Branched Chain Amino Acids as a Major Source of Alanine Nitrogen in Man

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SUMMARY

In vitro perfusion and incubation studies and recent investigations in dogs suggest that branched chain amino acids (BCAA) may be a major source of alanine nitrogen. To determine the contribution of BCAA nitrogen to the formation of alanine in man, seven postabsorptive adults received prime-dose constant infusions of ^{15}N-leucine, L-[6,6,6-$^{2}H_3$] leucine, and L-[2,3,3,3-$^{2}H_4$] alanine; isotopic enrichment was determined in arterialized venous plasma samples by gas chromatography-mass spectroscopy. At substrate and isotope steady state, alanine flux and the rate of ^{15}N alanine appearance were 5.4 ± 0.3 μmol/kg·min and 32 ± 2 nmol/kg·min, respectively. Leucine nitrogen flux was significantly greater than that of leucine carbon flux (2.54 ± 0.25 vs. 1.90 ± 0.10 μmol/kg·min, respectively; $P < 0.001$). The 30% greater flux of leucine nitrogen when compared with leucine carbon suggests significant recycling of the leucine carbon in vivo.

The percent of circulating alanine nitrogen derived from leucine was 12.5 ± 1.5%; however, the rate of leucine nitrogen transferred to alanine was 0.66 ± 0.05 μmol/kg·min, and represents a minimum of 28% of leucine nitrogen going to alanine. On the basis of these data, together with the percent of alanine and leucine in body protein, only 40% of circulating plasma alanine could come from endogenous protein, whereas 60% is derived from de novo synthesis. In addition, at least 20% of the nitrogen required for alanine synthesis is derived solely from leucine following an overnight fast. Therefore, if the contribution of isoleucine and valine nitrogen is similar to that of leucine, the BCAA may contribute to a minimum of 60% of the nitrogen required for alanine synthesis in postabsorptive man. DIABETES *31*:86–89, January 1982.

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In the postabsorptive state, plasma alanine, a potential gluconeogenic substrate, could be derived from the transamination of pyruvate with a nitrogen donor, as well as that released from protein. Available evidence suggests that a significant proportion of circulating alanine is derived from de novo synthesis: in rat muscle, a major portion of alanine carbon is derived from glucose;[1] in human forearm studies, alanine release is greater than can be accounted for by its representation in protein.[2] Exposure of rat muscle to the branched chain amino acids, leucine, isoleucine, and valine, increases alanine release, suggesting that these amino acids may serve as nitrogen donors for alanine synthesis in vivo.[3–5] Recently, we demonstrated isotopically that leucine nitrogen was a significant source of alanine nitrogen in the dog.[6]

Since alanine flux is significantly greater[6–8] than that reported for leucine in man[9–11] (and that presumed for isoleucine and valine), the utilization of branched chain amino acids as a nitrogen donor for alanine synthesis could lead to significant loss of these essential amino acids. However, release of the α-ketoacid of leucine, α-ketoisocaproate, from rat muscle,[12] arteriovenous differences of the branched chain α-ketoacid across muscle,[13] and the rapid interconversion of plasma leucine and α-ketoisocaproate in conscious dogs[14] suggest that there may be significant release of the branched chain α-ketoacids into the vascular space, which might be available for reamination and/or oxidation at distant tissue sites.

The present studies, utilizing leucine as a paradigm for the branched chain amino acids (BCAA), were designed to determine whether the BCAA are major nitrogen donors for alanine synthesis in man and, if so, to determine whether nitrogen loss from leucine is associated with irreversible loss of its carbon skeleton.

STUDY PROTOCOL

Seven young (23 ± 2 yr) adult volunteers were studied in the postabsorptive state. All subjects were consuming a diet

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containing at least 200 g of carbohydrate per day for 3 days prior to the study, none had a family history of diabetes, and all were of normal weight (90–110% ideal body wt., Metropolitan Life Insurance Tables). Subjects were admitted to the Clinical Study Unit on the evening prior to study and consumed a 600-kcal meal at 1800 h. Seven hours prior to blood sampling, a constant infusion of ^{15}N-leucine was begun (≈0200 h) at a rate to enrich the circulating plasma pool of leucine to approximately 5 mole %, to assure isotopic equilibration of ^{15}N in the circulating alanine pool, and continued to the end of the study. At −120 min, a primed-dose (30 times the minute infusion rate) of [$^2H_3$] leucine and [$^2H_4$] alanine was administered and constant infusion continued throughout the study. Arterialized venous blood was obtained from a scalp vein needle placed retrograde in a hand vein with the hand placed in a warming box maintained at 50–55°C.[15] Five milliliters of blood were drawn at 15-min intervals during the study and centrifuged, and the plasma was stored at −70°C until assayed for leucine and alanine concentrations and moles % enrichment of [^{15}N] alanine, [$^2H_4$] alanine, [$^2H_3$] leucine, and [^{15}N] leucine.

MATERIALS AND METHODS

Sterile [6,6,6-$^2H_3$] leucine, [^{15}N] leucine and L-[2,3,3,3-$^2H_4$] alanine (Merck, Sharp, and Dohme, Isotope Division, Quebec, Canada) were dissolved in 0.9% saline and administered via a syringe pump. The rate of isotope infusion was documented by multiplying the concentration of amino acid in the infusate by the moles % enrichment of the isotope (98% [$^2H_3$] leucine, 98% [^{15}N] leucine, 90% [$^2H_4$] alanine) and the infusion rate (ml/min). Forty microliters of 1.0 mM L-[4,5,5,5,6,6,6-$^2H_7$] leucine were added as an internal standard to 200 μl of plasma, the amino acids were isolated, and the trimethyl silyl derivative was prepared as previously described.[8,9]

The derivatized amino acids were injected onto a 2 m × 2 mm 3% OV 11 (Supelco) packed column interfaced with a jet separator to a 5985B Hewlitt Packard GC/MS. Molar % enrichments were determined by comparing the peak height ratios of the unknown samples with those obtained on the same day from a standard curve of known enrichment ([^{15}N] alanine, [$^2H_4$] alanine, [^{15}N] leucine, [$^2H_3$] leucine, and [$^2H_7$] leucine). The [^{15}N] leucine standard curve is depicted in Figure 1. The decarboxylated TMS derivative of ^{15}N leucine (m/e 159) has a M+2 fragment of 4% of the base peak height, which would contribute an error to the base peak of [$^2H_3$] leucine (m/e 161). The contribution of the [^{15}N] leucine to the 161 fragment was linearly related to the mole % enrichment of [^{15}N] leucine (Figure 1 insert). Appropriate correction of the peak height ratio of (m/e 161)/(m/e 158) was made for the moles % enrichment of ^{15}N leucine in each sample. [$^2H_3$] leucine provides estimates of leucine flux which are essentially identical to [U-^{14}C] leucine in dogs and is considered to trace the flux of leucine carbon.[8] Plasma leucine concentrations were determined from the peak height ratios of $^2H_7$ and natural leucine. The contribution of the M+3 fragment of [^{15}N] alanine to the base peak (m/e 120) of [$^2H_4$] alanine was far below the sensitivity of the method. The coefficient of variation of replicate analyses (N = 10) of sample enriched within the ranges observed in the present study for [^{15}N] alanine, [$^2H_4$] alanine, [$^2H_3$] leucine, [^{15}N] leucine, and [$^2H_7$] leucine was less than 3% in

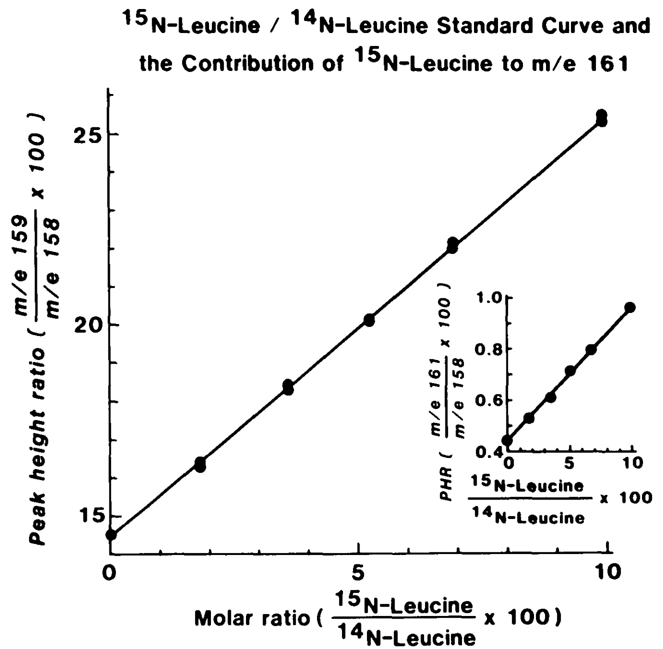

FIGURE 1. The peak height ratio (×100) of the base peak m/e of [^{15}N] and [^{14}N] leucine plotted against the molar ratio of [^{15}N] leucine/[^{14}N] leucine. The insert coordinates plot the contribution of a M+2 fragment of [^{15}N] leucine at various molar ratios to the peak height ratio of the base peaks of [$^2H_3$] and [$^2H_0$] leucine.

each case. Plasma alanine concentrations were determined microfluorometrically.[16]

Flux rates were determined using steady-state assumptions and standard formulas.[6] Since the ^{15}N was infused into the vascular space, these calculations assume that the isotopic enrichment of the ^{15}N in the intracellular and extracellular space are identical. Since ongoing proteolysis may result in some dilution of the [^{15}N] leucine in the intracellular space,[17] these calculations for interconversion reflect minimal estimates.[6] Assumptions and equations utilized in these calculations are found in the appendix.

RESULTS

In the postabsorptive state, arterialized venous plasma alanine and leucine concentrations were 254 ± 15 μM and 125 ± 8 μM, respectively, and changed little over the period of study (Figure 1). The mole percent enrichment of plasma [$^2H_4$] alanine, [$^2H_3$] leucine, and [^{15}N] leucine were 2.3 ± 0.1, 2.0 ± 0.1, and 5.7 ± 0.4, respectively, and remained constant throughout the study. Following 8 h of [^{15}N] leucine infusion, plasma [^{15}N] alanine mole percent enrichment was 0.73 ± 0.03 and remained constant for the duration of study (Figure 2).

At steady state, alanine flux was 5.4 ± 0.3 μmol/kg·min. The leucine N flux, which was 2.54 ± 0.25 μmol/kg·min, was greater ($P < 0.001$) than the leucine carbon flux (1.90 ± 0.10 μmol/kg·min) determined from the $^2H_3$ leucine tracer data. The rate of [^{15}N] alanine appearance was 32 ± 2 nmol/kg·min (Eq. 2, see APPENDIX). The percent of alanine nitrogen derived from leucine nitrogen was 12.5 ± 1.5% (Eq. 3). The rate of leucine nitrogen transferred to alanine was 0.66 ± 0.05 μmol/kg·min (Eq. 4) and represents a minimum of 28% of the leucine N flux going to alanine (Eq. 5) in postabsorptive man.

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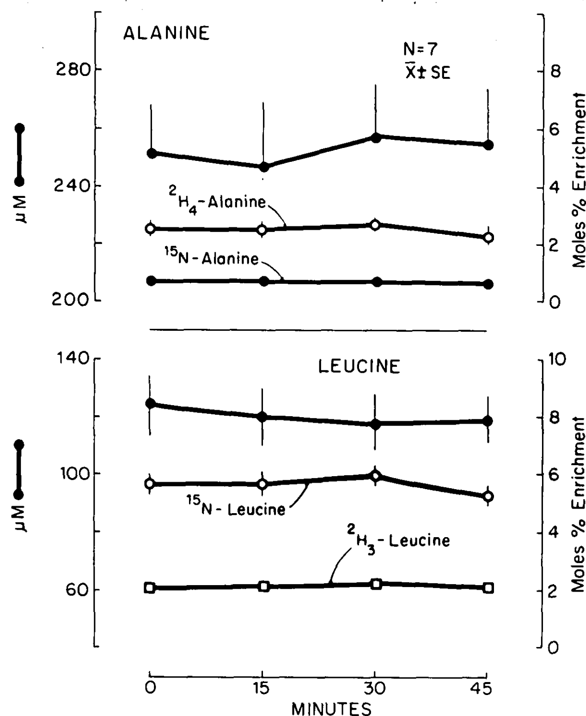

FIGURE 2. Plasma alanine and leucine concentrations, the moles % enrichment of [$^2H_4$] alanine, [^{15}N] alanine, [^{15}N] leucine, and [$^2H_3$] leucine at both substrate and isotope steady state.

DISCUSSION

The present studies demonstrate that leucine is a significant source of alanine nitrogen in overnight fasted man. The percent of circulating alanine nitrogen derived from leucine (12%) and the percent of leucine nitrogen going to alanine (28%) are considerably less than those observed previously in dogs (18 and 53%, respectively).[6] In these latter studies,[6] [^{14}C] leucine was used, in contrast to the [$^2H_3$] leucine employed in the present studies; however, since these two tracers provide essentially identical flux rates of leucine,[9] the differences observed between the present studies in man and those in dogs are most likely species related.

On the basis of the leucine content (8%) of protein,[18] and the rate of appearance of leucine carbon (1.90 μmol/kg·min) in the present study, the rate of proteolysis is calculated to be 3.1 mg/kg·min, assuming that the rate of appearance of leucine represents the rate of release of this essential amino acid from protein. Therefore, the rate of alanine release from protein would be 2.1 μmol/kg·min assuming the average alanine content in protein were 6%.[2,18] As a result, only 39% of the alanine flux may be derived from proteolysis; conversely, as much as 61% (3.3 μmol/kg·min) of the circulating alanine in postabsorptive man is derived from de novo synthesis. Since the rate of transfer of leucine N to alanine was 0.66 μmol/kg·min, nearly 20% of the nitrogen for alanine synthesis was derived from leucine alone.

Leucine nitrogen is presumed to be incorporated into alanine by a series of reversible transamination reactions involving the transfer of leucine nitrogen to glutamate via branched chain aminotransferase and the subsequent transamination of glutamate with pyruvate forming alanine via glutamate pyruvate transaminase.[19] Because of intracellular dilution of the [^{15}N] leucine by unlabeled leucine derived from proteolysis, the moles % enrichment of [^{15}N] leucine sampled in the plasma space may not reflect the true enrichment in the precursor pool at the site of transamination. For example, in dogs using a double labeled isotope model employing [4,5-^{3}H] leucine and [U-^{14}C] α-ketoisocaproate, the specific activity in the circulating pool of the reciprocal substrates (e.g., [4,5-^{3}H] α-ketoisocaproate and ^{14}C leucine) are approximately 50% of the specific activity of the circulating precursors,[14] whereas in man [1-^{13}C, α-^{15}N] leucine enrichment in muscle during constant infusion of this tracer is between 80 and 90% of that in the circulating pool.[17] As a result, the calculated rates and percent of leucine N going to alanine, as well as the percent of alanine nitrogen derived from leucine, represent minimal estimates. In addition, if the rates of transfer of nitrogen of isoleucine and valine to alanine are similar to that of leucine, more than 60% of the nitrogen involved in the de novo synthesis of alanine in overnight-fasted subjects may be derived from the BCAA. Whether the carbon skeleton of the pyruvate required for the de novo synthesis of alanine in man is derived from the catabolism of other amino acids,[20] or from glucose via glycolysis,[1,21] cannot be determined by the present studies.

The 35% higher apparent flux rate of leucine nitrogen when compared with leucine carbon in the present studies in man are similar to our previous observations in dogs.[6] These data, together with the demonstration of rapid interconversion of plasma leucine and ketoisocaproate in dogs,[14] provide strong evidence that significant recycling of leucine carbon via α-ketoisocaproate occurs in man. Whether this "recycling" of leucine carbon in vivo represents intraorgan "futile" transamination or transamination of leucine in one tissue and the transport of its α-ketoacid to distant tissues for reamination, as has been suggested,[13] cannot be determined from the present data. If the latter proposed mechanism applied to all the BCAA, the intra-organ exchange of these amino acids and their α-ketoacids would provide a unique mechanism for the redistribution of nitrogen between body organs and a possible means for conserving the carbon skeleton of essential amino acids during periods of decreased availability of dietary protein.

APPENDIX

Assumptions: (1) arterialized venous samples are drawn at a time when steady state is achieved in both plasma concentration and isotope enrichment of the substrates being measured; (2) 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 with the total amount of unlabeled substrate; (3) the isotopically labeled substances are handled in a manner identical to the unlabeled substrates.

Equations. Substrate flux (Q) is determined from the stable isotopically labeled tracer infusion by the following equation and expressed as μmol/kg·min.

$$Q = \frac{i \cdot E_i}{E_a} - i \tag{1}$$

where i is the tracer infusion rate (μmol/min·kg), E_i and E_a are the enrichments of stable isotopically labeled tracer in the infusate and arterial plasma, respectively, and −i re-

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moves the contribution of the stable isotopically labeled tracer infusion from the apparent flux.

The rate of appearance of [^{15}N] alanine in the plasma (nmol/kg·min) is

$$Q^{15N\,ala} = Q^{ala} \cdot E_a^{15N\,ala} \quad (2)$$

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

The percentage of alanine N derived from leucine N is

$$\frac{E_a^{[15N]\,ala}}{E_a^{[15N]\,leu}} \times 100 \quad (3)$$

where $E_a^{[15N]\,ala}$ and $E_a^{[15N]\,leu}$ are the isotope enrichment of [^{15}N] alanine and [^{15}N] leucine in arterialized venous plasma.

The rate of leucine N conversion to alanine N (μmol/kg·min) is

$$\text{Leu N} \rightarrow \text{Ala N} = \frac{Q^{15N\,ala}}{E_a} \quad (4)$$

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

The percentage of leucine N converted to alanine N is

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

where Q is the leucine flux measure with the [^{15}N] leucine

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