 2 ml of 6 N HCl and heating at 110^{o}C for 24 hours. The resulted protein hydrolysate was stored at -80^{o}C until further analyses. Protein hydrolysate equivalent to 1 mg of dry muscle weight was passed through cation-exchange column (AG 50W-8x 200-400-mesh; Bio-Rad Laboratories, Inc., Hercules, California) to isolate/purify the amino acids. The cation-exchange columns were conditioned with 3 ml of 2N NH_4OH and 3 ml of 1N HCl prior to adding the protein hydrolysate. Finally, the amino acids were eluted from the columns with 8 ml of 2N NH_4OH. Samples were then placed in SpeedVac Concentrator (Savant^{TM} SPD1010, Thermo Fisher Scientific) overnight at low heat to dry. Dried samples were reconstituted with 150 ul of Milli-Q® water and filtered using Spin-X® centrifuge filter tubes by centrifugation at 2,500 x g for 15 min, and in preparation for the mass spectrometry analyses (below).

Processing of Muscle for Isolation of Amino Acids from Mitochondrial Protein

Mitochondria were isolated from muscle using procedures comparable to those we have previously described (6, 7). These procedures were as follows: ~80 mg of muscle was used for the isolation of mitochondrial proteins. All procedures were performed on ice or at 4°C. After the muscle was cleaned from visible fat and connective tissue, the tissue was minced with scissors in 9 volumes of ice-cold solution (Solution I) containing (mM): 100 KCl, 40 Tris-HCl, 10 Tris-Base, 5 $MgCl_2$, 1 EDTA, 1 ATP, pH 7.5. It was then homogenized gently by hand using a ground glass-to-glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 800 x g for 10 min to obtain the supernatant containing mitochondria. The supernatant was then centrifuged at 14,000 x g for 10 min to obtain a mitochondrial pellet, which was re-suspended in 0.5 ml of solution (Solution II)

containing (mM): 100 KCl, 40 Tris-HCl, 10 Tris-Base, 5 $MgCl_2$, 1 EDTA, 0.2 ATP, pH 7.5. The re-suspended mitochondrial sample was centrifugated at 7000 x g for 10 min. After discarding the supernatant, the mitochondrial pellet was re-suspended in 0.5 ml of Solution II. After a final centrifugation at 4000 x g for 10 min, the final mitochondrial pellet was washed once with 500 ul of water. To hydrolyze the mitochondrial proteins, the mitochondrial pellet was reconstituted with 2 ml of 6 N HCl and heated at 110^{o}C for 24 hours. The entire mitochondrial sample was run through cation-exchange column (AG 50W-8x 200-400-mesh; Bio-Rad Laboratories, Inc., Hercules, California) to isolate/purify the amino acids. The rest of the procedures with respect to the isolation of the amino acids were identical to those described above for the isolation of the amino acids from mixed-muscle protein.

Processing of Blood for Isolation of Blood Amino Acids

Blood samples were transferred into glass tubes containing 1 ml of 15% SSA, and the samples were immediately mixed well. The samples were then centrifuged at 2,500 x g for 15 min at 4^{o}C, and the resultant supernatant was collected and stored at -80^{o}C until further analyses. Blood amino acids were isolated using cation-exchange columns (AG 50W-8x 100-200-mesh; Bio-Rad Laboratories, Inc., Hercules, California). Prior to adding 500 ul of the blood/SSA mixture supernatant sample into the cation-exchange columns, the columns were conditioned the same way that is described above for the mixed-muscle protein samples. The amino acids were eluted using 8 ml of 2N NH_4OH, and the samples were dried down overnight at low heat on a SpeedVac Concentrator (SavantTM SPD1010, Thermo Fisher Scientific). Dried samples were used for the mass spectrometry analyses (below).

Amino Acid Stable Isotope Enrichment Determination by Mass spectrometry

The isotopic enrichment of leucine, expressed as molar percent excess (MPE), was determined by LC-MS/MS (TSQ Quantiva™ Triple Quadrupole Mass Spectrometer, Thermo Fisher Scientific), using the isobutyl ester derivative of leucine. We followed experimental procedures comparable to those previously described (8), and which have also previously been utilized in our laboratory (5). For the determination of leucine enrichment in the blood samples, we applied selected reaction monitoring for transitions 188>86 and 197>95 for the quantification of m+0 and m+9 leucine isotopes, respectively. Due to the low level of enrichment in mixed-muscle and mitochondrial proteins, we monitored 190>88 (m+2) and 197>95 (m+9) for both samples, and used standard curve instead. The results were calculated against a curve with theoretical MPE on the x axis and m+9/m+2 ratios on the y axis. This 8-point enrichment calibration curve was constructed using unlabeled leucine and L-[2,3,3,4,5,5,5,6,6,6-$^{2}H_{10}$]leucine. It is noted that the L-[2,3,3,4,5,5,5,6,6,6-$^{2}H_{10}$]leucine standard is only 95.11% isotopically pure and contains 4.78% m+9 leucine. This enables us to use the d_{10}-leucine standard to construct an enrichment curve for d_{9}-leucine.

Protein Immunoblotting

Proteins were separated using SDS-PAGE and quantified by immunoblotting (1, 9). Specifically, muscle homogenate containing either 40 ug (eiF2α, PGC1α) or 50 ug (mTOR, S6K1) of protein was separated by electrophoresis using Any kD™ (eiF2α, PGC1α) or 4-20% (mTOR, S6K1) gradient polyacrylamide gels (Mini-PROTEAN, Bio-Rad Laboratories, Inc.). All samples from a given subject were run on the same gel. Following electrophoresis, protein was transferred to nitrocellulose (eiF2α) or polyvinylidene difluoride (mTOR, S6K1, PGC1α) membranes. Membranes were then incubated with appropriate antibodies to quantify phospho-protein and total protein abundances. Prior to incubating the membranes with primary antibodies, the membranes were washed with TBST and blocked with TBST + 5% BSA (eiF2α, PGC1α) or 5% non-fat dry milk (mTOR, S6K1) at room temperature for one hour. The membranes were incubated with primary antibodies overnight at 4^{o}C. The following primary antibodies were used: anti-mTOR (Cat. # 2972), anti-phospho-mTOR (Ser2448) (Cat. # 2971), anti-S6K1 (Cat. # 9202), anti-phospho-S6K1 (Thr389) (Cat. # 9205),

anti-eIF2α (Cat. # 2103), anti-phospho-eIF2α (Ser51) (Cat. # 9721) (all from Cell Signaling Technology). The anti-PGC1α antibody was from EMD Millipore Corporation (Cat. # AB3242). Anti-GAPDH antibody (Cat. # 600-401-A33, Rockland Immunochemicals) was used to quantify GAPDH, and was employed as a loading control. The next day, the membrane was washed with TBST and incubated for 1 hour at room temperature with a compatible secondary antibody. Secondary antibodies used were anti-rabbit (Cat. # 7074, Cell Signaling Technology, Inc.) and anti-mouse (Cat. # sc-516102; Santa Cruz Biotechnology) IgG HRP-linked antibodies. Secondary antibody was removed by washing with TBST. Positive bands were visualized using the Clarity™ Western ECL Blotting Substrate kit (Bio-Rad, Hershey, PA), and imaged on an ImageQuant LAS 4000 (GE Healthcare, Wauwatosa, WI). Membranes for phospho-eiF2α and eiF2α (~38 kDa) were stripped of primary and secondary antibodies using Restore™ Western Blot Stripping Buffer (Cat. # 21059, ThermoFisher Scientific, Inc.) and were subsequently re-probed for GAPDH (~37 kDa). The phospho-eIF2α-to-eIF2α ratio was calculated after the phospho and total eiF2α proteins were adjusted first to the loading control (i.e., GAPDH). Accordingly, phospho-mTOR and mTOR (~289 kDa) were normalized to GAPDH prior to the calculation of the phospho-mTOR-to-mTOR ratio. PGC1α (~91 kDa) was adjusted directly to the GAPDH signal intensity. Furthermore, to control for possible variability among blots that can impact the absolute band intensity (i.e. transfer efficiency, blocking, antibody/luminescent binding, etc.), every gel contained a well loaded with an identical sample (rat muscle homogenate). Densitometry data obtained from a given blot were normalized to the densitometry value of the internal control on that blot. Fold changes in response to the elevated plasma amino acids were calculated by dividing the value during elevated plasma amino acid concentrations by that in the fasted state.

Table S1 – Concentrations of individuals amino acids in plasma in the fasted state and in response to the amino acid mixture infusion

	Fasted	Amino Acids	Change (*Δ*)
Aspartic acid (μmol·L^{-1})			
Lean subjects	4.0 ± 0.4	19.4 ± 2.2†	15.4 ± 2.2
Subjects with obesity	4.1 ± 0.7	31.0 ± 3.3†*	26.9 ± 3.2*
Glutamic acid (μmol·L^{-1})			
Lean subjects	43.6 ± 5.1	123.9 ± 12.9†	80.3 ± 13.0
Subjects with obesity	59.9 ± 4.6	172.2 ± 18.6†*	112.3 ± 17.5
Asparagine (μmol·L^{-1})			
Lean subjects	39.3 ± 4.7	28.4 ± 4.0	-10.9 ± 3.6
Subjects with obesity	38.6 ± 3.4	26.8 ± 5.6†	-11.8 ± 5.2
Serine (μmol·L^{-1})			
Lean subjects	101.4 ± 4.1	189.4 ± 17.5†	88.1 ± 14.7
Subjects with obesity	93.0 ± 5.2	174.7 ± 20.9†	81.7 ± 18.5
Glutamine (μmol·L^{-1})			
Lean subjects	486. 2 ± 44.3	463.2 ± 52.4	-23.1 ± 40.5
Subjects with obesity	579.9 ± 49.7	513.7 ± 54.7	-66.3 ± 50.6
Threonine (μmol·L^{-1})			
Lean subjects	147.1 ± 20.6	311.3 ± 39.9†	164.1 ± 25.2
Subjects with obesity	141.9 ± 19.7	323.3 ± 38.9†	181.3 ± 21.1
Arginine (μmol·L^{-1})			
Lean subjects	76.8 ± 4.3	258.6 ± 21.3†	181.8 ± 19.2

Subjects with obesity	68.6 ± 2.6	266.3 ± 23.2†	197.7 ± 22.2
Tyrosine (μmol·L^{-1})			
Lean subjects	51.4 ± 3.3	63.7 ± 6.1	12.3 ± 7.1
Subjects with obesity	72.2 ± 5.8*	80.4 ± 7.5	8.2 ± 7.4
Methionine (μmol·L^{-1})			
Lean subjects	50.8 ± 7.7	192.0 ± 22.9†	141.1 ± 19.7
Subjects with obesity	54.5 ± 10.0	249.3 ± 21.9†*	194.8 ± 17.9
Valine (μmol·L^{-1})			
Lean subjects	186.0 ± 14.4	550.5 ± 31.7†	364.5 ± 36.1
Subjects with obesity	222.1 ± 18.5	583.3 ± 38.9†	361.2 ± 37.7
Phenylalanine (μmol·L^{-1})			
Lean subjects	53.4 ± 3.1	191.3 ± 13.3†	137.9 ± 13.7
Subjects with obesity	65.3 ± 4.7	241.8 ± 12.2†*	176.5 ± 11.7*
Isoleucine (μmol·L^{-1})			
Lean subjects	52.4 ± 5.4	200.4 ± 12.1†	148.0 ± 14.5
Subjects with obesity	65.8 ± 5.0	226.4 ± 18.3†	160.6 ± 18.2
Leucine (μmol·L^{-1})			
Lean subjects	119.1 ± 8.3	328.3 ± 13.3†	209.2 ± 17.3
Subjects with obesity	137.7 ± 10.0	377.0 ± 39.7†	239.3 ± 40.0

Values are mean ± SEM. *$P \leq 0.05$ versus Lean subjects, †$P \leq 0.05$ versus Fasted state.

Table S2 – Pearson correlation coefficients between measures of synthesis rate of muscle proteins in the fasted state and selected anthropometric and biochemical parameters

Variable	Mixed-muscle Protein synthesis (%/hr)	Mitochondrial Protein Synthesis (%/hr)
Mixed-Muscle Protein synthesis (%/hr)		
Total Mitochondrial Protein Synthesis (%/hr)	0.28	
Body Mass Index (kg/m^2)	-0.60*	-0.36
Body Fat Free mass (kg)	-0.43	-0.41
Body Fat Mass (kg)	-0.45*	-0.17
Body Fat (%)	-0.32	0.03
Waist Circumference (cm)	-0.55*	-0.39
Waist-to-hip Ratio	-0.37	-0.51*
VO_2max (ml/kg FFM/min)	-0.04	-0.06
Blood Glucose (mg/dl)	-0.50*	-0.20
Plasma Insulin (uIU/ml)	-0.44	-0.33

Plasma IGF-1	0.28	0.47*
Matsuda-ISI	0.25	0.21
HOMA-IR	-0.44*	-0.32
HbA1c (%)	-0.48*	-0.11
TSH (mIU/l)	0.12	-0.23
Plasma Triglycerides (mg/dl)	-0.36	-0.29
Plasma NEFA (mmol/1)	0.49*	0.53*
Plasma PUFA (mmol/l)	0.72*	0.20
Plasma SA (mmol/l)	0.58*	0.16
Plasma MUFA (mmol/l)	0.64*	0.49*
SFA/PUFA	-0.53*	-0.15
Palmitate/Oleate	-0.44	-0.46*

VO2max, maximal oxygen uptake; Matsuda-ISI, Matsuda insulin-sensitivity index; HOMA-IR, homeostatic model assessment of insulin-resistance; TSH, thyroid-stimulating hormone; NEFA, non-esterified fatty acids; PUFA, sum of concentrations of polyunsaturated NEFA (i.e., eicosapentaenoic acid, linolenic acid, docosahexaenoic acid, arachidonic acid, linoleic acid); SFA, sum of concentrations of saturated NEFA (i.e., myristic acid, palmitic acid, stearic

acid); MUFA, sum of concentrations of monounsaturated NEFA (i.e.,

palmitoleic acid, oleic acid); *$P \leq 0.05$.

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