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\textsuperscript{Ser2448}, p-AKT\textsuperscript{Ser473}, and p-AKT\textsuperscript{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\textsuperscript{Ser2448} by \(\sim 30\%\) above the values observed in the control (no amino acid ingestion) studies; p-AKT\textsuperscript{Ser473} and p-AKT\textsuperscript{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] \(\mu\)mol glucose/\(\mu\)U insulin \cdot mL\textsuperscript{-1} \cdot min\textsuperscript{-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...
the whey protein leucine content would impair insulin-mediated glucose disposal and be associated with decreased phosphorylated (p)-AMPKThr172 (and its downstream target p-ACC(Ser79)), increased p-mTORSer2448 (and its downstream target p-70S6KThr389), and decreased p-AKTSer473 and p-AKTThr308 (and their downstream target GSK(Ser9)) in skeletal muscle. To accomplish this goal, we had two groups of subjects complete two hyperinsulinemic-euglycemic clamp procedures: one with and 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).

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. 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.

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 [6,6-2H2]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 [6,6-2H2]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

Table 1—Subjects’ body composition and basic metabolic characteristics

|                      | Whey protein group (n = 11) | Leucine group (n = 11) | P*       |
|----------------------|---------------------------|-----------------------|----------|
| BMI (kg/m²)          | 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 (cm³) | 2,940 ± 118           | 3,197 ± 232           | 0.33     |
| Intra-abdominal fat (cm³) | 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.
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-AMPKThr172, p-ACCSer79, p-mTORSer2448, p-p70S6KThr389, p-AKTSer473, p-AKTThr308, and GSKβSer9 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 (Thr172) antibody (cat. no. 2531; Cell Signaling Technology), rabbit polyclonal anti–phospho-ACC (Ser79) antibody (cat. no. 3661; Cell Signaling Technology), rabbit polyclonal anti–phospho-mTOR (Ser2448) antibody (cat. no. 2971; Cell Signaling Technology), rabbit monoclonal anti–phospho-p70S6K (Thr389) antibody (cat. no. 9234; Cell Signaling Technology), rabbit monoclonal anti–phospho-AKT (Ser473) antibody (cat. no. 4066; Cell Signaling Technology), rabbit monoclonal anti–phospho-AKT (Thr308) antibody (cat. no. 4056; Cell Signaling Technology), rabbit monoclonal anti–phospho–GSK-3β (Ser9) antibody (cat. no. 9323; Cell Signaling Technology), and goat polyclonal anti–ACTIN antibody (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA). p-AKTSer473 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-AMPKThr172, p-ACCSer79, p-mTORSer2448, p-p70S6KThr389, p-AKTThr308, and p-GSKβSer9 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-AMPKThr172, p-ACCSer79, p-mTORSer2448, p-p70S6KThr389, p-AKTThr308, and p-GSKβSer9 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 Rg 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 Rg 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 $R_d$ 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 $R_d$ during the clamp was therefore calculated by subtracting the glucose infusion rate (dextrose plus tracer) from total glucose $R_d$. Leg glucose uptake was calculated as the product of leg plasma flow [i.e., blood flow $(1 - \text{hematocrit})$] 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 procedures 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-AMPKThr172, p-ACCSer79, p-mTORSer2448, p-p70S6KThr389, p-AKTSer473, p-AKThr308, and p-GSKSer389 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 $\leq 0.05$ 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 $R_d$ 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 $R_d$ 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).
Phosphorylation of Signaling Transduction Proteins in Muscle

Basal p-AMPK<sup>Thr172</sup>, p-ACC<sup>Ser79</sup>, p-mTOR<sup>Ser2448</sup>, p-p70S6K<sup>Thr389</sup>, p-AKT<sup>Ser473</sup>, and p-AKT<sup>Thr308</sup> and p-GSKB<sup>Ser9</sup> (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<sup>Ser2448</sup>, p-p70S6K<sup>Thr389</sup>, p-AKT<sup>Ser473</sup>, and p-AKT<sup>Thr308</sup> increased in all studies (P < 0.001), whereas p-AMPK<sup>Thr172</sup>, p-ACC<sup>Ser79</sup>, and GSKB<sup>Ser9</sup> (data not shown) were unchanged compared with basal values. The clamp-induced increases in p-mTOR<sup>Ser2448</sup> and p-p70S6K<sup>Thr389</sup> 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<sup>Ser473</sup> and p-AKT<sup>Thr308</sup> were not affected by whey protein or leucine ingestion, and neither whey protein nor leucine ingestion had an effect on p-GSKB<sup>Ser9</sup> (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<sup>Ser2448</sup>, p-p70S6K<sup>Thr389</sup>, p-AKT<sup>Ser473</sup>, and p-AKT<sup>Thr308</sup> 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

### 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 (μU · mL<sup>−1</sup>)** |                   |               |                   |         |
| 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 · min<sup>−1</sup>)** |                   |               |                   |         |
| 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 covariate revealed a significant main effect of clamp (P < 0.01) for glucose concentrations and plasma flow. 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).
increase in plasma leucine concentration and muscle p-mTORSer2448 and p-p70S6KThr389 contents but did not affect muscle p-AKTSer473 and p-AKTThr308. 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-mTORSer2448 and p-p70S6KThr389 in muscle without affecting p-AKTSer473 and p-AKTThr308. Moreover, whey protein, but not leucine, reduced glucose uptake in the absence of changes in skeletal muscle p-AKTSer473 and p-AKTThr308 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).
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 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.

**Figure 3** — Effect of leucine ingestion on p-AMPK$^{\text{Thr172}}$, p-ACC$^{\text{Ser79}}$, p-mTOR$^{\text{Ser2448}}$, p-p70S6K$^{\text{Thr389}}$, p-AKT$^{\text{Ser473}}$, and p-AKT$^{\text{Thr308}}$ (arbitrary units) in muscle ($\square$, 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) x condition (basal vs. clamp) interaction ($P < 0.05$) for p-mTOR$^{\text{Ser2448}}$ and p-p70S6K$^{\text{Thr389}}$ and a significant main effect of clamp ($P < 0.001$) for p-AKT$^{\text{Ser473}}$ and p-AKT$^{\text{Thr308}}$. Tukey post hoc analysis revealed the following significant differences. *Significantly different from corresponding basal value ($P < 0.05$); †significantly different from corresponding control value ($P < 0.05$).
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.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.

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