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Alanyl-glutamine and glutamine plus alanine supplements improve skeletal redox status in trained rats: Involvement of heat shock protein pathways

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ABSTRACT

Aims: We hypothesized that oral L-glutamine supplementations could attenuate muscle damage and oxidative stress, mediated by glutathione (GSH) in high-intensity aerobic exercise by increasing the 70-kDa heat shock proteins (HSP70) and heat shock factor 1 (HSF1).
Main methods: Adult male Wistar rats were 8-week trained (60-min/day, 5 days/week) on a treadmill. During the last 21 days, the animals were supplemented with either L-alanyl-L-glutamine dipeptide (1.5 g/kg, DIP) or a solution containing the amino acids L-glutamine (1 g/kg) and L-alanine (0.67 g/kg) in their free form (GLN + ALA) or water (controls).
Key findings: Plasma from both DIP- and GLN + ALA-treated animals showed higher L-glutamine concentrations and reduced ammonium, malondialdehyde, myoglobin and creatine kinase activity. In the soleus and gastrocnemius muscle of both supplemented groups, L-glutamine and GSH contents were increased and GSH disulfide (GSSG) to GSH ratio was attenuated ($p < 0.001$). In the soleus muscle, cytosolic and nuclear HSP70 and HSF1 were increased by DIP supplementation. GLN + ALA group exhibited higher HSP70 (only in the nucleus) and HSF1 (cytosol and nucleus). In the gastrocnemius muscle, both supplementations were able to increase cytosolic HSP70 and cytosolic and nuclear HSF1.
Significance: In trained rats, oral supplementation with DIP or GLN + ALA solution increased the expression of muscle HSP70, favored muscle L-glutamine/GSH status and improved redox defenses, which attenuate markers of muscle damage, thus improving the beneficial effects of high-intensity exercise training.

Introduction

Nutritionally classified as nonessential amino acid, L-glutamine is the most abundant free amino acid in the body, being primarily produced and released into the blood by the working skeletal muscles (Newsholme et al., 2003). However, high-output exercise (*i.e.*, high-intensity or long-term strenuous exercises) represents a catabolic situation that promotes a decrease in body L-glutamine pool (Cruzat and Tirapegui, 2009; Santos et al., 2007). This response is accompanied by the release into the plasma of substances indicative of muscle damage (Cruzat et al., 2010), mainly due to the production of reactive oxygen and nitrogen species (ROS/RNS) (Finaud et al., 2006). Hence, under these conditions, the organism faces a status of oxidative stress in which the overall oxidant potential is enhanced. To protect the cells from ROS/RNS, the tripeptide GSH (L-γ-glutamyl-L-cysteinylglycine) is the most important non-enzymatic soluble intracellular antioxidant and has many protective and metabolic functions in cellular metabolism including attenuation of oxidative stress (Roth, 2008).

Experimental evidence suggests that the L-glutamate moiety, needed to the *de novo* synthesis of the tripeptide GSH, is mostly derived from L-glutamine in a variety of tissues (Newsholme et al., 2003), including skeletal muscle (Flaring et al., 2003). However, the availability of intracellular L-glutamine is influenced by the accessibility to and transport of L-glutamine into the cell (Newsholme et al., 2003). Thus, a reduced availability of L-glutamine, as observed in threateningly stressful situations, such as intense, prolonged or exhaustive exercise, may reduce GSH concentration, leaving the body more vulnerable to oxidative stress and cell death (Kim and Wischmeyer, 2013; Rutten et al., 2005).

In order to improve L-glutamine "status" under physiological stresses, many researchers have attempted to employ dietary supplementations with this amino acid (Kim and Wischmeyer, 2013; Gleeson, 2008). Accordingly, the oral use of either L-glutamine dipeptides, such

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as L-alanyl-L-glutamine (DIP) (Rogero et al., 2008a; Rogero et al., 2006) or solutions containing L-glutamine and L-alanine, both in their free forms, has proven to be an effective non-invasive alternative to increase body L-glutamine pools (Cruzat et al., 2010; Cruzat and Tirapegui, 2009).

L-glutamine is also associated with the potentiation of the expression of the cytoprotective 70 kDa heat shock protein (HSP70), both *in vitro* (Hamiel et al., 2009) and *in vivo* (Singleton and Wischmeyer, 2007). Through the main heat shock transcription factor (HSF1), L-glutamine is able to facilitate transcription of HSP70 leading to the *de novo* synthesis of HSP70, in a process that depends, at least partially, on the activation of glucosamine pathway (Hamiel et al., 2009). However, the pathways by which L-glutamine enhances HSP70 expression are mostly unknown. Studies have shown that both local and systemic inflammatory injury leads to a deficit in HSP70 expression (Singleton et al., 2005; Singleton and Wischmeyer, 2007) which may impair recovery and/or survival from these injuries. The plausible cytoprotective effects of L-glutamine on HSP70-mediated defense against high-output exercise-elicited stress has not been addressed yet. Hence, in this work, we tested the hypothesis that oral supplementations with the L-glutamine in the DIP form or as a mixture of L-glutamine *plus* L-alanine (GLN + ALA, both in their free forms) could protect rats subjected to intense aerobic training (treadmill) against muscle damage via HSP70 expression and GSH antioxidant system.

Materials and methods

Animals and diet

This study was conducted with 24 Wistar rats, adult male, body weight weighing 204 ± 8 g, obtained from the Animal House of the Faculty of Pharmaceutical Sciences, University of São Paulo (FCF-USP). The study was approved by the FCF-USP Ethics Committee on Animal Experiments and was performed according to the standards of the Brazilian College of Animal Experimentation (CEEA protocol nº 154). The total period of experiment was nine weeks, including a week of adaptation of animals to cages. Throughout the period, the animals were kept in individual cages maintained under a reversed cycle of 12 h light, 12 h dark (lights on at 0700) at room temperature of 22 ± 2 °C and relative humidity of 60%. During the experimental period, animals were fed *ad libitum* diet prepared according to the American Institute of Nutrition (AIN-93 M) (Reeves et al., 1993) for adult rats.

Training protocol

The training protocol was performed on a treadmill for rodents following the experimental protocol proposed by Smolka et al. (2000). All animals were submitted to exercise training and its total duration was 8 weeks. The exercise sessions were 5 days/week (commencing from 8:00 am) and had progressive load intensity and duration with 3° grade. The grade of was always 3°. During the first week of training, all animals underwent a period of adaptation and familiarization treadmill, consisted of daily sessions of 20 min at a speed of 15 m/min. In the second and third weeks, the sessions progressed to 30 and 45 min long, with speeds of 20 m/min. and 22.5 m/min, respectively. During the subsequent weeks (fourth to eighth week) training sessions were performed for 60 min at a speed of 25 m/min, hence from 8:00 to 9:00 am. The present protocol was chosen based on previous studies that showed increased markers of oxidative stress accompanied of protective HSP72 expression by the skeletal muscle (Smolka et al., 2000).

Supplementation and experimental groups

Animals were daily supplemented with either L-alanyl-L-glutamine DIP (Cláris Pharmaceutical Products of Brazil Ltd., São Paulo, Brazil donated by Fórmula Medicinal Ltd., São Paulo, Brazil) at a dose of 1.5 g/kg, or free L-glutamine (1.0 g/kg) plus free L-alanine (0.67 g/kg) (GLN + ALA). Both free amino acids were supplied by Ajinomoto Interamerican Industry and Commerce Ltd., Brazil. The animals received supplements through gavage (1 mL/100 g body weight) for a total period of 21 days before euthanasia. Gavage was provided 1 h after the end of each session of exercise (so, approx 10:00 am, see above), and during this time animals had with free access to water and chow. In the last day of exercise, the same procedure was adopted in order to eliminate results that reflected an acute single dose effect (Rogero et al., 2004), since animals were killed 10 h after the last exercise session. The amount of DIP was calculated in such a way that the total amount of L-glutamine was the same as that of L-glutamine administered in its free form. This supplemented protocol was chosen because our previous studies have found effects on plasma and tissue L-glutamine concentration at this dosage (Cruzat and Tirapegui, 2009; Rogero et al., 2006). Control (CONTR) animals received water at the same volume by gavage.

Biochemical and molecular analysis

Animals were killed by decapitation 10 h after the last exercise session. This lag period was chosen because HSP70 protein expression has been found to be maximal at this time (Silver et al., 2012). Afterwards, blood was collected and plasma were stored at -80 °C for subsequent determination of L-glutamine, L-glutamate, ammonium, malondialdehyde (MDA), myoglobin (MYO) and creatine kinase (CK) activity. Afterwards, the soleus and gastrocnemius muscles were surgically excised and immediately frozen in liquid nitrogen for the determination of the concentration of L-glutamine, L-glutamate, total protein contents, GSH and glutathione disulfide (GSSG). Samples destined to be electrophoresed and immunoblotted for HSP70 and HSF1 were immediately freeze-clamped and frozen in liquid nitrogen in the presence of protease inhibitors.

L-glutamine and L-glutamate were determined spectrophotometrically using a commercial kit (Sigma-Aldrich Chemical) adapted for microplate reader (Bio-Rad) (Lund, 1970). Ammonium levels was measured using a commercial kit (Raichem Diagnostics), and as described by Neeley and Phillipson (1988). Total CK activity in plasma was carried out as described by Schumann et al. (2002). Measurement of serum MYO was performed using a commercial Myoglobin Enzyme Immunoassay Test Kit (MP Biomedicals Diagnostics Division, USA). Plasma lipid peroxidation was inferred from the analysis thiobarbituric acid-reactive substance (TBARS) in terms of malondialdehyde (MDA) equivalents according to the method described by Draper and Hadley (1990). GSH and GSSG contents in skeletal muscles were assessed by HPLC, as described by Cruzat and Tirapegui (2009).

Muscular tissues (soleus and gastrocnemius) were homogenized in appropriate volumes by using the NE-PER kit (Termoscientific - Pierce) for the extraction and separation of tissue cytoplasmic and nuclear fractions, following manufacturer's instructions for HSP70 and HSF1. By using this technique, a minimal (10%) cross-contamination between nuclear and cytosolic fraction was estimated to occur. After separation, protein contents in both fractions were quantified by using the BCA kit (Pierce Chemical). Equal amounts of protein (soleus muscle: cytoplasmic fraction, 23 μg; nuclear fraction, 9 μg; gastrocnemius muscle: cytoplasmic fraction, 23 μg; nuclear fraction, 17 μg) were prepared, SDS/PAGE-electrophoresed (Slab Gel Mini-Protean II, BioRad) and electrotransferred (BioRad) onto nitrocellulose membranes. For immunodetections, membranes were probed with anti-HSF1 (StressGen/Enzo Life Sciences, 1:500, which recognizes both the phosphorylated and unphosphorylated forms of HSF1), anti-HSP70 (clone BRM22, 1:2000, Sigma, which recognizes both the inducible HSP72 and the cognate HSP73 forms) and anti-β-actin (Sigma) by using the vacuum-filtration method and SNAP i.d. System (Millipore) following the manufacturer's instructions. Revelation was carried out by using biotin-labeled secondary anti-IgG antibodies (1:10000, Sigma) and streptavidin-horseradish peroxidase (GE HealthCare) and ECL Plus