Branched-chain Amino Acid Nitrogen Transfer to Alanine In Vivo in Dogs

DIRECT ISOTOPIC DETERMINATION WITH [^{15}N]LEUCINE

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ABSTRACT To investigate the contribution of branched-chain amino acids as a nitrogen source for alanine in vivo, dogs were infused with L-[^{15}N]leucine, L-[U-^{14}C]leucine, L-[2,3,3,3-$^{2}H_4$]alanine, and D-[6,6-$^{2}H_2$]-glucose. ^{14}C and ^{15}N isotopic equilibrium in plasma leucine, and deuterium enrichment in arterial and femoral plasma glucose and alanine were achieved within 3 h of initiation of the respective isotope infusion in all animals. The average flux of leucine determined by [^{15}N]-leucine was 5.4 μmol·kg^{-1}·min^{-1}, whereas using [^{14}C]-leucine it was 3.7 μmol·kg^{-1}·min^{-1}. Turnover rates for alanine and glucose were 11.0 and 17.2 μmol·kg^{-1}·min^{-1}, respectively.

[^{15}N]alanine was detected as early as 30 min, but nitrogen isotopic equilibrium in alanine was not achieved until 6 h. The absolute rate of leucine nitrogen transfer to alanine was 1.92 μmol·kg^{-1}·min^{-1}, which represented 41–73% (mean 53%) of leucine's nitrogen and 15–20% (mean 18%) of alanine's nitrogen.

Fractional extraction of alanine and leucine by the dog hindlimb was 35 and 24%, respectively. Average net alanine balance was −6.7 μmol·leg^{-1}·min^{-1}, reflecting a release rate (17.4 μmol·kg^{-1}·min^{-1}) that exceeded the rate of uptake (10.8 μmol·leg^{-1}·min^{-1}). Of the leucine taken up by the hindlimb, 34% transferred its nitrogen to alanine and 8% was oxidized to CO_2. Since the latter value reflects transamination as well as irreversible catabolism, the nitrogen derived from the oxidation of leucine by the hindlimb could account for only 25% of the observed ^{15}N incorporation into alanine.

The significantly faster flux of leucine nitrogen when compared with leucine carbon suggests significant recycling of the leucine α-ketoacid. These studies demonstrate that leucine is a major donor of nitrogen to circulating alanine in vivo.

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INTRODUCTION

During caloric deprivation, muscle releases alanine in excess of this amino acid's representative content in muscle protein (1). The released alanine is presumably synthesized within the myocyte from pyruvate and glutamate via glutamate-pyruvate transaminase (2, 3). Therefore, alanine production is dependent upon the relative availability of both pyruvate and glutamate. In vitro studies (2–4) have suggested that branched-chain amino acid (BCAA)[1] catabolism may be rate limiting for alanine production. Since BCAA are transaminated with α-keto glutarate forming the α-keto acid of the BCAA and glutamate, respectively (5), alanine production may be limited by the availability of glutamate as a nitrogen donor.

The decreased alanine production rate recently observed in children with maple syrup urine disease, an inborn error of branched-chain α-ketoacid dehydrogenase, supports a role of BCAA in alanine production in vivo (6). However, there is no direct evidence on the magnitude of BCAA nitrogen transfer to circulating alanine in vivo. The present study directly examines the incorporation of ^{15}N derived from leucine into alanine and other amino acids in the whole animal as well as across the isolated hindlimb of conscious sling-trained dogs.

METHODS

Materials. L-[^{15}N]leucine, L-[2,3,3,3-$^{2}H_4$]alanine ([$^{2}H_4$]alanine), and D-[6,6-$^{2}H_2$]glucose ([$^{2}H_2$]glucose) were obtained from Merck, Sharp & Dohme Canada Ltd, Quebec, Canada.

[1] *Abbreviations used in this paper*: [$^{2}H_4$]alanine, L[2,3,3,3,-$^{2}H_4$]alanine; BCAA, branched-chain amino acid; [$^{2}H_2$]glucose, D-[6,6-$^{2}H_2$]glucose.

Chemical and isotopic purity were confirmed by conventional gas chromatographic and mass spectrometric analytical techniques. L-[U-^{14}C]leucine (270 mCi/mmol) was obtained from New England Nuclear, Boston, Mass.

Routine analytical methods. Plasma glucose concentration was measured with a Beckman glucose analyzer (Beckman Instruments Inc., Fullerton, Calif.). Determination of plasma BCAA content was facilitated by a modification of the "physiologic" method routinely used with the automated Beckman 119 amino acid analyzer (7). Samples were run on Beckman PA-28 resin at 43°C using a 0.2 M sodium citrate buffer, pH 4.2. This method permitted rapid complete separation of leucine, isoleucine, and norleucine, the latter serving as internal standard for quantitation. Plasma alanine was determined microfluorometrically (8). Blood flow was calculated by established dye dilution principles using a constant indocyanine green infusion (9).

Radiotracer methods. Plasma [^{14}C]leucine content was determined using the same accelerated ion exchange procedure above with the exception that distilled water was run instead of ninhydrin. Column effluent fractions containing leucine were pooled, lyophilized to dryness, reconstituted with 1 ml of water to which 20 ml of scintillation cocktail was added, and radioactivity determined by conventional liquid scintillation counting corrected for quench by the external standard method. Specific activity of leucine was calculated by dividing the disintegrations per minute in leucine per milliliter plasma by the plasma leucine concentration (micromoles per milliliter). Recovery of [^{14}C]leucine added to plasma and run according to this procedure was 99±2%.

For determination of plasma $^{14}CO_2$ content, 1 ml of blood was anerobically injected into 1 ml of 3 M perchloric acid in a stoppered centrifuged tube with a hanging well (containing 200 μl of hyamine) attached to the inner aspect of the rubber stopper. The samples were agitated overnight in a 37°C incubation bath. The hanging well was then placed in 20 ml of scintillation cocktail and the radioactivity determined by liquid scintillation counting with external standard quench correlation. Recovery of $^{14}CO_2$ generated from ^{14}C sodium bicarbonate using this procedure was 96±1%.

Nonradioactive tracer methods. Isotopic enrichment of plasma glucose and amino acids was determined by selected ion monitoring gas chromatography mass spectrometry using either electron impact (10) or chemical ionization (11) techniques previously described which can measure ion current ratios with a relative precision of better than ±1%. A standard curve of known isotopic enrichments was run before samples of unknown enrichment.

Procedural methods. Healthy mongrel dogs were housed in the Animal Care Facility at Washington University and fed a Purina Dog Chow (Ralston Purina Co., St. Louis, Mo.) diet ad lib. 24 h before study, food was removed but the animals were allowed free access to water. On the morning of the experiment, dogs were given local anesthesia and five vascular catheters were placed: one in the saphenous vein, which was advanced to the femoral vein just distal to the bifurcation of the inferior vena cava; two percutaneous catheters in the femoral artery, one was threaded retrograde into the thoracic aorta for arterial blood sampling and the other advanced to a point distal to the bifurcation of the aorta on the ipsilateral side to the femoral vein catheter for indocyanine green infusion; and two were placed in peripheral forelimb veins for isotope infusions.

The conscious dog was then placed in a standing sling and rested for 30 min. At zero time a priming dose of L-[^{15}N]leucine (~2.5 μmol·kg^{-1}) was administered and immediately followed by a continuous infusion for 9 h at the rate of ~0.25 μmol·kg^{-1}·min^{-1}. At 3 h, a primed-constant infusion of indocyanine green (300 and 150 μg·h^{-1}, respectively) was initiated and continued to the end of the study. At 6 h of L-[^{15}N]leucine infusion, an additional primed-constant isotope infusion consisting of L-[^{14}C]leucine (~0.25 and ~0.025 μCi·kg^{-1}·min^{-1}), L-[$^{2}H_4$]alanine (~0.25 and ~0.15 μmol·kg^{-1}·min^{-1}), and [$^{2}H_2$]-glucose (~20 and ~0.3 μmol·kg^{-1}·min^{-1}) was begun and continued for the final 3 h of the study.

Blood was drawn simultaneously from the femoral vein and aorta into plastic syringes containing small amounts of heparin. 1.0 ml of blood was injected anaerobically in the rubber stoppered centrifuge tube for $^{14}CO_2$ determination immediately after blood sampling. The remaining blood was placed on ice, centrifuged at 4°C, separated, and the plasma stored at −80°C until assayed.

In a single dog, arterial blood alone was drawn throughout the 9 h of study to determine the time necessary to achieve ^{15}N isotopic equilibrium in circulating alanine and isoleucine. In three subsequent animals, simultaneous femoral vein and aortic samples were drawn at half-hourly intervals between 6 and 8 h and every 15 min during the final hour of study. In addition, 10 ml of blood were drawn from the femoral artery and vein at 8, 8½, and 9 h for blood flow determinations. Assumptions, definitions and equations used in the calculation of these data are found in the Appendix.

RESULTS

Fig. 1 shows the results of the single dog study to estimate attainment of isotopic steady state in both precursor and product during infusion of [^{15}N]leucine alone. Plateau ^{15}N enrichment in plasma leucine was reached after ~1 h of isotope infusion. Significant incorporation of ^{15}N into plasma alanine and isoleucine was detected by one-half hour. ^{15}N enrichment in these amino acids continued to rise to steady-state values at 4 h in the case of isoleucine and 7 h in the case of alanine. Thus, precursor-product relationships were studied over the last 2½-h interval of the 9-h multi-isotope infusion protocol in the remaining three animals (Fig. 2).

Average arterial glucose content of 5.6 mM remained unchanged for the duration of the study (Fig. 2). Mean plasma dideuteroglucose enrichment increased slightly

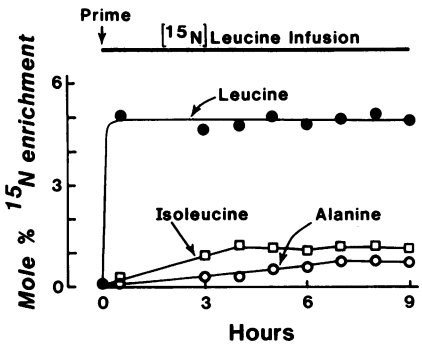

FIGURE 1 Mole percent enrichment of ^{15}N in plasma leucine, isoleucine, and alanine during a 9-h primed dose-constant infusion of [^{15}N]leucine (98 mol percent enrichment) in a single study.

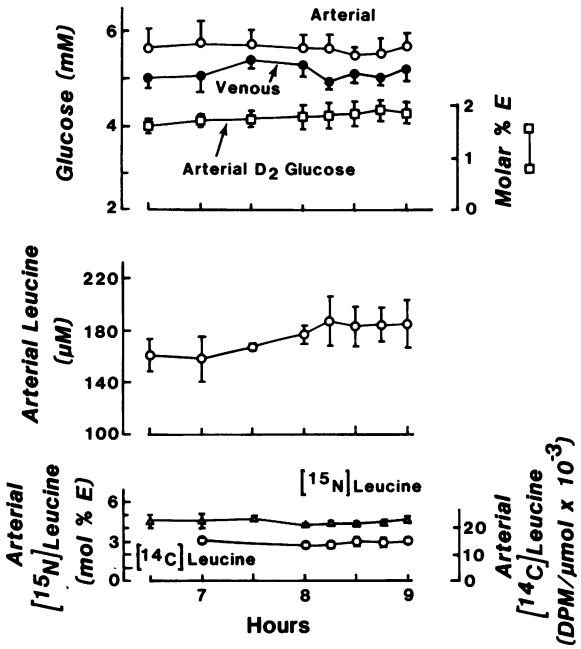

FIGURE 2 Plasma glucose and leucine concentrations, mole percent enrichment (E) of D-[6,6$^2H_2$]glucose (D_2 glucose) and [^{15}N]leucine and [^{14}C]leucine specific activity during the last 2½ h of simultaneous infusions of [$^2H_2$]glucose, [^{15}N]leucine, [$^2H_2$]alanine, and [U-^{14}C]leucine in three dogs. Data are presented as mean±SE.

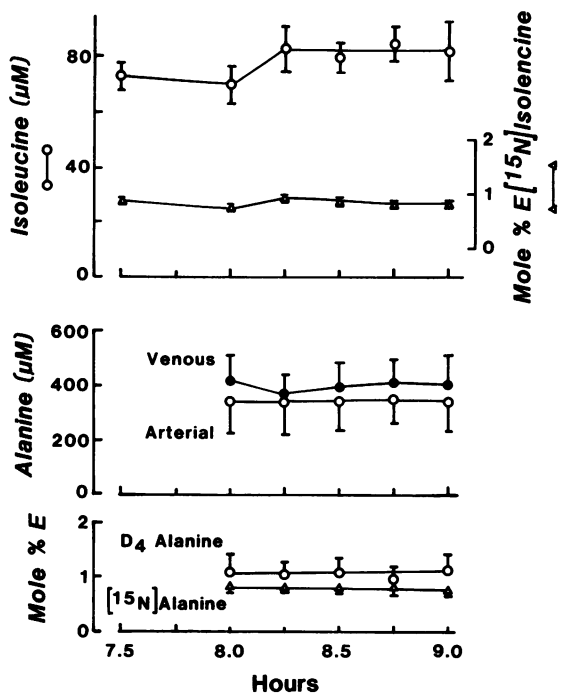

FIGURE 3 Plasma isoleucine and alanine concentrations and moles percent enrichment (E) of [^{15}N]isoleucine, [^{15}N]alanine and [2,3,3,3$^2H_2$]alanine (D_4 alanine) during the last 2½ h of a simultaneous infusion of [$^2H_2$]glucose, [^{15}N]leucine, [$^2H_4$]alanine, and [U-^{14}C]leucine in three dogs. Data are presented as mean±SE.

from the 6th to 8th h of study and remained constant during the final hour.

Plasma leucine concentration rose slightly from 160 μM at 6½ h to steady-state values of 176–184 μM between the 8th and 9th h of infusion whereas the plasma [^{15}N]leucine isotopic enrichment was constant (~4.5 mol percent excess) throughout the period of study. Likewise, plasma [^{14}C]leucine specific activity fluctuated about the steady-state mean of 14.8 dpm/μmol × 10^{-3} for the final 2 h of infusion.

Plasma isoleucine concentrations increased slightly during the course of the study (Fig. 3) from an average of 73 μM at the 7th h of infusion to 80–85 μM during the final 45 min. ^{15}N enrichment in plasma isoleucine was nearly constant (range, 0.75–0.90 mol percent excess) during the last 90 min of study.

There was a consistent negative plasma alanine arterial-venous difference with average venous and arterial alanine concentrations of 391 and 324 μM, respectively, during the last 60 min of study. During this hour, steady-state isotopic enrichment in both plasma [$^2H_4$]-alanine (~1.1 mol percent excess) and plasma [^{15}N]-alanine (~0.8 mol percent excess) were also maintained. The mean (±SE) for the final five substrate and isotopic determinations in each animal is presented in Table I and confirms that virtual steady state was achieved both in plasma content and in isotopic enrichment of glucose, alanine, and leucine during the final 60 min of study.

Since ^{15}N can be distributed throughout the entire body nitrogen pool by transamination, deamination, and reamination, the ^{15}N enrichment in other plasma amino acids at the 9th h of [^{15}N]leucine infusion was determined (Table II). In addition to ^{15}N present in the infused [^{15}N]leucine, significant incorporation of ^{15}N was found in the other two BCAA, isoleucine (1.01 mol percent excess) and valine (0.62 mol percent excess) as well as in serine (0.25 mol percent excess), glycine (0.17 mol percent excess), and ornithine (0.19 mol percent excess). No significant incorporation of ^{15}N was observed in threonine, aspartate, methionine, phenylalanine, tyrosine, or lysine.

Arterial substrate turnover. The average glucose (17.2 μmol·kg^{-1}·min^{-1}; range, 11.5–21.0) and alanine (11.0 μmol·kg^{-1}·min^{-1}; range, 8.9–13.4) fluxes were within the normal canine range (10) (Table III). Leucine nitrogen flux (4.96–6.15 μmol·kg^{-1}·min^{-1}) was 20–70% faster than the leucine carbon flux (3.29–4.24 μmol·kg^{-1}·min^{-1}, Table III).

Dividing the mole percent enrichment of product by that of precursor, the percent of alanine N derived from leucine was 18% (Eq. 6, Table IV). The rate of

TABLE I

Substrate Concentrations and Isotope Enrichments in Arterial and Venous Plasma

	Dog no.	Concentration		Mole percent excess			
		Artery	Vein	Artery	Vein	Artery	Vein
		mM				[$^2H_2$]Glucose	
Glucose	1	5.26±0.25*	4.93±0.18			1.68±0.18	1.72±0.07
	2	6.04±0.31	5.55±0.18			1.54±0.11	1.56±0.16
	3	5.52±0.42	5.18±0.36			2.25±0.11	2.15±0.11
	Mean	5.61±0.54	5.22±0.31			1.82±0.38	1.81±0.31
Alanine		*μM*		[$^2H_4$]Alanine		[^{15}N]Alanine	
	1	263±29	320±22	1.27±0.15	0.63±0.07	0.63±0.04	0.48±0.04
	2	204±13	271±33	1.32±0.11	0.67±0.07	0.93±0.04	0.58±0.04
	3	506±78	583±38	0.54±0.04	0.32±0.11	0.80±0.07	0.72±0.04
	Mean	324±159	391±168	1.04±0.43	0.54±0.11	0.79±0.16	0.59±0.12
Leucine		*μM*		[^{15}N]Leucine		[^{14}C]Leucine	
						dpm/μmol × 10^{-3}	
	1	152±13	142±11	4.24±0.38	3.26±0.18	14.66±2.17	13.54±1.90
	2	195±20	168±13	4.70±0.31	2.98±0.29	13.13±0.87	9.18±1.7
	3	207±11	188±7	4.45±0.31	3.75±0.29	15.55±1.52	13.90±1.77
	Mean	185±29	166±23	4.46±0.23	3.33±0.40	14.45±1.22	12.21±2.63

* Mean ± SE.

[^{15}N]alanine production averaged 0.086 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ (Eq. 3, Table IV) and reflected an average of 1.92 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ of leucine N transferred to alanine N. Using the flux of leucine N, ~36% of the leucine nitrogen went to alanine or alternatively using the flux of leucine C, 53% of the leucine flux donated N for alanine synthesis (Eq. 5, Table IV).

TABLE II

^{15}N Enrichment in Arterial Plasma Amino Acids after a 9-h Infusion of [^{15}N]*Leucine*

Amino acid*	Dog 1	Dog 2	Dog 3	Average
	mole percent enrichment			
Leucine	4.88	4.81	4.79	4.83‡
Isoleucine	0.82	0.89	1.31	1.01‡
Valine	0.63	0.56	0.66	0.62‡
Alanine	0.63	0.84	0.68	0.72‡
Serine	0.20	0.34	0.22	0.25‡
Glycine	0.13	0.04	0.35	0.17‡
Ornithine	0.32	0.16	0.09	0.19‡
Threonine	0.11	0.00	0.06	0.06
Aspartate	0.13	−0.08	−0.11	−0.02
Methionine	−0.06	0.01	0.06	0.00
Phenylalanine	−0.07	0.01	0.08	0.01
Tyrosine	−0.08	−0.09	0.03	−0.04
Lysine	−0.04	−0.07	0.00	−0.04

* Accurate estimate of ^{15}N enrichment in glutamine and glutamate could not be made using the current methods.
‡ Significant ($P < 0.05$) enrichment of ^{15}N above natural abundance.

Hindlimb substrate balance. Plasma dideuteroglucose enrichment in femoral artery and femoral vein were essentially identical within the error limits of the measurement; thus no net glucose was produced (as would be expected) and glucose and dideuteroglucose were extracted equally. The average extraction of glucose calculated stoichiometrically (6.8±0.6%, Eq. 7) was identical to that estimated from the deuterated tracer (7.1±1.8%, Eq. 8b). Glucose uptake (Eq. 9) averaged 39.4±7.1 $\mu mol \cdot min^{-1} \cdot leg^{-1}$, which could theoretically yield 78.8 μmol pyruvate $\cdot min^{-1} \cdot leg^{-1}$. Net hindlimb alanine balance was −6.7 $\mu mol \cdot min^{-1} \cdot leg^{-1}$ (Table V). Since arterial alanine was delivered to the hindlimb at an average rate of 31.3±5.5 $\mu mol \cdot min^{-1} \cdot leg^{-1}$, of which 35±3% was extracted (Eq. 8b), the alanine uptake by the extremity averaged 10.8±1.9 $\mu mol\ min^{-1}\ leg^{-1}$ (Eq. 9). As a result, hindlimb alanine release (Eq. 10) averaged 17.4±1.7 $\mu mol \cdot min^{-1} \cdot leg^{-1}$.

A small, but consistent, positive net leucine balance was observed across the hindlimb in each animal, averaging 1.9±0.5 $\mu mol \cdot min^{-1} \cdot leg^{-1}$ (Table V). The extraction fraction of leucine calculated from ^{14}C and ^{15}N were similar with the unexplained exception of dog 1, where the fractional extraction of leucine computed from the stable tracer was nearly twice that estimated by the radio-tracer. As a result, the average leucine uptake by the hindlimb was somewhat higher when

TABLE III
Arterial Substrate Flux During the final Hour of Isotope Infusion

Dog	Weight	Glucose	Alanine	Leucine carbon	Leucine nitrogen	$\frac{\text{Leucine nitrogen flux}}{\text{leucine carbon flux}} \times 100$
	kg			*μmol·kg⁻¹·min⁻¹*		%
1	20.0	21.0	10.8	3.61	6.15	170
2	17.3	19.1	8.9	4.24	5.08	120
3	23.0	11.5	13.4	3.29	4.96	152
Mean	20.1	17.2	11.0	3.71	5.40	147

calculated using [^{15}N]leucine (6.16±1.61 μmol·min^{-1}·leg^{-1} compared with the value obtained from [^{14}C]leucine (4.65±1.82 μmol·min^{-1}·leg^{-1}). Therefore, hindlimb leucine release was greater when estimated from the ^{15}N tracer (4.26±1.21 μmol·min^{-1}·leg^{-1}) than from the radiotracer (2.75±1.31 μmol·min^{-1}·leg^{-1}, Table V). Since [^{15}N]alanine is released from the hindlimb, a valid extraction fraction cannot be calculated from this labeled substrate.

To determine the rate of [^{15}N]alanine produced by the hindlimb, the [^{15}N]alanine delivered to but not extracted by the hindlimb must be substracted from the total [^{15}N]alanine efflux from the hindlimb (Eq. 11b, Table VI). Thus, the calculated rate of ^{15}N bypassing the hindlimb tissues averaged 0.158 μmol·min^{-1}·leg^{-1} and the observed total [^{15}N]alanine drainage was 0.229 μmol·min^{-1}·leg^{-1}. Therefore, the hindlimb released [^{15}N]alanine at the average rate of 0.071 μmol·min^{-1}·leg^{-1}. Alternatively, hindlimb [^{15}N]alanine release was estimated from the difference in alanine release computed from the extraction fraction of [$^{2}H_4$]alanine less that calculated from [^{15}N]alanine (average of 7%, data not shown) times the enrichment in venous plasma of [^{15}N]alanine. Such calculations yield a mean [^{15}N]alanine release of 0.058 μmol·min^{-1}·leg^{-1}, which is in reasonable agreement with the value of 0.071 μmol·min^{-1}·leg^{-1} calculated above. In either case, the hindlimb rate of appearance (R_a, Eq. 3) for [^{15}N]alanine of 0.058–0.071 μmol·min^{-1}·leg^{-1} represented only 3.4–4.1% of the whole body R_a for [^{15}N]alanine.

When similar estimates of hindlimb [^{15}N]leucine release were calculated (Table VI), the efflux of [^{15}N]-leucine from the hindlimb (0.546 μmol·min^{-1}·leg^{-1}) was entirely acounted for by the [^{15}N]leucine not extracted by the limb (0.548 μmol·min^{-1}·leg^{-1}) indicating, as anticipated, no net transfer of ^{15}N into leucine across hindlimb tissues.

Dividing the hindlimb [^{15}N]alanine release by the hindlimb [^{15}N]leucine uptake, one can estimate the fraction of leucine nitrogen converted to alanine nitrogen (Table VI). This value was quite different in each of the dogs. In dogs 1 and 3, the values thus calculated (29 and 65%, respectively) were virtually identical to the values of 31 and 66% calculated from the whole body flux data (Table IV). In dog 2, the hindlimb leucine nitrogen converted to alanine nitrogen was only 7% compared with a value of 38% computed for the whole animal.

At steady state, the average leucine oxidation rate in the hindlimb (Eq. 12) was 0.27 μmol·min^{-1}·leg^{-1} (Table VII), which represented, on the average, a fractional oxidation rate of 8% for leucine (Eq. 13, Table VII). If the only ^{15}N available for alanine transamination were derived from leucine oxidation, the total ^{15}N available from oxidation of [^{15}N]leucine could only yield 0.018 μmol·min^{-1}·leg^{-1} (Table VII). Since [^{15}N]-

TABLE IV
Relationship Between Leucine and Alanine Nitrogen in the Whole Body

Dog	R_a[^{15}N]alanine	Rate of leucine transferred to alanine N	Leucine N converted to alanine N	Alanine N derived from leucine N
	μmol ^{15}N·kg^{-1}·min^{-1}	*μmol·kg^{-1}·min^{-1}*	%	%
1	0.068	1.60	26* (44)‡	15
2	0.083	1.77	35 (42)	20
3	0.107	2.40	48 (73)	18
Mean	0.086	1.92	36 (53)	18

* Using the N flux of leucine as Q_{Leu} in Eq. 5.
‡ Using the carbon flux of Leu as Q_{Leu} in Eq. 5.

TABLE V
Substrate Balance Across the Leg

	Dog	Plasma flow	Net balance	[14C]Leucine			[15N]Leucine		
				Extraction fraction	Uptake	Release	Extraction fraction	Uptake	Release
		liters/min	*μmol·min⁻¹·leg⁻¹*	%	*μmol·min⁻¹·leg⁻¹*		%	*μmol·min⁻¹·leg⁻¹*	
Leucine	1	0.1178	1.18	14	2.50	1.28	29	5.19	3.87
	2	0.106	2.86	40	8.26	5.36	45	9.30	6.52
	3	0.0806	1.53	19	3.17	1.61	24	4.00	2.38
	Mean	0.101	1.86	24	4.65	2.75	33	6.16	4.26
Alanine				[2H4]Alanine					
	1	0.1178	−6.7	40	12.4	19.0			
	2	0.106	−7.1	33	7.1	14.1			
	3	0.0806	−6.2	32	13.0	19.1			
	Mean	0.101	−6.7	35	10.8	17.4			

alanine was released by the hindlimb at an average rate of 0.071 $\mu mol \cdot min^{-1} \cdot leg^{-1}$, the nitrogen available from leucine oxidation represents, at maximum, 25% of the total leucine nitrogen transfer to alanine.

The rate of N transfer from leucine to alanine (0.77–2.09 $\mu mol \cdot min^{-1} \cdot leg^{-1}$) at steady state reflects a minimal estimate of the rate of α-keto isocaproic acid formation (Table VIII). The rate of oxidation of leucine was only 28% (17–38%) of the rate of deamination of leucine and is consistent with net production of α-keto isocaproic acid by the hindlimb as has been reported in isolated hindlimb perfusion studies (12). However, the ultimate fate of this keto acid cannot be inferred in the present study because α-keto isocaproic acid balance across the limb was not measured.

DISCUSSION

Although muscle alanine release and hepatic glucose production decrease with prolonged starvation, the relative contribution of alanine carbon to glucose production increases (13). Therefore, the control and regulation of alanine production may play an important role in fasting glucose homeostasis. Because alanine is synthesized from both pyruvate and glutamate, either substrate could be rate limiting for alanine production. The *de novo* synthesis of alanine from pyruvate and glutamate in muscle by glutamate-pyruvate transaminase, and the reversal of this process in the liver, provides a unique interface between protein and carbohydrate metabolism. Considerable data have been presented in support of this glucose-alanine cycle in vivo (14). According to this concept, the carbon skeleton for alanine produced by muscle is derived almost exclusively from glycolysis of blood-borne glucose. Other investigators, however, have suggested that alanine carbon also may be derived from the catabolism of other amino acids within the myocyte with subsequent conversion of their carbon skeletons to pyruvate by way of the tricarboxylic acid cycle and malic enzyme

TABLE VI
[15N]Alanine and [15N]Leucine Relationships Across the Hindlimb

Amino acid	Dog	Arterial delivery	Uptake	Not taken up	Venous drainage	Amino acid production	Leucine N converted to alanine N
		μmol·min⁻¹·leg⁻¹					%
Alanine	1	0.195	0.078	0.117	0.181	0.064	29
	2	0.201	0.066	0.135	0.167	0.032	7
	3	0.326	0.104	0.222	0.338	0.116	65
	Mean	0.241	0.083	0.158	0.229	0.071	34
Leucine	1	0.759	0.220	0.539	0.545	0.006	
	2	0.972	0.437	0.535	0.531	0.004	
	3	0.743	0.178	0.565	0.568	0.003	
	Mean	0.825	0.278	0.546	0.548	0.002	

TABLE VII
[^{14}C]Leucine Oxidation and Nitrogen Generation from Leucine Across the Hindlimb

Dog	Rate of leucine oxidation	Percentage of leucine uptake	^{15}N available from the oxidation of [^{15}N]leucine	Total ^{15}N released in alanine
	$\mu mol \cdot min^{-1} \cdot leg^{-1}$		$\mu mol \cdot min^{-1} \cdot leg^{-1}$	
1	0.29	12	0.026	0.064
2	0.16	2	0.009	0.032
3	0.35	11	0.020	0.116
Mean	0.27	8	0.018	0.071

(4) or phosphoenolpyruvate carboxykinase and pyruvate kinase (15). In the present study, the glucose extracted by the hindlimb (39.4 $\mu mol \cdot min^{-1} \cdot leg^{-1}$) could generate a maximum of 79 $\mu mol \cdot min^{-1} \cdot leg^{-1}$ of pyruvate. This represents approximately four times the amount of pyruvate necessary to account for the alanine produced and is consistent with a primary role of glucose-derived pyruvate for alanine synthesis. Conversely, the amount of pyruvate necessary for the production of alanine was not insignificant and represented 23% of the pyruvate carbon theoretically provided by muscle glucose uptake. In any case, under conditions of the present study and excluding intracellular compartmentalization, pyruvate availability was most likely not rate limiting for alanine production.

The source of nitrogen for alanine synthesis is not well established. Since muscle appears to be the tissue primarily responsible for the catabolism of BCAA (3, 5), it has been proposed that muscle oxidation of BCAA may serve as an important local metabolic fuel source (16, 17) as well as a mechanism for the transport of the generated amino group (via alanine) to the liver for disposal as urea (1). A variety of in vitro muscle incubation and perfusion studies have indicated indirectly that branched chain amino acids might be a major source for alanine nitrogen (2, 15, 18). In addition, recent studies in this laboratory have demonstrated a 70% decrease in alanine production in children with maple syrup urine disease, an inherited deficiency of branched-chain α-keto acid dehydrogenase (6).

TABLE VIII
Rates of Transamination and Oxidation of Leucine Across the Hindlimb

Dog	Leucine uptake	Leucine N converted to alanine N	Rate of leucine transamination	Rate of leucine oxidation	Percent oxidation of deaminated leucine
1	2.56	30	0.77	0.29	38
2	7.61	7	0.53	0.16	30
3	3.22	65	2.09	0.35	17
Mean	4.46	34	1.13	0.27	28

In the present study, the flux of leucine nitrogen and carbon were different. If leucine transamination was rate limiting for catabolism, the leucine flux determined by these two isotopic methods should have been similar. Therefore, the 50% higher flux measured for leucine nitrogen suggests: (*a*) the rate-limiting step for leucine catabolism is distal to transamination (most likely the oxidative carboxylation of the α-keto acid) (5); (*b*) leucine nitrogen is lost to a larger intracellular nitrogen pool (mostly likely via glutamate), and (*c*) that the rate of leucine reversible (retransamination of the keto-acid) vs. irreversible disposable (oxidation of the α-keto acid) is ~3:2 (Table III).

Infusion of [^{15}N]leucine resulted in incorporation of ^{15}N into isoleucine and alanine (and also, presumably, valine) within 30 min. However, isotopic equilibrium in these circulating pools was not achieved until 6–7 h of the [^{15}N]leucine infusion. Assuming that glutamate is the major metabolic intermediate in the transfer of ^{15}N from leucine to alanine, isoleucine, and valine, these data are compatible with a very large and/or rapidly turning over intracellular pool of glutamate. Once isotopic equilibrium of ^{15}N was achieved in the isoleucine and valine pools, the rate of appearance of ^{15}N in these two amino acid pools would be offset by the rate of re-entry of isoleucine and valine ^{15}N into the glutamate pool, assuming a common metabolic fate of the nitrogen from all three BCAA and the irreversible loss of the labeled amino acids (e.g., protein synthesis). Despite some loss of ^{15}N to the other two BCAA, a reasonable estimate of the fate of [^{15}N]leucine can be calculated under steady-state conditions. Thus, nearly half of leucine's nitrogen was transferred to alanine and represented 18% of the nitrogen in alanine. Since endogenous proteolysis will dilute the [^{15}N]leucine enrichment in the intracellular space, the rates of conversion of leucine N to alanine and the percentage of alanine N derived from leucine N are minimal estimates. However, this error is most likely small (<10–15%) since the enrichment of free [1-^{13}C, α-^{15}N]leucine in muscle was observed to be 90% of the plasma enrichment in humans infused with this label for 5–11 h (19). Therefore, if the contribution of isoleucine and valine nitrogen were similar to that of leucine, more than 50% of alanine's nitrogen could be derived from the BCAA. These results support the concept that regulation of BCAA metabolism may be closely linked to the regulation of alanine production in vivo.

Measurement of ^{15}N balance across the hindlimb revealed results comparable to those observed in the whole animal. Although there was considerable individual variation, an average of 34% of the leucine nitrogen taken up by the hindlimb was released in alanine. This was in close agreement with the average

of 53% calculated from whole body measurements. Despite the similarity in the percent leucine N converted to alanine N calculated from the total body and hindlimb data, the hindlimb contributed less than 5% of the total ^{15}N from leucine incorporated into [^{15}N]-alanine using plasma flow measurements. Calculation of the amino acid flux across the hindleg using blood flow would increase the [^{15}N]alanine production by 60%. This would increase the contribution of [^{15}N]alanine production by hindlimb from 3.75 to 6% of the estimated total or 12% if both hindlegs were considered. If the primary site for alanine formation under the condition of this study was muscle, we would have anticipated a larger percentage of [^{15}N]alanine to have been released by the hind limb. These data suggest that a significant percentage of the leucine N is transferred to alanine in other body tissues. This could occur as the result either of the transfer of leucine N into alanine within a single tissue (e.g., fat [20], kidney [21], or brain [21, 22]), or as the result of transport of leucine N from muscle via a metabolic intermediate and subsequent incorporation of the N into alanine in other body tissues. A likely intermediate for this latter proposed mechanism would be glutamine since (*a*) it is released from muscle in excess of any other amino acid (18); (*b*) glutamate is an obligate intermediate in its *de novo* synthesis; and (*c*) the selective metabolism of glutamine by the intestine results in the production of significant amounts of alanine (23). Since the methods utilized in the present studies did not permit an accurate estimate of the ^{15}N enrichment in plasma glutamine and glutamate individually (11), the potential contribution of glutamine as an intermediate in the conversion of leucine N to alanine cannot be assessed.

The amount of ^{15}N necessary for the observed production of [^{15}N]alanine by the hindlimb could not be accounted for solely by the oxidation of leucine in the extremity. Approximately 0.018 μmol nitrogen·min^{-1}·leg^{-1} could have been generated from the hindlimb oxidation of [^{15}N]leucine. This represents only 25% of the observed ^{15}N released in alanine. Recent studies by Hutson and co-workers (12) using the perfused rat hindquarter demonstrated that 40–50% of leucine carbon was released into the venous circulation as the α-keto acid. In the present study, the rate of keto acid formation could be approximated from the percent [^{15}N]leucine incorporated into alanine (an indication of transamination rate) and the oxidation of [^{14}C]leucine (an index of net disposal). The difference between the rate of transamination of leucine (rate of N transferred from leucine to alanine, a minimal estimate of total transamination) and net disposal of leucine carbon via oxidation most likely reflects the rate of keto acid accumulation. Using these data, we estimate that a minimum of 24% of the leucine taken up by the hindlimb might be released as α-keto isocaproic acid, a value in reasonable agreement with that of Hutson et al. (12) when one considers differences in species and experimental design. The physiologic significance of the released branched-chain α-keto acids (12) is not fully known. However, exogenous administration of α-keto acids results in the formation of the respective BCAA (12, 24, 25) and improved nitrogen balance (26, 27). Such a system of transamination and reamination to form the essential BCAA may serve as a unique mechanism to conserve essential amino acids and to redistribute body nitrogen.

APPENDIX

Assumption

(*a*) Arterial and venous blood samples are drawn at a time when the animal is in the steady state both with regard to concentration and isotope enrichment of the substances being measured. (*b*) Deep venous blood is to the greatest extent, effluent drainage from the hindlimb muscles, and femoral venous samples are representative of total deep venous drainage on the whole. (*c*) Release of label from the muscle protein "sink" is small during the time-course of the experiment and, therefore, recycling of label is negligible when compared to the total amount of unlabeled substrate. (*d*) The isotopically labeled substances are handled in a manner identical to the unlabeled substrates.

Definition

[A], [V] Concentration (micromoles per milliliter) of a substrate in arterial (A) or venous (V) plasma, respectively.

S_a, S_v Specific activity of a radiotracer (disintegrations per minute per micromole) in arterial (a) or venous (v) plasma, respectively.

E_a, E_v, E_i Enrichment of stable isotopically labeled tracer or mole fraction in arterial (a) plasma, venous (v) plasma or the infusate (*i*). The mole fraction equals the moles of labeled substrate divided by the total moles of substrate (labeled + unlabeled).

[*A], [*V] Concentration of radiotracer (disintegrations per minute per milliliter) in arterial (A) and venous (V) plasma, respectively.

i Tracer infusion rate: for radiotracer, the units are disintegrations per minute; for stable isotope tracer, the units are micromoles per minute of total tracer. This latter i must be corrected to the actual amount of stable isotopically labeled material infused ($i \cdot E_i$) because the E of the tracers vary between 90 and 98%.

F_p Plasma flow through the femoral artery (milliliter per minute).

Equations

Whole animal studies. Leucine carbon flux (Q) is determined from the continuous infusion of L-[U-^{14}C] leucine infusion and corrected for body weight (micromoles per min per kilogram).

$$Q = \frac{i}{S_a kg} \quad (1)$$

Substrate flux (micromoles per minute per kilogram) for the whole animal is determined from the stable isotopically labeled tracer infusion ([$^2H_2$]glucose, [$^2H_4$]alanine, or [^{15}N]leucine)

$$Q = \frac{i}{kg}\left(\frac{E_i}{E_a} - 1\right), \quad (2)$$

where the second term (−1) removes the contribution of stable isotopically labeled tracer infusion from the apparent flux.

The rate of appearance of [^{15}N]alanine in the plasma (micromoles per minute per kilogram) is

$$R_a = Q \cdot E_a, \quad (3)$$

where Q is the apparent alanine flux measured from the [$^2H_4$]alanine label and E_a is the enrichment of [^{15}N]alanine in arterial plasma.

The rate of leucine N conversion to alanine N (micromoles per minute per kilogram) is

$$^{R}\text{Leu N} \rightarrow \text{Ala N} = \frac{R^a}{E_a}, \quad (4)$$

where E_a is the isotope enrichment of [^{15}N]leucine.

The percentage of leucine N converted to alanine N is

$$\frac{^{R}\text{Leu N} \rightarrow \text{Ala N}}{Q} \times 100, \quad (5)$$

where Q is the leucine flux measure with either the [U-^{14}C]-leucine or [^{15}N]leucine.

The percentage of alanine N derived from leucine N is

$$\frac{E_a{}^{[15N]Ala}}{E_a{}^{[15N]Leu}} \times 100. \quad (6)$$

For the product-precursor relationships determined by Eqs. 3–6, the actual rate of precursor converted to product requires measurement of the isotopic enrichment in the cellular free amino acid pool. Because proteolysis occurs in the intracellular space and tracer is infused into the vascular space, the intracellular enrichment in leucine is of necessity less than that in the arterial plasma. Thus, at isotope and substrate steady state, Eqs. 3–6 will be minimal estimates of the conversion. Measurement of muscle free amino acid isotopic enrichment after 6–11 h of [1-^{13}C, α-^{15}N]-leucine in humans demonstrates that the intracellular enrichment is 90% of that in the vascular space (19). As a result our measurement may underestimate the rates of conversion by ~10%.

Hindlimb studies. The overall leg balance (net uptake or increase) for a substrate in micromoles per minute per leg is

$$([A] - [V])F_p \quad (7)$$

The extraction fraction of the radiolabeled tracer presented to the hindlimb from the plasma is

$$f = \frac{[*A] - [*V]}{[*A]} \quad (8a)$$

and of the stable isotopically labeled trace is

$$f = \frac{[A]E_a - [V]E_v}{[A]E_a}. \quad (8b)$$

For the substrates to which Eqs. 8a and 8b apply (leucine, glucose, and alanine), there may be net substrate release from the hindlimb in the presence of net uptake of tracer. The infusion of tracer and the calculation of fractional extraction together with the measurement of net balance permits us to separate the two components contributing to net balance: the actual substrate uptake and substrate release. In other words, the net balance rate (Eq. 7) is equal to the actual substrate uptake minus the actual substrate release. For example, the net balance rate across a tissue is −40 μmol/min; without the use of isotope infusion and fractional extraction rate, it would be impossible to determine whether the tissue was taking up 20 and releasing 60 μmol/min or taking up 100 and releasing 140 μmol/min.

Substrate uptake (micromoles per minute) is the fractional extraction (Eq. 8a or 8b) times the substrate inflow or

$$f[A]F_p \quad (9)$$

The difference between Eqs. 9 and 7 represent substrate release (micromoles per minute): $f[A]F_p - ([A] - [V])F_p$, which for a radioisotopically labeled tracer reduces to

$$F_p[V]\left(1 - \frac{S_v}{S_a}\right) \quad (10a)$$

and for the stable isotopically labeled tracer to

$$F_p[V]\left(1 - \frac{E_v}{E_a}\right). \quad (10b)$$

For labeled substrates that can be synthesized by the tissue under study (e.g., formation of [^{15}N]alanine from the transfer of the ^{15}N from [^{15}N]leucine via transamination reactions in muscle), eqs. 9, 10a, and 10b do not apply. Therefore, overall balance of uptake and release of the labeled substrate (e.g., [^{15}N]alanine) must be calculated. Net balance across the hindlimb for a radioisotopically labeled tracer (disintegrations per minute) is

$$([*A] - [*V])F_p \quad (11a)$$

or for a stable isotopically labeled tracer (micromoles per minute) is

$$([A]E_a - [V]E_v)F_p \quad (11b)$$

The rate (micromoles per minute) of leucine oxidized to CO_2 by the hindlimb is

$$\frac{[*V] - [*A]}{S_a} \cdot F_p, \quad (12)$$

where [*V] and [*A] are the disintegrations per minute per milliliter plasma of $^{14}CO_2$ and S_a is the specific activity of [^{14}C]leucine. The fractional (percentage) oxidation of leucine by the hindlimb is

$$\frac{[*V_{CO_2}] - [*A_{CO_2}]}{[*A_{Leu}] - [*V_{Leu}]} \times 100, \quad (13)$$

where $[*V_{CO_2}] - [*A_{CO_2}]$ represents the venous arterial difference in $^{14}CO_2$ (disintegrations per minute per milliliter) and $[*A_{Leu}] - [*V_{Leu}]$ represents the arterial venous difference in [^{14}C]-leucine (disintegrations per minute per milliliter).

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