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Orjeta Kuci, Eliane Archambault, Agnes Dodacki, Esther Nubret, Jean-Pascal de Bandt, Luc Cynober

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## **Effect of citrulline on muscle protein turnover in an *in vitro* model of muscle catabolism**

**Kuci O.<sup>1</sup>, Archambault E.<sup>1</sup>, Dodacki A.<sup>2</sup>, Nubret E.<sup>1</sup>, De Bandt J.-P.<sup>1,3</sup> and Cynober L.<sup>1,3</sup>.**

<sup>1</sup>Laboratoire de Biologie de la Nutrition, EA 4466 PRETRAM, Université Paris Descartes, Sorbonne Paris Cité, <sup>2</sup>Variabilité de Réponse aux Psychotropes, UMR-s 1144, Université Paris Descartes, Sorbonne Paris Cité, <sup>3</sup>Service de Biochimie, Hôpital Cochin, HUPC, AP-HP, PARIS, France

**Corresponding author:** Prof L. Cynober  
Laboratoire de Biologie de la Nutrition EA 4466, Faculté de Pharmacie,  
4 avenue de l'Observatoire,  
75270 Paris Cedex 06, France.  
Phone: +33 1 53 73 99 47 Fax: +33 1 53 73 99 52  
e-mail address: [luc.cynober@parisdescartes.fr](mailto:luc.cynober@parisdescartes.fr)

24 **Abstract**

25 Muscle net catabolism, as seen after severe trauma or sepsis or in post-operative situations, is  
26 mediated by hormones, such as cortisol, and pro-inflammatory cytokines, such as tumor necrosis  
27 factor alpha (TNF- $\alpha$ ). Specific amino acids (AAs) may be able to limit this muscle mass loss. Citrulline  
28 (Cit) stimulates muscle protein synthesis in various situations, but there are few data on  
29 hypercatabolic situations, and its effects on protein breakdown are unknown. Our aim was to assess  
30 the effect of Cit on protein turnover in an *in vitro* model of muscle hypercatabolism.

31 Myotubes derived from C2C12 myoblasts were treated with 150 nM dexamethasone (DEX), 10 ng/ml  
32 TNF- $\alpha$ , or 0.006% ethanol (as control, CON) for 24 h. Myotubes were then incubated with or without 5  
33 mM Cit for 6 h. Muscle protein synthesis rate (PSR) was evaluated by the surface sensing of translation  
34 (SUnSET) method and by L-[3,5- $^3$ H]tyrosine (Tyr) incorporation. Muscle protein breakdown rate (PBR)  
35 was evaluated from Tyr release into culture medium. Cit action was analyzed by non-parametric  
36 Kruskal-Wallis and Mann-Whitney tests.

37 Cit treatment significantly increased PSR compared with the DEX group or TNF- $\alpha$  group (SUnSET  
38 method; DEX+CIT vs. DEX,  $p = 0.03$  and TNF- $\alpha$ +CIT vs. TNF- $\alpha$ ,  $p = 0.05$ ), and significantly decreased PBR  
39 in the CON and DEX groups (CON+CIT vs. CON  $p = 0.05$  and DEX + Cit vs. DEX,  $p = 0.05$ ).

40 Cit treatment regulated muscle protein turnover in an *in vitro* model of muscle net catabolism. It  
41 would be of interest to explore the underlying mechanisms.

42 **Key words:** Protein turnover; Glucocorticoids; Cytokines; Muscle hypercatabolism; Citrulline.

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## 48 **Introduction**

49 Muscle wasting occurs in net catabolic situations such as heavy surgery, trauma and sepsis [1, 2]. It is a  
50 consequence of increased glucose metabolism in the metabolic response to injury: since glycogen  
51 stores are very limited, and our bodies are unable to convert lipids into carbohydrates, most of the  
52 glucose required for energy supply comes from amino acids (AAs) [3], which in turn come from  
53 proteins, especially muscle proteins. In a healthy state, maintenance of muscle mass depends on a  
54 dynamic balance between anabolic and catabolic reactions. Conversely, in stress situations, there is an  
55 imbalance, with a relative or absolute reduction in protein synthesis and/or increase in protein  
56 breakdown [4]. The mobilization of muscle AAs is mainly driven by catabolic hormones (e.g. cortisol)  
57 [5] and mediators such as tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-1, or interleukin-6 [6].  
58 Severe prolonged net catabolism is a factor of morbidity and mortality among injured patients, and an  
59 appropriate nutritional support for these patients may be essential to limit the protein losses leading  
60 to muscle wasting and loss of muscle function. However, the effects of standard enteral or parenteral  
61 nutrition are limited by what is termed “resistance to renutrition” [7, 8]. Attention has accordingly  
62 focused on targeted nutritional strategies using specific nutrients such as leucine (Leu), which has  
63 been shown to regulate protein synthesis [9]. However, results on the use of Leu in intensive care unit  
64 (ICU) patients are disappointing [10]. More recently, another AA, citrulline (Cit) was shown to  
65 modulate nitrogen homeostasis (see [9, 11] for recent reviews) and protein synthesis. Osowska *et al.*  
66 [12], in a model of short bowel syndrome, demonstrated that Cit supplementation of enteral feeding  
67 was associated with an 80% increase in muscle protein synthesis rate and a 20% net muscle protein  
68 gain relative to rats fed a standard diet. These results have since been confirmed in various  
69 experimental models, and appear to be dependent on the mTOR (mammalian/mechanistic target of  
70 rapamycin) signaling pathway [13, 14]. Furthermore, supplementation with Cit stimulated muscle  
71 protein synthesis in young adults fed a hypoproteic diet [15]. There are few data on the effects of Cit  
72 in net catabolic situations: besides the already mentioned study on short bowel syndrome [12], only

73 three studies have yet been published: Wijnands *et al.* [16], in a model of endotoxemia in mice,  
74 showed improved intestinal microcirculation in animals supplemented with Cit; Batista *et al.* [17]  
75 showed that Cit preserved the integrity of the intestinal barrier, and also decreased the production of  
76 pro-inflammatory cytokines, such as IFN $\gamma$ , and maintained that of anti-inflammatory cytokines such as  
77 IL-10; finally, *in vitro* in isolated myotubes, Ham *et al.* [18] showed that Cit maintained myotube  
78 protein synthesis in catabolic conditions induced by hydrogen peroxide or LPS. Whereas *in vivo* and *in*  
79 *vitro* studies yield evidence that Cit stimulates protein synthesis, no study has evaluated the effect of  
80 Cit on muscle protein breakdown. In addition, a large majority of the studies on protein synthesis were  
81 performed *in vivo*, which does not tell us whether the action is direct (i.e. Cit-mediated) or indirect  
82 (e.g. through Cit conversion into arginine) [19]. Finally, the one *in vitro* study [18] did not consider  
83 hormones and mediators, which directly control muscle turnover.

84 The literature data being scant, the aim of our study was to evaluate the effect of Cit on the balance  
85 between protein synthesis and breakdown in hypercatabolic situations by an *in vitro* approach.  
86 Myotubes derived from C2C12 myoblasts were treated with glucocorticoids (GCs, e.g. dexamethasone,  
87 DEX) or TNF- $\alpha$ . GCs play a major role in muscle wasting by decreasing protein synthesis while  
88 increasing protein breakdown [20]. The net catabolic effect of GCs results mainly from two  
89 mechanisms. First, they inhibit the transport of AAs into muscle cells, which could limit the synthesis  
90 by reduction in substrate availability. Second, they exert an inhibitory effect on key effectors (e.g. Akt)  
91 of the initiation of protein translation. In addition, the catabolic effect of GCs results from the  
92 activation of the major proteolytic systems such as the ubiquitin/proteasome system (MuRF1 and  
93 Atrogin-1) [21]. TNF- $\alpha$  is a pro-inflammatory cytokine stimulating muscle catabolism, inducing a  
94 contractile dysfunction in muscle cells [22], and potentiating cortisol/corticosterone action [23].

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## 96 **Materials and Methods**

97 Materials: C2C12 (CRL-1172), high glucose-DMEM (Dulbecco's modified Eagle medium); 0.6 g/l L-Gln,  
98 4.5 g/l D-glucose, 1 mM sodium pyruvate, and 1.5 g/l bicarbonate were purchased from LGC  
99 Standards-ATCC (Molsheim, France). Fetal calf serum, antibiotics, horse serum, cytosine arabinoside,  
100 dexamethasone, TNF- $\alpha$ , bovine serum albumin and L-tyrosine were from Sigma-Aldrich (Saint-  
101 Quentin-Fallavier, France). Citrulline was a gift from Citrage (Créteil, France). Trypsin-EDTA solution  
102 was from Fisher Bioreagent™ (Illkirch, France). Matrigel and PBS were purchased from Dominique  
103 Dutscher (Paris, France). Puromycin and anti-puromycin antibody (clone 12D10) were purchased from  
104 Merck Millipore (Yvelines, France). Lysis buffer was purchased from Roche (Meylan, France). DC  
105 protein assay kit and mini-protean TGX™ precast protein gels were from Bio-Rad Laboratories  
106 (Marnes-la-Coquette, France). Horseradish peroxidase-conjugated anti-mouse antibody was from  
107 Jackson ImmunoResearch Laboratories Inc. (Newmarket, Suffolk, UK), and L-[3,5-<sup>3</sup>H]tyrosine (40-60  
108 Ci/mmol), was from PerkinElmer Life Sciences (Boston, MA, USA).

### 109 Culture cells

110 The protocol used was that of Menconi *et al.* [24], and C2C12 mouse cells were used between P2 and  
111 P5. The model of using C2C12-derived myotubes as a model of catabolism by exposure to DEX or TNF  
112 is well established; see for example Kimura *et al.* [25] and Mirza *et al.* [26].

113 Multiplication: After thawing, 310,000 myoblasts were incubated in a T25 culture flask in 7 ml of  
114 multiplication medium [high glucose-DMEM supplemented with 10% fetal calf serum (FCS) and 1%  
115 antibiotics (10,000 U.ml<sup>-1</sup> of penicillin and 10 mg/ml of streptomycin)] for 4 days at 37 °C with a  
116 humidified atmosphere and 5% CO<sub>2</sub>.

117 Differentiation: At 85% confluence, myoblasts were collected after trypsin treatment (0.25% trypsin-  
118 0.53 mM ethylenediaminetetraacetic acid), and 350,000 myoblasts were seeded in six-well plates  
119 coated with Matrigel in 3 ml of multiplication medium for 24 h. The culture medium was then

120 removed and replaced by 3 ml of differentiation medium [high glucose-DMEM supplemented with 2%  
121 horse serum (HS) and 1% antibiotics]. The culture medium was changed every day during which time  
122 the change in shape of the C2C12 was observed under optic microscopy. After 7 days, the culture  
123 medium was changed and supplemented with 10  $\mu$ M cytosine arabinoside (CA) for 3 days. CA enabled  
124 removal of the undifferentiated myoblasts [24]. Catabolism in myotubes was induced by adding 150  
125 nM dexamethasone (DEX) [24] or 10 ng/ml TNF- $\alpha$  [27] to the medium for 24 h [28] or 48 h [24] as  
126 shown in Figure 1. Control myotubes (CON group) were studied in parallel, and cells were incubated  
127 with 0.006% ethanol (vehicle).

128 For the study of Cit effects, cells were further incubated with/without 5 mM Cit for 6 h, as indicated  
129 below and in Figure 1.

#### 130 Protein breakdown rate (PBR)

131 *PBR was assessed by the measurement of Tyr [29] release into cell medium:* After 24 h of incubation  
132 with either DEX, TNF- $\alpha$ , or 0.006% ethanol, the medium was replaced, and the myotubes further  
133 incubated for 6 h with or without 5 mM Cit (*Figure 1A*). After 6 h, the culture medium was collected  
134 for the determination of tyrosine concentration. For this purpose, 1.5 ml of medium was  
135 deproteinized with 150  $\mu$ l of sulfosalicylic acid (SSA 30%) and left for 10 minutes in ice, followed by 5  
136 min centrifugation at 10,000 rpm at 4  $^{\circ}$ C. The supernatant was collected and kept at -80  $^{\circ}$ C until  
137 analysis by ion-exchange chromatography with post-column ninhydrin-based detection using a JLC-  
138 500/V AminoTac<sup>™</sup> apparatus (Jeol, Tokyo, Japan).

#### 139 Protein synthesis rate (PSR)

140 PSR was evaluated using two different approaches: surface sensing of translation (SUnSET), described  
141 [30, 31] as an alternative way to evaluate protein synthesis without using the radioactivity method,  
142 and cell incorporation of L-[3,5-<sup>3</sup>H] Tyr [24].

143 *SUnSET*: This method is based on the capacity of puromycin, a structural analogue of aminoacyl tRNA,  
144 to be incorporated in newly synthesized proteins and be revealed by Western blot (WB). Each well was  
145 incubated with 1  $\mu$ M of puromycin 30 min before the end of the protocol (*Figure 1A*). The plates were  
146 then placed in ice, and the wells rinsed once with 1 ml of cold PBS. Myotubes were lysed with 120  $\mu$ l  
147 ice-cold lysis buffer containing 40 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 5 mM ethylene  
148 glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid, 25 mM sodium glycerophosphate, 25 mM  
149 sodium fluoride, 1 mM sodium orthovanadate, 10  $\mu$ g/ml leupeptin, 1X concentrated anti-protease  
150 solution, and 0.5% Triton X-100. The samples were left at 4  $^{\circ}$ C for 15 minutes and centrifuged at 4  $^{\circ}$ C,  
151 14,000 rpm for 20 min. Supernatants were collected and stored at  $-80^{\circ}$ C. Protein concentrations in  
152 the samples were determined with a DC protein assay kit, and the same amounts of proteins (5  $\mu$ g)  
153 from each sample were dissolved in Laemmli buffer and subjected to electrophoretic separation on 4-  
154 15% SDS-PAGE acrylamide gels. Proteins were transferred to a nitrocellulose membrane. This step was  
155 verified by Ponceau red staining. The membrane was then incubated for 1 h with 5% powdered milk in  
156 Tris-buffered saline-0.05% Tween 20 (TBST). Nitrocellulose blots were then incubated overnight at 4  $^{\circ}$ C  
157 with anti-puromycin primary antibody (1:1000). After washing, membranes were incubated for 1 h at  
158 room temperature in 5% milk-TBST with horseradish peroxidase conjugated anti-mouse antibody  
159 (1:10,000). The signal was measured on an ImageQuant™ Las 4000 system using a CCD camera (GE-  
160 Healthcare, Vélizy-Villacoublay, France). Quantifications were performed using image analysis  
161 software (*Image J*). The optical density (OD) signal of puromycin was determined relative to the  
162 Ponceau red staining of the corresponding sample.

163 *Cell incorporation of L-[3,5- $^3$ H]tyrosine*: This measure was performed according to Menconi *et al.* [24].  
164 Following their results, DEX significantly decreased the size of myotubes after 48 h of treatment, and  
165 so at the end of cell differentiation, myotubes were incubated for 48 h with either 150 nM DEX, 10  
166 ng/ml TNF- $\alpha$ , or 0.006% ethanol (*Figure 1B*). After 48 h of incubation, the medium was replaced and  
167 the myotubes further incubated for 6 h with 5 mM Cit (*Figure 1B*). One hour before the end of the 6 h  
168 incubation period, the medium was removed in order to add L-[3,5- $^3$ H]tyrosine (2  $\mu$ Ci/ml) and

169 unlabeled L-tyrosine (2 mM) to the medium (*Figure 1B*). Thereafter, the cells were rinsed once with  
170 1 ml ice-cold PBS followed by 5 min incubation with 1 ml 10% trichloroacetic acid (TCA). After washing  
171 three times with 1 ml 10 % TCA, the myotubes were lysed with 120  $\mu$ l of ice-cold lysis buffer  
172 containing 40 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 5 mM EGTA, 25 mM sodium  
173 glycerophosphate, 25 mM sodium fluoride, 1 mM sodium orthovanadate, 10  $\mu$ g/ml leupeptin, 1X  
174 concentrated anti-protease solution, and 0.5% Triton X-100. The samples were left at 4 °C for 15  
175 minutes and then centrifuged at 4 °C and 14,000 rpm for 20 min. Supernatants were collected in  
176 specific plastic flasks filled with 3 ml Ultima Gold<sup>®</sup> liquid scintillation cocktail (Solvable<sup>™</sup> PerkinElmer  
177 Life Sciences, Boston, MA, USA). The radioactivity of each sample was counted on a scintillation  
178 counter (Tricarb<sup>®</sup>, PerkinElmer Life Sciences). PSR was expressed in dpm/ $\mu$ g protein.

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#### 180 Statistical analysis

181 Results are expressed as mean  $\pm$  SEM. The action of Cit was analyzed by non-parametric tests: Kruskal-  
182 Wallis followed by Mann-Whitney. Statistical analyses were performed using Graphpad Prism (version  
183 5.0; Graphpad Software Inc., San Diego, CA, USA). Differences were considered significant at  $p < 0.05$ .

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190 **Results**

191 Protein breakdown rate

192 We did not observe any effect of TNF- $\alpha$  treatment on PBR, compared with CON. By contrast, Cit  
193 treatment significantly decreased PBR, vs. the CON group and vs. DEX-treated cells even though that  
194 we did not observe any effect of DEX treatment on PBR, compared with CON (*Figure 2*).

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196 Protein synthesis rate

197 Only the DEX treatment significantly decreased PSR compared with CON using both methods (*Figure*  
198 *3A and 3B*). Cit treatment significantly increased PSR in the DEX-treated group using both methods  
199 (*Figure 3A and 3B*). TNF- $\alpha$  had no significant action on PSR, whether or not the cells were treated with  
200 Cit.

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## 213 Discussion

214 The disturbance of protein turnover in injured patients leads to net catabolism, which causes a  
215 decrease in muscle mass, and if protracted, muscle wasting and loss of function [1]. DEX and TNF- $\alpha$   
216 play an essential role in muscle wasting: during stress, plasma GC concentration rises to 3.0  $\mu\text{mol/l}$   
217 [32] and stimulates protein breakdown and/or decreases protein synthesis in muscle, and this leads to  
218 an increase in the rate of appearance of free AAs in the plasma [33]. Menconi *et al.* [24] confirmed the  
219 *in vitro* effect of GC on protein breakdown, something we did not observe in our model. We used the  
220 same range of DEX concentration as they did (Menconi *et al.* noted a peak effect at 100 nM of DEX,  
221 and we used 150 nM of DEX), and the same incubation time for PBR evaluation, but we did not use the  
222 same evaluation method. Using the HPLC method enabled us to evaluate only the release of Tyr into  
223 the culture medium. This can adversely affect the sensitivity of the method: the radioactivity-based  
224 method takes into account all cell compartments where free Tyr may occur. In our study, we did note  
225 a GC effect on PSR: we report the GC effect, testing two different times of exposure (24 h and 48 h)  
226 and using two different methods (SUnSET and radioactivity-based method) because the SUnSET  
227 method is relatively new, and its reliability needs confirmation. The effect of GC on protein synthesis is  
228 controversial: some studies have reported an effect of GC on protein synthesis [34], other not [24].  
229 Concerning PSR evaluation, Menconi *et al.* [24] and our study differ in the DEX concentration: they  
230 used a much higher DEX concentration than we did. The lack of effect of GCs in Menconi's study [24]  
231 may therefore be related to a cytotoxic effect induced by pharmacological levels of GCs.

232 Thus, taken as a whole, in our *in vitro* model, GC induces net catabolism by decreasing drastically PSR  
233 without effect on PBR. This indicates that our model reflects situation such as surgical stress rather  
234 than trauma, burn injury or sepsis [35].

235 A possible explanation of our results is that inhibition of PSR leads to an increase in intracellular free  
236 amino acid content which in turn blunts protein breakdown [33].

237 In general, muscle wasting appears in inflammatory conditions (sepsis, cancer, etc.). In these  
238 situations, TNF- $\alpha$  concentration can be as high as 6 ng/ml [28]. Buck *et al.* [36] have reported muscle  
239 wasting in a murine model of cachexia induced by chronically elevated serum TNF- $\alpha$  concentrations.  
240 Reid and Li, in a review paper [37], have reported that the action of TNF- $\alpha$  can be related to NF $\kappa$ B  
241 activation and to reactive oxygen species (ROS) production in the mitochondria of the cells. In our  
242 model, we did not observe any effect of TNF- $\alpha$  on protein turnover. The effects of TNF- $\alpha$  reported in  
243 the literature are generally from *in vivo* studies. It is likely that TNF- $\alpha$  effects are related to cross-talk  
244 between myocytes and macrophages and/or muscle adipocytes, and also collaboration with other  
245 mediators such as other cytokines or GCs. However, Li and Reid *et al.* [28], in an *in vitro* model of  
246 transfected C2C12 myoblasts overexpressing I- $\kappa$ B $\alpha$  proteins (endogenous NF $\kappa$ B inhibitor), treated by  
247 TNF- $\alpha$ , demonstrated that TNF- $\alpha$  induced protein breakdown by activating NF $\kappa$ B. Our method differed  
248 from theirs in using cytosine arabinoside during the cell culture protocol, which enables removal of  
249 undifferentiated myoblasts. The presence in the culture cells of both differentiated and  
250 undifferentiated myoblasts may have an influence on the action of TNF- $\alpha$ .

251 Concerning the effects of Cit, only one earlier study is relevant to ours: Ham *et al.*, [18] reported the  
252 direct beneficial effect of Cit on protein synthesis on C2C12 using different catabolic stimuli  
253 (starvation, inflammation and oxidative stress). After 48 h of starvation, PSR fell by 26%, and 1 h of Cit  
254 treatment restored it to the CON level. These conditions differed from ours, and the concentration of  
255 Cit was different (2.5 mM). Despite these differences, we obtained the same effect of Cit on protein  
256 synthesis. This agreement with Ham's findings [18], and the fact that we obtained the same results  
257 using our methods, is strong evidence that Cit improves myocyte protein synthesis in various net  
258 catabolic conditions, and is an important finding. We also examined the effect of Cit on protein  
259 breakdown, which Ham *et al.* [18] did not evaluate. Cit treatment decreased slightly (but significantly)  
260 the PBR in the DEX group in C2C12 cells. Our study is the first to be interested in the effect of a  
261 pharmaconutrient in these catabolic conditions (DEX or TNF- $\alpha$ ). Ham *et al.* [18] recommend the use of  
262 a dose of Cit above 1 mM, which we did with a dose of 5 mM in our study.

263 Taken as a whole, our data point out that the action of citrulline on muscle protein turnover relates  
264 primarily to an increase in protein synthesis with a marginal effect on muscle protein breakdown.

265 The action of Cit can be direct (i.e. through its own anti-oxidant properties [38]), or indirect (through  
266 its metabolism into arginine (Arg) and/or further metabolism into nitric oxide [39]). Our *in vitro*  
267 approach yields evidence that the action of Cit is direct, because it is known [40] that myocytes do not  
268 contain argininosuccinate synthase and lyase, which are required to convert Cit into Arg [41].

269 Our study has two limitations: first, we did not study the effect of a combination of DEX and TNF- $\alpha$ ,  
270 knowing that the latter acts primarily to modulate the action of the former [23], and second, we did  
271 not make any attempt to determine the underlying mechanism of Cit action. This point also deserves  
272 further study. However, we believe that our results taken as a whole represent a significant advance in  
273 the field.

274 In conclusion, our results indicate that Cit treatment leads to a regulation of muscle protein turnover,  
275 acting both on PSR and PBR, in an *in vitro* model of muscle catabolism. It will now be of interest to  
276 explore the underlying mechanisms of these effects.

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402 **Legends of figures**

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404 **Figure 1. Study design**

405 C2C12 cells were at the myoblast state; we started by inducing the differentiation of myoblasts in myotubes (*Figure 1A*).  
406 Myotubes were then treated with 150 nM of DEX or 10 ng/ ml of TNF- $\alpha$  or 0.006 % ethanol for 24 h, after which myotubes  
407 were treated with 5 mM of Cit for 6 h (*Figure 1A*). The Tyr released into the medium was measured by HPLC (PBR evaluation).  
408 For the SunSET protocol, myotubes were treated with 1  $\mu$ M of puromycin 30 min before the end of the 6 h protocol (PSR  
409 evaluation) (*Figure 1A*). Concerning the evaluation of L-[3,5- $^3$ H]Tyr (radioactivity protocol), myotubes were treated with 150  
410 nM of DEX or 10 ng/ml of TNF- $\alpha$  or 0.006 % ethanol for 48 h (*Figure 1B*). Myotubes were then treated with 5mM of Cit, and  
411 1 h before the end of the protocol with 2  $\mu$ Ci/ml labeled Tyr and 2 mM unlabeled Tyr (PSR evaluation). Each experiment was  
412 repeated at least four times, and each experimental point was run in duplicate within each experiment.

413 **Figure 2. Protein breakdown rate in C2C12 myotubes using HPLC method.**

414 Values are given as mean  $\pm$  SEM.

415 Analysis was performed by non-parametric tests: Kruskal-Wallis followed by Mann-Whitney.

416 \*Cit treatment significantly decreased PBR vs. CON and DEX groups at  $p < 0.05$  ( $n = 7$ /group).

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418 **Figure 3. Protein synthesis rate (PSR) in C2C12 myotubes (A) using SunSET method and (B) using radioactivity-based**  
419 **method.**

420 Values are given as mean  $\pm$  SEM.

421 Analysis was performed by non-parametric tests: Kruskal-Wallis followed by Mann-Whitney.

422 \*DEX significantly decreased PSR vs. CON [(**A**):  $p = 0.04$ ;  $n = 6$  and (**B**):  $p = 0.04$ ;  $n = 7$ ].

423 \*Cit treatment significantly raised PSR using the SunSET method [(**A**): DEX+CIT vs. DEX,  $p = 0.03$  and TNF- $\alpha$ +CIT vs. TNF- $\alpha$ ,  
424  $p = 0.054$ ;  $n = 6$ ]. Using the radioactivity-based method, Cit treatment significantly raised PSR only in the DEX group [(**B**):  
425 DEX+CIT vs. DEX,  $p = 0.01$ ;  $n = 4$ ].

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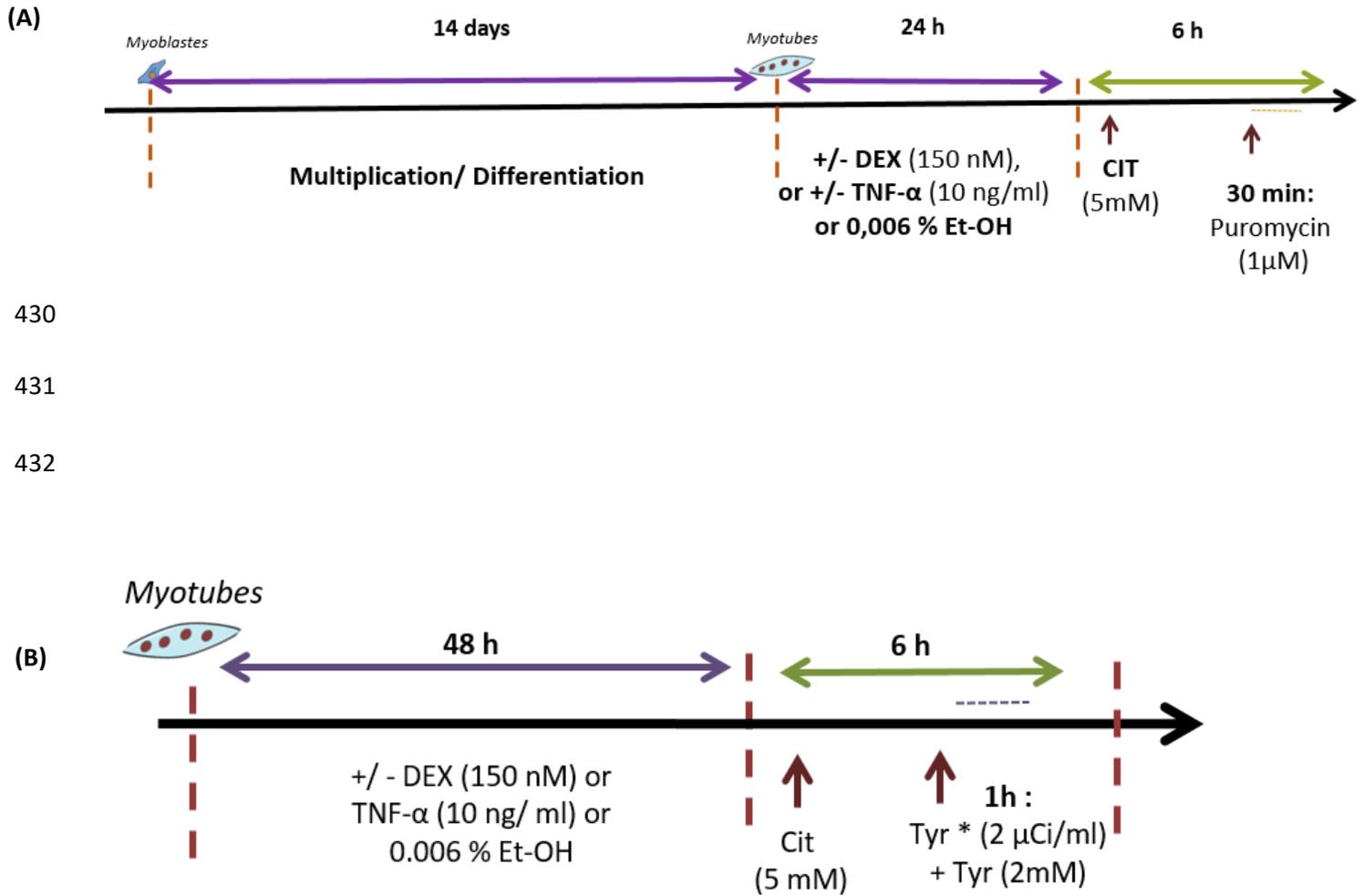
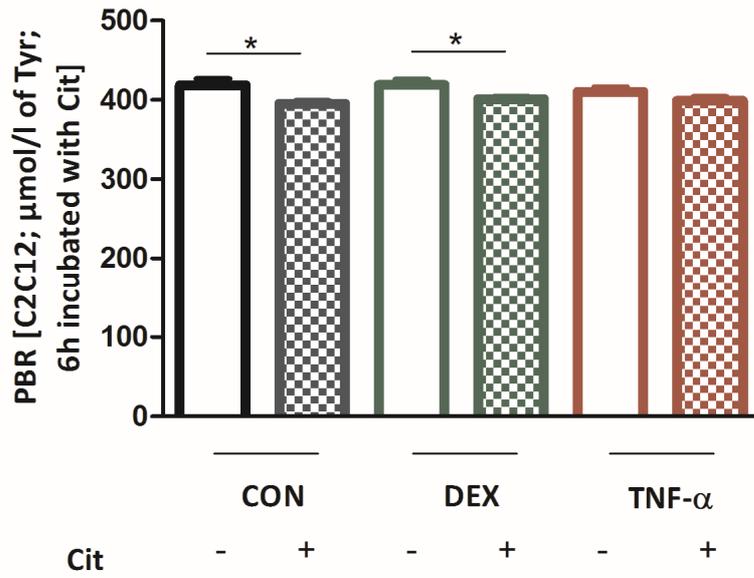


Figure 1

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451 **Figure 2**

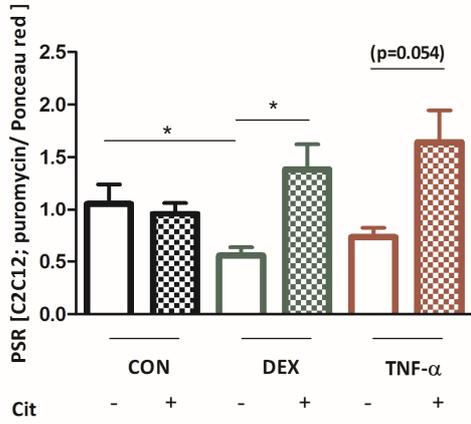
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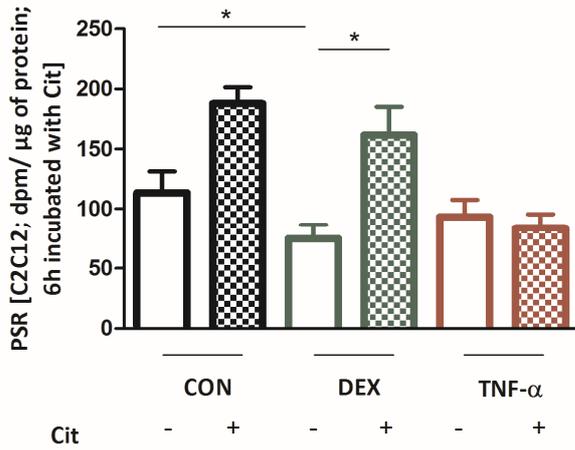
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(A)



(B)



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471 **Figure 3**

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