Citrulline modulates muscle protein metabolism in old malnourished rats

S. Osowska, T. Duchemann, S. Walrand, A. Paillard, Y. Boirie, L. Cynober, and C. Moinard. Citrulline modulates muscle protein metabolism in old malnourished rats. Am J Physiol Endocrinol Metab 291: E582–E586, 2006. First published April 11, 2006; doi:10.1152/ajpendo.00398.2005.— Protein energy malnutrition is common in the elderly, especially in hospitalized patients. The development of strategies designed to correct such malnutrition is essential. Our working hypothesis was that poor response to nutrition with advancing age might be related to splanchnic sequestration of amino acids, which implies that fewer amino acids reach the systemic circulation. Administration of citrulline, which is not taken up by the liver, can offer a means of increasing whole body nitrogen availability and, hence, improve nutritional status. Thirty old (19 mo) rats were given 50% of their spontaneous intakes recorded during the acclimatization period for 12 wk. They were randomized into three groups: 10 rats (R group) were killed and 20 others refed (90% of food intake) for 1 wk with a standard diet (NEAA group) or a citrulline-supplemented diet (Cit group). Before being killed, the rats were injected with $^{13}$C]-valine, and the absolute protein synthesis rate (ASR) was measured in the tibialis using the flooding-dose method. When the rats were killed, the tibialis was removed for protein content analysis. Blood was sampled for amino acid and insulin analysis. The standard diet did not have any effect on protein synthesis or on the protein content in the muscle. Citrulline supplementation led to higher protein synthesis and protein content in muscle ($117 ± 9, 120 ± 14,$ and $163 ± 4$ mg/organ for protein content in R, NEAA, and Cit groups, $P < 0.05$). The ASR were $0.30 ± 0.04,$ $0.31 ± 0.04,$ and $0.56 ± 0.10$ mg/h in the three groups, respectively (R and NEAA vs. Cit, $P < 0.05$). Insulinemia was significantly higher in the Cit group. For the first time, a realistic therapeutic approach is proposed to improve muscle protein content in muscle in frail state related to malnutrition in aging.

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THE RISING NUMBER OF ELDERLY PEOPLE in Western countries has made successful aging a major health policy concern. Due to medico-economic implications, it makes sense to focus on the malnourished at-risk elderly population, especially as malnutrition is frequent in the elderly (34), from 30 to 60% in hospitalized persons. This lengthens hospital stay and increases morbidity and mortality (6). Also, the elderly exhibit a decreased ability to recover from a malnourished state (17, 18, 33). It is clear that, at the molecular level, age-related malnutrition is accompanied by impaired protein metabolism (9, 13). In particular, it has been demonstrated (5, 36) that there is a significant increase in amino acid metabolism within the splanchnic area in the elderly compared with adults. This high splanchnic extraction of amino acids in the elderly leads to inadequate systemic plasma levels of amino acids in the post-prandial period (26). Because hyperaminoacidemia (together with insulin) is one of the main stimulating factors for protein synthesis (31), the insufficient increase in postprandial plasma concentration of amino acids would result in blunted protein synthesis rates (3). Recently, we observed the same effect in elderly rats (19). On the basis of these data, strategies were developed to saturate this age-related abnormal splanchnic extraction (named splanchnic sequestration): high-protein diet (38), pulsed diet [80% of protein in a single meal (1, 2)], or use of “fast proteins” (12). However, the practical application of these approaches is not necessarily easy in elderly malnourished patients. For this reason, we propose an alternative strategy. Our working hypothesis was that by using an amino acid that escapes splanchnic extraction, namely citrulline (43), it would be possible to deliver a more adequate amount of nitrogen [especially through citrulline conversion into arginine in the kidney (42)] to the peripheral tissues, including muscles, and thus to increase protein synthesis. Citrulline has a highly specific metabolism. The activity of the two enzymes that metabolize citrulline (argininosuccinate synthetase and argininosuccinate lyase) is very low in the intestine (37). It cannot be used in situ by enteroocytes, and so citrulline is released as such into the circulation (41). Also, citrulline is not taken up by the liver (29). To test our working hypothesis, we chose a well-validated model of old malnourished rats in which an impaired response to renutrition has been proven (38).

MATERIALS AND METHODS

Animals

Thirty 19-mo-old, male Sprague-Dawley rats (Charles River, L’Arbresle, France) were used in the experiment. They were housed individually and kept at 20–23°C in alternate 12:12-h light-dark cycles. They had free access to water. During an acclimatization period, all rats were fed a standard diet ad libitum (UAR, Villemoisson-sur-Orge, France) for 2 wk. Daily spontaneous intakes were determined: these old rats consumed 34.4 g/day.

Animal care complied with the French regulations for the protection of animals used for experimental and other scientific purposes (D 2001-486) and with European Community regulations (Official Journal of the European Community, L538 12:18:1986).

Experimental Procedures

Dietary restriction. After the acclimatization period, the rats were given 50% of their spontaneous intakes recorded during the acclimatization period for 12 wk.

Refeeding. After the dietary restriction period, 10 rats were killed and 20 rats were refed for 1 wk. Food was limited to 90% of their spontaneous intake measured during acclimatization period (see...
Table 1. Protein content and ASR in the splanchnic area

<table>
<thead>
<tr>
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<th>R NEAA</th>
<th>Cit</th>
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<tr>
<td>Liver protein, g/organ</td>
<td>2.75±0.14</td>
<td>3.51±0.12*</td>
</tr>
<tr>
<td>Liver ASR, mg/24 h</td>
<td>0.997±0.096</td>
<td>1.503±0.155†</td>
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</table>

Values are means ± SE. ASR, absolute protein synthesis rate; R, rats killed at the end of the restricted period; Cit, rats refed with a citrulline-supplemented diet; NEAA, rats refed with an NEAA-enriched diet. ANOVA + Duncan test: *P < 0.05 vs. R and Cit; †P < 0.05 vs. R.

above) to ensure that all rats ate all of their ration and, hence, received the same amounts of food. All the rats ate all their ration.

Experimental groups and plan. At the end of the acclimatization period, rats were randomized to the following groups. The “restricted” group (R, n = 10) underwent dietary restriction for 12 wk and were killed at the end of the period of restriction. In the “citrulline” group (Cit, n = 10), rats were refed for 1 wk, after 12 wk of dietary restriction, with a citrulline supplement (5 g·kg⁻¹·day⁻¹) and then killed. The dose of citrulline is the extrapolation of doses used in humans, taking into consideration that metabolic rate and nitrogen requirements are 10 times those of humans (10). In the “nonessential amino acid” group (NEAA, n = 10), rats were refed, after 12 wk of dietary restriction, for 1 wk with a standard diet supplemented with nonessential amino acids (alanine, glycine, proline, histidine, asparagine, and serine in equimolar ratios) so that their nitrogen intake was equal to that of the Cit group.

After 1 wk, rats were killed. During the last 2 days of the experiment, rats were placed in individual metabolic cages, and urine was collected for determination of nitrogen balance and 3-methylhistidine excretion. Before being killed, the rats were injected with [1-¹³C]valine to determine the protein synthesis rate (see Protein mass in the tissues).

Tissue removal. Rats in the postabsorptive state were anesthetized with isoflurane and killed by beheading. Blood was sampled in heparinized tubes, which were rapidly centrifuged. The liver was immediately removed and weighed, and a sample was cut off, frozen in liquid nitrogen, and stored at −80°C until analysis. For the jejunum and ileum, the intestine mucosa was washed with cold NaCl (0.9%), reinserted and scraped, rapidly frozen in liquid nitrogen, and stored at −80°C until analysis. A muscle of the hindlimbs (tibialis) was rapidly removed, weighed, frozen in liquid nitrogen, and stored at −80°C until analysis. Tibialis was selected because this muscle is rich in type II fibers (24), and it is known (22) that muscles rich in type I fibers (i.e., soleus) are only marginally affected by advancing age.

Parameters Studied and Analytic Methods

Free amino acid concentrations in plasma and tissues. Frozen tissues (muscles, liver, and intestinal mucosa) were homogenized in ice-cold 10% trichloroacetic acid (TCA) containing 0.5 mMol/L EDTA and 125 μM norvaline as a sample preparation internal standard. The acid-soluble fraction containing free amino acids was separated by centrifuging from precipitated proteins (10 min, 2,500 g, 4°C). Tissue and plasma amino acid concentrations were determined by ion exchange chromatography with ninhydrin detection (JLC-500V; Jeol, Tokyo, Japan) (28). Results are expressed in micromoles per liter of plasma or micromoles per gram of tissue.

Our laboratory is registered in the European Quality Control Program (ERNDIM), ensuring the reliability of measurements for all the amino acids studied.

Urinary excretion. Myofibrillar protein degradation was evaluated by measuring excreted urinary 3-methylhistidine (3-MH) (20). 3-MH is released during myofibrillar protein breakdown without being either metabolized or reutilized for protein synthesis (44). In the rat, 3-MH is excreted chiefly in acetylated form. Samples were thus hydrolyzed with HCl (6 mol/L, vol/vol) at 100°C for 12 h, centrifuged (30 min, 2,500 g, 4°C), and filtered on 0.44-μm filters before quantification by ion exchange chromatography with ninhydrin detection (L8500A; Hitachi, Tokyo, Japan) (21). Results are expressed in micromoles of 3-MH per millimole creatinine to take into account muscle mass in the evaluation of myofibrillar protein breakdown.

Urinary creatinine excretion was measured as previously described (30).

Nitrogen was quantified by chemiluminescence (29) using an Antek 9000 apparatus (Antek, Houston, TX), and nitrogen balance was calculated as the difference between nitrogen intake and nitrogen urinary output.

Plasma insulin and glucose. Plasma insulin concentrations were analyzed using a commercial RIA kit (INSIK-5; DiaSorin, Antony, France) with rat insulin as a standard (Linco, St. Louis, MO) (32).

Plasma glucose was measured by a hexokinase end point method at 340–380 nm (Olympus AU600, Rungis, France) (4).

Protein mass in the tissues. The frozen tissue was pulverized and homogenized in ice-cold 10% TCA (1 ml TCA/100 mg tissue) using an UltraTurrax T25 tissue disrupter (Ika Labortechnik, Staufen, Germany). After delipidation with ethanol-ether (1:1, vol/vol), the precipitate was dissolved in 1 N NaOH (4 ml/100 mg tissue) for 12 h at 40°C. The total protein content was then determined according to the method described by Fleury et al. (15) on a Genesyx spectrophotometer (ThermoSpectronic, New York, NY).

Tissue protein synthesis rates were measured by the flooding-dose method (7). Just before being killed, each rat (in postabsorptive state) was injected subcutaneously with a large dose of L-¹³C]valine (99 atom%), 300 μmol/100 g; Cambridge Isotope Laboratories, Andover, MA) to flood the precursor pool of protein synthesis. Tracer incorporation was determined using the kinetics of incorporation of L-¹³C[valine from 20 to 50 min, with rats from each group being killed at different times. A 200-mg piece of tissue was used. Measurement of L-¹³C[valine enrichment in hydrolyzed proteins was performed using GC-C-IRMS (µGas System; Fisons Instruments, VG Isotech, Middlewich, UK). Amino acids in the tissue fluid were derivatized, and valine enrichments were used for precursor pool enrichment to calculate fractional synthesis rates (FSR) as previously described.

The FSR of proteins was calculated using the equation FSR = (Eₜ × (1/Eₚₑₑ × t)), where E₀ represents the enrichment as atom percent excess of ¹³C derived from decarboxylation of valine from proteins at time t (minus basal enrichment), Eₚₑₑ is the mean enrichment in the precursor pool (tissue fluid ¹³C[valine), and t is the incorporation time in hours.

Finally, the absolute synthesis rate (ASR) is calculated as ASR = P × FSR, where P is the protein content. Data are expressed as milligrams per hour.
**RESULTS**

**Protein Mass**

In the duodenum, the protein mass did not change between the groups (data not shown). In the liver, protein mass was significantly higher in the NEAA group compared with the R and Cit groups (NEAA vs. R and Cit, \( P < 0.05 \); Table 1). It was also higher in the Cit group compared with the R group (Cit vs. NEAA, \( P < 0.05 \)). In the tibialis muscle, only supplementation with citrulline significantly increased protein mass in this muscle (Cit vs. R and NEAA, \( P < 0.05 \); Fig. 1).

With respect to the 3-MH-to-creatinine ratio (3-MH/creatinine), myofibrillar proteolysis was unaffected by the different diets (data not shown).

**Protein Synthesis Rates**

Regarding the splanchnic area, in the duodenum there was no difference in protein synthesis rate between the groups. In the liver, the protein synthesis rate was significantly increased only in the NEAA group compared with the R group (NEAA vs. R, \( P < 0.05 \); Table 1).

In the tibialis muscle, only supplementation with citrulline led to a significantly higher ASR in this muscle compared with the other groups studied (Cit vs. NEAA and R, \( P < 0.05 \); Fig. 2). Mean FSR was 27% higher in CIT-supplemented than in NEAA-supplemented rats, but the difference did not reach significance (Table 2).

**Nitrogen Balance**

Nitrogen balance in both refeed groups (NEAA: 1,308 ± 72; Cit: 1,035 ± 94 mg/48 h) was higher (NEAA and Cit vs. R, \( P < 0.05 \) compared with the restricted group (308 ± 50 mg/48 h).

**Plasma Insulin**

Insulin concentrations were higher in the NEAA group compared with the R group (NEAA vs. R, \( P < 0.05 \); Fig. 3). Citrulline supplementation led to even higher insulin levels (Cit vs. R and NEAA, \( P < 0.05 \)) without change in glycemia (data not shown).

**Amino Acid Concentrations**

Among the 25 amino acids measured, concentrations of citrulline, ornithine, and arginine were the only ones that displayed significant variations according to the diet manipulations. They were higher in the plasma and the tibialis muscle in the Cit group after the renutrition period (Cit vs. R and NEAA, \( P < 0.05 \); Table 3).

**DISCUSSION**

Protein-energy malnutrition is common in the elderly, especially in hospitalized patients. There is a close correlation between severity of protein-energy malnutrition and morbidity and mortality risk. However, the capacity of malnourished elderly persons to respond successfully to renutrition is lower than that of younger adults (17). The mechanism responsible for this response deficit (14) may include a higher splanchnic extraction of amino acids (5, 16) in older than in young adults, which implies that fewer amino acids reach the systemic circuits.

### Table 3. Amino acid concentrations in plasma and tibialis muscle

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>R</th>
<th>NEAA</th>
<th>Cit</th>
</tr>
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<tbody>
<tr>
<td>Citrulline</td>
<td>104±7</td>
<td>155±20</td>
<td>2394±279*</td>
</tr>
<tr>
<td>Ornithine</td>
<td>41±3</td>
<td>43±6</td>
<td>223±27*</td>
</tr>
<tr>
<td>Arginine</td>
<td>118±4</td>
<td>99±7</td>
<td>561±55*</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrulline</td>
<td>26±4</td>
<td>50±11</td>
<td>616±104*</td>
</tr>
<tr>
<td>Ornithine</td>
<td>6±0</td>
<td>3±0</td>
<td>20±4*</td>
</tr>
<tr>
<td>Arginine</td>
<td>66±3</td>
<td>22±3</td>
<td>203±35*</td>
</tr>
</tbody>
</table>

Values are means ± SE. ANOVA + Duncan test: \(* P < 0.05 \) vs. R and NEAA.
The increased levels of insulin could be a result of increased levels of arginine, which is well known to be a potent inducer of insulin secretion (23). However, this effect may also be related to citrulline per se; it was recently shown (27) that citrulline at a physiological concentration (0.1 mM) increased insulin release from rat isolated islets.

In conclusion, citrulline supplementation in the old malnourished rats increases protein content of the muscle by stimulating protein synthesis. Whether this effect is transposable to humans and whether this strategy can improve the clinical outcome of elderly malnourished patients requires further study. Also, further work is needed to determine the mechanisms (direct or indirect) involved in citrulline action. Also, there is a splanchnic sequestration of amino acids in a number of pathological situations (including trauma, cancer, and type 2 diabetes). Although the underlying mechanisms here are certainly different from those encountered with advanced age, evaluating the effects of citrulline supplementation in these various situations is of interest because they are all characterized by impaired muscle protein synthesis.

GRANTS

S. Osowska was the recipient of a fellowship from Laboratoires Biocodex. This work was supported in part by a grant from Laboratoires Biocodex and in part by a grant from the French Ministry of Research (contrat quadranniel EA 2498).

REFERENCES


