Amino Acids (2012) 43:1171–1178
DOI 10.1007/s00726-011-1172-z

ORIGINAL ARTICLE

Effects of leucine and citrulline versus non-essential amino acids on muscle protein synthesis in fasted rat: a common activation pathway?

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Received: 15 November 2011 / Accepted: 18 November 2011 / Published online: 13 December 2011
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Abstract Leucine (LEU) is recognized as a major regulator of muscle protein synthesis (MPS). Citrulline (CIT) is emerging as a potent new regulator. The aim of our study was to compare MPS modulation by CIT and LEU in food-deprived rats and to determine whether their action was driven by similar mechanisms. Rats were either freely fed (F, $n = 10$) or food deprived for 18 h. Food-deprived rats were randomly assigned to one of four groups and received per os, i.e., gavage, saline (S, $n = 10$), L-leucine (1.35 g/kg, LEU, $n = 10$), L-citrulline (1.80 g/kg CIT, $n = 10$) or isonitrogenous non-essential amino acids (NEAA, $n = 10$). After gavage, the rats were injected with a flooding dose of [^{13}C] valine to determine MPS. The rats were killed 50 min after the injection of the flooding dose. Blood was collected for amino acid, glucose and insulin determinations. Tibialis anterior muscles were excised for determination of MPS and for Western blot analyses of the PI3K/Akt, mTORC1, ERK1/2/MAPK pathways and AMP kinase component. MPS was depressed by 61% in starved rats (Saline vs. Fed, $P < 0.05$). Administration of amino acids (NEAA, LEU or CIT) completely abolished this decrease (NEAA, CIT, LEU vs. Fed, NS). Food deprivation affected the phosphorylation status of the mTORC1 pathway and AMP kinase (Saline vs. Fed, $P < 0.05$). LEU and CIT administration differently stimulated the mTORC1 pathway (LEU > CIT). LEU but not CIT increased the phosphorylation of rpS6 at serine 235/236. Our findings clearly demonstrated that both CIT and LEU were able to stimulate MPS, but this effect was likely related to the nitrogen load. LEU, CIT and NEAA may have different actions on MPS in this model as they share different mTORC1 regulation capacities.

Keywords mTORC1 pathway · Fasting · Insulin

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Introduction

Protein metabolism in skeletal muscle is tightly regulated. Many factors such as hormones and nutrients are implicated in this regulation and are recognized as strong determinants of protein homeostasis, particularly in fed state where plasma amino acid concentrations are increased. Consequently, many studies have reported the ability of insulin and amino acids to promote protein synthesis and/or inhibit proteolysis (Hamel et al. 2003; Prod'homme et al. 2004).

The amino acid leucine (LEU) is not only a substrate for protein synthesis, but also one of the main promoters of muscle protein synthesis in the fed state (Fujita et al. 2007; Stipanuk 2007). Models submitting rats to short fasting have clearly demonstrated that LEU modulates muscle protein synthesis by activating the mammalian target of

rapamycin complex 1 (mTORC1) pathway (Anthony et al. 2000, 2002; Baum et al. 2005; Crozier et al. 2005).

It has recently been established that citrulline (CIT) modulates muscle protein synthesis. We have shown that CIT is able to restore muscle protein content and stimulate muscle protein synthesis in malnourished aged rats (Osowska et al. 2006). Of note, CIT activates muscle protein synthesis by modulating the mTORC1 pathway (Le Plenier et al. 2011).

The question we now need to tackle is how both LEU and CIT—despite very different chemical structure and metabolism—are able to promote muscle protein synthesis through mTORC1 activation. Moreover, the available literature data on their action have not been obtained in the same model, thus precluding any meaningful comparison and making it difficult to identify whether these two amino acids share the same transduction pathway. To address this issue, we used an experimental model making it possible to investigate the acute effects of CIT and LEU on muscle protein synthesis. This model was previously developed (Anthony et al. 1999; Le Plenier et al. 2011) and it has been intensively used to study the biological properties of LEU (see Kimball and Jefferson 2006 for review). The model consists of 18-h food deprivation (which leads to a −65% drop in muscle protein synthesis). LEU is afterward administered by oral gavage. LEU administration activates muscle protein synthesis within 1 h (+165%), associated with an intense activation of the mTORC1 pathway (Kimball and Jefferson 2006).

The aim of our study was to compare muscle protein synthesis modulation by CIT and LEU in food-deprived rats and to determine whether these two amino acids share similar action on transduction pathways (i.e., PI3K/Akt, ERK1/2 MAPK, mTORC1 and AMP kinase). In parallel, we studied an isonitrogenous control group of rats receiving a mixture of non-essential amino acids and an absolute control group receiving saline.

Materials and methods

Materials

All primary antibodies were purchased from Cell Signaling (Ozyme, St Quentin-en-Yvelines, France): phosphoAkt (Ser 473 and Thr 308) (1:1,000), Akt (1:1,000), phospho4E-BP1 (Ser 65), (1:500), 4E-BP1 (1:1,000), phosphoS6K1(Thr 389) (1:500), S6K1 (1:500), phospho S6 ribosomal protein (Ser 240/244 and Ser 235/236) (1:2,000), S6 ribosomal protein (1:3,000), phospho-p42/44 MAP kinase (Thr 202/Tyr 204) (1:500), p42/44MAP Kinase (1:1,000), phospho-p90RSK (Ser 380) (1:500), p90RSK (1:1,000), phospho-AMPKα (Thr 172) (1:1,000), AMPKα (1:1,000), anti-rabbit IgG horseradish (1:2,000).

All L-amino acids were purchased from Sigma Aldrich Chemical (St Quentin Fallavier, France) except citrulline, which was a gift from Kyowa Hakko Co (Tokyo, Japan).

Animals

Fifty 3-month-old, male Sprague–Dawley rats (Charles River, L'Arbresle, France) were used in the experiment. They were maintained on a 12-h light:dark cycle with a standard diet (Dietex, Villemoisson-sur-Orge, France) with ad libitum access to water.

Animal care complied with the French regulations on the protection of animals used for experimental and other scientific purposes (D2001-486) and with European Community regulations (*Official Journal of the European Community*, L538 12:18:1986). The protocol was also reviewed and approved (under number P2.CM.032.07) by the regional ethics committee for animal testing.

Experimental procedures

The model of food-deprived rats was used as previously described (Kimball and Jefferson 2006). After a 1-week acclimatization period, rats were either freely fed (F, $n = 10$) or food deprived for 18 h. Food-deprived rats were randomly assigned to one of four groups receiving 0.155 mol/l of saline solution (S, $n = 10$), 1.35 g/kg body weight L-leucine (LEU, $n = 10$), 1.80 g/kg body weight L-citrulline (CIT, $n = 10$) or a non-essential amino acids mixture (NEAA, $n = 10$).

The amount of LEU given was equivalent to the amount consumed by rats of this age when fed an AIN-93 powdered diet (Harlan-Teklad, Madison, WI, USA) (Gautsch et al. 1998). The CIT solution was made equimolar with the LEU solution. The non-essential amino acid (NEAA) solution (alanine, glycine, proline, histidine, asparagine and serine) was made isonitrogenous with the LEU group. The saline group was studied to control for any possible volume-induced effects of oral gavage, i.e., gastric expansion-induced signaling.

Amino acids or saline were provided by oral gavage at under 2.5 ml/100 g body weight. After solution administration, the rats were returned to their cages. Both freely fed and food-deprived rats were allowed free access to water.

Immediately after solution administration, both freely fed and food-deprived rats were injected subcutaneously with a large dose of L-[1-^{13}C] valine (50% mol excess, 300 μM/100 g, Cambridge Isotope Laboratories, Andover, MA, USA) to determine protein synthesis rate, as previously described (Chanseaume et al. 2007; Tardif et al. 2011).

Exactly 50 min after administration of the flooding dose, the rats were killed by beheading. Trunk blood was

collected into heparinized tubes and rapidly centrifuged. The right and left *Tibialis anterior* muscles were excised, divided into two parts, and weighed, then quickly frozen in liquid nitrogen and stored at −80°C until analysis. *Tibialis* muscle was chosen for its high proportion of type II fibers, and it is known to be sensitive to CIT action (Osowska et al. 2006).

Parameters studied and analytical methods

Free amino acid concentrations in plasma

Plasma was deproteinized with 30% (w/v) sulfosalicylic acid solution and the supernatants were stored at −80°C until analysis. Amino acids were measured by ion-exchange chromatography using an amino acid analyzer with post-column ninhydrin derivatization (AminoTac, JLC-500/V, Jeol) (Neveux et al. 2004). The results of our participation in the European Quality Control Scheme (ERNDIM, Maastricht, The Netherlands) indicate the accuracy of our amino acid determinations.

Plasma insulin and glucose

Plasma insulin concentrations were analyzed using a commercial RIA Kit (RI-13K; Millipore, Labodia, Paris, France). Plasma glucose was measured by a hexokinase end-point method at 340–380 nm (Cobas 6000, Roche Diagnostics Systems, Meylan, France).

Measurement of total mixed proteins in skeletal muscle

MPS rates were measured using the flooding dose method, as previously described (Chanseaume et al. 2007; Tardif et al. 2011). We studied the incorporation of a stable isotope in the form of labeled amino acid (L-[1-^{13}C] valine) in *tibialis anterior* muscle. Rats were infused subcutaneously with a large dose of L-[1-^{13}C] valine (50% mol excess, 300 μmol/100 g;) to flood the precursor of protein synthesis. At 50 min after injection, the rats were killed. Fractional rates of protein synthesis were assessed from rate of L-[^{13}C] valine incorporation into total mixed muscle protein, as described previously (Chanseaume et al. 2007). After protein hydrolysis, amino acids were derivatized, and measurement of L-[^{13}C] valine enrichment in hydrolyzed proteins was performed using gas chromatography–combustion-isotope ratio mass spectrometry (*μ*Gas System; Fisons Instruments, VG Isotech, Middlewich, UK). L-[^{13}C] valine enrichment in muscle fluid was also assessed as described previously (Chanseaume et al. 2007) and used as precursor pool enrichment for the calculations of fractional synthesis rate (FSR).

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. Data are expressed as % h^{-1}.

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.

Preparation of muscle lysates

Frozen muscles (*tibialis anterior*) were ground in liquid nitrogen. This powdered muscle was weighed and homogenized in 10 volumes of buffer containing 150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1% NP 40, phosphatase inhibitors (20 mM sodium fluoride, 1 mM sodium pyrophosphate, 25 mM sodium glycerophosphate, 1 mM sodium orthovanadate) and protease inhibitors (complete protease inhibitor cocktail; Roche, Meylan, France). The samples were continuously homogenized (1 h, 4°C) and then centrifuged at 10,000×*g* for 30 min at 4°C. Supernatants were aliquoted and stored at −80°C. Protein concentrations were determined using the bicinchoninic acid assay (BC Assay UP 40840A kit Uptima, Interchim, France).

Immunoblotting for exploration of PI3K/Akt, ERK1/2 MAPK, mTORC1 pathways and AMPK

The supernatant was combined with an equal volume of SDS sample buffer (Laemmli Sample Buffer, Biorad, Marne-La-Coquette, France) and heated to 95°C. Total protein (50 μg) was separated by SDS-PAGE using a denaturing gel. After electrophoresis, proteins were transferred to nitrocellulose (GE-Healthcare, Orsay, France) and then blocked for 1 h with a blocking buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween20, 5% non-fat dry milk). Nitrocellulose blots were incubated overnight at 4°C with primary antibodies that recognize phosphorylated forms. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h in TBS/T with gentle shaking. The blots were developed using the enhanced chemiluminescence detection system (GE-Healthcare, Orsay, France) according to the manufacturer's protocol. Films were scanned and quantitated using the BioImaging System (Ozyme, Saint-Quentin-en-Yvelines, France). After quantification of the relative intensity of phosphorylated forms, membranes were stripped using a stripping buffer (0.5% acetic acid) and then probed with primary antibodies that recognize total forms.

Statistical analysis

All data were analyzed using Statview statistical software (SAS Institute, Cary, USA). All data are expressed as mean values ± SEM. Results were compared using ANOVA followed by Tukey–Kramer tests. Differences were considered statistically significant at $P < 0.05$.

Results

Leucine and citrulline concentrations (Table 1)

Food deprivation has no significant effect on plasma LEU and CIT concentrations (Saline vs. Fed, NS). CIT supplementation led to a 50-fold increase in plasma CIT concentration (CIT vs. all other groups, $P < 0.05$), whereas LEU supplementation led to a 13-fold increase in plasma LEU concentration (LEU vs. all other groups, $P < 0.05$).

Plasma insulin and glycemia (Table 1)

Food deprivation decreased plasma insulin concentrations and glycemia (Saline vs. Fed, $P < 0.05$) but provision of amino acids was without effect.

Muscle protein synthesis (Table 1)

Food deprivation was associated with a significant decrease in fractional synthesis rate (FSR) (Saline vs. Fed, $P < 0.05$). CIT, LEU and NEAA were able to restore FSR (NEAA, CIT, LEU vs. Fed, NS). The same pattern was observed on absolute synthesis rate (ASR).

Phosphorylation state of Akt (Fig. 1)

Neither food deprivation nor refeeding affected Akt phosphorylation levels.

Phosphorylation state of mTORC1 effectors

Food deprivation decreased the phosphorylation state of S6K1 at Thr 389 (Fig. 2a), of its target rpS6 at Ser 240/244 and Ser 235/236 (Fig. 2b, c, respectively) and 4E-BP1 at Ser 65 (Fig. 2d) (Saline vs. Fed, $P < 0.05$). Both CIT and LEU treatments affected the phosphorylation state of rpS6 at site 240/244 and 4E-BP1 (CIT and LEU vs. Saline group, $P < 0.05$). Of note, LEU had a stronger effect than CIT ($P < 0.05$). Finally, only LEU administration activated S6K1 and rpS6 at ser 235/236 (LEU vs. all groups, $P < 0.05$).

NEAA treatment did not lead to any significant changes in the phosphorylation state of these three signaling proteins.

Phosphorylation state of ERK 1/2 MAPK pathway

Neither food deprivation nor refeeding affected the phosphorylation state of p44/42 MAPK (data not shown) and its target, p90 RSK. Food deprivation decreased the phosphorylation state of rpS6 at 235/236 (Saline vs. Fed, $P < 0.05$, Fig. 2c) and only LEU administration was able to phosphorylate rpS6 at this site (LEU vs. all groups, $P < 0.05$).

Phosphorylation state of AMP kinase

Food deprivation is associated with an increase in AMP kinase phosphorylation (Saline vs. Fed, $P < 0.05$, Fig. 3), but it was not influenced by any supplementation regime.

Table 1 Plasma parameters [amino acids (citrulline (CIT) and leucine (LEU) (μmol/L)), insulinemia (ng/ml) and glycemia (mmol/L)] and muscle protein synthesis [%/h (FSR), mg/h (ASR)] in freely fed (Fed) rats and in food-deprived (Saline) rats subsequently given oral administration of LEU, CIT or a non-essential amino acid mixture (NEAA)

	Fed	Saline	NEAA	LEU	CIT
Plasma parameters					
CIT (μM)	159 ± 8	109 ± 8	122 ± 5	106 ± 4	7992 ± 648$^{\#}$
LEU (μM)	130 ± 15	170 ± 17	111 ± 13	1687 ± 166$^{\#}$	120 ± 13
Insulin (ng/ml)	0.84 ± 0.20	0.06 ± 0.02$^{\#}$	0.12 ± 0.05$^{\#}$	0.39 ± 0.11$^{\#}$	0.12 ± 0.03$^{\#}$
Glucose (mM)	8.90 ± 0.24	6.06 ± 0.32$^{\#}$	7.55 ± 0.31$^{\#}$	7.02 ± 0.23$^{\#}$	6.55 ± 0.37$^{\#}$
Protein synthesis					
FSR (%/h)	0.40 ± 0.02	0.28 ± 0.02$^{\#}$	0.35 ± 0.03	0.32 ± 0.01	0.30 ± 0.02
ASR (mg/h)	0.18 ± 0.01	0.11 ± 0.01$^{\#}$	0.16 ± 0.01	0.15 ± 0.01	0.14 ± 0.02

Mean values ± SEM. ANOVA + Tukey/Kramer test: $^{\#}$ $P < 0.05$ versus Fed group

FSR fractional synthesis rate, *ASR* absolute synthesis rate

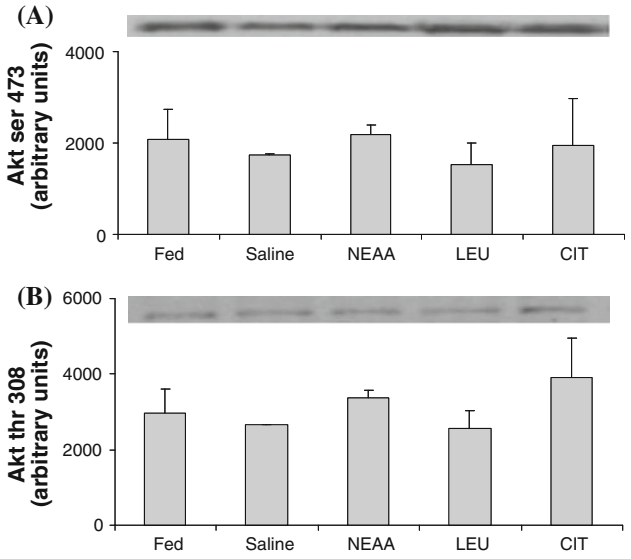

Fig. 1 Phosphorylation of Akt at ser 473 (A) and thr 308 (B) in skeletal muscle of food-deprived rats (Saline) and at 1 h after oral administration of non-essential amino acids (NEAA), leucine (LEU) or citrulline (CIT). Phosphorylation values were corrected by total forms. ANOVA + Tukey/Kramer test

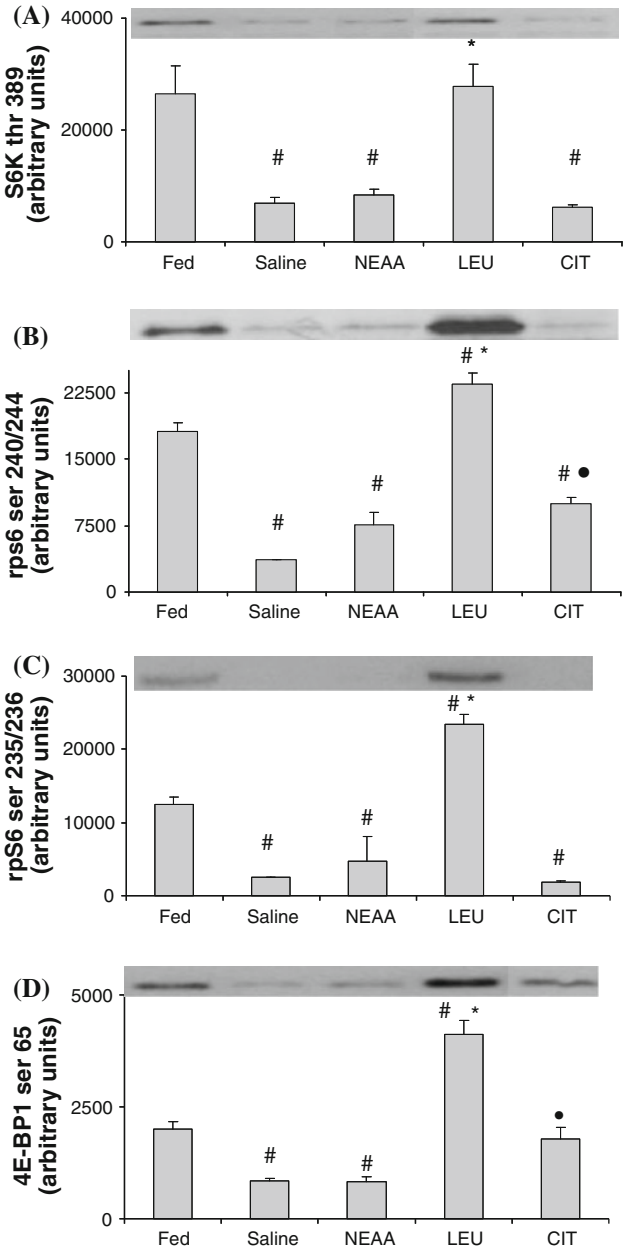

Fig. 2 Phosphorylation of 70-kDa ribosomal protein S6K1 at Thr 389 (**a**), rpS6 at Ser 240/244 (**b**) and at Ser 235/236 (**c**) and 4E-BP1 at Ser 65 (**d**) in skeletal muscle of food-deprived rats (Saline) and at 1 h after oral administration of non-essential amino acids (NEAA), leucine (LEU) or citrulline (CIT). Phosphorylation values were corrected by total forms. ANOVA + Tukey/Kramer test: $^{*}P < 0.05$ versus Saline, NEAA and CIT groups, $^{\#}P < 0.05$ versus fed group and $^{\bullet}P < 0.05$ versus Saline and NEAA groups

Discussion

Recent data have highlighted the ability of citrulline CIT to modulate nitrogen homeostasis in various physiological or pathological situations (Moinard and Cynober 2007). In particular, CIT is recognized as an activator of muscle protein synthesis (MPS) (Osowska et al. 2006). Until recently, only LEU was known to modulate MPS. If these two amino acids share the same MPS properties, the question that arises is do they also share similar mechanisms of action? To address this issue, it seemed important to compare their action not only on protein synthesis, but also on mTORC1 pathway activation in the same animal model. For this purpose, we used an experimental model developed by Layman ey al. and extensively used by Kimball and Jefferson (2006 for review) to investigate the acute effects of LEU on MPS.

As Kimball and Jefferson previously observed, fasting was characterized by a 60% decrease in MPS, and we further confirmed the previous report that oral administration of LEU was associated with a re-establishment of MPS (Anthony et al. 2000). Interestingly, CIT treatment led to a similar effect on MPS but, surprisingly, so did isonitrogenous administration, which suggests that the identified effect on MPS is not specific to LEU or CIT but is dependent on nitrogen load.

Several studies using isonitrogenous controls have clearly shown that NEAA treatment has no effect on MPS (Garlick and Grant 1988; Smith et al. 1998). To the best of our knowledge, only essential amino acid treatments have been reported to have an effect on MPS (Volpi et al. 2003). Several hypotheses can be put forward to explain this discrepancy. First, study designs differ from one study to another, especially in terms of length of fasting period [from 10 to 18 h (Garlick and Grant 1988; Kimball and

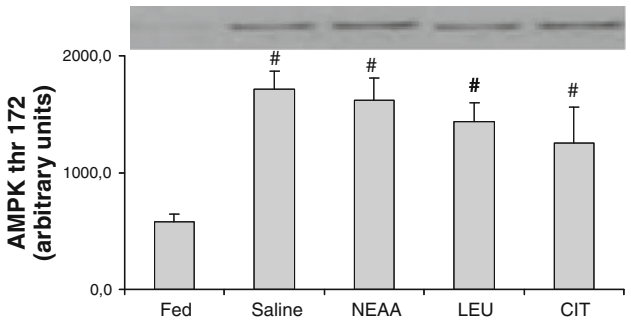

Fig. 3 Phosphorylation of AMPKα at Thr 172 in skeletal muscle of food-deprived rats (Saline) and at 1 h after oral administration of non-essential amino acids (NEAA), leucine (LEU) or citrulline (CIT). Phosphorylation values were corrected by total forms. ANOVA + Tukey/Kramer test: $^{\#}P < 0.05$ versus Fed group

Jefferson 2006)], composition of isonitrogenous solution [from one amino acid to a complex mixture (Garlick and Grant 1988; Kimball and Jefferson 2006)] or route of delivery [gavage, intravenous infusion, intra-peritoneal route, etc. (Garlick and Grant 1988; Kimball and Jefferson 2006; Smith et al. 1998)]. Second, in the fasted state, NEAA homeostasis is maintained by catabolism of essential amino acids (EAA)—alanine, for example, is produced in muscle from LEU and pyruvate—and limited EAA availability affects MPS since it is well known that a deficiency in one amino acids may be a limiting step for protein synthesis. Hence, in the fasted state, NEAA administration could spare EAA utilization and thereby preserve MPS. Further studies using multicatheterization methods and stable isotopes are required to confirm this hypothesis. Third, one or more amino acids in the NEAA mixture could display specific anabolic properties. For example, alanine has been shown to stimulate liver protein synthesis in starved rats (Perez-Sala et al. 1987), but to the best of our knowledge this effect has not been shown in muscle. Similarly, proline and glycine may possess pharmacological properties (Grimble et al. 1992; Ikejima et al. 1996) that could indirectly modulate protein synthesis. This raises the puzzling issue of choosing an adequate isonitrogenous control, which needs to be totally inert (Chambon-Savanovitch et al. 1999; Roth et al. 2003).

The modulation of MPS is usually accompanied by modified mTORC1 pathway activity. mTORC1 regulates the activity of translational machinery by promoting phosphorylation of two main substrates, 4E-BP1 and S6K1. Hyperphosphorylation of 4E-BP1 releases eIF-4E and allows cap-dependant mRNA translation. The activation of S6K1 promotes mRNA translation through several substrates, including phosphorylation of the ribosomal protein (rp) S6 at all sites (i.e., Ser 235, 236, 240 and 244). Our study confirmed previous results (Anthony et al. 2000) in which fasting was associated with a decrease in the phosphorylation status of mTORC1 kinase pathway. Moreover, phosphorylation of S6K1 (and its target rpS6) and 4E-BP1 increased in skeletal muscle 60 min after LEU administration, but it should be noted that rpS6 and 4E-BP1 were overactivated. Interestingly, our data demonstrate that CIT is also able to promote phosphorylation of rpS6 and 4E-BP1, but not to the same extent as LEU. However, these CIT-mediated changes still appear enough to increase MPS at 1 h, and it seems that LEU-mediated overactivation of this signaling pathway has no further effect on MPS.

Importantly, there are a variety of upstream pathways that either directly or indirectly regulate mTORC1 activity. Indeed, food intake is associated with hyperinsulinemia, which in turn stimulates the PI3K/Akt pathway. Here, administration of LEU or CIT had no effect on Akt phosphorylation status or insulin plasma concentration, which is in line with results obtained by Kimball and Jefferson's team (Anthony et al. 2000, 2002; Crozier et al. 2005; Gautsch et al. 1998). Another important regulator of the mTORC1 pathway is AMP kinase. This latter enzyme is the downstream component of a pathway that acts as a sensor of cellular energy (Hardie and Sakamoto 2006). Activation of AMP kinase in cells leads to an inhibition of mTORC1. Several studies have already underlined the implication of AMP kinase on LEU action on mTORC1 (Wilson 2011). Interestingly, in our model, AMP kinase was well activated as classically observed in the fasted state, but CIT and LEU were unable to normalize this intracellular mediator. These results suggest that AMP kinase may not be implicated in CIT and LEU action.

Finally, it has been proposed that LEU triggers ERK1/2 MAPK pathway activation, which is involved in the control of S6K1 (Duchêne et al. 2008; Lee et al. 2008). In the present work, we explored the implication of the ERK1/2 MAPK pathway in MPS regulation by LEU and CIT. We did not observe any activation of p44/42 MAPK or its target p90RSK. However, their involvement cannot totally be ruled out, since p90RSK is an activator of rpS6 at serine 235 and 236 (which are overactivated by LEU). However, rpS6 is equally a target of S6K1 at all phosphorylation sites (i.e., Ser 235, 236, 240 and 244). Further studies using specific ERK1/2 MAPK inhibitors (i.e., U0126) should definitively confirm or refute their putative involvement. Finally, other transductional pathways could be involved in MPS such as hVps34, MAP4K3 and Rag GTPases (Dinckinson and Rasmussen 2011), but at the present time there is no tool to explore these pathways in vivo. Furthermore, specific amino acid transporters may have a role upstream of mTORC1 (Nicklin et al. 2009). However, the in vivo implication of these new proteins is still subject to debate and additional studies are needed to take our findings further.

In conclusion, this is the first study to compare the effects of LEU and CIT on muscle protein synthesis. The results

clearly demonstrate that both CIT and LEU are able to stimulate MPS in this food-deprived rat model, and that this effect is mediated by the mTORC1 signaling pathway. Interestingly, our results suggest that CIT only activates mTORC1 whereas LEU activates both mTORC1 and the ERK1/2 MAPK pathway (as evidenced by phosphorylation of rpS6 at serine 235 and 236). The issue of why different amino acid supplementations (e.g., LEU, CIT, mixture of NEAA) activating different pathways leads to exactly the same increase in MPS warrants further study. We hypothesize the existence of a downstream element, common to the various pathways, which acts as a limiting/controlling step in activation on muscle protein synthesis.

Acknowledgments We thank Sylviane Darquy for conducting the insulin and glucose assays, Julie Marc for her help in amino acid determinations, and Thomas Camus for animal care and management. We thank also Harvard Apparatus and Minerve for providing devices used in this study. This work was supported by a grant (EA 4466) from the French Ministry of Research and Technology under a 4-year contract.

Conflict of interest S. Le Plénier, L. Cynober and C. Moinard are shareholders of Citrage Company.

References

Anthony JC, Anthony TG, Layman DK (1999) Leucine supplementation enhances skeletal muscle recovery in rats following exercise. J Nutr 129:1102–1106

Anthony JC, Anthony TG, Kimball SR, Vary TC, Jefferson LS (2000a) Orally administered leucine stimulates protein synthesis in skeletal muscle of postabsorptive rats in association with increased eIF4F formation. J Nutr 130:139–145

Anthony JC, Yoshizawa F, Anthony TG, Vary TC, Jefferson LS, Kimball SR (2000b) Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. J Nutr 130:2413–2419

Anthony JC, Lang CH, Crozier SJ et al (2002a) Contribution of insulin to the translational control of protein synthesis in skeletal muscle by leucine. Am J Physiol Endocrinol Metab 282:E1092–E1101

Anthony JC, Reiter AK, Anthony TG et al (2002b) Orally administered leucine enhances protein synthesis in skeletal muscle of diabetic rats in the absence of increases in 4E-BP1 or S6K1 phosphorylation. Diabetes 51:928–936

Baum JI, OConnor JC, Seyler JE, Anthony TG, Freund GG, Layman DK (2005) Leucine reduces the duration of insulin-induced PI 3-kinase activity in rat skeletal muscle. Am J Physiol Endocrinol Metab 288:E86–E91

Chambon-Savanovitch C, Felgines C, Farges MC et al (1999) Comparative study of glycine, alanine or casein as inert nitrogen sources in endotoxemic rats. J Nutr 129:1866–1870

Chanseaume E, Giraudet C, Gryson C et al (2007) Enhanced muscle mixed and mitochondrial protein synthesis rates after a high-fat or high-sucrose diet. Obesity 15:853–859

Crozier SJ, Kimball SR, Emmert SW, Anthony JC, Jefferson LS (2005) Oral leucine administration stimulates protein synthesis in rat skeletal muscle. J Nutr 135:376–382

Dinckinson JM, Rasmussen B (2011) Essential amino acids sensing, signalling, and transport in the regulation of human muscle protein metabolism. Curr Opin Clin Nutr Metab Care 14:83–88

Duchêne S, Audoin E, Crochet S, duclos MJ, Dupont J, Tesseraud S (2008) Involvement of the ERK1/2 MAPK pathway in insulin-induced S6K1 activation in avian cells. Domest Anim Endocrinol 34:63–73

Fujita S, Dreyer HC, Drummond MJ et al (2007) Nutrient signalling in the regulation of human muscle protein synthesis. J Physiol 582:813–823

Garlick PJ, Grant I (1988) Amino acid infusion increases the sensitivity of muscle protein synthesis in vivo to insulin. Effect of branched-chain amino acids. Biochem J 254:579–584

Gautsch TA, Anthony JC, Kimball SR, Paul GL, Layman DK, Jefferson LS (1998) Availability of eIF4E regulates skeletal muscle protein synthesis during recovery from exercise. Am J Physiol 274:C406–C414

Grimble RF, Jackson AA, Persaud C, Wride MJ, Delers F, Engler R (1992) Cysteine and glycine supplementation modulate the metabolic response to tumor necrosis factor α in rats fed a low protein diet. J Nutr 122:2066–2073

Hamel FG, Upward JL, Siford GL, Duckworth WC (2003) Inhibition of proteasome activity by selected amino acids. Metab Clin Exp 52:810–814

Hardie DG, Sakamoto K (2006) AMPK: a key sensor of fuel and energy status in skeletal muscle. Physiology 21:48–60

Ikejima K, Iimuro Y, Forman DT, Thurman RG (1996) A diet containing glycine improves survival in endotoxin shock in the rat. Am J Physiol 271:G97–G103

Kimball SR, Jefferson LS (2006) New functions for amino acids: effects on gene transcription and translation. Am J Clin Nutr 83:500S–507S

Le Plenier S, Walrand S, Cynober L, Moinard C (2011) Direct action of citrulline on muscle protein synthesis: role of the mTORC1 pathway. Clin Nutr 6(S1):20

Lee MY, Jo SD, Lee JH, Han HJ (2008) L-leucine increases [3H]-thymidine incorporation in chicken hepatocytes: involvement of PKC, PI3K/Akt, ERK1/2, and mTOR signaling pathways. J Cell Biochem 105:1410–1419

Moinard C, Cynober L (2007) Citrulline: a new player in the control of nitrogen homeostasis. J Nutr 137:1621S–1625S

Neveux N, David P, Cynober L (2004) Measurement of amino acid concentration in biological fluids and tissues using ion-exchange chromatography. In: Cynober L (ed) Metabolic and therapeutic aspects of amino acids in clinical nutrition, CRC Press, Boca Raton, pp 17–28

Nicklin P, Bergman P, Zhang B et al (2009) Bidirectional transport of amino acids regulates mTOR and autophagy. Cell 136:521–534

Osowska S, Duchemann T, Walrand S et al (2006) Citrulline modulates muscle protein metabolism in old malnourished rats. Am J Physiol Endocrinol Metab 291:E582–E586

Perez-Sala D, Parrilla R, Ayuso MS (1987) Key role of L-alanine in the control of hepatic protein synthesis. Biochem J 241:491–498

Prod'homme M, Rieu I, Balage M, Dardevet D, Grizard J (2004) Insulin and amino acids both strongly participate to the regulation of protein metabolism. Curr Opin Clin Nutr Metab Care 7:71–77

Roth E, Zellner M, Wessner B et al (2003) Glycine—an inert amino acid comes alive. Nutrition 19:817–818

Smith K, Reynolds N, Downie S, Patel A, Rennie MJ (1998) Effects of flooding amino acids on incorporation of labeled amino acids into human muscle protein. Am J Physiol 275:E73–E78

Stipanuk MH (2007) Leucine and protein synthesis: mTOR and beyond. Nutr Rev 65:122–129

Tardif N, Salles J, Landrier JF et al (2011) Oleate-enriched diet improves insulin sensitivity and restores muscle protein synthesis in old rats. Clin Nutr (Epub ahead of print)

Volpi E, Kobayashi H, Sheffield-Moore M, Mittendorfer B, Wolfe RR (2003) Essential amino acids are primarily responsible for the amino acid stimulation of muscle protein anabolism in healthy elderly adults. Am J Clin Nutr 78:250–258

Wilson GJ, Layman DK, Moulton CJ et al (2011) Leucine or carbohydrate supplementation reduces AMPK and eEF2 phosphorylation and extends postprandial muscle protein synthesis in rats. Am J Physiol Endocrinol Metab (Epub ahead of print)