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ORIGINAL ARTICLE

Effect of citrulline on muscle functions during moderate dietary restriction in healthy adult rats

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Abstract Low calorie diets are designed to reduce body weight and fat mass, but they also lead to a detrimental loss of lean body mass, which is an important problem for overweight people trying to lose weight. In this context, a specific dietary intervention that preserves muscle mass in people following a slimming regime would be of great benefit. Leucine (LEU) and Citrulline (CIT) are known to stimulate muscle protein synthesis (MPS) in post-prandial and post-absorptive state, respectively. This makes them interesting bioactive components to test in the context of dietary restriction. We tested the concept of combining LEU and CIT in adult female rats. We postulated that the sequential administration of LEU (mixed in chow) and CIT (given in drinking water before a rest period) could be beneficial for preservation of muscle function during food restriction. Sixty female rats (22 weeks old) were randomized into six groups: one group fed ad libitum with a standard diet (C) and five food-restricted groups (60 % of spontaneous intake for 2 weeks) receiving a standard diet (R group), a CIT-supplemented diet (0.2 or 1 g/kg/day, CIT0.2 group and CIT1 group, respectively), a LEU-supplemented diet (1.0 g/kg/day) or a CIT + LEU-supplemented diet (CIT + LEU 1.0 g/kg/day each). At the end of the experiment, body composition, muscle contractile properties and muscle protein synthesis (MPS) rate were studied in the *tibialis anterior* muscle. Dietary restriction tended to decrease MPS (R: 2.5 ± 0.2 vs. C: 3.4 ± 0.4 %/day, $p = 0.06$) and decrease muscle strength (R: 3,045 ± 663 vs. C: 5,650 ± 661 A.U., $p = 0.03$). Only CIT administration (1 g/kg) was able to restore MPS (CIT1: 3.4 ± 0.3 vs. R: 2.5 ± 0.2 %/day, $p = 0.05$) and increase muscle maximum tetanic force (CIT1: 441 ± 15 vs. R: 392 ± 22 g, $p = 0.05$) and muscle strength (CIT1: 4,259 ± 478 vs. R: 3,045 ± 663 A.U., $p = 0.05$). LEU had no effect and CIT + LEU supplementation had few effects, limited to adipose mass and fatigue force. The results of this study highlight the ability of CIT alone to preserve muscle function during dietary restriction. Surprisingly, LEU antagonized some effects of CIT. The mechanisms involved in this antagonistic effect warrant further study.

Keywords Dietary restriction · Muscle mass · Slimming diet · Protein synthesis

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Introduction

The incidence of overweight and obesity is around 20–40 % in industrialized nations, and this figure is expected to rise further in the next few decades. While the causes of this phenomenon are probably multiple, the precise chain of causation remains unclear. However, nutrition clearly plays a direct role, and one of the obvious solutions to lose weight is dieting. Unfortunately, while low calorie slimming diets induce a loss of fat mass, they also lead to a significant loss of lean tissue mass. This

muscle loss is a global health issue, and carries an associated decrease in strength that in worst-case scenarios can affect body mobility. In this context, a key challenge is to find alternative dietary interventions allowing to lose fat but not muscle when following a dietary restriction. Two amino acids (AA), i.e. leucine (LEU) and citrulline (CIT), are known to stimulate muscle protein synthesis (MPS). Thus, a strategy based on enriching a hypocaloric slimming diet with these AA could be a solution to stimulate muscle protein synthesis while limiting muscle loss during a slimming regimen. LEU has been shown to stimulate MPS in various physiological and pathological conditions (see Anthony et al. 2002; Nicastro et al. 2011; Stipanuk 2007 for recent reviews). CIT has been shown to stimulate MPS in malnourished aged rats (Osowska et al. 2006) and to improve nitrogen balance in rats with short bowel syndrome (Osowska et al. 2004). In addition, CIT stimulates MPS in fasted adult rats (Le Plénier et al. 2012) and in healthy adults fed a low-protein (8 % protein) diet (Jourdan et al. 2008). Moreover, experimental studies suggest that LEU and CIT share the same physiological targets of action on MPS, i.e. mTORC1 and its downstream pathways (Cynober et al. 2010; Stipanuk 2007).

It is also of major importance to underline that LEU and CIT undergo very limited catabolism in the splanchnic area: CIT is not metabolized in enterocytes and not taken up by the liver (Curis et al. 2005), while LEU is only minimally catabolized in the intestine (i.e. 10–20 %) (Abumrad and Miller 1983) and is not catabolized in hepatocytes (Harper et al. 1970). Consequently, these two AA remain highly available for peripheral tissue, in particular to sustain MPS in post-prandial (i.e. LEU) or post-absorptive state (i.e. CIT) (Cynober et al. 2010; Moinard and Cynober 2007).

Another interesting observation is that LEU stimulates protein synthesis in the post-prandial state whereas CIT is synthesized and stimulates MPS in the post-absorptive state or when protein availability is low (Cynober et al. 2010). The hypothesis arising from this feature is that a sequential administration of LEU and CIT should optimize the preservation of muscle mass in subjects following slimming diets. Hence, the ideal strategy would be to administer LEU within a meal and CIT in the post-absorptive state (i.e. before bed rest).

Here we evaluated the effect of sequential administration of LEU and CIT to preserve lean body mass during food restriction. We used the dose of 1.0 g/kg/day of CIT in a model of food-restricted rats. This dose has already shown beneficial effects on body composition in aged rats (Moinard et al. 2009) and in short bowel syndrome in adult rats (Osowska et al. 2004, 2008). Moreover, a group of rats receiving a dose of 0.2 g/kg/day of CIT was added to test for the first time whether the CIT effect is still observed at a lower dose. LEU was administered at a dose in the range classically used for studies on LEU effects (Anthony et al. 2000, 2002; Le Plénier et al. 2012).

Materials and methods

Animals

This experiment used female adult Sprague–Dawley rats (Charles River, L'Arbresle, France). All the rats were aged 26 weeks at the end of the experiment. The choice of female rats to perform this study is based on the fact that slimming diets are particularly popular in women. The rats were housed individually and kept at 20–23 °C in alternate 12:12-h light–dark cycles (6–18 h off, 18–6 h on). During an acclimation period, all rats were fed standard diet (Dietex, Saint-Gratien, France) ad libitum for 2 weeks. The protocol complied with French regulations on the care of animals used for experimental and other scientific purposes (D 2001-486) and with the EC regulations (OJEC, L538 12:18:1986), and the program was approved by the regional animal ethics committee (Comité Régional d'Ile-de-France) under No. CEEA34.CM.009.11.

Experimental procedure

The experiment used 60 animals purchased at 22 weeks old. After acclimation (see above), the rats were randomized into six groups ($n = 10$/group). A control group (C group) was euthanized at the end of the acclimation period. The other five groups were restricted to 60 % of spontaneous food intake over 2 weeks: a restricted control group fed a standard diet (R group), a group fed standard diet + CIT at 0.2 g/kg/day (CIT0.2), a group fed standard diet + CIT at 1.0 g/kg/day (CIT1), a group fed standard diet + LEU at 1.0 g/kg/day (LEU) and a group fed standard diet + CIT at 1.0 g/kg/day + LEU at 1.0 g/kg/day (CIT + LEU).

Spontaneous intake was measured during the acclimation period by weighing the food consumed. As food intake was assessed at 25 g/day, restricted intake was calculated as 15 g/day.

LEU was carefully mixed in the diet, whereas CIT was dissolved in limited volumes of drinking water to ensure the rats consumed the full CIT dose. This CIT-enriched water was proposed as indicated below at the beginning of the light period, which corresponds to the post-absorptive period in rats.

Valine (VAL, 130 mg/kg/day) and isoleucine (ILE, 220 mg/kg/day) were added to the diet in the LEU groups to maintain physiological balance between the three branched-chain AA (10). The LEU-enriched diet was prepared in Paris-Sorbonne University's 'Pharmacotechnic' laboratory

to ensure the homogeneity of the mixture, as previously published (Osowska et al. 2006).

A preliminary test, performed on six rats during 3 weeks, showed that after 4 h of food and water deprivation, the animals drank 4 mL of water within 1 h. Taking this into account, together with mean animal weight (350 g) and CIT solubility (200 g/L at 20 °C), two solutions with different CIT concentrations were prepared. 2 mL of CIT solution at 35.02 g/L of CIT was given to the CIT0.2 group and 3 mL of CIT solution at 116.67 g/L was given to the CIT1 group. Note that ion-exchange chromatography assays were run (data not shown) to check that CIT was stable in solution in our experimental conditions.

During the 2 weeks of the experiment, food (standard or LEU-supplemented diet restricted to 60 % of spontaneous intake) was given at 9 a.m. Since CIT was given in the drinking water before "bed rest" (1 h before the light cycle switches on, i.e. 5 p.m.), food and drink were removed 2 h before the CIT solution was made available (at 3 p.m.), and the rats were left with access to standard drinking water for 1 h (up to 6 p.m). We checked that all the water made available during this 1-h period was drunk. The experimental design is summarized in Fig. 1.

Rats were weighed twice a week and spontaneous food intakes were recorded daily.

On the last day of the experiment, rats in post-absorptive state were anesthetized by isoflurane inhalation (3 %) using a regulated airflow apparatus (Minerve, Esternay, France) and received a single subcutaneous injection of an analgesic drug (Temgesic, 20 % solution; 85 µl/100 g bw). Muscle contractile properties were evaluated (see below), and after laparotomy, blood was collected from the abdominal aorta into heparinized or EDTA tubes. The hindlimb muscles (*gastrocnemius*, *soleus* and *tibialis anterior)* were rapidly removed, weighed, frozen in liquid nitrogen, and stored at −80 °C until analysis. This means that tissue sampling was performed about 16 h after the food had been removed (the previous evening).

Morphometric and muscle parameters and metabolic evaluation

Muscle protein content

The frozen muscles were homogenized in ice-cold 10 % trichloroacetic acid (TCA) (10 volumes) 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. Total protein content was then determined by spectrophotometry according to the method described by Fleury et al. (1951).

Body composition assessment

After euthanasia, the rats were dissected to separate and weigh the carcass (reflecting fat free mass), skin with the fat attached to it (reflecting fat mass), and abdominal fat

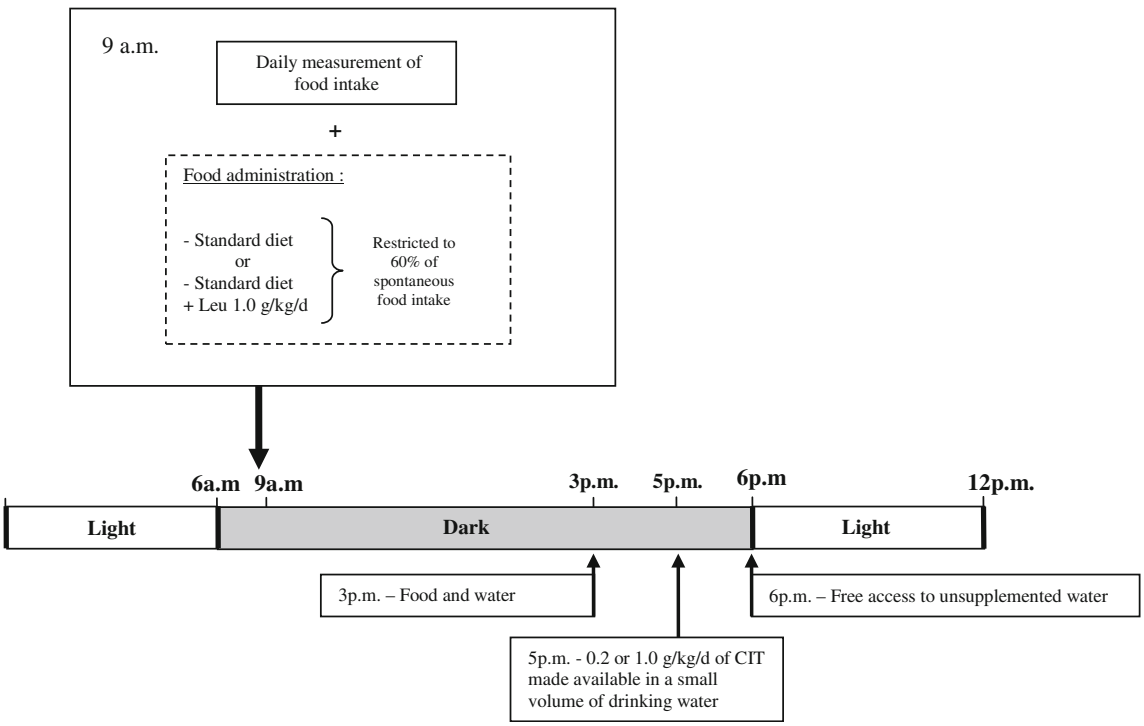

Fig. 1 Experimental design

mass (defined as mesenteric, perirenal, retroperitoneal and others).

Muscle contractile properties

At the end of the experiment, *tibialis anterior* muscle function was evaluated by measuring in situ muscle contraction response to nerve stimulation (Faure et al. 2011; Vignaud et al. 2003). After anesthetizing the animals, their knees and feet were fixed using clamps and pins. The distal tendons of the *tibialis* muscles were attached to an isometric transducer (Entran ELFS-T3 M-10 N, Devices Inc., Fairfield, NJ) using a silk ligature. All isometric transducer data output was recorded and analyzed by computer (Data translation 9,801, Bietigheim-Bissingen, Germany). The sciatic nerves were stimulated by bipolar silver electrodes by supramaximal square wave pulse of 0.2 ms duration. All isometric measurements were then made at an initial muscle length of L0 (length at which maximal tension was obtained during the twitch).

"Tetanic" responses were recorded at 100 Hz to determine maximal muscle strength. "Fatigue" resistance was then determined under continuous stimulation at 50 Hz for 30 s after a 5 min rest period. The duration corresponding to a 20 % decrease in muscle tension under this 50 Hz stimulation was noted "20 % fatigue decrease", or "50 % fatigue decrease" for a 50 % decrease. The area under this force–time curve was noted "Fatigue AUC" to represent muscle fatigue resistance.

Muscle protein synthesis

Tissue protein synthesis rates were measured at the end of the experiment by the flooding-dose method (Guillet et al. 2004a). A flooding dose of [U-^{13}C] Valine (150 µmol/100 g body weight, 90 Atom %; Eurisotop, Sain-Aubin, France) was intravenously injected into a lateral tail vein. A blood sample (200 µL) was taken 3 min later in a lateral tail vein. Animals were euthanized at 30, 35, 40, 45 and 50 min after injection of the flooding dose (two animals per timepoint in each group). Muscles were dissected and weighed. *Gastrocnemius* and *tibialis anterior* muscle were immediately frozen in liquid nitrogen and kept at −80 °C until analysis.

Muscle samples (100 mg) were homogenized in 5 % perchloric acid in a tissue lyser (2 × 1 min; Qiagen, Switzerland). After centrifugation, the supernatants were purified on AG 50 W-X8 cationic resin (Bio-Rad, Switzerland) and analyzed for ^{13}C enrichment of labeled free AA by gas chromatography-coupled mass spectrometry (GC–MS, Hewlett-Packard 6,890 gas chromatograph interfaced with a Hewlett-Packard 5,972 mass spectrometer) according to the method described by Faure et al. (2011). Briefly, analytical separation was performed on a DB-5MS (30 m × 0.25 mm I.D., 0.25 µm film thickness) fused-silica column. Helium was used as carrier gas at a pressure of 7 psi (0.9 mL/min). Pulsed splitless (30 psi) injection was used with a purge delay of 0.2 min. Injector temperature was 250 °C. The column was held at 60 °C for 1 min and then ramped up at 15 °C/min to 150 °C, 1.1 °C/min to 155 °C, and 25 °C/min to 300 °C, with a 1 min hold time, giving a total runtime of 18.3 min. Val retention time was around 8.8 min. The ions *m/z* 201 and 206, corresponding to fragment ions of unlabeled and [U-^{13}C] labeled Val, respectively, were monitored.

The protein pellet was washed twice in perchloric acid, then homogenized in a specific homogenization buffer allowing the separation of sarcoplasmic protein (in the supernatant from centrifugation at 5,000 *g* for 15 min) from the myofibrillar protein (in the pellet). After additional washing, the pellet was heated at 50 °C for 30 min in 0.3 M NaOH. The supernatant obtained after further centrifugation was precipitated again in perchloric acid, and the pellet washed twice in 70 % ethanol and finally hydrolyzed overnight at 110 °C in 6 M HCl before purification on cationic resin (AG50 W-X8, Biorad, Switzerland). Samples were then dried, dissolved in an aqueous solution of 0.2 % sulfuric acid (H_2SO_4), filtered with a nylon syringe filter and analyzed for ^{13}C isotopic enrichment by liquid chromatography on a 2.1 × 250 mm, 5 µm Primesep A column (Brechbühler, Switzerland) coupled to a Delta V Advantage isotope ratio mass spectrometer (LC-IRMS, Thermo, Bremen, Germany). The column was held at room temperature. LC flow rate was set at 300 µL/min. LC gradient was linear from 5 to 100 % of mobile phase A (water) for 20 min and held at this condition until 55 min before dropping back to the initial conditions. Mobile phase B was acidified water (0.2 % H_2SO_4). Total runtime was 61 min. Flow rates of the acid and oxidant reagents in the LC interface (LC Isolink, Thermo, Bremen, Germany) were set at 80 µL/min. Val was well separated from the other AA. For further calculation, the isotopic ratio of Val (^{13}C/^{12}C, expressed as δ^{13}C vs. VPDB, ‰) was transformed into atom percent excess (APE, %) (Godin et al. 2007).

Analysis of signal transduction factors in the mTORC1 pathway (4E-BP1, S6R, P70S6kinase)

The mTORC1 downstream pathway was analyzed using the following specific primary antibodies: phosphoAkt (Ser 473), Akt, phospho4E-BP1 (Ser 65), 4E-BP1, phosphoS6K1 (Thr 389), S6K1 (1:500), phospho-S6 ribosomal

protein (Ser 240/244), S6 ribosomal protein, as previously described (Le Plénier et al. 2012).

Plasma amino acids

Blood was collected into heparin tubes. Plasma was then aliquoted per 500 µL, frozen and stored at −80 °C. AA in deproteinized (sulfosalicylic acid) plasma were separated and quantified by ion-exchange chromatography with spectrophotometric detection after ninhydrin derivatization using an AminoTac JLC-500/V AA analyzer (Jeol, Croissy-sur-Seine, France; (Neveux et al. 2004).

Statistics

All statistical analysis were performed using R Software (version 2.9.2) with Tinn R (version 2.3.5.2) as editor.

Normally distributed data were analyzed with ANOVA followed by appropriate contrasts. Otherwise, data were analyzed with Kruskal–Wallis tests followed by rank sum Wilcoxon tests. When using the Wilcoxon tests, the Hodges Lehman estimator was used to obtain the estimated effects. Results are presented as median ± robust standard error computed from Rousseeuw's Sn.

Differences at $p < 0.05$ were considered significant. Significances at $p < 0.1$ were quoted as possibly interesting for future research.

Results

Weight monitoring (Table 1)

Weight monitoring clearly showed a restriction-related weight loss ($p = 0.04$ vs. control group). However, it is important to note that such body weight loss, even if significant, was of limited amplitude (around 15.5 g after 2 weeks of food restriction). The dietary intervention with LEU and/or CIT supplementation did not change this body weight loss (Table 1).

Body composition (Table 2)

Body composition scores showed significant effects of restriction on many parameters. Restriction significantly decreased total adipose tissue ($p < 0.001$), including cutaneous ($p = 0.043$), mesenteric ($p = 0.001$), perirenal ($p = 0.01$), retroperitoneal ($p < 0.001$), visceral ($p < 0.001$) and other adipose tissues ($p = 0.002$). Food-restricted animals also showed decreased visceral mass ($p = 0.03$), while CIT and LEU together had a synergistic effect on restoring viscera weight to control values.

Table 1 Body weights of rats before and during the restriction period, in of rats receiving a control diet ad libitum (basal control) or restricted (60 % of spontaneous intake; control restricted), restricted + LEU (1.0 g/kg/day; *R*–LEU), restricted + CIT (0.2 g/kg/day; *R*–CIT0.2 or1.0 g/kg/day; *R*–CIT1), or restricted plus LEU and CIT (each at 1.0 g/kg/day; *R*–LEU–CIT)

	Final body weight (g)	Body weight loss (g)
Basal control	293.0 ± 132.3	0 ± 40.2
Control restricted	287.5 ± 104.0	**15.5 ± 33.1****
R-LEU	283.0 ± 68.5	5.5 ± 16.5
R-CIT 0.2	279.5 ± 113.4	9.0 ± 30.7
R-CIT 1	298.0 ± 61.4	8.5 ± 33.1
R-LEU-CIT	286.0 ± 89.8	12.0 ± 26.0

Results are expressed as median ± standard error based on Rousseeuw's Sn ($n = 6$–10)

Statistically significant differences are highlighted in bold

** $p < 0.05$ vs. basal control group

Plasma concentrations of AA of interest (Table 3)

Plasma concentrations of AA of interest (i.e. leucine, citrulline and its metabolites) showed a large increase in CIT, arginine (ARG) and ornithine (ORN) plasma concentrations in CIT1 rats. The lower CIT concentration (i.e. 0.2 g/kg/day) had no incidence on plasma AA levels. LEU supplementation alone failed to increase LEU plasma levels.

Muscle metabolism exploration

Muscle weights and protein content

The results showed no significant effect of any diet on *tibialis*, *soleus* and *gastrocnemius* weights (data not shown). A similar pattern was found for the protein content of *gastrocnemius* muscle (data not shown).

Muscle protein synthesis (Fig. 2)

Although not statistically significant, fractional MPS rate tended to decrease in the *tibialis anterior* muscle of food-restricted rats compared to controls (2.51 vs 3.31 %, i.e. a 24 % decrease; $p = 0.061$). Moreover, CIT administration alone tended to restore MPS rate (CIT1 vs. *R*, $p = 0.051$), as control groups and CIT1 rats showed the same MPS levels (3.31 vs 3.40 %, respectively). Sarcoplasmic protein synthesis in the same muscle remained unaffected by the dietary manipulations.

mTORC1 pathway immunoblotting

There was no significant difference in any downstream element of the mTORC1 pathway in our experimental conditions. Indeed, no positive activation could be detected

Table 2 Body composition of rats receiving a control diet ad libitum (basal control) restricted (60 % of spontaneous intake; control restricted), restricted + LEU (1.0 g/kg/day; R-LEU), restricted + CIT (0.2 g/kg/day, R-CIT0.2 or 1.0 g/kg/dk R-CIT1), or restricted plus both LEU and CIT (each at 1.0 g/kg/day; *R*–LEU–CIT)

	Basal control	Control restricted	R-CIT 0.2	R-CIT 1	R-LEU	R-LEU-CIT
Total adipose tissue (g)	6.75 ± 8.90	**2.72 ± 4.65****	2.94 ± 2.19	2.52 ± 6.67	2.03 ± 2.95	1.97 ± 3.91
Cutaneous adipose tissue (g)	48.6 ± 30.5	**41.7 ± 16.6****	45.1 ± 18.2	46.0 ± 17.3	43.7 ± 14.5	41.6 ± 20.9
Mesenteric adipose tissue (g)	2.22 ± 3.12	**0.74 ± 0.84****	0.80 ± 0.76	0.77 ± 1.06	0.79 ± 0.98	0.74 ± 1.23
Perirenal adipose tissue (g)	0.63 ± 1.63	**0.34 ± 0.50****	0.37 ± 0.47	0.35 ± 0.76	0.31 ± 0.25	0.32 ± 0.50
Retroperitoneal adipose tissue (g)	1.37 ± 3.64	**0.44 ± 1.33****	0.49 ± 0.85	0.38 ± 1.32	0.26 ± 0.40	0.28 ± 1.03
Other adipose tissue (g)	2.85 ± 2.97	**1.02 ± 2.38****	1.19 ± 1.53	1.06 ± 3.55	0.68 ± 1.27	0.74 ± 2.12
Viscera (g)	30.5 ± 19.3	**26.1 ± 12.5****	26.6 ± 15.5	29.1 ± 8.4	26.5 ± 7.6	**29.7 ± 15.7**§,*
Lean mass with viscera (g)	206.2 ± 76.9	196.2 ± 49.2	193.0 ± 86.3	202.7 ± 76.8	198.5 ± 44.9	203.9 ± 50.8
Lean mass without viscera (g)	176.5 ± 62.7	172.7 ± 61.2	165.6 ± 79.4	174.1 ± 70.1	172.2 ± 41.5	173.1 ± 62.4
Weight (g)	277.8 ± 130.0	260.0 ± 97.6	257.0 ± 115.8	267.0 ± 92.8	259.0 ± 54.4	258.0 ± 66.2

Results are expressed as median ± standard error based on Rousseeuw's Sn ($n = 10$)

Statistically significant differences are highlighted in bold

R restricted

* $p < 0.05$ vs. restricted group

** $p < 0.05$ vs. basal control group

§ $p < 0.05$ vs. LEU

Table 3 Plasma amino acid concentrations (μmol/L) in rats receiving a control diet ad libitum (basal control), restricted intake (60 % of spontaneous intake; control restricted), restricted + LEU (1.0 g/kg/day; *R*–LEU), restricted + CIT (0.2 g/kg/day, *R*–CIT0.2 or 1.0 g/kg/day, *R*–CIT1), restricted plus LEU and CIT (each at 1.0 g/kg/day, *R*–LEU–CIT)

	Citrulline	Arginine	Ornithine	Leucine
Basal control	133 ± 87	154 ± 101	38 ± 53	253 ± 245
Control restricted	147 ± 123	162 ± 156	43 ± 43	260 ± 472
R-LEU	152 ± 125	156 ± 151	41 ± 13	278 ± 256
R-CIT 0.2	157 ± 209	153 ± 188	39 ± 47	232 ± 199
R-CIT 1	**410 ± 1,184**$^{*,\#}$	**243 ± 361**$^{*,\#}$	**56 ± 109**$^{*,\#}$	228 ± 330
R-LEU-CIT	**401 ± 657***,§	**302 ± 255***,§	**68 ± 39***,§	250 ± 280

Results are expressed as median ± standard error based on Rousseeuw's Sn ($n = 9$–10)

Statistically significant differences are highlighted in bold

R restricted

* $p < 0.05$ vs. restricted group

§ $p < 0.05$ vs. LEU

# $p < 0.05$ vs. CIT0.2 group

for any of the proteins tested in our samples. However, we confirmed that mTOR pathway was activated in a positive post-prandial control (data not shown).

Muscle contractile properties (Table 4)

Evaluation of *tibialis anterior* muscle function in response to nerve stimulation showed a significant increase on half-time relaxation ($p = 0.027$) and "Fatigue AUC" ($p = 0.029$) with dietary restriction. Moreover, sequential CIT + LEU administration enhanced "Fatigue force" (LEU-CIT vs. *R*, $p = 0.046$), and CIT administration alone significantly increased "Tetanus" ($p = 0.046$) and "Fatigue AUC" (CIT1 vs. *R*, $p = 0.052$ and CIT1 vs. CIT0.2, $p = 0.023$).

Discussion

Dietary restriction periods are classically associated with fat loss but are also characterized by a definitively harmful decrease in lean body mass. Numerous nutritional strategies have been proposed to counteract this regimen-related side-effect (Bopp et al. 2008; Chaston et al. 2007; McKnight et al. 2010; Wycherley et al. 2012). Muscle mass loss is generally associated with a decrease in strength.

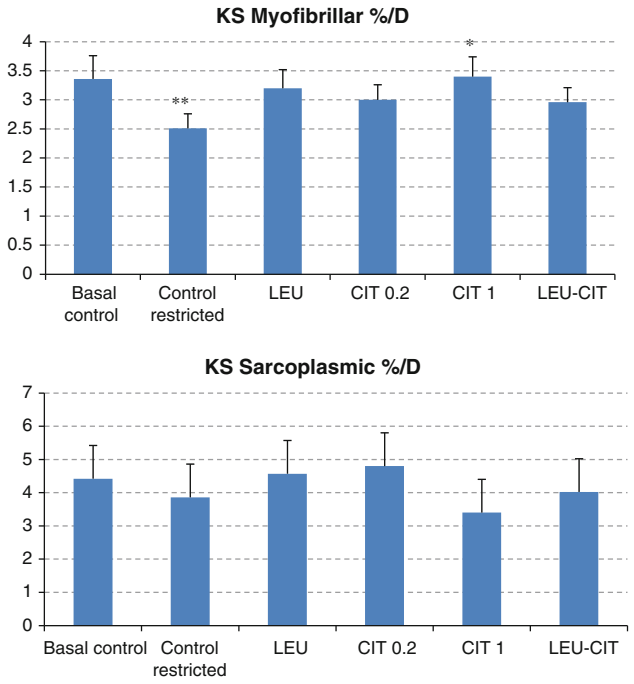

Fig. 2 Muscle myofibrillar and sarcoplasmic protein synthesis fractional rates (KS) in rats receiving a control diet ad libitum (basal control), restricted intake (60 % of spontaneous intake; control restricted), restricted + LEU (1.0 g/kg/day; *R*–LEU), restricted + -CIT (0.2 g/kg/day, *R*–CIT0.2 or 1.0 g/kg/day, *R*–CIT1), or restricted plus LEU and CIT (each at 1.0 g/kg/day, *R*–LEU–CIT). Results are expressed as median ± standard error based on Rousseeuw's Sn ($n = 9$–10). *$p < 0.051$ vs. restricted group and **$p < 0.061$ vs. basal control group. Trends for statistically significant differences are highlighted in *bold*

Moreover, it is always a struggle to recover muscle mass and strength once it is lost. LEU is considered one of the main signals for MPS in the post-prandial state, whereas CIT could play a major role in preserving MPS in the post-absorptive state (for reviews, see (Cynober et al. 2010; Moinard and Cynober 2007).

We hypothesized that sequential administration of CIT and LEU could effectively preserve muscle mass and function when on a hypocaloric diet. This study allowed us to test CIT and LEU sequential administration on a main feature of low calorie slimming diets, especially the preservation of lean mass, during a 2-week caloric restriction diet. This food restriction model displayed weight loss associated with a reduction in adipose tissue mass, which confirms previous observations (Chaston et al. 2007; Felgines et al. 1999). However, the reduction of lean body mass appears to be limited, since there was no decrease in muscle weights or protein contents in the restricted rats. Thus, a 40 % reduction of food intake for 2 weeks induced strong fat loss but little if any muscle loss. This contrasts with patterns reported in humans. However, we did find a reduction in MPS rate in *gastrocnemius* muscle which was accompanied by a decrease in contractile properties. Thus, it appears that in this study, the dietary restriction induced a reduction of protein synthesis that was not translated into muscle loss but that started to affect muscle function. Such a discrepancy between muscle loss and muscle function has already been reported in other muscle loss settings, such as during aging.

Table 4 Muscle contractile properties of rats receiving a control diet ad libitum (basal control), restricted intake (60 % of spontaneous intake; control restricted), restricted + LEU (1.0 g/kg/day; *R*–LEU), restricted + CIT (0.2 g/kg/day, *R*–CIT0.2 or 1.0 g/kg/day, *R*–CIT1), or restricted plus LEU and CIT (each at 1.0 g/kg/day, *R*–LEU–CIT)

	Twitch (g)	Half-time relaxation (sec.)	Tetanus (g)	Fatigue (g)	20 % fatigue decrease (sec.)	50 % fatigue decrease (sec.)	Fatigue AUC (AU)
Basal control	142 ± 165	43 ± 37	394 ± 302	369 ± 164	5.1 ± 8.6	13.0 ± 8.3	5,650 ± 6,608
Control restricted	133 ± 206	**33 ± 39****	392 ± 219	363 ± 527	3.8 ± 10.9	9.8 ± 16.1	**3,045 ± 6,630****
R-LEU	146 ± 85	36 ± 30	453 ± 316	442 ± 343	4.5 ± 10.1	10.1 ± 8.2	3,940 ± 7,131
R-CIT 0.2	144 ± 94	34 ± 23	459 ± 234	370 ± 331	2.8 ± 6.6	9.6 ± 13.9	3,084 ± 3,588
R-CIT 1	141 ± 79	36 ± 28	**441 ± 146***	394 ± 206	4.2 ± 11.1	11.1 ± 13.6	**4,259 ± 4,776***,#**
R-LEU-CIT	142 ± 47	34 ± 14	463 ± 356	**418 ± 307***	3.8 ± 7.8	10.6 ± 12.1	4,501 ± 8,260

Results are expressed as median ± standard error based on Rousseeuw's Sn ($n = 10$)

Statistically significant differences are highlighted in bold

AU arbitrary units

* $p < 0.05$ vs. restricted group

** $p < 0.05$ vs. basal control group

# $p < 0.05$ vs. CIT0.2 group

Interestingly, CIT administration alone appears to be able to preserve fractional MPS rate, as controls and CIT1 group rats showed identical values. Although the difference between restricted and control animals did not reach statistical significance, a 25 % mean decrease in muscle protein synthesis nevertheless has a real metabolic relevance. In addition, our data are in line with previous observations that the CIT effect on MPS is not global but is specific to myofibrillar protein, since this effect was not observed for fractional sarcoplasmic protein synthesis rate. This result concurs with a study on malnourished aged rats showing that CIT specifically upregulates the expression of the main myofibrillar protein (Faure et al. 2013). Moreover, this preservation of MPS is associated with improved muscle strength via an action on contractile function, as shown by a marked increase in "Tetanus" (100 Hz stimulation) in the CIT1-treated rats. This result is in line with work by Faure et al. (2011) performed in protein energy malnourished aged rats, in which CIT administration led to an increase in muscle mass that was strongly correlated to muscle protein content and maximal titanic isomeric force. This latter effect is of major importance. Indeed, it is known that maintaining or restoring muscle mass does not necessarily go along with an activation in the intrinsic force-generating capacity of skeletal muscle (Clark et al. 2006a, b).

LEU administration is widely recognized to increase MPS in various physiopathological settings (Baracos and Mackenzie 2006; Leenders and van Loon 2011). In our model of dietary restriction, LEU failed to preserve MPS and muscle contractile properties. This result could be explained by methodological issues. Indeed, all studies evaluating LEU effects on MPS are performed at the postprandial state, whereas here we measured MPS at the postabsorptive state. Indeed, access to food was removed about 15 h before measuring MPS rate, which is a relatively long delay. In addition, to the best of our knowledge, most studies reporting benefits of LEU on MPS were done in acute administration protocols, whereas chronic LEU administration failed to preserve MPS (Balage and Dardevet 2010; Verhoeven et al. 2009; Vianna et al. 2012). Unfortunately, we were unable to go deeper in the signaling pathway involved (i.e. mTORC1). Indeed, our experimental conditions, in particular the long-lasting postabsorptive state of animals before euthanasia and sampling, shut off this signaling pathway and thus did not allow us to detect potential activation levels of the proteins of interest. This lack of expression of mTOR downstream elements has already been observed in young adult male rats after 18-h fasting. These results are also in agreement with the study by Guillet et al. (2004b) which showed no activation of mTOR pathway in fasted young adults whereas elderly subjects showed increased basal activation of this pathway.

We also tested whether sequential CIT plus LEU administration had a synergistic effect on muscle function. CIT + LEU administration had an impact on muscle metabolism, as it significantly increased fatigue force, which corresponds to a 50 Hz muscle stimulation, without any similar effect on other muscular parameters measured. In this context, it appears that LEU blunted the CIT effects on MPS rates of myofibrillar proteins. This result is very surprising since, to our knowledge, this is the first study to report antagonism between CIT and LEU in terms of their regulatory effect on muscle protein metabolism. The design of our study does not allow us to confidently develop a hypothesis on the mechanisms involved. Further studies are warranted to evaluate the possible interactions between these two AA.

To conclude, this study showed that CIT administration is able to preserve muscle function during dietary restriction and that our concept of sequential administration of CIT + LEU is not suitable to preserve muscle function during dietary restriction. These results clearly point to the interest of CIT in this situation, and there is a strong rationale for performing clinical studies.

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