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The contribution of muscle, kidney and splanchnic tissues to leucine transamination in humans

Giacomo Garibotto^{1,2}, Daniela Verzola^{1,2}, Monica Vettore³, and Paolo Tessari³.

¹Nephrology, Dialysis and Transplantation Clinic, Dept. of Internal Medicine, University of Genova; ²IRCCS AOU San Martino-IST, Genova; and ³Metabolism Division, Dept. of Medicine, University of Padova, Italy.

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Correspondence to:

Giacomo Garibotto M.D. Nephrology, Dialysis and Transplantation Clinic, Dept. of Internal Medicine, Viale Benedetto XV n. 6, 16132 Genova. E-mail: gari@unige.it

G. Garibotto and P. Tessari equally contributed to this study.

ABSTRACT

The first steps of leucine utilization are reversible deamination to α -ketoisocaproic acid (α -KIC) and irreversible oxidation. Recently the regulatory role of leucine deamination over oxidation was underlined in rodents. Our aim was to measure leucine deamination and reamination in the whole-body, in respect to previously-determined rates across individual organs, in humans. By leucine and KIC isotope kinetics, we determined whole-body leucine deamination and reamination, and we compared these rates to those already reported across the sampled organs. As an *in vivo* counterpart of the “metabolon” concept, we analysed ratios between oxidation to either deamination or reamination. Leucine deamination to KIC was greater than KIC reamination to leucine in the whole-body ($p=0.005$), muscle ($p=0.005$) and the splanchnic area ($p=0.025$). These rates were not significantly different in the kidneys. Muscle accounted for $\approx 60\%$ and $\approx 78\%$, the splanchnic bed for $\approx 15\%$ and $\approx 15\%$, and the kidney for $\approx 12\%$ and $\approx 18\%$, of whole-body leucine deamination and reamination rates, respectively. In the kidney, percent leucine oxidation over either deamination or reamination was >3 -fold greater than muscle and the splanchnic bed. Skeletal muscle contributes by the largest fraction of leucine deamination, reamination and oxidation. However, in relative terms, the kidney plays a key role in leucine oxidation.

Keywords: Leucine, transamination, BCAA, ketoacids, skeletal muscle, kidney

Introduction

The control of the catabolic flux of most amino acids takes place at the first metabolic steps, i.e. at the transamination/deamination reactions (Nelson 2015). The term “transamination” is referred to the overall process of amino group transfer from a donor amino acid to an accepting ketoacid, to form a daughter amino acid. Conversely,

“deamination” is referred just to loss of an amino group by a given amino acid, whereas “reamination” refers to the capture of an amino group by a ketoacid to form an amino acid.

The transamination of leucine, valine and isoleucine (BCAA) plays a key role in nitrogen distribution among non-essential amino acids and in nitrogen shunting either to urea formation or to protein synthesis (Nelson 2015). At variance with most amino acids, which are predominantly utilized by the liver, BCAAs are thought to be mainly catabolized by extra-hepatic tissues (Nelson 2015) (Cohen 1941) (Ichihara 1985) (Miller 1961), above all skeletal muscle (Miller 1961) (Adibi 1976) (Brosnan 2006) and adipose tissue (Brosnan 2006). Nevertheless, the relative role of BCAA transamination vs. oxidation is incompletely defined. Recent observations suggest that transamination plays an more important role than that previously assumed, on the control of BCAA metabolism. Mitochondrial BCAA transaminases (BCAA-Tm) are ubiquitary in animal tissues (Taylor 1966) (Suryawan 1998). Furthermore, it has been recently demonstrated that mitochondrial BCAA-Tm and branched chain α -ketoacid dehydrogenase (BCKD) enzyme complexes associate to form a supra molecular entity (Islam 2007), of the type originally defined as “metabolon” (Robinson 1985), that provides a coordinated control of the BCAA metabolic flux and oxidation. According to this concept, the tissue specific level of BCKD dehydrogenase, and the phosphorylation state of the BCKD complex, regulate both BCAA-carbon irreversible loss (i.e. oxidation) and rates and direction of reamination/deamination. In BCAA-Tm knock-out mouse, it was concluded that BCAA-Tm actually plays a key role in BCAA signaling and anabolism, beyond that “classically” played by oxidation (Joshi 2006).

Among the three BCAAs, leucine plays a pivotal role both as an important anabolic signal and a regulator of protein turnover (Buse 1975) (Kimball 2004). In addition, leucine kinetic is commonly taken as an index of whole body and organ protein turnover (Matthews 1983) (Tessari 1994). So far, however, only few studies have provided data on whole body

and organ leucine transamination and oxidation in humans. Matthews et al. (1981) proposed a model of leucine deamination and reamination at whole body level, while Cheng et al. (1985) reported estimates of these rates across the human forearm. More recently, we reported the rates of leucine deamination, reamination and oxidation across the leg, the splanchnic area and the kidney (Tessari 1996). However, in that study, we didn't report some additional data, which were not analyzed at that time yet, on whole body leucine deamination and reamination, to which organ rates could be compared.

This study aims to complete the picture of leucine deamination, reamination and oxidation rates across three major organs in humans, in comparison to the corresponding ones determined at whole body level. In addition, this study reports on a new model to measure whole-body leucine deamination, based on the infusion of an independent KIC tracer, conceptually similar to that previously used to estimate first pass splanchnic leucine uptake and deamination (Biolo 1997). The contributions of skeletal muscle, the splanchnic bed and the kidneys, to whole body leucine deamination, reamination and oxidation, are also presented in the light of the "metabolon" concept, based on a combined analysis of leucine transamination and oxidation.

Material and methods

Subjects were enrolled between September 1993 and July 1994. The subjects' clinical characteristics, the inclusion criteria and the experimental design, had been previously reported (Tessari 1996), except for an additional subject (male gender, age 56 yrs, BMI 25.8 kg/m²), whose data became available after the publication of the original manuscript (Tessari 1996). In addition to the previously reported tracers, all subjects had also been infused with the [²H₃]- α -ketoisocaproic acid stable isotope tracer ([α -D₃-KIC] (>98% purity, obtained from Tracer Technologies, Somerville, MA, USA). All isotopes were dissolved in sterile

saline and proven to be sterile and pyrogen-free before use. Studies were performed in the post-absorptive state. At 08:00 am, primed-continuous infusions of the leucine tracers were started and carried out for 4 h (Tessari 1996). The D₃-KIC infusion rate was 0.0343 ± 0.0051 (Mean $\pm$ SE) $\mu\text{mol}/\text{kg}^1 \text{ min}$. Isotope priming doses were 30x the continuous infusion rates per min.

Sample processing and analytical methods were performed as described by Tessari et al. (1996) and Schwenk et al. (1984). We employed a compartmental model in the analysis of the regional leucine metabolic steps, as previously reported (Tessari 1996) (Tessari 1995) (see: Supplemental Methods and Supplemental Figure 1). Arterial and venous concentrations and enrichments (Supplemental Figure 2) of Leucine, Kic and CO₂ remained stable, indicating that tracers and tracees were at steady state throughout the study.

Statistical analysis

The data were reported as Means $\pm$ SE. Organ as well as whole body kinetics data were normalized per 1.73 m² of body surface (Tessari 1996). The Wilcoxon test for paired data was used to compare the rates of leucine deamination and reamination within each organ as well as in the whole body, using the Statistical Software (Version 7.1, StatSoft Italia). In addition, the data were analyzed also using the two tailed Student's t test for paired data. To compare the percent contribution of each organ to whole-body deamination or reamination rates, as well as the fraction of leucine oxidation over either leucine deamination to KIC or KIC reamination to leucine among the different organs, we employed the One Way ANOVA followed by the Newman Keuls post hoc test. A p value less than 0.05 was considered statistically significant.

Results

Leucine deamination and KIC reamination

The rates of whole-body leucine deamination to KIC were quite similar using either Model 1 as reported by Matthews (1981) or Model 2 (based on the data calculated from the infusion of the independent KIC tracer (Figure 1).

Leucine deamination to KIC was greater than KIC reamination to leucine at whole body level ($p=0.005$), in skeletal muscle ($p=0.005$) and in the splanchnic area ($p=0.025$), whereas these rates were not significantly different from each other in the kidneys (Figure 2A), as previously reported (Tessari 1996).

When expressed as percentage of whole-body rates, total skeletal muscle accounted for $\approx 60\%$ of leucine deamination and $\approx 78\%$ of leucine reamination, the splanchnic bed for $\approx 15\%$ and $\approx 15\%$, and the kidneys for $\approx 12\%$ and $\approx 18\%$, respectively (Figure 2B). The sum of the percent contributions by the three organs, to body leucine deamination to KIC was $\approx 87\%$, while that of KIC reamination to leucine was $\approx 112\%$.

Leucine flux through oxidation, deamination and reamination.

The sum of leucine deamination (the F7 model parameter) and leucine oxidation (F9), as well as the sum of reamination (F8) and oxidation, expressed both as $\mu\text{moles/min}$ and as percentage of whole body rates, are reported in Table 2. In absolute terms, skeletal muscle accounted for the largest portion of these rates in respect to those of the whole-body, about 4-fold greater than those of either the splanchnic bed or the kidneys. In relative terms, the sum of the three sampled districts accounted for $\approx 80\%$ (for F7+F8), and for 93% (for F8+F9), of the corresponding whole-body rates.

The ratios of leucine oxidation (F9) to deamination (F7), and that of leucine oxidation to reamination (F8), are reported in Figure 3. These ratios indicate the fraction of leucine irreversible loss (through oxidation), over either leucine deamination to KIC (Figure 3A), or KIC reamination to leucine (Figure 3B), and they can approximate the “metabolon” concept

in vivo across the three sampled organs. In the kidneys, percent leucine oxidation over either deamination ($55\pm 16\%$) or reamination ($119\pm 47\%$), was >3-fold greater than the corresponding values calculated across both the skeletal muscle ($15\pm 3\%$, and $23\pm 8\%$ for deamination and reamination, $p<0.04$ and $p<0.01$ respectively) and the splanchnic bed ($17\pm 7\%$, and $36\pm 17\%$, $p<0.005$ and $p<0.04$, respectively (Figures 3A and 3B). These data indicate that, in the kidneys, leucine is preferentially oxidized, to a much larger extent than that observed in either the skeletal muscle or the splanchnic area.

Discussion

BCAA metabolism in humans is tightly regulated to maintain levels sufficiently high to support major functions, such as protein synthesis, provision of N for the synthesis of non-essential amino acids and hormone-like signals, but at the same time excess levels are prevented by the upregulation of irreversible disposal pathways (Suryawan 1998). Four major observations can be drawn from this study. First, in post-absorptive human beings, skeletal muscle accounts for the largest fraction of body BCAA leucine deamination and reamination, even larger than that expected from tissue BCAA-Tm distribution (Suryawan 1998). Second, a relevant fraction ($\approx 30\text{--}35\%$) of these rates is accounted for by visceral organs (splanchnic bed and kidneys). Third, skeletal muscle and visceral organs together virtually account for body total leucine deamination and reamination. Fourth, in the kidneys leucine is preferentially channeled towards oxidation rather than to reamination.

Overall, our *in vivo* data demonstrate that skeletal muscle plays a major role in overall leucine metabolism. The high percentage of body leucine deamination and oxidation accounted for by skeletal muscle, in agreement with the concept that the BCAAs are predominantly catabolized by extra hepatic tissues, as also suggested by their low first-pass splanchnic extraction (Biolo 1992) (Matthews 1993) (Stoll 1998). Also leucine oxidation

took place to a large extent in skeletal muscle ($\approx 37\%$ of whole body rates), as previously reported (Tessari 1996). Of note, the high rates of leucine deamination and reamination in muscle can provide nitrogen (N) for the synthesis of non-essential amino acids such as alanine and glutamine, the key nitrogen carriers from the periphery (skeletal muscle) to the liver.

We observed that in the whole body leucine deamination is 6- to 7-fold faster than oxidation (Tessari 1996), a finding similar to what was previously observed for valine also (Staten 1984). A similar leucine deamination/oxidation ratio is observed in skeletal muscle and in splanchnic organs. These data suggest that in muscle and splanchnic organs the transamination step may regulate the free levels of the individual BCAA's, while decarboxylation limits their catabolism. Contrarywise, the leucine deamination and oxidation rates are similar in the kidney.

A new finding of this study is that the contribution by visceral organs to whole body leucine deamination and reamination rates is substantial (about one third of total), and almost equally shared by kidneys and splanchnic organs. In the fetal kidney BCAA transport is necessary for early nutrition and development (Guetg, 2015). The adult kidney, in particular in the medullary thick ascending limb, is rich in all the enzymatic machinery involved in leucine catabolism and/or oxidation. Oxidation of leucine in this nephron segment may provide energy to sustain active ion transport (Tring-Trang-Tan, 1988).

The liver is thought to be the primary site for the oxidation of branched-chain keto acid (BCKA) but not BCAA (Brosnan & Brosnan, 2006). In this study the contribution of splanchnic organs to whole body leucine deamination and reamination rate was greater than expected ($\sim 15\%$). However, we could not evaluate the oxidation of KIC to beta-hydroxy-beta-methylbutyrate (HMB) by the enzyme KIC dioxygenase, a reaction which takes predominantly in the liver. In liver rats it has been observed that a minor percentage (about

5%) of daily leucine metabolism is channeled through the dioxygenase pathway (Van Koeveering and Nissen 1992).

Sites other than muscle and visceral organs, such as adipose tissue, brain, heart and lung (that couldn't be sampled in our study), should contribute minimally to leucine metabolism. However, concerning body fat, it should be considered that the data derived from skeletal muscle (i.e. leg) catheterization also include the contributions of both intramuscular and subcutaneous adipose tissue (Frick 1988).

One original contribution of our study is the investigation of the relative roles of leucine deamination, reamination and oxidation, both in the whole body and in selected organs in humans. By such data presentation, we intended to depict a sort of leucine “metabolon” in humans. Nevertheless, we are fully aware that the transfer of the “metabolon” concept into the *in vivo* human setting is rather complex, and perhaps not entirely appropriate. One example and/or limitation of such a transfer is given by the precursor substrate(s) of renal leucine oxidation. Since in the kidneys leucine deamination and reamination rates were not statistically different from each other leucine oxidation in the kidneys ($\approx 4.5 \mu\text{mol/min}$, unreported data) should have predominantly derived from other, unaccounted sources: one would likely be KIC itself. Indeed, as reported previously (Garibotto 2002), there was a net KIC uptake by the kidneys, of $\approx 3.3 \mu\text{mol/min}$ (using plasma data), a figure that, added to the net (albeit insignificant) difference between deamination and reamination ($\approx 0.5 \mu\text{mol/min}$), yields a total of $3.8 \mu\text{mol/min}$, that would account for $\approx 85\%$ of renal total leucine oxidation. Therefore, should leucine oxidation in the kidneys predominantly derive from the KIC taken up (i.e. not from leucine deamination itself), thus directly entering the mitochondria for oxidation, this other oxidation route may not be strictly considered under the “metabolon” concept. On the other hand, the limitation of our model and/or approach is intrinsically linked to the complexity of the studies in humans.

Another feature of BCAAs is the role of leucine as an anabolic nutrient signal to stimulate protein synthesis by activation of mTORC1, an effect reinforced by physical exercise and protein feeding. Several conditions causing wasting, such as sarcopenia of aging and inflammation blunt the leucine-induced mTORC1 activation (Ham, 2014). A limitation of our study is that we evaluated leucine deamination only in the postabsorptive, basal state. In addition, our patients were studied at rest, and the effects of physical exercise on leucine metabolism have not therefore been addressed.

The understanding of BCAA/Tm-BCKD activity in individual organs and in the whole body in disease is of major importance in several clinical fields. Elevated levels of BCAAs are implicated in obesity, insulin resistance and type 2 diabetes (Adams, 2011). An emergent hypothesis is that in obese, insulin-resistant state or in T2DM, raised BCAA and BCKA levels reflect reduced BCAA/Tm-BCKD activity in a variety of metabolically relevant tissues such as liver, WAT, and possibly muscle (Adams, 2011). However this hypothesis still needs to be confirmed in human studies. It is interesting that in animal models, liver BCKD activity can be modulated by protein intake (Brosnan and Brosnan 2006). Should this take place also in humans, changes in the amounts or quality of ingested protein could influence BCKD activity in different tissues to correct the alterations in BCAA/BCKA pattern in insulin resistant states.

Further studies are required to understand mechanism and mediators which regulate deamination and reamination rates in sepsis. During sepsis, accelerated protein degradation is associated with increased transamination and oxidation of amino acids in skeletal muscle (Woolf 1979). Reamination of KIC to leucine has also been shown to be enhanced in the liver of starved and endotoxin treated rats (Holecek 2001).

BCAA deamination and transamination is also a potential major topic in patients with chronic kidney disease (CKD). Our finding of preferential leucine/KIC degradation in the

human kidney suggests that progressive CKD may be associated with reduced requirement for leucine. This observation may explain, at least in part, the good nutritional status achieved even with very low protein diets in non-dialyzed patients with CKD (Bellizzi, 2016). Of note, supplemented very low protein diets (SVLPDs) (0.28-0.40 g/kg) containing branched-chain ketoacids are offered to CKD patients to provide EAA precursors without the nitrogen load from EAAs. These SVLPDs appear to generate less toxic metabolic products than similar amounts of protein from LPDs (Gao 2010) and have proven to be effective and safe when postponing dialysis treatment in elderly CKD patients (Bellizzi 2016). However the optimal doses of keto-acids and the muscle and systemic adaptations to keto acid supplementation are still unresolved.

In conclusion, this study provides estimates of leucine deamination, reamination and oxidation in the skeletal muscle, the splanchnic bed and the kidneys, as well as in the whole body in the post-absorptive state, in humans. Whereas in absolute terms, the most relevant contributions are provided by skeletal muscle; in relative terms the kidney plays a remarkable role particularly in leucine oxidation. The data here presented could be of help in the understanding of whole-body as well as organ leucine metabolism, and they can ultimately lead to a better modeling of amino acid metabolism. They may also be important in the calculation of the amount of leucine effectively delivered to tissues, both from a nutritional standpoint and for the associated signaling effect.

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Statement of Authorship

G.G. and P.T. designed the protocol, recruited the subjects, contributed to study performance, data and statistical analyses and the overall data evaluation. P.T. developed the original model of whole body leucine deamination. D.V. and M.V. performed the laboratory analyses and critically reviewed the data. G.G. and P.T. wrote the manuscript and had the primary responsibility for the final content. All authors read and approved the final manuscript.

Conflict of Interest Statement and Funding sources

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Legends

Figure 1. Rates of whole body leucine deamination to KIC (i.e. of the KIC derived from leucine) calculated using either Model 1 (from Matthews 1993] or from Model 2 (Equations 4 to 6 of the compartmental model). Data are Means $\pm$ SE of 10 subjects. The deamination data calculated with the two models were virtually identical ($p>0.85$ by paired t-test).

Figure 2. (A): Rates of leucine deamination to α -ketoisocaproate, KIC (Leu $\rightarrow$ KIC), and of KIC reamination to leucine (KIC $\rightarrow$ Leu), across total Skeletal Muscle (n=10), the Splanchnic Bed (n=8), the Kidneys (n=7), as well as in the Whole Body. Rates are expressed as μ moles / min. Levels of the statistically significant difference between deamination and reamination (by the Wilcoxon paired t test) within each organ as well as in the whole body, are reported. Data are shown as Means $\pm$ SE.

(B): Percent contributions to the Whole Body, of leucine deamination to α -ketoisocaproate, KIC (Leu $\rightarrow$ KIC), and of KIC reamination to leucine (KIC $\rightarrow$ Leu), across total Skeletal Muscle (n=10), the Splanchnic Bed (n=8) and the Kidneys (n=7), as well as the Sum of the percentages of the three organs. The reported levels of statistical significance indicate the differences (by the One Way ANOVA and the Newman Keuls post hoc test) between rates in either the Splanchnic Bed or the Kidneys, and the corresponding ones in Skeletal Muscle. The Sum of rates from the three organs are reported as gross means (i.e. without SE), because not all subjects were studied across all the three organs. The sum of either deamination or reamination are not significantly different from 100% ($p>0.7$). Data are shown as Means $\pm$ SE.

Figure 3. (A) The percentage of leucine oxidation (F9 model parameter) vs. deamination (F7) of each organ as well as in the whole body (WB). The reported “p” values indicate the level of the significant differences between either total Skeletal Muscle (SM, n=10), or the Splanchnic Bed (SB, n=7) and the kidneys (KD, n=6), calculated by the One Way ANOVA and the Newman Keuls post hoc test). Whole body ratios (n=10) are also reported. Data are shown as Means $\pm$ SE.

(B) The percentage of leucine oxidation (F9 model parameter) vs. reamination (F8) of each organ as well as in the whole body (WB). The reported “p” values indicate the level of the significant differences between either total Skeletal Muscle (SM, n=10), or the Splanchnic Bed (SB, n=7) and the kidneys (KD, n=7), calculated by the One Way ANOVA and the Newman Keuls post hoc test). Whole body ratios (n=10) are also reported. Data are shown as Means $\pm$ SE.

Supplemental Figure 1. Schematic representation of the modified six compartment model (Tessari 1995). Briefly, the *tracee* model (with some added notations) is here depicted. A more detailed discussion can be found in the original paper. The *tracee* model is composed of six compartments, three of leucine and three of KIC. For each substrate, one compartment is in the artery, one is intracellular and one is in the vein. The notations F1 and F4 indicate the flux of tracee (e.g. unlabeled) leucine into the artery and out in the vein, respectively. F2, F3 and F12 indicate the leucine flux from artery to cell, from cell to vein and from artery directly to vein, respectively. F5 indicates the release of free leucine in the intracellular (i.c.) space from protein degradation. F6 indicates intracellular leucine incorporation into protein (e.g. protein synthesis). F7 and F8 indicate leucine conversion into KIC, and *vice versa*. F9 indicates the irreversible loss of the leucine-carbon (actually, of KIC) to oxidation. Finally, F10 and F11 indicate KIC flux into the artery and out into the vein, respectively.

At steady state, the sum of entries into each compartment (or sum of all compartments considered together) must be equal to the sum of exits from each compartment (or the sum of them). No tracer recycling from proteolysis is assumed to occur during the experiment.

The notation: [$^{15}\text{N}\text{-Leu} + ^{14}\text{C}\text{-Leu}$] *peripheral infusion* indicates the infusion of the two leucine tracers into the circulation; that of [$^{15}\text{N}\text{-Leu} + ^{14}\text{C}\text{-Leu}$] $\rightarrow$ *i.c.* indicates the flux of tracers from blood to the intracellular compartment(s); that of $^{14}\text{C}\text{-[Leu} \leftrightarrow \text{KIC]}$ indicates the reversible interconversion between ^{14}C -Leucine and ^{14}C -KIC; and, finally, the arrow leading to $^{15}\text{N}\text{-[Leu]}$ indicates the irreversible loss of ^{15}N from leucine through i.c. deamination.

Supplemental Figure 2. Steady-state values (reported as means $\pm$ SE) of plasma Leucine concentration (panel a), KIC concentration (panel b), ^{15}N -Leucine mole percent enrichment (MPE) (panel c), $^2\text{H}_3$ -KIC MPE (panel d), ^{14}C -Leucine specific activity (SA) (panel e), ^{14}C -KIC SA (panel f), and ^{14}C -Bicarbonate concentration (panel g), in the artery (n = 10), the femoral (n = 10), the hepatic (n = 8) and the renal veins (n = 7), and of expired $^{14}\text{CO}_2$ (n = 10) (panel h).

Table 1: Whole-body leucine kinetics either derived from Matthews et al. (1981) or determined in the present study.

<i>$\mu\text{mol/kg} \times \text{hr}$</i>	Matthews et al (1981)	Present data
Leu C Ra	120	122
Leu N Ra	155	174
Leu Ox	21	23
Leu to KIC (=deamination)	56	87
KIC to Leu (=reamination)	35	58

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Table 2: The sum of leucine deamination (F7 model parameter) and of leucine oxidation (F9 model parameter) (in $\mu\text{moles/min}$), as well as of leucine reamination (F8) and oxidation (F9), across each of the three sampled districts and in the whole body, and their percentage contribution to whole body rates. Results are shown as Means $\pm$ SE

	Total skeletal muscle (n=10)	Splanchnic area (n=8)	Kidneys (n=7)	Whole-body (n=10)
Leu Deam. (F7) + Ox (F9)	55.1 $\pm$ 7.7	13.8 $\pm$ 5.1	13.6 $\pm$ 2.3	125.1 $\pm$ 15.6
[F7+F9] as % whole body	53.1 $\pm$ 10.9	14.0 $\pm$ 6.3	12.9 $\pm$ 3.2	$\approx$ 80%
Leu Ream. (F8) + Ox (F9)	46.4 $\pm$ 6.7	8.9 $\pm$ 3.5	13.1 $\pm$ 2.3	91.5 $\pm$ 17.4
[F8+F9] as % whole body	62.1 $\pm$ 10.5	14.2 $\pm$ 7.3	17.3 $\pm$ 3.8	$\approx$ 93%

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Figure 1. Rates of whole body leucine deamination to KIC (i.e. of the KIC derived from leucine) calculated using either Model 1 (from Matthews 1993] or from Model 2 (Equations 4 to 6 of the compartmental model). Data are Means $\pm$ SE of 10 subjects. The deamination data calculated with the two models were virtually identical ($p>0.85$ by paired t-test).

254x190mm (300 x 300 DPI)

Figure 2. (A): Rates of leucine deamination to α -ketoisocaproate, KIC (Leu→KIC), and of KIC reamination to leucine (KIC→Leu), across total Skeletal Muscle ($n=10$), the Splanchnic Bed ($n=8$), the Kidneys ($n=7$), as well as in the Whole Body. Rates are expressed as $\mu\text{moles} / \text{min}$. Levels of the statistically significant difference between deamination and reamination (by the Wilcoxon paired t test) within each organ as well as in the whole body, are reported. Data are shown as Means $\pm$ SE.

(B): Percent contributions to the Whole Body, of leucine deamination to α -ketoisocaproate, KIC (Leu→KIC), and of KIC reamination to leucine (KIC→Leu), across total Skeletal Muscle ($n=10$), the Splanchnic Bed ($n=8$) and the Kidneys ($n=7$), as well as the Sum of the percentages of the three organs. The reported levels of statistical significance indicate the differences (by the One Way ANOVA and the Newman Keuls post hoc test) between rates in either the Splanchnic Bed or the Kidneys, and the corresponding ones in Skeletal Muscle. The Sum of rates from the three organs are reported as gross means (i.e. without SE), because not all subjects were studied across all the three organs. The sum of either deamination or reamination are not significantly different from 100% ($p>0.7$). Data are shown as Means $\pm$ SE.

197x158mm (300 x 300 DPI)

Figure 3. (A) The percentage of leucine oxidation (F9 model parameter) vs. deamination (F7) of each organ as well as in the whole body (WB). The reported “p” values indicate the level of the significant differences between either total Skeletal Muscle (SM, n=10), or the Splanchnic Bed (SB, n=7) and the kidneys (KD, n=6), calculated by the One Way ANOVA and the Newman Keuls post hoc test). Whole body ratios (n=10) are also reported. Data are shown as Means $\pm$ SE.

(B) The percentage of leucine oxidation (F9 model parameter) vs. reamination (F8) of each organ as well as in the whole body (WB). The reported “p” values indicate the level of the significant differences between either total Skeletal Muscle (SM, n=10), or the Splanchnic Bed (SB, n=7) and the kidneys (KD, n=7), calculated by the One Way ANOVA and the Newman Keuls post hoc test). Whole body ratios (n=10) are also reported. Data are shown as Means $\pm$ SE.

190x254mm (300 x 300 DPI)

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Supplemental Methods

Whole-body calculations

“*Conventional model*”. Whole-body KIC reamination to leucine [KIC → Leu] (expressed as $\mu\text{mol/kg} \times \text{min.i.73 m2}$) was calculated using the [^{15}N -Leu] enrichment and the [^{14}C -Leu] specific activity in arterial plasma by applying a published model (Matthews 1981):

Eq. 1: [KIC → Leu] = [^{15}N -Leu Ra] – [^{14}C -Leu Ra]

where: [^{15}N -Leu Ra] and [^{14}C -Leu Ra] are the rates of appearance (as $\mu\text{mol/kg} \times \text{min. 1.73 m2}$) of leucine calculated using plasma [^{15}N -Leu] enrichment as well as plasma [^{14}C -Leu] specific activity, and conventional steady-state formulas. At variance with the original model of Matthews et al. (1981), however, we used two distinct tracers of leucine, not the single, doubly labelled [^{15}N , ^{13}C -Leu] isotope. This choice was motivated mainly because of difficulties in the measurements of blood and plasma ^{13}C -bicarbonate, whereas we worked out and used a reliable method for the measurement of plasma ^{14}C -bicarbonate, which is required for the calculation of organ leucine oxidation, to be related to whole body rates. While our approach might have led to a possible, yet undetermined, recycling of ^{15}N -labeled amino groups from glutamate back to KIC to yield ^{15}N -leucine, such a potential bias would have been equally offset by a similar conceptual approach at organ level (Tessari 1996).

In the following table, we show the data of whole-body leucine kinetics as measured in the present and those derived from the referenced (Matthews 1981) study. As it can be seen, our approach results in similar rates of leucine-carbon Ra, a $\approx 10\%$ greater leucine-nitrogen Ra, similar oxidation rates, a $\approx 35\%$ lower leucine deamination to KIC, and a $\approx 39\%$ lower KIC reamination to leucine. Whether the observed differences are due to

subjects' population, to the different laboratories and methods of analyses, or to the different types of tracers, cannot be determined. Nevertheless, we used the same isotope couple (i.e. separate [^{15}N -Leu Ra] and [^{14}C -Leu Ra] tracers of leucine, for both the whole-body and the organ calculations (Tessari 1996) (Tessari 1995).

In addition, and in support to the validity of our approach, the data of the rate of leucine conversion to KIC calculated from Matthews' model using the two distinct leucine tracers here employed (Supplemental Table 1), turned out to be quite similar to the rate of KIC production from leucine (i.e. an alternative method to measure deamination), calculated from the independent D_3 -KIC tracer (see below) (Figure 1 of the main MS), thus reassuring about the reliability of our measurements.

Leucine oxidation [Leu Ox], that is required to estimate leucine deamination (Matthews 1981) was calculated as follows:

$$\text{Eq. 2:} \quad [\text{Leu Ox}] = \frac{\text{expired } [^{14}\text{CO}_2]}{\text{plasma } [^{14}\text{C}]\text{-KIC SA}}$$

where: expired [$^{14}\text{CO}_2$] is the rate of $^{14}\text{CO}_2$ expiration (in DPM/kg x min), corrected for 20% fixation into body bicarbonate pool, then divided over plasma ^{14}C -KIC specific activity (in DPM/nmol). We used in this instance plasma ^{14}C -KIC as a precursor pool because the same assumption is employed in the compartmental model (Tessari 1995) (see below).

Leucine deamination to KIC [Leu → KIC] was then calculated as the sum of leucine reamination and oxidation as follows:

Eq. 3: [Leu → KIC] = [KIC → Leu] + [Leu Ox]

The rates calculated with Eqs. 1 to 3, expressed as μmol/kg x min, were then referred to a whole-body basis by multiplying them for the body weight.

Alternative model.

Since independent tracers of leucine (¹⁴C-Leu) and of KIC (D₃-KIC) had been infused, we could calculate also the rate of appearance in plasma of ¹⁴C-KIC from the infused ¹⁴C-Leu by means of the D₃-KIC stable isotope. This rate represents another way to calculate the ¹⁴C-leucine deamination to ¹⁴C-KIC, and can be employed to calculate total leucine deamination. Such an approach is actually the same as that used previously with the simultaneous infusions of oral vs intravenous tracers leucine and KIC tracers (Kimball 2004) (Garibotto 2002).

Thus, the rate of appearance in plasma of [¹⁴C-KIC], defined as [¹⁴C-KIC_{pl}], from the infused [¹⁴C-Leu], estimated using the infused D₃-KIC isotope, was calculated as follows:

D₃-KIC inf

Eq. 4:
$$[^{14}\text{C-Leu} \rightarrow ^{14}\text{C-KIC}_{\text{pl}}] = \frac{\text{D}_3\text{-KIC enrichment}}{\text{plasma } ^{14}\text{C-KIC SA}}$$

where: $[\text{D}_3\text{-KIC inf}]$ is the rate of intravenous infusion of $[\text{D}_3\text{-KIC}]$ (in $\mu\text{mol/kg} \times \text{min}$); $[\text{D}_3\text{-KIC enrichment}]$ is arterial plasma enrichment of $\text{D}_3\text{-KIC}$; and $[^{14}\text{C-KIC SA}]$ is arterial plasma $[^{14}\text{C}]$ -KIC specific activity (in DPM/nmol). The final unit of measurement of Eq. 4 is in DPM/kg $\times$ min. To the value calculated with Eq. 4, the fraction of ^{14}C counts of the $^{14}\text{C-KIC}$ formed inside the cell from $^{14}\text{C-Leucine}$ deamination and disappearing into oxidation (i.e. to $^{14}\text{CO}_2$), should be added, since they are a product of leucine deamination as well. Therefore, the rate of $^{14}\text{CO}_2$ expiration was added to the: $[^{14}\text{C-Leu} \rightarrow ^{14}\text{C-KIC}_{\text{pl}}]$ calculated in Eq. 4, to yield the total rate of deamination of $^{14}\text{C-Leucine}$ to $^{14}\text{C-KIC}$, here defined as: $[^{14}\text{C-Leu} \rightarrow ^{14}\text{C-KIC}_{\text{tot}}]$

Eq. 5:
$$[^{14}\text{C-Leu} \rightarrow ^{14}\text{C-KIC}_{\text{tot}}] = [^{14}\text{C-Leu} \rightarrow ^{14}\text{C-KIC}_{\text{pl}}] + [^{14}\text{CO}_{2\text{exp}}]$$

where: $[^{14}\text{CO}_{2\text{exp}}]$ is the rate of expiration of $^{14}\text{CO}_2$ (in DPM/kg $\times$ min).

By dividing the result of Eq. 5 over plasma $^{14}\text{C-KIC SA}$, the rate of KIC appearance from leucine (i.e. leucine deamination to KIC), here defined as $[\text{Leu} \rightarrow \text{KIC}]$ is calculated:

Eq. 6: $[Leu \rightarrow KIC] = \frac{[^{14}C\text{-}Leu \rightarrow ^{14}C\text{-}KIC_{tot}]}{plasma\ [^{14}C]\text{-}KIC\ SA}$

Organ calculations

The rates of leucine conversion to KIC, as well as of KIC conversion to leucine, which we assume to represent leucine deamination to KIC, and KIC reamination to leucine, respectively, were calculated using the six compartment model previously reported (Tessari 1995). Briefly, the basic assumptions of this model are the following: (1) venous $^{14}C\text{-}KIC$ SA is representative of the leucine SA inside the cell; and (2) the rate of leucine inflow into cell is calculated from the net disappearance rate of $[^{15}N]\text{-leucine}$ across each organ. Thereafter, the various exchange rates between the six compartments are derived. The assumptions and limitations of this model are discussed elsewhere (Tessari 1995 and 1996).

The equations upon which Leucine deamination to KIC and of KIC reamination to leucine are calculated, are here reported:

Eq. 7: Reamination $[Leu \rightarrow KIC] = \frac{[Leu\text{-}N\ Ra] - [Leu\text{-}C\ Ra]}{\{1 - [(Leu\ SA_{ven} - KIC\ SA_{ven}) / Leu\ SA_{ven}]\}}$

Eq. 8: Net transamination (T) = $Leu\ Ox + KIC\ Ra_{ven} - KIC\ Ra_{art}$

Where : KIC Ra_{ven} and KIC Ra_{art} are the rates of appearance of KIC as measured using either venous or arterial plasma ^{14}C -KIC SA.

Then:

Eq. 9: Deamination = Net transamination + Reamination

A detailed description of these calculation is reported elsewhere (Tessari 1996).

In the light of the recently proposed “Metabolon” concept, we also calculated the sum of leucine oxidation (F9) and either leucine deamination (F7) and KIC reamination (F8), across each organ as well as in the whole body. We used the whole body deamination data obtained with our model (see Eqs. 4 to 6), whereas for the reamination data we used Matthews’s model (Matthews 1981). These data are presented in Table 1. In addition, we expressed organ leucine oxidation also a percent value of either deamination (F7) or reamination (F8) (Figure 3). However, since in one subject the deamination rate (F7) in the kidney was (slightly) negative, whereas in another the reamination rate (F8) in the splanchnic bed was (slightly) negative, the calculation of ratios of F9 over either F7 or F8 would yield large negative numbers. For this reason, we did not utilize these data for the calculations of the means. The organ data have been normalized per 1.73 m² of body surface_ (Tessari 1996). All data were expressed as $\mu\text{mol}/\text{min}$, either in the whole body or at each organ level. They are shown as Means $\pm$ SE.

Plasma vs. whole-blood measurements.

The kinetic data of this study were calculated using plasma specific activities and enrichments. The resulting estimates are somehow different (albeit quantitatively but not qualitatively) from those previously published (Tessari 1996), likely because the latter were calculated using whole-blood measurements. In the present study however, we couldn't use whole-blood data as well, because under our experimental conditions (i.e. the infusion of the two independent ^{15}N -leucine and the ^{14}C -leucine tracers), there was no significant difference between the rate of whole-body leucine appearance calculated upon measurements of the ^{15}N -leucine enrichment and that calculated from the ^{14}C -leucine specific activity in whole blood, thus preventing the application of Matthews's model (Matthews 1981). As a matter of fact, the initial equation of that model (Matthews 1981) is based on the difference between the rates of leucine appearance derived from measurements of plasma ^{15}N -, ^{14}C -leucine vs. ^{14}C -leucine enrichments, following the infusion of the doubly-labelled ^{15}N -, ^{14}C -leucine single tracer. Therefore, if there is no difference between the two estimates, no calculation of leucine deamination and reamination can be performed. In contrast, in our study a net and consistent difference between the leucine Ra data calculated with the two independent tracers (i.e. the ^{15}N -leucine and the ^{14}C -leucine) was observed using the plasma measurements.

The possible reason(s) for such an unexpected finding need to be discussed. There are two separate points to be addressed.

One concerns the type of the leucine tracer(s) employed in the studies. The use of two independent tracers of leucine (i.e. the stable isotope ^{15}N tracer and the radioactive ^{14}C tracer, as we did in our study), rather than the doubly-labelled ^{15}N -, ^{14}C -leucine single stable isotope tracer, originally employed in (Matthews 1981), might have led to some, yet unaccounted, recycling of ^{15}N from intracellular nitrogen pools back to unlabelled KIC,

to yield the ^{15}N -leucine again. In contrast, such a recycling (of ^{15}N back to ^{14}C -KIC to yield the double labelled ^{15}N -, ^{14}C -leucine again) might be minimized following the infusion of the doubly-labelled ^{15}N -, ^{14}C -leucine single stable isotope tracer as proposed in the original model, because it is highly unlikely that the ^{15}N would be transferred specifically to a ^{14}C -labelled KIC, rather than to the far more abundant unlabelled KIC (Matthews 1981). These potential, subtle differences in ^{15}N -leucine recycling using different tracers, possibly combined also with a somewhat larger analytical variation using both stable and radioactive isotope measurements in our study, vs. the use of only stable isotope analyses (Matthews 1981), might explain the above outlined limitation.

The second point concerns a possible, untoward effect derived from the combination of the ^{15}N isotope recycling with the specific site of isotope measurements (i.e. either plasma or whole-blood). Using plasma measurements (as we did in this study) Matthews's model (also developed on plasma measurements) (Matthews 1981) could be employed to our experimental conditions too, as anticipated above, and the resulting leucine deamination rate came out to be virtually identical to that determined with the simultaneous infusion of an independent KIC tracer, despite the use of different leucine tracers. We don't have a clear explanation for such a consistent and perhaps lucky result using plasma measurements. It is possible that whole-blood measurements (which required whole-blood deproteinization and had a somehow greater analytical variability), combined to possible specific effect due to the leucine isotopes employed, limited the feasibility of the use of whole-blood data in our experimental conditions. In addition, a specific role of red blood cells in leucine handling (both labelled and unlabelled) cannot be excluded (Garibotto 2002) (Tessari 2010).

[¹⁵N-Leu + ¹⁴C-Leu] peripheral infusion