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20 **Abstract:**

21 Muscle protein synthesis (MPS) is a complex and finely-regulated mechanism that plays a key role in
22 muscle homeostasis. Amino acid bioavailability is widely considered a major driver of MPS regulation
23 via mTOR pathway activation. However, recent results suggest that amino acid bioavailability affects
24 cellular energy status. Whatever the tool used to modulate energy status (amino acid depletion or
25 mild mitochondrial uncoupling), a decrease in cellular energy status decreases MPS, without
26 necessarily involving the mTOR pathway. Here we propose that energy status directly regulates one
27 or several energy-consuming step(s) during MPS. This new paradigm modifies our vision of protein
28 metabolism and raises prospects for new advances in therapeutics.

29

30 **Introduction:**

31 Human skeletal muscle is the main store of proteins at whole-body level. Skeletal muscles
32 drive the locomotor function but they also have an important metabolic role. Muscle protein
33 turnover (i.e. protein synthesis and proteolysis) is a tightly regulated process. Muscle growth occurs
34 when protein synthesis is higher than proteolysis, whereas muscle atrophy occurs when protein
35 synthesis is lower than proteolysis [1].

36 New proteins get synthesized through multiple reactions that occur in the nucleus, the
37 cytosol, or various subcellular locations [2]. The process begins by mRNA translation, which proceeds
38 in 3 phases: initiation, elongation, and termination. The level of muscle protein synthesis is governed
39 by two factors: translational efficiency (i.e. the rate of protein synthesized by ribosome) and
40 translational capacity (i.e. the number of ribosomes/units of tissue; see [3] for an extensive review).
41 Crucially, these different steps demand a large amount of energy to drive both chemical reactions
42 (such as peptide bonds and phosphorylation) and mechanical reactions (ribosome displacements) [4].
43 Protein synthesis is thought to be mainly, if not totally, under control of the mTOR signaling pathway,
44 which is where both exogenous (i.e. amino acids) and endogenous (i.e. AMPK) stimuli converge
45 (Figure 1). Based on the effects of the mTORC1 inhibitor rapamycin, there is no doubt that mTORC1
46 regulates protein synthesis [3]. There is ample literature showing that mTORC1 can regulate several
47 steps in protein synthesis, [1-3, 5, 6] but the molecular regulatory mechanisms involved are not
48 always fully understood.

49

50 **Regulation of muscle protein synthesis by amino acids**

51 Exogenous regulatory factors include amino acids and hormones, which are recognized as
52 major regulators of protein synthesis. After a meal, there is an increase in amino acid availability at
53 peripheral level associated with an increase of insulin, both of which are required to stimulate MPS

54 *via* direct activation of the mTORC1 pathway [5, 6]. A study using a model of isolated perfused
55 muscle showed that maximal stimulation occurred with the normal postprandial amino acid
56 concentrations [7], and *in vitro* studies using myotube cultures also found that high levels of amino
57 acids are associated with high levels of protein synthesis [8].

58 From a finalist point of view, it seems logical that amino acids stimulate protein synthesis,
59 and especially muscle synthesis. Indeed, the organism has to fight on two fronts. On one hand, there
60 is no storage capacity for amino acids (unlike glycogen which has no other function than the storage
61 of glucose, proteins are primarily synthesized to perform a function). On the other hand, amino acids
62 are neurotoxic and so the body needs to limit any excessive hyperaminoacidemia (amino acids are
63 eliminated via the urea cycle).

64

65 **Regulation of muscle protein synthesis by energy level**

66 In most cells, ATP is mainly produced by mitochondria during a process that couples oxygen
67 consumption to ATP production (OXPHOS machinery). Because the fluxes of ATP production and ATP
68 consumption are not directly measurable in a living cell, oxygen consumption is a surrogate of ATP
69 consumption in intact cells, tissues, and organisms.

70 It is assumed that the inhibition of an ATP-consuming process results in a decrease in cellular
71 oxygen consumption. In practice, this reduction in oxygen consumption is measurable when a major
72 metabolic pathway is inhibited. It has been shown under controlled conditions that during the
73 gradual inhibition of mitochondrial respiration, some ATP-consuming processes decrease rapidly (the
74 inhibition of the metabolic pathway has virtually no effect on remaining oxygen consumption) while
75 all the others are maintained [9]. These observations led to the conclusion that there is a hierarchy
76 between the various processes that consume ATP in cells, with some stopping when ATP production
77 decline and others persisting despite it [9]. Note that this experimental approach is based on two

88 assumptions: first, that all the ATP-consuming processes in a given pathway are inhibited; second,
89 that other ATP-consuming processes are not increased. With these hypotheses fresh in mind, note
90 that it has been reported that protein synthesis is strongly inhibited as soon as mitochondrial
91 respiration is weakly decreased [9], while Na⁺ cycling and Ca²⁺ cycling are almost unaffected [9]. It is
92 proposed that shutting down protein synthesis serves to save ATP for more immediately vital cells
93 functions.

84 The concept of a hierarchy between several ATP-consuming processes in cells implies the
85 existence of a signal capable of inhibiting certain metabolic pathways but not others. The progressive
86 inhibition of mitochondrial respiration not only leads to gradual inhibition of ATP synthesis rate, it
87 also results in a decrease in the ATP/ADP ratio.

88 By sensing the concentration of AMP, which is in equilibrium with the ATP/ADP ratio, the
89 AMP-activated protein kinase (AMPK) is an important sensor of cellular energy status [10]. Given that
90 AMPK can inactivate mTOR, it has been proposed that energy status controls protein synthesis via its
91 effect on AMPK. Although this proposal cannot be ruled out, recent data are not consistent with a
92 pure effect of energy status on mTOR, which leads us to propose that besides signaling regulation,
93 protein synthesis is also directly thermodynamically regulated by the ATP/ADP Ratio.

94 Using a model of primary myotubes, we evaluated the effect of amino acid starvation on MPS
95 (evaluated by the Sunset method), and energy status [4]. As expected, amino acid starvation led to a
96 decrease in MPS (-29%), the remaining MPS being made from endogenous amino acids. Surprisingly,
97 we also observed that amino acid starvation led to a decrease in energy status (ATP/ADP ratio) (-
98 13%) and to an AMPK activation. In order to distinguish the respective role of these two regulators
99 (amino acid starvation and energy status) in decreasing MPS, we used a model of myotubes with a
100 reduced cellular energy status induced by mild uncoupling of the OXPHOS machinery. We observed
101 that the same decrease in ATP/ADP ratio led to a decrease in protein synthesis (-21%) despite the

102 presence of a high concentration of amino acids in the medium. At this stage, these results remained
103 in line with the proposition that MPS was decreased due to mTOR regulation. We then tested the
104 effect of leucine or citrulline, which **both** activate MPS in cases of amino acid deficiency. We
105 observed that citrulline but not leucine was able to counteract the inhibition of MPS. Importantly,
106 citrulline did not normalize the ATP/ADP ratio and had no effect on AMPK. In other words, citrulline
107 has no effect on mTOR but increases MPS under energy stress. Importantly, however, citrulline did
108 not increase MPS under control conditions, indicating that citrulline as such is not a direct activator
109 of MPS.

110

111 **ATP for paying, not for signaling**

112 Citrulline only stimulates MPS when there is energy stress, which it does not improve. To
113 account for the fact, we propose a regulation of protein synthesis based on thermodynamic
114 considerations. Thermodynamics laws stipulate that a reaction can only take place when it is
115 accompanied by a decrease in its Gibbs' free energy (i.e. when the ΔG of the reaction is < 0).
116 However, non-thermodynamically favorable reactions ($\Delta G > 0$) can occur if they are coupled with
117 thermodynamically favorable reactions ($\Delta G < 0$) as long as the sum of the two ΔG remains < 0 . As a
118 rule, the reaction that “pays for” a non-thermodynamically favorable reaction is the hydrolysis of ATP
119 into ADP plus Pi (phosphate).

120 Remember that the direction of a reversible reaction depends on the respective
121 concentrations of the reagents and the products of the reaction. This phenomenon, known as the
122 “law of mass action”, means that the ΔG of the reaction can move from a negative value (when the
123 reaction occurs in the forward direction) to a positive value (when the reaction occurs in the reverse
124 direction). In other words, the ΔG of a given reaction changes according to the concentrations of its
125 reagents and products. In the case of ATP synthesis/hydrolysis ($ATP \leftrightarrow ADP + Pi$), this means that the

126 ΔG of ATP hydrolysis depends on the concentrations of ATP, ADP and Pi. If we apply the law of mass
127 action to this reaction, then the ΔG of ATP hydrolysis depends on the $\text{ATP}/(\text{ADP} \times \text{Pi})$ ratio. In other
128 words, when a molecule of ATP is hydrolyzed, the energy available depends not only on the
129 concentration of ATP but also on the concentrations of ADP and Pi. Physiologically, the concentration
130 of Pi is much higher than the concentrations of ATP and ADP, so it is mainly the ATP/ADP ratio that
131 influences the ΔG of the reaction. By analogy with money, ATP molecules are like coins, whose value
132 depends on the ATP/ADP ratio. This takes us to a counter-intuitive thermodynamic truth: it is not the
133 availability of ATP that controls reaction, but its value (i.e. its ATP/ADP ratio).

134 Protein synthesis involves many energy-consuming steps hydrolyzing either ATP or GTP
135 (which are at thermodynamic equilibrium *via* the nucleoside diphosphate kinase). It is thus likely that
136 one or several step(s) require a large amount of energy that cannot be released by the hydrolysis of
137 an ATP with a low ATP/ADP ratio (or a GTP with a low GTP/GDP ratio). In other words, the reaction
138 does not take place because the energy required is insufficient.

139 Although no reaction (alone or coupled to an energy-releasing reaction) can be performed if
140 its ΔG is not negative, some reactions do not occur despite a negative ΔG . More precisely, the
141 reaction occurs but so slowly that we do not perceive it. Thermodynamically, this apparent paradox
142 is explained by the existence of a “potential barrier” (sometimes called the “energy barrier”).
143 Importantly, the size of the potential barrier depends on the ΔG (it increases when the ΔG becomes
144 less negative). To be overcome (i.e. so that the reaction occurs), this potential barrier requires an
145 activation energy. In biology, the thermodynamically favorable reactions are regulated by enzymes
146 that decrease the potential barrier of reactions. In other words, enzymes facilitate
147 thermodynamically favorable reactions by decreasing their activation energy, but they are unable to
148 catalyze a non-thermodynamically favorable reaction. We propose that citrulline can also reduce the
149 potential barrier of one or more energy-consuming processes involved in protein synthesis.

150 Both thermodynamics and enzymes regulate metabolic pathways. However, the respective
151 share of the thermodynamic component and the enzymatic (kinetic) component varies according to
152 the reactions and, for a given reaction, with the conditions. In general, thermodynamic conditions
153 control a reaction when its enzyme is activated or in high concentration, whereas enzymes become
154 the main regulator when inhibited or in low concentration. This easily explains why citrulline does
155 not stimulate protein synthesis under the control conditions (when ATP/ADP ratio is high) because,
156 under these conditions, the potential barrier is already low.

157 It is abundantly clear that protein synthesis involves many enzymes or machineries that
158 require energy consumption. All these steps are potentially controlled by signaling pathways (mTOR
159 for example). However, it must be kept in mind that the activation of an enzyme has no effect if the
160 energy required for the reaction is insufficient. We believe that protein synthesis can be regulated
161 directly (thermodynamically) by the ATP/ADP ratio, independently of AMPK signaling. This
162 thermodynamic regulation explains the observations of no activation of protein synthesis despite the
163 activation of its signaling pathways.

164

165 **In vivo data supporting our proposal.**

166 It is well known that malnourished patients lose muscle proteins and that this process
167 contributes to morbidity and mortality (linked to time and intensity). Different strategies have been
168 tested to limit protein loss, but with limited results. For example, using a rodent model of traumatic
169 brain injury leading to muscle loss [11], we observed an impairment of energy status in tissues [12].
170 We were able to normalize caloric and protein intake using enteral nutrition. **However**, this strategy
171 did not restore energy status [12] nor **muscle protein content** [13]. **This observation is consistent**
172 **with our proposal that the normalization of protein intake is not sufficient to restore muscle mass**
173 **when energy status is impaired.**

174 Ageing induces a decrease of muscle mass by 50 % between 20 and 80 years old that can
175 possibly lead to sarcopenia. Several pieces of evidence suggest that mitochondrial function is
176 reduced during aging, both in muscle and in neurons (see [14, 15] for recent reviews). It is generally
177 assumed that such dysfunction reduces physical activity, leading to muscle atrophy. Besides this
178 mechanical hypothesis, a direct effect of energy status on protein synthesis has not yet been
179 proposed but is supported by indirect evidence. Indeed, ageing leads to a decrease in ATP content
180 [16, 17], while it decreases the rate of mitochondrial protein synthesis in muscle [18].

181 Several human studies concordantly conclude that a decrease in energy consumption leads
182 to a decrease in the rate of protein synthesis [19-22]. Unfortunately, none of these studies measured
183 energy status (the ATP/ADP ratio). Note however, that caloric restriction in rat led to a decrease in
184 energy status (as measured by the activation of the AMPK pathway) [23]. Together, these results are
185 consistent with our proposal that energy status directly regulates protein synthesis.

186

187 **Relevance to clinical nutrition.**

188 Here, we propose that energy status is a major factor in the regulation of muscle protein
189 synthesis. It acts indirectly via mTOR pathway signaling (regulation by AMPK) but also directly during
190 the consumption of energy. From a theoretical point of view, two strategies could restore the
191 synthesis of proteins when impaired by energy stress. The first is to restore energy homeostasis (i.e.
192 to normalize the ATP/ADP ratio). The second is to use a protein synthesis catalyst capable of
193 offsetting the effect of a low ATP/ADP ratio. For the time being, the first strategy is difficult to
194 implement because the measurement of the energetic status is not done routinely. However, this is
195 probably what happens during the healing of an acute disease. Alternatively, the use of citrulline (or
196 other compounds that would act similarly) is a pragmatic approach **that could be implemented**. The
197 development and systematic use of the measurement of the cellular energy status would be a

198 valuable tool to revisits our understanding of protein metabolism and lay the foundation for new
199 approaches to the management of undernutrition.

200 **Conclusion**

201 In this article, we propose that the energy status (i.e., the ATP/ADP ratio) is bona fide
202 regulator of protein synthesis. This proposal is supported by both direct and indirect evidence, but it
203 remains to be confirmed in larger scale experiments in humans.

204

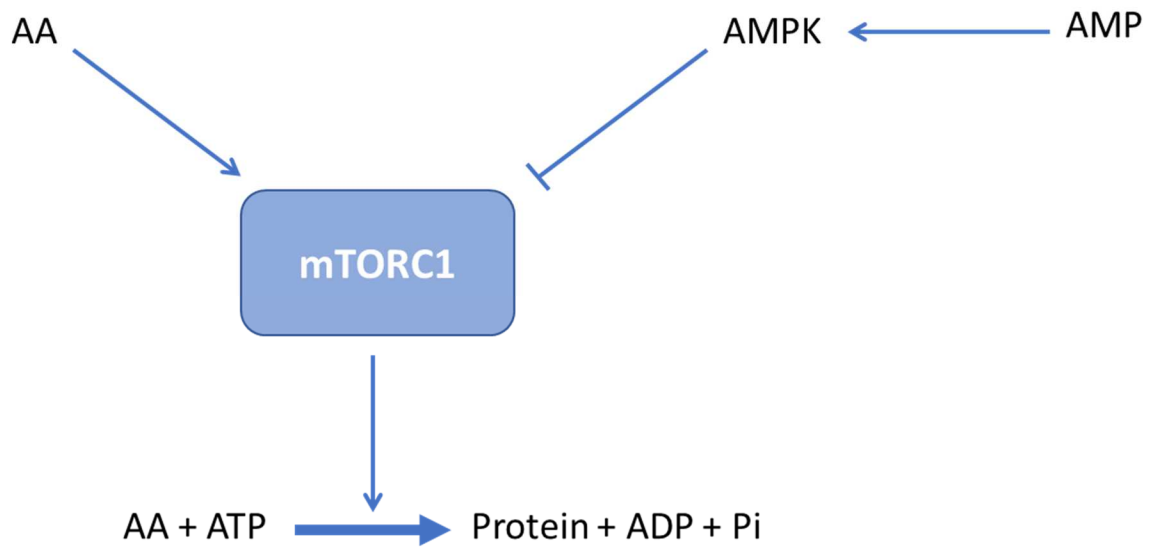
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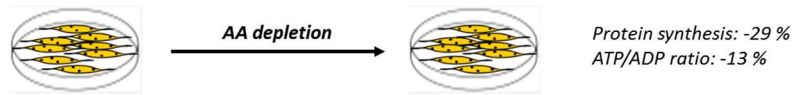
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269 **Figure 1:** General overview of regulation of protein synthesis by amino acids and energy status

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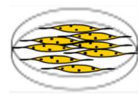
Complete medium

Depleted medium

Protein synthesis: -29 %
ATP/ADP ratio: -13 %

A

+ CIT

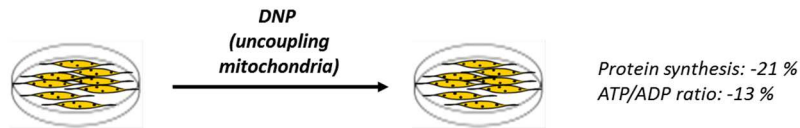


Depleted medium + CIT

Protein synthesis: +22 %
ATP/ADP ratio: no effect

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272



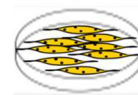
Complete medium

Complete medium + DNP

Protein synthesis: -21 %
ATP/ADP ratio: -13 %

B

+ CIT



Complete medium + DNP

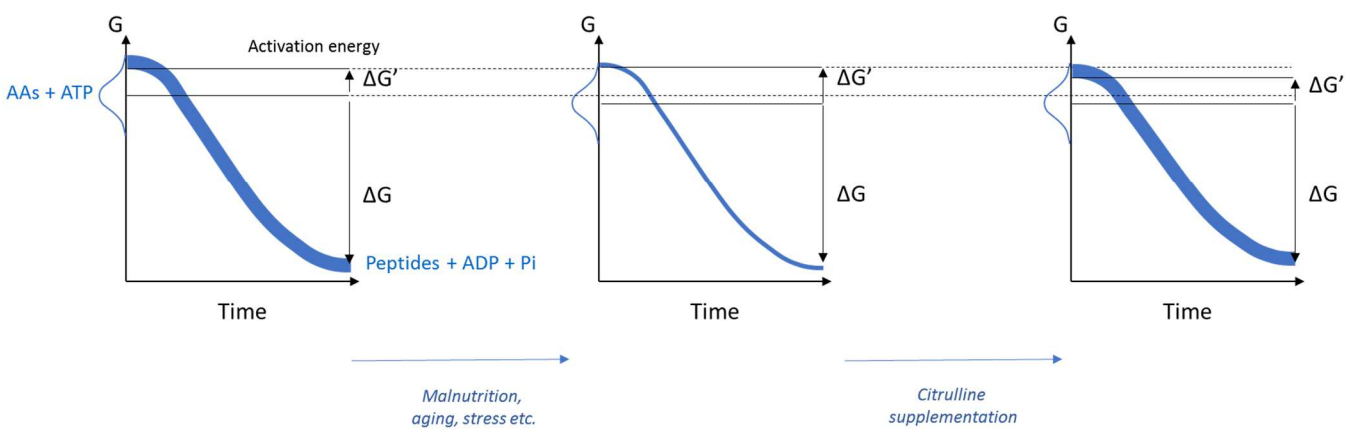
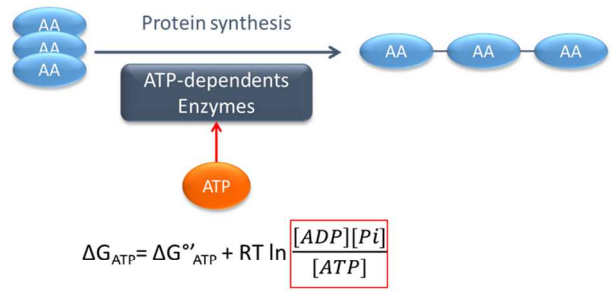
Protein synthesis: +11 %
ATP/ADP ratio: no effect

273

274

275 **Figure 2:** In myotubes, AAs deficiency for 16h decreases both protein synthesis and energy status. In
 276 these conditions, CIT is able to restore protein synthesis without modification of ATP/ADP ratio
 277 (Figure 2A). In a second set of experiments (Figure 2B), cellular energy state was decreased by mildly
 278 uncoupling mitochondria (as the same level as amino acid deficiency) but with a complete medium
 279 (with complete AA medium). Such conditions also decrease protein synthesis and CIT again
 280 stimulates protein synthesis (adapted from Goron et al., *J Cachexia Sarcopenia Muscle*, 2019).

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Figure 3: At the molecular level, the G of reagents is a value that fluctuates around an average state. Only reagents whose G exceeds the G of the activation energy are converted into product. As the average G decreases, the number of reagents exceeding the G of the activation energy decreases. As a result, the rate of reaction decreases. Thus, to accelerate a reaction, it is necessary to increase the average G or to decrease the activation energy.

When applied to protein synthesis, ATP-consuming processes (or GTP-consuming processes) slow down when the average G of ATP (or GTP) decreases. Citrulline would restore the initial velocity by decreasing the activation energy.