Citrulline directly modulates muscle protein synthesis via the PI3K/MAPK/4E-BP1 pathway in a malnourished state: evidence from in vivo, ex vivo, and in vitro studies

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RESEARCH ARTICLE

Citrulline directly modulates muscle protein synthesis via the PI3K/MAPK/4E-BP1 pathway in a malnourished state: evidence from in vivo, ex vivo, and in vitro studies

Servane Le Plénier,[1] Arthur Goron,[1] Athanassia Sotiropoulos,[2] Eliane Archambault,[1] Chantal Guihenneuc,[3] Stéphane Walrand,[4] Jérome Salles,[4] Marion Jourdan,[1] Nathalie Neveux,[1] Luc Cynober,[1,5] and Christophe Moinard[1]

[1]*Laboratoire de Biologie de la Nutrition, EA4466, Faculté de Pharmacie, Université Paris Descartes, Sorbonne-Paris-Cité, Paris, France;* [2]*Centre National de la Recherche Scientifique UMR 8104, Institut Cochin, Université Paris Descartes, Sorbonne-Paris-Cité, Paris, France;* [3]*Laboratoire d'épidémiologie environnementale, EA 4064, Faculté de Pharmacie, Université Paris Descartes, Sorbonne-Paris-Cité, Paris, France;* [4]*Unité de Nutrition humaine, UMR 1019, Institut National de la Recherche Agronomique/Université d'Auvergne, Centre de Recherche en Nutrition Humaine, Auvergne, Clermont-Ferrand, France; and* [5]*Service de Biochimie interhospitalier Cochin et Hôtel-Dieu, GH Hôpitaux universitaire Paris Centre, AP-HP, Paris, France*

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Le Plénier S, Goron A, Sotiropoulos A, Archambault E, Guihenneuc C, Walrand S, Salles J, Jourdan M, Neveux N, Cynober L, Moinard C. Citrulline directly modulates muscle protein synthesis via the PI3K/MAPK/4E-BP1 pathway in a malnourished state: evidence from in vivo, ex vivo, and in vitro studies. *Am J Physiol Endocrinol Metab* 312: E27–E36, 2017. First published November 8, 2016; doi:10.1152/ajpendo.00203.2016.—Citrulline (CIT) is an endogenous amino acid produced by the intestine. Recent literature has consistently shown CIT to be an activator of muscle protein synthesis (MPS). However, the underlying mechanism is still unknown. Our working hypothesis was that CIT might regulate muscle homeostasis directly through the mTORC1/PI3K/MAPK pathways. Because CIT undergoes both interorgan and intraorgan trafficking and metabolism, we combined three approaches: in vivo, ex vivo, and in vitro. Using a model of malnourished aged rats, CIT supplementation activated the phosphorylation of S6K1 and 4E-BP1 in muscle. Interestingly, the increase in S6K1 phosphorylation was positively correlated ($P < 0.05$) with plasma CIT concentration. In a model of isolated incubated skeletal muscle from malnourished rats, CIT enhanced MPS (from 30 to 80% CIT vs. Ctrl, $P < 0.05$), and the CIT effect was abolished in the presence of wortmannin, rapamycin, and PD-98059. In vitro, on myotubes in culture, CIT led to a 2.5-fold increase in S6K1 phosphorylation and a 1.5-fold increase in 4E-BP1 phosphorylation. Both rapamycin and PD-98059 inhibited the CIT effect on S6K1, whereas only LY-294002 inhibited the CIT effect on both S6K1 and 4E-BP1. These findings show that CIT is a signaling agent for muscle homeostasis, suggesting a new role of the intestine in muscle mass control.

eukaryotic initiation factor 4E-binding protein 1; mitogen-activated protein kinase; phosphatidylinositol 3-kinase; muscle; myotube; amino acids; protein synthesis; mammalian target of rapamycin

CITRULLINE (CIT) is a nonprotein amino acid. It takes its name from the watermelon (*citrullus vulgaris*). CIT is known mainly as an intermediary of ureagenesis in periportal hepatocytes (24) and at the whole body level is produced almost exclusively by enterocytes (4). Intestinal CIT production is controlled largely by dietary protein supply (37). CIT has also emerged as an important regulator of nitrogen homeostasis in both humans and animals, as reviewed recently (6). For example, in pioneering work, we used a model of aged malnourished rats to demonstrate that CIT-enriched diet stimulated muscle protein synthesis (MPS) (+80%), and led to a net muscle protein gain (+20%) (10, 33). Interestingly, this metabolic effect was accompanied by muscle histological change and an increase in maximum strength as well as increased traction in treated animals (11). Similar results were obtained in healthy aged rats, in which CIT increased muscle protein content (28). This was also found in other situations such as fasting or caloric restriction in adults rats (22, 38). Finally, our experimental work showing the ability of CIT to modulate MPS was confirmed in humans; in a crossover trial, Jourdan et al. (20) showed that CIT administration to healthy volunteers fed a hypoprotein diet increased MPS compared with a nonessential amino acid mixture. Thus all these studies confirm the ability of CIT to modulate MPS in vivo, but no work has yet determined the underlying mechanisms of CIT action (an increase in nitrogen disposal for muscle or a pharmacological effect) directly or indirectly through arginine and/or nitric oxide production (21). The transductional properties of this amino acid are not known, and many molecular targets may be involved. Amino acids induce anabolic responses through several transductional pathways, such as phosphatidylinositol 3-kinase (PI3K)/Akt, MAPK, and the major mammalian target of rapamycin complex 1 (mTORC1) (15).

We hypothesized that CIT might act directly on MPS through activation of these signaling pathways. To investigate the mechanisms by which CIT modulates muscle protein synthesis, we studied *1*) the involvement of transductional pathways (mTORC1, PI3K/Akt, and MAPK pathways) in the regulation of MPS by CIT and *2*) its possible direct action on these pathways.

For this purpose, we used a systematic approach using experiments conducted in vivo (in malnourished aged rats), ex

Address for reprint requests and other correspondence: S. Le Plénier, Lab Biologie de la Nutrition (case 16), Faculté de Pharmacie, 4, avenue de l'Observatoire, F-75270 Paris, France (e-mail: servane.le-plenier@parisdescartes.fr).

vivo (in rat epitrochlearis muscle), and in vitro (on myotubes in culture).

EXPERIMENTAL PROCEDURES

In vivo studies. We used a validated rat model of malnutrition (40). In brief, Sprague-Dawley male rats aged 23 mo were used. Thirty rats were subjected to a dietary restriction for 12 wk (50% of spontaneous food intake with a standard diet; UAR 04, Villemoisson-sur-Orge, France) and then assigned to three groups; one group was euthanized (R), and the others were refed for 1 wk (90% of spontaneous food intake) with either a citrulline-supplemented diet (CIT) or an isonitrogenous standard diet (Ctrl). The other rats (10 of them) were fed ad libitum (AL). Rats in the postabsorptive state were anesthetized with isoflurane and euthanized by beheading. Blood was sampled, and muscles of the hindlimbs (tibialis anterior) were rapidly removed, weighed, and frozen in liquid nitrogen. Soluble proteins were extracted, and immunoblotting was performed as described previously (22). Plasma was also collected to measure CIT concentration as described below (33).

Ex vivo studies. Our protocol was reviewed and approved by the Ile-de-France Regional Ethics Committee (no. P2.CM.032.07) for the use of animals in research. Eighty 3-mo-old male Sprague-Dawley rats (Charles River, L'Arbresle, France) were used. They were maintained in a 12-h light-dark cycle with a standard rodent diet (UAR, Villemoisson-sur-Orge, France), with ad libitum access to water. Spontaneous intakes were determined daily. At the end of the acclimatization period, the rats were randomized into two groups. A healthy group (n = 40) was fed ad libitum for 6 wk with the standard diet. A malnourished group (n = 40) was subjected to dietary restriction {50% of the spontaneous food intake with a 5% protein diet [5% (wt/wt) proteins, 3% (wt/wt) lipids, 59% (wt/wt) carbohydrates, and 33% (wt/wt) water, fiber, vitamins, and minerals];UAR} for 6 wk, as described previously (40). Animal weights and spontaneous intakes were recorded daily. After 6 wk of experimentation, rats in the postabsorptive state were euthanized by beheading after general anesthesia with isoflurane (Minerve, Esternay, France).

Muscle incubation. All of the chemicals named below were purchased from Sigma Aldrich Chemical (Saint-Quentin-Fallavier, France), except for L-citrulline (L-CIT), which was a gift from Kyowa Hakko. (Tokyo, Japan). The inhibitors used were purchased from Tebu-Bio (Le Perray-en-Yvelines, France). Muscles were incubated described as previously (26, 27). Briefly, epitrochlearis muscles were dissected intact for incubation. They were immediately preincubated for 30 min at 37°C in Krebs-Henseleit buffer (KHB; 120 mM NaCl, 4.8 mM KCl, 25 mM $NaHCO_3$, 1 mM $CaCl_2$, 1.2 mM NaH_2PO_4 and 1.2 mM $MgSO_4$) supplemented with 2 mM HEPES, pH 7.4, 8 mM glucose, and 10 mU/ml insulin, and saturated with 95% O_2-5% CO_2 gas mixture. The muscles were then transferred for 120 min in fresh media of the same composition containing 1 mM L-[*ring*-^{13}C]phenylalanine (99 atom%; Cambridge Isotope Laboratories, Andover, MA) with and without 2.5 mM L-CIT. The dose was chosen according to an in vivo study (33) showing that CIT supplementation in aged rats led to a CIT plasma concentration of ~2.5 mM. In additional experiments, the same studies were performed with selective inhibitors to explore the involvement of mTORC1/PI3K/MAPK pathways. Briefly, epitrochlearis muscles were preincubated for 30 min in KHB supplemented with 2 mM HEPES, pH 7.4, 8 mM glucose, 10 mU/ml insulin, and a selective inhibitor (INH) as described in Table 1. The muscles were then transferred for 120 min in fresh medium of the same composition with supplementation of 2.5 mM CIT and 1 mM L-[*ring*-$^{13}C_6$] phenylalanine. After incubation, the two epitrochlearis muscles were removed, blotted on filter paper, weighed, and frozen in liquid nitrogen, and the medium was aliquoted and stored at −20°C until analysis.

Determination of whole muscle protein synthesis. Tissue protein synthesis rates were evaluated by the flooding dose method, as described previously (35), measuring the incorporation of labeled L-[*ring*-$^{13}C_6$]phenylalanine in the protein pool. Fractional synthesis rates (FSR) of proteins were calculated using the following equation:

$$\mathrm{FSR} = (\mathrm{Ei} \times 100)/(\mathrm{Ep} \times t)$$

where Ei is the enrichment as atom percentage excess of L-[*ring*-$^{13}C_6$] phenylalanine derived from phenylalanine from proteins at time t (minus basal enrichment), Ep is the mean enrichment in the precursor pool (tissue fluid L-[*ring*-$^{13}C_6$]phenylalanine), and t is the incorporation time in hours. FSR data are expressed in percentage per hour (%/h).

Absolute synthesis rate (ASR) is calculated as ASR = P × FSR, where P is the protein content. ASR data are expressed as milligrams of proteins per 24 h (mg/24h).

Determination of subcellular protein synthesis. Protein synthesis of each protein fraction (sarcoplasmic, mitochondrial, and myofibrillar) was also assessed as described previously (16). Only FSR was measured, because all the samples were used up for the determination of enrichment, and no material was left for the determination of protein content. Data (FSR) are expressed in %/h.

Determination of free amino acid levels. Media were deproteinized with a 30% (wt/vol) sulfosalicylic acid solution. Frozen epitrochlearis muscles were homogenized in ice-cold 10% trichloroacetic acid containing 0.5 mM EDTA and 125 μM norvaline as a sample preparation internal standard. Amino acids were assayed as described previously (31). Muscle amino acid concentrations are expressed in micromoles per gram tissue.

In vitro studies. Primary cultures were derived from gastrocnemia and tibialis anterior muscles of 4-wk-old male mice, as previously described (32). Cells were plated at low density (100 cells/cm^2) on 0.02% gelatin-coated dishes (cold water fish skin; Sigma, Saint-Quentin-Fallavier, France) and grown in complete medium composed of DMEM-Ham's F-12 (GIBCO, Invitrogen, Saint-Aubin, France), 2% Ultroser G (Biosepra, Pall corporation, Saint-Germain-en-Laye, France), 20% fetal calf serum (Sigma-Aldrich), and antibiotic-antimycotic solution (10,000 U/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B; GIBCO, Invitrogen). To differentiate muscle cells into myotubes, myoblasts were plated on Matrigel-coated dishes (Dutscher, Brumath, France) in complete medium and, after 6-h adhesion, switched to DMEM-Ham's F-12 + 2% horse serum (Sigma-Aldrich). Myotubes were then serum- and amino acid-deprived for 16 h on *day 2* of differentiation in serum- and amino acid-free DMEM (Eurobio, Courtaboeuf, France) containing 0.2% BSA (Sigma-Aldrich, Saint-Quentin-Fallavier, France).

Experiment 1. To characterize the kinetic effect of CIT on the mTORC1 pathway, serum- and amino acid-deprived myotubes were incubated in serum- and amino acid-free DMEM with and without 5 mM CIT for 60, 90, 120, 180, 240, and 300 min. Incubation in a standard DMEM (DMEM-Ham's F-12; Gibco) was used as positive control. The dose of CIT was determined according to previous studies evaluating the effect of amino acids (i.e., leucine or glutamine) in vitro (2, 5, 14).

Table 1. *Treatment and experimental conditions of muscle incubations*

	Ctrl−	CIT	CIT + R	CIT + W	CIT + P
KHB, glc (8 mM), insulin (10 mU/ml), and 1 mM L-[*ring*-^{13}C]phenylalanine	+	+	+	+	+
CIT (2.5 mM)	−	+	+	+	+
R (200 nM)	−	−	+	−	−
W (200 nM)	−	−	−	+	−
P (20 μM)	−	−	−	−	+

Ctrl, control; CIT, citrulline; R, rapamycin; W, wortmannin; P, PD-98059; KHB, Krebs-Henseleit buffer; glc, glucose.

Experiment 2. Serum- and amino acid-deprived cells were pretreated with and without selective inhibitors, as described in Table 2, for 30 min in serum- and amino acid-free DMEM. After a preincubation period, cells were incubated for 120 min (i.e., optimal conditions as determined in *experiment 1*) in the conditions described in Table 2.

Extraction of soluble proteins. Cells were washed twice with cold PBS and then scraped from the culture dish into lysis buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 5 mM EDTA, phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM sodium fluoride), and protease inhibitors (complete protease inhibitor cocktail; Roche, Meylan, France)]. After lysis (30 min, 4°C), the samples were centrifuged at 10,000 *g* for 20 min at 4°C. The protein concentration of the clarified lysates was determined using the Lowry assay (DC Protein Assay Kit 2; Bio-Rad, Marne-La-Coquette, France).

Immunoblotting. All antibodies were purchased from Cell Signaling Technology (Ozyme, St. Quentin-en-Yvelines, France): phosphorylated eukaryotic initiation factor 4E-binding protein 1 (4E-BP1; Ser^{65}; no. 9451, 1:500), phospho-4E-BP1 ($Thr^{37/46}$; no. 2855, 1:500), 4E-BP1 (no. 9644, 1:1,000), phosphorylated p70 ribosomal protein S6 kinase 1 (S6K1; Thr^{389}; no. 9234, 1:500), S6K1 (no. 2708, 1:500), and anti-rabbit IgG horseradish peroxidase-linked antibody (no. 7074, 1:2,000). Samples were then standardized to 0.5 mg/ml by dilution with 3× Laemmli SDS sample buffer (Ozyme, Saint-Quentin-en-Yvelines, France) containing 30% glycerol, 0.625 M Tris (pH 6.8), 20% (wt/vol) SDS, 0.5% (wt/vol) bromophenol blue, dH_2O, and 1:9 dilution β-mercaptoethanol and heated at 95°C for 10 min. Protein at 20 μg/lane was loaded onto TGX 12% SDS-PAGE precast gels (Bio-Rad) and transferred to nitrocellulose membranes (Hybond-C Extra; GE Healthcare, Aulnay-sous-Bois, France) at 120 mA for 2 h. After membranes were blocked for 1 h in TBS-T buffer (10 mM Tris·HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) with 5% nonfat skimmed milk, blots were incubated overnight at 4°C with primary antibodies recognizing phosphorylated forms. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h in TBS-T with gentle agitation. Proteins were then visualized using enhanced chemiluminescence (ECL Prime; GE Healthcare) on ImageQuant Las 4000 system (GE-Healthcare) using a CCD camera. Band density was quantified using ImageJ software. For normalization, blots were stripped using antibody stripping buffer (Gene Bio-Application, Paris, France) and then reprobed for total proteins to verify the relative amount analyzed.

Statistics. In the in vivo and in vitro studies, nonparametric tests were used, avoiding Gaussian assumptions.

In ex vivo studies, relationships between absolute synthesis rate (ASR) with and without CIT and ASR under inhibitor were modeled by linear approaches. Bayesian inferences were chosen because they were well suited to small samples.

In the in vivo studies, differences in phosphorylation state of the mTORC1 targets were studied using nonparametric ANOVA among four groups (AL, R, Ctrl, and CIT) using a Kruskal-Wallis test.

Table 2. *Treatment and experimental conditions of primary myotube cultures*

	Ctrl−	Ctrl+	CIT	CIT + R	CIT + L	CIT + P
DMEM-Ham's F-12	−	+	−	−	−	−
Serum- and amino acid-free DMEM	+	−	+	+	+	+
CIT (5 mM)	−	−	+	+	+	+
R (20 nM)	−	−	−	+	−	−
L (50 μM)	−	−	−	−	+	−
P (10 μM)	−	−	−	−	−	+

Ctrl, control; CIT, citrulline; R, rapamycin; L, LY-294002; P, PD-98059.

Correlations between CIT concentrations and phosphorylation level of S6K1 were estimated and tested by a Spearman correlation test.

Ex vivo studies were analyzed using a Bayesian linear model (13). This model assesses associations between ASR with CIT and ASR under control for *group 1* and between ASR with CIT and ASR under inhibitor for *group 2*. Two models were implemented by a Bayesian approach: model 1 without constraints on slopes and model 2 assuming an inverse relationship between slopes for the two groups. More precisely, for rat *i*:

$$\text{Model 1: } ASR^{(CIT,G1)}{}_i = \alpha\, ASR^{(ctrl,G1)}{}_i + \varepsilon_i \text{ and } ASR^{(CIT+inh,G2)}{}_i = \beta\, ASR^{(CIT,G2)}{}_i + \varepsilon_i$$

$$\text{Model 2: } ASR^{(CIT,G1)}{}_i = \alpha\, ASR^{(ctrl,G1)}{}_i + \varepsilon_i \text{ and } ASR^{(CIT+inh,G2)}{}_i = (1/\alpha)\, ASR^{(CIT,G2)}{}_i + \varepsilon_i$$

where $ASR^{(T,\,G)}$ is ASR for rats under "treatment" T (*t* = CIT, Ctrl, or CIT + inh) in group G (G = *group 1* or *group 2*).

For each model, positivity of slopes was assumed with a null intercept and Gaussian residuals with different variances between groups. These models were estimated for healthy and malnourished rats separately. Vague positive prior distributions were chosen for slopes (exponential with parameter equal to 0.01) and for variances (inverse-γ with parameters equal to 0.01 and 0.01). Bayesian inference was done via Marko Chain Monte Carlo algorithms with 50,000 iterations and 1,000 iterations for burn-in, using Winbugs software (23). Convergence was assessed by checking good mixing between iterations and accurate approximations by MC error. Results are expressed as posterior means of slopes and a 95% posterior credibility interval. The advantage of a Bayesian approach is that it offers the possibility of estimating posterior distribution not only of parameters but also of all quantities of interest directly deduced from parameters. In particular, for *model 1*, we were interested in the product αβ; its posterior mean and 95% posterior credibility interval will also be given.

In the in vitro studies, the effects of CIT with and without inhibitors on phosphorylation status were tested with nonparametric rank sum tests. The effect of CIT on the phosphorylation state of S6K1 and 4E-BP1 was appraised via the study of the distribution of relative phosphorylated values: the median was then compared with value 1 with a Wilcoxon test. Concerning the effects of inhibitors, two groups were compared (1 group with CIT and another with CIT and inhibitor) with a Wilcoxon-Mann-Whitney test.

RESULTS

Effect of a CIT-enriched diet on muscular mTORC1 activation in aged malnourished rats. A previous study (33) had shown that in this aged rat model, CIT was able to increase MPS. We thus evaluated the phosphorylation status of S6K1 and 4E-BP1 in the tibialis of these rats by Western blot. Following caloric restriction, a marked decrease in S6K1 phosphorylation status (AL vs. R, $P < 0.05$; Fig. 1*A*) and a nonsignificant decrease in 4E-BP1 phosphorylation status (AL vs. R, $P = 0.116$; Fig. 1*B*) were observed. This latter result probably lacked power, as the figure clearly shows the decrease. Interestingly, only CIT-supplemented rats activated the phosphorylation status of those proteins in skeletal muscle to normal levels.

Correlations between citrullinemia and ratio of phosphorylation/total level of S6K1. As reported previously (33), CIT was largely increased by CIT supplementation (CIT: 2394 ± 279 vs. R: 104 ± 7, $P < 0.05$), and only ornithine and arginine patterns were modified. The increase in phosphorylation level of S6K1 was positively correlated with the CIT concentration in plasma from CIT-treated rats (Spearman

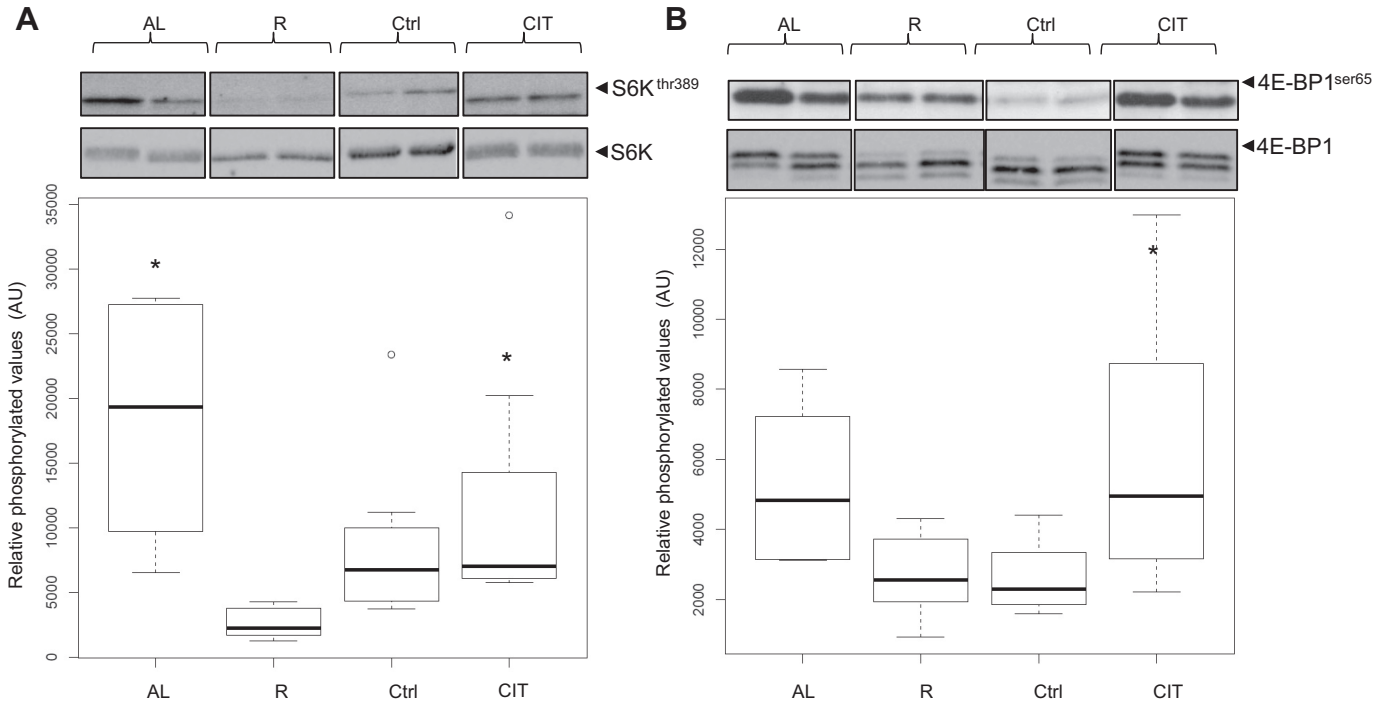

Fig. 1. Effect of a citrulline (CIT)-enriched diet on muscular mammalian target of rapamycin complex 1 (mTORC1) activation in aged, malnourished rats. One group of aged rats (20 mo old) was healthy [ad libitum (AL)]. The other 3 groups were restricted for 12 wk; 1 group was euthanized at the end of the restricted period (R), and 1 group was refed for 5 days with a control diet (Ctrl) or with a CIT-supplemented diet (CIT). Phosphorylation status of p70 ribosomal protein S6 kinase 1 (S6K1; *A*) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1; *B*) in skeletal muscles was measured by Western blot. Nonparametric ANOVA on S6K1 phosphorylation values and on 4E-BP1 phosphorylation values among 4 groups (AL, R, Ctrl, and CIT) rejected significantly means equality between groups (Kruskal-Wallis test, $P = 0.0023$ and $P = 0.0289$, respectively). Posterior comparison was made by a nonparametric Wilcoxon test. *$P < 0.05$ vs. R. AU, arbitrary units.

correlation coefficient between S6K1 and [CIT] in plasma was 0.72, $P < 0.05$; Fig. 2*A*). We found no correlation between CIT concentration in muscle and S6K1 phosphorylation level (Fig. 2*B*).

Effect of CIT on protein synthesis in isolated epitrochlearis muscle. We investigated whether the muscle was the direct target of CIT for regulation of muscle protein synthesis using an isolated incubated muscle model. For this purpose, epitrochlearis muscles from healthy or malnourished rats were incubated for 2 h with and without CIT. Whole muscle protein synthesis, fractional protein synthesis (i.e., myofibrillar, mitochondrial, and sarcoplasmic proteins), and amino acid patterns were determined. CIT treatment increased by 80% ($\alpha^M = 1.89$) on average the absolute synthesis rate (ASR) of malnourished rats (Fig. 3*B*), whereas FSRs were not modified (data not shown). However, although total FSR was not affected by CIT, it was increased specifically in subcellular fractions from healthy (Fig. 3*C*) and malnourished rats (Fig. 3*D*).

Moreover, incubation with CIT elevated muscle concentration of this amino acid 12-fold (CIT: 7.50 ± 1.00 vs. Ctrl: 0.65 ± 0.10 μmol/g, means ± SE, *t*-test: $P < 0.001$ vs. Ctrl), whereas concentrations of other amino acids in muscle, in particular CIT-related amino acids (i.e., ornithine and arginine), were not influenced by CIT incubation.

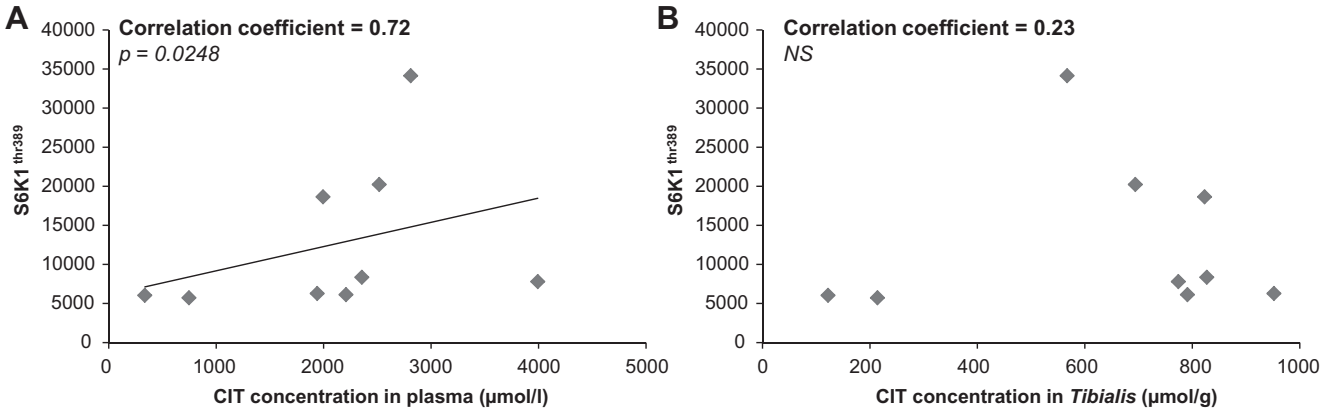

Fig. 2. Correlations between citrullinemia and ratio of phosphorylation to total level of S6K1 from CIT-treated rats (*A*) and between citrulline in tibialis muscle and phosphorylation level of S6K1 from CIT-treated rats (*B*). Correlations were estimated and tested by a Spearman correlation test.

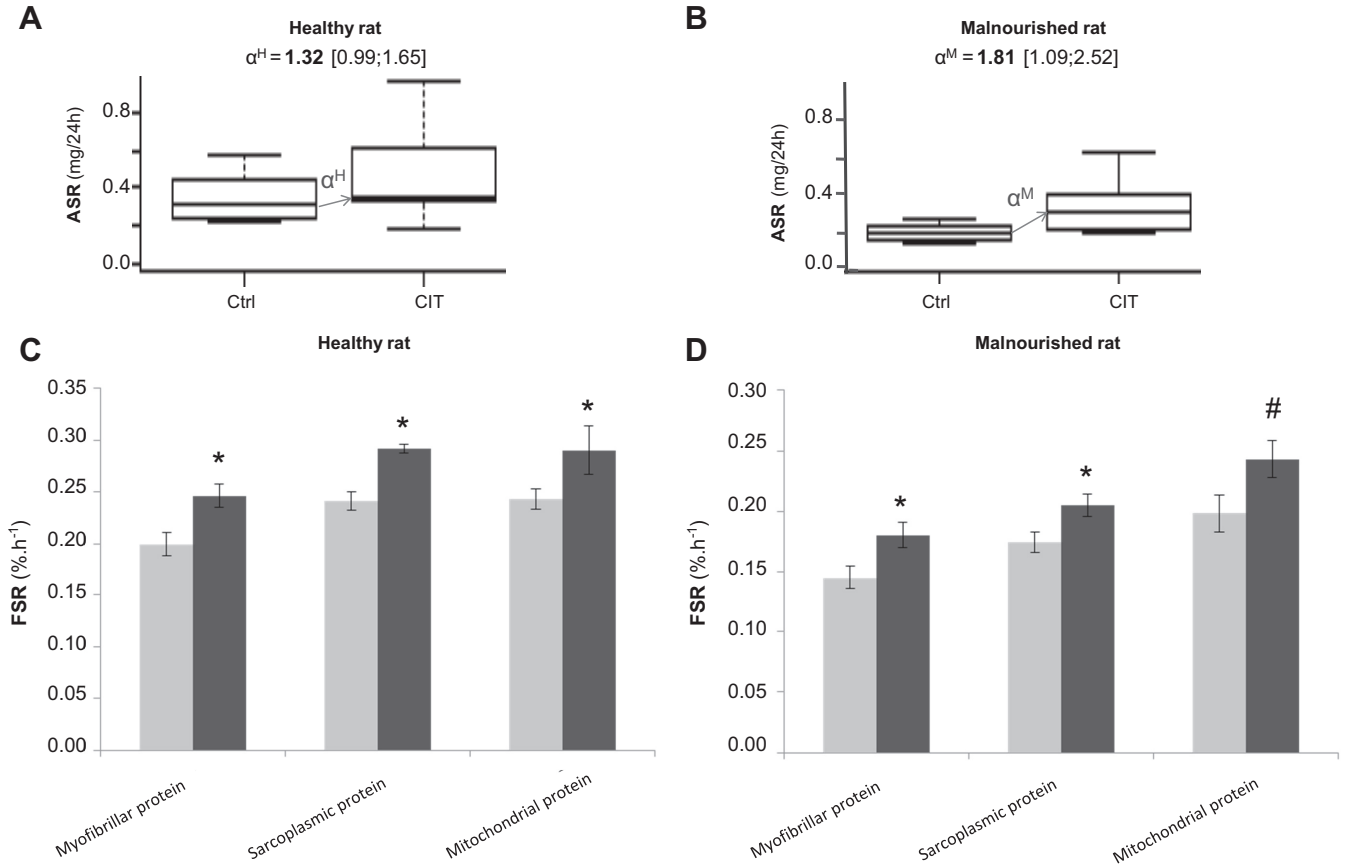

Fig. 3. Effect of CIT treatment on muscle protein synthesis in isolated epitrochlearis muscle. Epitrochlearis muscles of healthy and malnourished rats were incubated for 120 min with citrulline (CIT; 2.5 mM) and without CIT (Ctrl). Synthesis rates were estimated using rate of L-[*ring*-^{13}C]phenylalanine incorporation into proteins. *A* and *B*: box plot charting the distribution of absolute synthesis rate (ASR) in epitochlearis muscle of healthy (*A*) and malnourished rats (*B*) incubated with and without CIT. ASR is expressed in mg/24 h. The effect of CIT was analyzed by a Bayesian approach (model 1 without constraint), with Ctrl to CIT being estimated by the parameter-α. Parameter-α is expressed as posterior means of slopes and a 95% posterior credibility interval, and it is significantly different from whether one excluded from the interval. α^H, healthy; α^M, malnourished. *C* and *D*: fractional synthesis rate (FSR) of each protein fraction of epitrochlearis from healthy (*C*) and malnourished rats (*D*). Light gray bars, control; dark gray bars, CIT. FSR is expressed in %/h. Differences were studied using a nonparametric Mann-Whitney test. *$P < 0.05$ vs. Ctrl; #$P = 0.07$ vs. Ctrl.

Effect of selective inhibitors on protein synthesis of muscle incubated with citrulline. To investigate mechanisms by which CIT modulates MPS, we explored transductional pathways (i.e., mTORC1, PI3K/Akt, and MAPK pathways) in epitrochlearis muscle by an inhibitor-based approach. For this purpose, epitrochlearis of healthy and malnourished rats were incubated for 2 h with CIT and with CIT and selective inhibitors. Whole muscle protein synthesis was determined by the flooding dose method. We postulated that the action of the selective inhibitors used (rapamycin, wortmannin, and PD-98059) would lead to a return to baseline; i.e., we assumed that $\beta = 1/\alpha$. To test this assumption, we studied the product $\alpha\beta$ under *model 1* (Table 3). Because one is generally included in the 95% posterior credibility interval, the assumption that $\beta = 1/\alpha$ seems reasonable. Further results were obtained under *model 2* (with constraints), enabling a statistical power increase.

The presence of rapamycin (inhibitor of mTORC1 pathway; Fig. 4*A*) decreased absolute synthesis rate by 44% on average in muscle of malnourished rats. A decrease in 25% for healthy rats was weakly significant. The same results were obtained for wortmannin (inhibitor of the PI3K pathway; Fig. 4*B*) and for PD-98059 (inhibitor of the MAP kinase pathway) (Fig. 4*C*).

CIT-induced stimulation was completely inhibited in the presence of the inhibitors, demonstrating that the activation of

Table 3. *Comparison of passages from control to citrulline and from citrulline to citrulline with inhibitors*

	$\alpha\beta^R$	$\alpha\beta^P$	$\alpha\beta^W$
	CIT + INH of mTORC1	CIT + INH of MAPK kinase	CIT + INH of PI3K
Healthy rats	0.92 (0.24, 1.53)	1.26 (0.75, 1.83)	0.51 (0.06,0.92)
Malnourished rats	0.98 (0.13, 2.20)	1.03 (0.20, 2.10)	1.04 (0.12, 2.35)

αβ-product of each inhibitor is defined as follows: R, rapamycin; P, PD-98059; W, wortmannin; CIT, citrulline; INH, inhibitor; mTORC1, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase. The parameter-α corresponds to the passage Ctrl− to CIT and parameter-β to the passage CIT to CIT + INH. Results are expressed as posterior means of αβ product slopes and a 95% posterior credibility interval. When one is included in the interval, then the assumption $\alpha = 1/\beta$ is not rejected [Bayesian approach (model 1 without constraint) as described above].

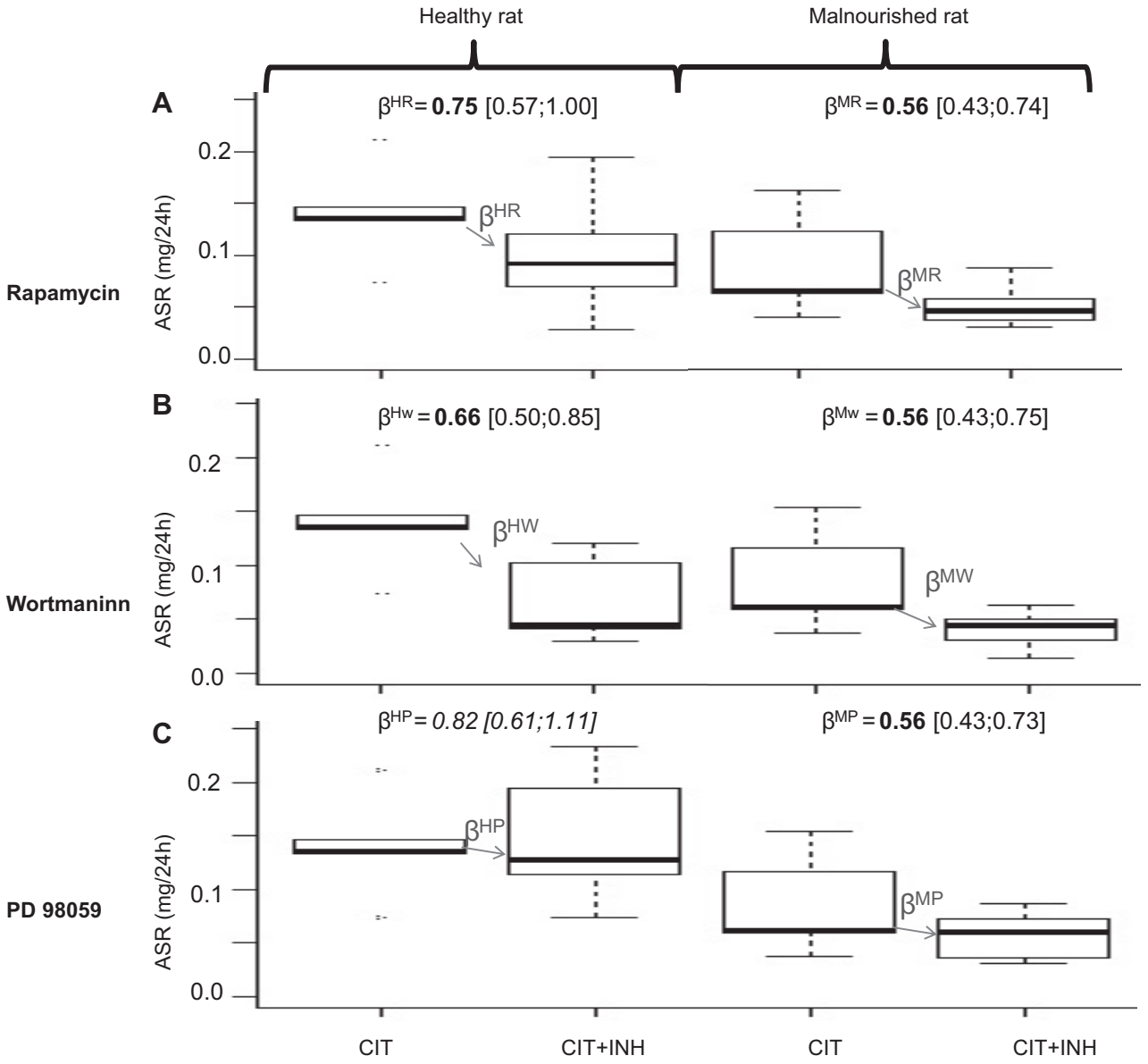

Fig. 4. Selective inhibitors totally blunted CIT effect in incubated epitrochlearis muscles. Epitrochlearis muscles of healthy and malnourished rats were incubated with CIT (2.5 mM) for 120 min with selective inhibitor (CIT + INH) and without selective inhibitor (CIT). Synthesis rates were estimated using rate of L-[*ring*-^{13}C]phenylalanine incorporation into proteins. Box plot charting the distribution of ASR in epitochlearis muscle of healthy and malnourished rats incubated with and without rapamycin (20 nM; *A*), with and without wortmannin (200 nM; *B*), or with and without PD-98059 (20 μM; *C*). ASR is expressed in mg/24 h. As described above, the effects were analyzed by a Bayesian approach (model 2 with constraint where $\beta = 1/\alpha$). Parameter-β is expressed as posterior means of slopes and a 95% posterior credibility interval, and it is significantly different from whether one is excluded from the interval. H, healthy; M, malnourished; R, rapamycin; W, wortmannin; P, PD-98059.

these pathways was necessary for the CIT-induced stimulation of muscle protein synthesis.

CIT activated mTORC1 pathway in culture of primary myotubes. To investigate the effect of CIT on the mTORC1 pathway, we used culture of primary muscle cells (Fig. 5). Thus serum- and amino acid-deprived myotubes were treated with 5 mM CIT for 120 min following a Western blot test to measure the phosphorylation status of 4E-BP1 and S6K1. Amino acid deprivation is associated with a decrease in mTORC1 activation (see Fig. 6). As shown in Fig. 5, CIT supplementation led to a 2.5-fold increase in the phosphorylation status of S6K1 (Fig. 5*A*) and a 1.5-fold increase in the phosphorylation status of 4E-BP1 (Fig. 5*B*) compared with untreated control. However, after 120 min of treatment of CIT, phosphorylation of 4E-BP1 at Thr$^{37/46}$ was unaffected (data not shown).

Effects of inhibitors on phosphorylation status of S6K1 and 4E-BP1. To explore the possible involvement of the mTORC1, PI3K/Akt, and MAP kinases/ERK1/2 pathways, cells were incubated with the specific inhibitors rapamycin, LY-294002, and PD-98059, respectively. We observed that both rapamycin and PD-98059 inhibited the CIT effect on S6K1 (Mann-Whitney, $P = 0.0039$ for both tests; Fig. 7, *A1* and *C1*). Only LY-294002 inhibited CIT effect on the two targets of mTORC1 (Mann-Whitney, $P = 0.0039$ and 0.0038; Fig. 7, *B1* and *B2*, respectively).

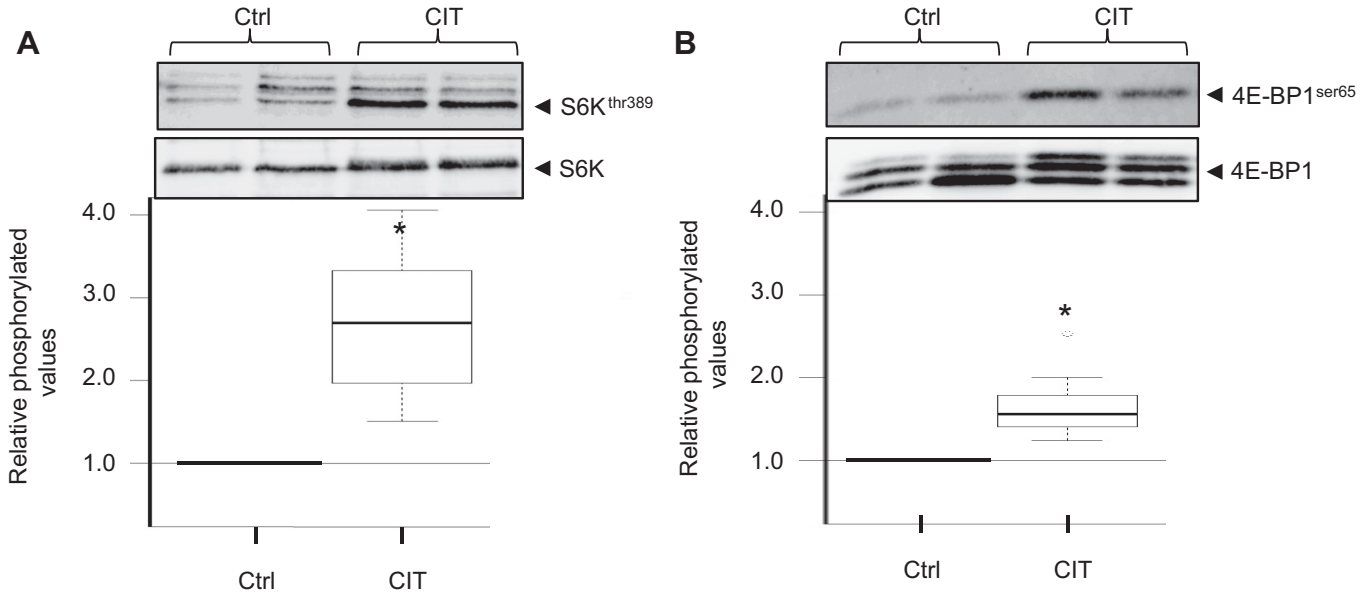

Fig. 5. CIT stimulated mTORC1 pathway in myotubes in culture. Primary myotubes were serum and amino acid deprived for 16 h and treated with CIT (5 mM) and without CIT (Ctrl) for 2 h. Phosphorylation status of mTORC1 targets (4E-BP1 and S6K1) was measured by Western blot. Box plot charting the distribution of relative phosphorylated values of S6K1 (*A*) and 4E-BP1 (*B*) in serum- and amino acid-deprived myotubes after 120 min of stimulation with CIT (5 mM). $*P < 0.05$ ($n = 12$) for nonparametric Wilcoxon test.

DISCUSSION

Citrulline has emerged as an important regulator of nitrogen homeostasis (6, 9). Over the last 10 yr, the ability of CIT to modulate protein metabolism has been shown in different situations (short bowel syndrome, fasting, aging, etc.) and in both animals and humans. However, the underlying mechanism of this action remains poorly understood. The aim of this study was to determine the precise mechanism by which CIT administration influences MPS.

The mTORC1 signaling pathway is considered as a nitrogen-sensing pathway regulating skeletal MPS (17) and the recent work by Guridi et al. (18) confirms that regulation of mTORC1 determines lean body mass. Experimental and clinical studies have shown that essential amino acids, particularly leucine, activate the mTORC1 pathway and consequently stimulate MPS (12).

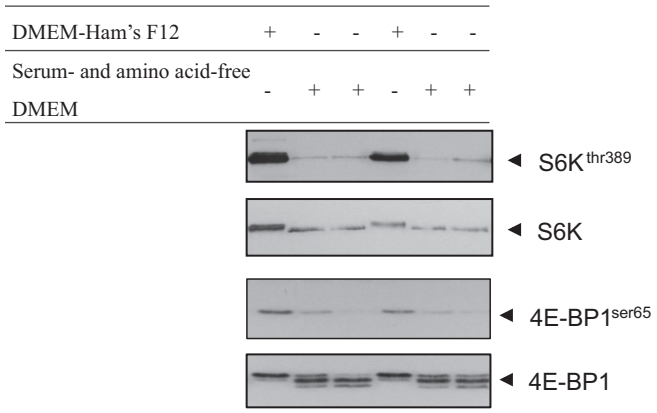

Fig. 6. Effect of amino acid deprivation on mTORC1 signaling pathway in myotubes in culture. Primary myotubes were serum and amino acid deprived for 16 h and treated without serum with a standard DMEM containing amino acids (DMEM-Ham's F-12) or with amino acid-free DMEM for 2 h. Phosphorylation status of mTORC1 targets (4E-BP1 and S6K1) was measured by Western blot.

In the present study, we show that CIT supplementation in malnourished aged rats is able to stimulate the mTORC1 pathway in the skeletal muscle. This experimental result is in line with those of others using a model of short fasting (22) but seems to conflict with those of Churchward-Venne et al. (7). These authors did not observe any effect of CIT on muscle protein synthesis or mTORC1 activation in elderly men. This discrepancy could be linked to marked differences between our work and the design of this study. First, Churchward-Venne et al. (7) compared the effect of a very high dose of protein (45 g of whey protein) or high dose of protein (15 g of protein) plus CIT. In these conditions, they were not exploring the effect of CIT but rather, how replacement of some protein by CIT would have the same effect (not the same question). Second, considering the effect on the mTORC1 pathway, they did not observe any effect of CIT (in combination with whey protein) between the two groups studied, but the authors did not evaluate mTORC1 activation in basal conditions, which does not allow any conclusion to be drawn about the possible effect of CIT. However, in vivo, the anabolic effect of CIT on muscle could be indirect and mediated by hormonal change, such as stimulation of insulin secretion. Insulin is known to stimulate MPS through activation of the PI3K/Akt pathway. However, this is unlikely because no effect of CIT was observed on insulin secretion in either animal (22) or human studies (20, 29). This hypothesis is supported at the transductional level, since we observed no CIT-related activation of Akt (the main insulin target). Taken together, these data show that the anabolic action of CIT is independent of insulin and the Akt pathway. Interestingly, we observed a positive correlation between plasma CIT concentration (but not muscle CIT concentration) and S6K1 phosphorylation. Similar results were obtained in a previous study (34) in a model of short bowel syndrome rats in

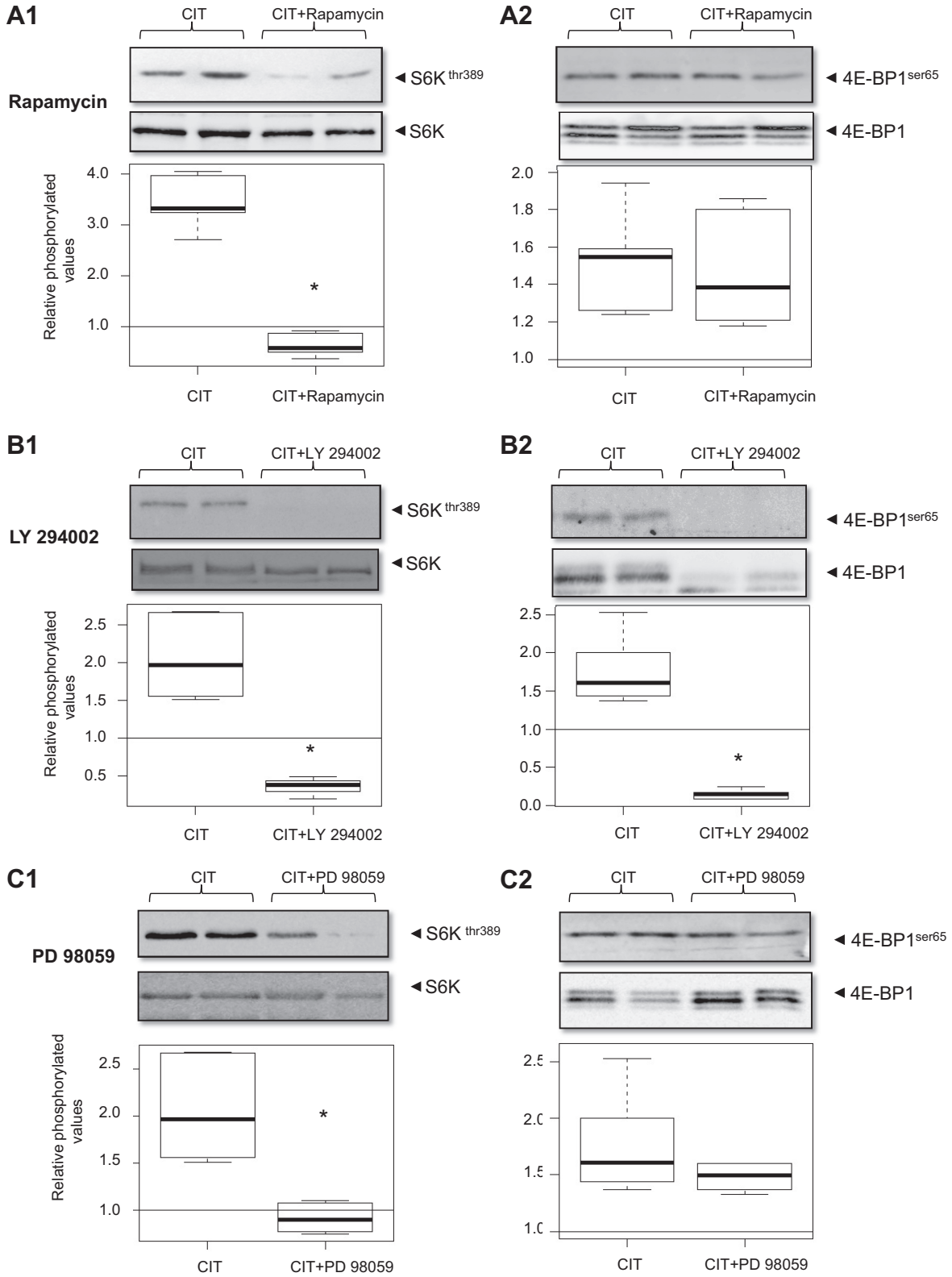

Fig. 7. Effect of selective inhibitors of CIT activation on mTORC1 in deprived myotubes. Primary myotubes were serum and amino acid deprived for 16 h and treated with CIT (5 mM), CIT + rapamycin (20 nM), CIT + LY-294002 (50 μM), and CIT + PD-98059 (10 μM) for 2 h. Phosphorylation status of mTORC1 targets (4E-BP1 and S6K1) was measured by Western blot. Box plot charting the distribution of relative phosphorylated values of S6K1 (*A1*, *B1*, and *C1*) and 4E-BP1 (*A2*, *B2*, and *C2*). *$P < 0.05$ for nonparametric Mann-Whitney test comparing CIT and CIT + INH.

which the increase in muscle weight was correlated with CIT concentration in plasma but not in muscle. These correlations suggest that the CIT effect could be direct on the muscle signaling pathway. However, measurement of MPS and phosphorylation state of targets of mTORC1 does not prove causality. In particular, CIT is subject to intense interorgan trafficking, generating glutamine, arginine, or other amino acids (8) that could be the true activation of MPS at the muscle level. For this reason, we used an ex vivo approach, enabling us to significantly improve our knowledge. As observed in vivo, CIT increases MPS in incubated epitrochlearis, suggesting a direct effect of this amino acid. In addition, the amino acid pattern was not affected by CIT in either the incubation medium or muscle. This result was expected because muscle does not possess the enzymatic equipment (i.e., argininosuccinate synthetase and argininosuccinate lyase) for the metabolization of CIT into arginine (39). A limitation of our work is that only CIT properties were explored. Thus, it would be of interest to compare CIT with its other metabolites (i.e., arginine, ornithine, or glutamine) to evaluate their respective effect.

Muscle is composed of several groups of proteins differing in their function: myofibrillar proteins (for contraction), mitochondrial proteins (for energy production), and sarcoplasmic proteins involved in numerous other functions (26). Interestingly, we demonstrate that CIT action did not affect all protein fractions. This result is in line with previous data (10) obtained in vivo indicating that CIT supplementation increases the expression of protein involved in muscle contraction. In this ex vivo study, we explored the possible involvement of the mTORC1 pathway with the use of metabolic inhibitors. Interestingly, pretreatment of incubated muscle with rapamycin clearly demonstrated that MPS activation by CIT is an mTORC1-dependent mechanism, confirming in vivo results. To better characterize the mechanism of action, the use of LY-294002 and PD-98059 supports the possible involvement of PI3K and MAP kinase in CIT action, since we observed that they were all efficient in suppressing CIT-induced increase in MPS. However, this model is not best suited to determining the precise relationship between MAP kinase, PI3K, and mTORC1. Also, muscle is a complex structure made up of different cell types, and our results based on the ex vivo model are not sufficient to assert that CIT directly affects MPS. For this reason, we used an in vitro approach. Consistent with our ex vivo studies, we report that CIT directly stimulates mTORC1 signaling via an overactivation of S6K1 and 4E-BP1 in myotubes. This last experiment confirms that CIT activates mTORC1 by a myotube autonomous system.

By characterizing this CIT effect at the molecular level, we show that rapamycin affects S6K1 but not 4E-BP1 phosphorylation status. This result is surprising since S6K1 and 4E-BP1 are downstream targets of mTORC1. However, this type of mTORC1-independent regulation of 4E-BP1 was recently described by others (41) and showed in a model of C_2C_{12} myoblasts that mTORC1 inhibition did not alter the phosphorylation state of 4E-BP1 (30). In particular, these authors showed that 4E-BP1 could be regulated by the PI3K/Akt pathway and S6K1 by both the PI3K/Akt and MAPK/ERK1/2 pathways. Such mechanisms would be consistent with our ex vivo observations and our in vivo observation (higher activation of 4E-BP1 than S6K1).

Thus PI3K inhibition abolishes the CIT action on 4E-BP1 and S6K1 and supports a CIT-mediated regulation of S6K1 and 4E-BP1 by this transduction pathway. To refine this result, the MAPK/ERK1/2 pathway was also studied (using PD-98059 as an inhibitor) because this pathway interacts with the PI3K/Akt pathway. Our results suggest that S6K1 activation is linked to both PI3K and MAPK/ERK1/2, whereas 4E-BP1 activation involves only PI3K.

Taken as a whole, our data suggest an mTORC1-dependent mechanism of CIT action on S6K1 involving MAPK/ERK1/2 and PI3K/Akt pathways. Whereas the relationship between PI3K and MAPK/ERK1/2 has been demonstrated (1, 25), their interrelationship is still not clear because it often depends on the cell type under study and on the experimental conditions.

Finally, our results show a relationship between the activation of PI3K/MAPK/4E-BP1 pathways by CIT and its action on MPS, because the inhibition of PI3K and MAP kinase profoundly disturbed the enhancing effect of CIT, confirming our in vitro observations.

In recent years, a new concept has emerged to explain some specific properties of amino acids, that of nutrient transceptors (36), i.e., amino acid transporters, which may regulate cellular trafficking and are also called amino acid transceptors (19). However, to date, very little is known about CIT membrane transporters (3), and further work will be necessary to confirm this last hypothesis.

To conclude, our combined data obtained in vivo, ex vivo, and in vitro clearly establish for the first time the role of CIT as an important signaling amino acid for the control of MPS.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

S.L.P., A.G., E.A., C.G., J.S., and M.J. performed experiments; S.L.P., A.G., A.S., J.S., and N.N. analyzed data; S.L.P., A.S., S.W., N.N., and C.M. interpreted results of experiments; S.L.P. prepared figures; S.L.P., L.C., and C.M. drafted manuscript; S.L.P., A.G., A.S., E.A., C.G., S.W., J.S., M.J., N.N., L.C., and C.M. edited and revised manuscript; A.G., A.S., C.G., S.W., J.S., M.J., N.N., L.C., and C.M. approved final version of manuscript

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