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Basic nutritional investigation

Whey protein modifies gene expression related to protein metabolism affecting muscle weight in resistance-exercised rats

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ABSTRACT

Objective: The aim of this study was to evaluate the effects of resistance exercise on the mRNA expression of muscle mammalian target of rapamycin (*mTOR*), muscle-specific RING finger-1 (*MuRF-1*), and muscle atrophy F-box (*MAFbx*) in the presence or absence of whey protein ingestion. We hypothesized that resistance exercise in combination with whey protein ingestion alters the gene expression of proteins related to muscle protein synthesis (*mTOR*) and/or degradation (*MuRF-1* and *MAFbx*), thus affecting muscle weight gain in rats.

Methods: Thirty-two male Fischer rats were randomly assigned to the following four experimental groups (n = 8/group): Control sedentary, control exercised, whey protein sedentary, and whey protein exercised. Exercise consisted of inducing the animals to perform sets of jumps for 8 wk. Body weight gain, muscle weights, food intake, and feeding efficiency were evaluated. Gene expressions were analyzed by quantitative real-time reverse transcription polymerase chain reaction. Statistical evaluation was performed using a two-way analysis of variance with a Tukey post hoc test.

Results: Whey protein exercised rats exhibited higher body and muscle weight gain compared with control-exercised rats ($P = 0.032$). The expression of *mTOR* was reduced by exercise but increased when whey protein was consumed as a dietary protein ($P = 0.005$). *MuRF-1* expression was reduced by exercise ($P < 0.001$), whereas *MAFbx* was reduced only by whey protein ingestion ($P = 0.008$) independent of exercise.

Conclusions: A reduction in *MAFbx* gene transcription induced by whey protein and the interaction between exercise and whey protein ingestion on *mTOR* gene expression contributed significantly to differences in body and muscle weight gain.

Introduction

Regular resistance exercise (RE), such as weightlifting, in combination with adequate protein consumption, efficiently stimulates muscle growth, which results from a cumulative increase in muscle protein synthesis, a decrease in muscle protein degradation, or a combination of the two. The rate of protein synthesis is mediated by the activation of a cellular network of

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signaling pathways involving the mammalian target of rapamycin (mTOR), a central serine/threonine kinase that integrates several different upstream and downstream signals that regulate mRNA translation [1,2]. Muscle protein degradation is mediated primarily (80%–90%) by the ubiquitin proteasome system (UPS) [3], which includes the following three components that participate in ubiquitin transfer reactions: Ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligases (E3). E3 ligases, particularly muscle atrophy F-box (MAFbx) and muscle-specific RING finger-1 (MuRF-1), are key enzymes mediating muscle protein loss and are overexpressed in numerous catabolic conditions [4,5].

The phosphorylation of mTOR can be enhanced by amino acids and proteins, especially those with high leucine content [2,6]. Therefore, whey protein (WP) deserves special attention because it is present in a variety of sports supplements, and several benefits of WP for athletes have been reported [7,8]. WP induces mTOR phosphorylation to a greater extent than other protein sources, such as soy, in treadmill-exercised rats [9] and prolongs mTOR signaling in response to RE in humans [10]. However, the effects of RE in combination with WP ingestion on *mTOR* gene expression are not well known.

Conflicting data have been reported on the effects of RE on the E3 ligases, *MAFbx*, and *MuRF-1*. Overexpression of E3 ligases mRNA was observed after a single RE session [11,12], whereas other studies reported different effects of chronic RE on *MuRF-1* and *MAFbx* mRNA expression [3,13]. Additionally, the effects of combined RE and WP ingestion on *MAFbx* and *MuRF-1* mRNA expression are less clear. We hypothesize that RE in combination with WP ingestion might alter the gene expression of proteins related to muscle protein synthesis and/or degradation in rats and thus affect muscle weight gain.

Therefore, this study aimed to evaluate the mRNA expression of *mTOR*, *MAFbx*, and *MuRF-1* in rats subjected to a RE protocol for 8 wk. Additionally, we evaluated the effects of WP on RE-induced gene expression and how these adaptations affect body and muscle weight gain.

Materials and methods

Animals and groups

Thirty-two male Fischer rats (60 d old) weighing approximately 110 g were used in the experiment. The animals were housed individually in galvanized wire metabolic cages in a room with controlled temperature (23 ± 1°C), relative humidity (55 ± 10%), and a 12-h light/dark cycle. The animals received care in accordance with the guidelines of the Canadian Council on Animal Care. Rats were randomly distributed into four experimental groups (n = 8/group) as follows: Control sedentary (CS) control exercised (CE) whey protein sedentary (WS) and whey protein exercised (WE).This research was approved by the Ethical Committee of Federal University of Ouro Preto–protocol no. 036/2008.

Diets

The CS and CE rats were fed the AIN-93 M standard diet [14], and the WS and WE animals received the AIN-93 M diet modified with WP instead of control protein. The compositions of the diets are presented in Table 1, and the amino acid compositions of the proteins (casein as a control and WP) are presented in Table 2. Food and water were provided ad libitum. Body weight and food consumption were measured weekly. Food consumption was corrected for losses from spilling.

Exercise training program and experimental procedures

Exercised rats were submitted to a RE program for 8 wk, as previously reported [15], to mimic a weightlifting-training model for hind limb muscles.

During an adaptation period, rats from each exercised group (CE and WE) were subjected to swimming without weights for 15 min in a 40-cm deep swimming pool with a water temperature of 32 ± 1°C for 1 wk. After the adaptation period, the exercise training consisted of inducing the animals to perform jumps in a circular plastic container with a water level corresponding to 150% of their body length. Weights were attached to the animal's chest to promote submersion. When the rats touched the bottom of the container, they had to jump to emerge from the water to breathe. Water in the container and the weights attached to the animal's chest generated resistance during the exercise. The animals performed four sets of 10 jumps per day, five times per week for 8 wk. A 1-min resting interval was included between each set of jumps. The exercise intensity was increased weekly by changing the weights according to the animal's body weight (25% of body weight in week 1, 30% in week 2, 35% in week 3, 40% in week 5, 50% in week 6, and 55% in weeks 7 and 8). During the RE sets, sedentary animals were kept in a similar aquatic environment consisting of a 5-cm deep swimming pool. At the end of the eighth training week, and within 24 h after the last training session, all rats were fasted for 12 h, anesthetized by isoflurane inhalation (4%), and sacrificed by cardiac puncture. The gastrocnemius and extensor digitorum longus (EDL) muscles were immediately excised, washed, weighed, and stored at −80°C until further analysis. All rats were evaluated for body weight gain, muscle weights, food intake, and feeding efficiency. For quantitative real-time reverse transcription polymerase chain reaction (PCR), six animals per group were randomly selected. However, for MuRF-1 mRNA expression, two rats from the WS group (WS n = 4) and one from the WE group (WE n = 5) were excluded because no amplification during real-time PCR was observed.

Table 1
Compositions of the experimental diets

Ingredients (g/1000 g)	CS/CE	WS/WE
Casein*	140	–
Whey protein†	–	150
Mineral mixture‡	35	35
Vitamin mixture§	10	10
Soybean oil	40	40
Sucrose	100	100
Cellulose	50	50
Choline	2.5	2.5
Cornstarch	622.5	612.5
Total	1.000	1.000

CE, control exercised; CS, control sedentary; WE, whey protein exercised; WS, whey protein sedentary

* Isofar (Rio de Janeiro, Brazil), containing 85% protein as determined by the Kjeldahl method.

† Probiótica, (São Paulo, Brazil), containing 80% protein as determined by the Kjeldahl method.

‡ Mineral mixture for the AIN-93M diet [14].

§ Vitamin mixture for the AIN-93M diet [14].

Total RNA isolation and cDNA synthesis

Total RNA was isolated from 40 to 50 mg of freeze-dried gastrocnemius muscle using the guanidine thiocyanate method with a lysis buffer solution

Table 2
Amino acid composition of casein (control) and whey protein used as a dietary protein source, and the minimum requirements for rodent diets (g/100 g protein)

Amino acid	Casein	Whey protein	Minimum requirements*
Threonine	3.7	8.0	3.7
Valine	5.5	6.3	5.6
Isoleucine	4.2	7.2	4.7
Leucine	8.4	11.2	8.7
Lysine	6.9	8.8	7.3
Methionine	2.6	2.2	2.6
Phenylalanine	4.3	3.1	4.9
Tryptophan	1.2	1.9	1.3
Tyrosine	4.8	3.2	–
Cysteine	0.4	2.7	–
Aspartic acid†	6.3	11.9	–
Arginine	2.9	2.2	–
Serine	4.9	5.3	–
Histidine	2.2	2.1	–
Glutamic acid‡	18.8	18.9	–
Glycine	1.7	1.7	–
Alanine	2.7	5.3	–
Proline	10.6	7.8	–

* Estimated minimal nutrient composition of the AIN-93M rodent diets [14].

† Aspartic acid: aspartic acid + asparagine.

‡ Glutamic acid: glutamic acid + glutamine.

(Promega Co., Madison, WI, USA). The final amount of RNA obtained was measured using a spectrophotometer. Two μg of RNA was used as a template to synthesize cDNA using a reverse transcriptase kit with random primers (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μL. The cDNA was stored at −80°C until further analysis.

Quantitative real-time reverse transcription PCR analysis

Real-time PCR was performed using an ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), a Power SYBR® Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA, USA) and the following primers: Rat *mTOR* (NM_019906) forward, 5'-TCTTCTTCCAGCAAGTTCAGC-3', and reverse, 5'-GAATCAGACAGGCACGAAGG-3' (product size 97 bp); rat *MAFbx* (NM_133521) forward, 5'-GCAAAACATAAGACTCATACG-3', and reverse, 5'-GTAGAGTGGTCTCCATTCG-3' (product size 83 bp); and rat *MuRF-1* (NM_080903) forward, 5'-AGGTGAAGGAGGAACTGAG-3', and reverse, 5'-AACTGCTCTCGGTACTGG-3' (product size 86 bp). The primers were derived from *Rattus Novergicus* genes (National Center of Biotechnology Information GenBank), and were constructed using the Prime-BLAST program (http://ncbi.nlm.nih.gov/tools/primer-blast/). The expression of the housekeeping genes, *GAPDH* (AF106860) forward, 5'-GGATGCAGGGATGATGTTCT-3', and reverse, 5'-AAGGGCTCATGACCACAGTC-3' (product size 116 bp) and *β-Actin* (NM_031144) forward, 5'-TCGTACCACTGGCATTGTGAT-3' and reverse, 5'-CGAAGTCTAGGGCAACATAGCA-3' (product size 233 bp), were determined as endogenous controls. The *GAPDH* gene was identified as the most reliable housekeeping gene in all experimental conditions. A two-way repeated-measures analysis of variance (ANOVA) revealed no effect of diet ($P = 0.503$), exercise ($P = 0.390$) or interaction (diet × exercise $P = 0.754$); thus, the level of *GAPDH* mRNA was not influenced by either WP or RE and was therefore suitable as an internal control. All reactions were performed in 96-well MicroAmp Optical plates. Each well contained 1 μL of the diluted cDNA, 6 μL of Power SYBR® Green PCR Master Mix, 0.48 μL of the corresponding primers and probes for each gene, and 4.52 μL of sterile dH_2O in a final volume of 12 μL per well. All samples were run in triplicate. The cDNA was initially denatured at 95°C for 5 min, followed by 40 PCR cycles consisting of denaturation at 95°C for 20 sec and primer annealing and extension at 55°C for 30 sec. The cycle threshold (C_t) values were averaged, and the ΔC_t values (target gene C_t minus *GAPDH* C_t) were calculated for each group. Additionally, the $\Delta\Delta C_t$ values (ΔC_t from the sample group minus ΔC_t from the control group) were calculated, and mRNA amplification was determined as $2^{-\Delta\Delta Ct}$ [16], with the results expressed as percent of the control.

Statistical analysis

All variables were tested for normal distribution using the Shapiro-Wilk test ($P > 0.05$). Data were analyzed by two-way ANOVA; the classification variables were diet (CS + CE × WS + WE), exercise (CS + WS × CE + WE), and the interaction between diet and exercise (CS × CE × WS × WE). Tukey's post hoc test was used to determine the differences between the four groups when a statistically significant diet × exercise interaction was observed. SigmaPlot version 11.0 was used for these analyses.

Results

Body weight gain and muscle weights

Body weight gain and gastrocnemius and EDL muscle weights were similar in all groups except for CE rats, which exhibited the lowest values. Although WE rats exhibited body and muscle weights similar to those of the CS and WS rats, these values were higher than those observed in CE rats (Table 3).

Food intake and feeding efficiency

During the experiment, food intake was slightly lower in exercised rats compared with sedentary rats and was not modified by WP ingestion. Additionally, WP ingestion promoted better feeding efficiency (Table 3).

Real-time PCR

The results of the expression of genes related to muscle protein synthesis and/or degradation are presented in Figures 1–3. The results indicate that RE induced a significant decrease in *mTOR* mRNA expression, whereas WP ingestion prevented the RE-mediated reduction in gene expression. WE animals exhibited *mTOR* mRNA expression levels similar to those in the CS and WS groups, but this expression was significantly higher than that in CE rats (Fig. 1). The mRNA expression of *MuRF-1* was significantly reduced by RE but not by WP ingestion (Fig. 2), whereas *MAFbx* mRNA expression was significantly decreased only in WP animals independent of RE (Fig. 3).

Discussion

The present study evaluated the influence of RE alone or in combination with WP ingestion on the gene expression of proteins related to muscle protein synthesis (*mTOR*) and degradation (*MuRF-1* and *MAFbx*) and aimed to determine how these adaptations affect body and muscle weight gain in rats. WP ingestion prevented the reduction of *mTOR* mRNA expression induced by RE and significantly reduced the expression of *MAFbx* mRNA in sedentary and resistance-exercised rats. As a result, rats exhibited different patterns of body and muscle weight gain.

The mTOR kinase plays a central role in a signaling pathway involved in muscle protein synthesis, where it transduces signals to eukaryotic initiation factor 4 E binding protein 1, p70 S6 k, or a combination of the two. Activation of p70 S6 k results in activation of ribosomal protein S6, which enhances mRNA translation, including mRNA involved in muscle protein synthesis [1,2]. Interestingly, RE resulted in lower body weight gain, suggesting that under controlled conditions, RE could decrease muscle mass; this behavior is commonly observed in rats subjected to different types of exercise [17,18], and data from the present study suggest that this effect is related to

Table 3
Initial body weight, food intake, body weight gain, gastrocnemius and extensor digitorum longus (EDL) weights, and feeding efficiency in the experimental groups

Variables	Experimental groups				*P*-value		
	CS	CE	WS	WE	D	E	D × E*
Initial body weight (g)	114 ± 16	113 ± 9	114 ± 8	113 ± 9	0.957	0.933	0.975
Food intake (g)	764 ± 38	675 ± 43	754 ± 74	701 ± 61	0.689	0.001	0.363
Body weight gain (g)	182 ± 9.6[a,†]	150 ± 16.8[b]	198 ± 19.8[a]	203 ± 33.3[a]	<0.001	0.026	0.032
Gastrocnemius (mg)	1734 ± 140[a]	1526 ± 98[b]	1667 ± 187[a]	1697 ± 109[a]	0.297	0.078	0.021
EDL (mg)	135 ± 7[a]	121 ± 7[b]	137 ± 10[a]	138 ± 9[a]	0.037	0.001	<0.001
Feeding efficiency[‡]	23.83 ± 0.92	22.92 ± 1.60	26.33 ± 1.27	26.75 ± 4.41	0.001	0.779	0.454

CE, control exercised; CS, control sedentary; D, diet; E, exercise; EDL, extensor digitorum longus; WE, whey protein exercised; WS, whey protein sedentary
Values are expressed as the mean ± SD, n = 8 rats/group
Statistical analysis was performed using a two-way ANOVA with a Tukey post hoc test
* D × E is the interaction between the corresponding parameters.
† Within a row, significantly different values are marked with different superscript letters when a significant interaction was observed ($P < 0.05$).
‡ Feeding efficiency = (weight gain/daily intake) × 100.

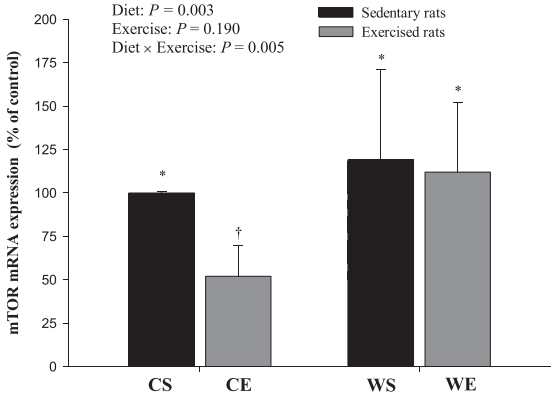

Fig. 1. *mTOR* mRNA expression in gastrocnemius muscle of control sedentary (CS), control exercised (CE), whey protein sedentary (WS), and whey protein exercised (WE) groups after 8 wk. Results are expressed as the mean ± SD; n = 6/group; statistical analysis was performed using a two-way ANOVA with a Tukey post hoc test. Significantly different values are marked with superscript symbols when a significant interaction was observed ($P < 0.05$). mTOR, mammalian target of rapamycin.

differences in *mTOR* mRNA expression. In fact, the inhibitory effects of acute exercise on the mTOR signaling pathway and consequently on protein synthesis in skeletal muscle during exercise were previously reported [19,20]. One possible mechanism appears to involve regulated in DNA damage and development (REDD1) protein, an upstream negative effector of the mTOR-signaling pathway. It has been observed that an acute bout of endurance exercise induced *REDD1* mRNA expression, suggesting that REDD1 is involved in inhibiting skeletal muscle protein synthesis during exercise [20]. Although *REDD1* mRNA was not evaluated in the present study, the reduction in *mTOR* expression induced by RE reported here could be related to modification in *REDD1* mRNA. Moreover, because both CE and WE rats performed the same RE protocol, differences in the dietary protein ingested could explain the differences observed in *mTOR* gene transcription between these two groups.

WP is a high-quality protein source, containing large amounts of branched-chain amino acids (BCAA), mainly leucine, a crucial signal for mTOR activation [2,21]; however, the exact mechanism by which leucine activates and/or induces *mTOR* mRNA

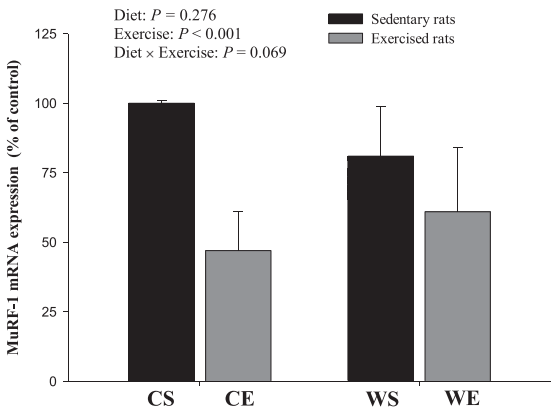

Fig. 2. MuRF-1 mRNA expression in the gastrocnemius muscle of control sedentary (CS), control exercised (CE), whey protein sedentary (WS), and whey protein exercised (WE) groups after 8 wk. Results are expressed as the mean ± SD; CS n = 6, CE n = 6, WS n = 4, and WE n = 5; statistical analysis was performed using a two-way ANOVA with a Tukey post hoc test. MuRF-1, muscle-specific RING finger-1.

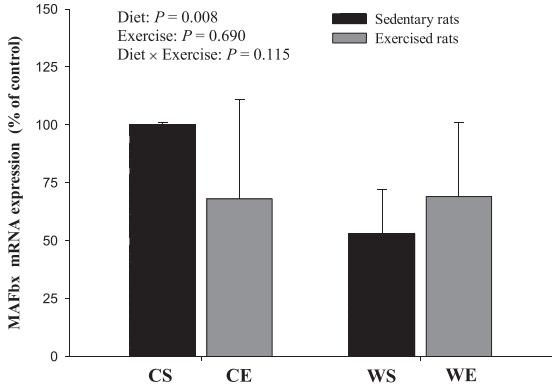

Fig. 3. *MAFbx* mRNA expression in the gastrocnemius muscle of control sedentary (CS), control exercised (CE), whey protein sedentary (WS), and whey protein exercised (WE) groups after 8 wk. Results are expressed as the mean ± SD; n = 6/group; statistical analysis was performed using a two-way ANOVA with a Tukey post hoc test. MAFbx, muscle atrophy F-box.

transcription remains unknown [22]. Moreover, differences in the amino acid composition of both proteins (casein as a control and WP) cannot entirely explain the difference in weight gain between the CE and WE groups because casein provides the minimum requirements for a rodent diet (Table 2), and no significant differences in body and muscle weight gain were observed between the CS and WS groups. Although WP contains deficient levels of phenylalanine and methionine, it has been demonstrated that WP fulfills more biological requirements than casein, exhibiting a greater digestibility, protein efficiency ratio, and net protein ratio, and it contains different bioactive peptides that can positively affect the utilization of protein for growth [23]. These benefits could also explain the better feeding efficiency observed with WP-based diets (Table 3). Additionally, when compared with casein, WP digestion/absorption kinetics promotes a blood leucine peak, resulting in different effects on protein turnover [24,25]. When associated with RE, WP increases muscle protein synthesis and fat-free body mass [26,27] in response to mTOR phosphorylation [10] and, as reported in the present study, in response to *mTOR* mRNA transcription.

Muscle protein degradation is primarily controlled by the UPS pathway [3] and contributes to protein net balance and consequently to muscle growth. Various results regarding the effects of RE on *MAFbx* and *MuRF-1* mRNA expression have been reported [3,11–13]; however, the effect of WP ingestion on RE-induced mRNA expression is poorly described. A single bout of RE was reported to increase *MuRF-1* mRNA expression [11,12], whereas chronic RE, as performed in the present study, decreases *MuRF-1* transcription, most likely in response to a phenomenon described as “training adaptation” [13]. However, it is unclear if the reduction in *MuRF-1* mRNA induced by RE would improve protein net balance and, consequently, muscle growth [2]. Data from the present study do not support this hypothesis. In fact, previous studies suggest that *MuRF-1* interacts with structural proteins, including myosin and titin [5,28]. Therefore, it is reasonable to speculate that RE would reduce the degradation of contractile proteins by down-regulating *MuRF-1* mRNA expression, but not regulatory proteins involved in protein synthesis, which seem to be targets of *MAFbx* [29]. However, CE rats exhibited a significant lower body weight gain compared with sedentary rats, likely resulting from lower food ingestion by exercised rats throughout the experiment (Table 3). Another possibility is that if RE increased proteolysis by both *MAFbx* and *MuRF-1* in CE rats, these E3 ligases might have been regulated by

RE at the translational level or even post-translationally and not at the mRNA transcription level.

MuRF-1 mRNA expression was not affected by WP ingestion. Although dietary protein types can affect the expression of genes related to protein breakdown [30], E3 ligases appear to be up-regulated only when dietary proteins with limited essential amino acids are provided [30]. However, gene expression of E3 ligases is not modified after the ingestion of high-quality dietary proteins, including soy [30]; after BCAA/leucine supplementation [31,32]; or, as demonstrated here, after WP ingestion. Notably, WE rats exhibited higher body and muscle weight gain compared with CE rats, although no significant differences in food ingestion between these two groups were observed. These data suggest that despite the decrease in *MuRF-1* mRNA induced by RE, modifications in *mTOR* gene expression induced by RE and WP were more relevant to the differences observed in body and muscle weight gain.

WP significantly reduced muscle *MAFbx* mRNA expression in both sedentary and RE rats. The different response to RE on *MAFbx* mRNA levels reported in the present study is in agreement with most previous studies [11,13,31]. However, the effect of WP on the transcription of E3 ligases is unclear. Previous studies involving the ingestion of BCAA or leucine alone and RE revealed that protein degradation of subjects at rest or of RE subjects is reduced [31,32]. Recently, it was reported that BCAA supplementation reduced the expression of *MAFbx* mRNA but not of *MuRF-1* mRNA in both resting and exercised participants [31]. The distinct effects of RE and WP ingestion on *MAFbx* and *MuRF-1* mRNA expression observed in the present study, and found by others [31,32], suggest that dietary proteins rich in leucine content have a strong influence on *MAFbx* mRNA expression independent of RE performance. Although no significant interaction between RE and WP in *MAFbx* gene expression was observed, a reduction in *MAFbx* mRNA induced by WP diets was associated with rats that exhibited significantly higher body and EDL muscle weight gain (Table 3).

Nutritional strategies to improve muscle mass, reduce muscle atrophy, or both are particularly important in older individuals, where sarcopenia is commonly observed and skeletal muscle protein synthesis in response to food intake is impaired [33]. Recent evidence suggests that WP could stimulate muscle protein synthesis in older individuals subjected to RE more than other protein sources [34]. The data reported here are in agreement with these findings and suggest that WP may represent an effective nutritional aid to favor muscle anabolism in this clinical condition, confirming its health-promoting benefits. However, this study had the following limitations:

1. A limited number of animals (n = 4–6) was used to determine *MuRF-1* mRNA expression.
2. The effect of RE and WP ingestion on muscle *mTOR*, *MuRF-1*, and *MAFbx* mRNA expression of Fischer rats observed in the present study may not reflect the same response in human muscle.
3. Further studies involving mRNA expression of others genes, such as upstream and downstream targets of mTOR and E3 ligases, in a larger number of animals could help to clarify the complex network of genes involved in the regulation of muscle growth.

Conclusion

In this study, we demonstrated that WE rats exhibited higher body and muscle weights gains compared with CE rats after 8 wk. The reduction of *MAFbx* mRNA transcription induced by WP ingestion and the interaction between RE and WP in *mTOR* mRNA expression contributed significantly to these differences.

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