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Original article

Oral citrulline does not affect whole body protein metabolism in healthy human volunteers: Results of a prospective, randomized, double-blind, cross-over study

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SUMMARY

Background & aims: Citrulline increases protein synthesis during refeeding in rodents with short bowel syndrome, aging and malnutrition, and improves nitrogen balance in fed healthy humans. The aim of the current study therefore was to determine whether citrulline had affected protein metabolism in healthy volunteers.

Methods: In a randomized, double-blind, cross-over study, 12 healthy adults received a 5-h intravenous infusion of L-[1-^{13}C]-leucine in the post-absorptive state, after a 7-day oral supplementation with 0.18 g/kg/day citrulline, or an iso-nitrogenous placebo. Treatment order was randomized, treatment periods were separated by 13-day wash out. Leucine appearance rate (Ra) was determined from plasma [1-^{13}C]-keto-iso-caproate enrichment and leucine oxidation from expired $^{13}CO_2$, and nitrogen balance was estimated from 6-h urinary urea excretion.

Results: Compared with placebo, oral citrulline supplementation increased plasma citrulline, arginine and ornithine concentrations, but failed to affect albumin, transthyretin, free insulin and insulin-like growth factor (IGF)-1 plasma concentrations, urinary nitrate excretion, or nitrogen balance. Citrulline supplementation did not alter leucine Ra, leucine oxidation, nor whole-body protein synthesis.

Conclusion: In healthy, well nourished volunteers, oral citrulline increases plasma citrulline and arginine availability but does not affect whole-body protein kinetics in the post-absorptive state.

1. Introduction

Citrulline is a non essential amino acid that is not incorporated into protein. Endogenous citrulline is produced from glutamine by enterocytes in the small bowel.[1–5] In the absence of renal failure, plasma citrulline concentration is an index of intestinal mass and/or absorptive function.[6–13] Citrulline is converted to arginine in kidney: in fasting humans 5–15% of overall arginine production is derived from citrulline – the remaining coming from proteolysis.[14] As citrulline escapes splanchnic uptake, oral citrulline supplementation might be an efficient way to enhance arginine availability and thus impact whole-body protein synthesis as arginine may have a protein anabolic effect.[15,16]

In addition, several studies suggest citrulline *per se* may have a protein anabolic effect: compared with an iso-nitrogenous mixture of non essential amino acids, enteral citrulline supplementation improved nitrogen balance in enterectomized rats[17]; and refeeding with enteral citrulline increased muscle protein synthesis, together with a rise in serum insulin, in old malnourished rats.[18] However, little is known about the effect of citrulline on protein metabolism in humans. Recently we showed that oral citrulline acutely increased plasma citrulline and arginine concentrations, and improved nitrogen balance in healthy volunteers in the fed state.[19] The aims of this study were: i) to determine whether a 7-day dietary citrulline administration would enhance whole-body protein synthesis in the

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post-absorptive state in healthy humans, and, ii) if so, whether this was associated with a rise in insulin or insulin-like growth factor (IGF) 1.

2. Subjects and methods

2.1. Citrulline and iso-nitrogenous placebo

All amino acids were supplied by INRESA Pharma, France. Our pharmacist prepared single-dose vials of L-Citrulline (Kyowa Hakko Kogyo, Japan) and iso-nitrogenous placebo constituted of an equimolar mix of L-alanine (34.3 mg/kg/day), glycine (28.9 mg/kg/day) (Degussa Rexim (Nanning) Pharmaceutical, China), L-aspartate (51.3 mg/kg/day) (Rexim SAS, Germany), L-histidine (59.8 mg/kg/day), L-proline (44.4 mg/kg/day) (Kyowa Hakko Kogyo, Japan), and L-serine (40.5 mg/kg/day) (Evonik Degussa GmbH, Germany).

Sterile, pyrogen free L-[1-^{13}C]leucine (Cambridge Isotope Laboratories, Woburn, MA) was tested and prepared as described.[20]

2.2. Experimental design

The study protocol was approved by the regional ethical committee for human experimentation (Comité de protection des personnes dans la recherche biomédicale) and by the 'Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSAPS)'. The study protocol was registered as #NCT00756080 on www.clinicaltrials.govwebsite. Twelve healthy volunteers were included fulfilling the following inclusion criteria: age between 18 and 45 years normal body mass index (BMI), absence of any earlier supplementation with citrulline, glutamine, or ornithine α-ketoglutarate, absence of any treatment with anabolic agents or corticosteroids during the month prior to inclusion in the study, no current artificial feeding, absence of renal, cardiac, respiratory or hepatic insufficiency, or chronic inflammatory disease, fasting blood glucose <6 mmol/L, and, for women, the use of oral contraceptive measure, and a negative pregnancy test. All subjects received detailed information on the purpose and potential risks of the study, and were enrolled after signing a written consent form. A dietary history was obtained by a post-graduate dietician, and subjects were instructed to maintain a daily intake of 1800–2000 kcal/day with 50% calories from carbohydrate, 35% from fat and 15% from protein. Subjects were provided meal plans with recipes and were trained on how to estimate and assign portions and food amounts. The compliance towards the dietary instructions was not monitored on a daily basis. Subjects were instructed to fill in the dietary record themselves, by making note, as specifically as possible, of every food item ingested during each 7-day period. The energy and macronutrient intakes were calculated at the end of each 7-day period by the same dietician using the Regal-micro® software (INRA, AFSSA, TEC & DOC ed., distributed by Lavoisier SAS, Cachan, France). Subjects were also asked to maintain their regular level of activity through the study. The study was designed as a prospective, randomized, double-blind, and cross-over study. Subjects received a 7-day oral supplementation with 0.18 g/kg/day citrulline (0.06 g/kg tid), as used in previous studies,[19] followed or preceded by a 7-day oral supplementation with 0.18 g/kg/day (0.06 g/kg tid) iso-nitrogenous placebo. Treatment order was randomized and treatment periods separated by a 13-day wash out.

2.3. Isotope infusion protocol

At the end of each 7-day period, each subject was admitted at 0700 h to the Clinical Investigation Unit after an overnight fast and voided to start a 6-h urine collection. Two short catheters were inserted, one in a forearm vein for isotope infusion, and the other one in a contralateral superficial hand vein for blood sampling.[21] At 0800 h a primed, continuous intravenous infusion of L-[1-^{13}C] leucine (6 μmol/kg/h) was then initiated, and continued for 5 h until 1300 h. Thirty and 15 min before the infusion, and at 180, 200, 220, 240, 260, 280 and 300 min, arterialized-venous blood samples were obtained to measure plasma ^{13}C-α-keto-isocaproate enrichment. Simultaneously, samples of expired air were collected to measure $^{13}CO_2$ enrichment. Total CO_2 production (VCO_2) was measured using indirect calorimetry (Deltatrac Metabolic Monitor, Datex, Helsinki, Finland). Throughout isotope infusion citrulline or iso-nitrogenous placebo were administered as q. 20 min oral sips according to randomization.

2.4. Analytical methods

Breath $^{13}CO_2$ enrichments were measured by gas chromatography-isotope ratio mass spectrometry (GC-IRMS; Isochrom III, VG, Ipswich, UK), and plasma ^{13}C-α-keto-isocaproate enrichment by GCMS (Hewlett–Packard MSD 5971) as described.[22]

2.5. Calculations

Leucine appearance rate into plasma (Ra_{LEU}; μmol kg^{-1} h^{-1}), leucine oxidation (Ox_{LEU}; μmol kg^{-1} h^{-1}) and non-oxidative leucine disposal (NOLD; μmol kg^{-1} h^{-1}) were calculated using standard isotope dilution equations for steady state conditions as described.[23] As leucine is an essential amino acid, the only source of leucine Ra in post-absorptive subjects is leucine release from the breakdown of body protein. Because leucine is assumed to be either oxidized or incorporated into protein, NOLD is an index of whole-body protein synthesis.

2.6. Assessment of nutritional status

Physical exam including the measurement of weight, height and the calculation of body mass index (BMI) were performed in all patients before each isotope infusion protocol. Body composition was determined by single-frequency bioelectrical impedance analysis (Bodystat® 1500, Bodystat Ltd, Man Island, Royaume Uni).

2.7. Hormone and substrate assays

Serum albumin, transthyretin, free insulin and IGF-1 plasma concentrations were determined using radio-immuno-assay. Amino acid concentrations were analyzed by liquid chromatography-tandem mass spectrometry using the amino reactive isotope coded tags (iTRAQ® Reagents, AB Sciex, Foster City, CA, USA) and the AB SCIEX iMethod™ test (AB Sciex, Foster City, CA, USA). In all subjects but two, urine was collected from 0700 h to 1300 h during isotope infusion. Urinary nitrate + nitrite excretion was determined using a colorimetric method (Roche-Diagnostic, Meylan, France).[19] Six-hour nitrogen balance was calculated as: nitrogen balance (g.6 h^{-1}) = [nitrogen intake (g.6 h^{-1})] - [0.028 × urinary urea (mmol.6 h^{-1})].

2.8. Statistical analysis

Statistical analyses were performed with Statview® 4.5 software (SAS Institute Inc., Cary, NC). Normality of the distribution was tested with the Kolmogorov–Smirnov test. Continuous variables were reported as mean ± SD, and compared between citrulline and placebo treatments by paired Student *t* test. *P*-values <0.05 were considered to be statistically significant.