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Higher insulin sensitivity in EDL muscle of rats fed a low-protein, high-carbohydrate diet inhibits the caspase-3 and ubiquitin-proteasome proteolytic systems but does not increase protein synthesis

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Abstract

Compared with the extensor digitorum longus (EDL) muscle of control rats (C), the EDL muscle of rats fed a low-protein, high-carbohydrate diet (LPHC) showed a 36% reduction in mass. Muscle mass is determined by the balance between protein synthesis and proteolysis; thus, the aim of this work was to evaluate the components involved in these processes. Compared with the muscle from C rats, the EDL muscle from LPHC diet-fed rats showed a reduction (34%) in the *in vitro* basal protein synthesis and a 22% reduction in the *in vitro* basal proteolysis suggesting that the reduction in the mass can be associated with a change in the rate of the two processes. Soon after euthanasia, in the EDL muscles of the rats fed the LPHC diet for 15 days, the activity of caspase-3 and that of components of the ubiquitin-proteasome system (atrogin-1 content and chymotrypsin-like activity) were decreased. The phosphorylation of $p70^{S6K}$ and 4E-BP1, proteins involved in protein synthesis, was also decreased. We observed an increase in the insulin-stimulated protein content of p-Akt. Thus, the higher insulin sensitivity in the EDL muscle of LPHC rats seemed to contribute to the lower proteolysis in LPHC rats. However, even with the higher insulin sensitivity, the reduction in p-E4-BP1 and $p70^{S6K}$ indicates a reduction in protein synthesis, showing that factors other than insulin can have a greater effect on the control of protein synthesis.

Keywords: Low-protein, high-carbohydrate diet; Protein synthesis; Proteolytic pathways; Extensor digitorum longus; Insulin sensitivity; Growing rats

1. Introduction

In developing countries, malnutrition is an early and usual event in the human life. Normally, children in these countries consume a great amount of carbohydrates and a small quantity of protein, which can lead to structural damage in several organs and increased mortality [1,2].

Skeletal muscle is considered the most abundant tissue in the body, making up approximately 40–50% of the total body mass and serving as the largest body protein pool [3,4]. Skeletal muscle exhibits a high resting metabolic rate and is one of the most adaptable tissues responding to numerous external and physiological stimuli [5], which induce changes in its phenotypic profile in terms of size and composition [6]. Thus, particularly in conditions of protein malnutrition, the adaptations of muscles are fundamental to body homeostasis.

Several authors have suggested that protein restriction during the critical stage of development results in a reduction in the skeletal muscle mass and in the number and size of fibers [7,8], along with higher vulnerability to atrophic reactions [9]. However, other authors have shown that muscle fibers are capable of adapting their metabolism, optimizing protein turnover to preserve the primary functions in malnutrition conditions [10–12].

Studies from our research group showed that rats that were fed a low-protein, high-carbohydrate diet (LPHC; 6% protein and 74% carbohydrate) for 15 days soon after weaning showed an increase in the diet and calorie intake with a reduction in the body weight gain compared to the corresponding factors in the rats fed a control diet (C; 17% protein and 63% carbohydrate) [13]. Even when the increase in the food intake was accounted for, the LPHC rats ingested 60% less protein than the rats fed the C diet at the end of 15 days, which was confirmed by the reduced postprandial amino acid concentration in the blood of these rats [13]. The protein-deficient state in LPHC rats could also be indicated by the hypoproteinemia and impairment in the body growth (evaluated by Lee index) compared to the condition of the rats receiving a C diet [13]. Moreover, LPHC rats showed a reduction in the body mass with a higher energetic gain as a consequence of an increase in the body lipid content and a reduction in

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the water and protein contents [13] (data are shown in Table S1 of the supplementary material). Additionally, the LPHC diet promotes an increase in serum epinephrine and norepinephrine levels, as well as 10 times higher levels of tumor necrosis factor alpha (TNF-α) and 100% higher levels of corticosterone and leptin [13,15,16] (data are shown in Table S2 of the supplementary material); thus, the LPHC diet seems to promote a highly catabolic environment. A previous study also showed a reduction in the mass of different skeletal muscles in LPHC rats compared with C rats [13].

The mass and protein content of skeletal muscle are determined by the dynamic equilibrium between the rates of protein synthesis and proteolysis [6]. In addition to the availability of essential amino acids, insulin and the insulin-like growth factor (IGF-1) are considered to be factors that mediate normal muscle development [17,18]. These factors bind with their respective receptors, resulting in activation/phosphorylation of protein kinase B, also known as Akt. Akt stimulates protein synthesis by activating the mammalian target of rapamycin (mTOR) and its downstream effectors and inhibits glycogen synthase kinase-3β (GSK-3β), a negative regulator of protein synthesis [19]. Activation of Akt also induces the phosphorylation of the forkhead box transcription factor (Foxo) and its subsequent nuclear exclusion, which results in the inhibition of atrogene transcription and consequently in the inhibition of proteolysis [20].

In turn, there are different proteolytic systems in cells. Intracellular proteolysis may occur in lysosomes after fusion with the autophagosome, which requires the expression of microtubule-associated protein 1 light chain 3 beta (LC3) and gamma-aminobutyric acid receptor-associated protein (GABARAP). The proteolytic capacity of the lysosomes is determined by the activity of cathepsins L, B, D and H [21]. Extralysosomal proteolytic pathways in the skeletal muscle cells of mammals include the calpains or Ca^{2+}-dependent proteases, caspase-3 and the ubiquitin-proteasome proteolytic system [14]. The specificity in protein breakdown by this last proteolytic system is dependent on the content of ubiquitin ligase enzymes (E3s). Two E3s, muscle F-box protein (atrogin-1 or MAFbx) and the protein muscle RING finger-1 (MuRF-1) are known as atrogenes, and they play a decisive role in mediating the loss of muscle mass [20]. Studies indicate the participation of calpains and caspase-3 in the dissociation of actin and myosin from myofibrils before they are degraded by the proteasome [22,23].

A previous study on soleus muscles of LPHC rats showed that the reductions in mass and protein content resulted from decreases in protein synthesis [14] and overall proteolysis. The proteolysis was reduced mainly due to inhibition of both the ubiquitin-proteasome system and caspase-3 activity [14]. The results also indicated higher insulin sensitivity in the soleus muscles of the LPHC rats, which was evidenced by increases in the insulin receptor content and in insulin-stimulated Akt phosphorylation [14], suggesting that the higher insulin sensitivity in the soleus muscle of the LPHC rats was responsible for the inhibition of the proteolytic processes.

However, the alterations observed in the protein metabolism of the soleus muscles from LPHC rats are not necessarily valid for other skeletal muscles because the metabolic response in different metabolic situations seems to be muscle-type specific. Differences in the sensitivity of skeletal muscles to dietary manipulations have also been reported by several authors. Mizushima *et al.* [24] showed that transgenic mice subjected to nutrient starvation experienced rapid and intense macroautophagy in the extensor digitorum longus (EDL) muscle, which almost exclusively contains fast-twitch fibers (glycolytic, type II), and experienced moderate and slow macroautophagy in the soleus muscles, which contains a high percentage of slow-twitch fibers (oxidative, type I). Furthermore, the regulation of extralysosomal proteolytic pathways can differ in skeletal muscles with different fiber-type compositions [25,26].

Thus, the aim of this study was to evaluate the effect of the LPHC diet on components of the systems for protein synthesis and proteolysis as the first stage in establishing possible mechanisms that explain the reduced mass of EDL muscle. For this purpose, we evaluated the following in EDL muscles from control and LPHC rats: (1) mass and protein content of the EDL muscle; (2) *in vitro* basal protein synthesis and proteolysis; (3) protein contents and enzyme activities related to proteolytic pathways; (4) insulin signaling pathway protein content; (5) basal and phosphorylated contents of 4E-BP1, $p70^{S6K}$, GSK-3β, CREB and AMPK, which are proteins involved in the regulation of protein synthesis; and (6) basal and phosphorylated content of Foxo1, which is involved in the regulation of proteolysis.

2. Methods and materials

2.1. Animals and treatment

The animals used in the experiments were provided by the Central Animal House of Universidade Federal de Mato Grosso (UFMT). The animals were handled according to the Brazilian College of Animal Experimentation Regulations, and the experiments were approved by the Animal Ethics Committee of UFMT (protocol no. 23,108.043335/08-1). Male Wistar rats (5–10 animals) with an initial body weight of approximately 90–100 g (~30 days old) were randomly distributed into two groups: (i) control group (C) that was fed a diet composed of 17% protein, 63% carbohydrate and 7% lipid and (ii) LPHC group that was fed a diet composed of 6% protein, 74% carbohydrate and 7% lipid. The reduction of protein in the LPHC diet, in term of calories, was compensated by carbohydrates (Table 1). The diets are isocaloric (16.3 kJ/g) and were administered for 15 days. The rats were housed in individual metabolic cages at 22 ± 1 °C with a 12 h:12 h light:dark cycle; they also received water and food *ad libitum*. The body weight and food intake of each rat were recorded daily. All rats were euthanized on the 15th day of treatment, and the EDL muscles were collected, weighed and stored at −80°C or immediately used in experiments.

2.2. Total protein content of the EDL skeletal muscle

For the LPHC and C fed rats, both EDL muscles (right and left) were used for the analysis. The muscles were minced and homogenized using a glass-Teflon homogenizer (Wheaton Overhead Stirrer) in buffer containing 50 mM potassium phosphate dibasic, 5 mM EDTA, 0.5 mM DTT, 1.15% KCl, 1 mM phenylmethyl sulfonyl fluoride, 5 μg/ml aprotinin and 1 μg/ml leupeptin at pH 7.4 in a proportion of 4% wt/vol. The homogenate was centrifuged at 600*g* for 10 min at 4°C. The total protein content was determined by the Bradford method [27], and the data are expressed in milligrams per gram muscle.

2.3. In vitro, basal protein synthesis and proteolysis

Groups of rats were treated with the C or LPHC diet. After 15 days of treatment, the animals in the fed state were euthanized, and the muscles were removed and incubated for the *in vitro* evaluation of the protein synthesis. The same process was performed with another set of rats from the C and LPHC groups for the evaluation of muscle proteolysis.

The EDL muscles were rapidly dissected, weighed and fixed by the tendon in appropriate supports (to maintain the muscles at the resting length) and were incubated in Erlenmeyer flasks (1 muscle/flask) containing Krebs-Ringer bicarbonate buffer (0.120 M NaCl, 0.015 M $NaHCO_3$, 4.828 mM KCl, 1.2 mM $MgSO_4$, 1.212 mM KH_2PO_4 and 2.4 mM $CaCl_2$ at pH 7.4) and 5 mM glucose, aerated with 95% O_2 and 5% CO_2.

Table 1
Compositions (g/kg) of the control and low-protein, high-carbohydrate diets

Ingredients	Control diet	LPHC diet
Casein (84% protein)	202	71.5
Cornstarch	397	480
Dextrinized cornstarch	130.5	159
Sucrose	100	121
Soybean oil	70	70
Fiber (cellulose)	50	50
Mineral mix (AIN 93 G)[a]	35	35
Vitamin mix (AIN 93 G)[a]	10	10
L-Cystine	3	1
Choline bitartrate	2.5	2.5

[a] For the detailed composition, see Reeves *et al.* (1993).