Diabetes Volume 64, May 2015

Gordon I. Smith,[1] Jun Yoshino,[1] Kelly L. Stromsdorfer,[1] Seth J. Klein,[2] Faidon Magkos,[1] Dominic N. Reeds,[1] Samuel Klein,[1] and Bettina Mittendorfer[1]

Protein Ingestion Induces Muscle Insulin Resistance Independent of Leucine-Mediated mTOR Activation

Diabetes 2015;64:1555–1563 | DOI: 10.2337/db14-1279

Increased plasma branched-chain amino acid concentrations are associated with insulin resistance, and intravenous amino acid infusion blunts insulin-mediated glucose disposal. We tested the hypothesis that protein ingestion impairs insulin-mediated glucose disposal by leucine-mediated mTOR signaling, which can inhibit AKT. We measured glucose disposal and muscle p-mTOR^{Ser2448}, p-AKT^{Ser473}, and p-AKT^{Thr308} in 22 women during a hyperinsulinemic-euglycemic clamp procedure with and without concomitant ingestion of whey protein (0.6 g/kg fat-free mass; n = 11) or leucine that matched the amount given with whey protein (n = 11). Both whey protein and leucine ingestion raised plasma leucine concentration by approximately twofold and muscle p-mTOR^{Ser2448} by ~30% above the values observed in the control (no amino acid ingestion) studies; p-AKT^{Ser473} and p-AKT^{Thr308} were not affected by whey protein or leucine ingestion. Whey protein ingestion decreased insulin-mediated glucose disposal (median 38.8 [quartiles 30.8, 61.8] vs. 51.9 [41.0, 77.3] μmol glucose/μU insulin · mL^{-1} · min^{-1}; $P < 0.01$), whereas ingestion of leucine did not (52.3 [43.3, 65.4] vs. 52.3 [43.9, 73.2]). These results indicate that *1*) protein ingestion causes insulin resistance and could be an important regulator of postprandial glucose homeostasis and *2*) the insulin-desensitizing effect of protein ingestion is not due to inhibition of AKT by leucine-mediated mTOR signaling.

Skeletal muscle insulin resistance is a common metabolic complication of obesity and is the key factor responsible for abnormal postprandial glucose clearance and increased risk for developing type 2 diabetes and cardiovascular disease in obese people (1–3). It has been suggested that branched-chain amino acids (i.e., leucine, isoleucine, and valine) (4,5), most likely leucine alone (6–8), are involved in the pathogenesis of obesity-associated insulin resistance because *1*) branched-chain amino acid concentrations in plasma and their metabolites are increased in obese compared with lean people (9,10) and have been identified as predictors of insulin resistance (9,11–14); *2*) data from studies conducted in cultured myotubes and isolated rat skeletal muscles have demonstrated that leucine can impair insulin-mediated glucose uptake (15,16), presumably via AMPK-mediated mTOR-p70S6K phosphorylation and subsequent serine phosphorylation of insulin receptor substrate (IRS)-1 (7,15–19), and *3*) infusing amino acids during a hyperinsulinemic-euglycemic clamp procedure can reduce glucose disposal in people (20–23). Collectively, these data suggest that dietary protein (or leucine) ingestion might be an important regulator of muscle insulin sensitivity, but we are unaware of any studies that have evaluated this issue. A better understanding of the interaction between dietary amino acid availability and insulin-mediated muscle glucose uptake could help elucidate the mechanisms responsible for obesity-associated abnormalities in glucose metabolism.

The goal of the current study was to test the hypothesis that protein ingestion impairs insulin-stimulated glucose disposal owing to leucine-mediated mTOR phosphorylation in muscle. Accordingly, we predicted that both whey protein ingestion and ingestion of leucine that matches

[1]Department of Medicine and Center for Human Nutrition and Atkins Center of Excellence in Obesity Medicine, Washington University School of Medicine, St. Louis, MO

[2]Department of Radiology, Washington University School of Medicine, St. Louis, MO

Corresponding author: Bettina Mittendorfer, mittendb@wustl.edu.

Received 19 August 2014 and accepted 24 November 2014.

Clinical trial reg. nos. NCT01538836 and NCT01757340, clinicaltrials.gov.

G.I.S. and J.Y. contributed equally to this article.

© 2015 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered.

the whey protein leucine content would impair insulin-mediated glucose disposal and be associated with decreased phosphorylated (p)-AMPK^{Thr172} (and its downstream target p-ACC^{Ser79}), increased p-mTOR^{Ser2448} (and its downstream target p-p70S6K^{Thr389}), and decreased p-AKT^{Ser473} and p-AKT^{Thr308} (and their downstream target GSKβ^{Ser9}) in skeletal muscle. To accomplish this goal, we had two groups of subjects complete two hyperinsulinemic-euglycemic clamp procedures: one with and another without simultaneous whey protein ingestion or one with and another without simultaneous ingestion of leucine that matched the amount present in whey protein. Furthermore, we selected a dose of whey protein (and leucine) that would elicit a rise in plasma leucine concentration similar to that observed after mixed-meal ingestion (24,25).

Table 1—Subjects' body composition and basic metabolic characteristics

	Whey protein group (*n* = 11)	Leucine group (*n* = 11)	*P**
BMI (kg/m^2)	33.6 ± 0.8	36.0 ± 1.5	0.16
Body mass (kg)	90.3 ± 2.3	96.4 ± 4.5	0.25
Body fat (%)	48.1 ± 1.0	50.3 ± 1.3	0.20
Subcutaneous abdominal fat (cm^3)	2,940 ± 118	3,197 ± 232	0.33
Intra-abdominal fat (cm^3)	1,431 ± 137	1,428 ± 235	0.99
FFM (kg)	46.8 ± 1.4	47.4 ± 1.2	0.77
Leg lean mass (kg)	15.1 ± 0.6	15.6 ± 0.6	0.49
Plasma concentrations			
Glucose (mmol/L)†	5.11 ± 0.10	5.43 ± 0.17	0.12
Glucose (2-h post-OGTT) (mmol/L)	7.00 ± 0.49	7.46 ± 0.50	0.52
Triglycerides (mmol/L)†	1.88 ± 0.30	1.27 ± 0.20	0.11
Total cholesterol (mmol/L)†	6.10 ± 0.30	5.12 ± 0.11	0.01
HDL cholesterol (mmol/L)†	1.49 ± 0.09	1.51 ± 0.10	0.87
LDL cholesterol (mmol/L)†	3.75 ± 0.27	3.02 ± 0.13	0.03

Data are means ± SEM. OGTT, oral glucose tolerance test. *Values were determined by using Student *t* test for independent samples. †Values were obtained after an overnight fast.

RESEARCH DESIGN AND METHODS

Subjects and Prestudy Testing

Twenty-two sedentary (<1.5 h of exercise/week) and weight-stable (<2 kg change for at least 6 months) 50- to 65-year-old (mean ± SD age 57.8 ± 4.2 years) postmenopausal women participated in this study, which was approved by the institutional review board of Washington University School of Medicine in St. Louis, Missouri. Written informed consent was obtained from all subjects before participation. All subjects completed a history and physical examination, a resting electrocardiogram, standard blood tests, and an oral glucose tolerance test. None of the subjects had evidence of chronic illness or significant organ dysfunction (e.g., diabetes, liver cirrhosis) or were taking medications (including hormone-replacement therapy) that could interfere with insulin or glucose metabolism, and none reported excessive alcohol intake or consumed tobacco products. Body fat mass and fat-free mass (FFM) were determined by using DEXA (Lunar iDXA; GE Healthcare Lunar, Madison, WI). Intra-abdominal and abdominal subcutaneous adipose tissue volumes were quantified by using MRI (1.5-T superconducting magnet; Siemens, Iselin, NJ) and Matlab software (Mathworks, Natick, MA) in the Washington University School of Medicine Center for Clinical Imaging Research.

Experimental Design

Each subject completed two hyperinsulinemic-euglycemic clamp procedures and was randomized to clamp procedures conducted with or without simultaneous whey protein ingestion (*n* = 11) or clamp procedures conducted with or without simultaneous leucine ingestion (*n* = 11) (Table 1). Before each clamp procedure, subjects were instructed to adhere to their usual diet and to refrain from vigorous physical activities for 3 days. Subjects were admitted to the Clinical Research Unit in the late afternoon, where they consumed a standard dinner between 1800 and 1900 h and then fasted, except for water, until the next morning. At 0600 h, a catheter was inserted into an arm vein for the infusion of a stable isotope labeled glucose tracer; catheters for blood sampling were inserted into the radial artery of the opposite arm and in retrograde fashion into the femoral vein of one leg. At ~0645 h, a primed, constant infusion of [6,6-$^2H_2$]glucose (priming dose: 22 μmol · kg body wt^{-1}, infusion rate: 0.22 μmol · kg body wt^{-1} · min^{-1}), purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA), was started, and 4 h later the hyperinsulinemic-euglycemic clamp procedure was initiated with two 5-min priming doses (first 200 mU · m^{-2} body surface area [BSA] · min^{-1} and then 100 mU · m^{-2} BSA · min^{-1}) of human insulin (Novolin R; Novo Nordisk, Princeton, NJ); for the remaining 230 min, insulin was infused at a rate of 50 mU · m^{-2} BSA · min^{-1}. Euglycemia (at blood glucose concentration ~5.6 mmol/L) was maintained by variable rate infusion of 20% dextrose (Baxter, Deerfield, IL) enriched to 2.5% with [6,6-$^2H_2$]glucose. Subjects in the whey protein trial consumed either 0.6 g whey protein (unflavored Unjury; ProSynthesis Laboratories, Inc., Reston, VA) per kg FFM (containing 0.0684 g leucine/kg FFM), dissolved in 360 mL water, or the same volume of water alone during the clamp procedure. In the leucine trial, subjects consumed either 0.0684 g leucine (Sigma-Aldrich, St. Louis, MO) per kg FFM, dissolved in 360 mL Kool-Aid (Kraft Foods, Inc., Northfield, IL), or the same volume of the Kool-Aid solution alone during the clamp

procedure. To minimize potential differences in plasma leucine concentration between studies that could arise from differences in the intestinal absorption rate of free compared with whey protein–derived leucine (26,27) and to elicit a rise in plasma leucine concentration similar to that after mixed-meal ingestion (i.e., sustained approximate doubling for ~3–4 h) (24,25) after both whey protein and leucine ingestion, we arranged for both the whey protein and leucine drinks to be consumed in small aliquots every 20 min. The whey/no whey and leucine/no leucine studies were conducted in randomized order 1–4 weeks apart.

Blood samples to determine plasma glucose, insulin, and leucine concentrations and glucose kinetics were obtained immediately before starting the glucose tracer infusion and every 6–7 min during the last 20 min of the basal period and the clamp procedure; additional blood samples were obtained every 10 min during the clamp procedure to monitor blood glucose concentration. Muscle tissue from the quadriceps femoris was obtained from 9 of 11 subjects in the whey protein group and 8 of 11 subjects in the leucine group by using Tilley-Henkel forceps 60 min after starting the glucose tracer infusion (basal period) and 180 min after starting the insulin infusion to determine the contents of p-AMPK^{Thr172}, p-ACC^{Ser79}, p-mTOR^{Ser2448}, p-p70S6K^{Thr389}, p-AKT^{Ser473}, p-AKT^{Thr308}, and GSKβ^{Ser9}. The basal and clamp biopsies were taken through separate incisions (~5 cm apart) on the same leg.

Leg blood flow in the common femoral artery was measured between 120 and 180 min after starting the glucose tracer infusion (basal period) and between 60 and 180 min after starting the insulin infusion (clamp period) by using Doppler ultrasound (M-Turbo; Sonosite, Inc., Bothell, WA) and a linear array 13–6 MHz frequency probe (Sonosite) (28).

Sample Collection, Processing, and Analyses

Blood samples were collected in chilled tubes containing heparin (to determine glucose and insulin concentrations) or EDTA (to determine amino acid concentrations and glucose enrichment). Samples were placed in ice, and plasma was separated by centrifugation within 30 min of collection and then stored at −80°C until final analyses. Muscle samples were rinsed in ice-cold saline immediately after collection, cleared of visible fat and connective tissue, frozen in liquid nitrogen, and stored at −80°C until final analysis.

Plasma glucose concentration was determined by using an automated glucose analyzer (Yellow Spring Instruments, Yellow Springs, OH). Plasma insulin concentration was measured by using a commercially available ELISA (EMD Millipore, St. Charles, MO).

The glucose tracer-to-tracee ratio (TTR) in plasma was determined by using gas chromatography–mass spectrometry (GC-MS) (Hewlett-Packard MSD 5973 system with capillary column) after derivatizing glucose with acetic anhydride. Plasma leucine concentration was determined by using GC-MS (MSD 5973 system) after adding a known amount of nor-leucine to aliquots of each plasma sample and derivatization with *t*-butyldimethylsilyl (29). The concentrations of all other amino acids in plasma were determined by using the EZ:faast physiological (free) amino acid kit (Phenomenex, Torrence, CA) and GC-MS analysis per the manufacturer's instructions.

Western analysis was used to quantify the contents of p-AMPK^{Thr172}, p-ACC^{Ser79}, p-mTOR^{Ser2448}, p-p70S6K^{Thr389}, p-AKT^{Ser473}, p-AKT^{Thr308}, and GSKβ^{Ser9} in muscle. Frozen muscle tissue was rapidly homogenized in ice-cold Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA), and proteins were extracted (30). Protein (20–30 μg) from each sample was loaded onto 7% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA), separated by SDS-PAGE, and transferred to Immobilon-FL polyvinylidene difluoride membranes (Millipore, Bedford, MA). The blotted membranes were incubated with the following primary antibodies: rabbit polyclonal anti–phospho-AMPKα (Thr^{172}) antibody (cat. no. 2531; Cell Signaling Technology), rabbit polyclonal anti–phospho-ACC (Ser^{79}) antibody (cat. no. 3661; Cell Signaling Technology), rabbit polyclonal anti–phospho-mTOR (Ser^{2448}) antibody (cat. no. 2971; Cell Signaling Technology), rabbit monoclonal anti–phospho-p70S6K (Thr^{389}) antibody (cat. no. 9234; Cell Signaling Technology), rabbit monoclonal anti–phospho-AKT (Ser^{473}) antibody (cat. no. 4060; Cell Signaling Technology), rabbit monoclonal anti–phospho-AKT (Thr^{308}) antibody (cat. no. 4056; Cell Signaling Technology), rabbit monoclonal anti–phospho–GSK-3β (Ser^{9}) antibody (cat. no. 9323; Cell Signaling Technology), and goat polyclonal anti-ACTIN antibody (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA). p-AKT^{Ser473} and ACTIN blots were incubated with LI-COR IRDye 800–labeled secondary antibodies (926–32213 and 926–32214, respectively; LI-COR Biosciences, Lincoln, NE) and developed by using the Odyssey Infrared Imaging System (LI-COR Biosciences). p-AMPK^{Thr172}, p-ACC^{Ser79}, p-mTOR^{Ser2448}, p-p70S6K^{Thr389}, p-AKT^{Thr308}, and p-GSKβ^{Ser9} blots were incubated with a horseradish peroxidase–conjugated secondary antibody (cat. no. 7074; Cell Signaling Technology) and developed by using the Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare Life Sciences, Piscataway, NJ). The contents of p-AMPK^{Thr172}, p-ACC^{Ser79}, p-mTOR ^{Ser2448}, p-p70S6K^{Thr389}, p-AKT^{Ser473}, p-AKT^{Thr308}, and p-GSKβ^{Ser9} were expressed relative to a single sample loading control and relative to ACTIN. The results were the same, irrespective of the control protein used.

Calculations

Endogenous glucose R_a in plasma in the basal state was calculated by dividing the glucose tracer infusion rate by the average plasma glucose TTR during the last 20 min of the basal period. During the clamp, total glucose R_a was calculated by dividing the glucose tracer infusion rate by the average plasma glucose TTR during the last 20 min of

the clamp procedure and adding the tracer infusion rate to this value. Total glucose R_a represents the sum of endogenous glucose production plus the rate of infused glucose (dextrose plus tracer) and equals the rate of glucose disappearance (R_d) from plasma. Endogenous glucose R_a during the clamp was therefore calculated by subtracting the glucose infusion rate (dextrose plus tracer) from total glucose R_a. Leg glucose uptake was calculated as the product of leg plasma flow [i.e., blood flow × (1 − hematocrit)] and the plasma glucose arterio-venous concentration difference.

Statistical Analyses

Statistical analyses were carried out with SPSS, version 21, for Windows (IBM, Armonk, NY). All data sets were tested for normality by using the Kolmogorov-Smirnov test, and skewed data sets were log transformed for further analysis. Student t test was used to compare basic characteristics of subjects in the whey protein and leucine groups. Three-way (group [whey protein vs. leucine] × study [control vs. whey protein or leucine ingestion] × condition [basal vs. clamp]) repeated-measures ANOVA and Tukey post hoc procedure were used to evaluate the effects of whey protein and leucine ingestion on plasma glucose, amino acid, and insulin concentrations; leg plasma flow; whole-body and leg glucose kinetics; and p-AMPK^{Thr172}, p-ACC^{Ser79}, p-mTOR^{Ser2448}, p-p70S6K^{Thr389}, p-AKT^{Ser473}, p-AKT^{Thr308}, and p-GSKβ^{Ser9} contents in muscle. In addition, ANCOVA with plasma insulin concentration as a covariate was used to compare the effects of whey protein and leucine ingestion on whole-body and leg glucose kinetics to account for small but potentially important differences in insulin concentration between studies. The glucose R_d-to-insulin ratio was compared by using two-way (group [whey protein vs. leucine] × study [control vs. whey protein or leucine ingestion]) ANOVA and Tukey post hoc procedure.

A P value of ≤0.05 was considered statistically significant. Unless otherwise noted, data are presented as means ± SEM or median (quartiles) for normally distributed and skewed data sets, respectively.

RESULTS

Arterial Plasma Glucose, Insulin, and Amino Acid Concentrations

Basal arterial plasma glucose, insulin, and amino acid concentrations were not different between the whey protein and leucine ingestion groups and between the whey protein or leucine ingestion and respective control studies within each group. During the clamp procedure, glucose concentration was maintained at the 5.6 mmol/L target (mean 5.62 ± 0.02 mmol/L) in all studies. Insulin concentration increased ~10-fold in all studies, and the clamp-induced increase was ~10% greater (P < 0.01) during whey and leucine ingestion than during their respective control studies. Leucine concentration decreased by ~40% during insulin infusion in the control studies and increased to ~70% above basal values during whey protein and leucine ingestion. Total branched-chain amino acid concentration decreased by ~40% during insulin infusion in the control studies and increased by ~60% above basal values during whey protein ingestion but did not change during leucine ingestion. Total amino acid, essential amino acid, and nonessential amino acid concentrations decreased during insulin infusion in the control studies and during leucine ingestion but increased during whey protein ingestion. Accordingly, total amino acid, branched-chain amino acid, essential amino acid, and nonessential amino acid concentrations during insulin infusion were 25–50% lower (P < 0.01) during leucine ingestion than whey protein ingestion (Table 2).

Whole-Body Glucose Kinetics

Basal endogenous glucose R_a was not different between the control and whey protein ingestion studies (837 ± 41 and 809 ± 38 μmol · min^{-1}, respectively) or the control and leucine ingestion studies (773 ± 37 and 772 ± 26 μmol · min^{-1}, respectively). During the clamp, endogenous glucose R_a was almost completely (by 90.5 ± 1.3%) suppressed in all studies (to 77 ± 24 and 65 ± 14 μmol · min^{-1} in the control and whey protein ingestion studies, respectively, and to 69 ± 22 and 93 ± 25 μmol · min^{-1} in the control and leucine ingestion studies, respectively; main effect of clamp, P < 0.001; no significant interactions and no significant main effect of group). Glucose R_d during the clamp was significantly lower during whey protein ingestion than during the control drink ingestion (P < 0.01), whereas leucine ingestion had no effect on glucose R_d during the clamp (Fig. 1). The difference in the effect of whey protein and leucine ingestion on glucose R_d during the clamp persisted even when the small differences in plasma insulin concentration among studies were taken into account by using ANCOVA or by evaluating the glucose R_d-to-insulin ratio, which was median 38.8 μmol/μU min^{-1} · mL^{-1} (quartiles 30.8, 61.8) and 51.9 μmol/μU min^{-1} · mL^{-1} (41.0, 77.3) in the whey protein and corresponding control studies, respectively (P < 0.01), and 52.3 μmol/μU min^{-1} · mL^{-1} (43.3, 65.4) and 52.3 μmol/μU min^{-1} · mL^{-1} (43.9, 73.2) in the leucine and corresponding control studies, respectively.

Leg Plasma Flow and Glucose Kinetics

Basal leg plasma flow was not different between the whey protein and leucine ingestion and respective control studies (Table 2). During the clamp, leg plasma flow significantly increased (compared with basal conditions) in all studies (main effect of clamp, P < 0.001), and neither whey protein nor leucine ingestion affected the clamp-induced increase (Table 2).

The basal rate of leg glucose uptake was very small and not different between the whey protein or leucine ingestion studies and their respective control studies. During the clamp, leg glucose uptake increased by >10-fold in all studies (P < 0.001); the clamp-induced increase was reduced (by ~20%) with whey protein (P < 0.05) but not leucine ingestion (Fig. 1).

Table 2—Effects of whey protein and leucine ingestion on arterial plasma amino acid and insulin concentrations, arterio-venous plasma glucose concentration differences, and leg plasma flow

	Whey protein group		Leucine group	
	Control	Whey protein	Control	Leucine
Leucine (μmol/L)				
Basal	113 ± 4	120 ± 3	115 ± 4	113 ± 5
Clamp	68 ± 5*	205 ± 10*†	70 ± 4*	203 ± 10*†
Branched-chain amino acids (μmol/L)				
Basal	368 ± 18	398 ± 12	368 ± 15	360 ± 17
Clamp	239 ± 21*	636 ± 30*†	244 ± 14*	369 ± 19†‡
Essential amino acids (μmol/L)				
Basal	807 ± 38	864 ± 33	758 ± 31	751 ± 34
Clamp	571 ± 38*	1,385 ± 54*†	566 ± 31*	687 ± 34*†‡
Nonessential amino acids (μmol/L)				
Basal	903 ± 40	931 ± 46	883 ± 37	919 ± 27
Clamp	713 ± 32*	1,043 ± 39*†	765 ± 31*	788 ± 58*‡
Total amino acids (μmol/L)				
Basal	1,711 ± 75	1,795 ± 75	1,640 ± 54	1,670 ± 51
Clamp	1,284 ± 66*	2,428 ± 88*†	1,331 ± 54*	1,475 ± 82*†‡
Insulin ($\mu U \cdot mL^{-1}$)				
Basal	5 ± 1	5 ± 1	6 ± 1	6 ± 1
Clamp	53 ± 2*	61 ± 4*†	49 ± 4*	53 ± 3*†
Glucose (mmol/L)				
Basal, artery	5.09 ± 0.05	5.12 ± 0.06	5.33 ± 0.16	5.33 ± 0.12
Clamp, artery	5.61 ± 0.04§	5.60 ± 0.06§	5.64 ± 0.05§	5.63 ± 0.04§
Basal, vein	5.00 ± 0.05	5.04 ± 0.06	5.25 ± 0.15	5.27 ± 0.03
Clamp, vein	4.42 ± 0.16§	4.59 ± 0.10§	4.43 ± 0.17§	4.44 ± 0.12§
A-V glucose difference (mmol/L)				
Basal	0.09 ± 0.02	0.08 ± 0.02	0.08 ± 0.03	0.05 ± 0.03
Clamp	1.19 ± 0.16*	1.01 ± 0.14*†	1.20 ± 0.17*	1.19 ± 0.13*
Plasma flow ($mL \cdot min^{-1}$)				
Basal	173 ± 25	178 ± 23	162 ± 34	193 ± 34
Clamp	219 ± 31§	224 ± 31§	204 ± 37§	218 ± 34§

Data are means ± SEM. Three-way ANOVA revealed a significant study (control vs. whey protein or leucine ingestion) × condition (basal vs. clamp) × group (whey protein vs. leucine groups) interaction ($P < 0.001$) for branched-chain, essential, nonessential, and total amino acid concentrations; a significant study × condition interaction ($P < 0.001$) for leucine and insulin concentrations; and a significant main effect of clamp ($P < 0.01$) for glucose concentrations and plasma flow. ANCOVA with plasma insulin concentration as a covariate revealed a significant study (control vs. whey protein) × condition (basal vs. clamp) interaction ($P < 0.05$) for the arterio-venous (A-V) glucose concentration difference. Tukey post hoc analysis revealed the following significant differences. *Significantly different from corresponding basal value ($P < 0.01$). †Significantly different from corresponding control values ($P < 0.01$). ‡Significantly different from corresponding value in the whey protein group ($P < 0.01$). §Significant main effect of clamp ($P < 0.001$).

Phosphorylation of Signaling Transduction Proteins in Muscle

Basal p-AMPK^{Thr172}, p-ACC^{Ser79}, p-mTOR^{Ser2448}, p-p70S6K^{Thr389}, p-AKT^{Ser473}, and p-AKT^{Thr308}, and p-GSKβ^{Ser9} (data not shown) contents in muscle were not different between groups and studies (whey protein or leucine ingestion and respective controls). During the clamp procedure, p-mTOR^{Ser2448}, p-p70S6K^{Thr389}, p-AKT^{Ser473}, and p-AKT^{Thr308} increased in all studies ($P <$ 0.001), whereas p-AMPK^{Thr172}, p-ACC^{Ser79}, and GSKβ^{Ser9} (data not shown) were unchanged compared with basal values. The clamp-induced increases in p-mTOR^{Ser2448} and p-p70S6K^{Thr389} were greater during whey protein and leucine ingestion relative to their respective control studies but not different during whey protein and leucine ingestion. The clamp-induced increases in p-AKT^{Ser473} and p-AKT^{Thr308} were not affected by whey protein or leucine ingestion, and neither whey protein nor leucine ingestion had an effect on p-GSKβ^{Ser9} (data not shown) (Figs. 2 and 3).

DISCUSSION

In the current study, we tested the hypothesis that protein ingestion impairs insulin-stimulated glucose disposal via leucine-mediated skeletal muscle mTOR-p70S6K phosphorylation and subsequent inhibition of the insulin signaling cascade. Accordingly, we evaluated rates of whole-body and leg glucose disposal and p-mTOR^{Ser2448}, p-p70S6K^{Thr389}, p-AKT^{Ser473}, and p-AKT^{Thr308} in skeletal muscle during basal conditions and during a hyperinsulinemic-euglycemic clamp procedure with and without concomitant whey protein or leucine ingestion. Ingestion of whey protein and leucine alone (which matched the amount of leucine present in whey protein) caused the same

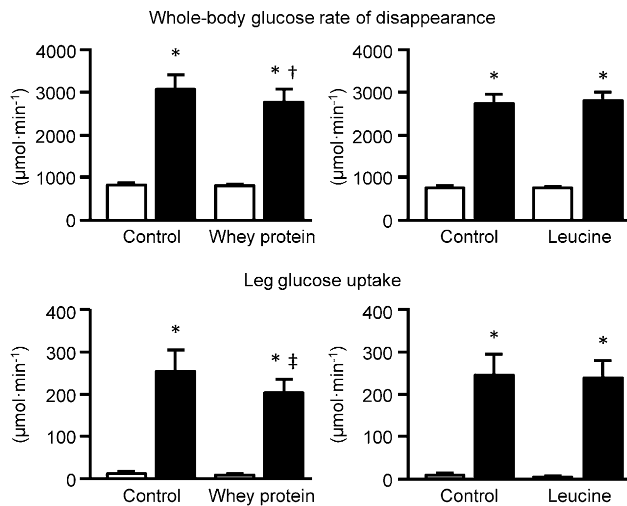

Figure 1—Effects of whey protein and leucine ingestion on whole-body glucose R_d (upper panel) and leg glucose uptake (lower panel) (□, basal; ■, clamp). Data are means ± SEM. Three-way ANOVA revealed a significant group (whey protein vs. leucine groups) × study (control vs. whey protein or leucine ingestion) × condition (basal vs. clamp) interaction ($P < 0.001$) for whole-body glucose R_d. ANCOVA with plasma insulin concentration as a covariate revealed a significant study (control vs. whey protein) × condition (basal vs. clamp) interaction ($P < 0.05$) for whole-body glucose R_d and leg glucose uptake. Tukey post hoc analysis revealed the following significant differences. *Significantly different from corresponding basal value ($P < 0.01$); †significantly different from corresponding control value ($P < 0.01$); ‡significantly different from corresponding control value ($P < 0.05$).

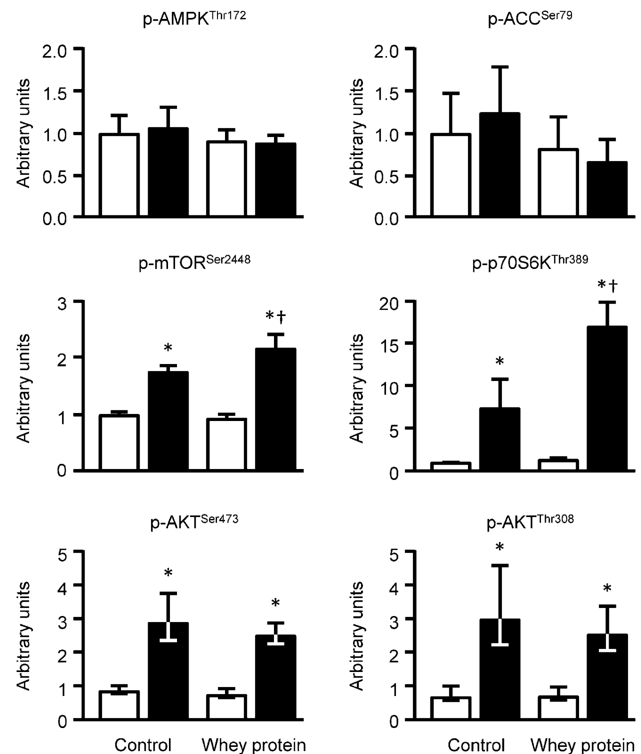

Figure 2—Effect of whey protein ingestion on p-AMPK^{Thr172}, p-ACC^{Ser79}, p-mTOR^{Ser2448}, p-p70S6K^{Thr389}, p-AKT^{Ser473}, and p-AKT^{Thr308} (arbitrary units) in muscle (□, basal; ■, clamp). AMPK, p-ACC, mTOR, and p-p70S6K data are means ± SEM; AKT data were log transformed for ANOVA and are presented as backtransformed geometric means and errors. Three-way ANOVA revealed a significant study (control vs. whey protein or leucine ingestion) × condition (basal vs. clamp) interaction ($P < 0.05$) for p-mTOR^{Ser2448} and p-p70S6K^{Thr389} and a significant main effect of clamp ($P < 0.001$) for p-AKT^{Ser473} and p-AKT^{Thr308}. Tukey post hoc analysis revealed the following significant differences. *Significantly different from corresponding basal value ($P < 0.05$); †significantly different from corresponding control value ($P < 0.05$).

increase in plasma leucine concentration and muscle p-mTOR^{Ser2448} and p-p70S6K^{Thr389} contents but did not affect muscle p-AKT^{Ser473} and p-AKT^{Thr308}. Moreover, whey protein, but not leucine, ingestion impaired glucose disposal. These results indicate that protein ingestion could be an important regulator of postprandial glucose homeostasis, but the adverse effect of protein ingestion on glucose disposal is not mediated by leucine and occurs independently of mTOR and AKT signaling in muscle.

The results from several studies conducted in cultured myotubes and isolated rat skeletal muscles suggest that leucine-mediated mTOR signaling has adverse effects on insulin sensitivity because they demonstrate that leucine stimulates mTOR and IRS serine phosphorylation (15,16) and impairs PI3K-AKT signaling and insulin-mediated glucose uptake (15,16,31). In addition, it has been demonstrated that treatment with rapamycin, an mTOR inhibitor, abolishes the adverse effect of hyperaminoacidemia on insulin-mediated glucose disposal both in vivo in people and in vitro in cultured myocytes (16,20). However, rapamycin does not directly inhibit mTOR kinase activity and has mTOR-independent effects throughout the body (32–34), which confounds the interpretation of those results. The data from our study suggest that mTOR-p70S6K signaling is not involved in protein-induced inhibition of glucose uptake during hyperinsulinemia in people. We found that both whey protein and leucine ingestion increased p-mTOR^{Ser2448} and p-p70S6K^{Thr389} in muscle without affecting p-AKT^{Ser473} and p-AKT^{Thr308}. Moreover, whey protein, but not leucine, reduced glucose uptake in the absence of changes in skeletal muscle p-AKT^{Ser473} and p-AKT^{Thr308} contents. In concert with our findings, data from previous studies conducted in human subjects (23,35) illustrate that an ~6 h infusion of an amino acid mixture (~80 g amino acids containing ~7 g leucine) and a 2 h infusion of ~2 g of leucine during hyperinsulinemia-euglycemia increased S6K activity and p70S6K and IRS serine phosphorylation and impaired glucose uptake (amino acid mixture only) without a decrease in AKT phosphorylation. Together, these data suggest that the cellular mechanism responsible for the adverse effect of hyperaminoacidemia on glucose disposal lies downstream of AKT or occurs independently of the inhibitory mTOR-p70S6K-IRS signaling pathway to AKT. It is possible that protein ingestion interfered with non–insulin-mediated glucose disposal, which accounts for 15–25% of total glucose disposal during hyperinsulinemic-euglycemic clamp conditions (36,37).

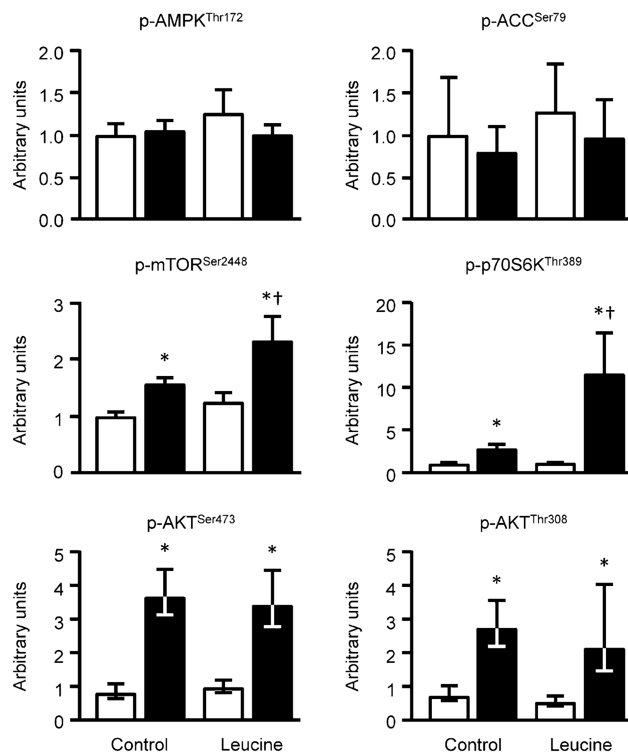

Figure 3—Effect of leucine ingestion on p-AMPK^{Thr172}, p-ACC^{Ser79}, p-mTOR^{Ser2448}, p-p70S6K^{Thr389}, p-AKT^{Ser473}, and p-AKT^{Thr308} (arbitrary units) in muscle (□, basal; ■, clamp). AMPK, p-ACC, mTOR, and p-p70S6K data are means ± SEM; AKT data were log transformed for ANOVA and are presented as backtransformed geometric means and errors. Three-way ANOVA revealed a significant study (control vs. whey protein or leucine ingestion) × condition (basal vs. clamp) interaction ($P < 0.05$) for p-mTOR^{Ser2448} and p-p70S6K^{Thr389} and a significant main effect of clamp ($P < 0.001$) for p-AKT^{Ser473} and p-AKT^{Thr308}. Tukey post hoc analysis revealed the following significant differences. *Significantly different from corresponding basal value ($P < 0.05$); †significantly different from corresponding control value ($P < 0.05$).

The mechanism(s) responsible for the effect of both whey protein and leucine ingestion on mTOR phosphorylation is unclear. The results from studies conducted in cultured rat muscles suggest that both glucose and leucine stimulate mTOR phosphorylation through downregulation of AMPK (7,31,38,39). However, we found that insulin-glucose infusion alone and the ingestion of whey protein and leucine increased muscle p-mTOR^{Ser2448} without an effect on muscle p-AMPK^{Thr172} content or its downstream target p-ACC^{Ser79}. This dissociation between AMPK and mTOR signaling is consistent with the results from several previous studies conducted in human subjects during glucose-insulin infusion (40–43) and suggests that other mechanisms (e.g., Vps34 or phosphatidic acid signaling [44–47]) were responsible for both the insulin-glucose– and whey protein– and leucine-mediated increases in muscle p-mTOR^{Ser2448} in our study.

The insulin-mediated suppression of endogenous glucose R_a was not affected by whey protein or leucine ingestion in our study, most likely because endogenous glucose production is very sensitive to insulin (48) and was almost completely suppressed by the hyperinsulinemia achieved in our study. However, it is also possible that the amount of protein given in our study (~23 g) was not enough to elicit an effect. Intravenous administration of amino acids in excess of ~80 g was found to blunt the insulin-mediated suppression of endogenous glucose production during both low- and high-dose insulin infusion (21,23,49), whereas administration of ~12 g had no effect (22).

Our study has some limitations that need to be considered. First, we studied only 50- to 65-year-old obese postmenopausal women. Although it is possible that the findings from our study cannot be extrapolated to other populations, we believe this is unlikely because amino acid–induced insulin resistance has previously been observed in nonobese men and women across a wide age range (i.e., 18–70 years) (21–23). Secondly, it is possible that our study did not contain an adequate number of subjects to detect a leucine-mediated decrease in whole-body glucose R_d and leg glucose uptake. However, this seems unlikely because mean whole-body glucose R_d and leg glucose uptake during the clamp were nearly identical after leucine and control drink ingestion, and the individual values were numerically greater after leucine than control drink ingestion in 6 and 8 of the 11 subjects, respectively.

In summary, we found that whey protein ingestion impairs glucose disposal during hyperinsulinemia, both at the whole-body level and across the leg, independent of leucine-mediated mTOR-p70S6K and AKT signaling. Protein intake could therefore be an important regulator of postprandial glucose clearance. Additional studies are needed to determine the precise mechanism(s) responsible for the adverse effect of protein ingestion on glucose disposal, the extent to which it may be counterbalanced by the stimulatory effect of protein ingestion on insulin secretion (8,50), and how long-term changes in dietary protein intake affect glucose homeostasis.

Acknowledgments. The authors thank Janet Winkelmann (Washington University School of Medicine, St. Louis, MO) for help with subject recruitment and scheduling; Kathryn Gratza, Jennifer Shew, Freida Custodio, Shannon Kelly, Kohsuke Kanekura, and Adewole Okunade (all from Washington University School of Medicine, St. Louis, MO) for technical assistance; the staff of the Clinical Research Unit for help in performing the studies; and the study subjects for participation.

Funding. This publication was made possible by National Institutes of Health grants DK-094483 and DK-056341 (Washington University School of Medicine Nutrition Obesity Research Center), DK-020579 (Washington University School of Medicine Diabetes Research Center), GM-103422 (Washington University School of Medicine Biomedical Mass Spectrometry Research Resource), and UL1-TR-000448 (Washington University School of Medicine Clinical and Translational Science Award) including KL2 sub-award TR-000450, a Central Society for Clinical and Translational Research Early Career Development Award, and a grant from the Longer Life Foundation.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. G.I.S. conducted the studies, processed study samples, collected data, performed data analyses, and wrote the manuscript.

J.Y. processed study samples, collected data, assisted with data analysis, and edited the manuscript. K.L.S. processed study samples and edited the manuscript. S.J.K., F.M., and D.N.R. assisted in conducting the studies and edited the manuscript. S.K. designed the studies, obtained funding for the studies, performed medical supervision of the studies, and edited the manuscript. B.M. designed the studies, obtained funding for the studies, was involved in conducting the studies, processed study samples, collected data, performed the final data analyses, and wrote the manuscript. B.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented in abstract form at the 2014 Experimental Biology meeting, San Diego, CA, 26–30 April 2014.

References

1. Prager R, Wallace P, Olefsky JM. Hyperinsulinemia does not compensate for peripheral insulin resistance in obesity. Diabetes 1987;36:327–334
2. Bornfeldt KE, Tabas I. Insulin resistance, hyperglycemia, and atherosclerosis. Cell Metab 2011;14:575–585
3. Bastien M, Poirier P, Lemieux I, Després JP. Overview of epidemiology and contribution of obesity to cardiovascular disease. Prog Cardiovasc Dis 2014;56: 369–381
4. Newgard CB. Interplay between lipids and branched-chain amino acids in development of insulin resistance. Cell Metab 2012;15:606–614
5. Adeva MM, Calviño J, Souto G, Donapetry C. Insulin resistance and the metabolism of branched-chain amino acids in humans. Amino Acids 2012;43: 171–181
6. Tremblay F, Jacques H, Marette A. Modulation of insulin action by dietary proteins and amino acids: role of the mammalian target of rapamycin nutrient sensing pathway. Curr Opin Clin Nutr Metab Care 2005;8:457–462
7. Saha AK, Xu XJ, Balon TW, Brandon A, Kraegen EW, Ruderman NB. Insulin resistance due to nutrient excess: is it a consequence of AMPK downregulation? Cell Cycle 2011;10:3447–3451
8. Tremblay F, Lavigne C, Jacques H, Marette A. Role of dietary proteins and amino acids in the pathogenesis of insulin resistance. Annu Rev Nutr 2007;27: 293–310
9. Newgard CB, An J, Bain JR, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab 2009;9:311–326
10. Felig P, Marliss E, Cahill GF Jr. Plasma amino acid levels and insulin secretion in obesity. N Engl J Med 1969;281:811–816
11. Xu F, Tavintharan S, Sum CF, Woon K, Lim SC, Ong CN. Metabolic signature shift in type 2 diabetes mellitus revealed by mass spectrometry-based metabolomics. J Clin Endocrinol Metab 2013;98:E1060–E1065
12. Würtz P, Soininen P, Kangas AJ, et al. Branched-chain and aromatic amino acids are predictors of insulin resistance in young adults. Diabetes Care 2013;36: 648–655
13. Menni C, Fauman E, Erte I, et al. Biomarkers for type 2 diabetes and impaired fasting glucose using a nontargeted metabolomics approach. Diabetes 2013;62:4270–4276
14. Thalacker-Mercer AE, Ingram KH, Guo F, Ilkayeva O, Newgard CB, Garvey WT. BMI, RQ, diabetes, and sex affect the relationships between amino acids and clamp measures of insulin action in humans. Diabetes 2014; 63:791–800
15. Tzatsos A, Kandror KV. Nutrients suppress phosphatidylinositol 3-kinase/Akt signaling via raptor-dependent mTOR-mediated insulin receptor substrate 1 phosphorylation. Mol Cell Biol 2006;26:63–76
16. Iwanaka N, Egawa T, Satoubu N, et al. Leucine modulates contraction- and insulin-stimulated glucose transport and upstream signaling events in rat skeletal muscle. J Appl Physiol (1985) 2010;108:274–282
17. Atherton PJ, Smith K, Etheridge T, Rankin D, Rennie MJ. Distinct anabolic signalling responses to amino acids in C2C12 skeletal muscle cells. Amino Acids 2010;38:1533–1539
18. Patti ME, Brambilla E, Luzi L, Landaker EJ, Kahn CR. Bidirectional modulation of insulin action by amino acids. J Clin Invest 1998;101:1519–1529
19. Anthony JC, Yoshizawa F, Anthony TG, Vary TC, Jefferson LS, Kimball SR. Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. J Nutr 2000;130:2413–2419
20. Krebs M, Krssak M, Bernroider E, et al. Mechanism of amino acid-induced skeletal muscle insulin resistance in humans. Diabetes 2002;51:599–605
21. Flakoll PJ, Wentzel LS, Rice DE, Hill JO, Abumrad NN. Short-term regulation of insulin-mediated glucose utilization in four-day fasted human volunteers: role of amino acid availability. Diabetologia 1992;35:357–366
22. Pisters PW, Restifo NP, Cersosimo E, Brennan MF. The effects of euglycemic hyperinsulinemia and amino acid infusion on regional and whole body glucose disposal in man. Metabolism 1991;40:59–65
23. Tremblay F, Krebs M, Dombrowski L, et al. Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability. Diabetes 2005;54:2674–2684
24. el-Khoury AE, Sánchez M, Fukagawa NK, Gleason RE, Tsay RH, Young VR. The 24-h kinetics of leucine oxidation in healthy adults receiving a generous leucine intake via three discrete meals. Am J Clin Nutr 1995;62:579–590
25. Kiskini A, Hamer HM, Wall BT, et al. The muscle protein synthetic response to the combined ingestion of protein and carbohydrate is not impaired in healthy older men. Age (Dordr) 2013;35:2389–2398
26. Boirie Y, Gachon P, Corny S, Fauquant J, Maubois JL, Beaufrère B. Acute postprandial changes in leucine metabolism as assessed with an intrinsically labeled milk protein. Am J Physiol 1996;271:E1083–E1091
27. Gropper SS, Gropper DM, Acosta PB. Plasma amino acid response to ingestion of L-amino acids and whole protein. J Pediatr Gastroenterol Nutr 1993; 16:143–150
28. Rådegran G, Saltin B. Nitric oxide in the regulation of vasomotor tone in human skeletal muscle. Am J Physiol 1999;276:H1951–H1960
29. Smith GI, Villareal DT, Mittendorfer B. Measurement of human mixed muscle protein fractional synthesis rate depends on the choice of amino acid tracer. Am J Physiol Endocrinol Metab 2007;293:E666–E671
30. Yoshino J, Conte C, Fontana L, et al. Resveratrol supplementation does not improve metabolic function in nonobese women with normal glucose tolerance. Cell Metab 2012;16:658–664
31. Saha AK, Xu XJ, Lawson E, et al. Downregulation of AMPK accompanies leucine- and glucose-induced increases in protein synthesis and insulin resistance in rat skeletal muscle. Diabetes 2010;59:2426–2434
32. Goodman CA, Frey JW, Mabrey DM, et al. The role of skeletal muscle mTOR in the regulation of mechanical load-induced growth. J Physiol 2011;589:5485–5501
33. Livingstone M, Bidinosti M. Rapamycin-insensitive mTORC1 activity controls eIF4E:4E-BP1 binding. F1000 Res 2012;1:4
34. Huo Y, Iadevaia V, Yao Z, et al. Stable isotope-labelling analysis of the impact of inhibition of the mammalian target of rapamycin on protein synthesis. Biochem J 2012;444:141–151
35. Greiwe JS, Kwon G, McDaniel ML, Semenkovich CF. Leucine and insulin activate p70 S6 kinase through different pathways in human skeletal muscle. Am J Physiol Endocrinol Metab 2001;281:E466–E471
36. Baron AD, Brechtel G, Wallace P, Edelman SV. Rates and tissue sites of non-insulin- and insulin-mediated glucose uptake in humans. Am J Physiol 1988; 255:E769–E774
37. Edelman SV, Laakso M, Wallace P, Brechtel G, Olefsky JM, Baron AD. Kinetics of insulin-mediated and non-insulin-mediated glucose uptake in humans. Diabetes 1990;39:955–964
38. Pruznak AM, Kazi AA, Frost RA, Vary TC, Lang CH. Activation of AMP-activated protein kinase by 5-aminoimidazole-4-carboxamide-1-beta-D-ribonucleoside prevents leucine-stimulated protein synthesis in rat skeletal muscle. J Nutr 2008; 138:1887–1894
39. Wilson GJ, Layman DK, Moulton CJ, et al. Leucine or carbohydrate supplementation reduces AMPK and eEF2 phosphorylation and extends postprandial

muscle protein synthesis in rats. Am J Physiol Endocrinol Metab 2011;301: E1236–E1242
40. Høeg LD, Sjøberg KA, Lundsgaard AM, et al. Adiponectin concentration is associated with muscle insulin sensitivity, AMPK phosphorylation, and ceramide content in skeletal muscles of men but not women. J Appl Physiol (1985) 2013;114:592–601
41. Atherton PJ, Etheridge T, Watt PW, et al. Muscle full effect after oral protein: time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling. Am J Clin Nutr 2010;92:1080–1088
42. Højlund K, Glintborg D, Andersen NR, et al. Impaired insulin-stimulated phosphorylation of Akt and AS160 in skeletal muscle of women with polycystic ovary syndrome is reversed by pioglitazone treatment. Diabetes 2008;57:357–366
43. Vendelbo MH, Clasen BF, Treebak JT, et al. Insulin resistance after a 72-h fast is associated with impaired AS160 phosphorylation and accumulation of lipid and glycogen in human skeletal muscle. Am J Physiol Endocrinol Metab 2012; 302:E190–E200
44. Dodd KM, Tee AR. Leucine and mTORC1: a complex relationship. Am J Physiol Endocrinol Metab 2012;302:E1329–E1342
45. Proud CG. Amino acids and mTOR signalling in anabolic function. Biochem Soc Trans 2007;35:1187–1190
46. Lang CH, Frost RA, Vary TC. Regulation of muscle protein synthesis during sepsis and inflammation. Am J Physiol Endocrinol Metab 2007;293:E453–E459
47. Avruch J, Long X, Ortiz-Vega S, Rapley J, Papageorgiou A, Dai N. Amino acid regulation of TOR complex 1. Am J Physiol Endocrinol Metab 2009;296: E592–E602
48. Conte C, Fabbrini E, Kars M, Mittendorfer B, Patterson BW, Klein S. Multiorgan insulin sensitivity in lean and obese subjects. Diabetes Care 2012;35: 1316–1321
49. Boden G, Tappy L. Effects of amino acids on glucose disposal. Diabetes 1990;39:1079–1084
50. Ranawana V, Kaur B. Role of proteins in insulin secretion and glycemic control. Adv Food Nutr Res 2013;70:1–47