The Hyperinsulinemic Amino Acid Clamp Increases Whole-Body Protein Synthesis in Young Subjects

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We propose that hyperinsulinemia stimulates protein synthesis when postabsorptive plasma amino acid (AA) concentrations are maintained. During a euglycemic hyperinsulinemic clamp, many AA, notably the branched-chain amino acids (BCAA), decline markedly. Therefore, we tested whether individual plasma AA could be maintained within the range of postabsorptive concentrations to assess the effects of insulin, infused at 40 mU/m^2 · min on whole-body protein and glucose metabolism, using [1-^{13}C]-leucine and [3-^{3}H]-glucose methodology. Validation studies of background [^{13}C] enrichment and breath $^{13}CO_2$ recovery factors were performed in a subset of 6 subjects. In 10 healthy, young men, infusion rates of an AA solution were based on fluorometric determinations of total BCAA every 5 minutes. All 21 plasma AA remained in the target range; 15, including the BCAA, alanine, and glycine were within 13% of baseline, and only 6 (Thr, His, Arg, Asn, Cit, Tyr) varied more (18% to 42%). Notably, both leucine flux and nonoxidative leucine R_d (protein synthesis) increased with insulin (2.36 ± 0.06 to 2.81 ± 0.10 and 1.79 ± 0.05 to 2.18 ± 0.10 μmol/kg fat-free mass (FFM) · min, respectively; $P < .0005$) while leucine oxidation only tended to increase ($P = .05$) and endogenous leucine R_a (protein breakdown) decreased by 18% (2.36 ± 0.06 to 1.94 ± 0.09 μmol/kg FFM · min; $P < .0005$), resulting in a marked elevation of net protein synthesis (−0.57 ± 0.02 to 0.24 ± 0.02 μmol/kg FFM · min; $P < .0000001$). Thus, in vivo protein anabolism was induced when maintaining postabsorptive plasma amino acid concentrations during hyperinsulinemia through a suppression of whole-body protein breakdown, no significant change in oxidation and an elevation of synthesis compared with postabsorptive conditions.
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IT HAS LONG been recognized that insulin is a key hormone in the regulation of not only glucose and lipid metabolism, but protein as well.[1] Indeed, studies indicate that by reducing endogenous proteolysis, insulin plays a role in meal-induced protein anabolism.[2-5] Although in vitro[6,7] and in vivo[8] studies in skeletal muscle show that insulin can stimulate protein synthesis, studies measuring whole body-protein kinetics suggest that the primary effect of insulin is to restrain proteolysis,[9,10] while its role on protein synthesis is less clear. The effects of insulin are usually studied during a hyperinsulinemic euglycemic clamp, which causes a decrease in concentrations of most plasma amino acids (AA).[9,11-13] Leucine and the other 2 branched-chain amino acids (BCAA), isoleucine and valine, are among those most sensitive to insulin[14,15] and are important determinants of protein metabolism. Studies of protein turnover during hyperinsulinemia with associated hypoaminoacidemia have reported either unchanged[16] or decreased whole-body protein synthesis,[9,17,18] possibly through reduced availability of plasma AA. Some investigators have attempted to overcome this effect by using a constant rate-intravenous infusion of a mixture of AA in conjunction with a hyperinsulinemic, euglycemic clamp, producing hyperaminoacidemia in most cases.[17-20] Under these latter conditions, protein synthesis was systematically increased. Thus, one of the major difficulties in studying the in vivo effects of insulin on amino acid metabolism is to maintain their plasma concentrations and pool sizes at baseline, which is crucial to compensate for the lowering effect of insulin.[16,19]

A spectrophotometric assay for measuring total BCAA concentrations within 3 minutes of blood withdrawal was recently reported.[21]. This assay uses leucine dehydrogenase that oxidatively deaminates leucine, isoleucine, and valine with a stoichiometric reduction of nicotinamide adenine dinucleotide (NAD). We have adapted this method for fluorimetry to establish a technique to maintain baseline concentrations of BCAA by varying infusion rates of an AA solution during a hyperinsulinemic, euglycemic clamp. We reasoned that by clamping BCAA, if the appropriate proportions of 19 AA were present in the solution infused, most of those known to be key regulators of protein metabolism might also be clamped. By controlling AA provision, this method should allow the determination of the respective roles of insulin and AA in protein turnover and of alterations in protein metabolism in humans whose conditions are associated with insulin resistance. Our data in healthy young male subjects show that glucose and the majority of plasma AA can be clamped simultaneously within the range of normal postabsorptive concentrations during hyperinsulinemia and that under these conditions, whole body-protein anabolism does occur.

MATERIALS AND METHODS

Subjects and Diets

Ten lean, healthy young men were recruited through advertisements in local newspapers, screened by medical history, physical examination, and laboratory investigation, as previously detailed.[22,23] They were admitted to the McGill University Health Centre (MUHC)-Royal Victoria Hospital's Clinical Investigation Unit (CIU) after giving written informed consent. This protocol was approved by the Human Ethics Review Committee of the Hospital. Body composition was assessed,

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Table 1. Subject Data

N	10
Age (yr)	26.5 ± 1.1
Height (cm)	178.5 ± 1.9
Weight (kg)	69.1 ± 1.9
BMI (kg/m^2)	21.7 ± 0.5
FFM (kg)	59.4 ± 1.2
Body fat (%)	13.8 ± 1.4
REE (MJ/d)	6.8 ± 0.2
Energy intake (MJ/d)	11.5 ± 0.3
Protein intake (g/kg · d)	1.5 ± 0.0
Nitrogen balance (g/d) (n = 8)	0.48 ± 0.30*

NOTE. Data are means ± SE.

Abbreviations: BMI: body mass index; FFM fat-free mass; REE: resting energy expenditure.

*Not significantly different from zero.

after an overnight fast and morning voiding, by bioelectrical impedance analysis (BIA) using the RJL-101A Systems (Detroit, MI) instrument, as described by Lukaski et al.[24]. The average of 2 measurements was used for calculation of fat-free mass (FFM) by using a sex-specific formula for estimating body composition.[25] The characteristics of the subjects are shown in Table 1.

Subjects received an isoenergetic diet, based on resting metabolic rate measured by indirect calorimetry (Deltatrac, Sensor Medics, Yorba Linda, CA) as previously described,[26] multiplied by a physical activity factor of 1.7. The diet consisted of a meal replacement liquid formula (Ensure, Abbott Laboratory, St. Laurent, QC, Canada) divided into 6 equal meals per day, given at 3 hour-intervals, in addition to a breakfast composed of 30 g bran cereal (All-Bran Cereal, Kellogg Canada, Etobicoke, ON, Canada) and 200 mL milk (2% fat). This diet provided 15% of energy as protein. All subjects consumed this diet for 7 days, 2 days at home followed by 5 days in the CIU. Nitrogen balance studies were conducted during the last 3 days of the adaptation diet, as previously described.[26]

Spectrofluorometric Assay Conditions

Leucine dehydrogenase catalyzes the oxidative deamination of the BCAA, with high specific activity, converting L-leucine, L-isoleucine and L-valine, into their respective keto analogues, α-ketoisocaproate, α-ketomethylvalerate, and α-ketovalerate. The reaction requires NAD^+ as a cofactor and produces NADH in amounts equimolar to the amount of substrate converted.[27] The production of NADH can be measured by fluorescence and quantified using a standard curve with standards of BCAA of known concentrations. The conditions determined for this assay were adapted from the spectrophotometric assay, described by Beckett et al,[21] although using a different source of enzyme.

Preparation of leucine dehydrogenase. Leucine dehydrogenase (E.C. 1.4.1.9, specific activity of 47.0 U/mg) from Bacillus cereus (Calbiochem, Calbiochem-Novabiochem, LaJolla, CA) was diluted in 25 mmol/L sodium phosphate buffer, pH 7.2, and frozen at −70°C in aliquots. Upon use, the enzyme was diluted in the same buffer containing 1 mg/mL bovine serum albumin (Sigma, Sigma Chemical, St Louis, MO).

Preparation of reagents. All the chemicals were from Fisher Chemicals (Fisher Scientific, St. Laurent, QC, Canada). Buffers were freshly prepared on the day of the study. The assay buffer was 0.1 mol/L potassium phosphate, pH 8.4, containing 2 mmol/L EDTA and 0.02% (vol/vol) mercaptoethanol. β-NAD (Roche, Roche Diagnostics, Laval, QC, Canada) was prepared in 0.1 mol/L sodium carbonate buffer, pH 10.7, at a concentration of 120 mmol/L.

Preparation of BCAA standards. Stock solutions of 10 mmol/L L-valine, L-leucine, and L-isoleucine (Sigma Chemical) in 0.01 mol/L potassium phosphate (monobasic) buffer, pH 6.0, were aliquoted and kept at −70°C until use. BCAA solutions were diluted to 1 mmol/L and standards of 0 to 250 μmol/L were prepared by mixing, in assay buffer, valine, leucine, and isoleucine in ratios of 50%, 30%, and 20%, respectively, to mimic physiologic proportions.

Fluorometric conditions. Appearance of NADH was determined at an excitation wavelength of 355 nm and emission wavelength of 485 nm on a spectrofluorometer (Turner model 430 equipped with a xenon lamp, Sequoia-Turner, Mountain View, CA). The cell chamber of the fluorometer was heated and maintained at 37°C with a circulating water system.

Enzymatic assay. The enzymatic assay contained 25 μL of standard or plasma, 4 mmol/L NAD, 0.5 U leucine dehydrogenase, and phosphate buffer containing EDTA and mercaptoethanol, for a total volume of 2 mL. After a blank reading, NADH fluorescence was recorded after exactly 4 minutes of enzyme reaction at 37°C and converted to micromoles of total BCAA using the standard curve.

Standard curve of the BCAA assay. The measurement of the change in NADH fluorescence was linear ($R = .9996$) for standards of mixed BCAA of concentrations ranging from 0 to 750 μmol/L. Repeated measures of the standards after an 8-hour clamp study showed an excellent correlation (slope 0.94, $R = .999$) with the standard curve established in the morning, indicating enzyme stability and maintenance of the assay conditions over time. This method showed an interassay variability of 4.2% for standards and 6.5% for plasma, while intra-assay variability was below 4%.

Validation of the fluorometric enzymatic assay against high-performance liquid chromatography. A significant ($P < .01$) correlation was found between plasma BCAA concentrations obtained with the enzymatic assay and those obtained with high-performance liquid chromatography (HPLC) (n = 28 samples). The slope of the relationship was 0.61, indicating an underestimation of total BCAA with the enzymatic assay. However, the Pearson's correlation coefficient of 0.8 ($P < .01$) confirmed reliability of the method for purposes of maintaining total BCAA concentrations stable during clamp studies, relative to the baseline period.

Determination of Plasma AA

Individual plasma AA was determined by ion-exchange HPLC with postcolumn ninhydrin detection[28] at the MUHC-Montreal Children's Hospital clinical laboratory. Briefly, plasma was deproteinized with sulfosalicylic acid, the supernatant was mixed with lithium buffer, pH 2.2, (Beckman 338084, Beckman Instruments, Palo Alto, CA) and filtered through a 0.2-μm filter before injection into the AA analyzer (Beckman System 6300, model GM802). Glucosaminic acid was used as internal standard. Calibration was performed using mixed standards of AA (Beckman) with glutamine, asparagines, and tryptophan prepared separately, as they are unstable on storage.

Hyperinsulinemic, Euglycemic, Isoaminoacidemic Clamp Protocol

All subjects were studied after an overnight fast. The hyperinsulinemic clamp experiment was performed with modifications to the original reported method,[29] as recently detailed by Banerji and Lebovitz[30] and Saad et al[31] and to the glucose turnover methodology to ensure precision in estimation of suppression of hepatic glucose output.[32] Target levels were 5.5 mmol/L glucose and maintenance of individual subject's basal plasma BCAA concentrations. At 8 AM, subjects had catheters inserted in an antecubital vein for infusions and in a contralateral hand vein (retrogradely) for blood sampling. The hand was placed in a heating box at 65°C to 70°C to arterialize the venous blood.[33] A primed [22 μCi (888 kBq)], continuous infusion [0.22

μCi/min (8.88 kBq/min)], of [3-^{3}H]-glucose was started 180 minutes before insulin and maintained for the duration of the clamp. A primed infusion of biosynthetic regular human insulin (Humulin R, Eli Lilly Canada, Toronto, ON, Canada) was started at 180 minutes and maintained for at least 3.5 hours at a rate of 40 mU/m^2 · min to achieve plasma insulin concentrations of 500 to 600 pmol/L. At 184 minutes, variable infusion rates of sterile 20% (wt/vol) potato starch-derived glucose (Avebe b.a., Foxhol, The Netherlands) in water with added [3-^{3}H]-glucose, (the "hot GINF" method) as specified in Finegood et al[32] was started to maintain glycemia constant and minimize changes in circulating glucose specific activity. This source of glucose was chosen because of its low natural ^{13}C content.[34] At the same time, a 10% AA mixture (10% TrophAmine without electrolytes, McGaw, Irvine, CA) was used at variable infusion rates to maintain plasma AA constant.

Measurements of arterialized plasma glucose and BCAA concentrations were performed every 5 minutes and infusion rates adjusted accordingly. Blood samples were collected for glucose, BCAA, insulin, and tritiated glucose specific activity every 10 minutes for 40 minutes before the insulin infusion, then every 30 minutes for insulin and tritiated glucose specific activity until the last 40 minutes, at which time they were again drawn at 10-minute intervals. Indirect calorimetry was performed for 20 minutes before and during the last 30 minutes of the insulin infusion, as described in Bogardus et al.[35] The nonprotein respiratory quotient was calculated using the known nitrogen excretion rates for the day previous to the test. Calculations of nonoxidative and oxidative components of glucose disposal were as in Bogardus et al.[35] Methods for the individual assays are as detailed in Finegood et al[32] and Sigal et al[36] for immunoreactive insulin and glucagon, and glucose turnover. Plasma urea was determined by the hospital clinical laboratory. Analyses of the clamp data were as specified in Saad et al[31] and Bogardus et al.[35]

Leucine turnover studies during the hyperinsulinemic clamp were performed as detailed in Pacy et al,[4] Lariviere et al,[37] and Matthews et al.[38] An oral bolus of 0.1 mg/kg of Na$H^{13}CO_2$ and an intravenous bolus of 0.5 mg/kg of [1-^{13}C]-leucine was followed by a constant infusion rate of 0.008 mg/kg · min of [1-^{13}C]-leucine in parallel with the tritiated glucose. Blood samples were collected every 10 minutes for 40 minutes before the insulin infusion, then every 30 minutes up to the last 40 minutes, at which time they were again drawn at 10-minute intervals. Expired air samples were collected into a breath collection balloon, transferred to 10 mL Vacutainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and stored at room temperature until analyzed for $^{13}CO_2$ enrichment by isotope ratio mass spectrometry on a Micromass 903D (Vacuum Generators, Winsforce, United Kingdom). Leucine kinetics were calculated according to the stochastic model of Matthews et al,[38] using ketoisoaproic acid (KIC) as an index of the precursor pool enrichment (reciprocal model). The [^{13}C]enrichment of KIC was analyzed by gas chromatography-mass spectrometry on a Hewlett Packard 5890 after derivatization with quinoxalitol tert-butyldimethylsilyl ether derivative.[39] During the AA infusion, leucine kinetics were calculated using the average rate of leucine infusion during the last 40 minutes to give exact measurements of leucine R_a and R_d.[40] Recovery factors, determined under our conditions (described in [^{13}C]bicarbonate recovery protocol) were used to correct for the fraction of $^{13}CO_2$ produced by oxidation of [^{13}C]leucine and not expired in breath, during the postabsorptive and clamped states.

The selection of a commercial AA solution was made on the following basis: that it had high proportions of BCAA and sufficient amounts of those essential AA known to be most sensitive to insulin,[13] moderate proportions of others likely to be associated with the smallest possible changes in the circulation, and low proportions of the nonessentials whose levels might increase with hyperinsulinemic glucose clamping. None had glutamine or asparagine, as these are unstable in solution, so their plasma concentrations reflected the changes in endogenous levels produced by the clamp. After study of composition of 7 possible candidate solutions, results of pilot studies (not shown) led to the selection of the brand used in this report, based upon analysis of individual plasma AA. The initial rate of amino acid infusion was set at 0.004 mL/kg FFM · min. The amino acid mixture contained: isoleucine 0.82 g; leucine 1.4 g; lysine 0.82 g (added as lysine acetate 1.2 g); methionine 0.34 g; phenylalanine 0.48 g; threonine 0.42 g; tryptophan 0.20 g; valine 0.78 g; cysteine <0.016 g (as cysteine HCl H_2O <0.024 g); histidine 0.48 g; tyrosine 0.24 g (as tyrosine 0.044 g and N-acetyl-L-tyrosine 0.24 g); alanine 0.54 g; arginine 1.2 g; proline 0.68 g; serine 0.38 g; glycine 0.36 g; L-aspartic acid 0.32 g; L-glutamic acid 0.50 g; taurine 0.025 g, all the foregoing per 100 mL; and sodium 5 mEq; chloride <3 mEq, and acetate 97 mEq/L.

Background [^{13}C] Enrichment of Plasma KIC and Expired CO_2 Validation Studies

Because the glucose and AA solutions used in our clamp protocol possibly have a different natural [^{13}C] enrichment than that of the body and could therefore affect the background isotopic enrichment, we conducted validation studies to verify this and correct for a potential effect. With the use of potato starch–derived glucose with a low [^{13}C] content, a dilution effect of $^{13}CO_2$ was most expected. Six young healthy men, of whom 3 were also part of the clamp study group, were studied under exactly the same conditions as in the clamp protocol described above, including the prior formula diet given for 4 days, with the exception that they did not receive any tracers.

[^{13}C] Bicarbonate Recovery Protocol

Published recovery factors of expired $^{13}CO_2$ were determined for the fasted and fed states only, and hence not validated to our clamp conditions of euglycemia and isoaminoacidemia. Moreover, any possible dilution effect of background $^{13}CO_2$ from the infusates would also have to be considered in the calculations of the recovery factors. Therefore, [^{13}C] bicarbonate recovery studies were conducted in 6 young healthy men (2 of whom were also part of the clamp and background [^{13}C] enrichment study groups) according to the methodology and calculations described by El-Khoury[41] to account for the proportion of ^{13}C retained in the bicarbonate pool under the present experimental conditions. Subjects underwent the same clamp protocol as described above, without the other tracer infusions, but received a primed, continuous infusion of [^{13}C] bicarbonate instead. On average, these 2 groups for validation and recovery studies did not differ in age, weight, body mass index (BMI), glucose and amino acid infusion rates from the study group.

Statistical Analysis

Data are presented as means ± SEM. Individual results between baseline and clamp periods were compared by repeated-measures analysis of variance (ANOVA) and means between baseline and clamp periods by paired *t* tests, with significance level less than .05. Results presented in tables under "basal" correspond to a mean of 2 measurements performed at −40 minutes and 0 minutes before insulin infusion, and "clamp" period represents a mean of 4 measurements made during each subject's steady-state period. Pearson's coefficient was used to correlate BCAA levels measured by fluorometry to those measured by HPLC.

RESULTS

Weight was maintained with the isoenergetic diet and nitrogen balance was at equilibrium with the dietary energy and protein provided (Table 1). No untoward effects were experienced by any subject.

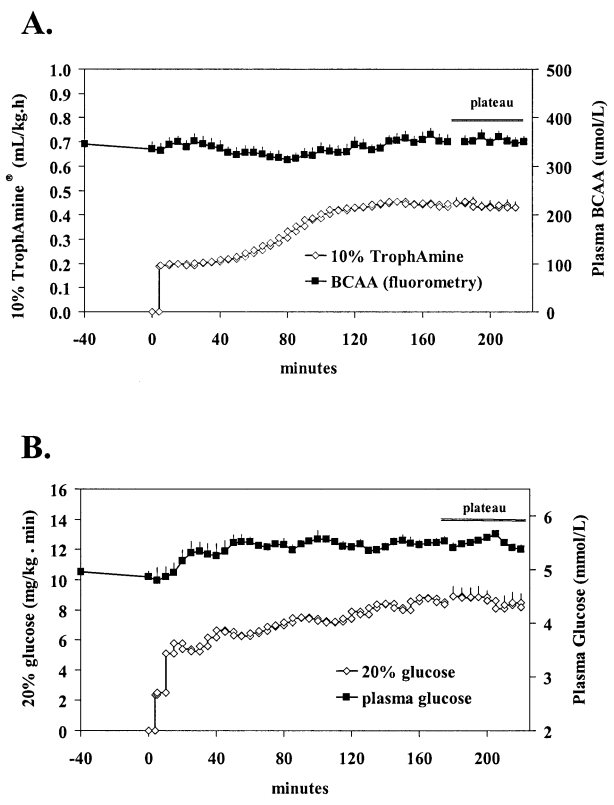

Fig 1. (A) Plasma BCAA concentrations and AA infusion rates during hyperinsulinemia. Data are mean ± SEM for 10 subjects. The BCAA were measured by the enzymatic fluorometric assay described in Materials and Methods. Insulin infusion was started at 180 minutes. (B) Plasma glucose and glucose infusion rates during the hyperinsulinemic clamp. In both graphs, "plateau" defines the last 40 minutes of steady-state for each individual.

Plasma BCAA and AA Solution Infusion Rates

Plasma concentrations of BCAA were maintained throughout the study, as illustrated in Fig 1A, by feedback adjustments of the infusion rate of AA solution, based on 5-minute BCAA determinations by enzymatic assay. The coefficient of variation of plasma BCAA concentrations was 4.1% for the last 30 minutes of the insulin infusion and the mean concentration of this plateau was not significantly different from that measured during the baseline period, whether measured by fluorometry or HPLC (Fig 1A and Table 2). The infusion rates of the AA solution were increased gradually up to about 90 minutes after the insulin infusion was initiated and remained stable thereafter at 0.71 ± 0.01 mg of total AA/kg · min or 1.65 ± 0.04 μmol of total BCAA/kg · min. The coefficient of variation at plateau, a time when calculations of leucine turnover are done, was 3.6%. Thus, both AA solution infusion rates and plasma BCAA concentrations were at a steady-state for the plateau period of the study.

Plasma Glucose, Infusion Rates, and Kinetics

After initiation of insulin infusion, euglycemia was maintained for all the subjects at 5.51 ± 0.01 mmol/L with concurrent infusion of the 20% glucose solution (Fig 1B). Glucose infusion rates reached a steady-state of 9.75 ± 0.63 mg/kg FFM · min (coefficient of variation [CV] of 9.1%) for the last 30 minutes of study, an essential condition for glucose turnover calculations. As shown in Table 2, glucose R_a and R_d were equal at baseline. With hyperinsulinemia, endogenous glucose production was completely suppressed, and glucose uptake was significantly increased. Both oxidative and nonoxidative glucose disposal increased significantly during hyperinsulinemic euglycemic clamp. The target level of hyperinsulinemia was achieved (553 ± 27 pmol/L) during plateau. Plasma glucagon decreased minimally, but significantly, from baseline during the clamp.

Individual Plasma AA During Clamp Studies

Using BCAA measurements as indicators with the aim of achieving stable levels of all AA, most essential and nonessential AA concentrations were maintained at values within the normal fasting range, with the AA solution used (Table 3). Due to remarkably little intersubject variability in responses, slight, but statistically significant, increases were seen in isoleucine, leucine, and phenylalanine and decreases in threonine, alanine, glutamine, and serine. However, with the exception of histidine, threonine, arginine, asparagine, citrulline, and tyrosine, the changes observed in plasma concentrations were less than 13%. The total of the 3 BCAA concentrations was not different from baseline to clamp period (Table 2), nor was the sum of all the individual AA concentrations (Table 3).

Table 2. Amino Acid and Glucose Infusion Rates and Metabolic Responses to Hyperinsulinemia

	Basal	Clamp
Plasma BCAA (μmol/L)	404.5 ± 14.0	411.2 ± 12.5
Infusion rates		
Total amino acid (mg/kg · min)	—	0.71 ± 0.01
BCAA (μmol/kg · min)	—	1.65 ± 0.04
Plasma glucose (mmol/L)	4.92 ± 0.07	5.51 ± 0.01*
20% glucose infusion rate (mg/kg FFM · min)	—	9.75 ± 0.63
Glucose R_a (mg/kg FFM · min)	2.39 ± 0.08	−0.28 ± 0.09*†
Glucose R_d (mg/kg FFM · min)	2.39 ± 0.08	9.34 ± 0.61*
Oxidative glucose R_d (mg/kg FFM · min)	1.39 ± 0.15	2.97 ± 0.21*
Nonoxidative glucose R_d (mg/kg · min)	0.97 ± 0.17	6.34 ± 0.61*
Plasma insulin (pmol/L)	62.4 ± 2.7	552.9 ± 26.5*
Plasma glucagon (pmol/L)	12.5 ± 0.9	9.9 ± 0.5‡
Plasma glucagon/insulin ratio	0.20 ± 0.01	0.02 ± 0.00*

NOTE. Data are means ± SEM for n = 10 subjects. Basal: mean of 2 measurements/subject before insulin infusion, clamp: mean of 4 measurements/subject in the last 30-minute period of clamp study. BCAA were analyzed by HPLC.

Abbreviations: R_a, rate of appearance; R_d, rate of disappearance.

*$P < .0001$ *v* basal; †not different from zero; ‡$P < .01$ *v* basal.

Table 3. Individual Plasma AA in the Postabsorbtive State and During Clamp Studies

	Basal (μmol/L)	Clamp (μmol/L)	% Change
Essential AA			
Isoleucine	54.1 ± 2.7	59.3 ± 2.7*	10.0 ± 3.1
Leucine	129.4 ± 4.3	145.6 ± 4.8*	12.9 ± 3.0
Valine	219.9 ± 7.7	207.0 ± 6.3	−5.3 ± 2.8
Tryptophan	59.0 ± 3.1	55.4 ± 3.5	−5.6 ± 5.3
Methionine	23.7 ± 0.9	24.9 ± 0.7	6.3 ± 3.9
Phenylalanine	49.8 ± 1.3	52.9 ± 1.4*	6.4 ± 2.2
Lysine	181.7 ± 10.5	191.4 ± 6.5	6.9 ± 3.5
Threonine	130.9 ± 7.1	107.5 ± 4.4*	−17.1 ± 2.4
Histidine	82.7 ± 5.2	102.1 ± 4.8†	25.1 ± 3.7
Nonessential AA			
Glycine	226.6 ± 5.1	233.7 ± 5.4	3.5 ± 2.5
Glutamic acid	60.3 ± 5.8	55.0 ± 4.1	−6.9 ± 3.9
Proline	188.2 ± 15.7	171.1 ± 11.1	−7.3 ± 4.1
Taurine	42.8 ± 2.2	38.6 ± 1.3*	−8.6 ± 3.5
Ornithine	58.3 ± 4.0	52.1 ± 1.6	−8.5 ± 4.2
Alanine	373.8 ± 14.6	330.1 ± 12.2*	−11.1 ± 4.4
Glutamine	537.7 ± 33.4	471.6 ± 14.8*	−10.6 ± 3.4
Serine	100.1 ± 5.3	87.3 ± 2.9*	−11.9 ± 2.4
Citrulline	33.6 ± 2.1	24.5 ± 2.1*	−26.2 ± 6.3
Tyrosine	52.4 ± 2.1	35.2 ± 2.1†	−33.0 ± 2.1
Asparagine	41.4 ± 1.8	26.6 ± 0.9†	−35.4 ± 1.5
Arginine	68.8 ± 7.0	92.6 ± 4.5†	41.6 ± 8.8
Total of AA	2,715.0 ± 89.8	2,564.2 ± 39.6	−4.9 ± 2.5

NOTE. Data are mean ± SEM for n = 10 subjects. Basal: mean of 2 measurements/subject before insulin infusion, clamp: mean of 4 measurements/subject in the last 30-minute period of clamp study. Measurements were made by HPLC analysis as described in Materials and Methods.

*$P < .05$; †$P < .001$ *v* basal.

Leucine Kinetics During Clamp Studies

[^{13}C] enrichment of plasma KIC decreased significantly as a result of insulin, glucose, and AA infusions, while [^{13}C] enrichment of expired CO_2 was not significantly changed, as illustrated in Fig 2. However, the additional studies on background $^{13}CO_2$ enrichment (Fig 3A) revealed a dilution effect (−10.1% ± 1.6%) of the combined glucose and AA infusions. Therefore, this average was taken as a correction factor for the calculation of leucine oxidation rates during the hyperinsulinemic clamp studies. In contrast, the background [^{13}C] enrichment of plasma KIC (Fig 3B) was not affected. The dilution effect of background $^{13}CO_2$ enrichment during the clamp period was also accounted for in the calculation of the $^{13}CO_2$ recovery factor obtained from the [^{13}C] bicarbonate recovery studies, which was 0.78, while that of the postabsorptive period was 0.67.

Leucine kinetics were calculated when both ^{13}KIC and $^{13}CO_2$ enrichments reached a steady state during the postabsorptive state (CV of 3.3% and 3.4%, respectively) and during the hyperinsulinemic clamp (CV of 4.8% and 3.2%, respectively). As shown in Table 4, whether expressed per kilogram of body weight or FFM, leucine flux was significantly increased during hyperinsulinemia with maintenance of basal plasma AA. Endogenous leucine R_a, a surrogate for protein breakdown, was

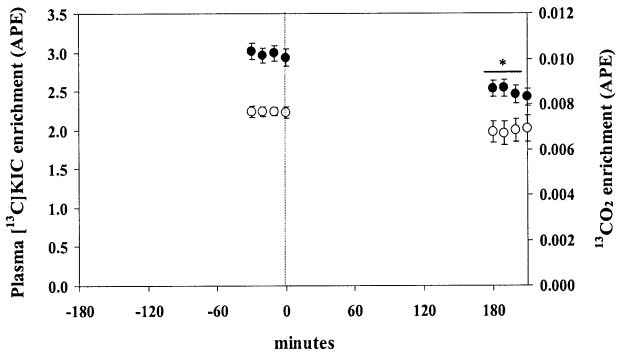

Fig 2. [^{13}C]-enrichment of plasma KIC and expired CO_2. Data are means ± SEM for 10 subjects. [^{13}C]-enrichment of plasma KIC (●) and expired CO_2 (○) were measured on samples collected during steady-states before infusions of insulin (shown by the dashed line), glucose and TrophAmine, and after 180 minutes of these infusions.

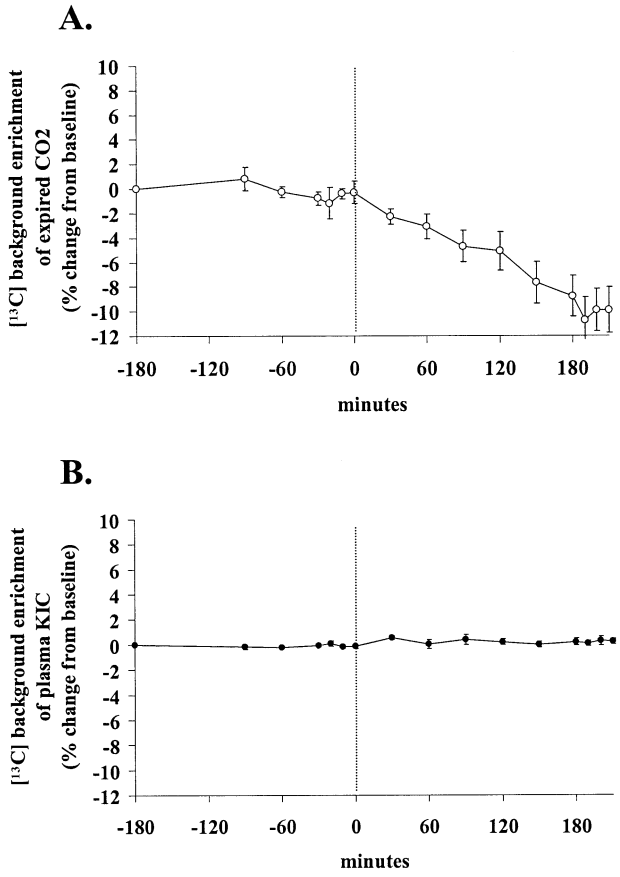

Fig 3. (A) Effect of the hyperinsulinemic, euglycemic, isoamino acid clamp on background [^{13}C] enrichment of expired CO_2. (B) Effect of the hyperinsulinemic, euglycemic, isoamino acid clamp on background ^{13}C enrichment of plasma KIC. Data are mean ± SEM for 6 subjects. These validation experiments were conducted under the same conditions as those of the hyperinsulinemic clamp studies described in Materials and Methods, except that the subjects did not receive any tracers (^{13}C-leucine and ^{3}H-glucose).

Table 4. Leucine Kinetics in the Postabsorbtive State and During Clamp Studies

	Basal (μmol/kg FFM · min)	Clamp (μmol/kg FFM · min)
Total R_a (flux)	2.36 ± 0.06	2.81 ± 0.10*
Oxidation	0.57 ± 0.02	0.64 ± 0.03
Endogenous R_a (protein breakdown)	2.36 ± 0.06	1.94 ± 0.09*
Exogenous R_a (leucine infusion)	—	0.91 ± 0.03
Nonoxidative R_d (protein synthesis)	1.79 ± 0.05	2.18 ± 0.10*
Nonoxidative R_d-R_a (net protein synthesis)	−0.57 ± 0.02	0.24 ± 0.02†

NOTE. Data are means ± SE for n = 10 subjects.
Abbreviation: R_a, rate of appearance.
*$P < .0005$ *v* basal; †$P < .00000001$ *v* basal.

significantly reduced by 18%. Even if leucine oxidation tended to increase ($P = .05$), nonoxidative leucine disposal, an index of protein synthesis increased significantly by 22%, resulting in a marked elevation of net whole-body protein retention with reversal from negative to positive values.

DISCUSSION

Demonstrating a role for insulin in regulating in vivo protein synthesis has been a considerable challenge, whereas its ability in suppressing protein breakdown is better established. Indeed, most studies of leucine kinetics in healthy subjects during hyperinsulinemia reported inhibition of protein breakdown by insulin. This has been observed when plasma concentrations of leucine were maintained as in the present study,[10,16,17,42-44] with hyperaminoacidemia,[17-20,45] and in the presence of hypoaminoacidemia.[9,11,12,18] In studies in which AA were infused together with the insulin, protein breakdown was suppressed further than with insulin alone, suggesting additive roles of insulin and AA.[16-19]

To our knowledge, the present study, using [^{13}C]-leucine methodology validated under our specific conditions, is the first to report a significant increase in whole-body protein synthesis with hyperinsulinemia, while maintaining most plasma AA in the range of normal postabsorptive values. The glucose and AA clamp technique, first developed by Abumrad et al,[16,42,46] was devised to compensate for the confounding reduction in plasma AA with insulin administration due to its suppressive effect on protein breakdown.[13,18,19] Our results show that in order to maintain most postaborptive plasma AA levels during the clamp, the exogenous AA had to be infused at a rate greater than the decrease in protein breakdown. Specifically, the mean infusion rate of leucine (0.91 μmol/FFM · min) was higher than the decrease of endogenous leucine R_a (0.42 μmol/FFM · min). Because leucine oxidation increased so little (if at all), the requirement for an exogenous leucine infusion rate beyond a replacement level had to be triggered by an increase in protein synthesis. Moreover, the validation studies designed to assess background [^{13}C]enrichment and $^{13}CO_2$ recovery factors gave us the opportunity to compare the within-subject variability of the AA infusion rates, and these were found to be highly reproducible. Thus, by effectively clamping plasma AA, the actual rate of AA infusion itself is quantitatively and physiologically meaningful.

Increases in in vivo whole body-protein synthesis in adult human subjects were observed in studies combining hyperinsulinemia with hyperaminoacidemia[17-20,45] and in hyperaminoacidemia with basal insulin.[17,19] In these cases, the increment in circulating AA not only promoted protein synthesis, but markedly increased oxidation, as well. Because protein synthesis is calculated by subtracting leucine oxidation from total flux, an accurate measure of $^{13}CO_2$ enrichment is critical, as is the $^{13}CO_2$ recovery factor for the hyperinsulinemic state. For this reason, we performed validation studies to control for the effect of the level of [^{13}C] in the infused solutions upon $^{13}CO_2$. The exogenous glucose contribution to the [^{13}C] pool was minimized by our use of low [^{13}C]-content potato starch as the source, instead of corn-derived glucose (with its higher [^{13}C] content), thus diluting the [^{13}C] pool rather than contaminating it. Not correcting for this dilution effect would have led to an underestimation of leucine oxidation rates and inversely, an overestimation of protein synthesis. We also defined the appropriate $^{13}CO_2$ recovery factors for our study protocol, according to the subjects' physiologic state and our experimental conditions, including the dilution effect on background $^{13}CO_2$ enrichment during the clamp period, as the factors previously available were determined during a fed state and at different plasma insulin levels.[47] The recovery factors obtained in the present study, 0.67 and 0.78 for the postabsorptive and clamp states, respectively, were very similar to those of Hoerr et al[47] (0.70 and 0.82). We calculated oxidation rates with these appropriate recovery factors and corrected for the dilution effect and found that protein synthesis rates are increased by 22% during the hyperinsulinemic clamp. Although we did not study leucine kinetics at different insulin and/or AA concentrations, this increase in synthesis must depend largely on the elevation in plasma insulin concentrations, provided that AA availability was maintained. These in vivo whole-body results are in accordance with in vitro data showing the coregulation of AA sufficiency and insulin in the stimulation of the initiation of mRNA translation within the cell.[48]

A single explanation for the different protein synthesis results of prior hyperinsulinemic, AA clamp studies in healthy subjects[10,16,17,43] and that of the present study is not apparent. This discrepancy may be contributed to by the higher statistical power resulting from studying 10 men, more than in the other studies, performed in a maximum of 5 or 6 subjects of the same gender. The homogeneity of our group probably contributed, as they had comparable BMI, % fat and FFM, and age, all factors implicated in insulin resistance. Furthermore, during the week before the clamp experiments, our subjects consumed a tightly controlled isoenergetic diet, rich in simple carbohydrates (from the liquid formula Ensure). It is recommended that a high carbohydrate intake be taken for 3 days before an oral glucose tolerance test,[49] as this leads to the best glucose tolerance for each individual by optimizing both insulin secretion and sensitivity.[50] Through the dietary control before the clamp, insulin sensitivity of both glucose and protein may thus have been maximized. Our subjects were adapted to a generous protein intake for the week before the leucine turnover experiments

that maintained their nitrogen balances at equilibrium. Protein intake per kilogram of FFM (1.82 ± 0.04 g/kg FFM · d) was similar for all subjects. This dietary strategy was chosen to stabilize protein balance and may have allowed detection of changes in leucine kinetics otherwise masked by an intersubject variability in prior protein intake, or in responses to insulin.

The AA concentrations achieved in our study were more stable than in previously reported studies of similar hyperinsulinemia that measured plasma leucine only and used different AA solutions. For example, at a similar insulin infusion rate as ours and using Travasol (Baxter, Toronto, Canada), substantial increases (40% and more) were reported for isoleucine, methionine, lysine, phenylalanine, histidine, as well as elevations of 100% and more in alanine and glycine.[10,17] With Vamin (Kabi Pharmacia, Milton-Keynes, UK), major increments were seen for methionine, phenylalanine, histidine, glycine, arginine, lysine, and serine.[44] Taking into consideration the physiologic effects of single AA, like the suppression of hepatic protein breakdown by alanine,[51] we felt it was important to minimize any imbalances in proportions of individual plasma AA concentrations that could occur even if the BCAA were clamped and the total of AA was unchanged. Thus, the TrophAmine solution appeared to provide the most appropriate composition for our purpose, compared with other preparations tested. With a higher proportion of essential AA, relatively less solution was needed to maintain plasma BCAA concentrations, therefore avoiding excesses in other AA. This is evidenced by most plasma AA having been maintained in the normal range of postabsorptive concentrations (as defined by combining postabsorptive ranges reported from similar studies in young adults[10,16,17,43]) during hyperinsulinemia. Of particular interest is that changes in the individual BCAA concentrations and those AA (histidine, arginine, asparagine, citrulline, and tyrosine) that showed greater proportional increases or decreases (ranging from 24% to 42%) were not statistically significantly correlated with any changes in leucine kinetic measurements (leucine flux, oxidation, protein synthesis, or breakdown).

As in most of the published studies in this area, we used plasma [^{13}C]-KIC (reciprocal pool model) as the preferred surrogate of intracellular leucine metabolism for the estimation of whole body protein turnover due to easier access of arterialized venous blood as opposed to tissue biopsies. Plasma [^{13}C]-KIC enrichment has been reported to best reflect cellular leucine enrichment in the postabsorptive state and during isotopic steady state.[52] But its validity as such an index, to estimate whole-body protein turnover in contrast to that of specific tissues, has never been assessed under conditions of hyperinsulinemia and exogenous supply of AA at a maintenance level. It is however probable that the enrichment of the plasma precursor under our conditions better reflects that of the intracellular leucine than in conditions of hyperaminoacidemia. This view is supported by a study reporting an overestimation of muscle protein synthesis with increased plasma concentrations of the tracee in dogs during hyperinsulinemia. In contrast, when AA were infused to maintain postabsorptive concentrations (with the same solution as the one we used, but a different tracer, phenylalanine), the estimation of protein synthesis was not different whether it was calculated from the enrichment of plasma phenylalanine, tissue fluid, or phenylalanyl-tRNA.[53]

The ability of insulin to stimulate protein synthesis is specific to organs, tissues, and protein fractions.[8,54,55] For instance, leg protein synthesis was increased by insulin combined with basal amino acid infusion, while splanchnic synthesis increased only with hyperaminoacidemia.[8] Boirie et al[55] demonstrated stimulation of muscle mitochondrial protein synthesis by insulin, but no effect on sarcoplasmic and myosin heavy chain protein synthesis in a swine model. In liver, fractional synthetic rates of mitochondrial and cytoplasmic proteins decreased with infusion of insulin with or without AA. This specificity of the action of insulin on different tissues makes it difficult to distinguish its effect on synthesis when it is estimated from the whole-body leucine kinetics. That we were able to show such an effect in 10 young healthy men, with a relatively noninvasive technique, suggests that our procedure should be sensitive enough to show alterations of protein metabolism in subjects characterized by insulin resistance. This technique may also be useful to demonstrate effects of gender, age, disease, dietary, and/or pharmaceutical treatments, as well.

In summary, we found increased protein synthesis with hyperinsulinemia during mixed AA infusion whose goal was to maintain postaborptive levels. This was made possible through 5-minute adjustments in the infusion rates of a commercial AA solution with a near optimal composition. Achieving a steady state in AA concentrations and infusion rates with hyperinsulinemia is as crucial for the study of protein metabolism as is the maintenance of euglycemia and rates of glucose infusion to study glucose metabolism and its degree of sensitivity to insulin. Under such conditions, hyperinsulinemia was associated with net protein anabolism through both suppression of protein breakdown and increased protein synthesis, which was also reflected by a need for greater rate of AA infusion than what would be required to correct for the decrease in protein breakdown. It remains to be investigated whether these findings are different in insulin-resistant states, including type 2 diabetes mellitus, in which protein metabolism is altered, as evidenced by negative nitrogen balance and elevated rates of protein turnover during hyperglycemia.[26,56]

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