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Functional effects of milk bioactive peptides on skeletal muscle of rats

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ABSTRACT

Whey protein hydrolysate (WPH) intake has shown to enhance heat shock proteins (HSPs). The presence of bioactive peptides has been suggested to affect the capacity of WPH to induce a HSP response. HSPs are responsible for maintaining normal cellular function and protecting cells from the damage induced by various stressors, including exercise. Bioactive peptides have been considered as food components that could be biofunctionality, however, studies focusing on potential bioactive peptides from WPH are still limited. The purpose of this study was to determine which WPH peptides could modulate the HSP response and other WPH abilities. Fifty-six male Wistar rats were divided into seven groups (n = 8): control, vehicle, Isoleucyl-leucine (Ile-Leu), Leucyl-isoleucine (Leu-Ile), Valyl-leucine (Val-Leu), Leucyl-valine (Leu-Val) and WPH. Each animal received 3 mmol/kg dose of peptide or WPH dissolved in water by oral gavage. Except for the control group, all groups were subjected to acute exercise for homeostasis alteration. Treadmill exercise has been established as a stimulus for an HSP response. The Leu-Val peptide increased the HSP70 and HSP25 muscle expression and restored the dihydrofolate reductase (DHFR) expression. Ile-Leu increased the muscle and serum HSP70 levels, but did not change the HSP25. Leu-Val increased and restored the expression of antioxidant system components. All peptides enhanced the lipase expression and reduced the cholesterol and triacylglycerol levels. These results indicated that the Leu-Val peptide is most likely involved in the WPH-induced stimulation of the HSP upregulation and antioxidant response, including the protective mechanism (DHFR) associated with the HSP70 effect. Additionally, the Ile-Leu could also contribute to the WPH effect.

1. Introduction

Heat shock proteins (HSPs) are proteins named according to molecular mass, and HSPs are responsible for maintaining normal cellular function and protecting cells from the damage induced by various stressors (Silverstein et al., 2014). HSPs participate in the restoration and/or stabilization of damaged proteins (Cumming, Paulsen, Wernbom, Ugelstad, & Raastad, 2014) thus stimulating higher cell resistance, tolerance and survival (Santoro, 2000). In skeletal muscle, HSPs are involved in the prevention of protein degradation and aggregation, the removal and stabilization of misfolded non-functional muscle proteins, the facilitation of correct folding, the maintenance of muscle integrity (Brinkmeier & Ohlendieck, 2014) and the stimulation of normal muscle function (Huey, Hilliard, & Hunt, 2013).

Current evidence suggests that the discovery of new HSP inducers could improve several health parameters (Silverstein et al., 2014), including muscle function (Brinkmeier & Ohlendieck, 2014; Huey et al., 2013; Silverstein et al., 2014). Previous studies showed that dietary proteins and supplementation with certain amino acids may influence the HSP response (De Moura, Lollo, Morato, Carneiro, & Amaya-Farfan, 2013; Moura et al., 2014; Wischmeyer, 2002). We previously reported that the consumption of whey protein hydrolysate (WPH) enhances exercise-induced HSP expression (De Moura et al., 2013; Moura et al., 2014).

Whey protein represents approximately 20% of the proteins of bovine milk, which is believed to stimulate muscular hypertrophy. Moreover, it has been reported that the consumption of whey protein hydrolysate (WPH) is associated with other health effects, such as an increase in the translocation of GLUT-4 (Morato et al., 2013), an improvement in satiety signals (Luhovyy, Akhavan, & Anderson, 2007) and an increase in glycogen content (Morifuji, Sakai, Sanbongi, & Sugiura, 2005). Several molecular effects caused by the consumption of WPH have been connected to the presence of bioactive peptides (Ichinoseki-Sekine, Kakigi, Miura, & Naito, 2014; Luhovyy et al., 2007), including the possible role of branched-chain amino acids (BCAAs) and peptides in the HSP response (De Moura et al., 2013; Moura et al., 2014). The presence of BCAA-containing peptides in WPH has been reported (Morifuji, Koga, Kawanaka, & Higuchi, 2009). Bioactive peptides have been considered as food component that could be biofunctionality. However, studies focusing on potential bioactive peptides from WPH are still limited.

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We tested the hypothesis that one or more BCAA-containing peptides presents in the WPH could be involved in the WPH-induced HSP response. Thus, the purpose of the present study was to investigate the effect of four potential bioactive BCAA-containing dipeptides on the HSP expression, antioxidant system and complementary parameters. This is an innovative study that shows new effects of milk-bioactive peptides in various biological responses.

2. Materials and methods

2.1. Ethics approval and animals

Animal manipulations were performed according to the guidelines of the Ethics Committee on the use of animals of the University of Campinas that approved all experimental protocols (CEUA-UNICAMP, protocol 2845-1). Fifty-six male Wistar rats (21 days old, specific-pathogen free) from the Multidisciplinary Center for Biological Research (University of Campinas, SP, Brazil) were remained in individual cages with access to chow (Labina, Purina, Brazil) and water ad libitum. Table 1 shows composition and amino acid profile of the chow. Animals were maintained under controlled conditions (55% humidity, 22 ± 1 °C, inverted 12-hour light/dark cycle).

2.2. Experimental procedures and design

After growth, animals (280 ± 11.33 g body weight) were randomized and divided into seven groups (n = 8 per group): Control (rest, without gavage), Vehicle (water), Isoleucyl-leucine (Ile-Leu), Leucyl-isoleucine (Leu-Ile), Valyl-leucine (Val-Leu), Leucyl-valine (Leu-Val) and whey protein hydrolysate (WPH). The presence of these dipeptides in WPH has been reported (Morifuji et al., 2009). All the groups, except for the control, were subjected to a single exercise bout on a treadmill without inclination at a speed of 18 m/min for 60 min (Milne & Noble, 2002). Treadmill exercise has been established as a stimulus for an HSP response (De Moura et al., 2013; Huey & Meador, 2008; Moura et al., 2014; Salo, Donovan, & Davies, 1991). The speed of 18 m/min is known to already influence HSPs in the soleus muscle (Milne & Noble, 2002). Immediately after exercising, the animals were gavaged with the respective peptides, WPH or water (vehicle) and were then sacrificed 3 h after the gavage.

Table 1
Chow composition and amino acid profile.

Composition (%)	
Protein	21.88 ± 0.79
Carbohydrate	55.75 ± 0.82
Fat	5.07 ± 0.07
Ash	8.27 ± 0.10
Humidity	9.01 ± 0.06
Amino acid profile (g/100 g of chow)	
Aspartate	2.03 ± 0.14
Glutamate	3.82 ± 0.13
Serine	0.98 ± 0.07
Glycine	0.97 ± 0.06
Histidine	0.59 ± 0.17
Arginine	1.62 ± 0.20
Threonine	0.77 ± 0.10
Alanine	1.03 ± 0.07
Proline	1.22 ± 0.01
Tyrosine	0.76 ± 0.05
Valine	1.06 ± 0.10
Methionine	0.45 ± 0.19
Cystine	0.21 ± 0.05
Isoleucine	0.98 ± 0.14
Leucine	1.73 ± 0.04
Phenylalanine	1.10 ± 0.07
Lysine	1.41 ± 0.02

2.3. Preparation of the oral solutions

Each animal received only one 3 mmol (0.75 g/kg) dose of peptide or WPH dissolved in water by oral gavage. All peptides (purity > 99.6%) were acquired from BioBasic (Markham, Ontario, Canada), and the WPH was obtained from Hilmar Ingredients (Hilmar, CA, USA).

2.4. Western blotting analysis

Soleus muscle sample was removed from the animals and immediately frozen in liquid nitrogen for subsequent analysis. The muscle samples were homogenized in antiprotease buffer as previously described (Moura et al., 2014). The protein concentration of the muscle homogenate was analyzed using the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). An equal amount of protein was loaded and separated by SDS-PAGE (8%) and then transferred to nitrocellulose membrane (Santa Cruz, 0.22 μm pore size) using a semi-dry system (Bio-Rad, CA, USA). The blots were incubated overnight at 4 °C with the appropriate antibodies to assess the protein level of the following: HSP70 (Enzo Life Sciences, Farmingdale, NY, USA; Ref. ADI-SPA 810 diluted 1:3000), HSP25 (Enzo Life Sciences, Farmingdale, NY, USA; Ref. ADI-SPA 801 diluted 1:2000), DHFR (Abcam, Cambridge, Ref. ab124814 diluted 1:2000) SOD (Abcam, Cambridge, Ref. ab51254 diluted 1:10.000), catalase (Santa Cruz, CA, USA, Ref. sc271803 diluted 1:1000), NOS (Abcam, Cambridge, Ref. ab15203), GPx (Abcam, Cambridge, Ref. ab22604 diluted 1:2000), GAPDH (Enzo Life Sciences, Farmingdale, NY, USA, Ref. ADI 905734 diluted 1:1000), VEGF (Abcam, Cambridge, Ref. ab46154 diluted 1:2000), lipase (Abcam, Cambridge, Ref. ab109251 diluted 1:2000) and PGC (Abcam, Cambridge, Ref. ab72230 diluted 1:1000). The appropriate secondary antibodies (Abcam, Cambridge mouse ab6789 and rabbit ab6721) were used for detection. The blots were visualized using a UVITEC Alliance LD2 instrument (Cambridge, UK) and quantified with the program Image J.

2.5. Determination of free amino acid profile in plasma and muscle samples

Blood samples were collected with heparin anticoagulant and centrifuged at 3000 ×*g* (4 °C, 15 min) to obtain plasma and stored at −20 °C. The soleus muscle was dissected and immediately frozen and stored in liquid nitrogen until analysis. The free amino acids from skeletal muscle soleus and plasma (100 mg) were extracted with a solution composed of methanol (≥99.9% for HPLC) and hydrochloric acid (0.1 M) at the rate of 80/20 (v/v) and then derivatized with phenylisothiocyanate. The derivatized samples were resuspended in anhydrous dibasic sodium phosphate buffer (5 mM, pH 7.4) and chromatographed using a Luna C-18, 100 Å; 3 μm, 250 × 4.6 mm (00G-4251-E0) column at 46 °C, detected at 254 nm (White, Hart, & Fry, 1986).

2.6. Biochemical analysis

Blood samples were collected and centrifuged at 3000 ×*g* (4 °C, 15 min) to obtain the serum. The following serum parameters were measured using clinical commercial kits (Laborclin, Vargem Grande, Paraná, Brazil): uric acid, total protein, globulin, triacylglycerol (TG), cholesterol, high density lipoprotein (HDL), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The serum glucose and albumin levels were also measured (Labtest, Lagoa Santa, Minas Gerais, Brazil). The total thiol content was analyzed using a 5,5 'dithiobis (2-nitrobenzoic acid) (DTNB) assay. Plasma samples (10 μL) were treated with 200 μL DTNB (0.5 mM diluted in 100 mM phosphate buffer, pH 7.4) and incubated for 30 min at room temperature in the dark. The reaction was measured at 412 nm. Glutathione solution (0.5 mM) was diluted in phosphate buffer (100 mM, pH 7.4) to obtain a standard curve. The results were expressed as mM GSH/mg protein. The serum HSP70 level was determined using an enzyme immunoassay kit (high