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Citrulline stimulates muscle protein synthesis, by reallocating ATP consumption to muscle protein synthesis

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Abstract

Background Animal studies and clinical data support the interest of citrulline as a promising therapeutic for sarcopenia. Citrulline is known to stimulate muscle protein synthesis, but how it affects energy metabolism to support the highly energy-dependent protein synthesis machinery is poorly understood.

Methods Here, we used myotubes derived from primary culture of mouse myoblasts to study the effect of citrulline on both energy metabolism and protein synthesis under different limiting conditions.

Results When serum/amino acid deficiency or energy stress (mild uncoupling) were applied, citrulline stimulated muscle protein synthesis by +22% and +11%, respectively. Importantly, this increase was not associated with enhanced energy status (ATP/ADP ratio) or mitochondrial respiration. We further analysed the share of mitochondrial respiration and thus of generated ATP allocated to different metabolic pathways by using specific inhibitors. Our results indicate that addition of citrulline allocated an increased share of mitochondrially generated ATP to the protein synthesis machinery under conditions of both serum/amino acid deficiency (+28%) and energy stress (+21%). This reallocation was not because of reduced ATP supply to DNA synthesis or activities of sodium and calcium cycling ion pumps.

Conclusions Under certain stress conditions, citrulline increases muscle protein synthesis by specifically reallocating mitochondrial fuel to the protein synthesis machinery. Because ATP/ADP ratios and thus Gibbs free energy of ATP hydrolysis remained globally constant, this reallocation may be linked to decreased activation energies of one or several ATP (and GTP)-consuming reactions involved in muscle protein synthesis.

Keywords Citrulline; Leucine; Energy metabolism; Protein metabolism; Mitochondria; Muscle

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Introduction

Citrulline (CIT) is a non-proteinogenic amino acid that has emerged as a major regulator of muscle protein synthesis and a promising therapeutic in sarcopenia challenge. In a pioneering work, Osowska *et al.*[1] demonstrated that CIT supplementation was associated with an increase of muscle protein synthesis in malnourished rats. Since then, several studies confirmed the positive effect of CIT on muscle protein synthesis, especially in the malnourished state.[2] Using a myotube *in vitro* model, Le Plénier *et al.*[3] confirmed a direct positive effect of CIT on protein synthesis in muscle

during amino acid/serum deficiency. They could link this effect to a stimulation of the mTORC1 pathway, a key activator of protein synthesis. Since also arginine (ARG) activates lysosome-located mTORC1 via a specific receptor (CASTOR1),[4–6] one may expect that conversion of CIT into ARG is involved in TORC1 activation. However, this hypothesis can be ruled out for two reasons: (i) *in vitro*[7] and *in vivo*[8] studies did not evidence any effect of ARG on muscle protein synthesis and (ii) CIT directly stimulates muscle protein synthesis in myotubes that do not quantitatively express argininosuccinate synthase nor argininosuccinate lyase (the two enzymes required for the transformation of CIT into ARG).[9]

Cellular protein synthesis has a very high energy requirement[10] in form of ATP and GTP, the latter generated from ATP by nucleoside diphosphate kinases. Thus, its activation by CIT should be supported by a corresponding up-regulation of energy metabolism. Indeed, there is indirect evidence to suggest that this is the case. CIT ingestion by healthy aged rats increased muscle mitochondrial density and TFAM (a mitochondrial transcription factor) expression.[11] CIT supplementation also increased energy expenditure in obese mice[12] and, as a malate salt, improved phosphocreatine regeneration after exercise in humans.[13] Moreover, CIT has been shown to affect lipid metabolism.[14–16]

Cellular ATP generation and consumption have to be constantly and closely matched to avoid energy deficits, because the cellular ATP pool itself has a very limited size that provides little reserve. A mismatch in this regulation can lead to limiting ATP supply that reduces the overall activity of cellular ATP-consuming pathways. The inhibitory effect of cell energetics can occur by reduced ATP turnover and, often but not always concurrent, by a decreased ATP/ADP ratio. The latter determines the usable Gibbs free energy effectively released during ATP hydrolysis. Under conditions where different ATP-consuming processes are in competition, both ATP turnover and ATP Gibbs free energy affect ATP allocation to these processes. For example, the pathways that require high ATP/ADP ratios tend to be inhibited first, thus making more ATP available for the pathways that can run at low ATP/ADP ratio. Thus, limiting ATP supply will not inhibit all pathways to the same degree, but rather in a hierarchical order.[17] As evidenced in thymocytes, protein synthesis and RNA/DNA synthesis fall rapidly as energy supply becomes limiting, while Na^+/K^+ pumping and Ca^{2+} cycling across the plasma membrane remain largely preserved.[10]

Here, we designed a study to delineate the role of energy metabolism in the beneficial effects of CIT on protein synthesis under different stress conditions. We report that protein synthesis decreases when ATP/ADP ratio decreases but that CIT can revert this inhibition by reallocating more respiration-generated ATP to the protein synthesis pathway, without affecting the global ATP/ADP ratio.

Materials and methods

Cell culture and treatments

Primary cultures were derived from *gastrocnemius* and *tibialis anterior* muscles of two 4-week-old male mice as previously described.[18] Experiments were performed on cells kept in culture for not more than 1 month (between 18–22 passages). Cells were plated at low density (100 cells/cm^2) on 0.02% gelatin-coated dishes (cold water fish skin; Sigma-Aldrich, Saint-Quentin-Fallavier, France) and grown in complete medium composed of DMEM-Ham's F12 (Gibco, Invitrogen, Saint-Aubin, France), 2% Ultroser G (Biosepra, Pall Corporation, Saint-Germain-en-Laye, France), 20% foetal 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). 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 F12 medium +2% horse serum (Sigma-Aldrich) at day 2. At day 5, myotubes were incubated either in amino acid-free and serum-free medium (Eurobio, Courtaboeuf, France) or in DMEM-Ham's F12 medium +2% horse serum for 16 h.

Myotubes were incubated under different conditions: (i) standard condition for 16 h with no further addition (Ctrl+), or addition of leucine (LEU, 5 mM), CIT (5 mM), or ARG (5 mM). (ii) Serum-deprived and amino acid-deprived for 16 h with no further addition (AA/$serum^-$), or addition of LEU (AA/$serum^-$ + LEU), CIT (AA/$serum^-$ + CIT), or ARG (AA/$serum^-$ + ARG). (iii) Standard condition for 16 h and addition of DNP, or further addition of LEU (DNP + LEU), CIT (DNP + CIT), or ARG (DNP + ARG). Before completion of the 16 h incubation period, the given amino acids (5 mM) were added for 90 min, and DNP (10 μM) for 15 min (i.e. 60 min after addition of a given amino acid). LEU was used as a positive control (as LEU is known to be a strong activator of muscle protein synthesis) and ARG was used as a negative control (as ARG is not known to stimulate muscle protein synthesis and allows to evaluate the nitrogen load effect).[7]

Myotube protein synthesis and immunoblotting

Myotube protein synthesis was quantified by the SUnSET method.[19] Briefly, after the different treatments, puromycin (1 μM) was added to the medium for 30 min. Cells were then collected, lysed in a RIPA buffer, and centrifuged at 3000 *g* for 10 min at 4°C, and the soluble protein fraction was collected. Immunoblotting of puromycin and analysis was then performed as previously described (quantification was made from the ratio puromycin-incorporated protein staining/Ponceau stain).[20] Total protein Ponceau staining of blots are illustrated in *Figure* S1.

Energy status

Adenine nucleotides were extracted from the myotubes in the presence of ice-cold 0.6 N perchloric acid +6.25 mM EDTA to precipitate proteins, centrifuged at 3000 *g* for 10 min at 4°C, neutralized to pH 6–7 with 2 N KOH + 0.3 M MOPS, and centrifuged again at 3000 g for 10 min at 4°C. [ATP] and [ADP] were then measured in the supernatant by HPLC to determine cell energy status as in.[21]

Mitochondrial respiration and glycolysis of myotubes (Seahorse)

Cellular respiration was measured in myotubes using the XF-96 analyser (Seahorse Bioscience, Billerica, MA) and based on published protocols.[22] The XF assay medium (HCO free modified DMEM, Seahorse Bioscience) was supplemented with 2 mM L-glutamine and 1 mM pyruvate and with further additions relevant to the experiment. The pH was adjusted to 7.4 at 37°C; 30 000 myoblasts/well were grown to confluence and differentiated into myotubes for 5 days in Seahorse assay plates as described earlier. Mitochondrial respiration was then measured under basal conditions followed by sequential additions of 1 μM oligomycin A (to inhibit the ATP synthesis) and 1 μM rotenone/antimycin A (to inhibit the respiratory chain). The following mitochondrial parameters were determined: basal respiration, basal mitochondrial respiration (basal cellular respiration minus non-mitochondrial respiration), ATP turnover-driven respiration (basal respiration minus oligomycin-inhibited respiration), resting respiration (oligomycin-inhibited respiration minus non-mitochondrial respiration), and non-mitochondrial respiration (rotenone/antimycin A-inhibited respiration). The results were expressed in pmol $O_2 \cdot min^{-1} \cdot well^{-1}$. Similarly, respiration was evaluated before and after the injection of specific inhibitors of protein synthesis (cycloheximide 40 μM), DNA/RNA synthesis (actinomycin D 10 μM), Na^+/K^+-ATPase pump activity (ouabain 380 μM), or Ca^{2+}-ATPase pump activity [lanthanum (III) chloride 2 mM]. To calculate the percentage of mitochondrial respiration allocated to each of these processes, the difference between mitochondrial respiration without and with specific inhibitor was divided by mitochondrial respiration without inhibitor (i.e. cycloheximide):

$$\begin{aligned} &\textit{trueem}\text{respiration for protein synthesis } (\%) \\ &= 100 \times \frac{(\text{total basal respiration } - \text{respiration with inhibitor})}{\text{total basal respiration}} \end{aligned}$$

Fatty acid oxidation in myotubes was determined in a Seahorse analyser according to the supplier's instructions.

Aerobic glycolysis in myotubes was also determined in a Seahorse analyser according to the supplier's instructions.

Interaction of citrulline and arginine with phosphate

^{31}P NMR (phosphorous nuclear magnetic resonance) spectra of samples containing phosphate (2 mM) and various amounts of either CIT or ARG (0 to 50 mM) in HEPES buffer (100 mM, pH 7.4) were registered on a Bruker Advance 400 spectrometer (^{31}P frequency 162 MHz) equipped with a QNP probe at 298 K. Deuterium oxide (10% vol.) was added to the samples as a lock. Spectra were acquired with no proton decoupling, 16 k acquisition points, a spectral window of 40 ppm, and a resolution of 0.81 Hz/point.

AMP-activated protein kinase activation

AMP-activated protein kinase (AMPK) activity was estimated by determining the phosphorylation status of acetyl-CoA carboxylase (ACC), a direct AMPK target. Myotubes were collected and lysed in a RIPA buffer and centrifuged at 3000 g for 10 min at 4°C. Soluble protein fraction was collected. Immunoblots were carried out as earlier, using mouse anti-phospho-ACC (Ser79) or anti-ACC antibodies (Santa Cruz, Heidelberg, Germany), and visualization of blots by exposure on film using ECL Plus reagent (Amersham).

Preparation of isolated mitochondria and permeabilized myofibres

Isolated mitochondria were used instead of myotubes, because the latter contain too few mitochondria for the more detailed analysis of mitochondrial respiration. Rat gastrocnemius muscles obtained from of both legs of 3-month-old Wistar male rats were collected and transferred into cold buffer containing sucrose 150 mM, KCl 75 mM, Tris 50 mM, KH2PO4 1 mM, MgCl2 5 mM, EGTA 1 mM, and lipid-free serum albumin 0.2%, pH 7.4 was used for mitochondrial extraction according to Fontaine *et al*..[23] Permeabilized skeletal muscle fibres were prepared from Wistar rat *plantaris* muscle according to Kuznetsov *et al*..[24]

Mitochondrial respiration (oxygen electrode) and complex I/II activities

The oxygen consumption rate of permeabilized myofibres and isolated mitochondria was measured using a Clark-type O_2 electrode (Oxygraph, Hansatech Instruments, Norfolk, United Kingdom). Mitochondria (0.2 mg/mL) were incubated at 37°C in a respiration buffer containing 125 mM KCl, 5 mM P_i, 20 mM Tris·HCl, 0.1 mM EGTA, and 0.1% fat-free BSA (pH 7.2). The suspension was stirred constantly with a built-in electromagnetic stirrer and stir bar. Mitochondria were

energized with either glutamate-malate (5 mM/2.5 mM) or succinate (5 mM), and oxygen consumption was recorded before and after adding 1 mM ADP. Oligomycin (1.25 μg/mL) was then added in order to measure the oxygen consumption in the absence of ATP synthesis. When succinate was used, 1 μM rotenone was added in the respiratory chamber in order to inhibit complex I activity. After addition of the substrates (glutamate-malate at 5 and 2.5 mM; succinate at 5 mM), maximal oxygen consumption rate (V_{max}) was measured under ADP stimulation (1 mM). Adding oligomycin then allowed to measure ATP synthase-independent oxygen consumption. After each measurement, fibre bundles were carefully removed, dried overnight at room temperature, heated at 100°C for 10 min, and weighed. Myofibre respiration rates were expressed as nmol $O \cdot min^{-1} \cdot mg\ dry\ wt^{-1}$, and mitochondrial respiration rates were expressed as nmol $O \cdot min^{-1} \cdot mg\ protein^{-1}$. Yield of oxidative phosphorylation (P/O) was measured using isolated mitochondria as described in Cano *et al*..[25] Briefly, ATP production was measured by HPLC and divided by the corresponding oxygen consumption.

Fluorescence measurement of mitochondrial membrane potential changes compared with mitochondrial respiration was performed in isolated mitochondria (*Figure* S2). Wastes (proton leak and/or redox slipping) were thus measured according to membrane potential as in Nicholls.[22] Finally, activities of complex I and II in muscle were determined as described in Mourmoura *et al*..[26] Enzymatic activities were expressed as $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$.

Statistics

All results are presented as means ± SEM. For cell measurements, statistics were performed to test differences between culture conditions (i.e. *Ctrl+*, *AA/serum*$^-$, and *DNP*) and between groups in the same culture conditions (e.g. *Ctrl+*, *LEU*, *CIT*, and *ARG*). The statistical significance of differences was analysed using a one-way ANOVA + Bonferroni test when three groups or more were compared or a Student's *t*-test when only two groups were compared. Values were considered significantly different at $P < 0.05$.

Results

Citrulline increases protein synthesis under stressed conditions without changing respiration

The effect of CIT (5 mM) on cellular protein synthesis in myotubes was assessed by comparison with LEU (5 mM) as positive control and ARG (5 mM) as negative control, with amino acids added for 90 min at the end of 16 h of culture. In muscle, LEU is known to stimulate protein synthesis, whereas ARG does not.[17]

Under our unstressed baseline conditions, LEU addition stimulated protein synthesis (+9%, LEU vs. Ctrl+, $P < 0.05$) whereas ARG and CIT did not (*Figure* 1, left panel). We then applied amino acid/serum deficiency (AA/serum$^-$) for 16 h, which decreased protein synthesis (−29%, AA/serum$^-$ vs. Ctrl+, $P < 0.05$). Under these stressed conditions, addition of both LEU and CIT stimulated cellular protein synthesis (+18%, AA/serum$^-$ + LEU vs. AA/serum$^-$, $P < 0.05$, +22%, AA/serum$^-$ + CIT- vs. AA/serum$^-$, $P < 0.05$, respectively), whereas ARG remained ineffective (*Figure* 1, middle panel). The same AA/serum$^-$ conditions decreased the ATP/ADP ratio by 13% (AA/serum$^-$ vs. Ctrl+, $P < 0.05$), and this decrease remained unaffected by the addition of LEU, CIT, or ARG (*Figure* 2, left and middle panels).

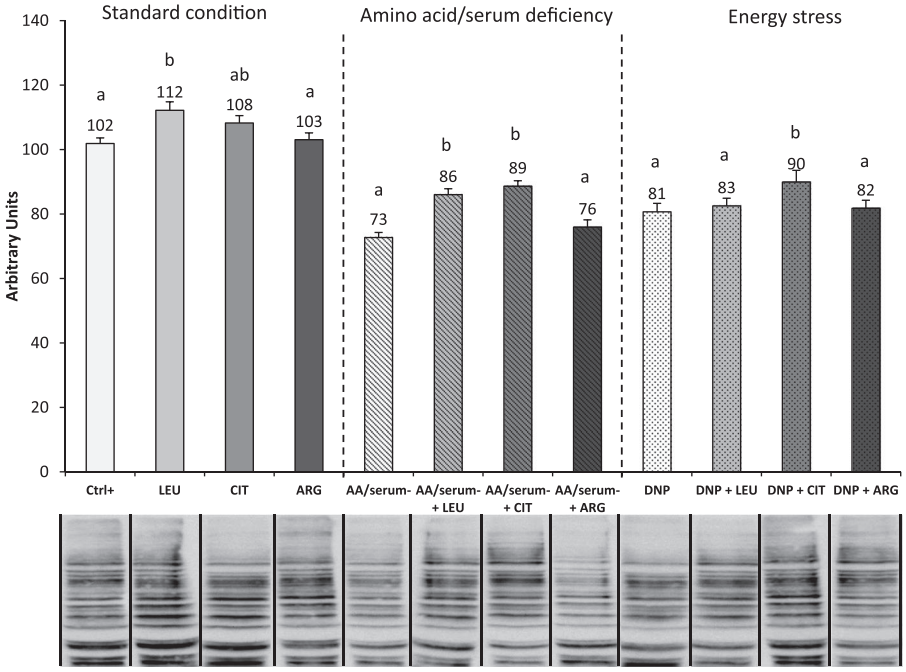
Next, we directly reduced cellular energy state by mildly uncoupling mitochondria. A concentration of the mitochondrial uncoupler DNP was used that led to a similar decrease in ATP/ADP ratio as that obtained under AA/serum$^-$ conditions (*Figure* 2, right panel). Such mild uncoupling also decreased protein synthesis (−21%, DNP vs. Ctrl+, $P < 0.05$) (*Figure* 1, right panel). Under this condition, CIT again stimulated protein synthesis (+11%, DNP + CIT vs. DNP, $P < 0.05$), whereas LEU and ARG addition failed to do so. Importantly, once again, the ATP/ADP ratio was decreased by uncoupling and remained unaffected by the addition of LEU, CIT, or ARG (*Figure* 2, right panel). Thus, energy requirements of CIT-induced protein synthesis are not covered by increased ATP/ADP ratios.

We then asked how mTORC1, the key regulator of protein synthesis, affects protein synthesis and energy state of myotubes. Under standard culture conditions, mTORC1 inhibition by rapamycin (200 nM) decreased protein synthesis (*Figure* S3) but had no effect on ATP/ADP energy status (*Figure* S4). This confirms that, also in myotubes, any regulation of energy state is upstream of mTOR. One of the key signalling hubs upstream of mTOR is AMPK, which senses the availability of cellular energy, in particular ATP/ADP and ATP/AMP ratios. We thus measured phosphorylation of the AMPK substrate ACC as a readout for AMPK activation. Consistent with our data on ATP/ADP ratios (*Figure* 2), AA/serum$^-$ conditions increased ACC phosphorylation status (+33%, AA/serum$^-$ vs. Ctrl+, $P < 0.05$), and CIT (or ARG) addition did not alter this effect (*Figure* S5). An important conclusion of this result is that the CIT effects on protein synthesis are not mediated by the AMPK signalling axis.

Citrulline and arginine do not interact with Pi to alter Gibbs free energy of ATP hydrolysis

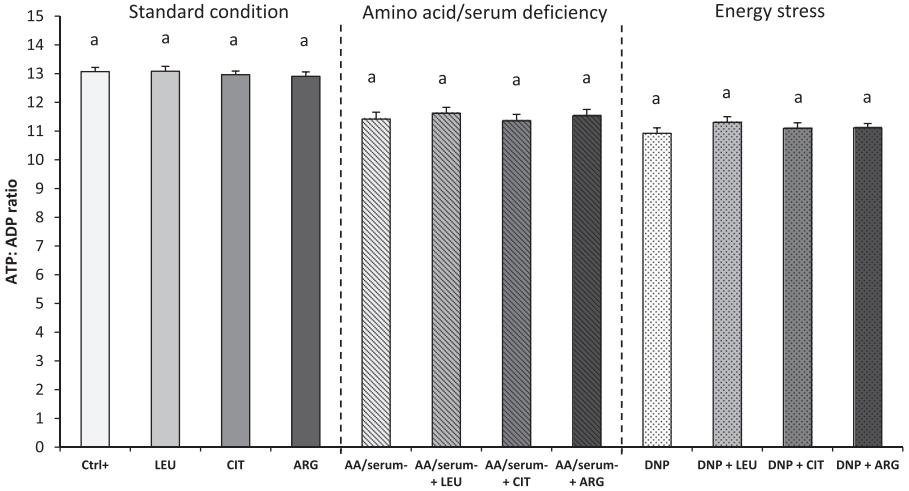
Next, because the Gibbs free energy of ATP depends on both ATP/ADP ratio and concentration of free Pi, we measured

Figure 1 Myotube protein synthesis evaluated by puromycin-incorporated protein staining in western blots. Data were calculated as the ratio of puromycin-incorporated protein immunostaining and total protein Ponceau staining (*Figure* S1). Concentration of amino acids addition (LEU, CIT, and ARG) were 5 mM. Results are presented as means ± SEM (n = 6). Significant differences within each culture condition (standard condition, amino acid/serum deficiency, and energy stress) are presented. Values with different letters in the same culture condition are statistically different ($P < 0.05$; one-way ANOVA and Bonferroni test). Blots presented are not necessarily from continuous lanes and have been chosen with similar protein loading (measured by Ponceau staining) to illustrate the differences between groups in the best way. ARG, arginine; CIT, citrulline; LEU, leucine.



whether CIT could interact somehow with Pi. The phosphorus chemical shift (δ) of phosphate (2 mM) did not show any significant change when adding CIT at pH 7.4, even in a 25-fold excess of CIT, indicating no interaction: $\delta_{(CIT\ 50\ mM)} - \delta_{(CIT\ 0\ mM)} = 0.01$ ppm for a resolution of 0.005 ppm/point. For comparison, ARG studied in the same conditions induced a slight change in δ, suggesting a slight interaction of the phosphate anion with the guanidinium function of ARG: $\delta_{(ARG\ 50\ mM)} -$

Figure 2 Myotube energy status as determined by ATP:ADP ratio measured by HPLC. Results are presented as means ± SEM (n = 6). Significant differences within each culture condition (standard condition, amino acid/serum deficiency, and energy stress) are presented. Values with different letters in the same culture condition are statistically different ($P < 0.05$; one-way ANOVA + Bonferroni test). ARG, arginine; CIT, citrulline; LEU, leucine.



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$\delta_{(ARG\ 0\ mM)}$ = 0.11 ppm. However, 50 mM ARG is a supraphysiological concentration, and there was no interaction at lower concentrations. The stability of the ARG-phosphate electrostatic interaction, that is proposed to be H-bond-driven, was indeed demonstrated to be large.[27]

Citrulline can reallocate respiration to protein synthesis under stress conditions

Given that ATP/ADP ratios, that is, the energy state, are altered by culture conditions, but not CIT addition, we determined overall basal respiration. Interestingly, whatever the culture conditions (standard, AA/serum$^-$, or energy stress/mild uncoupling), basal respiration remained unchanged and was neither affected by the addition of LEU, CIT, or ARG (*Table* 1, *Figure* 2). Thus, both basal respiration and ATP/ADP ratios cannot explain how more energy is provided to CIT-induced protein synthesis. We therefore wanted to know whether it may be the share of respiratory ATP generation that is allocated to protein synthesis that is changing with culture conditions. We thus repeated respiration experiments under AA/serum$^-$ and energy stress conditions while inhibiting protein synthesis (*Table* 1) or other major ATP-consuming pathways (*Table* 2). Indeed, respiration allocated to protein synthesis was significantly decreased by AA/serum$^-$ conditions (36% vs. 41%, AA/serum$^-$ vs. Ctrl+, $P < 0.05$), as well as by mild uncoupling (34% vs. 41%, DNP vs. Ctrl+, $P < 0.05$). Importantly, addition of LEU or CIT (but not ARG) normalized the percentage of the respiration allocated to protein synthesis (*Table* 1), consistent with the increased protein synthesis in myotubes under these conditions (*Figure* 1). In contrast, the respiration allocated to DNA synthesis and Na^+/K^+-ATPase or Ca^{2+}-ATPase pumps was not significantly affected under AA/serum$^-$ conditions (*Table* 2). Thus, the cellular activity receiving an increased share of cellular respiration under starved conditions, and which is reduced to normal levels by CIT, corresponds to a yet unidentified ATP consumer.

Beneficial citrulline effects do not involve direct effects on major bioenergetics pathways

Finally, we verified whether CIT has direct effects on some key metabolic pathways. Activities of the isolated mitochondrial complexes (complex I and II) were not modified by addition of 5 mM CIT (*Table* S1). In isolated skeletal muscle mitochondria, addition of 5 mM CIT had no effect on respiration, neither during ATP synthesis (state III) nor after ATP synthesis had been inhibited by oligomycin (state IV). Moreover, CIT did not affect oxidative phosphorylation efficiency (as assessed by P/O ratio). Similar results on respiration were observed using permeabilized fibres from Wistar rat

Table 1 In this table, some data are in 1 lines whereas the first columns are on 2 lines. Please increase the cell's size (clomuns Ctrl and +LEU, +CIT and +ARG) to be one 1 linesMyotube basal respiration and respiration allocated to protein synthesis evaluated in different culture conditions (standard condition, amino acid/serum deficiency, and energy stress) ± specific amino acid addition [CIT, LEU, or ARG (5 mM)]

Mitochondrial respiration (pmol $O_2 \cdot min^{-1} \cdot well^{-1}$)	Ctrl	+LEU	+CIT	+ARG	AA/serum$^-$	AA/serum$^-$ + LEU	AA/serum$^-$ + CIT	AA/serum$^-$ + ARG	DNP	DNP + LEU	DNP + CIT	DNP + ARG
Total basal	$73^a \pm 3$	$74^a \pm 3$	$73^a \pm 3$	$73^a \pm 2$	$72^a \pm 3$	$73^a \pm 3$	$72^a \pm 3$	$71^a \pm 2$	$77^a \pm 2$	$77^a \pm 3$	$74^a \pm 3$	76 ± 3^a
With inhibition of protein synthesis	$43^a \pm 2$	$43^a \pm 2$	$42^a \pm 2$	$44^a \pm 2$	$46^a \pm 2$	$43^{ab} \pm 2$	$39^b \pm 2$	$44^a \pm 1$	$51^a \pm 2$	$47^a \pm 3$	$44^a \pm 3$	$48^a \pm 3$
Allocated to protein synthesis (%)†	$41^a \pm 1$	$42^a \pm 1$	$43^a \pm 1$	$39^a \pm 2$	$36^a \pm 1$	$41^b \pm 1$	$46^b \pm 2$	$37^a \pm 1$	$34^a \pm 1$	$39^{bc} \pm 1$	$41^c \pm 2$	$36^{ab} \pm 1$

Results are presented as means ± SEM ($n = 8$). Significant differences between culture condition (standard condition, amino acid/serum deficiency, and energy stress) are presented. Values with different superscript letters within the same culture condition are statistically different ($P < 0.05$; one-way ANOVA and Bonferroni test). ARG, arginine; CIT, citrulline; LEU, leucine.
†Respiration because of inhibited protein synthesis, calculated as the difference between mitochondrial respiration without (line 1) and with specific inhibitor (line 2), divided by respiration without inhibitor (line 1), and expressed as percentage. See also Materials and Methods.

Table 2 Energy allocation to protein synthesis or other important energy-expenditure processes

Respiration allocated to (%)†	Ctrl	AA/serum$^-$	AA/serum$^-$ + CIT
Protein synthesis	41^a ± 1	36^b ± 1	46^a ± 2
DNA synthesis	16^a ± 1	13^a ± 1	14^a ± 1
Na^+/K^+ ATPase pump activity	17^a ± 1	15^a ± 1	15^a ± 1
Ca^{2+} ATPase pump activity	19^a ± 3	15^a ± 3	15^a ± 1
Other energy-expenditure processes	7	21	10

Results are presented as means ± SEM (n = 8). Values with different superscript letters are statistically different ($P < 0.05$; one-way ANOVA and Bonferroni test). CIT, citrulline.
†Respiration because of the inhibited process, calculated as the difference between mitochondrial respiration without (line 1) and with specific inhibitor (line 2), divided by respiration without inhibitor (line 1), and expressed as percentage. See also Materials and Methods.

plantaris muscle (*Table* S2). Because the yield of oxidative phosphorylation *in vivo* depends on the substrate used (it is lower with lipids than with carbohydrates), CIT could increase oxidative phosphorylation efficiency by decreasing β-oxidation. However, we observed the opposite under AA/serum$^-$ conditions, where respiration linked to β-oxidation increased from 12.1% to 16.2% ($P < 0.05$) in the presence of CIT (*Table* S3). Note that contrary to CIT, ARG had no effect on respiration linked to β-oxidation. Finally, we could neither observe an effect of CIT on basal aerobic glycolysis and maximum aerobic glycolysis capacity measured in myotubes (*Table* S3).

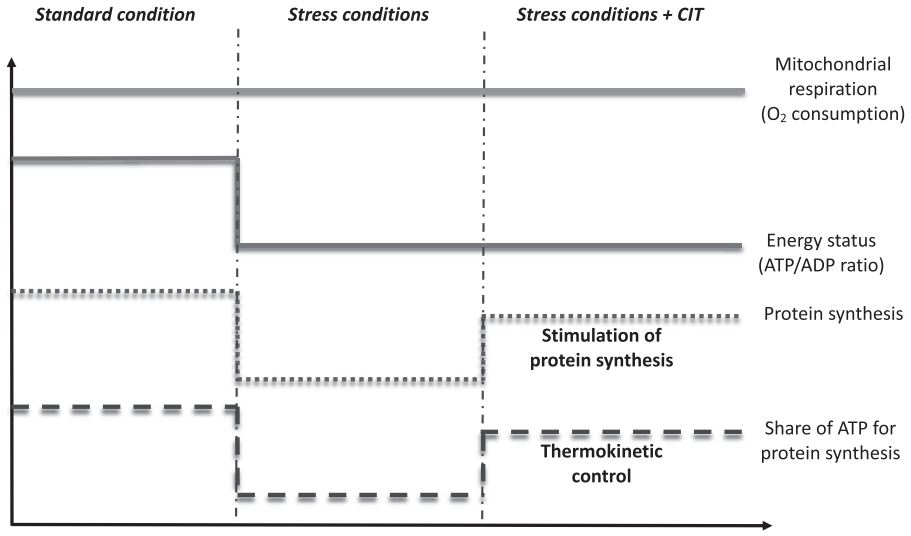
Discussion

Stress imposed to mouse myotubes by amino acid/serum deficiency or mild mitochondrial uncoupling decreased global ATP/ADP ratios and protein synthesis, without altering oxygen consumption. Addition of CIT (5 mM) under these conditions increased protein synthesis, but this was not supported by a concomitant change in bioenergetic parameters (oxygen consumption, ATP/ADP ratio, and ATP Gibbs free energy), bioenergetic pathways (oxidative phosphorylation efficiency and aerobic glycolysis), or energy-related cell signalling (AMPK activation). In contrast, addition of CIT reallocated oxygen consumption to protein synthesis, which had been deviated upon stress induction to some other, yet unidentified ATP consumer(s). These key results are summarized in *Figure* 3.

Less protein synthesis (*Figure* 1) for the same oxygen consumption (*Table* 1) suggests that amino acid/serum deficiency or mild uncoupling either stimulated ATP-consuming metabolic pathways other than protein synthesis or increased futile processes. Among the ATP-consuming processes, serum deprivation did not stimulate DNA synthesis, Na^+/K^+-ATPase pump activity or Ca^{2+}-ATPase pump activity (*Table* 2). However, cycloheximide might not inhibit all ATP-consuming steps in protein synthesis,[28] thus part of the 'respiration allocated to other energy expenditure' (*Table* 2) could still be related to protein synthesis, for example, upstream of the step inhibited by cycloheximide.

Nutrients such as amino acids not only serve for protein synthesis but also have important regulatory properties.

Figure 3 Summary of main results. CIT, citrulline.



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Among them, CIT is well known to stimulate muscle protein synthesis via an activation of the mTORC1 pathway.[1,3,29,30] However, the mechanism by which CIT provides the necessary energy for this process is not fully understood. Interestingly, most of the studies showing a positive effect of CIT on muscle protein synthesis observed this under conditions of protein/energy deficiency.[1,3,31] We observed a similar pattern here, with CIT-stimulated protein synthesis exclusively detected after amino acid/serum deprivation or mild uncoupling, when ATP/ADP ratios were decreased, but not in standard conditions (*Figure* 1). CIT stimulated protein synthesis while ATP/ADP ratios, respiration, and AMPK activation remained unchanged. This was not because of direct interaction of CIT with Pi, which could alter the Gibbs free energy of ATP hydrolysis. CIT did neither affect cellular ATP production nor increase cellular oxygen consumption, oxidative phosphorylation efficiency, or aerobic glycolysis. However, CIT may affect substrate utilization, because it stimulated fatty acid β-oxidation in myotubes, but this would not improve oxidative phosphorylation efficiency. This same stimulation was also reported for adipose tissue,[14,15] but the mechanisms involved remain unknown. Taken together, these observations lead us to propose that CIT may stimulate protein synthesis by decreasing the activation energy of one or several ATP-consuming (and GTP-consuming) reaction(s) involved in protein synthesis. This would occur under both standard and stressed conditions. However, only in the latter it would produce an effect on protein synthesis because the CIT-regulated enzyme(s) are no longer working at their V_{max}. At this stage, we do not know whether these enzymes regulate or just participate in protein synthesis.

The consensus view that amino acid availability is the major limiting step in protein synthesis has prompted the idea that CIT may stimulate protein synthesis after protein/energy deficiency by acting as a nitrogen source. However, this hypothesis can be ruled out, because ARG (the only known metabolite of CIT) did not stimulate protein synthesis in the relevant incubation condition, whereas CIT stimulated protein synthesis after mild uncoupling (a condition in which all the amino acids are present). Interestingly, LEU and CIT had some effects in common but differed in an important property. Both increased the percentage of mitochondrial respiration (i.e. ATP consumption) allocated to protein synthesis under both, amino acid deficiency and energy stress, and stimulated protein synthesis after amino acid deficiency. However, only CIT stimulated protein synthesis also after mild uncoupling, suggesting that CIT and LEU use different mechanisms to regulate protein metabolism. While the effects of CIT could be explained by a global mechanism linked to decreased energy status, as discussed earlier, the effect of LEU is specific to amino acid deficiency and likely involves dedicated signalling pathways not further analysed here.

This work adds decisive evidence to the earlier ideas of a hierarchical arrangement of ATP-consuming processes,[10,17] with protein synthesis being very sensitive to a decrease in ATP/ADP ratio. While many of these bioenergetic effects can be explained today by signalling pathways like those linked to AMPK,[32] the effects of CIT that we observed were not accompanied by any change in ATP/ADP ratio, respiration, or AMPK activation. However, in absence of any change in total ATP availability, the share allocated to protein synthesis was increased. We propose that it is the hierarchy of ATP allocation to different ATP-consuming processes that is altered, for example, by a decrease in the activation energy of one or several enzymatic step(s) involved in or controlling protein synthesis. More generally, this kind of thermokinetic control could explain why regulators are effective or ineffective at controlling a given metabolic step, depending on the energy status.

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Author contributions

A.G., E.F., and C.M. contributed to the conceptualization. A. G., E.F., and C.M. contributed to the methodology. A.G. and F.L. contributed to the investigation. A.G., E.F., and C.M. wrote the original draft. A.G., F.L., U.S., E.F., and C.M. contributed to writing review and editing. S.B. and P.D. contributed to the resources. A.G., E.F., and C.M. contributed to the supervision.

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Total protein ponceau staining of Western blots performed to measure the protein synthesis by puromycin incorporation in neosynthesized proteins.
Figure S2. Fluorescence measurement of mitochondrial membrane potential changes compared with mitochondrial

respiration (Nicholl's curve). Results (n = 6) are presented as means ± SEM.

Figure S3. Myotubes protein synthesis without or with mTORC1 inhibition evaluated by puromycin-incorporated protein staining in Western Blot. Results (n = 6) are presented as means ± SEM. Values with different superscript letters are statistically different ($p < 0.05$; Student t-test).

Figure S4. Myotubes energy status without or with mTORC1 inhibition determined by the ratio ATP: ADP measured by HPLC. Results (n = 6) are presented as means ± SEM. Values with different superscript letters are statistically different ($p < 0.05$; Student t-test).

Figure S5. Phosphorylation status of ACC determined by Western Blotting. Results (n = 6) are presented as means ± SEM. Values with different superscript letters are statistically different ($p < 0.05$; One-way Anova + Bonferroni test).

Table S1. Direct effect of citrulline (5 mM) on mitochondrial function

Table S2. Direct effect of citrulline (5 mM) on mitochondrial respiration in permeabilized muscle fibers

Table S3. Effects of citrulline or arginine (5 mM) on anaerobic glycolysis and β-oxidation in amino acid/serum deficiency

Conflict of interest

C.M. is a shareholder in Citrage. None of the other authors have any conflict of interest to report.

Ethical guidelines

The authors certify that they comply with the ethical guidelines for publishing in the Journal of Cachexia, Sarcopenia and Muscle: update 2017.[33]

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