Determination of the tolerable upper intake level of leucine in acute dietary studies in young men¹–⁴

Rajavel Elango, Karen Chapman, Mahroukh Rafii, Ronald O Ball, and Paul B Pencharz

ABSTRACT

Background: Leucine has been suggested to improve athletic performance. Therefore, the branched-chain amino acids (BCAAs), especially leucine, are popular as dietary supplements in strength-training athletes; however, the intake of leucine in excess of requirements raises concerns regarding adverse effects. Currently, the tolerable upper intake level (UL) for leucine is unknown.

Objective: The objective of the current study was to determine the UL for leucine in adult men under acute dietary conditions.

Design: Five healthy adults (20–35 y) each received graded stepwise increases in leucine intakes of 50, 150, 250, 300, 750, 1000, and 1250 mg · kg⁻¹ · d⁻¹, which corresponded to the Estimated Average Requirement (EAR) and the EAR ×3, ×5, ×10, ×15, ×20, and ×25 in a total of 29 studies. The UL of leucine was identified by the measurement of plasma and urinary biochemical variables and changes in leucine oxidation by using L-[1-¹³C]-leucine.

Results: A significant increase in blood ammonia concentrations above normal values, plasma leucine concentrations, and urinary leucine excretion were observed with leucine intakes >500 mg · kg⁻¹ · d⁻¹. The oxidation of L-[¹³C]-leucine expressed as label tracer oxidation in breath (F¹³CO₂), leucine oxidation, and α-ketoisocaproic acid (KIC) oxidation led to different results: a plateau in F¹³CO₂ observed after 500 mg · kg⁻¹ · d⁻¹, no clear plateau observed in leucine oxidation, and KIC oxidation appearing to plateau after 750 mg · kg⁻¹ · d⁻¹.

Conclusion: On the basis of plasma and urinary variables, the UL for leucine in healthy adult men can be suggested at 500 mg · kg⁻¹ · d⁻¹ or ~35 g/d as a cautious estimate under acute dietary conditions. This trial was registered at clinicaltrials.gov as NCT00972582.

INTRODUCTION

In the United States, ~3.4% of the population uses amino acid supplements, 62% of whom uses amino acid supplements on a daily basis (1), and thus, we must be concerned that these individuals may consume excessive amounts of amino acids. Excessive intakes of free amino acid may have adverse effects; however, there are very few data to either confirm or deny this position. Some amino acids have specific metabolic functions in addition to requirements for protein synthesis (eg, the stimulation of protein synthesis by leucine, synthesis of catecholamines from aromatic amino acids, methyl and sulfur donation from sulfur amino acids, and nitric oxide from arginine). Therefore, dietary supplementation with specific amino acids in excess of the requirement for protein synthesis may have adverse effects or it may be beneficial in some situations. For these reasons, additional knowledge is necessary regarding the highest possible intake of each amino acid at which no adverse effect occurs (2, 3).

The branched-chain amino acids (BCAAs)⁵ include leucine, valine, and isoleucine and are popular as dietary supplements in strength-training athletes. In particular, leucine has been implicated to improve athletic performance (4–11). Earlier reports (12, 13) have summarized studies of BCAA administration in athletes, normal adults, and patients with clinical disorders. Intakes of 15–60 g total BCAAs/d (~200–850 mg · kg⁻¹ · d⁻¹ for a 70-kg man) of total BCAAs did not result in adverse-event outcomes for the variables monitored. A significant problem with the interpretation of the reported human studies is the great variability in approaches; these experiments include supplements of all BCAAs at many different doses, ratios in BCAAs in intake, routes of infusion and consumption (intravenous compared with oral), and athletic training regimens.

Markers for the identification of an excess intake of an amino acid should have specific dose-response characteristics. In particular, a variation in intake should display an inflection point that would identify the onset of the amino acid–excess situation. Previously, in neonatal piglets, we observed an upper inflection point in the dose-response curve for phenylalanine retention and ¹⁴CO₂ production from phenylalanine oxidation with graded phenylalanine intake (14). Once the maximum level of phenylalanine oxidation was reached, plasma phenylalanine concen-

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3Supported by a grant from the International Council on Amino Acid Science. Mead Johnson Nutritional donated the protein-free powder for experimental diets.

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Abbreviations used: ALT, alanine aminotransferase; APE, atom percent excess; BCAA, branched-chain amino acid; EAR, Estimated Average Requirement; F¹³CO₂, label tracer oxidation in breath; KIC, α-ketoisocaproic acid; REE, resting energy expenditure; SickKids, The Hospital for Sick Children; UL, tolerable upper intake level.

Received July 29, 2011. Accepted for publication July 10, 2012.

First published online September 5, 2012; doi: 10.3945/ajcn.111.024471.
trations and retention rose rapidly. Hence, we reasoned that a suitable marker to define the upper limit of tolerance for a dietary amino acid would be the intake at which the maximum oxidation level was exceeded (15). We hypothesized that with increasing intakes of leucine above the Estimated Average Requirement (EAR of 50 mg · kg\(^{-1} \cdot d^{-1}\)) in adult men (16), the oxidation of leucine would increase and reach a maximum, after which the leucine oxidation would achieve a plateau. This metabolic limit to oxidize leucine may be used as a marker of an intake after which increasing intakes may result in increasing risk of adverse effects. The following 3 models of leucine oxidation were used in the current study: label tracer oxidation in breath (\(^{13}\)CO\(_2\)), leucine oxidation, and \(\alpha\)-ketoisocaproic acid (KIC) oxidation. For reasons that are not clear, discordant answers were obtained in the different models. Hence, we were forced to place most emphasis on the changes in metabolite concentrations for the determination of the leucine tolerable upper intake level (UL).

**SUBJECTS AND METHODS**

**Subjects**

Five healthy, young men participated in the study at the Clinical Investigation Unit, The Hospital for Sick Children (SickKids), Toronto, Canada. Subject characteristics, body composition, and energy intakes are summarized in Table 1. The subjects who participated in the study did not have any recent history of weight loss or illness, and none of the subjects used any medication during the study period. The purpose of the study and the potential risks involved were explained in detail to each subject, and written informed consent was obtained from all subjects. Subjects received financial compensation for costs incurred for participation in the study. The study underwent a clinical trial application review for safety and efficacy by Health Canada’s Natural Health Products Directorate to ensure subject safety. All procedures were also approved by the Research Ethics Board at SickKids.

**Experimental design**

Each subject participated in a dose-escalation study design in which graded stepwise increases in leucine intake were provided on each study day. This study design was chosen to ensure that subject safety could be monitored with each increased dose of leucine intake. A registered nurse was present at all times during the study day to monitor subjects and ensure safety. Each subject was initially studied at a leucine intake of 50 mg · kg\(^{-1} \cdot d^{-1}\), which has previously been estimated to be the EAR for leucine in adult men by using the indicator amino acid oxidation technique (16). After this baseline study, subjects received increased dietary leucine in a graded stepwise intake of 150, 250, 500, 750, 1000, and 1250 mg · kg\(^{-1} \cdot d^{-1}\), which corresponded to the EAR and the EAR ×3, ×5, ×10, ×15, ×20, and ×25 on separate study days. All study days were separated by a minimum of 2 wk to ensure a sufficient washout period between leucine-excess study-day diets.

Before the studies commenced, all subjects fasted overnight (~12 h), and an initial assessment was conducted at the Clinical Investigation Unit, SickKids, for body-composition analysis and measurements of resting energy expenditure (REE). Body composition was measured by using bioelectrical impedance analysis (BIA model 101A; RJL Systems). REE was measured by using continuous open-circuit indirect calorimetry (Vmax Encore, Metabolic cart; VIASYS).

**Study diets**

For 2 days before each study day, subjects were fed a milkshake-based standardized maintenance diet. Dietary intakes of the 2-d maintenance diets were provided in the form of milkshakes (Scandishake; Scandipharm) supplemented with carbohydrate (Polycose; Abbott Nutrition), protein (Beneprotein; Nestle Clinical Nutrition), and homogenized milk (3.25% fat) to meet the requirement of each subject. Except for water, no other food or beverages were consumed during the adaptation period. The maintenance diet provided protein at 1.0 g · kg\(^{-1} \cdot d^{-1}\) and energy at 1.7 × the REE. Before studies commenced, all subjects fasted overnight (~12 h). The study-day diet was consumed as hourly isocaloric meals (Figure 1). Each meal was formulated to represent one-twelfth of the daily energy requirement, which

| Table 1: Subject characteristics and energy intake

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>27.2 ± 2.1</td>
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<tr>
<td>Weight (kg)</td>
<td>71.4 ± 3.4</td>
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<tr>
<td>Height (cm)</td>
<td>173.6 ± 1.8</td>
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<td>BMI (kg/m(^2))</td>
<td>23.7 ± 1.2</td>
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<tr>
<td>LBM (kg)(^a)</td>
<td>50.2 ± 2.0</td>
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<tr>
<td>REE (kcal/d)(^b)</td>
<td>1704 ± 59</td>
</tr>
<tr>
<td>Energy intake (kcal/d)(^c)</td>
<td>2556 ± 89</td>
</tr>
</tbody>
</table>

\(^{a}\) All values are means ± SEMs (n = 5). LBM, lean-body mass; REE, resting energy expenditure.

\(^{b}\) Determined by using bioelectrical impedance analysis.

\(^{c}\) Determined by using open-circuit indirect calorimetry.

FIGURE 1. Study-day protocol for each oxidation study. Test meal: the experimental diet was given as a test meal hourly for 8 h. Isotopes: priming doses of NaH\(^{13}\)CO\(_3\) and L-[\(^1\)\(^3\)C]leucine were given at the fifth meal; an hourly dose of L-[\(^1\)\(^3\)C]leucine was simultaneously given and continued throughout the remaining 4 h. Samples: before the first meal, a fasted Bld and urine sample was collected. An hourly check of vital signs and Bld glucose was performed. Three baseline breath samples were collected at 45, 30, and 15 min, and one baseline plasma sample was collected at 15 min before the isotope protocol began. Four plateau breath and 2 plasma samples were collected at isotopic steady state every 30 min and 1 h, respectively, beginning 2.5 h after the start of the tracer protocol. VCO\(_2\) was measured by using indirect calorimetry after the fifth hourly meal. Bld, blood; Leu, leucine; VCO\(_2\), rate of carbon dioxide production.
depicted a 12-h fed condition. The experimental diet consisted of a protein-free liquid formula made with protein-free powder (PFD1; Mead Johnson) flavored with drink crystals (Tang and Kool-Aid; Kraft Foods), corn oil, and protein-free cookies. Energy was provided at 1.5× the REE on the basis of each subject’s measured REE after a 12-h fast, as described earlier. Protein was given as a crystalline L-α-amino acid mixture on the basis of the egg protein pattern at 1 g · kg⁻¹ · d⁻¹. Test leucine doses were provided as crystalline L-leucine divided into 12 equal doses, and each of the 8 hourly meals contained one-twelfth of the dose of test leucine.

Tracer protocol

On each study day, subjects consumed 4 hourly meals before the oral tracer-infusion protocol (Figure 1). A priming dose of 0.176 mg NaH¹³CO₃/kg [99 atom percent excess (APE); Cambridge Isotope Laboratories] and 2.38 mg L-[¹-¹³C]leucine/kg (99 APE; Cambridge Isotope Laboratories) was given at the fifth meal. Hourly doses of L-[¹-¹³C]leucine (1.19 mg · kg⁻¹ · h⁻¹) were given with subsequent meals until the end of the study to maintain a constant steady state in isotope kinetics.

Sample collection

On each study day, subjects arrived fasted at the Clinical Investigation Unit at 0800. Body weight was measured by using a digital scale, and height was measured by using a digital stadiometer. A registered nurse inserted a 21-gauge peripheral catheter into the antecubital fossa vein of the left arm for blood sample collection. A baseline blood sample and urine sample were collected and forwarded immediately on ice to the SickKids core laboratory for analysis (Figure 1). Baseline vital signs (blood pressure, heart rate, and body temperature) and blood glucose (One Touch Ultra2; LifeScan Inc) were measured. Three baseline breath samples were collected 45, 30, and 15 min before the tracer protocol began. Four plateau breath samples were collected at isotopic steady state every 30 min beginning 2.5 h after the start of the tracer protocol. The CV between the 4 plateau values of enrichment was <5%. Breath samples were collected in disposable Extainer tubes (Labco Ltd) by using a collection mechanism that permits the removal of dead-space air. Blood samples were collected 15 min before the isotope dose for baseline enrichment and 3 and 4 h after the first isotope dose for isotope enrichment at steady state. At the end of each study day, a blood sample and urine sample were collected and forwarded to the SickKids core laboratory for blood and urine biochemistry. During each study day, the rate of carbon dioxide production was measured immediately after the fifth meal for a period of 20 min by using an indirect calorimeter (Vmax Encore, Metabolic cart; VIASYS). Breath samples were stored at room temperature, blood samples at −80°C, and urine samples at −20°C until analyzed.

Sample analysis

The core laboratory at SickKids analyzed blood samples for ammonia, urea, creatinine, alanine aminotransferase (ALT), glucose, insulin, electrolytes (sodium, potassium, and chloride), and complete blood count including hematocrits, white blood cells, red blood cells, and hemoglobin using routine clinical analytic procedures. Urine samples were analyzed for creatinine and urea by the core laboratory.

Expired CO₂ enrichment was measured by using a continuous-flow isotope-ratio mass spectrometer (CF-IRMS 20/20 isotope analyzer; PDZ Europa Ltd). Enrichments were expressed as APE compared with a reference standard of compressed CO₂ gas.

Plasma L-[¹-¹³C]leucine enrichment was measured by using a triple-quadrupole mass spectrometer (API 4000; Applied Biosystems/MDS SCIEX) operated in positive-ionization mode with a TurbolonSpray ionization probe source (operated at 5800 V and 600 C), which was coupled to an Agilent 1100 HPLC system (Agilent Technologies Canada Inc). For deproteinization, 25-µL plasma samples were mixed with 200 µL methanol and centrifuged at 9000 × g for 5 min. The supernatant fluids were transferred to derivatizing tubes and dried under nitrogen at 45°C. The dried samples were derivatized with 50 µL HCl-butanol derivative reagent (Regis Technologies Inc). Vortexed samples were topped with nitrogen gas and heated at 55°C for 20 min. Derivatized samples were dried under nitrogen at 45°C. The dried amino acids were reconstituted in 5 mL 0.1% formic acid. The maximum sensitivity for the butylated L-[¹-¹³C]leucine was achieved by measuring product ion multiple reaction monitoring from the fragmentation of the protonated [M+H]⁺ molecule. A solution of pure butylated L-leucine (200 pg/µL) was infused into the mass spectrometer and the declustering potential optimized to maximize the intensity of the [M+H]⁺ precursor (parent) ion (m/z of 188:unenriched and 189:enriched). The collision energy was then adjusted to optimize the signal for the most abundant product (daughter) ion (m/z of 86) by using nitrogen as the collision gas. The individual components were separated by using a Hypercarb column (Hypersil 5 µm, 4.6 × 50-mm column) (Thermo Electron Corporation) at 50°C and eluted with a binary liquid chromatography gradient (10–50% aqueous acetonitrile that contained 0.025% formic acid and 0.05% trifluoroacetic acid in 5 min). The mean retention time was 1.2 min.

Plasma [¹³C]KIC enrichment and concentrations were measured by using methane-negative chemical ionization gas chromatography–mass spectrometry (Hewlett-Packard 5890, GC; Hewlett-Packard 5988A MS system; Hewlett-Packard). The urinary and plasma KIC, which is an index of intracellular leucine enrichment, was derivatized to its pentafluorobenzyl ester on the basis of an extractive derivatization process (18). A highly polar column [SP-2380 (0.20 µm × 0.25 mm × 30 m); Supelco Inc] was necessary to separate KIC from the keto acid of isoleucine α-keto-β-methylvalerate, which has the same molecular weight as KIC. Selected-ion chromatograms were obtained by monitoring [M-H-PFB]⁻ ions at m/z of 129 for KIC and 130 for [¹³C]KIC.

Plasma free BCAA and urinary leucine concentrations were determined by using HPLC analysis. A total of 200 µL plasma and urine and 80 µL of 0.25 mmol norleucine/L as the internal standard were extracted by using a cation-exchange column (Dowex 50 W-X8, 100–200 mesh H+ form; Bio-Rad Laboratories). Plasma and urinary amino acids were derivatized with phenyl isothiocyanate (adapted from PicoTag: Waters) (19–21), and their phenylisothiocyanate derivatives were separated and analyzed against an amino acid standard mix (Sigma) by reversed-phase (C18, 2.9 mm × 300 mm Pico Tag column; Waters Corp) column by using HPLC (Dionex Summit HPLC System; Dionex; operated under HPLC pump model PS80A LPG and ultraviolet-visible 170S). The areas under the peaks were integrated with Chromelon software (version 6.2; Dionex).
Estimation of isotope kinetics

Whole-body leucine and KIC flux were calculated from the dilution of isotope in the body amino acid pool at isotopic steady state (22) as follows:

\[
Q = i(E_i + E_p - 1)
\]

where \(Q\) is the rate of leucine or KIC flux (\(\mu\)mol \(\cdot\) kg\(^{-1}\) \(\cdot\) h\(^{-1}\)), \(i\) is the isotope-infusion rate (\(\mu\)mol \(\cdot\) kg\(^{-1}\) \(\cdot\) h\(^{-1}\)), and \(E_i\) and \(E_p\) are enrichments as mole fractions of the infused isotope (APE) and plasma leucine or KIC at the isotopic plateau (APE).

The rate of leucine and KIC oxidation were calculated by using the following equation (22):

\[
O = F^{13}CO_2(1 + E_p - 1 + E_i)
\]

where \(O\) represents leucine or KIC oxidation (\(\mu\)mol \(\cdot\) kg\(^{-1}\) \(\cdot\) h\(^{-1}\)) and \(F^{13}CO_2\) represents the rate of \(^{13}\)CO\(_2\) released by leucine tracer oxidation (\(\mu\)mol \(\cdot\) kg\(^{-1}\) \(\cdot\) h\(^{-1}\)) calculated by using the following equation:

\[
F^{13}CO_2 = (FCO_2)(ECO_2)(44.6)(60) + (W)(0.82)(100)
\]

where FCO\(_2\) is the CO\(_2\)-production rate (mL/min), ECO\(_2\) is the enrichment in expired breath at the isotopic steady state (APE), the constants 44.6 (\(\mu\)mol/mL) and 60 (min/h) converted FCO\(_2\) to micromoles per hour, \(W\) is the weight (kg) of the subject, the factor 0.82 is the correction for CO\(_2\) retained in the body because of the bicarbonate fixation (23), and the factor 100 changes APE to a fraction.

Statistical analysis

Results are expressed as means ± SDs or means ± SEMs. A mixed linear model with the subject as a random variable by using the Proc Mixed program (Statistical Analysis Systems–SAS/STAT version 8.2; SAS Institute) was used to analyze the changes APE to a fraction.

Dotted lines indicate normal blood ammonia concentrations in young men (35 \(\mu\)mol/L). Post, end-of-study-day blood samples; Pre, fasted blood samples.

RESULTS

Subject characteristics

Five healthy, young men (27.2 ± 2.1 y) participated in the study. Subject anthropometric measures and energy intakes were in the normal range (Table 1). All subjects maintained body weight during the study period. Two subjects participated in all 7 (50, 150, 250, 500, 750, 1000, and 1250 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\)) test intakes, and 3 subjects participated in 6 (50, 250, 500, 750, 1000, and 1250 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\)) test intakes, and 4 (50, 250, 750, and 1000 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\)) test intakes, respectively, in a total of 29 leucine oxidation studies. During the leucine-intake studies of 50–500 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\), subjects did not have any discomfort or side effects that were attributed to the study-day diet, although some subjects complained of general tiredness toward the end of the study day. At higher leucine intakes (750–1000 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\)), one subject complained of gastrointestinal discomfort, and the discomfort subsided once the study day ended. No other physical symptoms were observed during the study day.
Biochemical variables

Blood ammonia concentrations at the end of all study days were significantly higher (*P < 0.0001*) than fasting blood ammonia concentrations, except at a leucine intake of 50 mg·kg⁻¹·d⁻¹ (Figure 2A). With increasing intakes of leucine, blood ammonia concentrations rose significantly (*P < 0.0001*) by the end of the study day and were above the normal range of 35 μmol/L after leucine intakes of 500 mg·kg⁻¹·d⁻¹ (Figure 2B). When blood ammonia concentrations at the end of the study were above the upper limit of the normal range, subjects were contacted and requested to come back to the clinical investigational unit the next day to provide a blood sample to check ammonia concentrations. In all cases, blood ammonia concentrations were within the normal range (<35 μmol/L) when tested on the following day, which indicated that the adverse effect because of increased leucine intake was not observed when subjects returned to their normal diets. All studies were separated by a minimum of 2 wk, and this ensured that there was a sufficient washout period between any 2 studies for each subject.

Plasma glucose was significantly decreased at the highest intake of leucine (1250 mg·kg⁻¹·d⁻¹) compared with at 50 mg·kg⁻¹·d⁻¹ (*P = 0.0004*), although values stayed within the normal range of 3.3–6.1 mmol/L (Table 2). Plasma insulin was not affected because of increased leucine intakes (*P = 0.1632*). No other measured blood and urine biochemical variables including plasma ALT (as a marker of liver function), blood urea, creatinine, electrolytes, complete blood count, urinary creatinine, or urea showed any clear pattern because of increased leucine intakes (Table 2).

Plasma BCAAs, KIC, and urinary leucine

Plasma BCAAs concentrations were measured in the fed state in all subjects. With increasing intakes of leucine, plasma leucine concentrations increased significantly (*P < 0.0001*) (Figure 3A). Plasma valine (*P = 0.0055*) and plasma isoleucine (*P < 0.0001*) concentrations decreased significantly with increasing intakes of leucine (Figure 3, B and C). Urinary leucine excretion (Figure 4) increased significantly (*P < 0.0001*) with increasing leucine intakes and exhibited a similar pattern as observed in plasma leucine concentrations. Plasma KIC concentrations also increased with increasing leucine intakes, although concentrations were significantly (*P = 0.0033*) increased only at 1250 mg leucine·kg⁻¹·d⁻¹ (Figure 5).

### TABLE 2

<table>
<thead>
<tr>
<th>Biochemical variables</th>
<th>Leucine intake</th>
<th>Blood</th>
<th>Urea (mmol/L)</th>
<th>ALT (UL) (0–40)</th>
<th>Glucose (μmol/L)</th>
<th>Insulin (μmol/L)</th>
<th>HCT (0.42–0.5)</th>
<th>RBC (4.5–5.7)</th>
<th>Hemoglobin (g/L)</th>
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<tbody>
<tr>
<td></td>
<td>50 mg·kg⁻¹·d⁻¹</td>
<td>150 mg·kg⁻¹·d⁻¹</td>
<td>250 mg·kg⁻¹·d⁻¹</td>
<td>500 mg·kg⁻¹·d⁻¹</td>
<td>750 mg·kg⁻¹·d⁻¹</td>
<td>1000 mg·kg⁻¹·d⁻¹</td>
<td>1250 mg·kg⁻¹·d⁻¹</td>
<td>P</td>
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<tr>
<td>Urea (mmol/L) (2.9–7.1)</td>
<td>4.6 ± 0.2</td>
<td>4.2 ± 0.5</td>
<td>5.1 ± 0.3</td>
<td>5.7 ± 0.5</td>
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<td>ALT (UL) (0–40)</td>
<td>40.2 ± 12.1</td>
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<td>61.0 ± 31.0</td>
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<td>Glucose (μmol/L) (3.3–6.1)</td>
<td>6.2 ± 0.3</td>
<td>5.1 ± 0.3</td>
<td>5.4 ± 0.3</td>
<td>5.3 ± 0.2</td>
<td>5.2 ± 0.2</td>
<td>4.7 ± 0.2</td>
<td>4.9 ± 0.03</td>
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<td>Insulin (μmol/L)</td>
<td>282 ± 67</td>
<td>139 ± 62</td>
<td>289 ± 86</td>
<td>345 ± 64</td>
<td>294 ± 63</td>
<td>404 ± 123</td>
<td>366 ± 125</td>
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<td>HCT (0.42–0.5)</td>
<td>0.44 ± 0.01</td>
<td>0.52 ± 0.04</td>
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<td>RBC (4.5–5.7)</td>
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<td>Hemoglobin (g/L) (146–175)</td>
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<td>155.7 ± 2.6</td>
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**Note:** All values are means ± SEMs (n = 2–5 per mean). Values with different superscript letters within a row are significantly different, *P < 0.05*. The post hoc analysis was conducted using Tukey-Kramer multiple-comparison tests. ALT, alanine aminotransferase; HCT, hematocrit; RBC, red blood cell counts.

1 Normal range in parentheses (all such values).
plateau after 750 mg ⋅ kg\(^{-1}\) ⋅ d\(^{-1}\), although a breakpoint in KIC oxidation could not be determined reliably (Figure 6C).

**DISCUSSION**

The objective of the current study was to identify the UL for leucine in young men under acute dietary conditions. In all subjects with intakes of leucine >500 mg ⋅ kg\(^{-1}\) ⋅ d\(^{-1}\), there was an increase in blood ammonia concentrations (Figure 2B) above normal values (<35 \(\mu\)mol/L) with simultaneous and significant increases in plasma leucine concentration (Figure 3A) and urinary leucine excretion (Figure 4), which suggested that higher intakes might be harmful. Leucine oxidation was measured with increasing intakes of dietary leucine, and kinetics of L-[\(^{13}\)C]leucine were expressed as \(^{13}\)CO\(_2\), leucine oxidation, and KIC oxidation (Figure 3, B and C, and Figure 6A). Two-phase linear regression analysis identified a breakpoint in \(^{13}\)CO\(_2\) at a leucine intake of 550 mg ⋅ kg\(^{-1}\) ⋅ d\(^{-1}\), and KIC oxidation appeared to plateau after 750 mg ⋅ kg\(^{-1}\) ⋅ d\(^{-1}\), although leucine oxidation continued to rise even after 750 mg ⋅ kg\(^{-1}\) ⋅ d\(^{-1}\). Thus, a reliable UL could not be measured on the basis of kinetic data alone. With consideration of plasma and urinary data together, as a cautious estimate, 500 mg ⋅ kg\(^{-1}\) ⋅ d\(^{-1}\) could be the UL for leucine under acute dietary conditions.

In the current study, blood ammonia concentrations significantly increased with increasing leucine intakes (Figure 2B), although no significant changes were observed for urea, creatinine, ALT, glucose, insulin, electrolytes (sodium, potassium, and chloride), and complete blood count including hematocrit, white blood cells, red blood cells, and hemoglobin (Table 2). The increase in blood ammonia concentrations above normal physiologic values (<35 \(\mu\)mol/L) with leucine intakes >500 mg ⋅ kg\(^{-1}\) ⋅ d\(^{-1}\) was surprising because healthy adult men should have the ability to convert the potentially toxic ammonia into the less harmful product urea. The physiologic reason remains unclear for a potential mechanism to explain the rise in ammonia, although the rise was transient because the values had returned to within normal ranges the following day. Previous studies on BCAA administration (12, 13, 26) did not report such increases in blood ammonia, although no study reported test intakes >850 mg total BCAAs ⋅ kg\(^{-1}\) ⋅ d\(^{-1}\), with the maximum leucine dose ranging from 200–350 mg ⋅ kg\(^{-1}\) ⋅ d\(^{-1}\).

Leucine has been suggested to act as an insulin secretagogue (27–29), but we did not observe significant changes in plasma insulin because of increasing leucine intakes (Table 2), and plasma glucose remained within the normal range of 3.3–6.1 \(\mu\)mol/L. The plasma leucine concentration increased significantly with leucine intakes >500 mg ⋅ kg\(^{-1}\) ⋅ d\(^{-1}\), and plasma valine and isoleucine concentrations decreased significantly with increasing leucine intakes (Figure 3, A–C), as reported previously (30–33). BCAAs share a common catabolic pathway with the branched-chain ketodehydrogenase controlling the irreversible catabolic step, which commits the carbon skeleton

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**FIGURE 3.** Influence of graded dietary excess leucine intake on mean (±SD) plasma branched-chain amino acid concentrations (n = 2–5 per mean). A: Plasma leucine concentrations. B: Plasma valine concentrations. C: Plasma isoleucine concentrations. Symbols with different superscript letters are significantly different, \(P < 0.05\). The statistical analysis was performed by using mixed-models ANOVA, and the post hoc analysis was conducted by using Tukey-Kramer multiple-comparison tests.

**FIGURE 4.** Influence of graded dietary excess leucine intake on mean (±SD) urinary leucine concentrations (n = 2–5 per mean). Symbols with different superscript letters are significantly different, \(P < 0.05\). The statistical analysis was performed by using mixed-models ANOVA, and the post hoc analysis was conducted by using Tukey-Kramer multiple-comparison tests.
different superscript letters are significantly different, the post hoc analysis was conducted by using Tukey-Kramer multiple-comparison tests. KIC, α-ketoisocaproic acid.

of BCAAs to the tricarboxylic acid cycle. Leucine concentrations have been shown to stimulate branched-chain ketodehydrogenases and compete with the other 2 BCAAs for metabolism in vivo (34); this phenomenon, referred to as BCAA antagonism, is well documented (30) and might explain the imbalance of plasma BCAA concentrations in the current study.

In a previous neonatal piglet study, we observed that, with increasing intakes of phenylalanine (0.2–1.2 g · kg⁻¹ · d⁻¹), an upper inflection point in the dose-response curve for ¹⁴CO₂ production from ¹⁴C-phenylalanine was observed at 0.8 g · kg⁻¹ · d⁻¹ (14). The phenylalanine plasma concentration increased significantly with intakes >0.8 g · kg⁻¹ · d⁻¹. We hypothesized that one approach to identify the UL for an amino acid would be the level at which the maximum oxidative potential was reached (15). Sakai et al (35) used this approach to identify leucine excess in rats. The authors identified the metabolic limit by measuring ¹³CO₂ production from U-¹³C-leucine with an increasing leucine intake in the excess range. The maximum limit to oxidize excess leucine was reached at 10% of the dietary intake or 8900 mg · kg⁻¹ · d⁻¹; the oxidation achieved a plateau, and this inflection point was identified as the UL of leucine in rats (35). In the current study, the kinetics of l-[¹³C]leucine expressed as F¹³CO₂, leucine oxidation, and KIC oxidation (Figure 3, A–C) led to different results. Although a plateau in F¹³CO₂ was observed at >500 mg · kg⁻¹ · d⁻¹, no clear plateau was observed in leucine oxidation, and KIC oxidation appeared to plateau after 750 mg · kg⁻¹ · d⁻¹. A significant dilution of the tracer would have occurred because the dose of l-¹³C-leucine was kept constant with increasing intakes of leucine. A 5-fold increase in breath ¹³CO₂ enrichment (Figure 6A) and a 10–25 fold dilution because of an increased leucine intake are not fully explained physiologically, and future studies need to be performed to explore the kinetic differences observed.

Previously, in a controlled experiment, Tsubuku et al (36) studied oral supplements of leucine excess in 4-wk-old rats and estimated that ~3500 mg · kg⁻¹ · d⁻¹ was the no-observed-adverse-effect amount on the basis of body weight, food consumption, and hematologic measurements. Similarly, Mawatari et al (37) in 10-wk-old female rats estimated that oral leucine at 1000 mg · kg⁻¹ · d⁻¹ did not affect the outcome of pregnancy and did not cause fetal toxicity. To our knowledge, there have been no such controlled studies reported with graded doses of excess leucine intake in humans until the current study.

In the recent Dietary Reference Intake report (3), which was based on distribution data from the 1988–1994 NHANES III, the mean daily intake for all life-stage and sex groups of leucine from food and supplements was reported to be 6100 mg/d. Men 51–70 y of age had the highest intake at the 99th percentile for leucine at 14,100 g/d (~201 mg · kg⁻¹ · d⁻¹). Also, an analysis of the current habitual leucine intake was conducted in strength-training athletes who were chronic amino acid-supplement users. The average protein intake in these athletes was ~2 g · kg⁻¹ · d⁻¹ (38). The mean leucine content in food is ~15% (26). Therefore, for an athlete who weighs 80 kg, the dietary leucine intake is 300 mg · kg⁻¹ · d⁻¹. Available BCAA supplements contain a maximum of 1800 mg/serving (39), with a suggested dose of 3 doses/d; this intake equals ~67.5 mg · kg⁻¹ · d⁻¹. Therefore, the habitual total exposure of adults who consume 2 g protein · kg⁻¹ · d⁻¹ and this amino acid supplement is ~367 mg · kg⁻¹ · d⁻¹. These calculations revealed that most people, including athletes, consume less than the UL for leucine oxidation determined in the current study (500 mg · kg⁻¹ · d⁻¹). However, there is probably a range in protein and leucine intakes in athletes, with some individuals who consume more than the recommended dose and, thus, are at potential risk of adverse effects. The impact of chronic consumption by humans of excess leucine remains unknown, as the current studies were conducted with an acute dietary

**TABLE 3**

<table>
<thead>
<tr>
<th>Leucine intake</th>
<th>F¹³CO₂</th>
<th>Oxidation</th>
<th>Flux (µmol · kg⁻¹ · h⁻¹)</th>
<th>Oxidation (µmol · kg⁻¹ · h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg · kg⁻¹ · d⁻¹</td>
<td>5</td>
<td>1.05 ± 0.03³</td>
<td>124.5 ± 7.5⁴</td>
<td>13.6 ± 1.1⁴</td>
</tr>
<tr>
<td>150 mg · kg⁻¹ · d⁻¹</td>
<td>2</td>
<td>2.51 ± 0.12², b</td>
<td>197.0 ± 37.4², b</td>
<td>55.2 ± 13.0², c</td>
</tr>
<tr>
<td>250 mg · kg⁻¹ · d⁻¹</td>
<td>5</td>
<td>3.44 ± 0.43³</td>
<td>267.8 ± 24.9³</td>
<td>95.8 ± 9.6³</td>
</tr>
<tr>
<td>500 mg · kg⁻¹ · d⁻¹</td>
<td>4</td>
<td>4.47 ± 0.37</td>
<td>358.7 ± 38.3³</td>
<td>176.6 ± 30.0³</td>
</tr>
<tr>
<td>750 mg · kg⁻¹ · d⁻¹</td>
<td>5</td>
<td>5.30 ± 0.22²</td>
<td>463.8 ± 59.6², c, d</td>
<td>261.5 ± 35.1², d</td>
</tr>
<tr>
<td>1000 mg · kg⁻¹ · d⁻¹</td>
<td>5</td>
<td>5.23 ± 0.27³</td>
<td>544.0 ± 53.7³, d</td>
<td>310.0 ± 33.8³, d</td>
</tr>
<tr>
<td>1250 mg · kg⁻¹ · d⁻¹</td>
<td>3</td>
<td>5.33 ± 0.23³</td>
<td>667.8 ± 98.5³</td>
<td>388.3 ± 47.9³, d</td>
</tr>
</tbody>
</table>

¹All values are means ± SEMs. Values with different superscript letters within a column are significantly different, P < 0.05. The post hoc analysis was conducted by using Tukey-Kramer multiple-comparison tests. F¹³CO₂, label tracer oxidation in breath; KIC, α-ketoisocaproic acid.
supply of leucine. A high chronic intake may either reduce risk of adverse effects by increasing the basal leucine oxidation rate or increase risk of adverse effects by the gradual accumulation of metabolic events associated with excess intake, although such effects could not be determined from our acute-study conditions.

In conclusion, simultaneous and significant increases in blood ammonia concentrations, plasma leucine concentrations, and urinary leucine excretion were observed with leucine intakes >500 mg kg⁻¹·d⁻¹. Leucine oxidation measured by using L-[1-¹³C]leucine and expressed as F¹³CO₂, leucine oxidation, and KIC oxidation led to different results, and future studies need to be conducted to determine the reasons for the differences. On the basis of the plasma and urinary variables, the UL for leucine in healthy adult men can be suggested to be 500 mg kg⁻¹·d⁻¹ or ~35 g/d as a cautious estimate under acute dietary conditions.

We thank all of the subjects who participated in the study and Jasmine Donohue in the Department of Nutrition and Food Services (The Hospital for Sick Children) for preparing protein-free cookies.

The authors’ responsibilities were as follows—RE: study design, data collection, sample and data analyses, and manuscript writing; KC: data collection and data analysis; MR: sample analysis; and ROB and PBP: study design, data analysis, and manuscript writing. The authors had no conflicts of interest.

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