Exercise training reverses the negative effects of chronic L-arginine supplementation on insulin sensitivity

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A B S T R A C T

L-Arginine has emerged as an important supplement for athletes and non-athletes in order to improve performance. Arginine has been extensively used as substrate for nitric oxide synthesis, leading to increased vasodilatation and hormonal secretion. However, the chronic consumption of arginine has been shown to impair insulin sensitivity. In the present study, we aimed to evaluate whether chronic arginine supplementation associated with exercise training would have a beneficial impact on insulin sensitivity. We, therefore, treated Wistar rats for 4 weeks with arginine, associated or not with exercise training (treadmill). We assessed the somatotropic activation, by evaluating growth hormone (GH) gene expression and protein content in the pituitary, as well as GH concentration in the serum. Additionally, we evaluate whole-body insulin sensitivity, by performing an insulin tolerance test. Skeletal muscle morpho-physiological parameters were also assessed. Insulin sensitivity was impaired in the arginine-treated animals. However, exercise training reversed the negative effects of arginine. Arginine and exercise training increased somatotropic axis function, muscle mass and body weight gain. The combination arginine and exercise training further decreased total fat mass. Our results confirm that chronic arginine supplementation leads to insulin resistance, which can be reversed in the association with exercise training. We provide further evidence that exercise training is an important tool to improve whole-body metabolism.

1. Introduction

L-Arginine (Arg) is a versatile amino acid present in all animal cells, where it plays an essential role in multiple metabolic processes as ammonia detoxification, immune modulation, polyamine synthesis, and secretion of hormones [5,16,38]. Furthermore, Arg is a natural substrate for the nitric oxide synthase enzyme (NOS) and consequently the main biological precursor of nitric oxide (NO) [58].

In the sports field Arg is extensively used in high protein supplements in order to improve athlete's performance, increasing vasodilatation mediated by NO production [4,11,47]. In muscle, NO production induced by Arg activates satellite cells [6], and in combination with exercise training it improves angiogenesis in skeletal and cardiac muscles [48]. Furthermore, this amino acid is a potent secretagogue of insulin and growth hormone (GH) [1,16,20]. These hormones are intrinsically associated with glucose disposal and metabolism in several tissues.

Arg-induced NO production in somatotrophs increases GH gene and protein expression, and secretion [40]. Several other mechanisms have been described for Arg role in regulating GH expression, such as the suppression of somatostatin release [35], increment of calcium influx in GH3 cells and hemi-pituitaries [40,51] and NOS/NO/GC/cGMP pathway activation, required for GHRH-induced GH release from somatotrophs [45]. We have previously shown that rats chronically treated with Arg presented increased GH levels and developed insulin resistance. We provide molecular evidence connecting the increased GH content in the genesis of the insulin resistance observed in the arg-treated animals [15,16].

Arg stimulates insulin secretion by directly activating pancreatic β-cells membrane depolarization, and consequently inducing PKA and PKC activity [33,49]. Long-term insulin stimulation due to chronic arginine treatment could lead to decreased insulin sensitivity [15,61].
Physical activity stimulates the somatotropic axis function. As a physiological stress, exercise increases GHRH release and diminishes somatostatin release via adrenergic/cholinergic pathways [10,55], which further result in increased concentration of GH in the blood [53]. Exercise also stimulates liver and muscle IGF-I expression by increasing GH secretion, leading to secretion of other hormones and metabolites [25,31,44,46,54], which together improve skeletal muscle mass gain, as well as fat and glucose utilization.

Furthermore, physical activity is a well-known therapeutic strategy to improve the glucose metabolism. Muscle contraction induces glucose transporter (GLUT-4) trafficking to cell surface, by a mechanism dependent on the AMPK activation [32]. More recently, it has been shown that exogenous ATP, derived from the contractile activity of skeletal muscle, acting through purinergic receptors, increases AKT phosphorylation and GLUT-4 translocation to muscle cell surface [41]. Therefore, physical activity in association with body weight control could improve insulin sensitivity in diabetic patients, diminishing exogenous insulin treatment and improving glucose homeostasis [50].

In the present study, we aimed to investigate whether exercise training would counteract the insulin resistance caused by chronic Arg supplementation and whether their interaction could increase the somatotropic axis activity. For that, male Wistar rats were chronically treated with 35 mg of Arg per day for 4 weeks in the drinking water, in combination or not with aerobic exercise training. We evaluated the effects of Arg and exercise on GH expression and secretion, as well as its repercussions on insulin sensitivity. Biometric parameters were also evaluated.

2. Material and methods

2.1. Animal care

Twelve weeks old male Wistar rats (∼250 g) obtained from our own breeding colony (Institute of Biomedical Science, University of Sao Paulo, Brazil) were used in the experiments. The animals were housed in collective cages (5 animals per cage), in an inverted light-cycle room (12 h dark/12 h light cycle; lights off at 07h00), under a controlled temperature environment (23 ± 1 °C). The rats were maintained on standard chow diet, containing 22% of protein (Nuvilab CR1, Nuvital Nutrientes S/A, Colombo, PR, Brazil). Food and water were offered ad libitum.

2.2. Procedures

2.2.1. Exercise protocol and arginine treatment

In order to initiate the training protocol with a minimal variation between groups, all animals were subjected to 4 weeks adaptation to aerobic exercise training in ergometric treadmill (Inbramed, KT-300). The adaptation training was performed in a progressive speed increment (5, 10, 15 and 20 m/min), 0% grade, for periods of time that varied from 30 to 60 min, for 5 days a week. After the adaptation period, the rats were selected for their ability to run in a treadmill (16/30 animals) and further subjected to the training protocol (20 m/min, for 60 min, for 5 days a week), for additional 4 weeks (Table 1) combined or not with Arg treatment. Training experiments were carried out during the dark cycle at ZT13–15 Zeitgeber’s time.

The Arg supplementation was performed by adding L-arginine to the drinking water in a concentration of 1.25 g/L for 4 weeks, providing a dose of approximately 35 mg of arginine per day [16]. The control groups received plain water. The animals were divided into 4 groups: Control-Sedentary (CS), Arginine-Sedentary (AS), Control-Exercised (CE) and Arginine-Exercised (AE), by a randomized double-blind method.

At the endpoint, the rats were anesthetized with ketamine and xylazine (100 and 10 mg/kg of body weight, respectively) and euthanized by decapitation at ZT3–4 Zeitgeber’s time. Final body weight and naso-anal length were assessed to determine the Lee index. The extensor digitorum longus (EDL) and soleus muscles, as well as the heart, liver and the pituitary gland were removed and weighed. Tissues were snap-frozen in liquid nitrogen and stored at −80 °C until use.

The experimental protocol is in accordance to the ethical principles in animal research adopted by the Committee of Ethics in Animal Experimentation of the Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil (# 023, p. 43 and book 02, year 2007).

2.2.2. Gh expression

Total RNA was isolated from pituitaries using the acid guanidinium thiocyanate–phenol–chloroform extraction method as described [12]. The Gh mRNA expression was determined by Northern blot analysis using a 32P-labelled rat Gh cDNA by random priming (Random Primers DNA Labeling System Kit - Gibco BRL); and the variability in RNA loading was corrected by using a 32P–labelled RNA probe specific for IBS ribosomal RNA (18S rRNA), as previously described [17]. The results were expressed as mean ± SEM of Gh mRNA/18S rRNA ratio.

2.2.3. GH protein content

The pituitaries were homogenized in 0.25 M sucrose, 20 mM Tris-HCl buffer and 2 mM MgCl2, and centrifuged at 100g for 10 min at 4 °C. The supernatant was removed for protein content quantification by Bradford method [9]. Exactly 30 μg of protein were electrophoresed on 15% SDS-PAGE mini gel and transferred to nitrocellulose membrane (Bio-Rad, Richmond, CA). The membranes were blocked to avoid unspecific binding with 5% non-fat milk in PBS–Tween, for 1 h, and incubated overnight with rat anti-GH antibody (1:5000) (National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA) as described previously [24]. The membranes were washed in PBS–Tween, 4 times, for 10 min each, and incubated for 1 h with appropriated secondary peroxidase-conjugated antibody (1:5000, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Immunoreactivity was assessed by chemiluminescence reaction (ECL) and quantified by densitometric analysis using the ImageJ software. Sample loading was normalized by Ponceau staining of the nitrocellulose membrane. The results were expressed as mean ± SEM.

2.2.4. Serum GH concentration

Rats serum GH concentration was determined by the Milliplex® MAP - Rat Pituitary Magnetic Bead Panel kit (Cat® RPTMAG-86 K; Merck Millipore, MA, USA), following the manufacturer’s instructions.

2.2.5. Igf-1 mRNA expression

Total RNA was extracted from liver using the acid guanidinium thiocyanate–phenol–chloroform extraction method as described [12]. The synthesis of the first-strand complementary DNA and the following real-time quantitative PCR amplification were performed as described previously [15]. The sequences of the primers used are listed in Table 2.

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**Table 1**

<table>
<thead>
<tr>
<th>Adaptation and training protocol.</th>
<th>Day 1</th>
<th>Day 2</th>
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(Adapted from Martins et al. [36]).
2.2.6. Insulin tolerance test (ITT)

Rats were subjected to food deprivation for 2 h prior to the experiment. The animals received an intravenous injection of regular insulin (0.75 U/kg BW), and glucose blood levels were measured on samples obtained from the tail vein using a glucometer (Precision QIR*, Medience Products, Bedford, MA, USA) at 0 (T0), 4 (T4), 8 (T8), 12 (T12) and 16 (T16) minutes [23]. The values obtained were used to calculate the constant rate for plasma glucose disappearance (kITT) using the linear regression of the neperian logarithm of the blood glucose values obtained from 0 to 16 min of the kITT [8,23].

2.2.7. Biometric analysis

2.2.7.1. Body weight gain

All the animals were weighed weekly in order to calculate the average body weight and weight gain.

2.2.7.2. Tissues weight

Cardiac and skeletal muscles (EDL and Soleus) were weighed at the end of experimental period in order to evaluate the effects of Arg treatment and/or exercise training on muscle hypertrophy.

2.2.7.3. Lee-Index

The body fat content of the rats was estimated by the Lee-Index. For that, the naso-anal length (centimeters) and final body weight (grams) were measured and used to calculate the Lee index; weight 0.53/Naso-Anal Length [7].

2.2.8. Statistical analysis

Data were expressed as mean ± SEM. The significance level was set at 5% (p < 0.05). All the results were analyzed by Two-way ANOVA, followed by Tukey post-hoc test (Prism GraphPad Software version 6.05, San Diego, CA, USA). Insulin tolerance test was analyzed with regression curve and compared by unpaired Student’s t-test (Prism Graph Pad Software version 6.05, San Diego, CA, USA).

3. Results

3.1. GH mRNA, protein expression and serum concentration and Igf-I mRNA expression

The Gh mRNA content was significantly higher in the pituitary gland of trained rats when compared to the sedentary groups (p < 0.005). Arg supplementation, per se, increased Gh mRNA content independently of the training program (p < 0.05) (Fig. 1A). The GH protein content was unaltered among groups (Fig. 1B). However, as expected, GH concentration in the serum was increased in the exercise trained groups when compared to the sedentary (p < 0.005). Although not statistically significant, Arg supplementation alone tend to increased GH concentration in the serum in 3.9-fold when compared to control. Interestingly, arginine treatment in combination with exercise did not present further effect GH concentration in the blood.

Hepatic Igf-I mRNA content was unaltered in trained animals when compared to the control sedentary group (CE and AE vs CS; Fig. 1D). Arg supplementation increased hepatic Igf-I mRNA content by 3-fold when compared to the control group (AS vs CS) (p < 0.0001).

3.2. Insulin tolerance test (ITT)

Arg supplementation reduced insulin sensitivity in sedentary animals (p < 0.001) and exercise training improved insulin sensitivity in both control and Arg-supplemented groups (p < 0.0001), as expected (Fig. 2A and B).

3.3. Biometric parameters

3.3.1. Body weight gain

Body weight gain was similar among all groups before training intervention (Fig. 3A). Exercise training protocol reduced body weight gain in almost 50% in the both control and Arg-supplemented groups (Fig. 3A and B), while Arg supplementation did not affect significantly the body weight gain (Fig. 3A and B).

Neither training nor Arg treatment affected the linear growth (Fig. 4A). In order to investigate whether Arg supplementation, combined or not with training intervention affected body composition, we estimated the body fat content using the Lee-Index (Fig. 4B). Interestingly, Arg supplemented rats presented reduced Lee-Index when compared to controls, independently of the exercise intervention (p < 0.0001). As expected, exercise training induced a remarkable decrease in the Lee-Index in both control and Arg-supplemented groups (p < 0.0001).

3.3.2. Muscle mass

Soleus mass was increased in Arg-supplemented rats versus non-supplemented (p < 0.005). However, the exercise protocol had no additional significant effect on this parameter (Fig. 5A). Trained rats presented an increase in EDL mass when compared to sedentary rats (p < 0.005), while Arg supplementation had no significant effect (Fig. 5B). Exercise training increased cardiac mass in both control and Arg-supplemented groups (p < 0.0001), whereas Arg treatment alone did not this parameter (Fig. 5C).

4. Discussion

We have previously shown that chronic Arg supplementation induced insulin resistance in rats. Our findings indicated that chronic Arg consumption increased growth hormone synthesis and secretion, which presents diabetogenic effects, leading to insulin resistance [15]. In the present study, we investigated whether the association of exercise training with Arg supplementation would reverse the negative effects of Arg on insulin sensitivity. We also evaluated the effects of Arg and/or exercise training intervention on modulating growth hormone synthesis in pituitary gland and its physiological repercussions.

Our findings corroborate previous results from our group showing that chronic Arg supplementation increases Gh mRNA expression [15,16,40]. We also observed that Gh mRNA content was augmented by exercise training, as already reported [21,56]. Interestingly, the association of Arg supplementation with exercise training led to a further increase on Gh expression, indicating that Arg has an additive effect on increasing Gh expression in combination with exercise training. Notably, exercise training as well as Arg has been shown to increase Gh mRNA by inhibiting the hypothalamic somatostatin secretion [14]. Moreover, nitric oxide (NO) could also be playing a direct role in increasing Gh synthesis in pituitary. The NO generated in vivo by nitric oxide synthase (NOS) during the conversion of L-Arg to L-citrulline or by exercise-induced NOS activation, leads to an increase in cGMP levels by activating guanylate cyclase. This induces the PKG1-α phosphorylation of CREB at Ser133; a well-known downstream transcription factor that increases transcription of Gh gene [19,51,57,59].

Despite an increment on Gh mRNA expression, we show no difference in GH content in the pituitary of Arg-supplemented rats. This data instigate our group to search reasons for those discrepant results comparing our previously data published [15]. Our hypothesis was that the
protein was translated at pituitary gland stage and had being simulta-
neously secreted, which could result in a further increment on blood-
stream concentration and not inside the somatotropic cells level. For
this, we investigate the serum GH concentration and we observed that
all groups had higher serum GH concentration compared to control
group, indicating the Arg up regulates the pituitary GH secretion, as
well demonstrated in literature[1,3,10,31,60].

We also detected enhanced Igf-I mRNA content in liver of rats
subjected to Arg treatment, which reinforces Arg-induced effect on GH
secretion, as previously reported by our group [15,16]. Surprisingly,
Arg supplementation associated to exercise present no further effect on
hepatic Igf-I mRNA expression when compared to just trained rats.

Possibly, GH concentration is maintained at higher levels during the
exercise period, leading to a decrease in hepatic GH receptors content,
and consequently, to an impaired response of the liver to this hormone
[46,52].

Fig. 1. Effects of Arg supplementation associated or not with exercise on growth hormone (GH) gene expression, protein content in pituitary, GH concentration in serum and hepatic Igf-I gene expression.

A) Top-panel: autoradiogram representative of Gh and 18S rRNA transcripts obtained by Northern blotting; Bottom-panel: Gh mRNA abundance (AU). The 18S rRNA was used as internal control. Data are presented as mean ± SEM of the ratio Gh mRNA/18S rRNA. Two-way ANOVA followed by Tukey pos-hoc test was used. *p < 0.005 Arg supplemented vs non-supplemented; #p < 0.005 Exercise vs Sedentary (CS: n = 10; AS: n = 12; CE: n = 8; AE: n = 7). B) Top-panel: representative blot of GH protein, obtained by western blotting; Central-panel: membrane representative total protein content plotted in the western blotting obtained by Ponceau staining; Bottom-panel: GH content normalized by Ponceau staining of the membranes; data are represented as mean ± SEM of the AU. Two-way ANOVA followed by Tukey post-hoc test was used (CS: n = 9; AS: n = 5; CE: n = 7; AE: n = 7). C) Serum GH concentration; data are represented as mean ± SEM of the pg/ml. Two-way ANOVA followed by Tukey post-hoc test was used. *p < 0.005 Exercise vs Sedentary (CS: n = 5; AS: n = 6; CE: n = 6; AE: n = 8). D) Igf-I mRNA expression was normalized by the expression of Cyclophilin mRNA and expressed as mean ± SEM. Two-way ANOVA followed by Tukey pos-hoc test was used. *p < 0.0001 AS vs CS (CS: n = 4; AS: n = 4; CE: n = 4; AE: n = 4).
Fig. 2. Effects of Arg associated or not with exercise training on whole-body insulin sensitivity.  
A) Blood glucose (mg/dL) decay following insulin administration. Two-way ANOVA followed by Tukey post-hoc test was used. (CS: n = 20; AS: n = 20; CE: n = 6; AE: n = 9).  
B) Blood glucose decay constant rate (%/min) during ITT. Data is represented as % of glucose decay/min. Two-way ANOVA followed by Tukey post-hoc test was used.  
⁎p < 0.05 AS vs CS or AE vs CE; #p < 0.0001 Exercise vs Sedentary; (CS: n = 20; AS: n = 20; CE: n = 6; AE: n = 9).

Fig. 3. Effects of Arg supplementation associated or not with exercise training on body weight gain before, during and after the exercise training period.  
A) Body-weight gain (g) curve during the entire experimental period.  
⁎p < 0.05 CE vs CS;  
¤p < 0.05 AE vs AS.  
B) Accumulative weight gain (g) during the training protocol. Two-way ANOVA followed by Tukey post-hoc test was used.  
#p < 0.05 Exercise vs Sedentary (CS: n = 20; AS: n = 20; CE: n = 19; AE: n = 23).

Fig. 4. Effects of Arg supplementation associated or not with exercise training on naso-anal length and whole-body fat content.  
A) Naso-anal length (cm).  
B) Estimated fat content using the Lee-Index.  
⁎p < 0.05 Exercise vs Sedentary;  
⁎⁎p < 0.0001 AS vs CS. Two-way ANOVA followed by Tukey post-hoc test were used.  
(CS: n = 20; AS: n = 20; CE: n = 19; AE: n = 23).
somatotropic axis, altering the morpho-physiological parameters, it was not surprising that exercise training induced body weight and fat mass reduction, as shown by the Lee-Index results [13]. Arg-supplemented animals showed a slight increment in body length and body weight, despite the greater reduction on fat mass, in accordance with previous reports [22,34]. The significant drop of the Lee index and the increased muscle mass weight observed in all experimental groups may be a response to the recognized lipolytic and protein anabolic effects of GH [31,37].

In addition to these effects, NO synthesis caused by Arg supplementation has been shown to increase fat oxidation and reducing its synthesis [28]. Later studies by the same authors showed that Arg supplementation increased the glycerol release, and glucose and fatty acid oxidation in white adipose tissue, reducing adipocytes size [27,29]. Arg supplementation by itself did not increase cardiac muscle mass in control animals, although in combination with exercise it seems to attenuate cardiac muscle hypertrophy. This data corroborates past findings that shows Arg increasing eNOS and cystathionine γ lyase genes expression [2]. These Arg-induced effects protect left ventricle from hypertrophy, altering different aspects of cardiac muscle structure, as increasing the number of smaller arterioles, ameliorating the oxidative parameters, which improves the hemodynamic and cardiac function [2,28,42,43].

In our study, exercise training presented largely effect on lean mass gain, as shown by the Lee-Index ratio, and by also enhancing EDL and cardiac muscle weight gain, though the soleus mass remained unchanged by this condition. These results are in accordance with findings by Clavel and collaborators (2002), which observed a significant increment on rats EDL and heart muscles mass and not at soleus mass after an endurance-training program, suggesting that the fast-twitch muscle (EDL) and heart muscle had been adapted to the aerobic training, by increasing their aerobic capacity. Additionally, chronic physical exercise increases heart rate and cardiac output, among other important factors which might induce cardiac muscle hypertrophy [18,30].

In summary, we provide evidence that exercise training improved insulin sensitivity in Arg-supplemented rats, showing to be a potent tool to mitigate the adverse effects of chronic Arg supplementation on whole-body glucose homeostasis. Additionally, we confirm that chronic Arg supplementation activates the somatotropic axis and promotes lean mass gain and fat mass reduction. However, the association of Arg and exercise did not promote additional significant beneficial morphophysiological effects, since it rather attenuated the exercise-induced improvement on insulin sensitivity.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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