Effect of Insulin and Plasma Amino Acid Concentrations on Leucine Metabolism in Man

Role of Substrate Availability on Estimates of Whole Body Protein Synthesis

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Abstract

We examined the effect of insulin and plasma amino acid concentrations on leucine kinetics in 15 healthy volunteers (age 22±2 yr) using the euglycemic insulin clamp technique and an infusion of [1-^{14}C]leucine. Four different experimental conditions were examined: (*a*) study one, high insulin with reduced plasma amino acid concentrations; (*b*) study two, high insulin with maintenance of basal plasma amino acid concentrations; (*c*) study three, high insulin with elevated plasma amino acid concentrations; and (*d*) study four, basal insulin with elevated plasma amino acid concentrations. Data were analyzed using both the plasma leucine and alpha-ketoisocaproate (the alpha-ketoacid of leucine) specific activities.

In study one total leucine flux, leucine oxidation, and nonoxidative leucine disposal (an index of whole body protein synthesis) all decreased ($P < 0.01$) regardless of the isotope model utilized. In study two leucine flux did not change, while leucine oxidation increased ($P < 0.01$) and nonoxidative leucine disposal was maintained at the basal rate; endogenous leucine flux (an index of whole body protein degradation) decreased ($P < 0.01$). In study three total leucine flux, leucine oxidation, and nonoxidative leucine disposal all increased significantly ($P < 0.01$). In study four total leucine flux, leucine oxidation, and nonoxidative leucine disposal all increased ($P < 0.001$), while endogenous leucine flux decreased ($P < 0.001$).

We conclude that: (*a*) hyperinsulinemia alone decreases plasma leucine concentration and inhibits endogenous leucine flux (protein breakdown), leucine oxidation, and nonoxidative leucine disposal (protein synthesis); (*b*) hyperaminoacidemia, whether in combination with hyperinsulinemia or with maintained basal insulin levels decreases endogenous leucine flux and stimulates both leucine oxidation and nonoxidative leucine disposal.

Introduction

In vitro studies have demonstrated that insulin enhances amino acid uptake (1, 2) and stimulates protein synthesis (3, 4) by muscle. Moreover, many investigators have shown that insulin exerts an antiproteolytic action on both muscle (5) and liver (6). These in vitro data provide strong evidence that insulin plays an important anabolic role in the physiologic regulation of protein synthesis/degradation and amino acid metabolism. However, similar effects have been difficult to demonstrate in vivo. In type 1 diabetic subjects, insulin withdrawal is associated with a negative nitrogen balance (7), an increase in the plasma concentrations of the branched chain amino acids (leucine, isoleucine, and valine), and an increase in total leucine flux and leucine oxidation (8, 9). All of these metabolic disturbances are reversible with appropriate insulin treatment (10). In normal subjects, insulin induces a dose-related decrease in leucine flux and a marked decrease in the estimated rate of protein synthesis (11, 12).

The discrepancy between in vivo (11, 12) and in vitro (1–4) results may be explained in part by the well known amino acid lowering effect of insulin that occurs in the intact organism (13). Thus, the resultant hypoaminoacidemia may lead to limitation in substrate availability for protein synthesis and antagonize a protein anabolic effect of insulin in vivo. This hypothesis is supported by the studies from O'Keefe et al. (14) in postsurgical patients and from Nissen et al. (15) in dogs. These investigators demonstrated an anabolic effect on protein metabolism only in the presence of increased plasma insulin concentration and maintainance of plasma amino acid concentrations. The present study was undertaken to examine the separate effects of insulin and plasma amino acid concentrations on leucine metabolism in normal subjects.

Methods

Subjects. 15 healthy volunteers participated in the four experimental protocols. All subjects were within 20% of their ideal body weight (mean±SEM = 100±2%) (based upon the midpoint for medium frame individuals from the Metropolitan Life Insurance Tables, 1959). Their weight and height were 72±3 kg and 178±4 cm, respectively. Subjects ranged in age from 18 to 39 yr (mean = 22±2 yr). There were 10 males and 5 females. No subject had any history of renal, cardiovascular, or endocrine disease and all were consuming a weight-maintaining diet containing at least 250 g of carbohydrate/d and between 80 to 120 g of protein/d for at least 3 d before each study.

The purpose and potential risks of the study were explained to all subjects, and their voluntary written consent was obtained before their participation. The study protocol was reviewed and approved by the Human Investigation Committee of the Yale University School of Medicine.

Experimental protocol (Fig. 1). All tests were performed in the postabsorptive state beginning at 0800 hours following a 12-h overnight fast. A polyethylene catheter was inserted into an antecubital vein for the infusion of all test substances. A second catheter was placed retrogradely into a wrist vein for blood sampling. The hand was kept in a heated box at 70°C to ensure arterialization of the venous blood.

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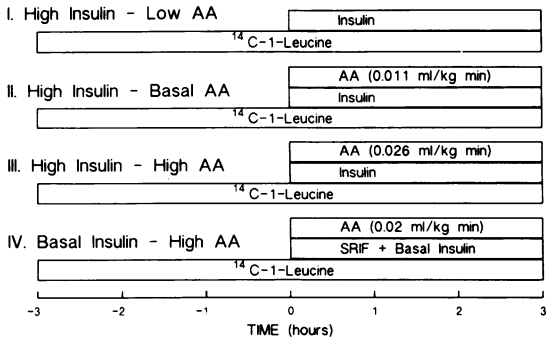

Figure 1. Study protocol. [^{14}C]-1-Leucine infusion was started 3 h before the initiation of insulin and/or amino acid infusion and continued for 3 h thereafter.

In each study protocol a primed-continuous infusion of [1-^{14}C]-leucine was administered (8 μCi bolus followed by 0.09 μCi/min) in combination with a priming dose of [^{14}C]bicarbonate (1.8 μCi). After 2 h of isotope equilibration, samples were drawn from 120 to 180 min for basal leucine and alpha-ketoisocaproic specific activities, and plasma hormone and substrate determinations. Expired air samples were collected at 15-min intervals and bubbled through a carbon dioxide trapping solution (hyamine hydroxide/absolute ethanol: 0.1% phenolphthalein 3:5:1). The solution was titrated to trap 1 mmol of CO_2 per 3 ml of solution. The ^{14}C radioactivity was subsequently determined using a tricarb scintillation counter (model 320, Packard Instrument Co., Downers Grove, IL) and the expired $^{14}CO_2$ specific activity calculated. Total $^{14}CO_2$ expired per minute was determined by multiplying the $^{14}CO_2$ specific activity by the total CO_2 production rate determined as described below.

At the end of the 3-h basal period one of the following study protocols was performed.

Study one, insulin clamp. Eight subjects were studied in this protocol. After the 180-min equilibration period, a prime-continuous infusion of crystalline porcine insulin (Eli Lilly & Co., Indianapolis, IN) was administered at the rate of 40 mU/m2 · min for an additional 180 min to achieve and maintain an increment in plasma insulin concentration of ~ 80 μU/ml. The plasma glucose was maintained constant at the basal level by determination of the plasma glucose concentration at 5-min intervals and periodic adjustment of a 20% glucose infusion as previously described (16). The insulin infusion lasted for 180 min.

Study two, insulin clamp plus basal amino acids. In five subjects who participated in study one, the insulin clamp was performed exactly as described above except that an amino acid solution (10% Travasol without electrolytes; Travenol Laboratories, Deerfield, IL) was infused with insulin and continued throughout the study. The composition of the Travasol solution (in μmol/ml) was as follows: leucine, 55.6; isoleucine, 45.7; lysine, 39.7; valine, 49.5; phenylalanine, 33.9; histidine, 30.9; threonine, 35.3; methionine, 26.8; tryptophan, 8.8; alanine, 232.3; arginine, 66.0; glycine, 137.2; proline, 59.1; serine, 47.6; tyrosine, 2.2; total amino acids, 870.6. The rate of amino acid infusion was 0.011 ml/kg · min (leucine infusion rate of 0.65 μmol/kg · min) and was chosen empirically on the basis of our previous experience to maintain the plasma amino acid concentrations constant at the basal, postabsorptive level.

Study three, insulin clamp plus increased plasma amino acid concentration. In seven subjects who participated in study one, the insulin clamp was performed as described above but the rate of amino acid infusion was increased to either 0.02 ml/kg · min (n = 3) or 0.033 ml/kg · min (n = 4) (leucine infusion rates of 1.11 and 1.83 μmol/kg · min, respectively) to achieve plasma amino acid concentrations two- to threefold above basal.

Study four, amino acid infusion plus basal insulinemia. After 180 min of [1-^{14}C]leucine equilibration, a primed-continuous amino acid infusion was administered to seven subjects. The priming dose was administered over the initial 25 min at the rate of 0.04 ml/kg · min and was followed by a continuous amino acid infusion at the rate of 0.02 ml/kg · min (leucine infusion rate of 1.11 μmol/kg · min). Concomitantly with the amino acid infusion, a 180-min infusion of cyclic somatostatin (Serono, Rome, Italy) (350 μg/h) and basal replacement of insulin (0.10 mU/kg · min) and glucagon (0.5 ng/kg · min) was initiated.

Respiratory exchange measurement. In all studies, total CO_2 production rate was performed as previously described (17). Briefly, a plastic ventilated hood was placed over the head of the subject and made air tight around the neck. A slight negative pressure was maintained in the hood to avoid loss of expired air. Ventilation was measured by means of a dry gas meter. The carbon dioxide content of expired air was continuously measured by an infrared carbon dioxide analyzer (model CD-3A; Applied Electrochemistry Inc., Sunnyvale, CA).

Analytical determinations. Plasma leucine specific activity was determined in Dr. DeFronzo's laboratory in all 27 studies using an amino acid analyzer (D500; Dionex Corp., Sunnyvale, CA). To precipitate plasma proteins, 2.5 ml of 10% sulfosalicylic acid was added to 2.5 ml of plasma and a 1-ml aliquot of the supernatant was analyzed in duplicate for amino acid concentration. 1 ml of the remaining supernatant was placed in duplicate on a cation exchange resin column (Dowex 50G; Bio-Rad Laboratories, Richmond, CA) and the free amino acid fraction was eluted with 4 N NH_4OH, dehydrated, and reconstituted in water (14). 10 ml of hydrofluor scintillation fluid was added to each vial and ^{14}C radioactivity was measured in a scintillation counter. The recovery of [^{14}C]leucine added to plasma was 98±2%. The interassay and intraassay variations for the determination of [^{14}C]leucine specific activity were 4±2 and 5±2%, respectively. More than 98% of the radioactivity collected in the amino acid fraction was in the leucine peak after separation by ion exchange chromatography.

Recent studies by Matthews et al. (18) and Schwenk et al. (19) have indicated that the total leucine carbon flux and the rate of leucine oxidation may be underestimated if calculations are performed using the plasma leucine specific activity. Because oxidation of leucine takes place only after transamination to alpha-ketoisocaproate (alpha-KIC)[1] and since intracellular alpha-KIC and leucine are in rapid equilibration, some investigators have suggested that the plasma alpha-KIC specific activity may provide a better estimate of the intracellular precursor pool when calculating rates of leucine oxidation and leucine flux (18–20). In 20 subjects (5 in study one, 5 in study two, 4 in study three, 6 in study four) sufficient plasma was available to measure the plasma alpha-KIC, as well as leucine specific activity in simultaneously drawn samples in the laboratory of Dr. Haymond (21, 22). Because of differences in the determination of radioactivity between the two laboratories, (i.e., R. A. DeFronzo and M. Haymond) and because $^{14}CO_2$ samples were not available for counting by Dr. Haymond, direct substitution of data values was not possible. However, on the basis of the ratio between [^{14}C]leucine and [^{14}C]KIC specific activities determined by high-performance liquid chromatography (HPLC) in the laboratory of Dr. Haymond, it was possible to calculate an "estimated" [^{14}C]KIC specific activity for all 27 subjects (see subsequent discussion under Calculations and in Results).

Plasma insulin concentration was determined using a double antibody radioimmunoassay technique (23). Plasma glucagon concentration was determined by radioimmunoassay using the 30K antibody of Unger (24). Plasma glucose concentration was determined using a glucose analyzer (Beckman Instruments, Inc., Fullerton, CA).

Calculations. Whole body leucine flux was calculated using a stochastic model for protein metabolism. This analysis assumes near steady state conditions. The validity and assumptions of the model have been previously discussed in detail by Waterlow (25) and Golden (26). Briefly, the model generates the following equations in which the

1. *Abbreviations used in this paper:* alpha-KIC, alpha ketoisocaproate; BCAA, branched chain amino acid.

total leucine turnover or flux (Q) equals: $Q = S + C = B + I$, where S is the rate of leucine incorporation into protein (or nonoxidative leucine disposal); C is the rate of leucine oxidation; B is the rate of leucine release from protein (endogenous leucine appearance); and I is the rate of exogenous leucine input.

The rate of leucine turnover (Q) is calculated as follows: $Q = F/leu\ sp\ act$, where F is the infusion rate of [^{14}C]leucine (in counts per minute per minute) and *leu sp act* is the specific radioactivity of leucine in the plasma compartment under equilibrium conditions.

The leucine oxidation rate (C) is calculated as follows: $C = O/(K \times leu\ sp\ act)$, where O is the rate of appearance of $^{14}CO_2$ in the expired air (in counts per minute per minute) and K is a correction factor (0.81) that takes into account the incomplete recovery of labeled [^{14}C]carbon dioxide from the bicarbonate pool (27).

An estimate of the rate of leucine incorporation into protein (S) can be calculated as follows: $S = Q - C$.

An estimate of the rate of leucine release into the plasma space from endogenous protein (B) can be calculated as follows: $B = Q - I$.

When the subjects are in the postabsorptive state, the leucine intake (I) equals zero and $B = Q$. When amino acids are being infused intravenously (i.e., as in study protocols 2–4), the rate of leucine infusion (I) must be subtracted from the total leucine flux (Q) in order to calculate the rate of endogenous leucine release from protein. The rate of exogenous leucine infusion was calculated as the product of the infusate leucine concentration in micromoles per milliliter and the infusion rate in milliliters per minute. Finally, the net balance of leucine flux into or out of protein was calculated as the difference between the nonoxidative leucine disposal (an estimate of protein synthesis) and the endogenous rate of leucine appearance (an estimate of protein degradation).

The above described model assumes that the plasma leucine specific activity accurately reflects the intracellular specific activity of leucine. Recent data, however, suggest that the plasma specific activity of the transaminated product of leucine, alpha-ketoisocaproate (KIC) may provide a better indicator of the specific activity in the intracellular mixing pool (18–20). Therefore, estimates of leucine kinetics were also carried out using estimates of the plasma [^{14}C]KIC specific activity. Thus, all of the calculations of leucine turnover were repeated using the above equations but with substitution of the "estimated" plasma [^{14}C]KIC specific activity for the plasma leucine specific activity. In this study (see Table II) the ratio of KIC to leucine specific activity was quite constant during each of the four experimental protocols (0.68±0.02) and was identical to the ratio observed in the basal state (0.68±0.02). In additional studies performed in insulin-dependent diabetic subjects (28), we also have shown that neither insulin nor amino acid infusion alters the ratio of KIC/leucine specific activity. Similar results have been reported by Tessari et al. (11) and Fukagawa et al. (12).

Ideally, one should use the intracellular leucyl t-RNA specific activity when calculating total leucine flux. Unfortunately, however, the specific activity of this precursor pool can not be determined in man. Six studies (29–34) from three different laboratories have examined the intracellular leucyl t-RNA pool in vitro and in vivo. Five of six have reported the t-RNA specific activity to be quite similar to the plasma leucine specific activity (29, 31–34), whereas one found a somewhat lower specific activity (30). Thus, at present, it can not be known with certainty what is the actual precursor specific activity to be used when calculating rates of leucine turnover. Taken in this context, the leucine flux rates determined from the plasma leucine and KIC specific activities might better be viewed as providing minimum and maximum estimates, respectively. Of particular importance with respect to the present work, none of the major conclusions are altered whether one uses the plasma leucine or estimated KIC specific activities.

Glucose metabolism. During the euglycemic insulin clamp studies, the glucose infusion rate was averaged over the final 60- to 180-min time period. Under similar conditions of hyperinsulinemia we have previously shown that endogenous glucose production is suppressed > 90–95% (35). Therefore, the total amount of glucose taken up by the entire body closely approximates the exogenous glucose infusion rate required to maintain euglycemia. Steady state plasma glucose and insulin concentrations represent the mean of values taken during the 60–180-min time period.

Statistical analysis. All values are expressed as the mean±SEM. Comparisons between the basal and insulin stimulated state within a group were performed using the t test for paired data. Intergroup comparisons were performed by analysis of variance.

Results

Plasma amino acid concentrations and plasma leucine specificity activity. During study one we observed a consistent decrease in most plasma amino acid concentrations with the notable exception of alanine (Table I, Fig. 2). The total plasma amino acid concentration decreased from 1913±58 to 1427±50 μmol/liter ($P < 0.001$). Branched chain amino acids (BCAA) declined from 406±15 to 217±4 μmol/liter ($P < 0.001$); in particular, plasma leucine fell from 127±5 to 57±3 μmol/liter ($P < 0.001$). A steady state plateau concentration was reached for all BCAA during the last hour of study. Between 120 and 180 min of insulin infusion the plasma concentrations of leucine (59±2 vs. 56±3 μmol/liter), isoleucine (25±3 vs. 21±2 μmol/liter), and valine (140±4 vs. 135±4 μmol/liter) were not significantly different ($P > 0.50$) (Fig. 2).

In study two both total and branched chain amino acid concentrations did not change significantly from their basal values (1901±117 vs. 2368±123 and 409±27 vs. 419±22 μmol/liter, respectively). Specifically, the plasma leucine concentration was maintained close to the basal level (127±3 and 114±4 μmol/liter at 0 and 180 min, respectively) (Fig. 2). A steady state plateau for all BCAA was reached during the last hour of study.

In study three a significant increase in both total and branched chain amino acid concentrations (1,972±84 vs. 3,504±304 and 417±18 vs. 835±34 μmol/liter, respectively) was observed. Plasma leucine concentration increased twofold, from a baseline value of 122±4 to 214±25 μmol/liter at 180 min ($P < 0.001$, Fig. 2).

In study four the total plasma amino acid concentration increased from 2127±103 to 4536±238 μmol/liter ($P < 0.001$) and the branched chain amino acid concentration from 434±21 to 970±21 μmol/liter ($P < 0.001$). Basal plasma leucine concentration was 122±6 μmol/liter and increased to 284±10 μmol/liter during the amino acid infusion period ($P < 0.001$, Fig. 2).

During the last hour of the basal equilibration period and during the 120–180-min of the experimental period a steady state plateau of plasma leucine specific activity (counts per minute per nanomole and disintegrations per minute per nanomole carried out in Dr. DeFronzo's and Dr. Haymond's laboratories, respectively) was reached in all four study protocols (Table I and Fig. 2).

Plasma alpha-KIC concentration and specific activity. In five patients in study one, five in study two, four in study three, and six in study four plasma alpha-KIC concentrations and specific activity were determined in the laboratory of Dr. Haymond (19). Basal plasma alpha-KIC concentration was similar in all study protocols (38±5, 34±3, 35±3, and 29±4 μmol/liter, respectively). In studies one and two they declined to 18±3 and 21±2 μmol/liter, respectively, whereas during study three they did not change (32±3 μmol/liter) and increased

Table I. Individual, Total, and Branched Chain (BCAA) Amino Acid (AA) Concentrations (μmol/liter) in the Four Study Protocols

	Study 1		Study 2		Study 3		Study 4	
	Basal	120–180 min	Basal	120–180 min	Basal	120–180 min	Basal	120–180 min
Taurine	4.9±2	39±3*	54±4	43±3	56±3	58±4	53±4	55±4
Aspartate	6±1	5±3	6±1	6±1	5±2	6±1	4±1	6±1
Threonine	148±14	92±8*	142±13	206±46*	144±10	275±8*	147±25	351±44‡
Serine	130±13	82±6*	130±13	145±16	121±12	255±11*	111±7	306±22‡
Asparagine	53±2	34±2*	51±3	34±1	48±4	50±6	47±6	64±7*
Glutamic acid	37±4	28±2*	38±4	29±3	38±4	39±3	35±9	42±9*
Glutamine	408±11	324±13*	411±27	385±27	409±18	503±34‡	472±49	609±54‡
Glycine	227±19	200±17*	213±30	359±44*	228±18	512±11‡	232±15	698±35‡
Alanine	215±13	259±20	227±23	490±34*	284±25	604±52‡	350±47	954±77‡
Citrulline	33±2	13±1*	31±3	28±5	34±4	41±8	33±1	50±2*
AABA	22±1	14±1*	12±2	18±1	14±3	19±5	19±2	42±4*
Valine	215±10	137±4	221±8	229±14	231±12	489±21‡	249±15	507±13‡
Cysteine	64±2	61±3	61±3	51±2	60±3	58±4	59±2	55±2
Methionine	27±2	16±2	24±2	49±4*	25±4	94±12‡	24±2	110±9‡
Isoleucine	62±3	23±3‡	60±3	78±5	64±4	131±14‡	61±1	180±3‡
Leucine	127±6	56±2‡	127±7	112±4	122±4	215±25‡	122±6	281±10‡
Tyrosine	46±6	24±4*	52±6	36±6*	48±4	51±5	57±8	73±12*
Phenylalanine	45±3	28±3*	46±2	74±5	41±3	104±8‡	44±1	146±7‡
BCAA	405±15	217±4‡	409±27	419±22	417±18	835±34‡	434±21	970±21‡
Total AA	1,913±58	1,427±50‡	1,901±117	2,368±123	1,972±84	3,504±304‡	2,127±103	4,536±238‡

* $P < 0.05$ vs. basal, ‡ $P < 0.01$ vs. basal.

slightly (37±3 μmol/liter) during study four. During the last hour of the equilibration period and during the 120–180-min period of the experimental period, the alpha-KIC specific activity (counts per minute per micromole) remained constant in all four study protocols.

During the basal equilibration period (120 to 180 min) the ratio of [^{14}C]KIC specific activity/[^{14}C]leucine specific activity was essentially identical in each of the 20 individuals in whom plasma was available for assay in Dr. Haymond's laboratory and this ratio averaged 0.68±0.02 (Table II). In each of the four study protocols the ratio of alpha-KIC to leucine specific activity during the last hour (120–180 min) of the experimental period was quite similar to that observed during the postabsorptive state. This observation is consistent with a number of previous publications in which the KIC to leucine specific activity has been shown to remain constant after a number of experimental maneuvers, including insulin infusion (11, 12), protein feeding (36), and lipid infusion (37).

For comparison of data for the entire study population (i.e., all 27 subjects), plasma KIC specific activity (counts per minute per millimole) was estimated for each individual by multiplying the leucine specific activity determined in Dr. DeFronzo's laboratory by the mean KIC/leucine specific activity ratio determined in Dr. Haymond's laboratory for each of the four groups (Table II). Using this "estimated" [^{14}C]KIC specific activity, the values for total leucine flux, leucine oxidation, nonoxidative leucine disposal, and endogenous leucine flux were recalculated.

The actual mean data for the [^{14}C]leucine and [^{14}C]KIC specific activities, as well as the expired $^{14}CO_2$ counts and the [^{14}C]leucine infusion rates, are presented in Table III for each of the four experimental groups. As can be seen, the changes in leucine specific activity determined in the laboratories of Drs. DeFronzo and Haymond agreed quite well with one minor exception. In study two the plasma leucine specific activity determined in Dr. DeFronzo's laboratory showed no change, whereas it decreased slightly when determined in Dr. Haymond's laboratory. At present, we have no explanation for this small difference in measured leucine specific activity. It should be noted, however, that while this difference may quantitatively alter the absolute rates of leucine turnover calculated in study two, it does not affect the qualitative changes that were observed.

Total leucine flux. In the postabsorptive state the leucine turnover rate was similar in all four study protocols and averaged 1.12±0.04 μmol/kg·min (Fig. 3). Under conditions of euglycemic hyperinsulinemia (study one), we observed a 40% decrease in the rate of leucine turnover using the [^{14}C]leucine specific activity (1.16±0.05 to 0.69±0.03 μmol/kg·min, $P < 0.01$). A similar decrease was observed using the estimated [^{14}C]KIC specific activity (1.73±0.08 to 1.08±0.07 μmol/kg·min, $P < 0.001$). In study two, with the same degree of hyperinsulinemia but with maintenance of basal plasma amino acid levels, the leucine turnover rate remained unchanged (1.08±0.05 vs. 1.06±0.05 μmol/kg·min) during the insulin infusion using either the [^{14}C]leucine specific activity or the estimated [^{14}C]KIC specific activity (1.58±0.04 vs. 1.54±0.04 μmol/kg·min). In study three (combined hyperinsulinemia and hyperaminoacidemia) and in study four (basal insulinemia and hyperaminoacidemia), the total leucine turnover rate increased from 1.08±0.04 to 1.80±0.09 and from 1.10±0.05 to 1.74±0.09 μmol/kg·min, respectively, using the [^{14}C]leucine specific activity ($P < 0.001$ vs. basal and $P < 0.001$ vs. both studies one and two; Fig. 3), and from 1.61±0.08 to

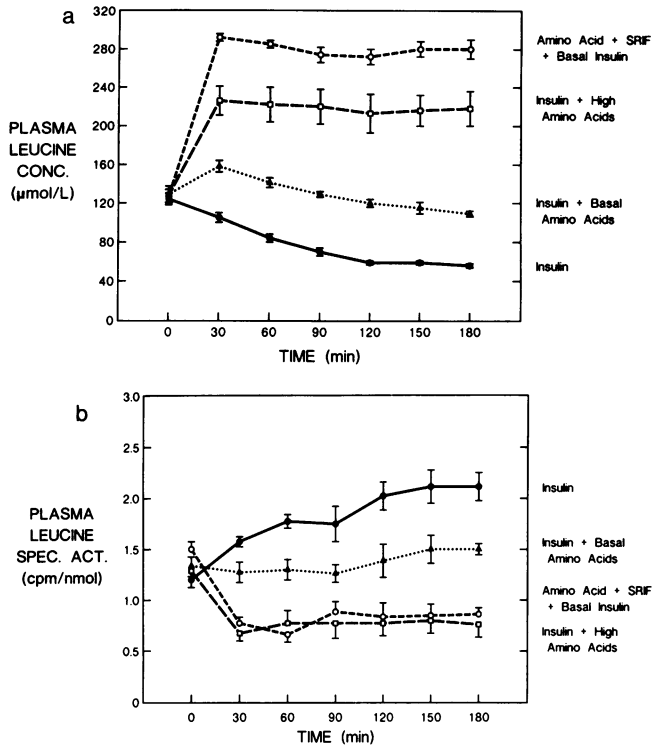

Figure 2. Time related change in plasma leucine concentration and leucine specific activity during each of the four study protocols.

2.68±0.05 and from 1.62±0.05 to 2.48±0.05 μmol/kg · min, respectively, using the [^{14}C]KIC specific activity.

Endogenous leucine flux. In study one amino acids were not infused (Fig. 4). Therefore, the endogenous leucine flux equals the total leucine turnover rate. In the postabsorptive state, endogenous leucine flux decreased from 1.16±0.05 to 0.69±0.03 μmol/kg · min during the euglycemic insulin clamp using the [^{14}C]leucine specific activity (or 1.73 to 1.08 μmol/kg · m, using the estimated [^{14}C]KIC specific activity) (both $P < 0.001$ vs. basal). In studies two through four a balanced amino acid solution was infused to maintain or to increase plasma amino acid concentrations. Therefore, the endogenous leucine flux was calculated as the difference between the total leucine flux and the leucine infusion rate. In study two endogenous leucine flux decreased from 1.08±0.05 to 0.38±0.05 μmol/kg · min ($P < 0.01$) using the [^{14}C]leucine specific activity. Using the estimated [^{14}C]KIC specific activity, a 0.71-μmol/kg · min decrease ($P < 0.01$) in endogenous leucine flux was observed. A similar decrease ($P < 0.01$) was observed during study three (1.08±0.04 to 0.39±0.06 μmol/kg · min, $P < 0.01$) using the [^{14}C]leucine data (or 1.58±0.05 to 1.24±0.05 μmol/kg · min using the [^{14}C]KIC data). In study four hyperaminoacidemia, while maintaining basal insulin levels, caused a decline in endogenous leucine flux from 1.10±0.05 to 0.64±0.09 μmol/kg · min ($P < 0.01$) using the leucine specific activity (or 1.62 to 1.37 μmol/kg · min using the [^{14}C]KIC specific activity).

Table II. Ratio between [^{14}C]KIC and [^{14}C]Leucine Specific Activities in the Four Study Protocols during the Last Hour of the Baseline and Experimental Periods (Mean of Four Determinations) (Mean±SEM).

Study	Number	Study period	
		Basal	120′–180′
I	5	0.67±0.02	0.63±0.04
II	5	0.68±0.04	0.69±0.07
III	4	0.67±0.07	0.69±0.07
IV	6	0.71±0.02	0.68±0.04

All determinations in this table were performed in Dr. Haymond's laboratory.

Leucine oxidation. Basal leucine oxidation was similar in all four study protocols and averaged 0.18±0.02 μmol/kg · min (Fig. 5). When hyperinsulinemia was created but amino acid levels were allowed to drop (study one), leucine oxidation fell significantly to 0.13±0.01 μmol/kg · min ($P < 0.01$). In contrast, when the same degree of hyperinsulinemia was created while maintaining the plasma amino acid concentrations constant at the basal level (study two), leucine oxidation was enhanced (0.29±0.02 μmol/kg · min) ($P < 0.01$). When hyperaminoacidemia was created, either with hyperinsulinemia (study three) or with euinsulinemia (study four), leucine oxidation was markedly stimulated (both $P < 0.01$ versus study two) (Fig. 5). Similar relative changes were observed in leucine oxidation using the [^{14}C]KIC data.

Nonoxidative leucine disposal. Using the [^{14}C]leucine specific activity, basal nonoxidative leucine disposal was similar in all four studies and averaged 0.93±0.01 μmol/kg · min (Fig. 6). During study one nonoxidative leucine disposal decreased by 40% to 0.55±0.03 μmol/kg · min ($P < 0.01$ vs. basal). In study two, when plasma amino acid levels were maintained at or slightly below postabsorptive levels, the decline in nonoxidative leucine disposal (0.90±0.04 to 0.76±0.03 μmol/kg · min) was significantly less than that observed in study one ($P < 0.01$). Finally, in both studies three (1.20±0.05 μmol/kg · min) and four (1.30±0.08 μmol/kg · min) a significant increment (both $P < 0.01$ vs. basal) (both $P < 0.01$ vs. studies one and two) in the rate of nonoxidative leucine disposal was observed (Fig. 6).

Using the [^{14}C]KIC specific activity basal nonoxidative leucine disposal averaged 1.37±0.04 μmol/kg · min. In study one, insulin alone resulted in a decrease ($P < 0.01$ vs. basal) in nonoxidative leucine disposal. In contrast, in study two the rate of nonoxidative leucine disappearance did not change significantly from baseline. In studies three and four, the rate of nonoxidative leucine disappearance increased ($P < 0.01$ vs. basal and studies one and two) by 0.42 and 0.55 μmol/kg · min, respectively.

Net leucine protein balance. In the basal state the net leucine balance with respect to flux into and out of protein was similar in all four study protocols and averaged −0.20±0.02 μmol/kg · min using the [^{14}C]leucine specific activity (Fig. 7). During study one the net balance increased slightly, but still remained in the negative range, −0.13±0.12 μmol/kg · min. In study two, when plasma amino acid concentrations were maintained at the basal postabsorptive level, the net leucine protein balance increased significantly and became positive, 0.38±0.04 μmol/kg · min ($P < 0.01$ vs. study one). Finally, during both studies three and four, the net leucine balance increased further to 0.81±0.04 and 0.66±0.05 μmol/kg · min, respectively ($P < 0.01$ vs. studies one and two).

Table III. Plasma Leucine and KIC Specific Activities, Expired $^{14}CO_2$ Production, [^{14}C]Leucine Infusion Rate, and Cold Leucine Infusion Rate in the Four Experimental Protocols

	Study I		Study II		Study III		Study IV	
	Basal	120–180 min	Basal	120–180 min	Basal	120–180 min	Basal	120–180 min
Number of subjects	8		5		7		7	
[^{14}C]Leucine, cpm/nmol	1.27±0.06	2.16±0.10	1.44±0.07	1.46±0.04	1.41±0.04	0.81±0.06	1.49±0.26	0.93±0.17
[^{14}C]Leucine, dpm/nmol	1.95±0.22	2.35±0.25	2.13±0.23	1.76±0.17	2.01±0.18	1.22±0.16	2.12±0.31	1.71±0.24
[^{14}C]KIC, cpm/nmol	0.85±0.04	1.36±0.09	0.98±0.05	1.01±0.04	0.99±0.09	0.56±0.04	1.06±0.19	0.66±0.10
[^{14}C]KIC, dpm/nmol	1.27±0.13	1.49±0.20	1.44±0.16	1.21±0.10	1.47±0.04	0.83±0.09	1.51±0.23	1.25±0.15
Expired $^{14}CO_2$, cpm/min	13,410±1,118	14,781±1,221	14,197±1,007	23,048±962	12,223±2,481	26,664±2,954	12,969±1,588	24,535±3,954
[^{14}C]Leucine infusion rate, cpm/kg · min	1,471±84		1,548±64		1,482±93		1,593±176	
[^{14}C]Leucine infusion rate, dpm/kg · min	3,149±129		2,688±122		2,653±368		2,446±309	
Cold leucine infusion rate, μmol/kg · min			0.67±0.00		1.47±0.14*		1.11±0.00	

Plasma leucine and KIC specific activities (dpm/nmol) and [^{14}C]leucine infusion rate (dpm/kg · min) were determined in Dr. Haymond's laboratory in five, five, four, and six subjects in studies I, II, III, and IV, respectively. Plasma KIC specific activities (CPM/nmol) were extrapolated. All values represent mean±SEM. *It should be noted that the four subjects in study III, whose samples were analyzed by HPLC, received 1.11 μmol/kg · min of cold leucine.

Using the [^{14}C]KIC specific activity, leucine balance in the postabsorptive state was negative, −0.29 μmol/kg · min. During study one, when only insulin was infused, the net leucine balance remained negative, −0.19 μmol/kg · min. In study two, leucine balance increased from −0.18 to +0.24 μmol/kg · min using the estimated [^{14}C]KIC specific activity. In studies three and four net leucine balance increased from about −0.23 to +0.56±0.03 and +0.48±0.03 μmol/kg · min, respectively.

Glucose metabolism. During the 60- to 180-min time period, the glucose infusion rates required to maintain euglycemia in the 40 mU/m^2 · min insulin clamp studies were 7.6±1.0, 8.1±1.0, and 7.4±1.0 mg/kg · min during studies one, two, and three, respectively.

Plasma glucose, insulin, and glucagon concentrations. The fasting plasma glucose concentration was similar in all four study protocols and averaged 86±2 mg/dl. During the euglycemic insulin clamp, the steady state plasma glucose levels (60–180 min) were maintained close to the basal values: 85±2 (study one), 86±2 (study two), 87±2 mg/dl (study three) with coefficients of variation of 4.7±1.0, 4.8±1.0, and 4.7±1.0, respectively. In study four the plasma glucose concentration (85±2 mg/dl) remained close to the basal level.

The mean fasting plasma insulin concentration was similar in all four studies and averaged 8±2 μU/ml. The steady state plasma insulin levels during the 60–180-min time period of the insulin clamp were 76±2, 74±2, and 80±2 μU/ml during studies one, two, and three, respectively. In study four the plasma insulin concentration did not change from baseline (10±1 vs. 12±1 μU/ml).

The mean fasting plasma glucagon concentration was similar in the four studies and averaged 174±14 pg/ml. During the 60- to 180-min time period of the insulin clamp, plasma glucagon either decreased slightly or did not change in the four study protocols, averaging 157±31, 137±12, 184±40, and 216±27 pg/ml, respectively (all P = NS).

Discussion

In this study we have investigated the effects of changes in the plasma insulin and amino acid levels on leucine metabolism in

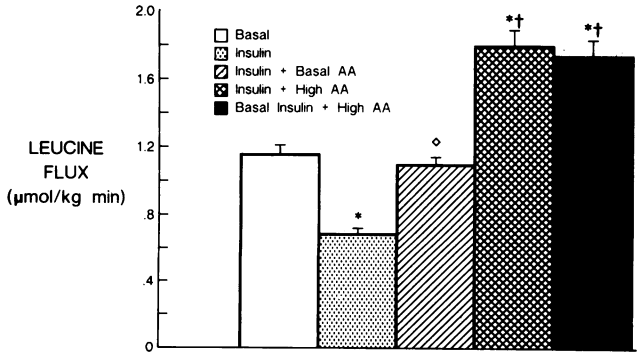

Figure 3. Effect of insulin and plasma amino acid levels on total leucine flux in the basal state and during each of the four study protocols. *P < 0.01 vs. basal; ◇ P < 0.01 vs. study one (*insulin*); †P < 0.01 vs. study one (*insulin*) and study two (*insulin + basal AA*).

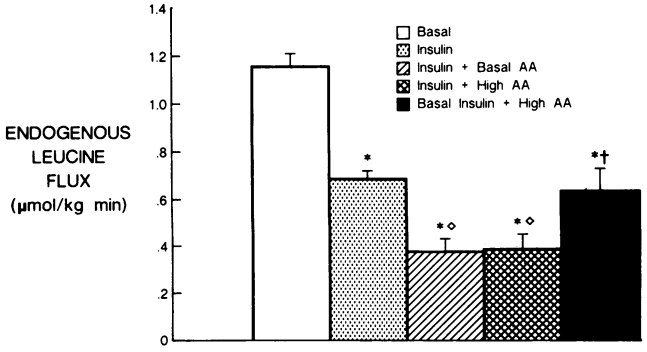

Figure 4. Effect of insulin and plasma amino acid levels on endogenous leucine flux in the basal state and during each of the four study protocols. *P < 0.01 vs. basal; ◇ P < 0.01 vs. study one (*insulin*): †P < 0.05 vs. study two (*insulin + basal AA*) and study three (*insulin + high AA*).

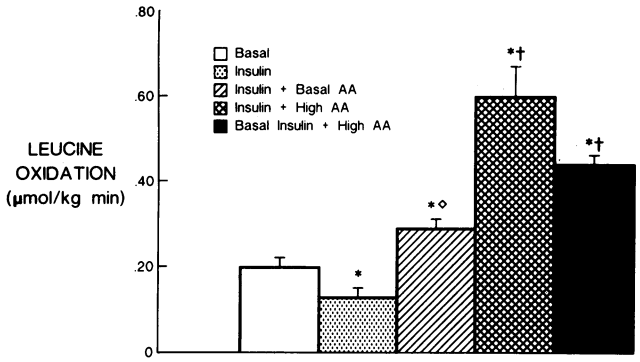

Figure 5. Effect of insulin and plasma amino acid levels on leucine oxidation in the basal state and during each of the four study protocols. *$P < 0.01$ vs. basal; ◇ $P < 0.01$ vs. study one (*insulin*); †$P < 0.01$ vs. study one (*insulin*) and study two (*insulin + basal AA*).

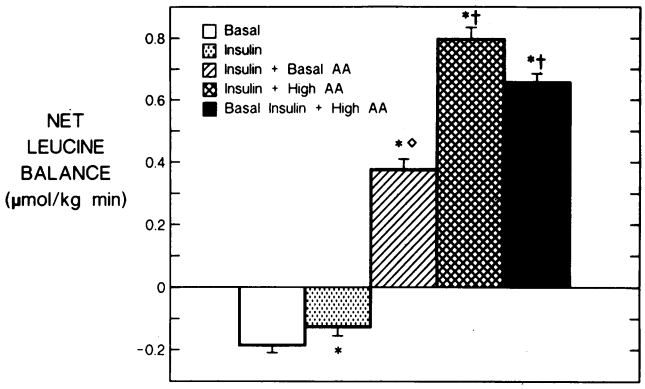

Figure 7. Net balance between nonoxidative leucine disposal (protein synthesis) and endogenous leucine flux (proteolysis) in the basal state and during the four experimental protocols. *$P < 0.01$ vs. basal; ◇ $P < 0.01$ vs. study one (*insulin*); †$P < 0.01$ vs. study one (*insulin*) and study two (*insulin + basal AA*).

normal subjects. In order to evaluate the separate roles of hyperinsulinemia and plasma amino acid concentrations on the regulation of leucine turnover, we employed the euglycemic insulin clamp technique in combination with the infusion of a balanced amino acid solution. This allowed us to independently manipulate the desired variables, i.e., the plasma insulin and amino acid concentrations.

In the first study protocol insulin was infused to create a state of physiologic hyperinsulinemia (70–80 μU/ml) and the plasma amino acid concentrations were allowed to drop spontaneously (Table I). The plasma branched chain amino acid concentrations declined by 30–40%, a result similar to previous reports (13, 38, 39), and the plasma KIC concentration decreased by 50%. With the notable exception of alanine, a similar decline in the circulating levels of most other amino acids was also documented. However, from the plasma concentration of amino acids alone, it is not possible to define the mechanism(s), namely stimulation of protein synthesis/oxidation or inhibition of protein degradation, responsible for the amino acid lowering effect of insulin. To address this question, the present studies were performed with [^{14}C]leucine and the data analyzed using a stochastic model (25, 26). A major advantage of this type of analysis is that all measurements are made under near steady state conditions (i.e., in the postabsorptive state and after 3 h of hyperinsulinemia), when plasma leucine and other amino acid concentrations and plasma leucine and KIC specific activities are essentially constant. Within the limitations and assumptions (see references 18, 25, and 26 for a detailed review) of these experimental methods, we observed a significant decrease in the total leucine flux following 3 h of insulin infusion (study one). This result is consistent with recent data reported by Fukagawa et al. (12), Tessari et al. (11), and Tsalikian et al. (40) in normal man and with studies by Abumrad et al. (41) and Nissen et al. (15) in the dog, and indicates that under euglycemic conditions insulin infusion is associated with a major inhibitory action on the rate of endogenous leucine appearance. In the postabsorptive state, endogenous proteins are the only source for the appearance of the essential amino acid leucine in the systemic circulation. Therefore, it is reasonable to conclude that insulin suppresses proteolysis in man. Since both the rate of leucine oxidation and the rate of nonoxidative leucine disposal (an index of protein synthesis) decreased significantly during the infusion of insulin alone, it must be concluded that, under the present experimental conditions, the primary amino acid lowering effect of insulin is related to its inhibitory effect on protein degradation. This is, however, at variance with much in vitro data (3, 4) that indicates that the primary action of insulin is to stimulate protein synthesis.

It should be emphasized that the circulating levels of all measured plasma amino acid concentrations except alanine declined after insulin infusion. This hypoaminoacidemia might limit a stimulatory effect of insulin on protein synthesis. This speculation is further strengthened by the observation that the intracellular muscle concentrations of all measured amino acids except alanine decline significantly under comparable experimental conditions of hyperinsulinemia (13). It is possible, therefore, that the reduction in intracellular amino acid concentrations may have limited amino acid availability for protein synthesis and, thus, obscured a potential stimulatory effect of insulin. To examine this possibility subjects participated in an additional study in which amino acids were infused to maintain the plasma amino acid concentrations constant at the basal level during the same hyperinsulinemic stimulus (study two). Under hyperinsulinemic conditions in

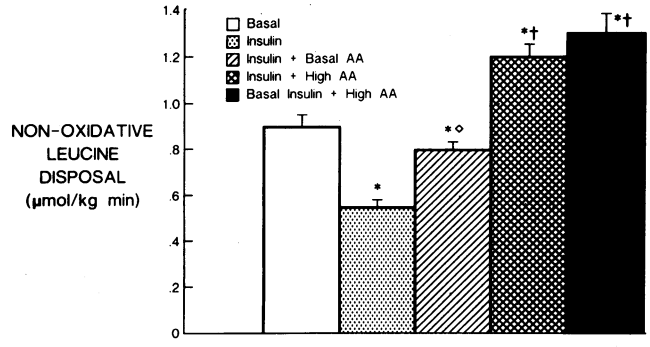

Figure 6. Effect of insulin and plasma amino acid levels on leucine turnover. Nonoxidative leucine disposal in the basal state and during each of the four study protocols. *$P < 0.01$ vs. basal; ◇ $P < 0.01$ vs. study one (*insulin*); †$P < 0.01$ vs. study one (*insulin*) and study two (*insulin + basal AA*).

which the basal amino acid concentrations were maintained constant, the decline in nonoxidative protein disposal observed during study one was largely prevented. However, a stimulatory effect of combined insulin/amino acid infusion on nonoxidative protein disposal could not be demonstrated in study two. Thus, to the extent that nonoxidative protein disposal provides an index of protein synthesis, we were unable to demonstrate a stimulatory action of hyperinsulinemia on this metabolic parameter.

Despite the above findings, it is well known that protein feeding is associated with repletion of muscle nitrogen (36). The primary difference between this situation and the experimental conditions in study two is the presence of hyperaminoacidemia following protein loading. Therefore, we repeated the insulin clamp study but doubled the amino acid infusion rate to cause combined hyperaminoacidemia/hyperinsulinemia (study three). Under these experimental conditions nonoxidative leucine disposal was increased above basal rates ($P < 0.001$) and was significantly greater than when insulin was infused alone (study one; $P < 0.01$) or with amino acids (study two; $P < 0.01$) to simply maintain the basal plasma amino acid concentration constant (Fig. 6). These results indicate that the stimulation of net protein synthesis in vivo in man is facilitated by the presence of hyperaminoacidemia. This conclusion is consistent with results obtained in study four where hyperaminoacidemia, in the presence of basal insulinemia, significantly enhanced nonoxidative leucine disposal.

The present results also help to elucidate the effects of insulin and plasma amino acid concentration on endogenous leucine flux (Fig. 4). When amino acids were infused with insulin (studies two and three), the decrease in endogenous leucine appearance was greater than observed in study one ($P < 0.01$). Furthermore, during study four, when plasma amino acid concentrations were raised while maintaining basal insulinemia, the rate of endogenous leucine appearance declined significantly from baseline ($P < 0.01$). Taken collectively, these results indicate that the plasma amino acid concentration per se (most likely by maintaining intracellular amino acid levels constant) exerts an inhibitory effect on protein degradation.

Perhaps the most interesting result from the present study is the response of leucine metabolism to hyperaminoacidemia in the presence and absence of hyperinsulinemia. Using the plasma [^{14}C]leucine specific activity, the combination of hyperinsulinemia and hyperaminoacidemia caused a greater inhibition ($P < 0.05$) of protein degradation than did hyperaminoacidemia plus maintenance of basal plasma insulin concentrations (Fig. 4). In contrast, the increase in nonoxidative leucine disposal was similar in studies three and four (Fig. 6). These data would suggest that amino acid availability may play an additive role to insulin in the suppression of endogenous proteolysis, and thus their mechanism of action appears to be different. Such is not the case for estimates of protein synthesis. These conclusions, however, are predicated on the isotope model employed. Recent studies by Schwenk et al. (19) suggest that the total leucine carbon flux and rate of leucine oxidation may be underestimated if calculations are performed using the plasma leucine specific activity during infusion of a leucine tracer. These (19) and other (20) studies suggest that the transaminated product of the infused leucine tracer, [^{14}C]KIC, may provide a more accurate estimate of the intracellular specific activity of leucine. Using the estimated [^{14}C]KIC specific activity, hyperaminoacidemia plus hyperinsulinemia and hyperaminoacidemia plus basal insulinemia produced similar increments in nonoxidative leucine disposal (+0.42 and +0.55 μmol/kg · min, respectively) and similar decrements in endogenous leucine flux (−0.35 and −0.25 μmol/kg · min, respectively), suggesting that insulin has no additive effect to hyperaminoacidemia to inhibit protein degradation and therefore may be operating through similar mechanisms to affect protein metabolism. In order to resolve this issue, it will be necessary to measure the intracellular specific activity of the t-RNA of leucine.

In summary, the primary effect of insulin on protein metabolism appears to be an inhibition of proteolysis. The decline in protein synthesis following insulin infusion is secondary to the amino acid lowering effect of the hormone. In contrast, hyperaminoacidemia not only inhibits proteolysis, but also has a major stimulatory effect on protein synthesis. Under the present experimental conditions insulin does not appear to enhance protein synthesis in the presence of either normal or decreased circulating amino acid concentrations.

Our inability to demonstrate any stimulatory effect of insulin on protein synthesis is somewhat surprising and deserving of further comment. Previous in vitro studies have clearly demonstrated that insulin augments the protein synthetic rate (1–4). However, all of these studies were carried out in rats and employed much higher insulin concentrations than in the present investigation. Thus, species differences and the pharmacologic insulin levels utilized in the in vitro studies may explain the seemingly conflicting results. It should also be noted that these previous in vitro studies compared the effect of no insulin versus very high insulin levels on protein synthesis, whereas our in vivo studies compared the effect of basal versus physiologic insulin levels on protein synthesis. It is possible that insulin does exert a stimulatory effect on protein synthesis but that this effect is maximal at basal levels of the hormone. This would explain its stimulatory action in vitro when insulin is increased from zero to pharmacologic levels but the lack of effect in vivo when insulin is raised from basal to physiologic levels.

Our results also demonstrate that both insulin and plasma amino acid concentrations can affect leucine oxidation. When hyperinsulinemia was created and the plasma amino acid concentration allowed to fall (study one), leucine oxidation declined (Fig. 5). Similar observations have been reported by Tessari et al. (11) and Robert et al. (9) in healthy subjects and in insulin-dependent diabetics by ourselves (28). In the study by Fukagawa et al. (12) insulin was reported to have no effect on leucine oxidation but the actual data were never displayed. In the present study infusion of amino acids with insulin to maintain basal amino acid concentrations constant resulted in a small, but significant increase in leucine oxidation. These results suggest that the decline in leucine oxidation observed in study one was due to a decrease in intracellular leucine concentration. When amino acids were infused while maintaining euinsulinemia (study four), the increase in leucine oxidation was significantly greater than in study two, indicating that hyperaminoacidemia per se has a marked stimulatory effect on leucine oxidation. Nissen et al. (15) also have demonstrated an increase in leucine oxidation following amino acid ingestion. Finally, the combination of hyperaminoacidemia plus hyper-

insulinemia (study three) stimulated leucine oxidation to a greater extent than observed with hyperaminoacidemia alone, suggesting that insulin per se augments leucine oxidation. This conclusion is consistent with the results obtained in study two in which hyperinsulinemia plus basal amino acidemia significantly increased leucine oxidation.

In summary, it would appear that the primary effect of insulin in man is to inhibit the endogenous leucine flux (protein degradation) while hyperaminoacidemia both stimulates nonoxidative leucine disposal (protein synthesis) while inhibiting the endogenous leucine flux. Both insulin and amino acid infusion augment leucine oxidation.

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