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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

reagents. Images were acquired by using the ImageQuant 350 chemiluminescence system (GE) and online stacking imaging software ImageQuant TL 7.0 (GE).

Statistical analysis

Statistical analyses were carried out using one-way ANOVA with post-test Tukey HSD (*Honestly Significant Differences*) and Levene's test of homogeneity. The level of significance was adopted $p < 0.001$ and SPSS 15.0 for Windows was used with data expressed as the mean ± S.E.M.

Results

Weight, food intake and plasma parameters

The animal's final body weight (weighing 293 ± 22 g) and food intake (20.5 ± 4.2 g/day) did not differ between the groups during the experimental protocol. As presented in Table 1, L-glutamine supplements significantly increased plasma L-glutamine concentrations (by 59% in GLN + ALA animals and by 62% in DIP rats) compared to controls. On the contrary, plasma glutamate concentrations were not affected by glutamine supplementations. Therefore, glutamine to glutamate plasma ratio, which is an indicator of the potential ($\Delta G < 0$) for glutamine flux through glutaminase and glutamate dehydrogenase pathway, was also recovered by both glutamine supplementations. This was paralleled by a similar decrease in plasma ammonium levels (by 23% in GLN + ALA and by 28% in DIP groups). Concerning stress/muscle damage indicators, both GLN + ALA and DIP treatments reduced plasma levels of MDA (by 38% and 47% respectively), MYO (by 35% and 44% respectively) and CK (by 24% and 25% respectively), thus suggesting a beneficial effect of L-glutamine supplements. It is of note, however, that the diminishing effects of DIP on MYO plasma levels were less than those found for GLN + ALA group ($p < 0.001$, Table 1).

L-Glutamine content in skeletal muscle

In the predominantly oxidative-type muscle soleus and fast-twitch fiber (mostly glycolytic) gastrocnemius muscle, both supplementations increased tissue L-glutamine concentration (Fig. 1A and B, respectively). When compared to GLN + ALA group, DIP supplementation increased glutamine concentration in gastrocnemius muscle ($p < 0.001$).

GSH and GSSG status in skeletal muscle

As described in Table 2, both DIP and GLN + ALA treatments doubled GSH contents in the soleus (94% and 109% increases, respectively) and in the gastrocnemius muscles (89% and 105% rises, respectively). GSSG muscle concentrations did not differ between groups, thus leading to a decrease in GSSG to GSH ratio (by approx 40%, Table 2), which led to a more reducing intracellular "voltage" (redox status) in both muscle types ($p < 0.001$). These results suggest an enhance in GSH synthesis over GSH conversion to GSSG under the present oxidative and stressful conditions imposed by high-intensity exercise.

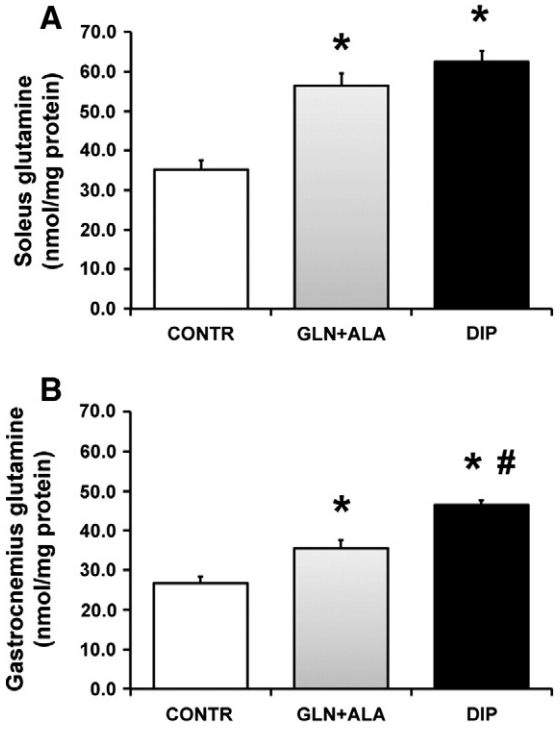

Fig. 1. L-Glutamine content in the soleus (A) and gastrocnemius skeletal muscle (B). Wistar rats (8 per group) were trained for 8 weeks and orally treated with either DIP or GLN + ALA or an equivalent amount of water (CONTR) for the last 21 days. Data are expressed as mean ± SEM. * $P < 0.001$ for the comparison with CONTR group. # $P < 0.001$ for the comparison with GLN + ALA and DIP group.

Heat Shock Protein Pathways

As shown in Fig. 2A, cytosolic HSP70 (HSP72 + HSP73) in slow-twitch fiber soleus muscle was increased in DIP group than in the controls and GLN + ALA group ($p < 0.001$). On the other hand, GLN + ALA supplementation reduced HSP70 in cytosol compared to control animals. However, nuclear localization of HSP70 was enhanced by 94% in DIP and by 112% in GLN + ALA group than in controls (Fig. 2B, $p < 0.001$). In the cytosol and nucleus of soleus muscle, HSF1 were increased by both supplementations ($p < 0.001$), although this response was more pronounced in the cytosol of GLN + ALA group than in DIP group (Fig. 2C and D, respectively, $p < 0.001$).

In the mainly fast-twitch fiber (mostly glycolytic) gastrocnemius muscle, GLN + ALA and DIP treatments enhanced cytosolic localization of HSP70, more in GLN + ALA than in the DIP group (Fig. 3A, $p < 0.001$),

Table 1
Plasma glutamine, glutamate, ammonium, malondialdehyde, myoglobin, and creatine kinase activity. Wistar rats (8 per group) were trained for 8 weeks and orally treated with either DIP or GLN + ALA or an equivalent amount of water (CONTR) for the last 21 days. Data are expressed as mean ± SEM. * $P < 0.001$ for the comparison with CONTR group. # $P < 0.001$ for the comparison with GLN + ALA and DIP group.

	CTRL	GLN + ALA	DIP
L-Glutamine (mmol/L)	0.79 ± 0.03	1.26 ± 0.03*	1.28 ± 0.03*
L-Glutamate (mmol/L)	0.36 ± 0.04	0.38 ± 0.01	0.41 ± 0.03
L-Glutamine/L-Glutamate	2.19 ± 0.06	3.31 ± 0.04*	3.12 ± 0.05*
Ammonium (μmol/mL)	5.30 ± 0.16	4.1 ± 0.22*	3.8 ± 0.08*
Malondialdehyde (μmol/L)	27.7 ± 1.18	17.3 ± 2.55*	14.8 ± 0.75*
Myoglobin (ng/mL)	114.9 ± 1.69	74.3 ± 1.88*	64.5 ± 2.06*#
Creatine kinase activity (U/mL)	378. 3 ± 1.6	286.2 ± 2.5*	283.0 ± 1.8*

Table 2
GSH and GSSG content in soleus and gastrocnemius skeletal muscles. Wistar rats (8 per group) were trained for 8 weeks and orally treated with either DIP or GLN + ALA or an equivalent amount of water (CONTR) for the last 21 days. Data are expressed as mean ± SEM. * $P < 0.001$ for the comparison with CONTR group.

	CTRL	GLN + ALA	DIP
SOLEUS			
GSH (μmol/g fresh tissue)	0.33 ± 0.06	0.64 ± 0.12*	0.69 ± 0.09*
GSSG (μmol/g fresh tissue)	0.12 ± 0.03	0.14 ± 0.03	0.14 ± 0.01
[GSSG]/[GSH] ratio	0.36 ± 0.07	0.22 ± 0.12*	0.20 ± 0.09*
GASTROCNEMIUS			
GSH (μmol/g fresh tissue)	0.19 ± 0.03	0. 36 ± 0.05*	0.39 ± 0.02*
GSSG (μmol/g fresh tissue)	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
[GSSG]/[GSH] ratio	0.47 ± 0.03	0.28 ± 0.65*	0.26 ± 0.02*

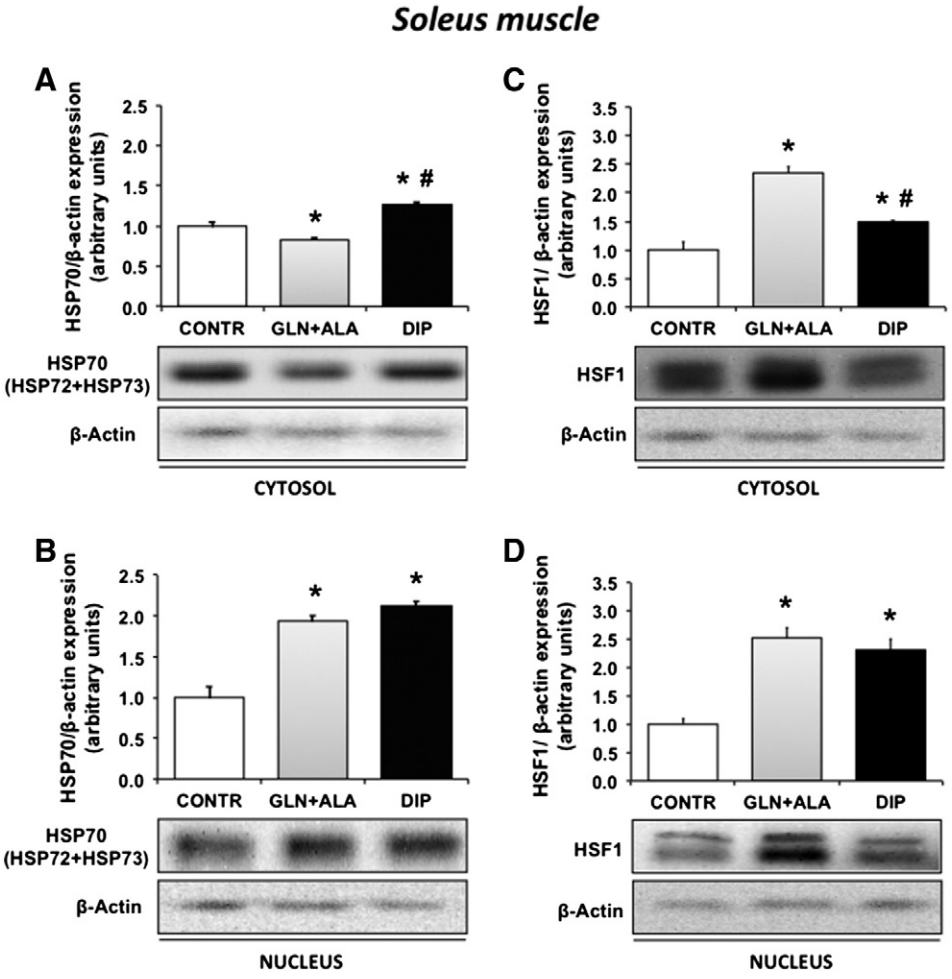

Fig. 2. Cytosolic (A) and nuclear (B) HSP70 expression and cytosolic (C) and nuclear (D) HSF1 in the soleus skeletal muscle. Wistar rats (8 per group) were trained for 8 weeks and orally treated with either DIP or GLN + ALA or an equivalent amount of water (CONTR) for the last 21 days. Tissues were surgically excised and immediately freeze-clamped under liquid nitrogen for Western blot analysis as described in the Methods section. Data are expressed as mean ± SEM. * $P < 0.001$ for the comparison with CONTR group. # $P < 0.001$ for the comparison with GLN + ALA and DIP group.

while no significant effect on nuclear localization (Fig. 3B). In the same tissue, cytosolic and nuclear localizations of HSF1 (Fig. 3C and D) were increased by GLN + ALA and DIP supplementations when compared to the controls ($p < 0.001$). However, the response of HSF1 in cytosol was more pronounced in GLN + ALA group, than in DIP group ($p < 0.001$).

Discussion

During metabolic stresses, such as long-term or high intensity physical exercise, the rise in catabolic processes stimulates cells to consume high amounts of the body's most abundant amino acid, glutamine, leading to an imbalance of whole body defenses (Curi et al., 2007; Newsholme, 2001). In the present study, 8-week trained rats subjected to chronic oral supplementation with L-glutamine in the DIP form or in it's free form along with free L-alanine showed an increase in plasma glutamine concentration as compared to controls. Since plasma glutamate remained unaltered, glutamine to glutamate plasma ratio was high in both supplemented groups than in the controls.

Augmented plasma concentrations of glutamine can stimulate its uptake by the cells and increase their availability to the intracellular environment (Williams et al., 1998). This can be seen in soleus and gastrocnemius skeletal muscles, which presented an increase in glutamine concentration promoted by both nutritional supplementations. Since glutamine is the immediate precursor of glutamate, even if cysteine and glycine were maintained at relatively constant levels (Rutten et al., 2005), muscle *de novo* synthesis of GSH were increased in DIP and GLN + ALA groups than in the controls. Moreover, the rise in GSH content evoked by both supplementations and the consequent decrease in GSSG to GSH ratio, an index of intracellular redox status (Silveira et al., 2007) make soleus and gastrocnemius skeletal muscle redox status much more balanced.

The GSH system is quantitatively the most important ROS/RNS scavenger and has many metabolic functions with the ability to protect cells against oxidative damage, such lipid peroxidation caused by hydrogen peroxide (H_2O_2) and free radicals (Roth, 2008). MDA concentration in plasma, a parameter of lipid peroxidation was higher in CONTR exercised animals, and L-glutamine supplementations reversed this pro-oxidant scenario. On the other hand, reduced levels of ammonium in the plasma of both GLN + ALA and DIP-treated animals are in line with the notion that both supplementations may increase plasma L-glutamine concentration, thus favoring L-glutamine supply to the kidneys (Bassini-Cameron et al., 2008). Hyperammonemia is induced by heavy exercise, and other studies in humans and animals confirm the present observations (Bassini-Cameron et al., 2008; Cruzat and Tirapegui, 2009).

Through diverse mechanisms, such as mitochondrial respiratory chain, xanthine oxidase or inflammation, oxidative stress is always a hallmark of heavy periods of exercise, which led to oxidative damage (Finaud et al., 2006). Once the tissue lesion is established, the rupture of contractile structures and the cytoskeletal components of the muscles release intracellular proteins in the blood, such MYO and CK, which are

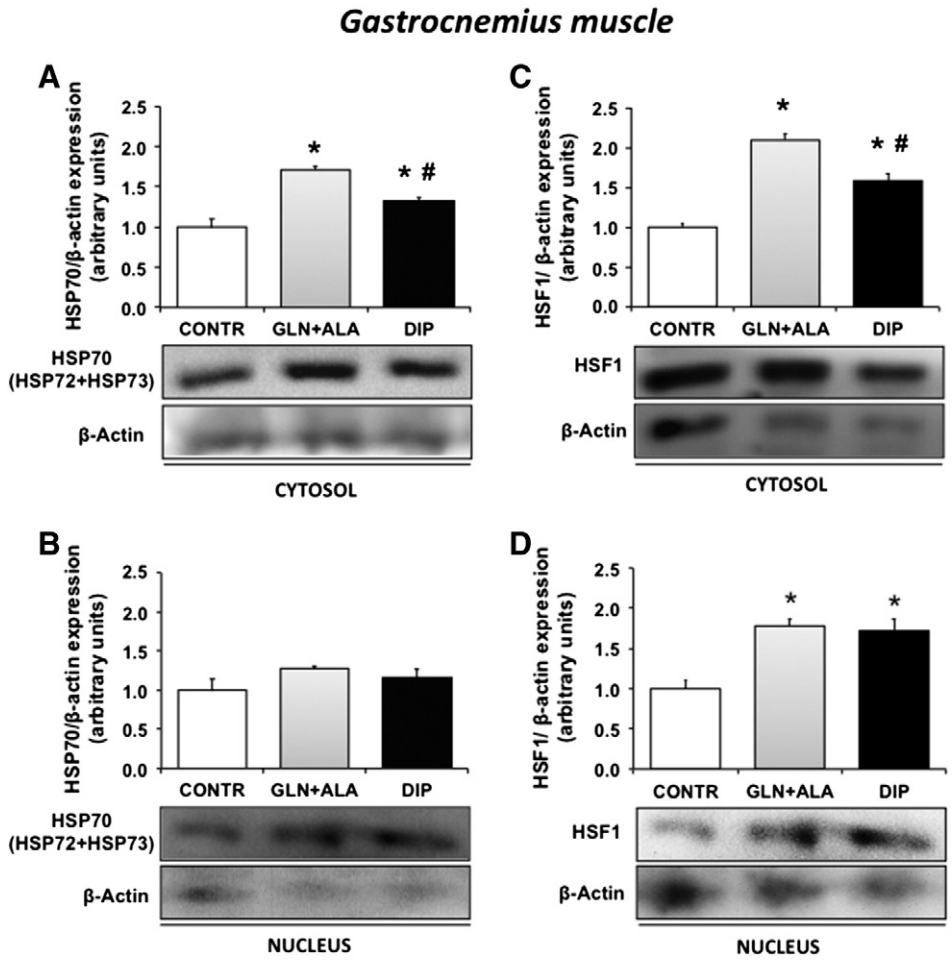

Fig. 3. Cytosolic (A) and nuclear (B) HSP70 expression and cytosolic (C) and nuclear (D) HSF1 in the gastrocnemius skeletal muscle. Wistar rats (8 per group) were trained for 8 weeks and orally treated with either DIP or GLN + ALA or an equivalent amount of water (CONTR) for the last 21 days. Tissues were surgically excised and immediately freeze-clamped under liquid nitrogen for Western blot analysis as described in the Methods section. Data are expressed as mean ± SEM. * $P < 0.001$ for the comparison with CONTR group. # $P < 0.001$ for the comparison with GLN + ALA and DIP group.

frequently used as muscle lesion markers (Malm, 2001). According to our results, animals subjected to both L-glutamine supplementations showed lower concentration of MYO and CK when compared with controls. These results are in accordance with studies from our laboratories with animals exposed to intense and prolonged swimming exercise in which oral supplementation with L-glutamine in the DIP form or in its free form along with L-alanine may reestablished total glutamine stores (Rogero et al., 2006), thus improving the intracellular redox status (Cruzat and Tirapegui, 2009) and reducing muscle damage and inflammation (Cruzat et al., 2010).

Although L-glutamine supplementations may have important antioxidant effects, as shown herein, other studies suggested that the administration of the amino acid serve as novel and potentially clinically relevant inducer of HSP70 expression, protecting against a variety of cell/tissue injuries (Kallweit et al., 2012; Kim and Wischmeyer, 2013). Local and systemic inflammatory injury leads to a deficit in HSP70 expression, which may impair recovery from these injuries (Singleton et al., 2005; Singleton and Wischmeyer, 2007). The conservation among different eukaryotes suggests that HSPs response is essential for survival in a stressful environment which the main function is act as a molecular chaperones (Akerfelt et al., 2010), especially in pro-oxidant situations, such heavy exercise (Heck et al., 2011). Therefore, one cannot discard the possibility that part of the beneficial effects of high-intensity exercise training may be due to the enhancement of HSP70 expression which is exacerbated by glutamine supplementations. A possible mechanistic explanation for such an improvement in glutamine-dependent HSP70 expression is the activation of the O-linked glycosylation (O-GlcNAc) pathway by glutamine. Specifically, glutamine after its entrance in glucosamine biochemical pathway, can induce O-GlcNAc modification which activates HSF1 and other proteins such as SP1, by reciprocal phosphorylation, then leading to nuclear translocation (Singleton and Wischmeyer, 2008). Once in the nucleus, the heat shock response is mediated at the transcriptional level by *cis*-acting sequences called heat shock elements (HSEs) that are present in multiple copies upstream of the HSP genes, such HSP70 family expression (Akerfelt et al., 2010).

Our *in vivo* results indicate that both oral L-glutamine supplementations enhance total protein of HSP70 family and the same effect can be observed in HSF1 in cytosol and nucleus. However differential interpretation of these results need to be considered. HSF1 is an inducible transcriptional factor, and an increase in its localization to the nucleus suggests an activation of the transcription factor, which promote HSP70 family response in cytosolic and nuclear localization of slow- and fast-twitch fiber skeletal muscles. The reason why, however, it was found a GLN + ALA supplementation-induced reduction in the cytosolic localization of HSP70 expression in the soleus muscle while no similar effect was observed in gastrocnemius muscle from both supplementation groups, remains to be established. Maybe these differences are related to the fact that slow-twitch fiber muscles (oxidative) express high levels of HSP70 under basal conditions than fast-twitch fiber

muscles (mixed or predominantly glycolytic). This could be related to the role of chaperone proteins against muscle oxidative damage through the stabilization of ionic channels and myotube development after frequent activation, such physical training (Tupling et al., 2007).

Considering the protecting role of HSP70 over oxidative muscle damage, our result may suggest that glutamine supplementations to exercised individuals may increase HSP70 response in order to protect cells under high oxidative stress profile that predominates during the first stages of exercise training. Expression of HSPs provides stress tolerance (Kim and Wischmeyer, 2013), by blocking the activation and nuclear binding of nuclear factor-κB (NF-κB) to the promoters of inflammatory genes that could otherwise cause cell death and/or impaired tissue recovery (Singleton and Wischmeyer, 2007). Indeed, HSP70's are now widely accepted as anti-inflammatory proteins by virtue of turning NF-κB off, thus extinguishing the production of inflammatory mediators. Therefore, one cannot refuse the possibility that glutamine supplementations may improve whole-body protective performance in high-intensity exercise training due to enhanced anti-inflammatory response mediated by glutamine-evoked enhancement of HSP70 expression.

It is also of note that the present findings slightly differ from those reported by Smolka et al. (2000), which described adaptions induced by the same continuous exercise model, but did observed elevation in citrate synthase parameters with no alterations in HSP72. Also, this is the first work to demonstrate glutamine effects on HSP70 pathways in both dipeptide form and in a stoichiometric combination with L-alanine, which is relevant, since alanine and glutamine metabolic routes often work in parallel, particularly in the active muscle (Felig, 1975). Whether, however, glutamine exerts its protective effects by saving alanine to muscle metabolism or acting together with alanine is currently under investigation in our laboratories.

Oral supplementation with L-glutamine has been suggested in various studies as a way to mitigate the reduction of this amino acid in the plasma and intracellular environment induced by high-intensity and prolonged exercise, or sports that have high frequency training (Castell et al., 1996; Cruzat and Tirapegui, 2009; Santos et al., 2007). However, oral administration of L-glutamine in its free form to healthy human or animals has shown low efficacy (Castell et al., 1996; Rogero et al., 2006; Valencia et al., 2002). Such ineffectiveness has been credited, at least to the fact that about 50-80% of L-glutamine in the free form is utilized by the enterocytes (D'Souza and Powell-Tuck, 2004; Dechelotte et al., 1991), and only small amounts of L-glutamine would be available to other cells and tissues, such as the skeletal muscle. On the other hand, DIP forms of L-glutamine, such L-alanyl-L-glutamine, are usually preferred as they could be metabolized more slowly by the enterocytes allowing high glutamine and alanine concentrations to be rapidly achieved in the plasma. These effects have been attributed to Pept-1, which is located exclusively in the luminal membrane and has a broad substrate specificity and actively transports dipeptides and tripeptides in the intestines of humans and animals (Adibi, 2003). However, the findings of the present study suggest that L-glutamine along with L-alanine solution have similar metabolic effects compared with DIP which seems that other mechanisms may be involved in the transport of dipeptides and amino acids, such paracellular movement and cell-penetrating peptides and amino acids (Gilbert et al., 2008). *In vivo* studies support this mechanistic effect, since both DIP and L-glutamine in its free form, in conjunction with other amino acids, can enhance whole body glutamine status in health and catabolic situations (Cruzat et al., 2010; Cruzat and Tirapegui, 2009; Harris et al., 2012; Rogero et al., 2008b).

The results presented herein also indicate that both supplementations influenced intracellular GSH stocks, diminishing tissue vulnerability to oxidative stress and leading to reduced plasma MYO and CK contents in trained rats. Moreover, the rise in HSP70 expression observed in both muscles may contribute to this beneficial effect of glutamine supplementations.

Conclusions

The present study indicates that in trained rats, chronic oral supplementation with L-glutamine, whether in its dipeptide form or in the free form along with L-alanine, represents an effective nutritional method to maintain L-glutamine stores, which attenuate the release of substances indicative of muscle damage and oxidative stress by enhanced GSH antioxidant system and HSP70 response, thus improving the beneficial effects of high-intensity exercise training.

Conflict of interest

Authors declare no conflict of interest.

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