Differential Regulation of Amino Acid Exchange and Protein Dynamics Across Splanchnic and Skeletal Muscle Beds by Insulin in Healthy Human Subjects

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To define the mechanism of insulin's anticatabolic action, the effects of three different dosages of insulin (0.25, 0.5, and 1.0 mU · kg^{-1} · min^{-1}) versus saline on protein dynamics across splanchnic and skeletal muscle (leg) beds were determined using stable isotopes of phenylalanine, tyrosine, and leucine in 24 healthy subjects. After an overnight fast, protein breakdown in muscle exceeded protein synthesis, causing a net release of amino acids from muscle bed, while in the splanchnic bed protein synthesis exceeded protein breakdown, resulting in a net uptake of these amino acids. Insulin decreased (P < 0.003) muscle protein breakdown in a dose-dependent manner with no effect on muscle protein synthesis, thus decreasing the net amino acid release from the muscle bed. In contrast, insulin decreased protein synthesis (P < 0.03) in the splanchnic region with no effect on protein breakdown, thereby decreasing the net uptake of the amino acids. In addition, insulin also decreased (P < 0.001) leucine nitrogen flux substantially more than leucine carbon flux, indicating increased leucine transamination (an important biochemical process for nitrogen transfer between amino acids and across the organs), in a dose-dependent manner, with the magnitude of effect being greater on skeletal muscle than on the splanchnic bed. In conclusion, muscle is in a catabolic state in human subjects after an overnight fast and provides amino acids for synthesis of essential proteins in the splanchnic bed. Insulin achieves amino acid balance across splanchnic and skeletal muscle beds through its differential effects on protein dynamics in these tissue beds. *Diabetes* 47:1824–1835, 1998

Insulin's anticatabolic effect has been extensively demonstrated in people with type 1 diabetes (1–3). Mechanistic studies performed in people with type 1 diabetes have demonstrated that insulin achieves its anticatabolic effect mainly by its inhibitory effect on protein breakdown (3–6). In healthy subjects, insulin has been shown to decrease whole body protein breakdown in a dose-dependent manner (5,7). In contrast, infusion of insulin directly into the brachial artery has been reported to achieve the maximal effect on protein breakdown at a low dosage of insulin (8). These observations are consistent with a hypothesis that insulin has a differential effect on protein metabolism in different tissues (9). Animal studies also provide support for the differential effect of insulin on protein metabolism in various body tissues (10,11). Studies in streptozotocin-induced diabetic rats have shown that insulin deficiency is associated with muscle protein loss but that there is a net increase of gut protein content (12). A hypothesis emerging from all these studies is that certain tissues such as skeletal muscle are more sensitive to insulin than tissues in the splanchnic bed (9).

Insulin deprivation has been shown not only to increase protein breakdown but also to increase leucine transamination in people with type 1 diabetes (3). It could be argued that since insulin deprivation is associated with hyperglucagonemia and glucagon is known to enhance branched-chain-2-oxo-acid dehydrogenase (13), increased leucine transamination during insulin deprivation results from hyperglucagonemia rather than insulin deficiency per se (3). A dose effect of insulin on leucine transamination in postabsorptive normal healthy subjects would favor the hypothesis that insulin per se causes an inhibition of leucine transamination. Also, it is unclear whether leucine transamination in skeletal muscle and splanchnic tissue exhibits a differential sensitivity to insulin in human subjects.

Studies were therefore performed in 24 healthy human subjects to test the hypotheses *1*) that insulin decreases protein breakdown in the whole body, but that the magnitude of effect is different for splanchnic and skeletal muscle tissues, *2*) that insulin does not affect protein synthesis in muscle but may affect splanchnic tissues, and *3*) that insulin decreases leucine transamination in a dose-dependent manner and that this effect also shows a differential sensitivity to splanchnic and skeletal muscle tissues.

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ANOVA, analysis of variance; KIC, α-ketoisocaproate.

RESEARCH DESIGN AND METHODS

Materials. L-[1-^{13}C,^{15}N]leucine (99 atom percent excess) and L-[^{15}N]phenylalanine (99 atom percent excess) were purchased from Cambridge Isotope (Andover, MA), and L-[ring-^{2}H$_4$]tyrosine (99 atom percent excess) and L[^{15}N]tyrosine (99 atom percent excess) were purchased from Isotec (Miamisburg, OH). The chemical, isotopic, and optical purity of these compounds were confirmed before use. Solutions were prepared under sterile precautions in the pharmacy and were shown to be bacteria- and pyrogen-free before use in human subjects.

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Subjects. Study participants were 24 healthy adults (12 women, 12 men) between the ages of 21 and 38 years. Each participant had a BMI between 18 and 27 and had normal hepatic and renal functions; no biochemical abnormalities were detected. Fasting blood glucose levels were normal (70–100 mg/dl) in all participants. The participants were not taking any medicines with the exception of birth control pills taken by four women.

The study protocol was reviewed and approved by the institutional review board of the Mayo Clinic and Foundation. The purpose and potential risks of the study were explained to all subjects, and informed, written consent was obtained from each subject before participation.

Experimental design. The subjects received a weight-maintenance diet, which consisted of 20% protein, 50% carbohydrate, and 30% fat, for 3 days before the study. All subjects maintained their usual level of physical activity. The 24 subjects were randomly assigned to four separate groups of 6 subjects each, and each group received saline or insulin at dosages of 0.25, 0.5, or 1.0 mU · min^{-1} · kg^{-1} each.

The studies were conducted after a 12-h overnight fast in the General Clinical Research Center at Mayo Clinic. Catheter sheaths were placed in the right femoral artery and femoral vein. Hepatic vein catheters were inserted under fluoroscopic guidance, and appropriate position was confirmed by nonionic contrast injection (14). A femoral artery catheter was inserted through the arterial sheath. A slow infusion of normal saline was used to maintain patency of the catheters. The femoral artery sheath was used for infusion of indocyanine green to measure blood flow in the leg and splanchnic regions with indicator-dilution techniques (14,15). The femoral artery, femoral vein, and hepatic vein catheters were used to collect blood samples.

Dosages of L-[1-^{13}C,^{15}N]leucine (1 mg/kg), L-[^{15}N]phenylalanine (0.75 mg/kg), L-[ring-$^{2}H_4$]tyrosine (0.6 mg/kg), and L-[^{15}N]tyrosine (0.3 mg/kg) were given intravenously to prime the respective pools to achieve an early plateau. A continuous infusion of L-[1-^{13}C,^{15}N]leucine (1 mg · kg^{-1} · h^{-1}), L-[^{15}N]phenylalanine (0.75 mg · kg^{-1} · h^{-1}), and L-[ring-$^{2}H_4$]tyrosine (0.6 mg · kg^{-1} · h^{-1}) was started and maintained for 5 h. Blood samples from the femoral artery, femoral vein, and hepatic vein were collected at baseline, 120, 130, 140, 150, 270, 280, 290, and 300 min. Each participant was randomly assigned to receive an intravenous infusion of insulin through a peripheral vein lasting between 150 and 300 min. The dosages of insulin infused were 0, 0.25, 0.5, or 1.0 mU · kg^{-1} · min^{-1}. The blood glucose was clamped at each participant's baseline blood glucose value by infusing 20% dextrose via a peripheral vein in each subject who received insulin infusion (16). The study was stopped at 300 min, the catheters were removed, and homeostasis was obtained.

Analysis of samples. Blood glucose was measured by the glucose oxidase method using a glucose analyzer (Beckman, Fullerton, CA). Hormonal assays were performed as previously reported (17). Plasma insulin and growth hormone were measured by a chemiluminescent sandwich assay (Sanofi Diagnostics, Chaska, MN), and glucagon and cortisol were measured by a radioimmunoassay technique (Diagnostic, Los Angeles, CA). Indocyanine green concentration was measured by spectrophotometry. Whole blood arterial amino acid concentrations were measured by reverse-phase high-performance liquid chromatography (18).

Plasma enrichment levels of [1-^{13}C,^{15}N]leucine, [1-^{13}C]leucine, [^{15}N]phenylalanine, [ring-$^{2}H_4$]tyrosine, and [^{15}N]tyrosine were determined by gas chromatography/mass spectrometry. An internal standard solution (50 µl) containing 30 µg/ml norleucine, 60 µg/ml β-methyl-phenylalanine hydrochloride (Sigma-Aldrich, St. Louis, MO), and 20 µg/ml [$^{13}C_6$]tyrosine (Isotec) was added to six standards containing leucine, phenylalanine, and tyrosine (all from Sigma-Aldrich Chemicals) in proportions ranging from 0 to 300 µmol/l, as well as to 100-µl plasma samples. The amino acids from plasma samples were isolated by ion-exchange chromatography and dried down. All standards and samples were derivatized with N-methyl-N(t-butyldimethylsilyl)-trifluoroacetamide plus 1% t-butyl-dimethylchlorosilane in acetonitrile (both from Regis Technologies, Morton Grove, IL) at room temperature overnight. The derivatives were injected onto a 30 mm × 0.25 mm × 0.25 µm DB5MS column (J&W Chromatography, Folsom, CA) in a gas chromatograph oven at a constant flow of helium (1.1 ml/min) with the following temperature programming: 120°C initially, then to 210°C at 25°C/min, to 225°C at 10°C/min, to 255°C at 25°C/min, to 265°C at 10°C/min, to 300°C at 25°C/min, and finally to 325°C at 10°C/min; this final temperature is held for 2 min. Under electron ionization, ions were mass monitored at m/z 200, 201, 302, 303, 304 for leucine and norleucine, at m/z 336, 337 for phenylalanine, at m/z 322 for β-methyl-phenylalanine, and at m/z 466, 467, 470, 472 for tyrosine using a Hewlett-Packard 6890 MSD (Avondale, CA) to determine the isotopic enrichment as well as concentrations of leucine, phenylalanine, and tyrosine.

Plasma α-ketoisocaproate (KIC) enrichment was measured after derivatizing the extracted KIC as quinoxalinol-trimethyl silyl derivative (19) under electron ionization conditions in a gas chromatograph/mass spectrometer monitoring m/z 233/232. KIC concentration was measured using ketovalaric acid as an internal standard.

Calculations. Isotopic plateau was observed during the baseline period (120–150 min) and during the insulin infusion (270–300 min). Determination of the plateau status was based on the observation that when the isotopic enrichment values of leucine, KIC, phenylalanine, and tyrosine in different sites were plotted against time, the slopes of each of these measurements were not different from zero. Mean values of isotopic enrichment values at each plateau were used for all calculations of amino acid kinetics.

Leucine carbon flux (QL_c), leucine nitrogen flux (QL_n), and leucine appearance from KIC (i.e., KIC reamination to leucine) (L_k) were calculated as follows (20):

$$QL_c \text{ or } QL_n = I\left[\left(\frac{E_i \text{ leu}}{E_p \text{ leu}}\right) - 1\right]$$

where I represents the rate of infusion of leucine (expressed in micromoles per kilogram per hour), E_i leu represents the isotopic enrichment of the labeled leucine, and E_p leu represents the isotopic plateau in plasma of the tracer ([^{13}C]KIC in the case of leucine carbon flux and [1-^{13}C, ^{15}N]leucine in the case of leucine nitrogen flux).

$$L_k = QL_n - QL_c$$

For calculations of leucine carbon flux, leucine nitrogen flux, and leucine transamination (KIC reamination to leucine) across leg and splanchnic bed, we used the equations as previously described (3,21). Leucine carbon flux and leucine nitrogen flux across leg or splanchnic region were calculated as $L_{AC} \times (E_A$ leu/E_V leu – 1) $\times F$, where L_{AC} is leucine concentration in the artery; E_A leu and E_V leu represent isotopic enrichment of [^{13}C]leucine or [1-^{13}C, ^{15}N]leucine in artery and vein, respectively; and F is blood flow. Leucine reamination from KIC was calculated as ($QL_c - QL_n$)/[1 – (E_V leu – E_V KIC)/E_V leu], where E_V leu and E_V KIC represent venous enrichments of leucine and KIC, respectively.

Whole body phenylalanine flux (Q_{Phe}) and tyrosine flux were calculated using an equation similar to the one used for leucine carbon flux and as previously described (3,22).

Phenlylalanine conversion to tyrosine (I_{PT}) was calculated as follows:

$$I_{PT} = Q_{Tyr} \times \frac{[^{15}N]P_{Tyr}}{[^{15}N]P_{Phe}} \times \frac{Q_{Phe}}{[I_{Phe} + Q_{Phe}]}$$

where [^{15}N]P_{Tyr} and [^{15}N]P_{Phe} are plasma isotopic enrichment of the tyrosine and phenylalanine, respectively.

Whole body phenylalanine incorporation into protein is calculated by subtracting phenylalanine conversion to tyrosine from whole body phenylalanine flux, because in addition to conversion to tyrosine and eventual catabolism, the only other fate of phenylalanine is incorporation into protein. The details of these equations are discussed elsewhere (3,22).

Regional dynamics of phenylalanine. We used the equations originally described elsewhere (23).

The rate of appearance of phenylalanine (R_p) (representing protein breakdown) in both splanchnic and leg regions is calculated as follows:

$$R_p = \left[\left(\frac{[^{15}N]Phe_a}{[^{15}N]Phe_v}\right) - 1\right] \times Phe_A \times F$$

where [^{15}N]Phe_a and [^{15}N]Phe_v represent isotopic enrichment of [^{15}N]phenylalanine in artery and vein, respectively, Phe_A is arterial concentration of phenylalanine, and F is plasma flow. Calculation of phenylalanine incorporation into leg protein is the same as the rate of disappearance of phenylalanine (R_d), as follows:

$$R_d = R_p + (Phe_A - Phe_V) \times F$$

Since phenylalanine also disappears across the splanchnic bed (specifically, liver) by conversion to tyrosine, phenylalanine conversion to tyrosine is subtracted from the rate of disappearance of phenylalanine.

$$I_{PT} = \left[\left(\frac{[^2H_4]Tyr_{EA}}{[^2H_4]Tyr_{EV}}\right) \times \frac{[^{15}N]Tyr_{EV}}{[^{15}N]Phe_{EV}} - \frac{[^{15}N]Tyr_{EA}}{[^{15}N]Phe_{EV}}\right] \times Tyr_A \times F$$

Statistics. All values are given as means ± SE. First, we performed pairwise comparisons at each dosage (baseline versus intervention). We performed a one-way analysis of variance (ANOVA) and tested whether the mean of the three insulin groups differed from that of the saline group using the ANOVA mean square error term. When a significant insulin effect was found, the differences from baseline among the groups were determined by unpaired t test. We also performed Spearman's rank correlation of percentage of change from baseline against dose levels of insulin to determine the dose effect.

Similar analyses were undertaken for whole body, splanchnic, and leg regions and also for the differences between splanchnic and leg regions. The correlations appear as values of r_s, and the attained significance levels are shown as P values proximal to the values of r_s given in all figures.

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TABLE 1
Plasma blood flows for the leg and splanchnic beds

Insulin dosage ($mU \cdot kg^{-1} \cdot min^{-1}$)	Leg (ml/min)	Splanchnic bed (ml/min)
0		
Baseline	267 ± 22	934 ± 87
Insulin	314 ± 38	973 ± 47
0.25		
Baseline	261 ± 53	925 ± 54
Insulin	252 ± 32	1,093 ± 153
0.5		
Baseline	311 ± 17	778 ± 70
Insulin	374 ± 42	830 ± 72
1.0		
Baseline	280 ± 38	793 ± 88
Insulin	335 ± 54	889 ± 105

Data are means ± SE.

RESULTS

Blood flow, hormones, and glucose. Leg and splanchnic blood flow values, which are given in Table 1, were not significantly affected by insulin at different dosages and during the control period.

Table 2 shows mean plasma hormone concentrations, glucose infusion rates, glucose levels, and glucose infusion rates to maintain steady-state glucose levels during the control and insulin treatment periods. Growth hormone and cortisol did not differ significantly during the infusion of different dosages of insulin. Glucagon levels decreased during the two high-dosage insulin infusions. The amount of glucose infused increased progressively as a function of the insulin dosage infused. Glucose levels remained constant while plasma insulin levels increased progressively with increasing dosages of insulin infused.

Amino acids. Plasma concentrations of serine, glutamine, histidine, threonine, arginine, tyrosine, valine, methionine, phenylalanine, isoleucine, leucine, and lysine decreased during the insulin treatment periods compared with the control periods ($P < 0.01$) (data not shown), whereas alanine concentration increased with the last two dosages of insulin ($P < 0.05$). Arterial blood concentrations of selected amino acids during the control and insulin treatment periods are shown in Fig. 1. A greater percentage of decline was noted in these amino acid concentrations at the higher dosage of insulin infused. There were no differences between the two high insulin dosages (0.5 and 1.0 $mU \cdot kg^{-1} \cdot min^{-1}$).

Arterial and venous values of isotopic enrichment and concentrations of leucine, phenylalanine, and tyrosine are shown in Tables 3–5. All values are the means of four measurements obtained between the time points indicated (i.e., 120–180 min and 270–300 min). The values given are used for calculations of protein dynamics across leg and splanchnic beds.

Protein dynamics

Whole body. Whole body values of phenylalanine, leucine carbon, and tyrosine did not change with saline but decreased with insulin at different dosages (Table 6). All of the above three flux values are measures of whole body protein breakdown. Similarly, phenylalanine-to-protein flux (i.e., phenylalanine disappearance rate minus phenylalanine conversion to tyrosine), a measure of whole body protein synthesis, also declined with insulin with no change during saline infusion. Figure 2 gives the percentages of change during different dosages of insulin infusion from the baseline of leucine carbon flux, phenylalanine flux, tyrosine flux, and phenylalanine-to-protein flux. There was significant insulin effect with significant decrease of all four parameters. There was also a significant dose effect based on regression analysis (Fig. 2). Like the amino acid concentrations (Fig. 1), the flux values of these amino acids showed no differences between the two high dosages of insulin.

Leucine transamination. Values for whole body leucine nitrogen flux and leucine transamination to KIC (leucine deamination to KIC; leucine to KIC) are given in Table 7. Leucine nitrogen flux and KIC-to-leucine reamination significantly increased during saline infusion but decreased during different dosages of insulin infusion. Figure 3 presents the percentages of change in whole body leucine nitrogen flux, leucine transamination from KIC to leucine, and transami-

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TABLE 2
Plasma, hormone, and glucose concentrations and glucose infusion rates of the study subjects during baseline and during infusions at different dosages of insulin

Insulin dosage ($mU \cdot kg^{-1} \cdot min^{-1}$)	Insulin (pmol/l)	Cortisol (µg/dl)	Glucagon (pg/ml)	Growth hormone (ng/ml)	Glucose infusion ($mg \cdot kg^{-1} \cdot min^{-1}$)
0					
Baseline	28 ± 2	12.4 ± 1.2	56 ± 4.7	1.3 ± 0.4	0
Insulin	25 ± 2	13.4 ± 1.3	58 ± 5.8	3.0 ± 1.3	0
0.25					
Baseline	48 ± 5	13.9 ± 3.4	56 ± 3.1	2.5 ± 0.9	0
Insulin	145 ± 21*†	19.7 ± 5.6	54 ± 6.2	8.3 ± 6.6	103 ± 22*
0.5					
Baseline	47 ± 5	16.8 ± 7.7	53 ± 2.3	1.8 ± 0.4	0
Insulin	194 ± 7*†‡§	16.6 ± 4.0	44 ± 1.7*‡	4.0 ± 1.3	171 ± 29‡
1.0					
Baseline	41 ± 5	14.1 ± 2.7	54 ± 4.1	1.3 ± 0.5	0
Insulin	404 ± 26*†‡§	16.1 ± 2.6	41 ± 3.3*‡	9.2 ± 3.9	363 ± 55§

Data are means ± SE. *$P < 0.01$ vs. baseline; †$P < 0.05$ vs. same time point at 0 $mU \cdot kg^{-1} \cdot min^{-1}$ insulin infusion; ‡$P < 0.01$ vs. 0.25 $mU \cdot kg^{-1} \cdot min^{-1}$ insulin infusion; §$P < 0.01$ vs. 0.5 $mU \cdot kg^{-1} \cdot min^{-1}$ insulin infusion.

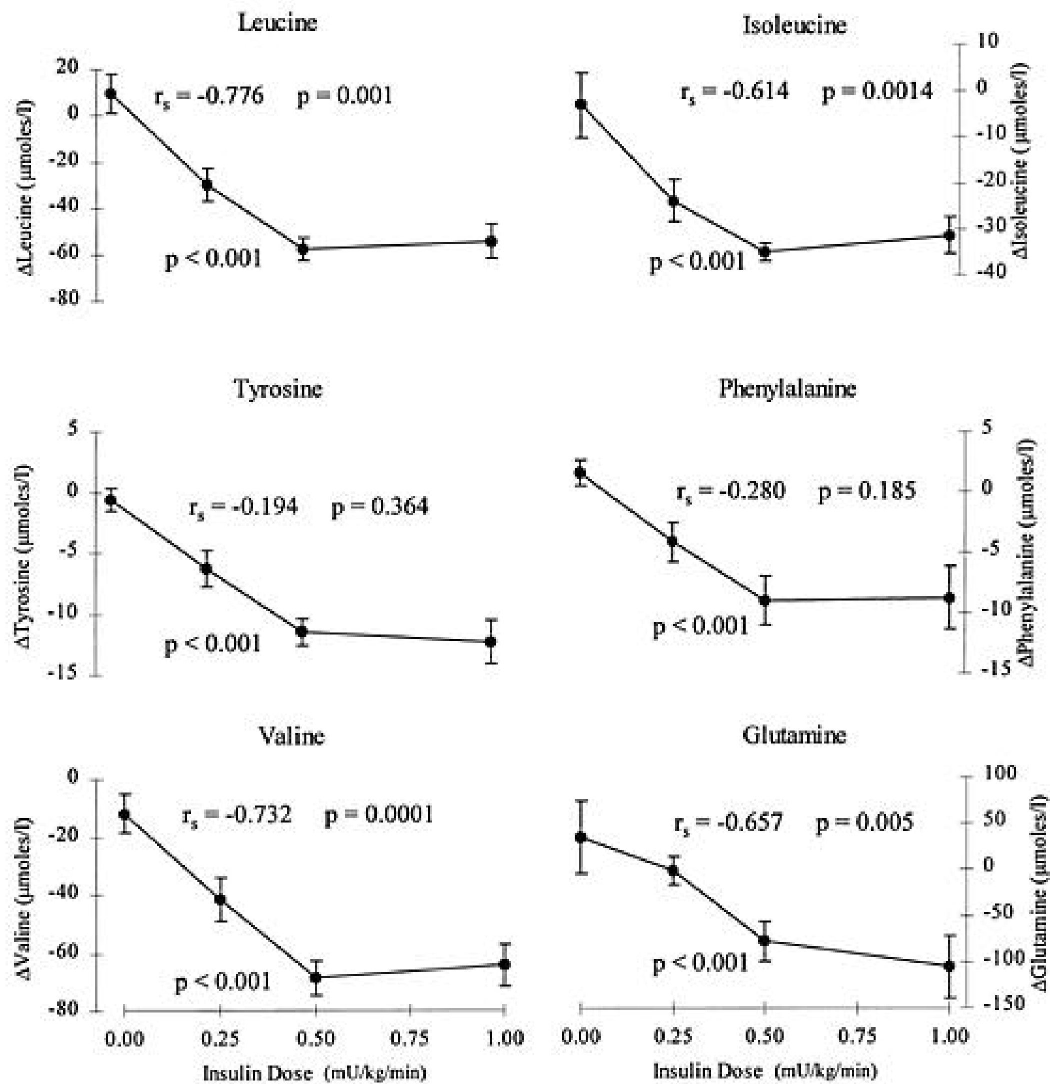

FIG. 1. Changes (Δ) in systemic concentrations of representative amino acids (leucine, isoleucine, tyrosine, phenylalanine, valine, and glutamine) as a function of the dosage of insulin infused. All of these amino acid concentrations decreased in a dose-dependent manner, although no changes were found between the insulin dosages 0.5 and 1.0 mU · kg^{-1} · min^{-1}. *P* values are based on one-way ANOVA and demonstrate the effect of insulin versus normal saline. The r_s values represent the correlations based on Spearman's rank correlation analysis, and the *P* values adjacent to the r_s values show the levels of significance based on this analysis.

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TABLE 3
Isotopic enrichment of leucine and KIC in the arterial, femoral, and hepatic veins at baseline (120–180 min) and during the last 30 min (270–300) of infusion at different dosages of insulin or saline

Insulin dosage	[1-^{13}C,^{15}N]leucine			Total [^{13}C]leucine			[^{13}C]KIC		
(mU · kg^{-1} · min^{-1})	AV	FV	HV	AV	FV	HV	AV	FV	HV
0.0									
At 120–180 min	3.629 ± 0.091	2.099 ± 0.055	2.376 ± 0.046	7.153 ± 0.188	5.570 ± 0.149	5.722 ± 0.141	5.292 ± 0.094	4.946 ± 0.142	5.531 ± 0.106
At 270–300 min	3.334 ± 0.078	2.055 ± 0.032	2.237 ± 0.044	7.056 ± 0.134	5.956 ± 0.114	5.947 ± 0.126	5.557 ± 0.091	5.190 ± 0.116	5.753 ± 0.089
0.25									
At 120–180 min	3.771 ± 0.068	2.177 ± 0.054	2.542 ± 0.053	7.092 ± 0.229	5.890 ± 0.070	6.049 ± 0.066	5.417 ± 0.170	4.939 ± 0.072	5.683 ± 0.102
At 270–300 min	4.719 ± 0.070	2.638 ± 0.063	3.116 ± 0.065	8.262 ± 0.189	6.574 ± 0.063	6.768 ± 0.067	5.925 ± 0.065	5.392 ± 0.058	5.978 ± 0.205
0.5									
At 120–180 min	3.485 ± 0.132	2.049 ± 0.070	2.332 ± 0.091	7.214 ± 0.190	5.715 ± 0.117	5.700 ± 0.134	5.472 ± 0.092	5.146 ± 0.089	5.540 ± 0.130
At 270–300 min	5.242 ± 0.111	3.283 ± 0.065	3.548 ± 0.114	8.944 ± 0.197	7.156 ± 0.142	6.802 ± 0.121	6.283 ± 0.108	5.751 ± 0.104	6.414 ± 0.107
1.0									
At 120–180 min	3.649 ± 0.185	2.085 ± 0.076	2.472 ± 0.076	7.148 ± 0.189	5.713 ± 0.131	5.811 ± 0.120	5.383 ± 0.082	5.037 ± 0.073	5.582 ± 0.091
At 270–300 min	5.660 ± 0.210	3.763 ± 0.141	3.602 ± 0.063	9.060 ± 0.190	7.410 ± 0.188	6.789 ± 0.105	6.359 ± 0.088	6.022 ± 0.087	6.514 ± 0.087

Data are means ± SE of four measurements obtained between the time points indicated. AV, arterial vein; FV, femoral vein; HV, hepatic vein.

TABLE 4
Isotopic enrichment of phenylalanine and tyrosine in the arterial, femoral, and hepatic veins at baseline (120–180 min) and during the last 30 min (270–300) of infusion at different dosages of insulin or saline

Insulin dosage	[15N]phenylalanine			[15N]tyrosine			[2H4]tyrosine		
(mU · kg^{-1} · min^{-1})	AV	FV	HV	AV	FV	HV	AV	FV	HV
0.0									
At 120–180 min	8.418 ± 0.088	6.581 ± 0.135	6.791 ± 0.112	1.327 ± 0.034	1.048 ± 0.035	1.330 ± 0.041	7.879 ± 0.112	6.030 ± 0.141	6.177 ± 0.123
At 270–300 min	8.594 ± 0.108	6.942 ± 0.104	7.042 ± 0.104	1.187 ± 0.035	0.878 ± 0.030	1.266 ± 0.029	8.155 ± 0.085	6.489 ± 0.124	6.566 ± 0.097
0.25									
At 120–180 min	8.531 ± 0.111	6.612 ± 0.093	7.025 ± 0.080	1.387 ± 0.037	1.073 ± 0.035	1.423 ± 0.039	8.271 ± 0.163	6.302 ± 0.118	6.753 ± 0.123
At 270–300 min	9.372 ± 0.115	7.250 ± 0.083	7.647 ± 0.099	1.297 ± 0.053	0.938 ± 0.045	1.292 ± 0.047	9.245 ± 0.131	7.020 ± 0.100	7.447 ± 0.081
0.5									
At 120–180 min	8.356 ± 0.187	6.826 ± 0.146	6.622 ± 0.134	1.300 ± 0.040	1.055 ± 0.035	1.293 ± 0.052	8.124 ± 0.194	6.536 ± 0.169	6.411 ± 0.152
At 270–300 min	9.526 ± 0.133	7.965 ± 0.140	7.721 ± 0.098	1.281 ± 0.056	1.019 ± 0.039	1.241 ± 0.058	9.392 ± 0.140	7.672 ± 0.158	7.694 ± 0.124
1.0									
At 120–180 min	8.841 ± 0.240	7.007 ± 0.146	7.025 ± 0.087	1.397 ± 0.054	1.257 ± 0.052	1.324 ± 0.047	8.168 ± 0.257	6.532 ± 0.189	6.670 ± 0.162
At 270–300 min	10.110 ± 0.222	8.502 ± 0.149	7.966 ± 0.110	1.350 ± 0.049	1.150 ± 0.043	1.144 ± 0.048	9.991 ± 0.313	8.234 ± 0.227	8.182 ± 0.218

Data are means ± SE of four measurements obtained between the time points indicated. AV, arterial vein; FV, femoral vein; HV, hepatic vein.

nation when normalized for leucine concentration (demonstrating that the changes in leucine transamination are not entirely related to the fall in leucine concentration) are given in Fig. 3. All three of these parameters were decreased significantly by insulin.

Regional protein dynamics. Figure 4 shows the percentages of change of protein synthesis (phenylalanine to protein) and protein breakdown (protein to phenylalanine) as a function of insulin dosage. It is shown that insulin had no significant effect on protein synthesis in the leg, whereas insulin significantly decreased protein synthesis in the splanchnic region. The maximal insulin effect on splanchnic protein synthesis was achieved at 0.5 mU · kg^{-1} · min^{-1} of insulin. Regional phenylalanine appearance (protein breakdown) demonstrated a different pattern. Insulin had no significant effect on protein breakdown in the splanchnic region, but it decreased protein breakdown significantly in the leg, and a dose effect was observed. The percentages of change of tyrosine appearance rate in the leg (another measure of protein breakdown) also decreased progressively with the increasing insulin dosage (0 insulin = −7.6 ± 2.5, 0.25 mU · kg^{-1} · min^{-1} insulin = 11.4 ± 1, 0.5 mU · kg^{-1} · min^{-1} insulin = 16.3 ± 2, and 1.0 mU · kg^{-1} · min^{-1} = 22.3 ± 1; $P < 0.01$; $r = 0.87$). Tyrosine disappearance rate, a measure of protein synthesis, did not show any changes with insulin. Insulin had no significant effect on the conversion of phenylalanine to tyrosine in the splanchnic region (data not shown).

Leucine carbon flux across leg and splanchnic regions are given in Fig. 5. Leucine carbon flux across the leg region decreased significantly only at the maximal dosage of insulin ($P < 0.05$), although it tended to decrease with increasing dosage of insulin. No such changes were noted across the splanchnic region. Figure 5 also shows the percentages of change of regional leucine nitrogen flux and transamination (reamination) as a function of the dosage of insulin infused. Leucine nitrogen flux decreased significantly in both the leg and splanchnic regions. Leucine transamination decreased significantly as the dosage of insulin infusion increased. However, the declines in leucine nitrogen flux and transamination rate were significantly higher in the leg than in the splanchnic region as the dosage of insulin infusion was increased ($P < 0.01$).

TABLE 5
Concentrations of leucine, phenylalanine, and tyrosine (expressed as micromoles per liter) in the arterial, femoral, and hepatic veins at baseline (120–180 min) and during the last 30 min (270–300) of infusion at different dosages of insulin or saline

Insulin dosage	Leucine			Phenylalanine			Tyrosine		
(mU · kg^{-1} · min^{-1})	AV	FV	HV	AV	FV	HV	AV	FV	HV
0.0									
At 120–180 min	135.34 ± 4.95	145.75 ± 4.68	141.68 ± 5.74	54.77 ± 1.36	59.63 ± 1.63	50.14 ± 1.50	44.34 ± 3.80	48.43 ± 4.05	35.33 ± 3.33
At 270–300 min	149.61 ± 5.12	154.70 ± 11.71	141.65 ± 5.16	55.94 ± 1.11	59.83 ± 4.64	49.49 ± 1.27	43.93 ± 3.97	45.86 ± 4.70	34.20 ± 3.42
0.25									
At 120–180 min	129.29 ± 6.62	134.84 ± 6.43	129.68 ± 7.20	50.24 ± 2.19	54.44 ± 2.59	44.69 ± 2.20	37.60 ± 3.02	41.05 ± 3.35	29.17 ± 2.45
At 270–300 min	95.64 ± 5.49	98.62 ± 6.83	95.49 ± 5.21	46.55 ± 2.27	49.49 ± 3.75	42.96 ± 2.26	32.59 ± 2.64	35.77 ± 4.05	27.23 ± 2.15
0.5									
At 120–180 min	126.33 ± 2.99	134.37 ± 3.28	130.58 ± 4.08	59.27 ± 1.16	64.72 ± 1.35	50.06 ± 1.44	60.40 ± 2.03	64.85 ± 1.80	44.74 ± 1.42
At 270–300 min	78.92 ± 3.06	81.99 ± 2.83	79.41 ± 3.71	49.62 ± 1.21	51.75 ± 1.44	45.27 ± 1.56	48.25 ± 1.80	49.52 ± 1.83	37.03 ± 1.22
1.0									
At 120–180 min	131.86 ± 5.05	135.00 ± 4.32	131.79 ± 5.60	54.76 ± 2.16	60.61 ± 2.04	49.16 ± 1.94	55.03 ± 3.52	59.40 ± 3.62	43.04 ± 2.64
At 270–300 min	74.81 ± 3.98	75.74 ± 6.32	71.49 ± 3.36	45.34 ± 1.51	45.75 ± 4.42	44.00 ± 1.27	41.67 ± 3.50	41.66 ± 4.88	35.00 ± 3.06

Data are means ± SE of four measurements obtained between the time points indicated and expressed as micromoles per liter. AV, arterial vein; FV, femoral vein; HV, hepatic vein.

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TABLE 6
Whole body flux values of leucine carbon, phenylalanine, tyrosine, and phenylalanine to protein

	Saline		Insulin ($mU \cdot kg^{-1} \cdot min^{-1}$) 0.25		0.50		1.0	
	Baseline	Infusion	Baseline	Infusion	Baseline	Infusion	Baseline	Infusion
Leucine carbon flux	87 ± 6	88 ± 3	90 ± 8	75 ± 4*	88 ± 6	69 ± 4*	89 ± 6	66 ± 4*
Phenylalanine flux	44 ± 1	43 ± 1	43 ± 1	39 ± 1*	43 ± 2	38 ± 1*	41 ± 2	36 ± 2*
Tyrosine flux	33 ± 1	31 ± 1	32 ± 2	28 ± 1*	32 ± 2	27 ± 1*	33 ± 2	26 ± 2*
Phenylalanine to protein	39 ± 1	39 ± 1	38 ± 1	35 ± 1*	39 ± 2	34 ± 1*	37 ± 2	33 ± 2*

Data are means ± SE. *$P < 0.01$ vs. baseline.

Figure 6 shows the protein synthesis and breakdown in the muscle mass (assuming that the leg constitutes 25% of whole body muscle mass) and splanchnic regions during either saline or insulin infusion. These measurements are based on the phenylalanine model. During the normal saline infusion, protein breakdown in skeletal muscle was significantly higher than protein synthesis, whereas in the splanchnic region, protein synthesis was significantly higher than protein breakdown. These differences disappeared progressively with the increasing dosages of insulin, so that at the maximal dosage of insulin (1.0 $mU \cdot kg^{-1} \cdot min^{-1}$), there were no differences between protein synthesis and breakdown in both leg and splanchnic regions.

Net balance of phenylalanine and tyrosine as a function of dosage of insulin infused is shown in Fig. 7. The splanchnic region was in a positive net balance of amino acids in the postabsorptive state. As the dosage of insulin infused was increased, the positive net balance of phenylalanine and tyrosine in the splanchnic region was significantly decreased, so that phenylalanine balance was not different from zero at the maximal dosage of insulin. Conversely, the leg was in a negative net balance of phenylalanine and tyrosine in the postabsorptive state. Insulin decreased this negative balance, and at the maximal dosage of insulin, both phenylalanine and tyrosine balances were not different from zero. Similar insulin effects were observed on all of the above parameters when

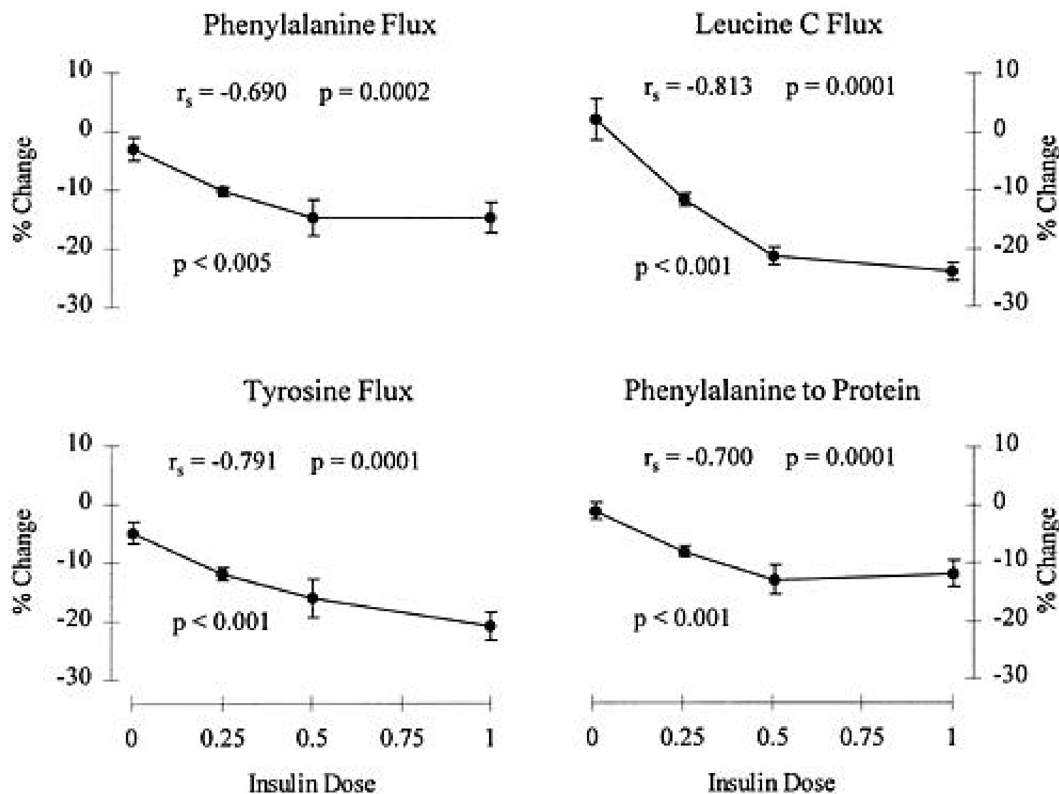

FIG. 2. Whole body amino acid flux and phenylalanine-to-protein (incorporation). Phenylalanine, tyrosine, leucine carbon flux, and phenylalanine incorporation into protein decreased as the dosage of insulin infused increased, although no changes were found between the insulin dosages 0.5 and 1.0 $mU \cdot kg^{-1} \cdot min^{-1}$. *P* values are based on one-way ANOVA and demonstrate the effect of insulin versus normal saline. The r_s values represent the correlations based on Spearman's rank correlation analysis, and the *P* values adjacent to the r_s values show the levels of significance based on this analysis.

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TABLE 7
Whole body flux values of leucine nitrogen and transamination (leucine to KIC)

	Saline		Insulin (mU · kg^{-1} · min^{-1}) 0.25		0.50		1.0	
	Baseline	Infusion	Baseline	Infusion	Baseline	Infusion	Baseline	Infusion
Leucine nitrogen flux	174 ± 10	190 ± 11*	168 ± 6	132 ± 4†	184 ± 16	117 ± 5†	181 ± 19	108 ± 9†
Leucine to KIC	87 ± 10	101 ± 10*	77 ± 5	56 ± 5†	95 ± 11	48 ± 5†	91 ± 14	40 ± 7†

Data are means ± SE. *$P < 0.05$ vs. baseline; †$P < 0.01$ vs. baseline.

changes in circulating insulin were used instead of insulin infusion rates (data not shown).

DISCUSSION

The current study demonstrated that during the postabsorptive state, skeletal muscle was in a catabolic state with protein breakdown exceeding protein synthesis. In contrast, protein synthesis exceeded protein breakdown in the splanchnic bed. As a result, there was a net release of amino acids from skeletal muscle and a net uptake of amino acids in the splanchnic bed. Insulin decreased the net release of amino acids from leg and the net uptake of amino acids in the splanchnic bed by differential effects on protein breakdown and synthesis in skeletal muscle and splanchnic beds. Whereas insulin decreased protein breakdown in the skeletal muscle in a dose-dependent manner, insulin had no effect on protein breakdown in the splanchnic bed. In contrast, whereas protein synthesis in skeletal muscle was not affected by insulin, protein synthesis in the splanchnic bed was significantly reduced. The overall effect was to abolish the differences between protein breakdown and synthesis in both splanchnic and muscle tissues (Fig. 6), thereby achieving net amino acid balance in both of these tissue beds (Fig. 7).

The current study clearly demonstrated that insulin has differential effects on skeletal muscle and splanchnic tissues. Several previous studies demonstrated that insulin decreases whole body protein breakdown in people with type 1 diabetes (3,5,6,24,25) and in healthy subjects (7,26–28). Whole body studies in which insulin was administered systemically were performed, and a dose-related decline in protein breakdown was reported (7,26). When insulin was administered intra-arterially into the forearm muscle bed (8), the maximal inhibitory effect on protein breakdown was reported to occur at an insulin level similar to that achieved in our study with the 0.25 mU · kg^{-1} · min^{-1} insulin infusion. In another study, intra-arterial insulin administration was reported to have no effect on protein breakdown in the leg muscle bed (29). The difference in the conclusions derived from these two studies (8,29) cannot be explained entirely by the different mathematical models used. It is unlikely that the differences between the studies could be due to the differences between the two muscle beds into which insulin was infused, because the muscle fiber types in human subjects do not differ substantially among various muscle groups.

In the current study, we administered insulin systemically and measured protein breakdown across the leg and the splanchnic region. We observed that protein breakdown in the leg was decreased in a dose-dependent manner. The significant effect on protein breakdown was based on measure-

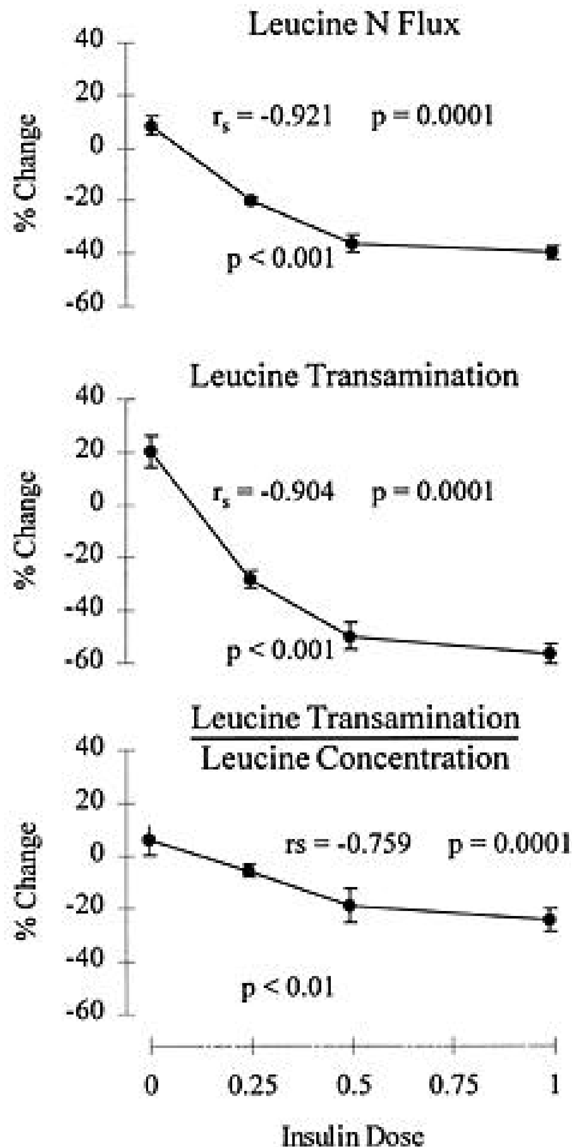

FIG. 3. Whole body leucine nitrogen flux and transamination as a function of dosage of insulin infused. *P* values represent the significance based on one-way ANOVA and demonstrate the effect of insulin versus normal saline. The r_s values represent the correlations based on Spearman's rank correlation analysis, and the *P* values adjacent to the r_s values show the levels of significance based on this analysis. Leucine nitrogen flux and transamination decreased as the dosage of insulin infused was increased. Leucine transamination decreased with increasing insulin dosage even after normalization for leucine concentration.

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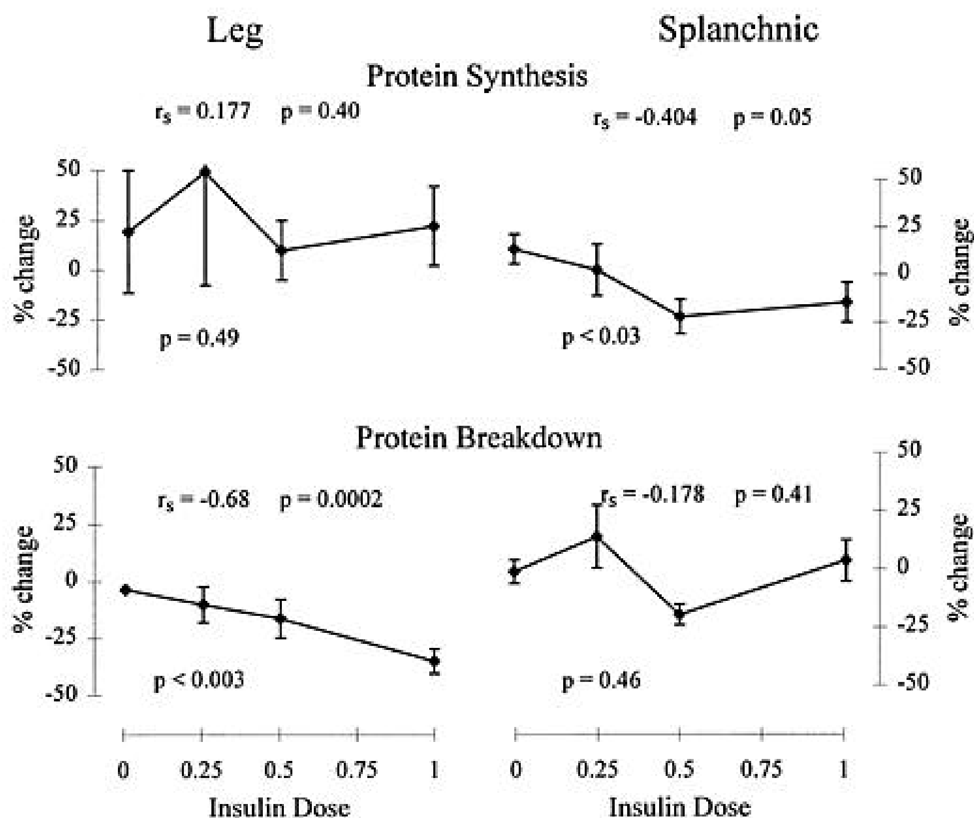

FIG. 4. Regional protein synthesis and breakdown (phenylalanine model) as a function of dosage of insulin infused. *P* values represent the significance based on one-way ANOVA and demonstrate the effect of insulin versus normal saline. The r_s values represent the correlations based on Spearman's rank correlation analysis, and the *P* values adjacent to the r_s values show the levels of significance based on this analysis. Insulin decreased protein synthesis in the splanchnic region but not in the leg. Insulin decreased protein breakdown in the leg but not in the splanchnic region.

ments using tracers of both phenylalanine and tyrosine. Based on leucine carbon flux across the leg, there was a significant effect of insulin only at the maximal dosage. The use of phenylalanine and tyrosine as tracers of protein dynamics across muscle bed has several advantages over leucine (30). These two amino acids are not synthesized de novo and are not metabolized in skeletal muscle. The only fate of these amino acids in the muscle tissue is their incorporation into muscle protein. In addition, these two amino acids have low intracellular concentrations, thereby allowing faster equilibration with extracellular compartments. It is likely that arteriovenous studies using isotopes of phenylalanine and tyrosine in the leg are more representative of intracellular events than are studies based on isotopes of amino acids such as lysine and leucine. Lysine and leucine have relatively higher intracellular pools, thereby making it less likely for them to equilibrate with extracellular compartments during a short study period. In addition, not only protein breakdown but also leucine transamination determines leucine concentration, and leucine concentration is an important factor in the calculation of protein breakdown. It is therefore likely that the results based on the dilution of phenylalanine and tyrosine are more reliable as measures of muscle protein breakdown than are those based on leucine.

In contrast to its effect on muscle protein breakdown, insulin had no significant effect on protein breakdown in the splanchnic bed. The only previous study of the effect of insulin on protein breakdown in the splanchnic region was performed in people with type 1 diabetes (3). In C-peptide–negative individuals with type 1 diabetes, it was demonstrated that protein breakdown and synthesis in the splanchnic bed increased during insulin deprivation and that insulin replacement decreased both the breakdown and synthesis of splanchnic proteins (3). Taken together, these data suggest that increasing insulin above the baseline level during the postabsorptive state has no additional inhibitory effect on protein breakdown in the splanchnic region. In contrast, a progressive decline in muscle protein breakdown was observed as the insulin dosage was increased, indicating that protein breakdown is differentially regulated in the splanchnic and skeletal muscle beds.

The current study also demonstrated that insulin has differential effects on protein synthesis in splanchnic tissues and skeletal muscle. There was no effect of insulin on protein synthesis in the skeletal muscle, whereas in the splanchnic bed, protein synthesis was inhibited by insulin. Several previous studies performed in type 1 diabetic patients (3,31,32) as well as healthy subjects (27,33,34) demonstrated that insulin has no effect on muscle protein synthesis, which was contradicted by one human study (35) and several animal studies (12,36–38). The current study demonstrated that insulin in concentrations spanning the physiological range failed to stimulate muscle protein synthesis. The reported increase in muscle protein synthesis in rodents appears to be related to the fact that the initial studies were performed in growing animals and that when older rats were studied, as in studies of

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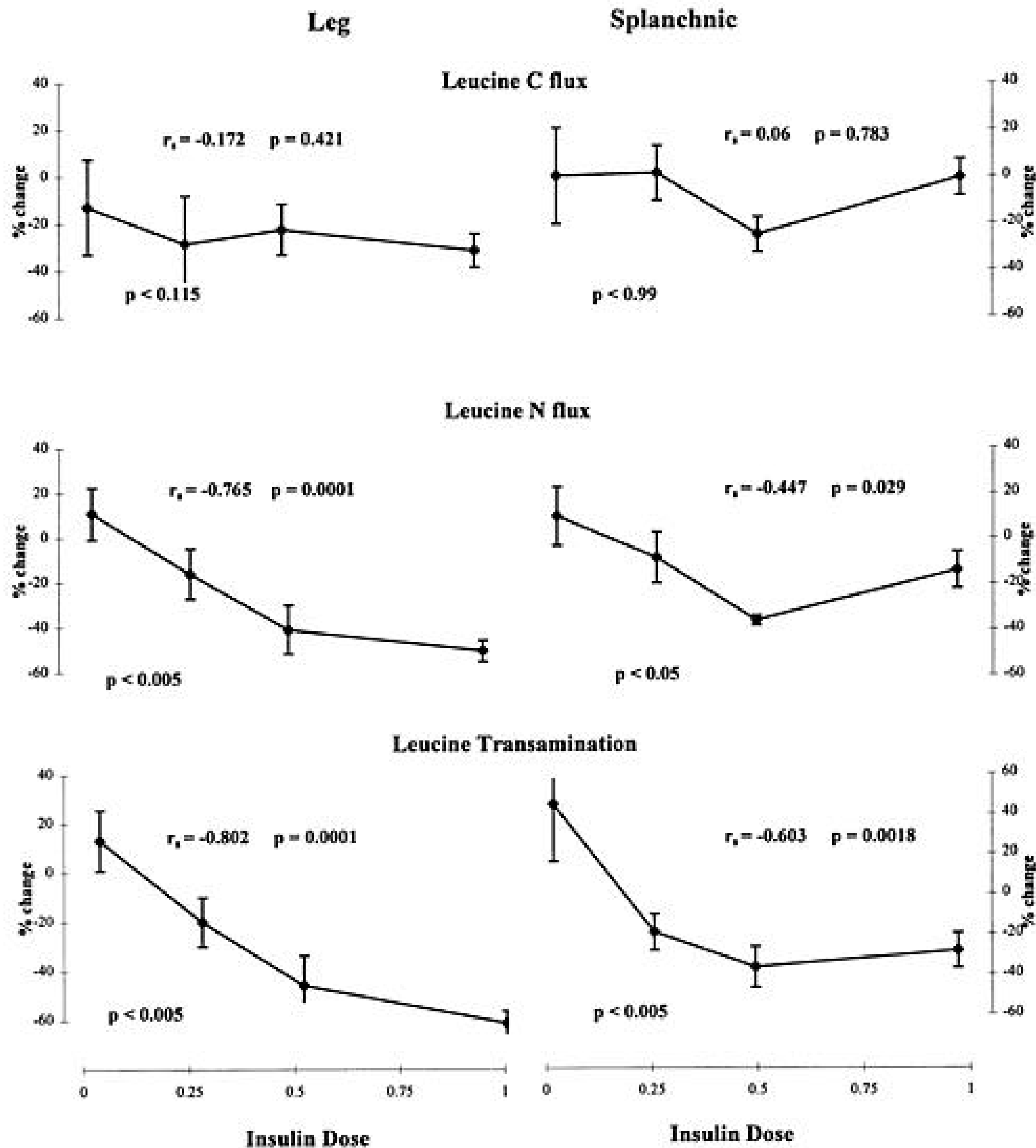

FIG. 5. Regional leucine carbon flux, regional leucine nitrogen flux, and leucine transamination as a function of dosage of insulin infused. *P* values represent the significance based on one-way ANOVA and demonstrate the effect of insulin versus normal saline. The r_s values represent the correlations based on Spearman's rank correlation analysis, and the *P* values adjacent to the r_s values show the levels of significance based on this analysis. Leucine transamination decreased as the dosage of insulin infused increased, but the magnitude of decrease was greater in the leg than in the splanchnic region.

adult humans, insulin had no effect on muscle protein synthesis (39). In contrast to findings in the muscle tissue, insulin decreased protein synthesis in the splanchnic bed in the current study. This differential effect of insulin on protein synthesis in two different tissues is intriguing. We previously hypothesized that insulin deprivation in type 1 diabetic patients causes an increase in flux of amino acids from the muscle bed, which in turn stimulates protein synthesis in the splanchnic bed (3,9). The above hypothesis was supported by the observation in animal studies demonstrating that gut protein synthesis was higher during insulin deprivation than during insulin treatment (10). In addition, an increased synthesis rate of fibrinogen was reported in people with type 1 diabetes during insulin deprivation (40). It is possible, therefore, that the insulin-induced fall in the protein synthesis rate of the splanchnic bed is related to the decline in circulating amino acids, and that this decline in amino acids is related to inhibition of protein breakdown.

Prevention of amino acid fall by simultaneous infusion of amino acid mixture with insulin administration may help to determine whether insulin-induced fall in protein synthesis in splanchnic bed is a direct effect of insulin or is secondary to a fall in circulating amino acids. The current experiment demonstrated that the protein conservation in the body that occurs with insulin administration is achieved by a complex mechanism. The overall effect of insulin appears to be the reduction of the difference between protein breakdown and synthesis in different tissues.

These studies demonstrating a positive amino acid balance across splanchnic bed should not be interpreted as evidence of increased protein mass occurring in this tissue bed. It is more likely that this positive amino acid balance repre-

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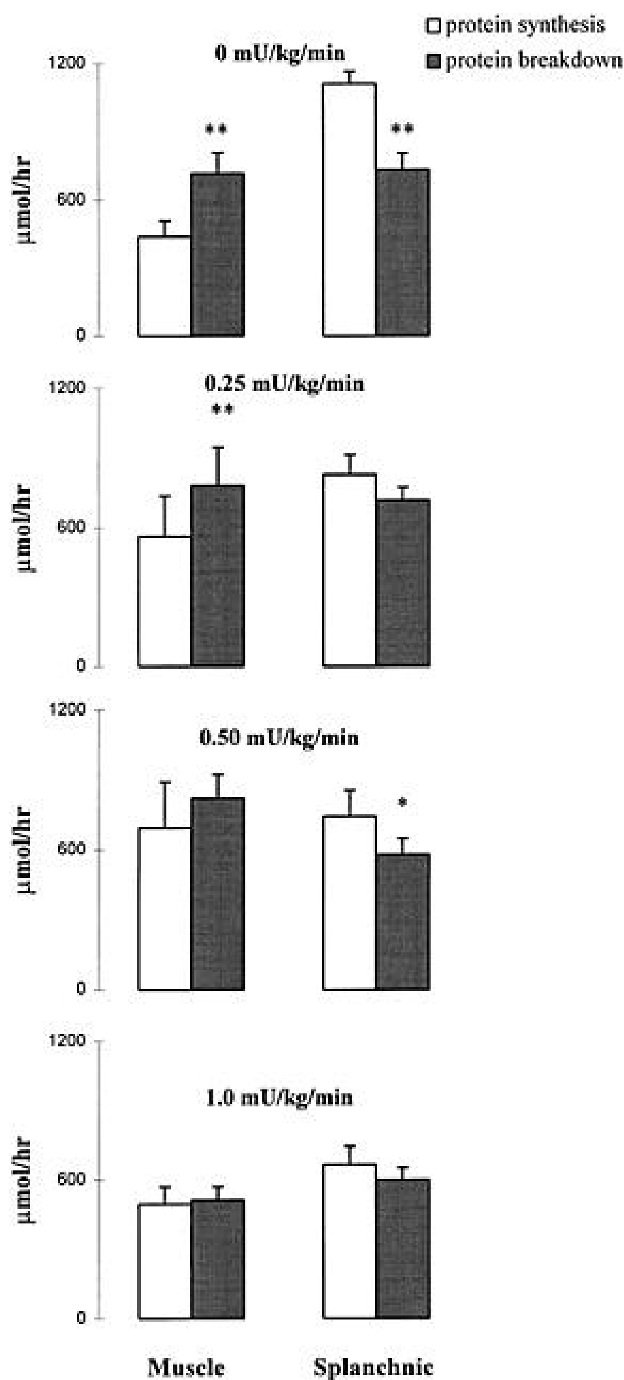

FIG. 6. Muscle protein synthesis (□) and breakdown (■) from phenylalanine model demonstrating greater protein breakdown than synthesis in the leg and greater protein synthesis than breakdown in the splanchnic region during saline infusion (0 mU · kg^{-1} · min^{-1} insulin). Significant differences between protein breakdown and synthesis are indicated: *P < 0.001; **P < 0.05. With increasing insulin dosages, these differences disappeared, with the same breakdown and synthesis occurring at 1.0 mU · kg^{-1} · min^{-1} insulin.

sents the production of circulating proteins in liver and other splanchnic tissues. Circulating proteins such as coagulation factors, other acute reaction proteins, and many other transfer proteins binding to hormones are essential for body functions, and their synthesis should be continued without interruption, irrespective of whether or not the whole organism is in a positive nitrogen balance. The current study demonstrated that skeletal muscle plays a crucial role in providing amino acids during the postabsorptive state to the splanchnic bed. Only measurements of amino acids and flow in the portal vein will allow us to separately measure the events in the liver and splanchnic bed. Such studies are not yet possible in human subjects; therefore, the relative changes in liver and gut during insulin infusion remain to be defined.

Insulin also has an inhibitory effect on leucine transamination. Leucine transamination is indicated by a greater leucine nitrogen flux than leucine carbon flux, and the differences between the two fluxes are a putative measure of KIC reamination to leucine. Leucine deamination to KIC can be measured only if leucine oxidation is also measured. The natural ^{13}C abundance in carbohydrates and the dextrose infusion of variable amount make the measurements of leucine oxidation unreliable. Leucine nitrogen flux minus leucine carbon flux, however, is a reliable index of leucine transamination. This crucial biochemical process, by which amino groups are transferred from one amino acid to another, plays a key role in nitrogen economy and the interorgan transfer of nitrogen (41). Part of insulin's effect on transamination may be due to a decline in the concentration of leucine, which is related to reduced protein breakdown. However, even after normalization for leucine concentration (Fig. 3), leucine transamination continued to decrease, thereby suggesting that insulin has a direct effect on leucine transamination that is independent of the decrease in leucine concentration. It is possible, however, that leucine concentration at the site of transamination (the intracellular compartment) is not truly reflected by the plasma levels. Current studies show that insulin's effect on leucine transamination is more pronounced in the skeletal muscle than in the splanchnic tissue. The relative insensitivity of leucine transamination to insulin in the splanchnic tissue and the inability of insulin to inhibit protein breakdown in the splanchnic tissue suggest that although circulating insulin levels are high, the amino acid flux from the splanchnic tissue remains uninterrupted.

Given that leucine transamination is a key biochemical process for providing amino nitrogen for glutamine and alanine synthesis (41,42), the relative lack of an insulin effect on leucine transamination in the splanchnic region may be crucial for the synthesis of alanine and glutamine. These amino acids are also provided by protein breakdown, which is also unaffected by insulin in the splanchnic region. Continuous supply of these amino acids, which are precursors of gluconeogenesis, ensures that gluconeogenesis continues without interruption, even during insulin-induced hypoglycemia. Recent studies in animals indicate that the amino acid supply from gut proteins is the major source for glucose recovery from hypoglycemia (43,44). In addition, amino acid availability is crucial for synthesis of many acute reaction proteins in liver. The relative lack of an insulin effect on amino acid appearance in the splanchnic bed ensures this amino acid supply in the presence of a progressive fall in muscle protein breakdown.

In summary, the current study demonstrated that insulin has a differential effect on protein metabolism in splanchnic and skeletal muscle tissues. During the postabsorptive state, there is a net loss of protein from the skeletal muscle because protein breakdown is higher than protein synthesis. Insulin decreases this net protein loss of skeletal mus-

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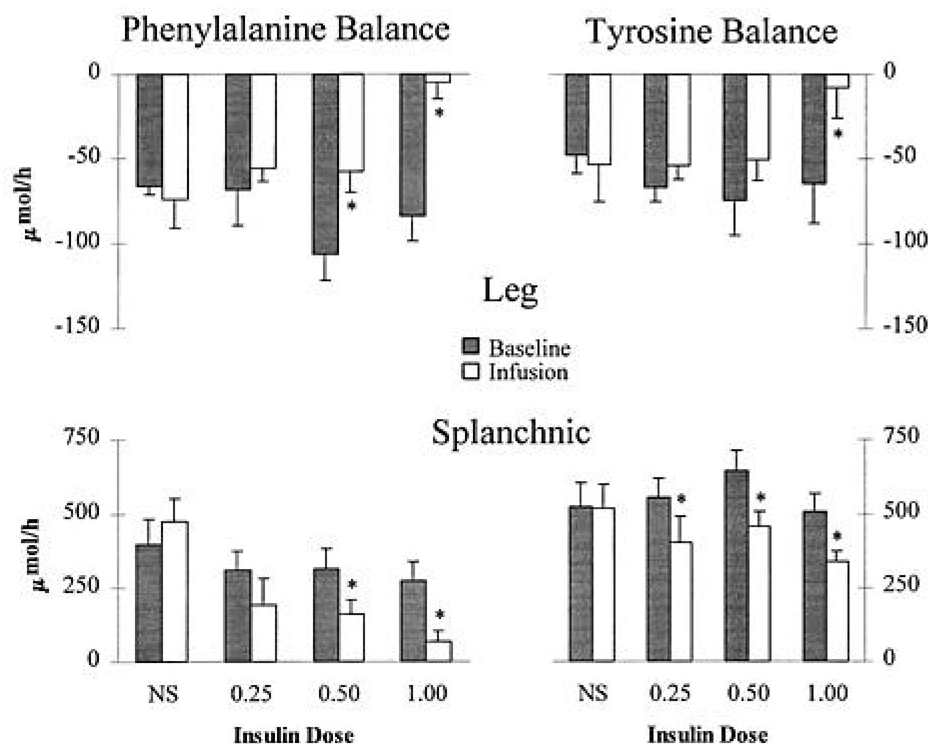

FIG. 7. Net balance of phenylalanine and tyrosine as a function of dosage of insulin infused. Asterisks indicate significant differences from the baseline value ($P < 0.01$). The splanchnic region was in a positive net balance of phenylalanine and tyrosine, and these positive amino acid balances were reduced with insulin infusion. Phenylalanine balance decreased to a level not different from zero at the highest dosage of insulin. The leg was in a negative net balance of these amino acids, and as the insulin dosages were increased, these negative balances were reduced to a level not different from zero.

cle by inhibiting protein breakdown without affecting protein synthesis. In splanchnic tissue, there is a net increase of protein accretion during the postabsorptive state because protein synthesis exceeds protein breakdown. This net positive balance of amino acids across splanchnic bed may represent continued synthesis of essential circulating proteins such as coagulation factors. Insulin decreases protein synthesis in the splanchnic tissue without affecting protein breakdown. It is likely that this decrease in protein synthesis in splanchnic bed occurs because of decreased amino acid supply from muscle. Insulin also has an inhibitory effect on whole body leucine transamination in skeletal muscle and, to a lesser extent, in the splanchnic tissue. In conclusion, insulin's effects on protein metabolism in the human body is complex, and the effects of insulin on splanchnic and skeletal muscle beds while achieving net amino acid balance are substantially different.

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Author Queries (please see Q in margin and underlined text)

Q1: OK to add "mg/dl" to "70–100" [fasting blood glucose]?

Q2: Is "lasting between 150 and 300 min" as meant?

Q3: For clarity, please replace slash in "gas chromatography/mass spectrometry with appropriate term (i.e., "and," "or," or "and/or"). Thanks.

Q4: Should "b" be Greek beta in "b-methyl-phenylalanine hydrochloride" (throughout)?

Q5: OK to change "um" to "μm" in "30 mm × 0.25 mm × 0.25 μm"?

Q6: Please provide expansion of the abbreviation "EI." Thanks.

Q7: Please confirm that equations are correct as given throughout entire "Calculations" section, and indicate any needed revisions. Thanks.

Q8: Does "[^{15}N,1-^{13}C]leucine" appear correctly?

Q9: OK to change chem dots to multiplication signs for consistency in "LAC × (EA leu/EV – 1) × F"?

Q10: Is "EA leu and EV represent isotopic enrichment of [^{13}C]leucine or [^{15}N,^{13}C]leucine in artery and vein, respectively" as meant?

Q11: OK to make "d" subscript for rate of disappearance of phenylalanine (R_d)?

Q12: Should definition be given for "r^s"?

Q13: Replace "given in the figures" with "given in Figs. 1–5"?

Q14: Tables 3 and 4—Are there any units that should be specified for isotopic enrichment values?

Q15: Tables 3–5: Please confirm that statements given in table footnotes ("Data are…") are correct.

Q16: Table 6—Correct that all P values for this table were <0.01? A single asterisk was keyed on original version as <0.05, but there are no single asterisks on original version, only double asterisks. Also, is wording of table footnote correct as given?

Q17: Is wording of table footnote correct as given?

Q18: Figure 2 legend—"phenylalanine-to-protein flux" OK? (As in text)

Q19: Figure 3 legend—Please refer to the bottom part of figure, "Leucine Transamination/Leucine Concentration," in the legend.

Q20: Is second part of the sentence beginning "Based on leucine carbon flux across the leg, there was…" OK as edited? As meant?

QUERIES CONTINUED NEXT PAGE

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Q21: Figure 5 legend—OK to add "Regional leucine carbon flux" to beginning of legend (both carbon and nitrogen flux are shown)?

Q22: Is "simultaneous infusion of amino acid mixture with insulin administration" edited as meant? Original text read: "simultaneous infusion of amino acid mixture insulin insulin administration."

Q23: Does "acute reaction proteins" mean "acute phase proteins"? (Could find only the latter term in our sources.) If so, do you wish to change both uses of the term in text?

Q24: OK to change "Since there is a" to "The" in the sentence "The natural ^{13}C abundance in carbohydrates and…unreliable"? As meant?

Q25: See query 23 re: "acute reaction proteins."

Q26: Acknowledgments—Please provide expansion for "GCRC," if possible. Thanks.

Ref. 1: Please confirm that journal title abbreviation and year are correct.

Ref. 30—If possible, please provide page range.

Ref. 38—Please provide volume number.

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