

Mechanism of insulin's anabolic effect on muscle: measurements of muscle protein synthesis and breakdown using aminoacyl-tRNA and other surrogate measures

Lisa S. Chow,¹ Robert C. Albright,² Maureen L. Bigelow,¹
Gianna Toffolo,³ Claudio Cobelli,³ and K. Sreekumaran Nair¹

Divisions of ¹Endocrinology, Nutrition and Metabolism, and ²Nephrology, Mayo Clinic College of Medicine, Rochester, Minnesota; and ³Department of Information Engineering, University of Padova, Padua, Italy

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Chow, Lisa S., Robert C. Albright, Maureen L. Bigelow, Gianna Toffolo, Claudio Cobelli, and K. Sreekumaran Nair. Mechanism of insulin's anabolic effect on muscle: measurements of muscle protein synthesis and breakdown using aminoacyl-tRNA and other surrogate measures. *Am J Physiol Endocrinol Metab* 291: E729–E736, 2006. First published May 16, 2006; doi:10.1152/ajpendo.00003.2006.—Despite being an anabolic hormone in skeletal muscle, insulin's anticatabolic mechanism in humans remains controversial, with contradictory reports showing either stimulation of protein synthesis (PS) or inhibition of protein breakdown (PB) by insulin. Earlier measurements of muscle PS and PB in humans have relied on different surrogate measures of aminoacyl-tRNA and intracellular pools. We report that insulin's effect on muscle protein turnover using aminoacyl-tRNA as the precursor of PS and PB is calculated by mass balance of tracee amino acid (AA). We compared the results calculated from various surrogate measures. To determine the physiological role of insulin on muscle protein metabolism, we infused tracers of leucine and phenylalanine into 18 healthy subjects, and after 3 h, 10 subjects received a 4-h femoral arterial infusion of insulin ($0.125 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), while eight subjects continued with saline. Tracer-to-tracee ratios of leucine, phenylalanine, and ketoisocaproate were measured in the arterial and venous plasma, muscle tissue fluid, and AA-tRNA to calculate muscle PB and PS. Insulin infusion, unlike saline, significantly reduced the efflux of leucine and phenylalanine from muscle bed, based on various surrogate measures which agreed with those based on leucyl-tRNA (-28%), indicating a reduction in muscle PB ($P < 0.02$) without any significant effect on muscle PS. In conclusion, using AA-tRNA as the precursor pool, it is demonstrated that, in healthy humans in the postabsorptive state, insulin does not stimulate muscle protein synthesis and confirmed that insulin achieves muscle protein anabolism by inhibition of muscle protein breakdown.

leucine; phenylalanine; amino acids; stable isotopes

INSULIN PLAYS A PIVOTAL ROLE in regulating body protein metabolism. Insulin deficiency, as seen in untreated type 1 diabetes mellitus (T1DM), is associated with increased skeletal muscle catabolism (14, 18) and urinary nitrogen loss (3, 41). In vitro evidence indicates that insulin decreases skeletal muscle protein breakdown (2, 22, 24, 31) and increases muscle protein synthesis (2, 22, 23, 33, 35). In humans, systemic insulin administration inhibits muscle protein breakdown in patients with T1DM (6, 8, 30, 34) and in healthy subjects (13, 29) but not confirmed by all studies (38). Systemic insulin administration causes hypoaminoacidemia, which may potentially affect muscle protein kinetics, since it has been shown that amino

acids (AA) play a key regulatory role on insulin effect on protein synthesis and breakdown (16, 21, 27). AA in combination with insulin have been shown to enhance muscle protein synthesis and to reduce muscle protein breakdown (7, 32). To overcome the systemic effect of insulin on AA levels, interarterial insulin has been directly infused into forearm (17, 26) and leg (10) to determine the effect of insulin on muscle protein metabolism. The first two forearm studies (17, 26) demonstrated that insulin at physiological levels decreases muscle protein breakdown and that the maximal effect is achieved at modest physiological levels, whereas the leg study (10) showed that insulin has no effect on muscle protein breakdown but increases muscle protein synthesis. The different effects of insulin on leg and forearm muscle may explain the difference, and there is continuing uncertainty whether the use of different precursor pools in these two studies may have caused the contradictory results.

We also sought to determine whether precursor pool selection may be a reason for the contradictory reports on insulin's effects on muscle protein metabolism. Either one-compartment (17, 26) or three-compartment (10) models have been used to study insulin's effect on muscle protein metabolism. The one-compartment model of protein metabolism calculates the AA rate of appearance (R_a) and disappearance (R_d) from the plasma compartment (4). The three-compartment model of protein metabolism calculates protein synthesis (PS) and protein breakdown (PB) from an approximation of the intracellular AA pool (9). To our knowledge, the obligatory precursor of protein synthesis is aminoacyl-tRNA (AA-tRNA); therefore, we used AA-tRNA as precursor of protein synthesis in a three-compartment model. Protein breakdown was calculated simply by mass balance of tracee AA assuming a steady-state situation. Using AA-tRNA as the precursor pool, we demonstrated superior precision in measurements and higher rates of muscle protein synthesis and breakdown than the results based on other surrogate precursor pools (40).

Because of the difficulty in isolating AA-tRNA, no previous human studies to date have used AA-tRNA as the precursor pool in measuring insulin's effect on muscle protein synthesis. Previously, phenylalanyl-tRNA has been used in measuring insulin's effects on skeletal muscle protein synthesis in rats, reporting that systemic insulin infusion does not increase protein synthesis (42). Here, we report the results from a study

Address for reprint requests and other correspondence: K. S. Nair, Mayo Clinic College of Medicine, 200 First St. SW, Rochester, MN 55905 (e-mail: nair.sree@mayo.edu).

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in 18 healthy adult volunteers receiving either insulin ($n = 10$) or saline ($n = 8$) infusion into the femoral artery. We used two independent tracers (L-[U- 13 C]leucine and [15 N]phenylalanine) and compared several surrogate measurements of precursor pools of protein metabolism, including enrichment and concentrations of plasma phenylalanine in the femoral artery, femoral vein, and ketoisocaproate (KIC) as well as tracer-to-tracee ratio (TTR) of muscle leucyl-tRNA, and tissue fluid free phenylalanine and phenylalanyl-tRNA, to resolve the discrepant reports of insulin's effects on protein synthesis and breakdown.

RESEARCH DESIGN AND METHODS

Materials. Stable isotope tracers L-[U- 13 C]leucine [98 atom percent excess (APE)] and L-[15 N]phenylalanine (99 APE) were purchased from Cambridge Isotope Laboratories (Woburn, MA). The chemical, isotopic, and optical purity was tested before use. Sterile solutions of the isotopes were prepared and confirmed to be bacteria and pyrogen free before use in humans.

Subjects. Eighteen normal, healthy volunteers were studied (age 28.1 ± 1.8 yr, BMI 24.6 ± 0.7 kg/m 2 , venous glucose 91.9 ± 1.6 mg/dl). Eight subjects were allocated to saline infusion and 10 subjects to insulin infusion. The demographic details of the subjects are reported in Table 1. All subjects underwent a complete history and physical examination, including biochemical and hematological tests, to confirm their health prior to participation. Inclusion criteria included men and women between the ages of 18 and 45 yr. Exclusion criteria included pregnancy and use of any medication that might interfere with bleeding, clotting, or body metabolism within the previous 2 wk. The subjects were placed on a weight-maintaining diet and were advised to avoid alcohol, large amounts of dietary protein, and strenuous exercise. Before beginning any procedure, all subjects provided their informed oral and written consent after the risks and benefits had been fully explained. The study protocol was approved by the Institutional Review Board of the Mayo Clinic and Foundation.

Protocol. The subjects were instructed by a dietician to consume a standard weight-maintaining diet (carbohydrate-fat-protein 55:30:15%) for 3 days before the study. Subjects were admitted to the General Clinical Research Center at St. Mary's Hospital the evening prior to the study. All subjects received a standard meal at 6:00 PM and had a 500-kcal snack at 10:00 PM. The purpose of the snack was to prevent a prolonged fasting state, as prolonged fasting may alter protein metabolism and be inconvenient for volunteers.

On the evening of admission, all subjects had an intravenous line inserted into the forearm vein with a continuous saline infusion to maintain patency. The following morning, baseline venous samples for isotopic measurements were taken, and priming doses of L-[U- 13 C]leucine (1 mg/kg), [15 N]phenylalanine (0.75 mg/kg), and [13 C]sodium bicarbonate (0.2 mg/kg) were given. A continuous isotope infusion was maintained with L-[U- 13 C]leucine (1 mg·kg $^{-1}$ ·h $^{-1}$), and [15 N]phenylalanine (0.75 mg·kg $^{-1}$ ·h $^{-1}$). The subjects receiving insulin also received TrophAmine (10% solution; B. Braun, Bethlehem, PA) at 0.15

ml·kg $^{-1}$ ·h $^{-1}$ to avoid hypoaminoacidemia associated with the insulin infusion. This was instituted after initial studies showed a slight fall in branched-chain amino acid (BCAA) concentration in blood following intra-arterial infusion of insulin. A 20% dextrose solution was infused if needed to maintain blood glucose at postabsorptive levels. The infusion rate of dextrose was 1.48 ± 0.21 mg·kg $^{-1}$ ·h $^{-1}$ during the last 2 h of steady state. Between 8:00 and 9:00 AM, one catheter was inserted into the femoral vein and another into the femoral artery. Catheter patency was maintained by a continuous infusion of 0.9% saline (29). Blood flow from time 120–180 min was measured by an intra-arterial infusion of indocyanine green (Cardiogreen), using the principle of indicator dye dilution as previously described (1). Blood samples were collected from the femoral artery and vein at the baseline state (at 0 min) and before the first muscle biopsy (at 165, 170, 175, and 180 min). The first muscle biopsy was performed 3 h (at 180 min) after initiation of the isotope infusion. The muscle biopsy was collected by the needle biopsy technique after local anesthesia and immediately placed over a glass slide kept over liquid nitrogen while excess fluid was blotted and connective tissue and fat were trimmed. The sample was then weighed, snap-frozen using isopentane cooled by liquid nitrogen, and stored at -85°C until analysis (25). After the first muscle biopsy was completed, each subject received an intravenous infusion of either saline or regular insulin (0.125 mU·kg $^{-1}$ ·min $^{-1}$) via the femoral arterial catheter for the next 4 h. Blood flow was again measured using Cardiogreen from time 360 to 420 min, and four blood samples were collected (at time 405, 410, 415, and 420 min) from both the femoral artery and vein. A second muscle biopsy was performed 7 h (at time 420 min) after initiation of the isotope infusion, which was ~ 4 h after the first biopsy. To minimize local trauma and hemotoma effects, the two needle biopsy sites were 4–6 in. apart and separated by a 4-h time interval. In addition, the first biopsy was collected from a more distal site; the second biopsy was collected from a more proximal site. Although the needle biopsy itself might still confound the results, we had normal saline controls to observe any biopsy-related changes in protein dynamics. Approximate wet weight of each biopsy specimen was 310 mg.

The collected blood was kept on ice and analyzed for glucose and indocyanine green. Other samples were centrifuged at 5°C and stored at -85°C until measurements of insulin, AA concentration, and AA TTR were performed, as previously described (25, 29, 40).

Sample processing. Muscle tissue fluid was obtained by homogenizing the muscle biopsy tissue in 1 M perchloric acid. After centrifugation, the supernatant containing the free pool of AA was removed and purified with a cation exchange column. The acidified samples were applied to the column and the AA eluted with 2 ml of 4 N NH $_4$ OH. A detailed description of this process has been reported previously (25).

A detailed description of AA-tRNA isolation has been reported previously (25).

AA concentrations were measured using reverse-phase high-performance liquid chromatography (19). Leucine, KIC, phenylalanine, and tyrosine concentrations were also measured by mass spectrometry and those values used for flux calculations. Arterial plasma, venous plasma, and muscle tissue fluid were processed to measure TTRs of acyl-tRNA, leucine, phenylalanine, and KIC, as previously described (15, 25). The AA-tRNA samples were derivatized to their trifluoroacetyl-isopropyl esters and underwent positive ion chemical ionization using ammonia as the reactant gas before being analyzed on a Hewlett-Packard 5989B GC-MS (MS Engine). The concentration of phenylalanine was only one-third the concentration of leucine because of the differences in tissue concentration (25). As the concentrations for phenylalanine extracted from tRNA of needle biopsy samples were extremely low in some of the processed samples and the precision of the measurement was unacceptable, results using phenylalanine tRNA were excluded from our analysis. On the basis of the extensive purification steps, the likelihood of the AA-tRNA pool being contaminated by other pools following the extensive extraction

Table 1. Patient demographics

	Saline	Insulin
Age	26.9 \pm 2.2	29.1 \pm 2.7
Sex (M/F)	6/2	4/6
Height, cm	178.6 \pm 5.1	173.5 \pm 3.7
Weight, kg	81.8 \pm 7.5	73.5 \pm 5.4
BMI, kg/m 2	25.2 \pm 1.3	24.1 \pm 0.9
Fasting glucose, mg/dl	92.9 \pm 3.2	91.2 \pm 1.4

Values are means \pm SE.

procedure was low and was confirmed by adding radiolabeled AA initially and then noting its absence after hydrolyzing AA from tRNA (25). The precision of the isotope measurement was substantially enhanced by the use of [U-¹³C]leucine rather than [1-¹³C]leucine.

Modeling of protein metabolism. Formulas to derive the rates of AA disappearance (R_d) and appearance (R_a) by the one-compartment model (Fig. 1, *left*) are described, respectively, by Eqs. 1 and 2. The difference between the V-A mode and the A-V mode is selection of the precursor pool using arterial blood or venous blood, respectively.

$$R_d = \Phi[ca - cv]/TTR_{a,V-A \text{ mode}} \quad (1)$$

or

$$R_d = \Phi[ca - cv]/TTR_{v,A-V \text{ mode}} \quad (2)$$

$$R_a = R_d - \Phi[Ca - Cv]$$

where Φ is regional blood flow, ca is tracer concentration in artery, cv is tracer concentration in vein, Ca is tracee concentration in artery, Cv is tracee concentration in vein, TTR_a is TTR in artery, and TTR_v is TTR in vein.

Rather than measuring AA R_d and R_a from the blood, which approximates protein synthesis (PS) and protein breakdown (PB), respectively, a more precise measurement would be to measure AA R_d and R_a from an intracellular precursor pool. This is possible with the three-compartment model, which separates out the arterial, venous, and tissue compartments (Fig. 1, *right*). We assumed that AA-tRNA is the source pool of AA for PS. Although AA-tRNA is used to estimate PS (Eq. 3 assumes that AA-tRNA is the precursor of PS), PB is calculated simply by mass balance of tracee AA (Eq. 4). The only assumption is that tracee is in a steady state, so that AV difference represents the balance between PS and PB, irrespectively from the site where these processes take place.

$$PS = \Phi[ca - cv]/TTR_t \quad (3)$$

$$PB = PS - \Phi[Ca - Cv] \quad (4)$$

As the formulas for PS and PB can be derived by applying Fick's principles to the arteriovenous difference across the selected tissue bed (Fig. 1, *right*), specific criteria about enrichment levels between the artery, vein, and tissue, necessary for intercompartmental flux calculations, need not be met (40). We applied the mass balance equation to the endogenous AA (tracee) so as to express the difference between AA inflow (F_{in}) and outflow (F_{out}) as equal to the difference between PS and PB:

$$F_{in} - F_{out} = \Phi[Ca - Cv] = PS - PB \quad (5)$$

Likewise, the AA tracer is described by a similar formula:

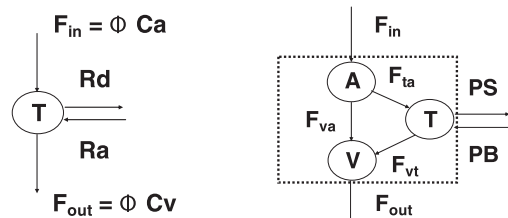


Fig. 1. One-compartment model and 3-compartment model of amino acid (AA) kinetics. *Left*: 1-compartment model showing the relationship between AA inflow (F_{in}), AA outflow (F_{out}), rate of disappearance (R_d), and rate of appearance (R_a). *Right*: 3-compartment model showing AA flow between artery (A), vein (V), and tissue (T) compartments. Box demarcates the intracellular compartment used in calculating protein synthesis and breakdown (PS and PB). F_{ta} , AA transport from A to T; F_{va} , AA transport from A to V; F_{vt} , AA transport from T to V; Φ , regional blood flow; Ca , AA concentration in artery; Cv , AA concentration in vein.

$$f_{in} - f_{out} = \Phi[ca - cv] = ps \quad (6)$$

where f_{in} is tracer inflow, f_{out} is tracer outflow, and ps is rate of tracer utilization.

The relationship of ps to PS is described by Eq. 7 and is dependent on the TTR for the selected intracellular precursor of protein synthesis (TTR_t):

$$ps/PS = TTR_t \quad (7)$$

By using Eq. 7, Eqs. 5 and 6 can be solved for PS (Eq. 3) and PB (Eq. 4). We use Eq. 7 with the following assumptions: the steady state is established, stable isotope-labeled tracer and tracee have the same metabolic fate with reference to protein metabolism, and the selected intracellular precursor pool is the site of intracellular protein synthesis.

Note that the calculation of PS (Eq. 3) is similar to R_d (Eq. 1) and the calculation of PB (Eq. 4) is similar to R_a (Eq. 2). The difference depends on the TTR of the selected precursor pool (3-compartment model: KIC, tissue fluid, AA-tRNA; 1-compartment model: arterial blood, venous blood).

Calculation of the arterial tracer concentration (ca), arterial tracee concentration (Ca), venous tracer concentration (cv), and venous tracee concentration (Cv) involves measuring TTRs and total AA concentrations (tracer + tracee) in the artery (TTR_a , C_{total}) and vein (TTR_v , C_{totv}). These values were calculated as the average of the four values measured at the time of the first muscle biopsy (times 165, 170, 175, and 180 min) and as the average of the four values measured at the time of the second muscle biopsy (times 405, 410, 415, and 420 min). From TTR_a , TTR_v , C_{total} , and C_{totv} , the concentrations of ca , Ca , cv , and Cv can be derived (40).

At baseline and at the end of infusion, we confirmed steady-state AA concentration and TTR by sampling four blood samples at each time point. As multiple muscle biopsies at baseline and at the end of infusion were not logistically feasible due to the quantity of required tissue, we extrapolated the observation of steady state in the plasma to assume steady state of the intracellular precursor pools on the basis of previous animal model evidence showing correlation (5).

Estimation of AA intercompartmental fluxes requires specific assumptions of AA transport in the organ. The three-compartment model (Fig. 1, *right*) assumes unidirectional AA transport from artery to tissue (F_{ta}), from tissue to vein (F_{vt}), and from artery to vein (F_{va}). By writing mass balance equations for the tracer and tracee at steady-state conditions, the specific AA intercompartmental fluxes can be derived (40).

Assay variability. TTR and concentration reproducibility were expressed as the standard deviation (SD) and coefficient of variation (CV) for an average value. We described our results as average value \pm SD (CV). Specifically for our study, the intra-assay variation for tissue fluid TTR was found to be $2.13 \pm 0.16\%$ (CV 7.63%), with the interassay variation for leucine tissue fluid TTR being $2.2 \pm 0.21\%$ (CV 9.36%) and interassay variation for phenylalanine tissue fluid TTR being $0.85 \pm 0.10\%$ (CV 11.3%). The interassay variability of leucyl-tRNA TTR from muscle samples has been previously reported as $4.5 \pm 0.2\%$ (25). The interassay variability for plasma leucine TTR and plasma leucine concentration (μM) was $6.94 \pm 0.28\%$ (CV 3.99%) and $91.3 \pm 8.2\%$ (CV 9.0%), respectively. Similar reproducibility was found for plasma phenylalanine TTR and plasma phenylalanine concentration (μM), the interassay variability being $4.77 \pm 0.26\%$ (CV 5.5%) and $52.5 \pm 3.16\%$ (CV 6.02%), respectively. The interassay variability for plasma KIC TTR was $3.44 \pm 0.06\%$ (CV 6.22%), and plasma KIC concentration (μM) was $44.93 \pm 1.50\%$ (CV 2.07%).

As previously described, a Monte Carlo analysis was performed to quantify the effect of both analytic errors and plateau variability on the precision of estimated fluxes (40).

Statistical analysis. A standard power calculation was performed to determine the appropriate number of subjects necessary to achieve discrimination of the predicted 20% change in protein dynamics expected with insulin infusion. On the basis of our published data of PS and PB across the leg in subjects with T1DM (30), we estimated that eight subjects in each group would be sufficient to demonstrate the 20% change. The effect of insulin could be detected with a power of 0.90. To determine whether insulin had any effect on outcome measures, we compared the baseline period (165–180 min) and the end of the insulin infusion period (400–420 min) by paired *t*-test. To determine whether any time-related changes occurred, we compared baseline and end of the saline infusion period also by paired *t*-test.

RESULTS

Subject data. Table 1 shows demographic data, and no differences in any parameters between the two groups were noted.

Figure 1 shows the model used for calculations that was discussed under RESEARCH DESIGN AND METHODS.

Insulin levels. Figure 2 (top) shows that femoral venous insulin concentration during insulin infusion significantly increased (5-fold) from 180 to 420 min, whereas no significant changes occurred during saline infusion. Arterial concentration at 420 min was significantly lower during saline infusion than at 180 min but was significantly higher during insulin infusion. Arteriovenous difference in insulin concentration during insulin infusion increased from 0.6 ± 0.2 to 13.4 ± 1.3 $\mu\text{mol/ml}$ ($P < 0.0012$), whereas no significant change (0.7 ± 0.2 to 0.3 ± 0.1) occurred during saline infusion.

Blood flow. Although blood flow showed a trend to increase during saline infusion (314 ± 39 vs. 376 ± 61 ml/min, $P = 0.15$), the increase (282 ± 27 vs. 375 ± 25 , $P < 0.002$) reached statistical significance only during insulin infusion.

Isotopic enrichment and concentrations of leucine, phenylalanine, and KIC. Isotopic enrichment values used for calculations of protein kinetics and AA and KIC are given in Table 2. No changes in phenylalanine, leucine, or KIC concentrations occurred during either insulin or saline infusion.

Table 3 shows total essential AA and BCAA levels during the plateau period when protein kinetics were measured. These values representing mean values of four time points in each subject showed no differences between the two groups.

Figure 2 (bottom) shows the arteriovenous balances during saline vs. insulin infusion. A negative leucine balance became positive by insulin infusion and negative phenylalanine balance became significantly less so and was not different from zero during insulin infusion. Glucose balance increased significantly with insulin, demonstrating substantially higher glucose uptake by muscle bed.

Protein kinetics. Figure 3 showed protein breakdown and protein synthesis using leucine as a tracer. The data show the calculated protein breakdown values using the following precursor pools—leucine tRNA (tRNA), leucine in tissue fluid (t-fluid), venous KIC enrichment (KICV), arterial leucine (AV mode), venous leucine (VA mode). The results indicate that saline treatment had no significant effect on PB and PS estimated based on various precursor pools. In contrast, insulin treatment significantly decreased PB, irrespective of the different pools used. The magnitude of differences differed (tRNA = -27.7% , $P < 0.02$, tissue fluid = -29.7% , $P = 0.01$, KICV =

Fig. 2. Top: plasma insulin concentrations at 180 and 420 min in femoral artery (A180 and A420) and femoral vein (V180 and V120). Arterial insulin concentrations were similar at 180 min but were higher during insulin infusion (filled bar) than during saline infusion (open bar) at 420 min ($P < 0.0004$). Venous insulin concentrations were not different at 180 min but were different between saline and insulin group at 420 min ($P < 0.0001$). At 420 min, arterial insulin concentrations decreased from 180 min in the saline group ($P < 0.004$) and increased from 180 min in the insulin group ($P < 0.0001$). At 420 min, venous insulin concentrations decreased from 180 min in the saline group ($P < 0.002$) but increased from 180 min in the insulin group ($P < 0.00001$). Net balance of phenylalanine and leucine was negative during saline infusion but the leucine balance became positive ($P < 0.002$) and phenylalanine balance became less negative ($P < 0.001$) with insulin infusion. Glucose balance became more positive during insulin infusion than during saline infusion ($P < 0.002$). #Difference from 180 min; *difference from saline period at the same time point.

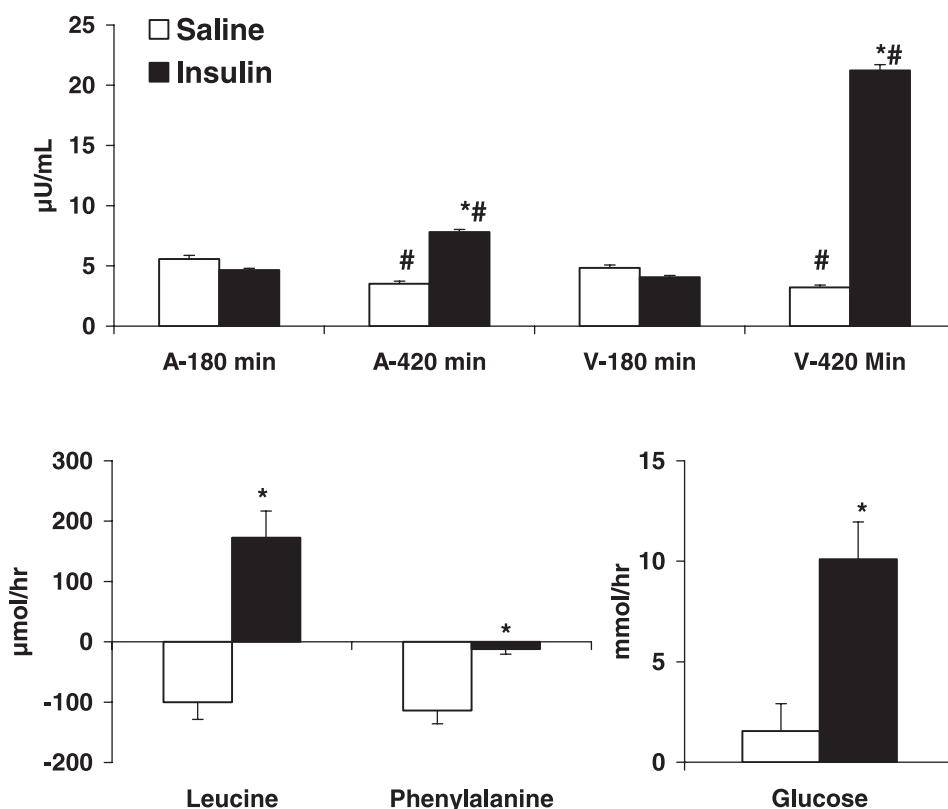


Table 2. Phenylalanine, leucine, and KIC concentrations or TTRs in molar percent in artery and vein

	Phenylalanine		Leucine		KIC		Tyrosine	
	Saline	Insulin	Saline	Insulin	Saline	Insulin	Saline	Insulin
Conc., $\mu\text{mol/l}$								
Artery								
180 min	50.50 \pm 2.62	47.23 \pm 1.95	119.2 \pm 7.88	118 \pm 6.84	35.22 \pm 2.61	31.22 \pm 1.68	52 \pm 6.2	49.6 \pm 3.22
420 min	51.39 \pm 3.10	50.85 \pm 1.95	118.5 \pm 7.99	126.5 \pm 6.99	33.34 \pm 2.66	31.12 \pm 1.72	48.5 \pm 6.2	44 \pm 2.65
Vein								
180 min	55.97 \pm 2.83	53.19 \pm 1.84	122.7 \pm 6.51	120.2 \pm 6.10	37.35 \pm 2.75	31.62 \pm 2.07	56.3 \pm 6.53	54 \pm 3.78
420 min	56.68 \pm 3.53	51.38 \pm 1.70	124.1 \pm 7.99	118.6 \pm 6.86	35.56 \pm 2.71	29.4 \pm 1.40	51.9 \pm 6.32	43.5 \pm 2.79
TTR (MP)								
Artery								
180 min	12.16 \pm 0.27	11.29 \pm 0.40	6.89 \pm 0.19	6.55 \pm 0.41	4.61 \pm 0.09	4.75 \pm 0.20	1.3 \pm 0.04	1.3 \pm 0.19
420 min	12.08 \pm 0.44	11.01 \pm 0.46	7.02 \pm 0.29	6.07 \pm 0.44	4.98 \pm 0.12	4.41 \pm 0.19	1.5 \pm 0.05	1.5 \pm 0.19
Vein								
180 min	9.23 \pm 0.28	8.59 \pm 0.43	5.35 \pm 0.13	5.03 \pm 0.37	4.21 \pm 0.09	4.38 \pm 0.19	1 \pm 0.05	1 \pm 0.17
420 min	9.55 \pm 0.33	9.37 \pm 0.45	5.75 \pm 0.21	5.19 \pm 0.38	4.57 \pm 0.11	4.46 \pm 0.20	1.2 \pm 0.07	1.3 \pm 0.17
Tissue fluid								
180 min	7.20 \pm 0.35	7.86 \pm 0.63	3.27 \pm 0.37	3.71 \pm 0.40				
420 min	7.78 \pm 0.43	9.22 \pm 0.43	3.76 \pm 0.34	4.38 \pm 0.36				
tRNA								
180 min			4.7 \pm 0.19	4.91 \pm 0.34				
420 min			5.25 \pm 0.24	5.48 \pm 0.28				

Values are means \pm SE. MP, molar percent; KIC, ketoisocaproate; TTR, tracer-to-tracee ratio.

-14.5%, $P < 0.03$, VA mode = 19%, $P = 0.04$ and AV mode = 21.06%, $P < 0.01$). The insulin showed no effect on PS based on tRNA, tissue fluid, KICV and A-V mode but showed significant increase based on V-A mode. Estimation of protein breakdown and protein synthesis based on arterial KIC showed decrease and increase respectively (data not shown since this approach is theoretically not valid).

Using phenylalanine as the AA tracer, Fig. 4 shows that insulin infusion decreases PB. This was observed regardless of using tissue fluid (t-fluid: -23.95%, $P = 0.01$), arterial phenylalanine (VA-R_a: -19.53%, $P = 0.01$) or venous phenylalanine (AV-R_a: -20.43%, $P = 0.02$) as the precursor pool. In terms of PS, only the use of arterial phenylalanine (V-A) as the precursor pool showed an increase in the rate of leucine disappearance (R_d: +38.92%, $P = 0.03$), suggesting an increase in PS. We could not use phenylalanine tRNA to calculate PS and PB due to low concentrations of phenylalanine derived from tRNA in several muscle samples (phenylalanine content is ~3% vs. >7% leucine).

DISCUSSION

The current study demonstrated that intra-arterial insulin infusion into healthy human subjects, which increased insulin to normal physiological postprandial levels, suppressed muscle protein breakdown with no significant effect on muscle protein

synthesis. The above finding is supported by measurements using leucyl-tRNA, the obligatory precursor of protein synthesis. The reduction in muscle protein breakdown by insulin was also supported by measurements based on other surrogate precursor pools and two different amino acid tracers. Insulin at the dose we infused that increased to normal postprandial concentrations in femoral vein had no stimulatory effect on muscle protein synthesis based on any of the precursor pools, with the exception of arterial TTR of leucine, KIC, and phenylalanine.

Muscle protein synthesis and breakdown were measured by simultaneous infusion of two separate AA tracers. Stable-isotope tracers of leucine and phenylalanine have previously been used to study protein metabolism of the whole body (28) (39), forearm (12, 39), and leg (29). The disappearance of the free leucine from skeletal muscle can be due to either deamination into KIC or incorporation into muscle proteins. However, once leucine is acylated to tRNA, the only known fate of leucine is protein synthesis, as transamination does not occur from leucyl-tRNA. Thus the rate of leucine disappearance from leucyl-tRNA is the most direct measure of protein synthesis. In contrast to leucine, phenylalanine's fate in skeletal muscle is limited to incorporation into protein. Thus the phenylalanine disappearance rate from intracellular and plasma compartment represents the rate of protein synthesis. It is also reassuring that the main conclusion from this study is supported by measurements using both tracers.

Isotopic enrichment values of tissue fluid AA, KIC, and arterial and venous AA have been used as surrogate measures of the muscle intracellular precursor pool. Previously, it has been shown that muscle tissue fluid most closely approximates AA-tRNA (5, 25). Nevertheless, tissue fluid AA tracer enrichment is likely less precise than that of AA-tRNA, as the compartmentalization of tissue fluid between the extracellular and intracellular spaces cannot be assumed to be equal in the

Table 3. Concentrations of total AA, essential AA, and BCAA during the last 2 h

	Saline	Insulin	P Value
Total AA	2,117 \pm 164.0	1,806 \pm 153.3	0.187
Essential AA	791 \pm 71.0	683 \pm 62.1	0.270
BCAA	378 \pm 38.2	296 \pm 29.5	0.115

Values are means \pm SE in $\mu\text{mol/l}$. AA, amino acids; BCAA, branched-chain amino acids.

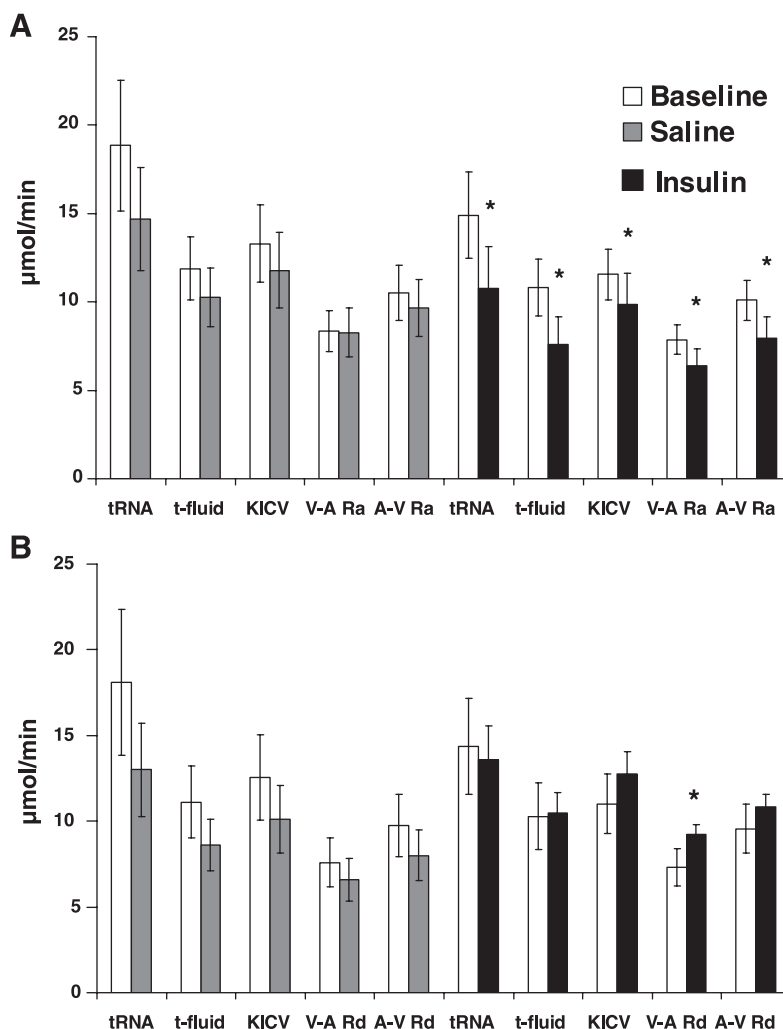


Fig. 3. Protein metabolism using leucine tracer. *A*: PB and leucine R_a . PB ($\mu\text{mol}/\text{min}$) was calculated using different precursor pools, including aminoacyl-tRNA (AA-tRNA), tissue fluid (t-fluid), and arterial α -ketoisocaproate KIC (KICv). AA R_a was derived using either arterial leucine concentration (A-V mode) or arterial leucine concentration (V-A mode) as precursor pool. *B*: PS and leucine R_d . In all cases, saline infusions caused no significant differences (P value ranged between 0.15 and 0.93). PS was significantly different ($P = 0.017$) in case V-A R_d . *Significantly different vs. baseline.

setting of saline or insulin infusion. Tissue fluid AA enrichment is also subject to contamination from blood (40). Despite these limitations, the tissue fluid-based measurement agreed best with the AA-tRNA-based conclusions.

Modeling of protein synthesis and protein breakdown using either venous (AV mode) or arterial AA TTR (VA mode) calculates the AA R_a and R_d from the plasma compartment. Using either the VA mode or the AV mode, we report a consistent decrease in R_a with insulin infusion. Insulin, however, increased R_d as described with the VA mode compared with the decline reported by the AV mode (Figs. 3 and 4). The approximation of protein synthesis in the VA mode as measured by the AA R_d from the arterial compartment does not consider the changes in intracellular compartment occurring with the influx of AA from protein breakdown. In contrast, the AV mode, based on venous AA isotopic enrichment, is more representative of the intracellular compartment, since the femoral vein drains muscle bed. Indeed, the calculations of the AV mode using both venous leucine and phenylalanine agreed with the results from AA-tRNA (26, 36, 40). Similarly, the measurements based on venous KIC were comparable to results obtained from AA-tRNA, in contrast with those using arterial KIC. Because leucine is transaminated mostly in muscle,

venous KIC enrichment is clearly a better measure of the intracellular pool than arterial KIC. Insulin infusion into artery is likely to reduce transamination; thus venous KIC and these effects occurring in the intracellular compartment is better represented by venous KIC. Moreover, the results based on leucyl-tRNA agreed with the results based on venous KIC enrichment.

Our results demonstrate the variability in measurement of protein synthesis and protein breakdown using different precursor pools, especially when the magnitude of change is considered. Measurements of protein synthesis and breakdown using these different precursor pools are more consistent when only the direction of change is considered. When comparing leucyl-tRNA other precursor pools such as tissue fluid leucine, tissue fluid phenylalanine, plasma KICv, venous leucine, and venous phenylalanine, we found agreement in the direction of change for protein synthesis and protein breakdown.

With insulin infusion, we found that leucine balance became positive (net increase in leucine uptake) and phenylalanine balance became less negative. One explanation for leucine balance to become positive, unlike phenylalanine, is the inhibitory effect of insulin on leucine transamination (29) in addition to inhibition of protein breakdown.

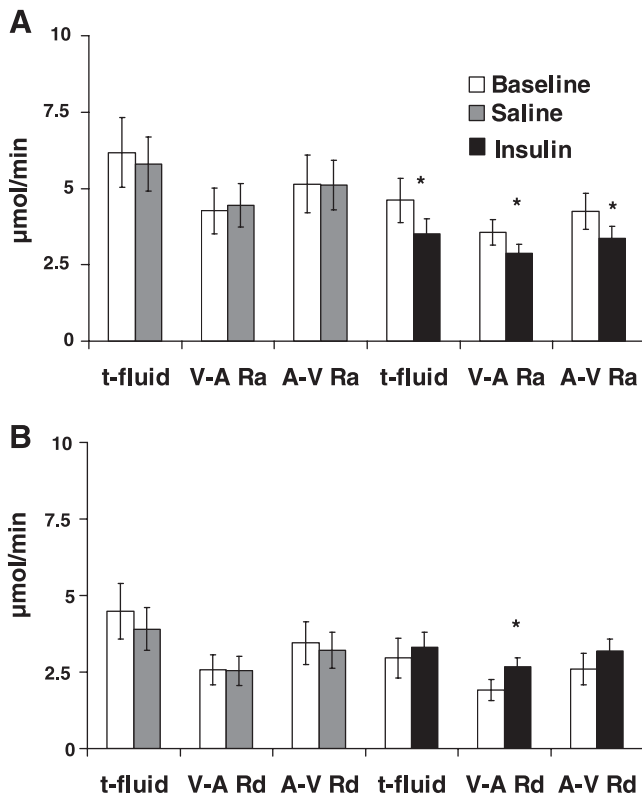


Fig. 4. Protein metabolism using phenylalanine tracer. A: PB and phenylalanine R_a . PB ($\mu\text{mol}/\text{min}$) calculated using different precursor pools, including AA-tRNA, t-fluid, and KICv. AA R_a was derived using either venous leucine concentration (A-V mode) or arterial leucine concentration (V-A mode) as precursor pool. B: PS and phenylalanine R_d . Results are reported as means \pm SE. *Significantly different ($P = 0.0024$) in case t-fluid, $P = 0.005$ in case V-A R_d , $P = 0.009$ in case A-V R_a . PS showed significant difference only in case of V-A R_d ($P = 0.001$). No significant differences ($P > 0.10$) were noted comparing saline infusion vs. baseline.

We performed an intra-arterial insulin infusion to minimize systemic insulin effects. From our measurements (Fig. 2), however, systemic insulin level was modestly elevated. We prevented any systemic decline in AA or glucose by infusing very low rates of dextrose ($1.4 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) and AA mixture infusions. In this way, we ensured that muscle was exposed to similar glucose and AA concentrations in both study groups.

The current study clearly demonstrated that insulin infusion directly into the femoral artery failed to stimulate muscle protein synthesis. We achieved a more than fivefold increase (4.1 ± 0.0 to $21.2 \pm 0.5 \mu\text{U}/\text{ml}$) in the femoral venous insulin level, whereas in the control group the level decreased by 33%. With current radioimmunoassay used for these measurements, the levels of insulin that we achieved were what we usually observe in the postprandial state for lean healthy subjects. In contrast, an earlier study using tissue fluid TTR reported physiological hyperinsulinemia stimulating muscle protein synthesis (10). Our results using tissue fluid TTR did not agree with this earlier finding. We did not assume tissue fluid TTR, a mixture of intracellular and extracellular fluid, to be equivalent to intracellular TTR as previously proposed and made no corrections for the mixing of extracellular fluid to estimate the intracellular fluid (10). We are unsure about the validity of the above assumption, as insulin may shift fluid and electrolytes

between the intra- and extracellular compartments (25). Another potential reason is that, in the previous study, femoral venous insulin levels were increased more than sevenfold (10), as opposed to fivefold in the current study. A supraphysiological insulin infusion (1,000-fold above basal level) stimulated muscle protein synthesis, with a modest decline in muscle protein breakdown (20). A dose-effect relationship of insulin infused into a brachial artery demonstrated that even a 20-fold increase (6 ± 1 to $124 \pm 11 \mu\text{U}/\text{ml}$) in insulin failed to stimulate muscle protein synthesis but inhibited muscle protein breakdown (26). The dose of insulin used in the current study is well within normal physiology, and the reported results are based on the best available precursor pools and theoretically are the most accurate ones.

In the current study, the effect of a 4-h insulin infusion on muscle protein breakdown and synthesis was determined. It remains to be determined whether insulin effect on muscle protein metabolism is time dependent. The current approach only measured the effect of insulin on average changes on synthesis and breakdown of several muscle proteins. Previous studies have shown that insulin effect on protein synthesis is not only tissue specific but also protein specific (11). Insulin has been shown to stimulate muscle mitochondrial protein synthesis with no significant effect on myosin heavy chain (37). Insulin also stimulates muscle protein synthesis when amino acid levels are high (32). Currently, it remains to be determined whether insulin effect on muscle protein breakdown is specific to any protein or proteins.

In conclusion, we demonstrated that intra-arterial insulin infusion into healthy human subjects that increased insulin to normal physiological postprandial levels did not significantly increase muscle protein synthesis but significantly suppressed muscle protein breakdown. This study used the amino-acyl tRNA as the obligate precursor pool for protein synthesis, and the protein breakdown was calculated based on mass balance of tracee amino acids. It is reassuring that the conclusions are supported by the results derived from theoretically valid surrogate measures of aminoacyl tRNA and intracellular pool. Moreover, the conclusion is in agreement using two different tracers of amino acids. We conclude normal physiological levels of insulin achieve muscle protein anabolism by inhibiting muscle protein breakdown. This conclusion does not contradict results demonstrating stimulatory effect of insulin on muscle protein synthesis when amino acid levels are elevated or when supraphysiological doses of insulin are administered.

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