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Citrulline modulates muscle protein metabolism in old malnourished rats

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Osowska, S., 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 submitted to dietary restriction (50% of food intake) 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.

sarcopenia; malnutrition; protein synthesis

THE RISING NUMBER OF ELDERLY PEOPLE in Western countries has made successful aging a major health politic 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 enterocytes, 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

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Table 1. *Protein content and ASR in the splanchnic area*

	R	NEAA	Cit
Liver protein, g/organ	2.75±0.14	3.51±0.12*	3.35±0.15†
Liver ASR, mg/24 h	0.997±0.096	1.503±0.155†	1.397±0.089

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, aspartate, 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 [^{13}C]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%), reverted 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 Ultra-Turrax 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 Genesys spectrophotometer (ThermoSpectronic, New York, NY).

Tissue protein synthesis rates were measured by the flooding-dose method (7). Just before being killed, each rat (in postabsortive state) was injected subcutaneously with a large dose of L-[^{13}C]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-[^{13}C]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-[^{13}C]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 = $(E_i \times 100)/(E_{prec} \times t)$, where E_i represents the enrichment as atom percent excess of ^{13}C derived from decarboxylation of valine from proteins at time *t* (minus basal enrichment), E_{prec} is the mean enrichment in the precursor pool (tissue fluid [^{13}C]valine), and *t* is the incorporation time in hours.

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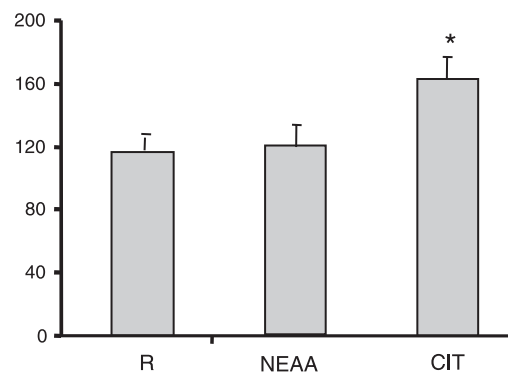

Fig. 1. Protein content in tibialis muscle (mg/organ). The 3 groups of rats were restricted for 12 wk. R, rats killed at the end of the restricted period; CIT, rats refed with a citrulline-supplemented diet; NEAA, rats refed with an NEAA-enriched diet. ANOVA + Duncan test: *$P < 0.05$ vs. R and NEAA.
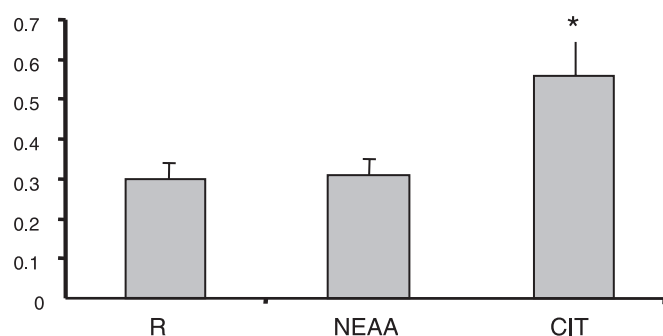
Fig. 2. Absolute protein synthesis rate in tibialis muscle (mg protein/h). ANOVA + Duncan test: *$P < 0.05$ vs. R and NEAA.

Fig. 3. Insulin concentration in the plasma (IU/l). ANOVA + Duncan test: *$P < 0.05$ vs. R; #$P < 0.05$ vs. R and NEAA.

Statistical Analysis

Data are expressed as means ± SE. Comparisons between sets of data were made using one-way analysis of variance followed by the Duncan test. The PCSM software was used (Deltasoft, Grenoble, France). Differences at $P < 0.05$ were considered significant.

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 refed 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

Table 2. *Weight and FSR of tibialis muscle*

Muscles	R	NEAA	Cit
Weight, mg	0.74±0.10	0.90±0.05*	0.85±0.12*
FSR, %/h	0.26±0.02	0.28±0.04	0.34±0.05

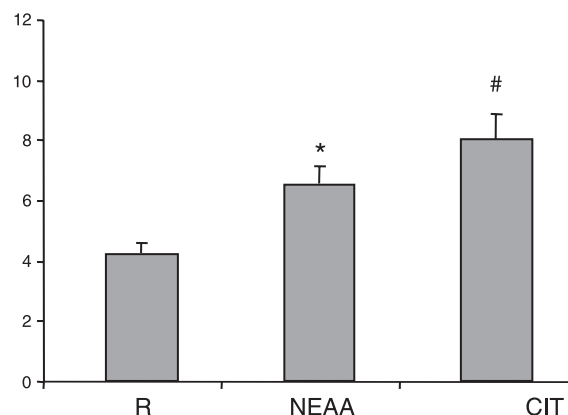Values are means ± SE. FSR, fractional synthesis rate. ANOVA + Duncan test: *$P < 0.05$ vs. R FSR.

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

	R	NEAA	Cit
Plasma			
Citrulline, μmol/l	104±7	155±20	2394±279*
Ornithine, μmol/l	41±3	43±6	223±27*
Arginine, μmol/l	118±4	99±7	561±55*
Muscle			
Citrulline, nmol/g	26±4	50±11	616±104*
Ornithine, nmol/g	6±0	3±0	20±4*
Arginine, nmol/g	66±3	22±3	203±35*

Values are means ± SE. ANOVA + Duncan test: *$P < 0.05$ vs. R and NEAA.

circulation (26). Some nutritional manipulations are promising (1, 2, 12, 38) but are difficult to perform in practice. The choice of citrulline as a potential tool to increase muscle protein synthesis appears to be an elegant and very logical strategy. As a matter of fact, among amino acids citrulline has a unique metabolism: it is not used by the intestine, and it is not taken up by the liver (11). Hence, administering citrulline is a tool to deliver nitrogen (through endogenous metabolism into arginine) available for protein synthesis in the peripheral tissues, including the muscle.

The results obtained herein confirm our hypothesis. We have shown that standard nutrition led to increased protein mass and synthesis only in the liver and had no effect on the muscle. The lack of stimulation of muscle protein synthesis is in agreement with the only study, to our knowledge, performed on old, 10-day-starved rats in which refeeding with a standard diet showed no effect on the muscle protein synthesis rate (25).

Conversely, citrulline supplementation did not affect either protein mass or protein synthesis in the splanchnic area, which confirms that it bypasses this area. The hepatic citrulline pool is strictly compartmentalized in the mitochondria of hepatocytes and does not mix with circulating citrulline (11). Conversely the citrulline-enriched diet exerted a remarkably stimulating effect on the protein synthesis in the muscle, and as a consequence the protein mass of the studied muscle was also significantly increased. This result is very impressive, as there is so far no study available in the literature showing such a protein synthesis stimulation in the old malnourished rat. The fact that the citrulline-supplementd diet increased the muscle synthesis whereas the NEAA-enriched diet increased liver protein synthesis probably explains why nitrogen balance was not different between groups. Further experiments using tracers exploring more tissues are required to confirm this hypothesis.

An increase in protein content is a result of increased protein synthesis only, as the 3-MH/creatinine ratio, which reflects myofibrillar proteolysis, remained unchanged in the three studied groups.

This study did not aim to determine the precise mechanisms by which citrulline administration influences protein synthesis. However, several hypotheses can be advanced that deserve further study.

Citrulline may simply be the vehicle taking nitrogen to the muscle, directly or through conversion into arginine in the kidney, and a greater availability of the latter would explain why protein synthesis increases.

Citrulline can act directly (or via arginine production) on protein synthesis. To date, the transductional properties of this amino acid are not known. Many molecular targets may be involved, and it would be of major interest to evaluate the effect of citrulline (vs. arginine) on the phosphorylation of signaling proteins causing anabolism, such as the mammalian target of rapamycin, ribosomal protein S6-kinase-1, Akt, etc. In the context of these future studies, it would be of interest to test in parallel amino acids that have proved to modulate protein synthesis in the elderly, e.g., a mixture of essential amino acid (35) and leucine alone (16).

Citrulline can act indirectly by stimulating insulin secretion. It is noteworthy that insulin levels were significantly higher in citrulline-supplemented rats than in controls, even though insulinemia still remained lower than in the old healthy rats (8).

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.

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