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Conflict of interest: C. Moinard is a shareholder in Citrage. Eric Fontaine has no conflict of interest to report.

Abstract:

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

Introduction:

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

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

Regulation of muscle protein synthesis by amino acids

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

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

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

Regulation of muscle protein synthesis by energy level

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

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

assumptions: first, that all the ATP-consuming processes in a given pathway are inhibited; second, that other ATP-consuming processes are not increased. With these hypotheses fresh in mind, note that it has been reported that protein synthesis is strongly inhibited as soon as mitochondrial respiration is weakly decreased [9], while Na^+ cycling and Ca^{2+} cycling are almost unaffected [9]. It is proposed that shutting down protein synthesis serves to save ATP for more immediately vital cells functions.

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

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

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

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

ATP for paying, not for signaling

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

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

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

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

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

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

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

In vivo data supporting our proposal.

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

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

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

Relevance to clinical nutrition.

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

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

Conclusion

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

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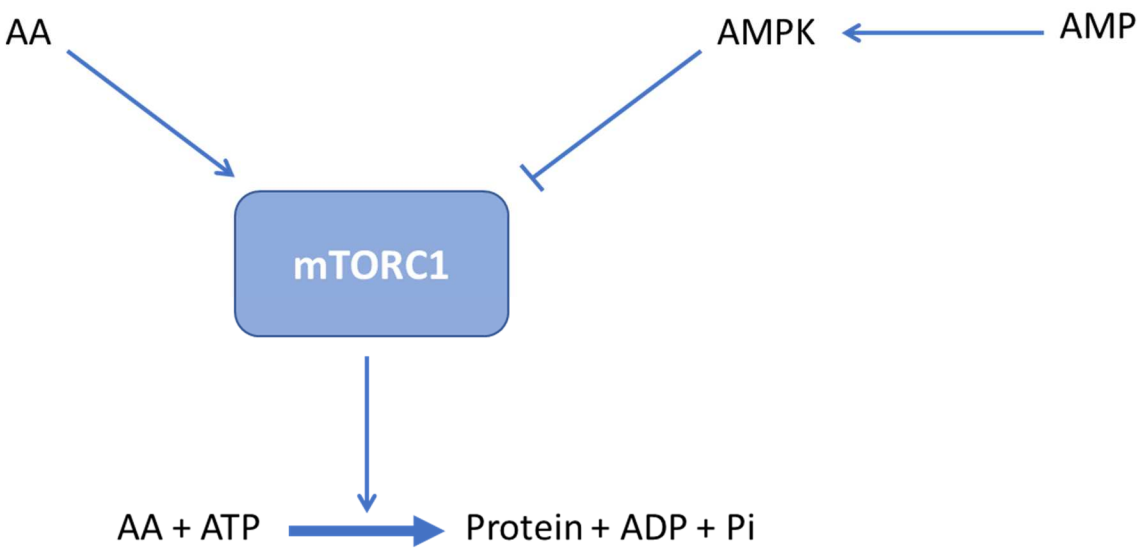
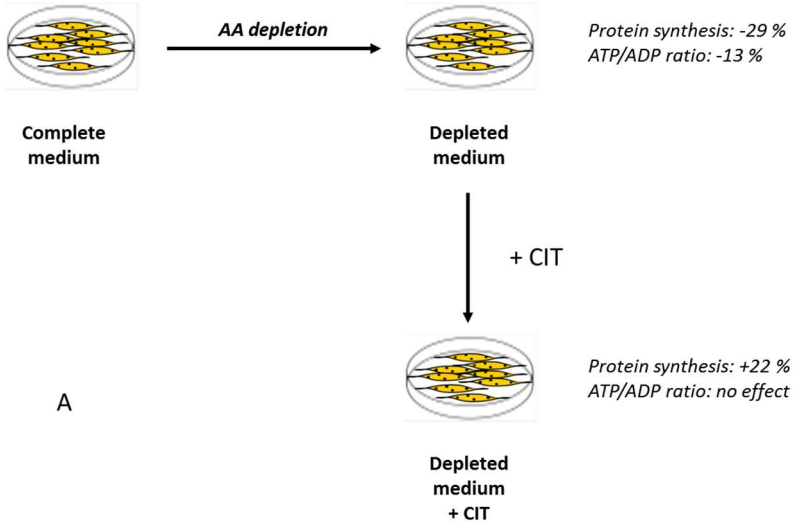


Figure 1: General overview of regulation of protein synthesis by amino acids and energy status



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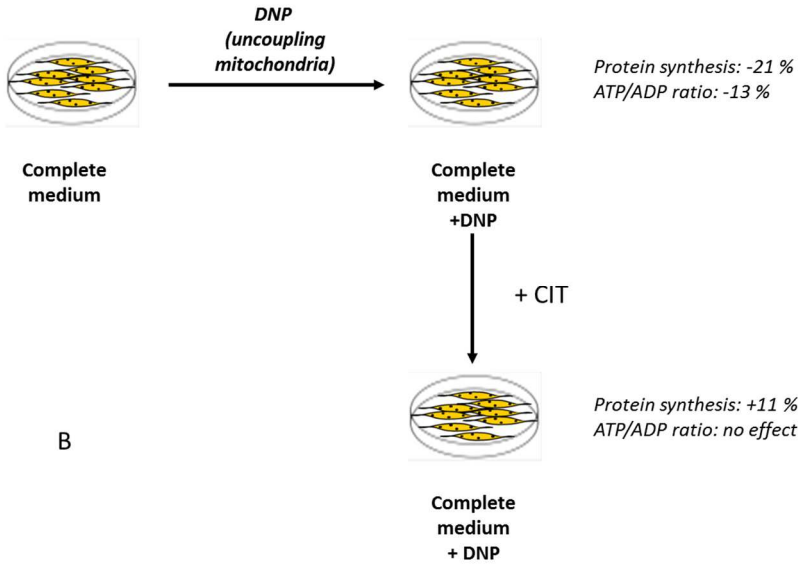

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275 **Figure 2**: In myotubes, AAs deficiency for 16h decreases both protein synthesis and energy status. In
276 these conditions, CIT is able to restore protein synthesis without modification of ATP/ADP ratio
277 (Figure 2A). In a second set of experiments (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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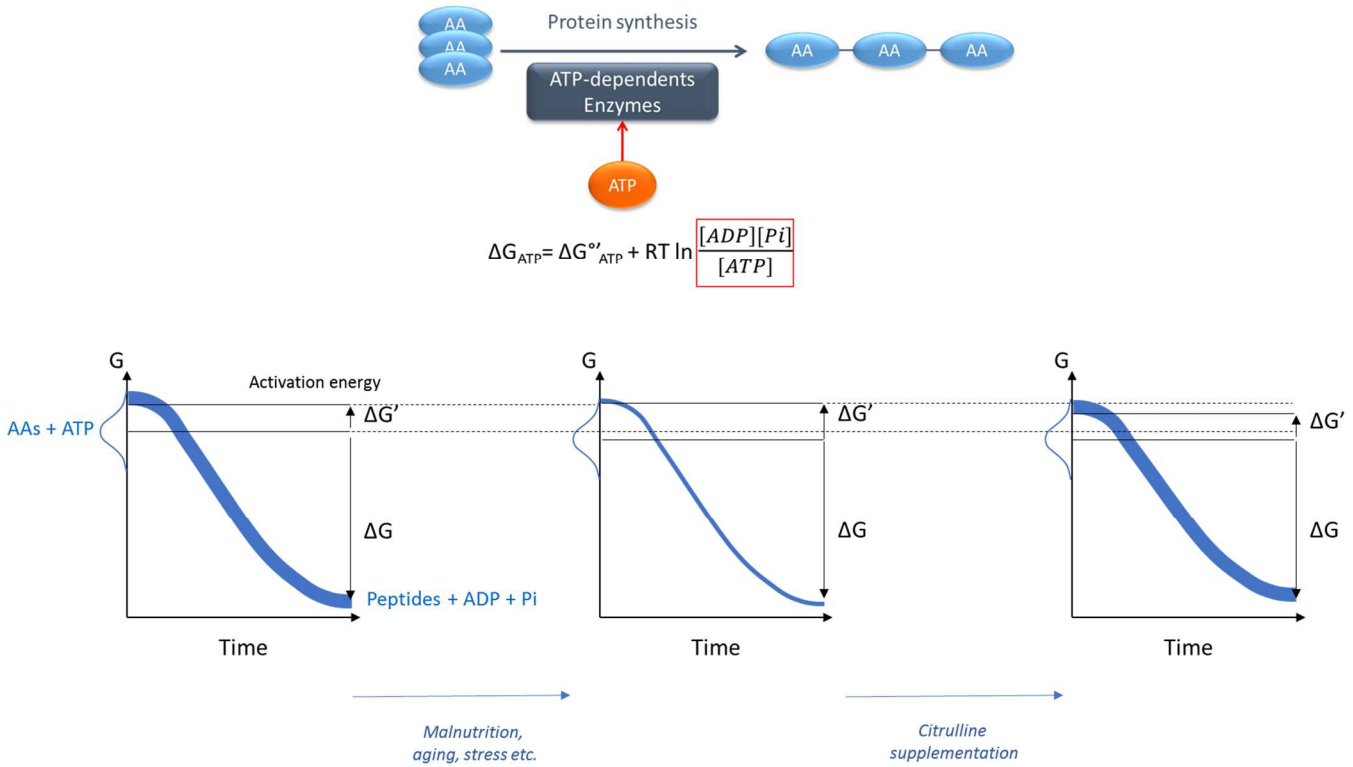

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285 **Figure 3**: At the molecular level, the G of reagents is a value that fluctuates around an average state.
286 Only reagents whose G exceeds the G of the activation energy are converted into product. As the
287 average G decreases, the number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

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

293