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L-Leucine supplemented whey protein. Dose–response effect on heart mTOR activation of sedentary and trained rats

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ABSTRACT

The aim of the study was to investigate the effect of leucine supplementation combined with exercise and whey protein in cardiac mTOR anabolic pathway. Ninety-six weanling male Wistar rats were divided into eight groups and fed diets containing either casein or WP plus increasing levels (0, 3, 4.5 and 6% of diet) of L-leucine for 30 days. A parallel set of eight groups was exercised for comparison. Serum aspartate amino transferase, alanine aminotransferase, creatine kinase, lactate dehydrogenase and branched chain amino acids were determined by standard methods, and mammalian target of rapamycin (mTOR) and p70S6K by the Western blot analysis. Chronic L-leucine supplementation was capable of increasing both mTOR and p70S6K phosphorylation in the heart in a dose-dependent fashion, independent of the type of dietary protein in both groups, sedentary and exercised, but the exercise potentialized the activation of the anabolic pathway. The content of protein in heart increase with L-leucine supplementation and the heart mass relativized by body mass did not change. In conclusion, the combination of L-leucine and milk proteins (casein or whey protein) has the potential to increase the mTOR pathway in the cardiac muscle without increasing the heart mass. The novelty of this study is to show the effectiveness of a blend of leucine and whey protein as a viable alternative to maximize the activation of the anabolic pathway of cardiac muscle and that the exercise can improve this process.

1. Introduction

Cardiac hypertrophy involves increased heart size due to increased cardiomyocyte size. This is initially an adaptive response to an increased workload or to defects in the efficiency of the contractile machinery, but in the long term it contributes to the development of heart failure and sudden death (Frey & Olson, 2003). Increased protein synthesis is a key feature of cardiac hypertrophy and probably underlies the increased cell and organ size observed under this condition (Hannan, Jenkins, Jenkins, & Brandenburger, 2003). Several other features are also seen, including changes in gene expression and reorganization of the contractile machinery, and it should be borne in mind that the protein accumulation characteristic of hypertrophy may reflect effects on protein breakdown as well as protein synthesis. Heart hypertrophy is linked to activation of mTOR pathway, and this fact can improve the muscle function, which is a common process in athletes. On the other hand, the pathological hypertrophy is associated with an activation increase of calmodulin, which is associated with loss-of-function of the heart muscle (McMullen & Jennings, 2007).

L-Leucine is a branched-chain amino acid that has been the subject of much investigation and is considered capable of stimulating cell protein synthesis (Anthony, Anthony, Kimball, & Jefferson, 2001; Drummond & Rasmussen, 2008). Nevertheless, its effectiveness has been reported to fail under such situations as sepsis, alcohol intoxication and high (excess) glucocorticoid levels in old rats (Anthony & Anthony, 2008). The attenuation of in vivo skeletal muscle proteolysis by L-leucine has also been described, but is less documented than for protein synthesis. Anthony, Anthony, and Layman (1999) reported that L-leucine increased skeletal muscle protein synthesis when given alone to rats following exercise. The main mechanism by which L-leucine exerts its protein synthesis stimulating function is by activating mTOR, a key protein in the process of cell proliferation (Proud, 2007). Once activated, mTOR phosphorylates and activates S6K1 and 4E-BP1 is inactivated. Activation of p70s6k is linked to preferential translation of messenger RNAs containing a polypyrimidine stretch in their 5′ untranslated region. While, the phosphorylation of 4E-BP1 decreases its binding to eIF4E and release of 4E-BP1 from

Abbreviations: ALT, alanine aminotransferase; AST, aspartate amino transferase; BCAAs, branched-chain amino acids; CAS, casein; CK, creatine kinase; ISO, L-isoleucine; LDH, lactate dehydrogenase; LEU, L-leucine; mTOR, mammalian target of rapamycin VAL — L-valine; WP, whey protein.

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eIF4E allows eIF4E to become part of the translation complex eIF4F. Active eIF4E is associated with higher translation of RNAs, thus increasing the cell capacity to synthesize proteins (Proud, 2007). Besides the anabolic effect gained through the activation of mTOR, L-leucine also stimulates insulin secretion (Bolster, Kimball, & Jefferson, 2003), thereby giving a further boost to anabolism. There is also evidence that supplementation with L-leucine for prolonged periods of time may have an anti-catabolic effect in skeletal muscle (Sugawara, Ito, Nishizawa, & Nagasawa, 2007).

The principal effects of the activation of protein synthesis by L-leucine have been described for skeletal muscle, and its characteristics are maintained even in animals undergoing training (Anthony et al., 2001). Despite this, little attention has been given to the effects of this supplementation on the cardiac muscle. It is well known that high quality proteins can stimulate protein synthesis at higher rates than lower quality proteins (Yoshizawa, Kido, & Nagasawa, 1999). The whey proteins possess high nutritive quality and a high speed of digestion and absorption, thus providing a fast and profuse supply of amino acids and small peptides (Morato et al., 2013), with a significant impact on the turnover of body proteins. For this reason, whey proteins have been recognized as promoting a positive metabolic balance, being classified by some researchers as rapid metabolism proteins (Fruhbeck, 1998), making them attractive for situations of metabolic stress, including that caused by physical exercise (de Moura, Lollo, Morato, Carneiro, & Amaya-Farfan, 2013), muscle mass loss (Lollo et al., 2012) and immune deficiencies, among others (Marshall, 2004). It may be relevant to note that the concentrations of BCAA in whey proteins, including L-leucine, are high. Altogether, BCAAs account for 21.2% of the amino acids that make up the whey proteins, or exactly 50% of the total of indispensable amino acids therein, a feature that is shared with few other proteins besides the skeletal muscle proteins (Etzel, 2004; Ha & Zemel, 2003). Hulmi, Lockwood, and Stout (2010) also reported that chronic (21 weeks) supplementation with whey proteins favored resistance training-induced muscle hypertrophy in young healthy individuals as compared to a non-energetic placebo. These data suggest that chronic 'high-quality protein' supplementation is able to improve exercise-induced muscle mass gain and performance.

Elucidating the signaling connections underlying cardiac hypertrophy is important both for our fundamental understanding of the process and for developing potential therapeutic strategies for this condition, which is a major risk factor for cardiac failure. This is a vigorous research area and several signaling pathways have been implicated in hypertrophic responses in cardiomyocytes (Frey & Olson, 2003; Molkentin & Dorn, 2001). The effect of the L-leucine plus whey protein combination in skeletal muscle is clear, hypertrophy, but not in the cardiac muscle. The aim of the study was to investigate the effect of leucine supplementation combined with exercise and whey protein on a cardiac protein synthesis activation (mTOR anabolic pathway).

2. Methods

2.1. Design

Male Wistar (21-day old, specific-pathogen free; n = 96) rats, bred in the Multidisciplinary Center for Biological Research, University of Campinas, SP, Brazil, were housed (~22 °C, 55% RH, inverted 12-hour light cycle) in 96 individual growth cages (one per animal), with free access to commercial chow (Labina, Purina, Brazil) and water at all times, until they weighed 120.2 ± 4.7 g. The animals were randomly assigned to any of sixteen groups according to the protein source in the diet, L-leucine supplementation and whether they were exercised or sedentary (Fig. 1). The research methodology was approved by the Ethics Committee on Animal Experimentation (CEEA-UNICAMP, protocol 1835-1).

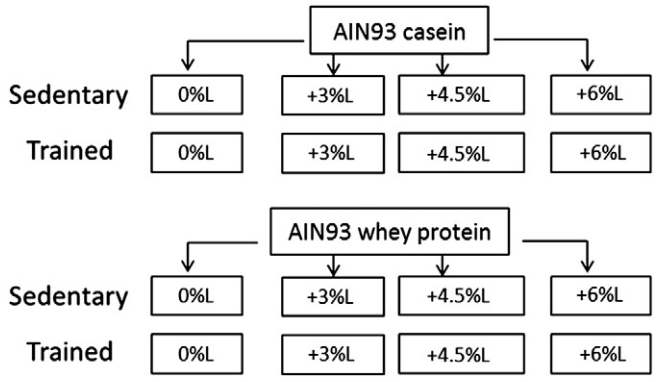

Fig. 1. Distribution of groups (n = 6). The AIN93-G WP diet was made by substituting the whey protein (WP) for the casein (CAS) of the AIN93 (standard) diet. The controls contained no added L-leucine.

2.2. Training protocol

The rats were selected beforehand on the treadmill, considering as not fit those that remained without departing from the baseline for 30 s. The animals began training on the day they started to consume the experimental diets, and continued for 4 weeks following the progressive protocol of Hohl et al. (2009), each week had five training sessions, and one session per day between 01:00 and 03:00 pm, the training sessions was, week 1): 20 min at 15 m/min; week 2): 30 min at 20 m/min; week 3): 45 min at 22.5 m/min; week 4): 60 min, at 25 m/min.

2.3. Experimental diets

The experimental diets were isonitrogenous (approximately 17% protein, dry basis), isolipidic and isocaloric (approximately 360 kcal/100 g), formulated following the recommendations of the American Institute of Nutrition, AIN93 (for growing rats) diet (Reeves, Nielsen, & Fahey, 1993). The two diets differed with respect to the nature of the protein source, casein or whey protein (Table 1), the L-leucine added was at least 99.7% pure (Ajinomoto, São Paulo). All the animals received the diets ad libitum.

2.4. Protein extraction and immunoblotting

The samples were prepared by homogenizing ~100 mg of frozen tissue in Triton buffer (100 mM Tris, pH 7.4, 1% Triton X-100) containing 100 mM sodium pyrophosphate, 100 mM NaF, 10 mM EDTA, 10 mM Na3Vo4, 2 mM PMSF and 0.1 mg/ml aprotinin (Lollo et al., 2012). The total protein content of the heart muscle was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). For immuno blotting, tissue homogenates were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane, using a wide Biocom Western blot system (Bridge of Weir, UK). The blots were probed with the appropriate antibodies to determine the total mTOR (dilution 1:1000, ref#2972) and phosphor-mTOR (Ser 2448, dilution 1:1000, ref#2971S) using Cell Signaling Technology (Danvers, MA) and total p70S6K (dilution 1:1000, ref#sc-9379) and phosphor-p70S6K (Thr389, dilution 1:1000, ref#sc-11759) using Santa Cruz (Santa Cruz, CA). Beta-actin was the loading control (dilution 1:2000, ref#ADI-905-733-100), obtained from Enzo Life Sciences (Farmingdale, NY), and used to assess the level of the proteins in the heart tissue. The appropriate secondary antibody conjugated to peroxidase and the BM chemiluminescence blotting system was used for detection. The bands were visualized by chemiluminescence (GE — I mageQuant LAS4000, Piscataway, NJ, USA) and the band intensities were quantified by processing with the program ImageJ (v. 1.47J for Windows).

Table 1
Composition of the diets (g/kg diet).

Ingredient	AIN93-G (casein)				AIN93-G (whey protein)			
	Control 0%L	+3%L	+4.5%L	+6%L	Control 0%L	+3%L	+4.5%L	+6%L
Leucine added	–	30	45	60	–	30	45	60
Corn starch	403.4	373.4	358.4	343.4	407.3	377.3	362.3	347.3
Casein (87.6% protein)	194.1	194.1	194.1	194.1	–	–	–	–
WP (89.4% protein)	–	–	–	–	190.2	190.2	190.2	190.2
Dextrinized corn starch	132	132	132	132	132	132	132	132
Sucrose	100	100	100	100	100	100	100	100
Soybean oil	70	70	70	70	70	70	70	70
Fiber	50	50	50	50	50	50	50	50
Mineral mix	35	35	35	35	35	35	35	35
Vitamin mix	10	10	10	10	10	10	10	10
L-cystine	3	3	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Tert-butylhydroquinone	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014

The AIN93-G WP diet was made by substituting the whey protein for the casein of the AIN93 (standard) diet plus leucine (L) added.

2.5. Biochemical parameters

Kits for blood sampling. Six hours after the training session, blood samples were collected in Vacutainers, kept at 4 °C, and then centrifuged at 3000 ×*g* (4 °C, 12 min) to obtain the serum. The following determinations were carried out to assess the sera: uric acid, aspartate amino transferase (AST), alanine aminotransferase ALT, creatine kinase (CK) and lactate dehydrogenase (LDH). Standard enzymatic spectrophotometric determinations of glucose and cholesterol were also carried out employing Laborlab kits (São Paulo, Brazil).

2.6. Determination of plasma free amino acids

Serum free amino acids were extracted with methanol and derivatized with phenylisothiocyanate (White, Hart, & Fry, 1986) and the PTH-derivatives chromatographed using a Luna C-18, 100 Å; 5 μ, 250 × 4.6 mm (00G-4252-EQ) column, at 50 °C, and quantified by comparison with a standard mixture using DL-2-aminobutyric acid as the internal standard (Sigma-Aldrich Corp, St Louis, MO, USA). The free amino acids were extracted in 80% ethanol and 0.1 M HCl, with 500 μL of 2-aminobutyric acid added as the internal standard. The mixture was sonicated for 10 min and further homogenized for 1 h, followed by centrifugation at 8500 ×*g* for 15 min. The supernatant was filtered through a 0.22 mm membrane and a 40 μL aliquot derivatized as described above for the subsequent injection of 20 μL into the liquid chromatograph.

2.7. Statistical analysis

The results were subjected to a statistical analysis using the SPSS software (Statistical Package for the Social Sciences), version 17.0. The data were tested for normality (Kolmogorov–Smirnov test) and homogeneity using the tools available therein. For parametric data, the two way multivariate analysis of variance (ANOVA) was used and the means compared (Duncan test), adopting the value of $P \leq 0.05$ as a criterion for statistical significance.

3. Results and discussion

Cardiac hypertrophy is an increase in heart mass and is a poor prognostic sign, being a characteristic of most forms of heart failure. On the other hand, in athletes, cardiac hypertrophy is known as a positive physiological hypertrophy and is associated with heart health improvements. Signaling pathways play unique roles in the regulation of pathological and physiological cardiac hypertrophy, mTOR activation is associated with physiological hypertrophy while calmodulin pathway is linked to pathological hypertrophy (McMullen & Jennings, 2007). In general, supplementing both casein and the whey protein with L-leucine promoted the expression of mTOR total and phosphorylated and p70S6K total and phosphorylated in the heart muscle, at the level of 4.5% and 6% (Figs. 2 and 3). However, the degree of response varied according to whether the protein was casein or whey protein, and to whether the animal belonged to a sedentary or trained group. The supplementation with L-leucine by itself can increase the activation of the mTOR pathway (CAS plus leucine supplementation), this result is coherent with that of other authors (Sanchez Canedo et al., 2010; Suryawan et al., 2012). This result was similar with whey protein like source of protein of the diet.

For instance, supplementation of the casein-trained group (Fig. 2B) with 4.5% L-leucine resulted in an increment in mTOR, however, at the 6% level the increment decreased, although it was still above the basal level. The mTOR activation observed in the sedentary groups supplemented with casein followed a similar pattern (although slightly higher) as compared to the trained groups (Fig. 2E–H), suggesting that the supplementation with L-leucine is more effective in the mTOR phosphorylation of sedentary rats. The mTOR activation is associated with physiological hypertrophy, which is a beneficial event for the heart. The training alone exerted the activation of the mTOR pathway, so trained animals had a higher level of mTOR activation as compared to sedentary rats. Furthermore, the supplementation with L-leucine slightly increased the mTOR phosphorylation. Perhaps the activation of signaling pathways of protein synthesis in healthy animals, for a long period, would cause a negative feedback and an increase in the activation of catabolic pathways, such as the ubiquitin proteosome pathway. This would be a possible explanation for the fact that the heart mass remains unchanged even with greater activation of anabolic pathways.

With regard to the stimulatory effect of supplementation on the expression of the phosphorylated form of mTOR (S2448), it was observed that regardless of the type of protein, the effect only showed a tendency to peak with the addition of 4.5% in the sedentary groups, whereas in the trained groups, the effect increased with every successive addition (Fig. 2), different of diaphragm response magnitude (Lollo et al., 2012). In the heart, mTOR is an important regulator of cardiac hypertrophy and rapamycin, an inhibitor of mTOR, can attenuate load-induced cardiac hypertrophy in mice (Shioi et al., 2003). L-leucine is a very effective amino acid with an insulinotropic effect (Anthony et al., 2002), and the insulin can increase the activation of mTOR on serine 2448, leading to increased protein synthesis via phosphorylation of the downstream targets p70S6K and 4EBP1 (Proud, 2007). The over-expression of Akt/PKB by insulin, an upstream regulator of mTOR, results in cardiac hypertrophy (Shioi et al., 2003), and the regulation of cell size by Akt is thought to be mediated by its phosphorylation and by the subsequent downstream phosphorylation of mTOR on serine 2448.

The expression of phosphorylated p70 S6 kinase seemed to be higher in animals supplemented with WP as compared to those supplemented

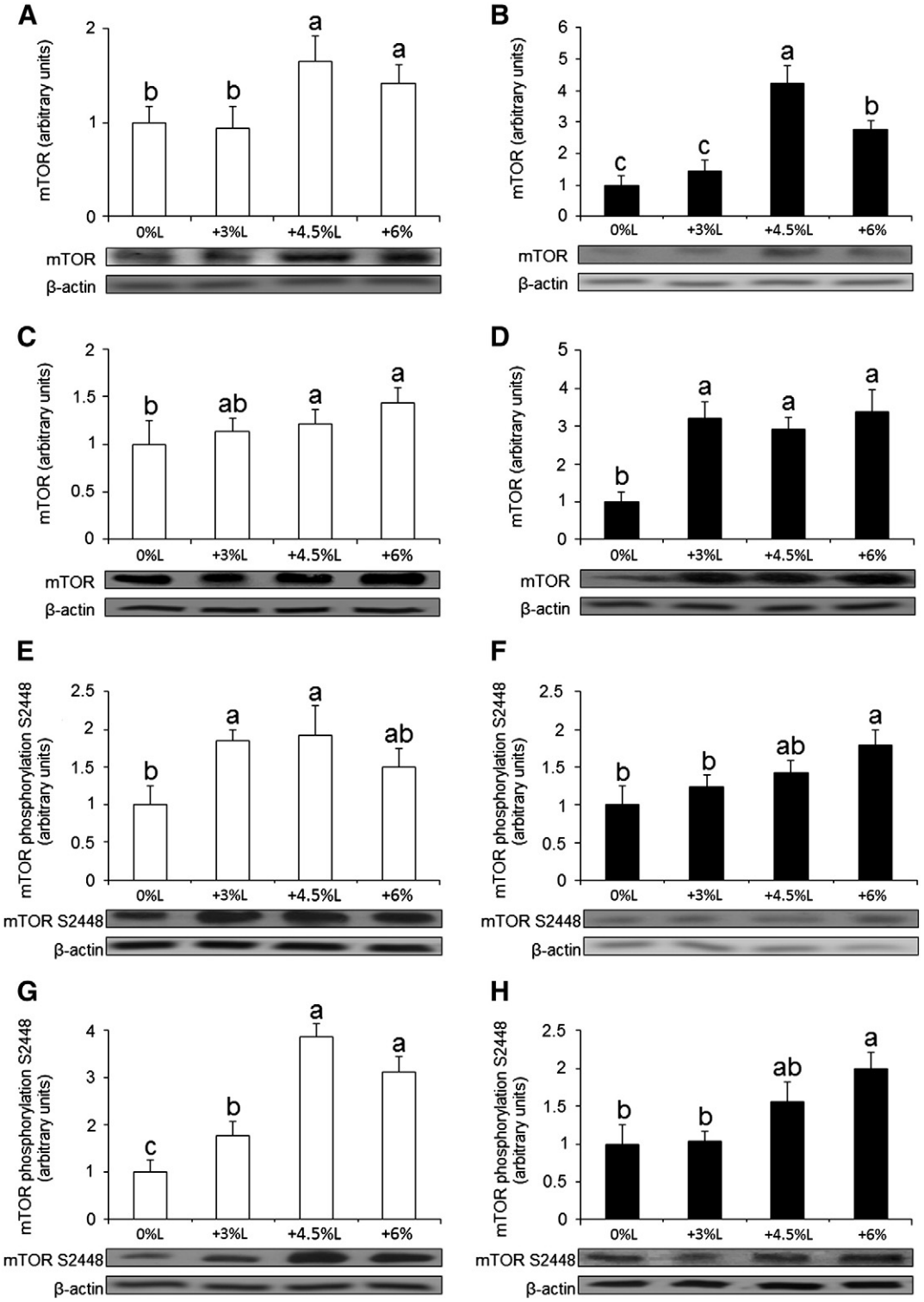

Fig. 2. mTOR and phosphorylated (S2448) mTOR response for casein (CAS [A, B, E and F]) and whey protein (WP [C, D, G and H]) to the three degrees of supplementation with L-leucine in both the sedentary □ (A, C, E and G) and trained ■ (B, D, F and H) groups (n = 6). No different capital letters above the bars denote no significant differences between diets– exercise, different small letters indicate significant differences between groups, two-way ANOVA was used and means were compared (Duncan test), adopting the value of $P < 0.05$ as a criterion for statistical significance.

with casein, and this followed a dose-dependent trend in both trained and sedentary groups (Fig. 3F and 3G), while the sedentary group supplemented with casein reached the peak of activation with 4.5% of L-leucine. The peak of activation occurred when 6% was supplemented for trained rats (for both WP and casein groups). We observed that the increased activation of p70 occurred in trained animals, suggesting that supplementation with L-leucine is more effective if given to animals subjected to exercise, and these data are consistent with the results of heart mass/body mass (Fig. 4A) and heart protein/body mass (Fig. 4D). All this information demonstrates that there is a significant anabolic effect of training associated with L-leucine supplementation on markers of cardiac hypertrophy. In animal models, L-leucine was shown to have a stimulatory effect on protein synthesis, independent of insulin (Anthony et al., 1999; Anthony et al., 2002). Anthony et al. (2002)

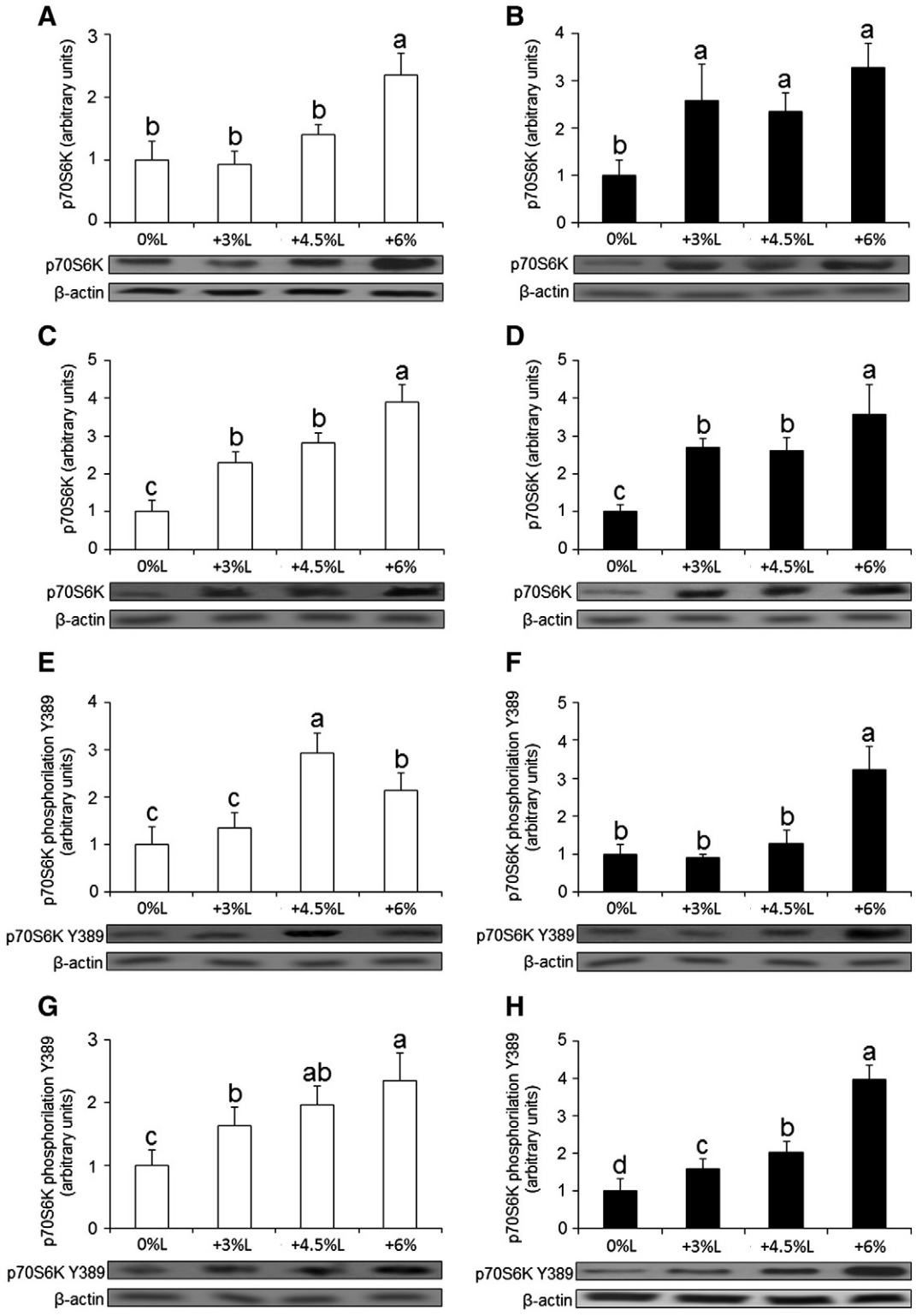

Fig. 3. p70S6K and p70S6K phosphorylated (Y389) response for casein (CAS [A, B, E and F]) and WP (whey protein [C, D, G and H]) to the three degrees of supplementation with L-leucine in both the sedentary □ (A, C, E and G) and trained ■ (B, D, F and H) groups (n = 6). CAS: casein like source of protein of the diet; WP: whey protein like source of protein of the diet; +3: diet with 3% of L-leucine; 4.5%: diet with 3% of L-leucine; 6%: diet with 3% of L-leucine. No different capital letters above the bars denote no significant differences between diets–exercise, different small letters indicate significant differences between groups, two-way ANOVA was used and means were compared (Duncan test), adopting the value of $P < 0.05$ as a criterion for statistical significance.

showed that the infusion of L-leucine resulted in the stimulation of protein synthesis in rats, even if the concentration of insulin in the blood was maintained low by the concomitant infusion of somatostatin. Thus the data presented suggest that although L-leucine itself can stimulate the release of insulin, the greater part of the anabolic effect is probably not due to the action of insulin (Anthony et al., 2002).

Finally, it is noteworthy to observe the high concentrations of BCAA, including L-leucine, present in the whey proteins. Altogether,

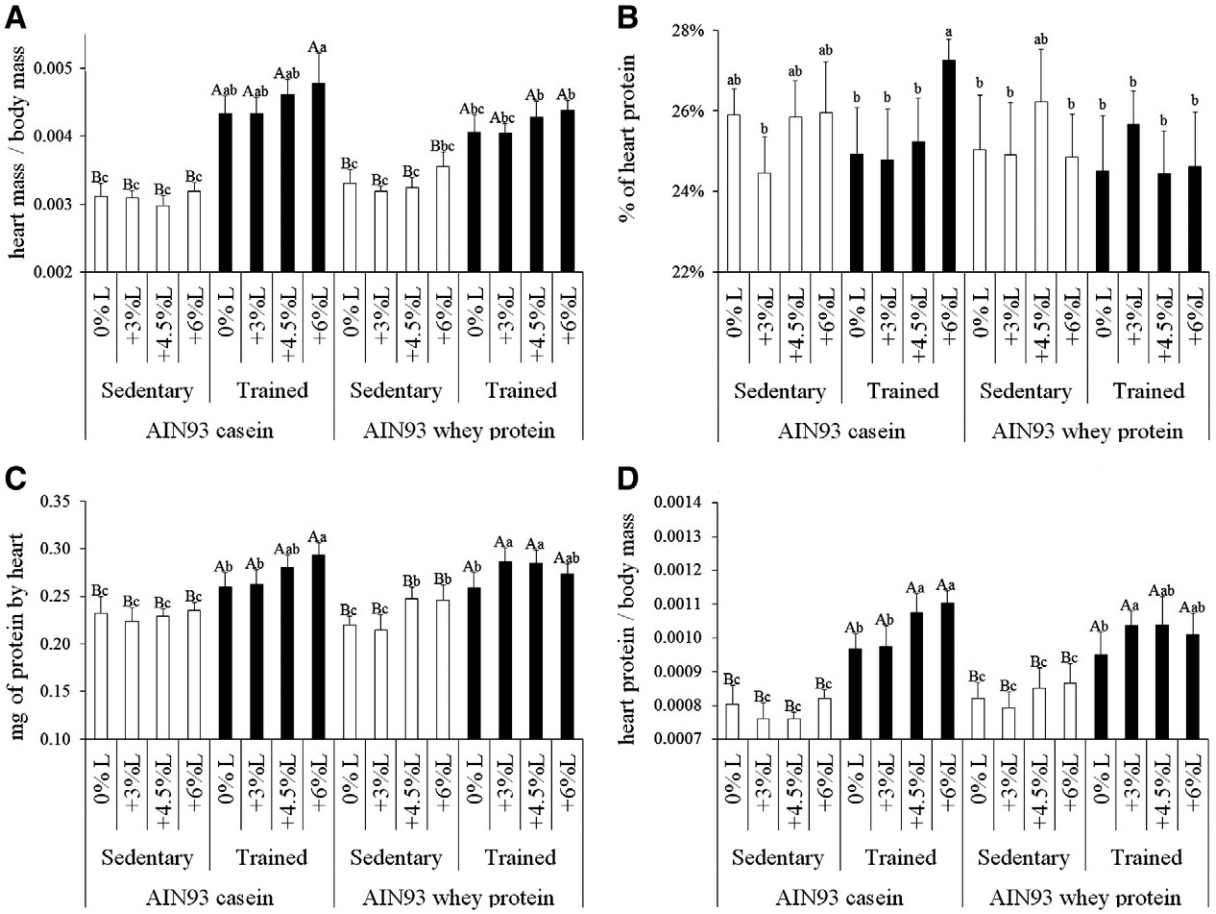

Fig. 4. Response of heart mass and protein contend, total and relativized for body mass of sedentary □ and trained ■ (n = 6). Casein (CAS): casein like source of protein of the diet; whey protein (WP): whey protein like source of protein of the diet; +3: diet with 3% of L-leucine; 4.5%: diet with 3% of L-leucine; 6%: diet with 3% of L-leucine. No different capital letters above the bars denote no significant differences between diets–exercise, different letters indicate significant differences between groups, two-way ANOVA was used and means were compared (Duncan test), adopting the value of $P < 0.05$ as a criterion for statistical significance.

BCAAs account for 21.2% of the amino acids that make up the whey proteins, or exactly 50% of the total of indispensable amino acids contained therein. This signifies that BCAAs are predominantly contained in these proteins in contrast to most other proteins (Etzel, 2004). This particular feature of the whey proteins means that they approach the amino acid profile of the skeletal muscle proteins (Ha & Zemel, 2003).

Another characteristic of the whey proteins is their capacity to stimulate the secretion of the anabolic hormone insulin into the bloodstream, thereby increasing the transport of amino acids into the cell and setting up the necessary conditions for protein synthesis (Calbet & MacLean, 2002). L-leucine has been proved to reduce lyzosomal and ATP-dependent protein degradation (Busquets et al., 2000).

At the cellular level, cardiomyocyte hypertrophy is characterized by an increase in cell size, enhanced protein synthesis and heightened organization of the sarcomere. Hypertrophic growth accompanies many forms of heart disease, including ischemic disease, hypertension, heart failure and valvular disease (Frey, Katus, Olson, & Hill, 2004), and ventricular hypertrophy is associated with a significantly increased risk of heart failure and malignant arrhythmia (Koren, Devereux, Casale, Savage, & Laragh, 1991). The body-mass adjusted lean heart mass increased as the body mass departed from a minimum of about 300 g, while in turn, the body mass varied between the set extremes. This function also showed that the mass of the heart was more influenced by a high body mass than by a high content of L-leucine in the diet. The minimum relative heart mass was found at around a body mass of 290 g and a total L-leucine intake of about 20 g/kg of diet. Overall, Fig. 4 shows that trained rats presented higher heart/body mass, signaling physiological hypertrophy, the content of protein in the heart (estimated by % of heart protein x mass tissue) suggests that the L-leucine supplementation improves this content in rats supplemented with 4.5% and 6% in trained animals that received casein in diet. It is not yet known what the significance of this outcome could be, but one possibility is that since increases in the total contents of L-leucine promote increases in body mass, beyond a certain point the corresponding increases in heart mass become disproportionately greater, with no associated bearings on the general health of the animals.

The consequences of supplementation on the activities of the AST and ALT enzymes, plus the serum levels of glucose, uric acid, creatinine and cholesterol were determined, in order to further assess the impact of the supplemental L-leucine in liver healthy parameters (AST and ALT), no alterations were observed. In addition, there were no alterations in the heart cell damage indicators, CK and LDH (Dawie, Chawla, Worku, & Azazh, 2011) (Table 2) suggesting no damage to the heart cells due to whey protein or L-leucine supplementation, suggestion of a safety strategy.

No significant difference was observed in serum insulin among groups (Table 2), but it must be noted that 12 hour fasted animals were used, that is, rats had no intake of L-leucine. It is known that the insulinotropic effect of insulin occurs within 2 or 3 h after ingestion of foods (Anthony et al., 2002), we emphasize that the skeletal muscle seemed to be more sensitive to the stimulatory effects of insulin than the cardiac muscle (Forsyth & Vary, 2008).

Table 2
Mean (M) and standard deviation (SD) of body mass gain and biochemical blood parameters (n = 6).

		AIN93 casein						AIN93 whey protein					
		Sedentary			Trained			Sedentary			Trained		
		0%L	+3%L	+4.5%L	+6%L	+3%L	+4.5%L	+6%L	0% L	+3%L	+4.5%L	+6%L	0% L	+3%L	+4.5%L	+6%L
Body mass gain[1]	M	201.3^{bcd}	219.7^{ab}	235.8^{a}	212.4^{bc}	202.0^{cd}	208.1^{bc}	202.2^{cd}	199.1^{cd}	191.4^{d}	209.5^{b}	227.1^{ab}	213.2^{b}	194.8d	208.7^{bc}	208.1^{bc}	215.1^{bc}
	SD	5.8	6.1	8.7	6.0	5.5	6.7	7.4	4.8	4.7	5.4	6.9	5.2	5.1	4.7	5.9	7.0
AST[2]	M	21.2	23.8	25.2	22.4	24.1	22.5	20.8	22.9	20.7	23.20	24.0	23.9	21.8	23.9	20.7	19.8
	SD	2.8	2.1	4.0	2.8	2.7	3.4	2.5	3.1	3.3	4.0	2.5	3.5	3.6	2.7	3.2	2.7
ALT[3]	M	68.1	75.4	69.8	70.8	67.1	65.1	62.9	65.9	70.3	74.1	73.2	71.2	70.9	72.8	68.5	72.1
	SD	6.4	7.4	8.1	5.9	7.5	9.2	7.2	6.8	12.1	10.5	9.6	7.4	5.9	6.9	7.2	11.0
CK[4]	M	389.1	400.1	405.6	398.8	412.8	419.1	405.4	408.5	399.8	407.4	402.2	399.7	410.5	417.5	408.5	403.5
	SD	20.4	22.9	19.8	28.1	25.4	18.7	24.1	23.8	17.4	15.9	22.4	21.5	25.1	29.5	26.4	21.3
LDH[5]	M	604.5	589.3	546.8	559.2	578.2	560.2	598.1	584.1	591.3	569.8	550.2	600.3	558.5	600.2	575.6	580.1
	SD	40.2	35.9	19.8	32.9	40.3	29.7	37.9	32.9	29.7	34.5	30.6	35.8	38.1	33.8	25.8	24.9
Insulin[6]	M	5.4	5.7	4.6	6.6	3.6	4.3	6.0	5.7	4.3	4.1	5.8	7.2	3.6	5.2	5.4	5.8
	SD	1.4	1.5	0.2	0.3	0.6	1.4	0.3	1.3	0.4	0.5	0.3	1.4	1.1	0.2	1.5	0.4
VAL[7]	M	0.191^{a}	0.150^{b}	0.101^{c}	0.095^{c}	0.190^{a}	0.157^{b}	0.127^{b}	0.108^{c}	0.205^{a}	0.161^{b}	0.134^{c}	0.101^{c}	0.221^{b}	0.154^{c}	0.132^{c}	0.95^{c}
	SD	0.11	0.08	0.09	0.11	0.12	0.09	0.08	0.6	0.14	0.08	0.09	0.07	0.16	0.11	0.09	0.10
ILE[8]	M	0.070^{a}	0.050^{b}	0.028^{c}	0.019^{c}	0.081^{a}	0.066^{b}	0.060^{b}	0.031^{c}	0.092^{a}	0.073^{a}	0.047^{c}	0.032^{c}	0.094^{a}	0.080^{b}	0.059^{b}	0.030^{b}
	SD	0.02	0.01	0.01	0.01	0.02	0.02	0.01	0.02	0.10	0.02	0.01	0.01	0.02	0.02	0.01	0.01
LEU[9]	M	0.110^{e}	0.235^{d}	0.389^{bc}	0.587^{a}	0.102^{e}	0.248^{d}	0.401^{c}	0.492^{ab}	0.109^{e}	0.300^{c}	0.397^{bc}	0.455^{b}	0.117^{e}	0.289^{cd}	0.429b^{c}	0.505^{a}
	SD	0.12	0.18	0.25	0.46	0.08	0.34	0.35	0.41	0.09	0.22	0.24	0.40	0.10	0.17	0.15	0.42

1 — in grams; 2 — aspartate amino transferase, U/L; 3 — alanine amino transferase, U/L; 4 — creatine kinase; 5 — lactate dehydrogenase, U/L; 6 — ng/mL 7 — valine mmol/mL; 8 — isoleucine, mmol/mL; 9 — leucine mmol/mL. The AIN93-G WP diet was made by substituting the whey protein for the casein of the AIN93 (standard) diet plus leucine (L) added. No different capital letters above the bars denote no significant differences between diets–exercise, Different small letters indicate significant differences between groups. two-way ANOVA was used and means were compared (Duncan test), adopting the value of $P < 0.05$ as a criterion for statistical significance.

4. Conclusions

Supplementation with L-leucine and whey protein effectively activated the cardiac mTOR pathway, the heart mass/body mass increase in the trained groups supplemented with 4.5% and 6% of L-leucine, feed with casein. The novelty of this study was to show the effectiveness of the blend of leucine and milk proteins as a viable alternative to maximize the activation of the anabolic pathway of the cardiac muscle of trained rats. Additionally, this combination of nutrients can provide essential amino acids for synthesis of heart protein, and this can be useful especially in circumstances of weakness of the heart muscle. AST and ALT, which are enzyme markers of acute hepatocellular injury, and CK and LDH, which are indicators of cardiac cell integrity, were not affected by the supplementation. Higher doses of L-leucine supplementation resulted in less body weight gains.

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