Insulin Stimulates Human Skeletal Muscle Protein Synthesis via an Indirect Mechanism Involving Endothelial-Dependent Vasodilation and Mammalian Target of Rapamycin Complex 1 Signaling

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Objective: Our objective was to determine whether endothelial-dependent vasodilation is an essential mechanism by which insulin stimulates human skeletal muscle protein synthesis and anabolism.

Subjects: Subjects were healthy young adults (n = 14) aged 31 ± 2 yr.

Design: Subjects were studied at baseline and during local leg infusion of insulin alone (control, n = 7) or insulin plus the nitric oxide synthase inhibitor NG-monomethyl-L-arginine (L-NMMA, n = 7) to prevent insulin-induced vasodilation.

Methods: We measured skeletal muscle protein metabolism with stable isotope tracers, blood flow with indocyanine green, capillary recruitment with contrast enhanced ultrasound, glucose metabolism with stable isotope tracers, and phosphorylation of proteins associated with insulin (Akt) and amino acid-induced mammalian target of rapamycin (mTOR) complex 1 (mTORC1) signaling (mTOR, S6 kinase 1, and eukaryotic initiation factor 4E-binding protein 1) with Western blot analysis.

Results: No basal differences between groups were detected. During insulin infusion, blood flow and capillary recruitment increased in the control (P < 0.05) group only; Akt phosphorylation and glucose uptake increased in both groups (P < 0.05), with no group differences; and mTORC1 signaling increased more in control (P < 0.05) than in L-NMMA. Phenylalanine net balance increased (P < 0.05) in both groups, but with opposite mechanisms: increased protein synthesis (basal, 0.051 ± 0.006 %/h; insulin, 0.077 ± 0.008 %/h; P < 0.05) with no change in proteolysis in control and decreased proteolysis (P < 0.05) with no change in synthesis (basal, 0.061 ± 0.004 %/h; insulin, 0.050 ± 0.006 %/h; P value not significant) in L-NMMA.

Conclusions: Endothelial-dependent vasodilation and the consequent increase in nutritive flow and mTORC1 signaling, rather than Akt signaling, are fundamental mechanisms by which insulin stimulates muscle protein synthesis in humans. Additionally, these data underscore that insulin modulates skeletal muscle proteolysis according to its effects on nutritive flow. (J Clin Endocrinol Metab 95: 3848–3857, 2010)

Abbreviations: 4E-BP1, Eukaryotic initiation factor 4E-binding protein 1; eNOS, endothelial nitric oxide synthase; FSR, fractional synthesis rate; ICG, indocyanine green; L-NMMA, NG-monomethyl-L-arginine; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; PI, pulsing interval; PKB, protein kinase B; S6K1, 4E-binding protein 1; TSC2, tuberous sclerosis complex 2.
The longstanding debate on whether insulin stimulates skeletal muscle protein synthesis in humans has yet to be settled. Insulin has been reported to stimulate skeletal muscle protein synthesis in animals (1–4) and humans (5, 6). Conversely, some human studies could not demonstrate significant changes in muscle protein synthesis with hyperinsulinemia while reporting an inhibitory effect on proteolysis (7–9). We have hypothesized that these discrepancies may be due to differences in muscle perfusion, nutritive flow, and/or amino acid availability, which then modulate insulin’s effect on muscle protein synthesis and breakdown (10–12). However, the fundamental question of how insulin stimulates human skeletal muscle protein synthesis and anabolism remains unanswered.

At the cellular level, insulin induces phosphorylation of Akt/protein kinase B (PKB) and mammalian target of rapamycin (mTOR) with a subsequent increase in the phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and p70 ribosomal protein S6 kinase 1 (S6K1). That is, insulin increases mTOR complex 1 (mTORC1) signaling, promoting translation initiation and accelerating muscle protein synthesis (13). Insulin also stimulates endothelial-dependent vasodilation by activating endothelial nitric oxide synthase (eNOS) (14–16), increasing capillary recruitment, microvascular volume, and nutritive flow to skeletal muscle in healthy young adults (17). This increased muscle perfusion raises the amount of muscle tissue exposed to insulin, nutrients, and amino acids, which can increase Akt and mTORC1 signaling, stimulating muscle protein synthesis (10). Conversely, skeletal muscle protein synthesis is resistant to insulin in healthy non-diabetic older subjects, a defect associated with both reduced vasodilation (12) and blunted Akt/mTORC1 signaling, which a single bout of aerobic exercise can reverse (18). In general, the ability of insulin to promote muscle protein synthesis and net deposition correlates positively with muscle blood flow and amino acid delivery in young and older subjects (10–12, 18). Amino acids activate mTORC1 signaling (19), making it very difficult to determine the main mechanism through which insulin stimulates human skeletal muscle protein synthesis, i.e., directly, via Akt signaling, or indirectly, via endothelial-dependent vasodilation increasing tissue exposure to insulin and amino acids and, consequently, mTORC1 signaling, or both.

The purpose of the present study was to determine the role of insulin-induced endothelial-dependent vasodilation on the insulin stimulation of muscle protein synthesis and net protein anabolism. We hypothesized that pharmacological inhibition of endothelial-dependent vasodilation would attenuate the stimulatory effect of insulin on skeletal muscle protein synthesis in young healthy subjects. To test this hypothesis, we measured skeletal muscle blood flow, perfusion, anabolic signaling, protein synthesis, and glucose kinetics in young healthy subjects at baseline and during local hyperinsulinemia in one leg. During insulin infusion, one group also received a concomitant infusion of the nitric oxide synthase inhibitor NG-monomethyl-l-arginine (l-NMMA) to prevent insulin-induced vasodilation (20).

**Subjects and Methods**

**Ethical approval**

After approval by the Institutional Review Board of the University of Texas Medical Branch (Galveston, TX) and the U.S. Food and Drug Administration (IND 73,870), all subjects read and signed a written informed consent form before enrollment.

**Subjects (Table 1)**

Fourteen young subjects from the Houston/Galveston, TX, area participated in a single acute experiment after random assignment to a control group receiving insulin only or an experimental group receiving insulin plus l-NMMA. All subjects were healthy and had normal glucose tolerance based on clinical history, physical examination, and laboratory tests, including a 2-h 75-g oral glucose tolerance test.

**Study design**

We measured skeletal muscle blood flow, perfusion, anabolic signaling, protein synthesis, phenylalanine kinetics, and glucose kinetics in the postabsorptive basal state (0–240 min) and during infusion of insulin (240–420 min) alone (control) or with l-NMMA (l-NMMA). Subjects were admitted to the University of Texas Medical Branch Clinical Research Center the afternoon before the experiment. They received a standardized meal at 1900 h (one third of their estimated daily energy requirements) and a snack at 2200 h, after which they were allowed only water until the end of the study (1400 h).

The next morning at 0600 h, polyethylene catheters were inserted into a forearm vein for stable isotope tracers (Isotec Inc., Sigma-Aldrich, Miamisburg, OH) and dextrose infusion, a central hand vein for arterialized blood sampling, and the common femoral artery and vein of one leg for stable isotope tracers (Isotec Inc., Sigma-Aldrich, Miamisburg, OH) and dextrose infusion, a central hand vein for arterialized blood sampling, and the common femoral artery and vein of one leg for blood sampling. The arterial line was also used for infusion of indocyanine green (ICG) (IC-Green; Akorn, Lake Forest, IL), insulin, and l-NMMA. At 0700 h, we drew a background blood sample for phenylalanine and glucose enrichment and ICG concentration and started (time = 0 min) a primed-continuous infusion of l-[ring-13C6]phenylalanine agnostically.

**TABLE 1. Subjects’ characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>l-NMMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Sex</td>
<td>5 females, 2 males</td>
<td>4 females, 3 males</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>32 ± 2</td>
<td>32 ± 3</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>67.7 ± 5.5</td>
<td>70.8 ± 5.8</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>1.63 ± 0.04</td>
<td>1.68 ± 0.05</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25 ± 1</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>Leg volume (liters)</td>
<td>9.09 ± 0.6</td>
<td>9.90 ± 0.7</td>
</tr>
</tbody>
</table>

Values are the mean ± se.
A detailed description of the study design is provided in the text. (prime, 2 μmol/kg; infusion, 0.05 μmol/kg · min) and D-[6,6-\(^{2}\)H_2]glucose (prime, 19 μmol/kg; infusion, 0.22 μmol/kg · min), which was maintained until the end of the study (Fig. 1). After 120 min, a first muscle biopsy was taken from the vastus lateralis of the leg bearing the femoral catheters, using aseptic technique, local anesthesia (1% lidocaine), and a 3-mm Bergström needle. The tissue (100–200 mg) was quickly rinsed with ice-cold saline to remove excess blood, blotted gently with a sterile sponge, frozen in liquid nitrogen (100–200 mg) was quickly rinsed with ice-cold saline to remove excess blood, blotted gently with a sterile sponge, frozen in liquid nitrogen, and stored at -80 °C until analyzed.

At 150 min ICG (0.5 mg/min) was started into the common femoral artery. After 10 min, four sequential blood samples were drawn at 10-min intervals from both femoral vein and hand vein to measure blood flow. At 180 min, after stopping the ICG infusion, we infused perfluor lipid microsphere (Definity; Lantheus Medical Imaging, N.Billerica, MA) in the wrist vein to measure muscle perfusion of the vastus lateralis. Subsequently, four blood samples were drawn at 5-min intervals from the hand vein and femoral vein and artery to determine insulin concentrations as well as phenylalanine and glucose concentrations and enrichments. At 240 min, the second muscle biopsy was taken from the same incision of the first, at a different needle angle.

After the second biopsy, we started an insulin infusion (Novolin R; Novo Nordisk, Princeton, NJ; 0.15 mU/min · 100 ml of leg) in the femoral artery of both groups and continued it until the end of the experiment (420 min). This insulin infusion rate was used to increase leg insulin concentration and availability to the same postprandial levels in both groups, while avoiding hyperinsulinemia (7, 9, 21). Dextrose (20%) enriched 2% with D-[6,6-\(^{2}\)H_2]glucose was infused at a variable rate to maintain blood glucose at the basal concentration (hyperinsulinemia) due to systemic hyperinsulinemia (7, 9, 21). Dextrose (20%) enriched 2% with D-[6,6-\(^{2}\)H_2]glucose was infused at a variable rate to maintain blood glucose at the basal concentration (hyperinsulinemia) due to systemic hyperinsulinemia (7, 9, 21).

FIG. 1. Study design. Blood and muscle sampling is indicated by arrows. A detailed description of the study design is provided in the text.

Analitical methods

Plasma glucose concentration was measured using an automated glucose analyzer (Yellow Springs Instrument Co., Yellow Springs, OH). ELISAs were used to determine insulin (Linco, St. Charles, MO) and endothelin-1 (R&D Systems, Inc., Minneapolis, MN) concentrations with a microplate reader (Bio-Rad, Hercules, CA).

Blood flow was measured based on ICG concentration in femoral and wrist veins by spectrophotometrical determination (Beckman Coulter, Fullerton, CA) at λ = 805 (22). Muscle perfusion was measured at baseline and during hyperinsulinemia using contrast enhanced ultrasound as described by others (23, 24). Ultrasound imaging of the vastus lateralis muscle was performed in a transaxial plane approximately 15–20 cm above the patella over the midportion of the muscle using a P4-2 phased array transducer. An octafluoropropane gas-filled albumin microbubbles suspension (Definity, St. Louis Park, MN) was infused iv (3.5 ml/min for 8 min), using a mechanical index of 1.3 and compression of 80%. Once the systemic microbubble concentrations reached steady state (~2 min), background images were obtained at a frame rate of 1/sec. Intermittent imaging was performed using an internal timer at pulsing intervals (PI) ranging from 1–25 sec, allowing progressively greater replenishment of the ultrasound beam elevation between destructive pulses. Depth, focus, and gain were optimized at the beginning of each experiment and held constant throughout. Data were recorded on a SVHS tape and digitized for analysis using an offline system. A minimum of three images were acquired at each PI. The background-subtracted video intensity at each PI was measured from a region of interest within the vastus lateralis muscle. PI vs. video intensity data were curve fitted to the function y = A × (1 − e^(-t)), where y is the video intensity at PI time t, A is the plateau video intensity (an index of microvascular blood volume), and β is the rate of microvascular refilling (an indicator of microvascular flow velocity) (25).

Total and phosphorylated Akt, tuberous sclerosis complex 2 (TSC2), mTOR, 4E-BP1, and S6K were measured in skeletal muscle samples collected at 120, 330, and 420 min using SDS-PAGE and immunoblotting (Bio-Rad) as previously described (26). Primary antibodies were purchased from Cell Signaling Technology (Beverly, MA). The concentrations and binding sites were as follows: phospho-Akt (Ser473, 1:1000, and Thr308, 1:1000), phospho-TSC2 (Thr1462, 1:500), phospho-mTOR (Ser2448, 1:1000), phospho-4E-BP1 (Thr37/46, 1:1000), and phospho-p70 S6K1 (Thr389, 1:500). Anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody (Amersham Bioscience, Piscataway, NJ) was used at a concentration of 1:2000. For each protein, total content was detected using an antibody dilution of 1:1000. Both phosphorylated and total proteins were normalized to a rodent internal loading control. Data are expressed as normalized phosphorylated protein.

Muscle tissue was ground, and free amino acids and proteins were extracted as previously described (27). After protein hydrolysis and amino acid purification, mixed muscle protein-bound phenylalanine enrichment was determined using gas chromatography-mass spectrometry (Agilent Technologies, Palo Alto, CA) and the external standard curve approach (27). Blood and intracellular free phenylalanine concentrations and enrichments, and blood glucose enrichments were determined by gas chromatography-mass spectrometry as previously described (27).

Calculations

The fractional synthesis rate (FSR) of mixed muscle proteins was calculated between 120–240 and 240–420 min from the incorporation rate of l-[ring-\(^{13}\)C_6]phenylalanine into the pro-
tein and the free-tissue phenylalanine enrichment using the precursor-product model: $FSR = \frac{\Delta[\text{EA}]}{t} \left( \frac{E_{M1(1)} + E_{M2(2)}}{2} \right)$, where $\Delta[\text{EA}]$ is the increment of protein-bound phenylalanine enrichment between two sequential biopsies, $t$ is the time interval between the two sequential biopsies, and $E_{M1(1)}$ and $E_{M2(2)}$ are phenylalanine enrichments (tracer/tracer ratio) in the free muscle pool in two subsequent biopsies. Results are presented as percentage per hour.

Muscle phenylalanine kinetics was calculated using two- and three-pool models that provide unique information regarding leg plasma and intracellular phenylalanine kinetics, respectively (28). The parameters include the following: delivery to the leg = $CA \times BF$; output from the leg = $CV \times BF$; net balance (NB) = $(CA - CV) \times BF$; leg rate of appearance (Ra) = $BF \times C_{A} - [E_{A} - E_{V}]$; leg rate of disappearance = $EA + NB = BF \times [(CA - CV) - C_{A}]$; release from proteolysis = $F_{M1(0)} = \frac{EM_{1(1)} - EV}{(CA - CV) \times CA + [E_{A} - E_{V}] \times BF}$; and utilization for protein synthesis = $F_{M1(0)} + NB$, where $C_{A}$ and $C_{V}$ are phenylalanine concentrations in the femoral artery and vein, respectively; $E_{A}$, $E_{V}$, and $EM_{1(2)}$ are phenylalanine enrichments (tracer to tracer ratio) in the femoral artery, femoral vein, and muscle; and $BF$ is blood flow. Data are expressed as per 100 ml of leg volume.

Basal whole-body endogenous glucose production and utilization were calculated using the single-pool model (27): endogenous glucose production = whole-body glucose utilization = $i/GE_{A}$, where $GE_{A}$ is glucose arterial enrichment and $i$ is tracer infusion rate. During clamp, endogenous glucose production was calculated by subtracting the exogenous glucose infusion rate from whole-body glucose utilization.

Leg glucose utilization was calculated as the product of blood flow by the arteriovenous difference in glucose concentration ($G_{A} - G_{V}$); leg glucose utilization = $(G_{A} - G_{V}) \times BF$.

Insulin delivery to the leg was calculated as the product of femoral vein insulin concentration by blood flow.

**Statistical analysis**

Subjects’ characteristics and baseline values for all measured variables were analyzed using one-way ANOVA. To determine the effects of inhibition of insulin-dependent vasodilation, comparisons were performed using ANOVA with repeated measures. The factors were subject, time (basal and insulin), and group (control and L-NMMA). Post hoc pairwise multiple comparisons were performed using the Bonferroni $t$ test. The level of significance was set at $P < 0.05$ and the trend level at $P < 0.10$. Statistical procedures were performed using SigmaStat 3.5 (Systat Software Inc., San Jose, CA).

**Results**

No baseline differences were detected between groups for any measured parameters.

**Blood flow, muscle perfusion, and endothelin-1 (Fig. 2)**

Blood flow and muscle perfusion increased significantly during insulin infusion in the control group only ($P < 0.05$) but did not change in the L-NMMA group.

Endothelin-1 decreased significantly ($P < 0.05$) with hyperinsulinemia only in the control group and did not change in the L-NMMA group.

**Insulin and glucose kinetics (Table 2)**

During hyperinsulinemic-euglycemic clamp, arterial glucose concentration did not significantly change in either group, whereas systemic and femoral insulin concentrations, insulin delivery to the leg, and leg and whole-body glucose utilization increased significantly ($P < 0.05$) in both groups with no differences between groups. Endogenous glucose production decreased significantly ($P < 0.05$) in both groups during hyperinsulinemia, with no between-group differences.

**Anabolic signaling (Fig. 3)**

There were no significant differences in total protein across biopsy times for any proteins measured. During in-
TABLE 2. Insulin and glucose concentrations and kinetics in two groups of healthy young subjects at baseline and during local insulin infusion in one leg with (l-NMMA) or without (control) concomitant infusion of the eNOS inhibitor l-NMMA

<table>
<thead>
<tr>
<th></th>
<th>Control Baseline</th>
<th>Insulin</th>
<th>l-NMMA Baseline</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insulin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systemic concentration (pmol/liter)</td>
<td>35 ± 4</td>
<td>65 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28 ± 7</td>
<td>68 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Femoral vein concentration (pmol/liter)</td>
<td>27 ± 4</td>
<td>248 ± 33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29 ± 6</td>
<td>282 ± 28&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Delivery to leg (pmol/min · 100 ml leg)</td>
<td>0.10 ± 0.02</td>
<td>1.00 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.12 ± 0.04</td>
<td>1.03 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arterial concentration (mmol/liter)</td>
<td>4.9 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>5.4 ± 0.1</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td>Leg uptake (µmol/min · 100 ml leg)</td>
<td>0.3 ± 0.2</td>
<td>2.3 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.4 ± 0.1</td>
<td>3.5 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Endogenous production (µmol/kg · min)</td>
<td>9.9 ± 0.8</td>
<td>7.1 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.3 ± 0.5</td>
<td>7.6 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Whole-body uptake (µmol/kg · min)</td>
<td>9.9 ± 0.8</td>
<td>14.4 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.3 ± 0.5</td>
<td>17.0 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are the mean ± SE.
<sup>a</sup> P < 0.05 vs. baseline.

Insulin infusion, Akt<sup>Ser473</sup> phosphorylation increased significantly from baseline (P < 0.05) in both groups, with no differences between groups. A similar pattern was observed for Akt<sup>Thr308</sup>, whereas TSC2<sup>Thr1462</sup> phosphorylation did not change significantly in either group. mTOR<sup>Ser2448</sup> phosphorylation significantly increased from baseline with hyperinsulinemia only in the control group (P < 0.05). S6K1<sup>Thr389</sup> phosphorylation increased during insulin infusion in both groups (P < 0.05) but was larger in the control group than in l-NMMA at the end of the infusion (P < 0.05). 4E-BP1<sup>Thr37/46</sup> phosphorylation increased significantly with insulin at biopsy 3 in the control group only (P < 0.05).

**Mixed-muscle FSR (Fig. 4)**

During insulin infusion, FSR increased significantly in the control group (P < 0.05) but did not change in the l-NMMA group.

**Phenylalanine kinetics (Table 3)**

During hyperinsulinemia, phenylalanine arterial, venous, and muscle concentrations slightly but significantly decreased (P < 0.05), whereas phenylalanine arterial, venous, and muscle enrichments increased significantly (P < 0.05), with no differences between groups. Phenylalanine delivery tended to increase (P = 0.07) in the control group (+16.0 ± 9.4%) but did not change in the l-NMMA group (−7.2 ± 6.9%). Conversely, phenylalanine output from the leg did not change in the control group but decreased significantly (P < 0.05) in the l-NMMA group. As a result, phenylalanine net balance increased significantly in both groups (P < 0.05) with no group differences, but opposite mechanisms underlay this effect. The Control group had a significant increase (P < 0.05) in phenylalanine utilization for protein synthesis, with no change in proteolysis. The l-NMMA group showed a significant decrease (P < 0.05) in phenylalanine release from muscle, with no change in utilization for protein synthesis.

**Discussion**

Our results provide a first mechanistic demonstration that the endothelial-dependent increase in blood flow and muscle perfusion is a fundamental mechanism by which insulin stimulates skeletal muscle protein synthesis in young, healthy subjects. Our novel data indicate that insulin-stimulated vasodilation promotes muscle protein synthesis by increasing nutritive flow and, consequently, mTORC1 signaling, whereas Akt/PKB signaling is either not directly involved or only facilitates the process.

Specifically, local hyperinsulinemia at physiological postprandial levels induced significant increase in leg blood flow (10, 12) and muscle perfusion (17), enhancing Akt and mTORC1 signaling while also increasing skeletal muscle protein synthesis, resulting in an overall net anabolic effect. Conversely, administration of the eNOS inhibitor l-NMMA during local insulin infusion prevented the insulin-induced increase in blood flow and muscle perfusion, resulting in decreased amino acid delivery to the muscle, reduced mTORC1 signaling, and complete obliteration of the insulin-stimulatory effect on muscle protein synthesis. Interestingly, l-NMMA infusion did not prevent Akt signaling and the net muscle protein anabolic effect of insulin. However, the insulin’s anabolic effect with l-NMMA was due to inhibition of proteolysis rather than an increase in synthesis.

Our findings that insulin enhances blood flow and microvascular recruitment, which can be blocked by l-NMMA, are consistent with previous reports (14, 20, 29–31). In our experiment, microvascular perfusion underwent a greater relative increase than total blood flow. This is in agreement with a previous report that the insulin effect on...
microvasculature precedes and is not necessarily associated with changes in total blood flow (17). Besides stimulating vasodilation directly, nitric oxide synthesis through eNOS activation reduces production of the potent vasoconstrictor endothelin-1 (32), which is typically elevated in conditions associated with endothelial dysfunction, such as aging and insulin resistance (18, 33). We observed significantly lower endothelin-1 concentrations during isolated hyperinsulinemia, whereas administration of L-NMMA with insulin prevented this effect, mimicking the endothelial dysfunction of aging (12, 18).

An exciting finding of our study is that L-NMMA inhibition of insulin-induced vasodilation did not prevent the physiological increase in skeletal muscle Akt/PKB phosphorylation and glucose uptake, whereas it blunted mTORC1 signaling and protein synthesis. These results have several implications. Recent studies in vitro have shown that to reach the target tissues, insulin must first stimulate its own transport across the endothelium. This process requires an intact insulin signaling apparatus (34, 35). Although we did not directly measure insulin endothelial transport, our data suggest that eNOS activation and nitric oxide production are not involved in insulin transport across the endothelium, because the same insulin dose given alone or with L-NMMA induced similar increases in skeletal muscle Akt/PKB phosphorylation. While animal data have shown that inhibition of vasodilation during hyperinsulinemia can blunt the early muscle response of Akt/PKB and glucose uptake (31), our results indicate that endothelial-dependent vasodilation is not an essential contributor to muscle Akt/PKB signaling and glucose uptake in the later phases of hyperinsulinemia (1.5–3 h). Furthermore, a positive effect of insulin-induced vasodilation on glucose metabolism is more evident at higher glucose uptake levels than that achieved in our study, when increased perfusion and flow provide more uniform tissue exposure to glucose and reduce the artery-to-vein glucose gradient (14, 29, 31).

Conversely, our data clearly prove that the insulin-induced increase in mTORC1 signaling relies upon the hormone’s effects on nutritive flow, which increases both nutrient delivery and the amount of muscle tissue exposed to circulating nutrients. Increased amino acid availability has been reported to stimulate mTOR phosphorylation independent of Akt/PKB phosphorylation (19, 36). Although the exact mechanisms underlying the ability of amino acids to regulate mTORC1 phosphorylation are not completely understood, the ability of amino acids to regulate the interaction of Rheb GTP with the mTOR complex may play a pivotal role because the binding of Rheb to mTOR appears to be necessary for mTORC1 activation (37, 38).
Thus, the observed increases in mTOR\textsuperscript{Ser2448}, S6K1\textsuperscript{Thr389}, and 4E-BP1\textsuperscript{Thr37/46} phosphorylation and muscle protein synthesis in our control group likely resulted from the blood flow-mediated increase in amino acid delivery, a phenomenon that did not occur in the L-NMMA group. Because Akt/PKB can also phosphorylate mTORC1 (19), it might have exerted a facilitating effect on mTORC1 signaling and protein synthesis. However, phosphorylation of TSC2\textsuperscript{Thr1462}, a mTORC1 inhibitor that can be directly phosphorylated and inactivated by Akt (39, 40), did not change in either group, suggesting that Akt played a minor role under these experimental conditions. Akt\textsuperscript{Ser473} and Akt\textsuperscript{Thr308} data also suggest that upstream signaling through phosphatidylinositol 3-kinase and phosphoinositide-dependent kinase 1 was not different between the two groups (41), allowing us to exclude a significant role of phosphoinositide-dependent kinase 1 on mTORC1 signaling (42). The slight discrepancies between mTOR\textsuperscript{Ser2448}, S6K1\textsuperscript{Thr389}, and 4E-BP1\textsuperscript{Thr37/46} phosphorylation patterns could be due to the fact that the phosphorylation status at one site does not always predict mTOR activity or that we missed the peak of mTOR\textsuperscript{Ser2448} phosphorylation due to the timing of the muscle biopsies. Regardless, mTOR activity increased in both groups, but more so in the control group as shown by the increased phosphorylation of its primary downstream effector, S6K1. We have also reported an alternate, differential mTOR regulation of 4E-BP1 and S6K1 phosphorylation in humans during exercise (26), which is analogous to the current findings. These results also suggest that our findings are unlikely due to a direct effect of L-NMMA on skeletal muscle cell signaling. It was recently shown in myotubes that NOS blockade by L-NMMA prevents the nitric oxide-induced attenuation of mTOR signaling and downstream translation (43). If L-NMMA had a direct effect on muscle, it should have enhanced, rather than blocked, mTOR phosphorylation and protein synthesis. In the L-NMMA group, prevention of the insulin-induced increase in nutritive flow and amino acid delivery perhaps outweighed any direct effect of nitric oxide availability on mTORC1 signaling. However, this area war-

### TABLE 3. Leg free phenylalanine concentrations, enrichments, and kinetics in two groups of healthy young subjects at baseline and during local insulin infusion in one leg with (L-NMMA) or without (control) concomitant infusion of the eNOS inhibitor L-NMMA

<table>
<thead>
<tr>
<th>Phenylalanine concentration (µmol/liter)</th>
<th>Control</th>
<th>Insulin</th>
<th>L-NMMA</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femoral artery</td>
<td>59 ± 3</td>
<td>56 ± 3(^a)</td>
<td>60 ± 4</td>
<td>58 ± 3(^a)</td>
</tr>
<tr>
<td>Femoral vein</td>
<td>63 ± 3</td>
<td>55 ± 3(^a)</td>
<td>65 ± 4</td>
<td>57 ± 3(^a)</td>
</tr>
<tr>
<td>Muscle</td>
<td>72 ± 5</td>
<td>62 ± 4(^a)</td>
<td>77 ± 8</td>
<td>66 ± 4(^a)</td>
</tr>
<tr>
<td>Phenylalanine enrichment (tracer/tracee, %)</td>
<td>7.9 ± 0.3</td>
<td>9.0 ± 0.5(^a)</td>
<td>7.6 ± 0.3</td>
<td>8.3 ± 0.4(^a)</td>
</tr>
<tr>
<td>Femoral artery</td>
<td>6.4 ± 0.3</td>
<td>7.6 ± 0.3(^a)</td>
<td>6.0 ± 0.6</td>
<td>6.7 ± 0.3(^a)</td>
</tr>
<tr>
<td>Femoral vein</td>
<td>5.6 ± 0.2</td>
<td>6.5 ± 0.4(^a)</td>
<td>5.0 ± 0.2</td>
<td>6.0 ± 0.2(^a)</td>
</tr>
<tr>
<td>Net balance</td>
<td>-14 ± 3</td>
<td>3 ± 3(^a)</td>
<td>-15 ± 2</td>
<td>4 ± 3(^a)</td>
</tr>
<tr>
<td>Delivery to the leg</td>
<td>211 ± 30</td>
<td>240 ± 31(^b)</td>
<td>217 ± 24</td>
<td>202 ± 29</td>
</tr>
<tr>
<td>Output from the leg</td>
<td>225 ± 31</td>
<td>237 ± 34</td>
<td>232 ± 25</td>
<td>198 ± 27(^a)</td>
</tr>
<tr>
<td>Leg rate of appearance</td>
<td>49 ± 8</td>
<td>44 ± 9</td>
<td>58 ± 5</td>
<td>46 ± 4(^a)</td>
</tr>
<tr>
<td>Leg rate of disappearance</td>
<td>35 ± 7</td>
<td>47 ± 7(^a)</td>
<td>43 ± 5</td>
<td>51 ± 7</td>
</tr>
<tr>
<td>Release from proteolysis</td>
<td>55 ± 9</td>
<td>54 ± 11</td>
<td>66 ± 7</td>
<td>52 ± 5(^a)</td>
</tr>
<tr>
<td>Utilization for protein synthesis</td>
<td>40 ± 8</td>
<td>56 ± 10(^a)</td>
<td>51 ± 7</td>
<td>56 ± 8</td>
</tr>
</tbody>
</table>

Values are the mean ± se.  
\(^a\) \(P < 0.05\) vs. baseline.  
\(^b\) \(P = 0.07\) vs. baseline.
rants further investigations. Finally, 1-NMMA is not eNOS specific and could interfere with muscle contraction via inhibition of nNOS (44). Additionally, 1-NMMA attenuates exercise-induced vasodilation (45), reducing the anabolic effect of exercise. However, our experiments were performed at rest, making it unlikely that these mechanisms were responsible for our findings. Collectively, these data suggest that insulin-induced increases in blood flow, microvascular flow, and amino acid delivery are key, interconnected, components of insulin’s anabolic properties on skeletal muscle proteins.

The notion that insulin stimulates skeletal muscle protein synthesis in adult humans has long been debated because some studies reported that insulin administration was unable to stimulate muscle protein synthesis while it decreased protein breakdown (8, 9, 21), whereas several others reported an increase in protein synthesis with no change in breakdown (6, 46–48). We have previously discussed this issue at length (10–12, 18), suggesting that differences in vasodilation and amino acid delivery might have explained these discrepancies. Studies reporting reduced proteolysis with no change in protein synthesis employed systemic insulin administration in the absence of exogenous amino acid administration, resulting in decreased amino acid concentration and delivery (9, 21, 49). Conversely, studies reporting increased protein synthesis prevented hypoaminoacidemia using local insulin administration (as in the present study) or systemic infusion with amino acid coinfusion (6, 46–48, 50). The present study provides the mechanistic proof that our original hypothesis is correct, showing not only that blockade of insulin-induced vasodilation prevents the increase in protein synthesis but also that when this occurs in a subject with an otherwise intact insulin signaling apparatus, proteolysis decreases with a consequent protein-sparing effect. This finding does not appear to be an artifact of the mathematical models employed to calculate protein breakdown because both the two- and the three-pool models provided qualitatively comparable results. We can only speculate on the basic mechanisms underlying these opposite responses of muscle protein breakdown to hyperinsulinemia, but future studies should examine the role of both mTOR complexes in the control of muscle protein turnover.

In summary, this is the first study to mechanistically demonstrate that insulin stimulates muscle protein synthesis not through Akt/PKB signaling but through increases in capillary recruitment, nutritive flow, and mTORC1 signaling. It also underscores that although insulin exerts a protein-sparing effect on the skeletal muscle of young individuals with normal insulin sensitivity, the mechanism is dependent on its ability to stimulate muscle perfusion. Further studies are needed to determine the mechanisms by which insulin controls skeletal muscle protein breakdown in relation to nutritive flow and whether endogenous meal-stimulated hyperinsulinemia acts through similar mechanisms to stimulate skeletal muscle protein synthesis.

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